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Cyclin-dependent Kinase 9 Links RNA Polymerase II Transcription to Processing of Ribosomal RNA*[S]

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Background: Processing of ribosomal RNA is sensitive to Cdk inhibitors.
Results: Inhibition of Cdk9 activity blocks 47 S rRNA processing and stabilizes a 3′ extended 47 S primary transcript. Defective 3′ processing negatively feeds back on RNAPI transcription.
Conclusion: Cdk9 facilitates processing of 47 S rRNA by RNAPII-dependent synthesis of U8 snoRNA.
Significance: Cdk9 may be a critical regulator of rRNA processing to harmonize RNAPII transcription activity with a ribosome biogenesis rate.

Ribosome biogenesis is a process required for cellular growth and proliferation. Processing of ribosomal RNA (rRNA) is highly sensitive to flavopiridol, a specific inhibitor of cyclin-dependent kinase 9 (Cdk9). Cdk9 has been characterized as the catalytic subunit of the positive transcription elongation factor b (P-TEFb) of RNA polymerase II (RNAPII). Here we studied the connection between RNAPII transcription and rRNA processing. We show that inhibition of RNAPII activity by α-amanitin specifically blocks processing of rRNA. The block is characterized by accumulation of 3′ extended unprocessed 47 S rRNAs and the entire inhibition of other 47 S rRNA-specific processing steps. The transcription rate of rRNA is moderately reduced after inhibition of Cdk9, suggesting that defective 3′ processing of rRNA negatively feeds back on RNAPI transcription. Knockdown of Cdk9 caused a strong reduction of the levels of RNAPII-transcribed U8 small nucleolar RNA, which is essential for 3′ rRNA processing in mammalian cells. Our data demonstrate a pivotal role of Cdk9 activity for coupling of RNAPII transcription with small nucleolar RNA production and rRNA processing.

Ribosome biogenesis is a highly conserved and energy-consuming process in the nucleolus of proliferating cells. In mammalian cells, several hundred rDNA genes are organized and transcribed in repeat clusters on different chromosomes. First, a 47 S precursor is synthesized by RNAPI containing the sequences for mature 18 S, 5.8 S, and 28 S rRNAs, which are flanked and interspersed by external (5′ETS, 3′ETS) and internal (ITS-1, ITS-2) transcribed spacers, respectively. The 47 S transcript is subject to extensive co-transcriptional modification by methylation and pseudouridylation and a cascade of endo- and exonucleolytic cleavage and degradation steps. These steps are tightly connected with the association and dissociation of processing factors to and from pre-ribosomes and finally result in the production of mature 40 S and 60 S ribosomal subunits (1–3).

The 47 S rRNA becomes extensively modified particularly at the catalytically active sites of the 18 S, 5.8 S, and 28 S rRNAs, which participate in the decoding and peptidyltransferase center of translating ribosomes (4). The two major rRNA modifications are methylation of the 2′ oxygen of the ribose (2′-*O*-Met) and pseudouridylation of uridine residues (5–8). Modification of rRNA is catalyzed by small nucleolar ribonucleoproteins (snoRNPs)[2] (9, 10), which are composed of a snoRNA, the modifying enzyme (methyltransferase or pseudouridine synthase), and further proteins. snoRNAs can be divided into two families according to their structural similarities and conserved sequence elements. Box C/D snoRNAs guide rRNA methylation and form a box C/D snoRNP, whereas box H/ACA snoRNAs guide rRNA pseudouridylation and form a box H/ACA snoRNP (7, 11, 12). Correct placement of rRNA modifications is guided by complementary base pairing of snoRNAs to the 47 S rRNA. Besides this guidance to rRNA modification sites, snoRNAs also facilitate the correct processing of rRNA. They are required for the formation of specific secondary structures in the rRNA, which allow the recruitment and function of endonucleolytic enzymes (13). Although guidance snoRNAs for rRNA modification are usually nonessential, the deletion of processing-specific snoRNAs in yeast is incompatible with cell viability. In yeast, most snoRNAs are expressed

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[2] The abbreviations used are: snoRNP, small nucleolar ribonucleoprotein; Cdk, cyclin-dependent kinase; 4-sU, 4-thiouridine; Pes1, Pescadillo 1; FL, flavopiridol; CTD, C-terminal domain; RNAPII, RNA polymerase II; ETS, external transcribed; ITS, internal transcribed; ActD, actinomycin D.

from independent transcription units. In higher eukaryotes snoRNAs are often embedded in intron sequences and are transcribed from promoters of the corresponding gene. Here, snoRNA biogenesis is physically and functionally linked to pre-mRNA synthesis. In the nascent transcript intron-embedded snoRNAs are protected from degradation by association with cellular factors and the subsequent formation of stable snoRNPs (14–16). Recent expression analyses revealed that snoRNAs levels are subject to regulation, and only a fraction of transcribed snoRNA sequences are processed and stabilized (17, 18).

A wealth of helicases, nucleases, and GTPases has been characterized as regulators of rRNA synthesis in yeast (19, 20). However, less is known about kinases, particularly of kinases involved in the regulation of rRNA processing. Proteome analysis of human nucleoli revealed up to 700 proteins, including 15 kinases, which reproducibly localize to the nucleolus (21). The function of these kinases is largely unknown. In addition, previous work suggested that cyclin-dependent kinases (Cdks) are involved in rRNA synthesis. Cdk inhibitors can quantitatively block rRNA processing, whereas rRNA transcription is almost unaffected (22–24).

In this study we have analyzed the contribution of Cdks to transcription and processing of rRNA. Our data show that Cdk9 activity is crucial for rRNA processing and that the phenotype of Cdk9 inhibition on rRNA processing can be phenocopied by α-amanitin, a highly specific inhibitor of RNAPII transcription. We further show that snoRNA U8, which is specifically required for rRNA processing, is diminished after Cdk9 knockdown. Our data suggest a model in which inhibition of Cdk9 prevents the proper production of pre-mRNA-embedded snoRNAs and thereby inhibits rRNA processing. This model links RNAPII activity directly to ribosome biogenesis.

EXPERIMENTAL PROCEDURES

Tissue Culture—U2OS cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 8% CO_2. Mouse embryonic fibroblasts and 2fTGH cells were cultured at 5% CO_2. Recombinant HeLa cells, harboring an α-amanitin-resistant RNAPII, were created as described (25, 26). HeLa cells carrying the resistant polymerase were cultured in medium containing α-amanitin (2 μg/ml). Dicer-deficient mouse embryonic fibroblast cells were generated as previously described (27). In brief, fibroblasts from wild-type and Dicer fl/fl mouse embryos were generated and immortalized through infection with an ecotropic retrovirus that expresses SV40 large T. Cells were reinfected with an amphotropic retrovirus that expresses Cre-recombinase and confers puromycin resistance. Cells were treated with puromycin (2 μg/ml) for 5 days, after which clones were selected by limiting dilution. Clones were evaluated for genomic deletion and lack of microRNAs (27, 28).

siRNA Transfections—8×10^4 U2OS cells were seeded in 6-well plates. 0.9 μl of 100 μM luciferase control, CALM interacting protein expressed in thymus and spleen (CATS)-, Cdk-, Nop56-, or Pescadillo 1 (Pes1)-specific siRNAs (Eurofins or Qiagen flexi tube) were used for transfection/well using Oligofectamine and Opti-MEM (Invitrogen). Cells were incubated for 6 h and transfected on two consecutive days. siRNA sequences (5′ to 3′) were as follows: siLuc (GL2), CGUACGCGGAAUACUUCGAdTdT; siCATS, CUUGACCUUGAGCCUUCUAUUdTdT; siCdk1, GAUCAACUCUUCAGGAUUUdTdT; siCdk2, AAGGUGGUGGCGCUUAAGAAAdTdT; siCdk3, GAGCAAAGCACUAAGGAAUdTdT; siCdk4, AACCCACACAAGCGAAUCUCUdTdT; siCdk5, GAGGAUCUUUCGACUGCUAdTdT; siCdk7, GCCUACAUGUUGAUGACUCdTdT; siCdk8, CUACAAAGCCAAGAGGAAAdTdT; siCdk9, GCAAGGGUAGUAUAUACCUGGUGUUdTdT; siCdk9$_{55}$, CCGUCGGUUGCCAUGUCAAdTdT; siCdk12, Qiagen FlexiTube siRNA (SI00288442); siCdk13, Qiagen FlexiTube siRNA (SI02621969); siCdk16, UGAGAUUGGCUUUGGGAAAdTdT; siNop56, CAAUAUGAUCAUCCAGUCCAUdTdT; siPes1, AGGUCUUCCUGUCCAUCAAdTdT.

[^{32}P]Orthophosphate Metabolic Labeling and RNA Extraction—2.5×10^5 U2OS cells were incubated in phosphate-free Dulbecco's modified Eagle's medium, 10% fetal bovine serum for 1 h and then incubated for 1 h in the presence 15 μCi/ml [^{32}P]orthophosphate (pulse). Labeling medium was removed, and cells were further cultivated for 3 h in Dulbecco's modified Eagle's medium, 10% fetal bovine serum (chase). Labeled RNA was extracted using the PeqGOLD total RNA kit (PeqLab). 1 μg of total RNA was separated on a 1% agarose-formaldehyde gel. The gel was dried on a Whatman paper using a regular gel drier (Bio-Rad) connected to a vacuum pump for 4 h at 80 °C. Metabolically labeled RNA was visualized by autoradiography and quantified by phosphorimaging and AIDA software.

4-Thiouridine (4-sU) Tagging—1.5×10^6 2fTGH cells were cultured in the presence of 4-sU (10 μM) and lysed, and total RNA was extracted as described for metabolic *in vivo* labeling. 50 μg of total RNA was incubated with biotin-HPDP (Pierce, 1 mg/ml; 2 μl/μg of RNA) in biotinylation buffer (100 mM Tris, 10 mM EDTA, pH 7.4, 1 μl/μg RNA) for 1.5 h at room temperature. An equal volume of chloroform was added, mixed, and incubated with biotinylated RNA for 3 min. The mixture was separated in pre-spun Phase Trap Gel heavy tubes (5 min, 16,000 rpm). For RNA precipitation and removal of unincorporated biotin-HPDP, a 1/10 volume 5 M NaCl and an equal volume of absolute isopropyl alcohol were added to the aqueous phase and centrifuged (20 min, 16,000 rpm). The pellet was washed in an equal volume of 75% ethanol and centrifuged (10 min, 16,000 rpm). RNA was resuspended in 100 μl of RNase-free H_2O. For separation, untagged and 4-sU-tagged RNA was first heated to 65 °C for 10 min and cooled on ice for 5 min. RNA was incubated with 75 μl of streptavidin-coated magnetic beads (Miltenyi) for 15 min with rotation. The reaction volume was applied to μMACS columns (Miltenyi), placed in an OctoMACS Separator magnetic stand, and equilibrated with 900 μl of μMACS washing buffer (100 mM Tris, 10 mM EDTA, 1 M NaCl, 0.1% Tween 20, pH 7.5). The columns were washed with μMACS washing buffer. 4-sU-biotin-streptavidin-tagged RNA was eluted in 700 μl of RLT lysis buffer (PeqLab) with dithioerythritol (100 mM). 4-sU-tagged RNA was recovered with the PeqGOLD total RNA kit as described above. 4-sU-tagged RNA was separated on a 1.5% agarose gel containing ethidium bromide (37.5 μg/100 ml). Signals of RNA under UV light were quantified by AIDA software.

Northern Blot Hybridization—5 μg of U2OS total RNA was separated on a 1% agarose-formaldehyde gel and blotted on Hybond N+ membranes (Amersham Biosciences). Probes (5′ to 3′) were as follows: 5′ETS (1), CGGAGGCCCAACCTCTCCGACGACAGGTCGCCAGAGGACAGCGTGTCAGC; 5′ETS (2), CGGTACCCCCAAGGCACGCCTCTCAGATCGCTAGAGAAGGCTTTTCTC; ITS-1 (3), AGCGCGGACACCACCCCACAGGCGCCCGGGGGTTCC; ITS-1 (4), TCCCGACGACGCACCGGGAGGAGGCCCTTCCTGGCGCGGCACGTCCCC; ITS-2 (5), CTCTCTTTCCCTCTCCGTCTTCCGGCGGCGGCGCCGCCCTCCCCGTCT; ITS-2 (6), TACGCGCGGGGAGGGCGAGGAGGACGGCGGGGCCTCGGAGGA; 3′ETS (7), AACGCGCACGCCCGCCGGGCCCCCCGCACGCAC; 3′ETS (8), CTCCCAAACCACGCTCCCCGGACCCCGTCCCGGCCCGGAG; 3′ETS (9), ACGGGGAGGAGGCGGGAACCGAAGAAGCGGGGCGGCCGACCGGGGTC; 3′ETS (10), TCGACCCGTGCGGAGGAGCGAGGAGGAAGGACG; 3′ETS (11), GCTAAGTCCGGAGCTCGCGGGCGGCAGCTGGTC; 3′ETS (12), GAGAGGGAGTTCCGCGTGGTCCCAGCTCCACCGCG; 3′ETS (13), CGCGGACGCAAACTCGCGGTGGGGCTGAA; 3′ETS (14), GCGAGAGGGCGAGAGCGACAGAGAGAGAGAG; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CCAGCAGTGAGGGTCTCTCTCTTCCTCTTG; C-MYC, GGAGGCTGCTGGTTTTCCACTACCCGAAAAAAATCCA; U8 (SNORD118), CAAGTCCTGATTACGCAGAGACGTTAATCACGTTTCATGC.

Quantitative Real-time PCR—8×10^4 U2OS cells were double transfected with siRNA (100 nM), and total RNA was extracted as described above. cDNA was produced using 2 μg of total RNA using random hexamer primers (0.2 μg/μl) (Fermentas) and the Superscript reverse transcriptase kit (Invitrogen). Subsequently, cDNA was diluted at 1:20 for quantitative real-time PCR using a LightCycler PCR analysis system (Roche Applied Science) according to the manufacturer's recommendations. The following primers were used for detection of Nop56 mRNA: 5′-AATTCCACAGCATCGTTCG-3′ and 5′-GCGGAGGTCCTCATGAAC-3′. Relative cDNA levels were calculated by the ΔΔCp-method.

Immunoblotting—2.5×10^5 U2OS cells were washed with phosphate-buffered saline and directly lysed in 2× SDS loading buffer (100 mM Tris/HCl, 200 mM dithioerythritol, 4% SDS, 10 mM EDTA, 0.2% bromphenol blue, 20% glycerol). Whole cell lysates were separated by SDS-PAGE and blotted on nitrocellulose membranes (Amersham Biosciences). Immunodetection was performed with the following antibodies: human anti-CATS (29) human anti-Cdk2 (Santa Cruz, sc-163, M2); human anti-Cdk4 (Santa Cruz, sc-260, C22); human anti-Cdk5 (Santa Cruz, sc-173, C8); human anti-Cdk7 (Santa Cruz, sc-529, C19); human anti-Cdk8 (Santa Cruz, sc-13155, D-9); human anti-Cdk9 (Santa Cruz, sc-484, C20); human anti-c-Myc (Roche Applied Science, 11667149001, 9E10); human anti-p53 (Santa Cruz, sc-126, DO-1); human anti-Pes1 (30); human anti-α-tubulin (Sigma, TG199, DM1A); human anti-RNAPII antibodies (3E10, 3E8, Pol-3–3) (31) and mouse anti-Dicer1 (polyclonal). Signals were quantified by AIDA software.

RESULTS

Processing of Ribosomal RNA Requires Cdk9—Previous studies showed that Cdk inhibitors (*e.g.* 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), roscovitine, or flavopiridol) specifically inhibit processing of ribosomal (r)RNA but not the steady-state levels of the 47 S rRNA precursor (22–24) (supplemental Fig. S1). To investigate the requirement of Cdks for rRNA processing, we applied an RNAi approach for the selective knockdown of individual Cdks. U2OS cells were transfected with siRNA targeting mRNAs of Cdk1 to Cdk16 or with control siRNA for mRNAs of luciferase, CATS, and the rRNA processing factor Pes1 (29, 30) (Fig. 1, supplemental Fig. S2). Knockdown efficiency of siRNAs was monitored by Western blot (supplemental Fig. S3). The impact of Cdk knockdown on rRNA synthesis was evaluated after metabolic labeling of rRNA with [^{32}P]orthophosphate and quantitative measurement of mature and immature rRNA forms. The majority of Cdk knockdowns affect neither rRNA production nor rRNA processing rates. Only the knockdown of Cdk5 and Cdk9 displayed a clear phenotype on rRNA synthesis. Cdk5 depletion diminished the levels of the 47 S primary transcript, suggesting that Cdk5 is crucial for rRNA transcription. The knockdown of Cdk9 largely mimicked the effect of Cdk inhibitors on rRNA synthesis. The synthesis of the 47 S rRNA was largely unaffected, whereas a strong reduction was observed for labeling rates of intermediate 32 S and mature 18 S and 28 S rRNAs. The result is consistent with previous reports that Cdk9 has the highest sensitivity to flavopiridol, whereas other Cdks (Cdk2, Cdk5, Cdk7) are explicitly less sensitive (32, 33). Thus, Cdk9 is the most likely candidate for the regulation of 47 S rRNA processing.

Knockdown of the Nucleolar Form of Cdk9 Does Not Impair rRNA Processing—In mammalian cells two forms of Cdk9 with molecular masses of 42 and 55 kDa were described. $Cdk9_{55}$ carries an N-terminal extension of 117 amino acids and is generated from an mRNA that originates from a second promoter located upstream of the start point of transcription used to generate mRNA encoding $Cdk9_{42}$ (34, 35). The relative abundance of $Cdk9_{55}$ and $Cdk9_{42}$ changes in various cell types upon differentiation. Interestingly, epitope-tagged $Cdk9_{42}$ localizes diffusely to the nucleoplasm, whereas $Cdk9_{55}$ accumulates in the nucleolus (36). We asked, therefore, if the knockdown of the nucleolus-specific $Cdk9_{55}$ inhibits processing of rRNA. Transfection of U2OS cells with a siRNA for the first exon of $Cdk9_{55}$ reduced the steady-state levels of the nucleolar form of Cdk9 >70%, whereas expression of $Cdk9_{42}$ remained unaffected (supplemental Fig. S3). Surprisingly, we did not detect an rRNA processing defect after knockdown of $Cdk9_{55}$, indicating that depletion of the large form of Cdk9 apparently does not block rRNA processing and that the nucleoplasmic $Cdk9_{42}$ is sufficient for rRNA processing.

Flavopiridol and Cdk9 Knockdown Induce a Specific 3′ Processing Defect of rRNA—Although 47 S rRNA production remains functional in flavopiridol (FL)-treated cells, its processing into intermediate and mature rRNAs is entirely blocked. The basis for this block is unclear. Therefore, we studied whether 47 S rRNA shows structural abnormalities in FL-treated cells, which might be causative for the processing

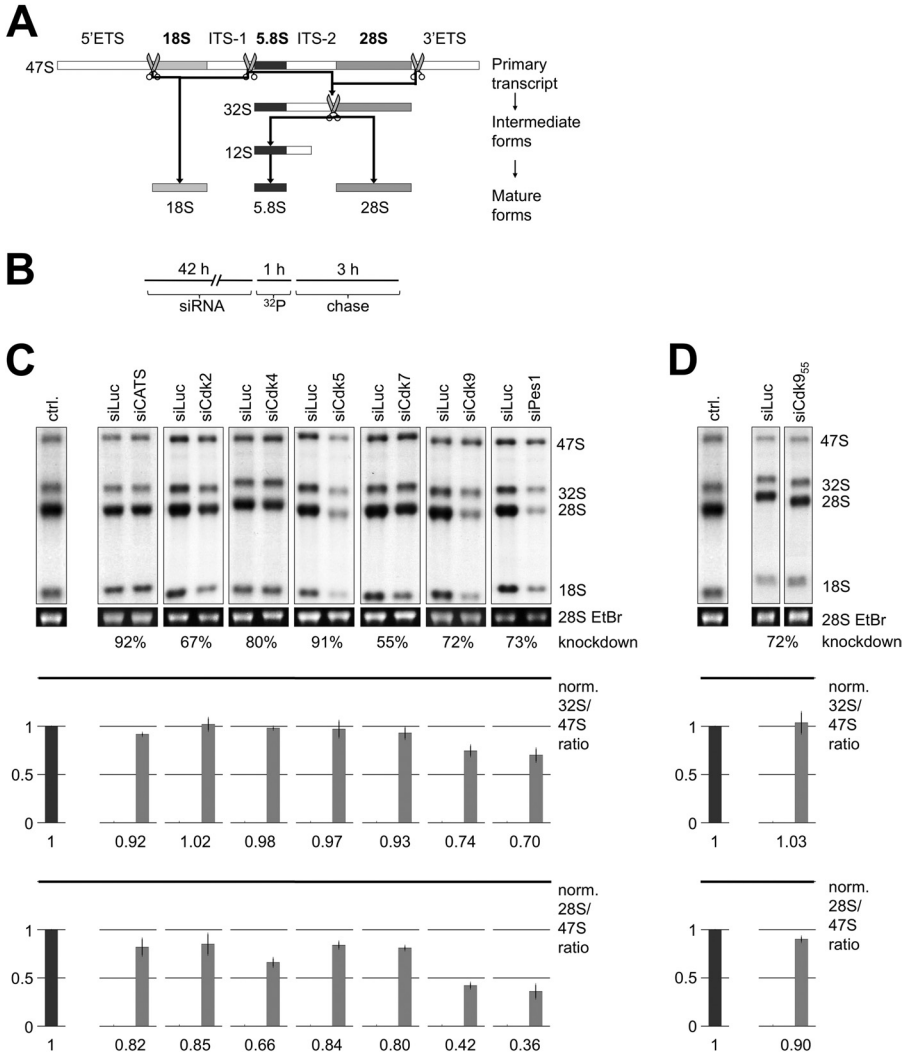

FIGURE 1. **Knockdown of Cdk9 inhibits rRNA processing.** *A*, transcription and processing of ribosomal RNA is shown. The polycistronic transcription unit encodes a 47 S precursor rRNA containing 5′ and 3′ external transcribed spacers (*5′ETS*, *3′ETS*), internal transcribed spacers 1 and 2 (*ITS-1*, *ITS-2*), and 18 S, 5.8 S, and 28 S rRNAs. The 47 S rRNA undergoes a cascade of endonucleolytic cleavages (scissors) and exonucleolytic degradation steps. *B*, ^{32}P *in vivo* metabolic labeling workflow is shown. Cells were pre-exposed to Cdk-specific siRNAs for 42 h, labeled with [^{32}P]orthophosphate for 1 h (*pulse*), and chased for 3 h (*chase*). *C*, knockdown of Cdk9 inhibits 47 S rRNA processing. U2OS cells were seeded and cultured overnight. Cells were transfected twice with siRNA (100 nM) for 6 h at two consecutive days. Analysis of labeled rRNA was performed as described under "Experimental Procedures." Inhibition of rRNA processing was measured by autoradiography. Knockdown efficacy of Cdks is indicated in % (see also supplemental Fig. S3). Labeled rRNAs were quantified using phosphorimaging and AIDA software. rRNA ratios were calculated and normalized to luciferase knockdown (*siLuc*) set as 1. Pes1 and CATS knockdown served as positive and negative controls, respectively. *ctrl.*, untreated cells. *D*, knockdown of Cdk9$_{55}$ does not inhibit rRNA processing. 28 S rRNA serves as the loading control in this and subsequent experiments.

defect. The primary ribosomal transcript is first processed downstream of the 3′ETS. Removal of the 3′ETS and parts of the 5′ leader are subsequent processing steps leading to the formation of the 45 S rRNA. Unfortunately, the 47 S and 45 S rRNAs have similar sizes and cannot be separated by agarose gel electrophoresis. Therefore, the 47 S signal in Northern analysis always represents a mixture of 45 S and 47 S rRNAs if hybridization experiments are performed with 45 S rRNA-specific probes. In the following experiment we studied the sequence composition of the 47 S rRNA with the help of 14 hybridization probes, which are distributed along the 47 S rRNA sequence (probes 1–9) and downstream thereof (probes 10–14) (Fig. 2, *upper panel*). Cells were treated with FL for 6 h, total RNA was isolated, and the structure of rRNA precursors was studied by Northern analysis. The fraction of rRNAs migrating at 47 S was positive for hybridization probes 1–9 in control cells. The observed signals are indicative for 47 S rRNA properly processed/terminated at the termination site (T-site), as signals for probes 10–14 downstream of the T-site were not detected in control cells. The signals obtained for rRNA from FL-treated cells differed in several aspects from control cells. Each of the signals for probes 1–6 was reduced in rRNA derived from FL-treated cells. Because FL-treated cells have a 47 S rRNA processing defect, the observed reduction may be explained by a lack of a properly processed 45 S rRNA in the fraction of 47 S rRNA. This assumption is in line with the observation that signals for probes 7–9, which correspond to the 3′ETS sequence and are absent in the 45 S rRNA, were not reduced in FL-treated cells. Therefore, we conclude that the ratio of 45S/47 S rRNA has significantly shifted toward 47 S

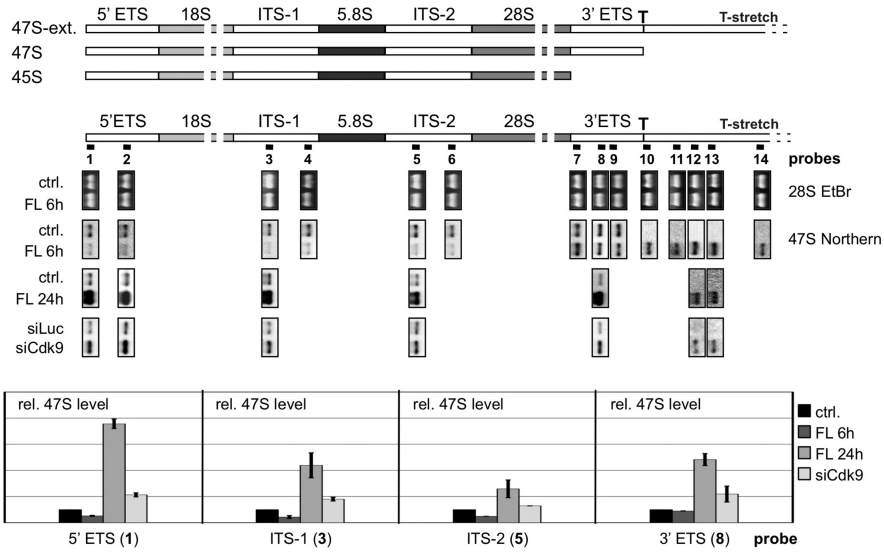

FIGURE 2. **Inhibition or knockdown of Cdk9 causes a processing defect of 47 S rRNA.** U2OS cells were treated with FL (0.8 μM) or siRNA against Cdk9. Total RNA was isolated and analyzed by Northern blot hybridization with probes for 5′ETS, ITS-1, ITS-2, 3′ETS, or sequences downstream of 3′ETS, respectively (probes 1–14). Signals were analyzed by autoradiography and quantified with phosphorimaging. T, termination site. 47 S rRNA signals were plotted relative to signals from control or siLuc transfected cells. Note: only signals hybridized by the same probe can be compared.

rRNA in FL-treated cells. The second remarkable difference for 47 S rRNA in FL-treated cells is the appearance of hybridization signals for probes 10–14. Apparently, FL-treated cells express a new, extended form of 47 S (47 S-extended) rRNA, which is not processed/terminated at the T-site. Because 47 S-extended rRNA displays a size similar to 47 S rRNA, we expect this extension to be limited to sequences between the T-site and the downstream T-stretch. Termination of RNAPI transcription at a T-stretch has recently been described in yeast as a failsafe mechanism if processing at the T-site is inhibited (37). The FL-induced processing defect is further pronounced if cells are treated with FL for 24 h (Fig. 2). We conclude a defect in proper 3′ processing of 47 S rRNA in FL-treated cells downstream of the T-site.

We next asked whether knockdown of Cdk9 was able to induce the same rRNA 3′ processing defect. U2OS cells were transfected with Cdk9-specific siRNA or control luciferase siRNA, and total RNA was isolated. As in FL-treated cells, we observed hybridization signals for probes 12 and 13 downstream of the T-site after knockdown of Cdk9 that were not seen for control cells (Fig. 2). In summary, FL and the knockdown of its cellular target Cdk9 both induce a specific 3′ processing defect of rRNA.

Inhibition of RNAPII Activity Blocks 3′ Processing of rRNA—Cdk9 is the catalytic subunit of the positive elongation factor b (P-TEFb). The kinase activity of positive elongation factor b controls transcript elongation by RNAPII. Serine 2 residues in the C-terminal domain (CTD) of the large subunit have been reported as a major target of Cdk9. We detected a strong and rapid reduction of CTD serine 2 but not serine 5 phosphorylation upon flavopiridol treatment, indicating that elongation of RNAPII is impaired (supplemental Fig. S4). Therefore, we wondered if RNAPII transcription is crucial for 47 S rRNA processing. The activity of RNAPII can selectively be inhibited by α-amanitin, a toxin of the mushroom *Amanita phalloides* with negligible impact on RNAPI and RNAPIII transcription. U2OS cells were treated with α-amanitin to study the function of RNAPII in 47 S rRNA processing using ^{32}P-labeling experiments. As expected, the global transcriptional inhibitor ActD blocked the activity of all RNA polymerases, and signals for the 47 S rRNA and other rRNA forms were not detectable (Fig. 3*A*, *lane 2*). Notably, α-amanitin and FL did not significantly affect the levels of the 47 S rRNA but strongly reduced the levels of premature 32 S and mature 28 S and 18 S rRNAs. The α-amanitin-induced rRNA processing defect was not observed if cells expressing an α-amanitin resistant RNAPII were treated with α-amanitin (supplemental Fig. S5). Importantly, α-amanitin induced the same processing defect in the 3′ region of 47 S rRNA (Fig. 3*B*), as has been observed for FL treatment and knockdown of Cdk9. These results further corroborate the notion that the FL-induced processing defect in the 3′ region of 47 S rRNA is caused by an RNAPII transcription defect. But which of the diverse RNAs transcribed by RNAPII are the most crucial for 47 S rRNA 3′ processing?

Impact of RNAPII Transcripts on rRNA 3′ Processing—Therefore we asked which subclass of RNAPII-specific transcripts might be of particular importance for rRNA 3′ processing and becomes limiting when RNAPII transcription is inhibited (supplemental Fig. S6*A*). We first tested the class of coding RNAs (mRNAs) in an indirect assay. If an affected mRNA for a highly labile protein is required for 47 S rRNA 3′ processing, inhibition of protein synthesis should deplete such a factor rapidly from cells and cause an rRNA processing defect. To test this hypothesis we treated U2OS cells with two translational inhibitors, cycloheximide and homoharringtonine, and studied rRNA processing in ^{32}P-labeling experiments. Both cycloheximide and homoharringtonine displayed a strong rRNA processing defect, yet the phenotype of this defect differs markedly from the phenotype observed in cells treated with FL. Although the ratio of 32 S/47 S is strongly reduced in FL-treated cells, this ratio was unaffected in cells treated with cycloheximide or homoharringtonine (supplemental Fig. S6*B*). Effective inhibi-

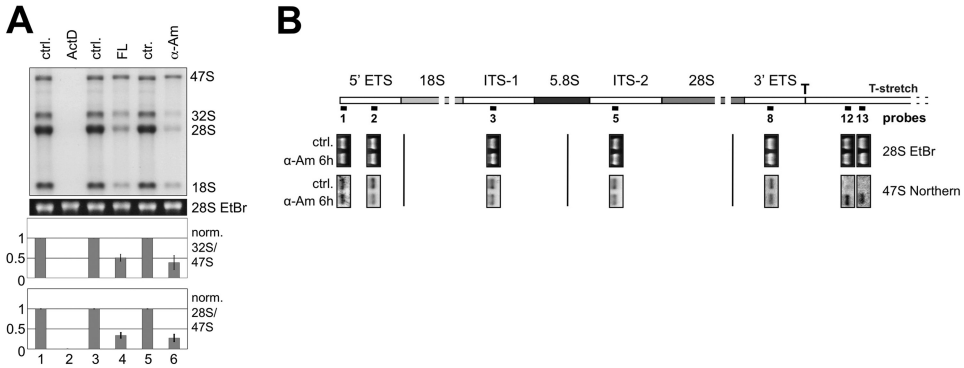

FIGURE 3. **Processing of 47 S rRNA is dependent on RNAPII transcription.** *A*, shown is inhibition of 47 S rRNA processing in U2OS cells after treatment with α-amanitin (α-*Am*). Cells were treated with ActD (5 μM), FL (0.8 μM), or α-amanitin (10 μg/ml) for 6 h followed by labeling with ^{32}P. rRNA ratios were calculated and normalized to rRNA ratios derived from control cells. *B*, inhibition of RNAPII transcription induces extended forms of 47 S rRNA. Cells were treated with α-amanitin (10 μg/ml). Total RNA was isolated and analyzed by Northern blot hybridization with the indicated probes.

tion of translation was monitored for the short-lived c-Myc protein (supplemental Fig. S6*C*). Also, translational inhibitors did not induce the extended form of 47 S rRNA observed in FL-treated cells (supplemental Fig. S6*D*). We conclude that translational inhibitors display a strong inhibitory effect on rRNA processing, but the phenotype is different to that observed in cells after knockdown of Cdk9 or treatment with FL and α-amanitin.

Dicer Knock-out Induces a Severe Defect in Processing of 18 S and 28 S rRNA—Small non-coding RNAs, including microRNAs, siRNA, snRNA, and others regulate cellular processes at multiple levels. They are transcribed by RNAPII and subsequently processed into premature forms by the Drosha complex. After export into the cytoplasm, small RNAs undergo final maturation steps by Dicer in the RNA-induced silencing complex (RISC). We next asked, if 3′ processing of 47 S rRNA might require the function of small RNAs and if rRNA processing is thus affected in Dicer knock-out cells. Mouse embryonic fibroblasts conditional for Dicer1 knock-out were retrovirally infected with a vector expressing Cre recombinase, and individual clones were selected. Depletion by Cre recombinase reduced endogenous Dicer levels >90% (Fig. 4*A*) and reduced the proliferation rate of cells >2-fold (data not shown). Next, rRNA synthesis rates were compared in Dicer-deficient and control cells. Although the labeling index for 47 S and 32 S rRNAs was almost unchanged in knock-out and control cells, the labeling index for 18 S and 28 S rRNA was strongly reduced in knock-out cells (Fig. 4, *B* and *C*). The impaired maturation of 18 S and 28 S rRNAs is in line with the observed proliferation defect of Dicer knock-out cells. However, Dicer knock-out cells were apparently defective neither in the synthesis of 47 S rRNA nor in early processing steps leading to the formation of 32 S rRNA. The ratio of 32 S/47 S rRNAs, which is strongly reduced in FL-treated cells, remains unchanged in knock-out cells. We conclude that knock-out of Dicer has a severe impact on rRNA processing steps and the formation of 18 S and 28 S rRNA but not on generation of 47 S rRNA and its proper 3′ processing and formation of 32 S rRNA. Thus, small RNAs produced in a Dicer-dependent manner are probably not crucial for 3′ processing of 47 S rRNA.

Reduced U8 snoRNA Levels Observed after Cdk9 Knockdown—The human genome encodes ~400 annotated snoRNAs

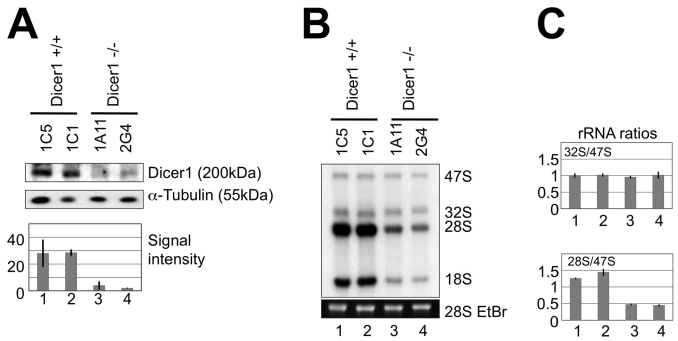

FIGURE 4. **Dicer1 knock-out does not inhibit 47 S rRNA processing.** *A*, mouse embryonic fibroblasts with floxed Dicer1 alleles were incubated with Cre recombinase to create a Dicer1 knock-out cell line. Two knock-out (*1A11*, *2G4*) and two wild-type cell clones (*1C5*, *1C1*) were single cell-cloned, and whole cell extracts were prepared. Dicer1 knock-out was monitored by Western blot analysis with Dicer-specific antibodies. *B*, Dicer1 knockout inhibits maturation of 28 S and 18 S rRNA but not levels of 47 S and 32 S rRNA. *C*, 32 S rRNA levels are normal in Dicer1 knock-out cells.

(snoRNABase). Mammalian snoRNAs are almost exclusively transcribed by RNAPII. The majority of mammalian snoRNAs map to intron sequences of pre-mRNAs, whereas some snoRNAs are transcribed from their own promoters. snoRNAs regulate various modification and processing steps of rRNA, and a subset of them are essential in yeast and mammals. Processing of snoRNAs occurs in the nucleus largely independently of Dicer activity. We, therefore, asked whether knockdown or inhibition of Cdk9 could affect the steady-state levels of snoRNAs. For our analysis we focused on mammalian specific U8 snoRNA (SNORD118), which plays a crucial role in the endonucleolytic cleavage of the primary ribosomal transcript and has been shown to be essential for 3′ processing of rRNA (38–40). We first tested if steady-state levels of U8 snoRNA are affected in ActD- and FL-treated cells. Treatment of cells with both drugs for 6 h had no impact on the levels of GAPDH mRNA, whereas the levels of U8 snoRNA and short-lived c-*myc* mRNA were strongly reduced (Fig. 5*A*). This suggests that steady-state levels of U8 snoRNA are highly sensitive to inhibition of RNAPII transcription.

Next we asked whether the decrease in U8 snoRNA levels is also seen after knockdown of Cdk9 (Fig. 5*B*). U2OS cells were treated on two consecutive days with Cdk9-specific siRNA, and U8 snoRNA levels were analyzed after 24 h. Depletion of Cdk9

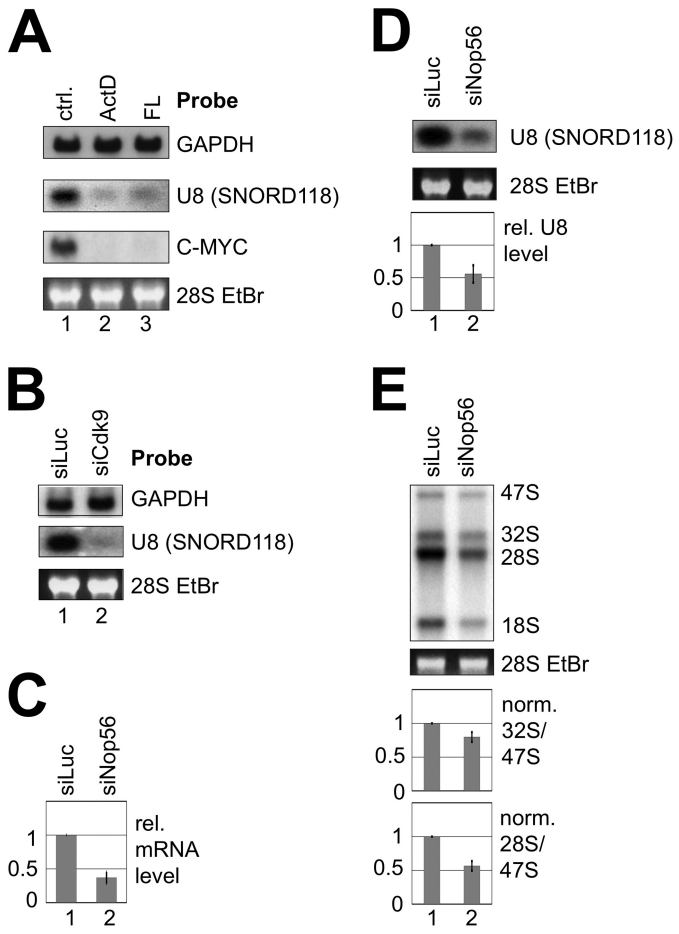

FIGURE 5. **Down-regulation of U8 snoRNA after Cdk9 inactivation.** *A* and *B*, shown are U8 snoRNA levels in U2OS cells after treatment with ActD (5 μM) or FL (0.8 μM) for 6 h (*A*) or after knockdown of Cdk9 (*B*). Cells were transfected with Cdk9-specific siRNA (see Fig. 1). Northern blot analysis of RNA was performed with probes recognizing U8 snoRNA (*SNORD118*), GAPDH, or c-*myc* RNA. *C*, Nop56 mRNA depletion after knockdown is shown. Quantitative real time PCR with specific primers was used to monitor knockdown efficacy. Nop56-specific siRNA sequence was used as described elsewhere (61). *D*, Nop56 knockdown reduces U8 (*SNORD118*) snoRNA levels. *E*, Nop56 knockdown blocks 47 S rRNA processing.

strongly reduced U8 snoRNA, as did treatment of cells with ActD and FL.

For proof of concept, rRNA processing efficacy was assessed in the absence of U8 snoRNA. To efficiently deplete U8 snoRNA, we performed knockdown of box H/ACA snoRNP protein Nop56. Nop56 is required for snoRNP assembly and stabilization of box H/ACA snoRNAs including U8 (41–43). Importantly, transfection with Nop56-specific siRNA efficiently diminished Nop56 mRNA levels (Fig. 5*C*), significantly reduced U8 snoRNA levels (Fig. 5*D*), and markedly impaired processing of 47 S rRNA (Fig. 5*E*). Our data suggest that reduced U8 snoRNA levels may contribute to the 3′ processing defect.

Inhibition of rRNA Processing Feeds Back on rDNA Transcription—The synthesis rate of 47 S rRNA is only slightly affected by knockdown of Cdk9 or after treatment of cells with flavopiridol, whereas processing of the primary transcript is entirely abolished. But why does inhibition of rRNA processing not lead to accumulation of the 47 S rRNA precursor? We approached this question with the help of different labeling experiments.

We performed an rRNA labeling experiment with 4-sU. 4-sU is a photoreactive ribonucleoside analog that is rapidly taken up by living cells and incorporated into RNA (44). After RNA isolation the incorporated 4-sU ribonucleoside can be covalently modified by biotinylation, which allows the subsequent purification of the newly synthesized RNA with streptavidin-coated beads (45). The fraction of 4sU-labeled RNA was separated in a denaturing agarose gel and stained with ethidium bromide. A faint band corresponding to the 47 S rRNA became visible in control and FL-treated cells 45 min after labeling, increased in intensity after 60 min, and reached steady-state levels after 120–180 min in control and FL-treated cells (Fig. 6*A*). A 32 S rRNA processing intermediate became visible in control cells after 60 min, before the appearance of the mature 18 S and 28 S rRNAs after 2 h and 3 h. In control cells >90% of 4-sU-labeled rRNA was processed to 32 S, 28 S, and 18 S rRNA after 3 h. In contrast, even though the 47 S rRNA precursor appeared with similar kinetics in FL-treated cells, its processing into immature 32 S rRNA or mature 28 S rRNA and 18 S rRNA did not occur. From this result we conclude that the synthesis and/or turnover rates of 47 S rRNA in FL-treated cells could be significantly reduced compared with control cells.

To confirm this assumption, a second labeling experiment was performed with [^{32}P]orthophosphate. We pulse-labeled U2OS cells for 1 h followed by a 0.5-, 1-, 2-, or 3-h chase (Fig. 6*B*). Control cells already showed a rapid incorporation of the ^{32}P label in 47 S rRNA after a 30-min chase, with a maximal incorporation of label after 2 h and a decline after 3 h. In contrast, incorporation of label in FL-treated cells was low after 30 min but steadily increased and reached a level similar to control cells after 3 h. The diminished incorporation of ^{32}P label in 47 S rRNA is consistent with a reduced transcription rate of RNAPI in FL-treated cells. We conclude that the FL-induced rRNA processing defect negatively feeds back on the transcription rate of rRNA and that this feedback may explain at least partly the missing accumulation of the 47 S precursor. To what extent degradation of the 47 S-extended rRNA occurs is currently unclear.

DISCUSSION

Cdk9-mediated RNAPII Transcription Is Required for 47 S rRNA Processing—Processing of rRNA is highly sensitive to Cdk inhibition by FL. Here we show that knockdown of Cdk9 can mimic the inhibitory effect of FL on rRNA processing and is in line with the high sensitivity of Cdk9 toward FL (32, 33). Two forms of Cdk9 are expressed in cells. Combined knockdown of nucleolar Cdk9$_{55}$ and nucleoplasmatic Cdk9$_{42}$ inhibited rRNA processing, whereas knockdown of Cdk9$_{55}$ alone was not sufficient. Cdk9$_{42}$ is the catalytic subunit of the positive elongation factor b that functions in the control of RNAPII transcription. Cdk9 is a major kinase for phosphorylation of the C-terminal domain of RNAPII and for phosphorylation of DRB sensitivity-inducing factor (DSIF) and negative elongation factor (NELF), which regulate RNAPII elongation. We, therefore, wondered if the cellular target affected by Cdk9 knockdown and responsible for the rRNA processing defect could be RNAPII transcription.

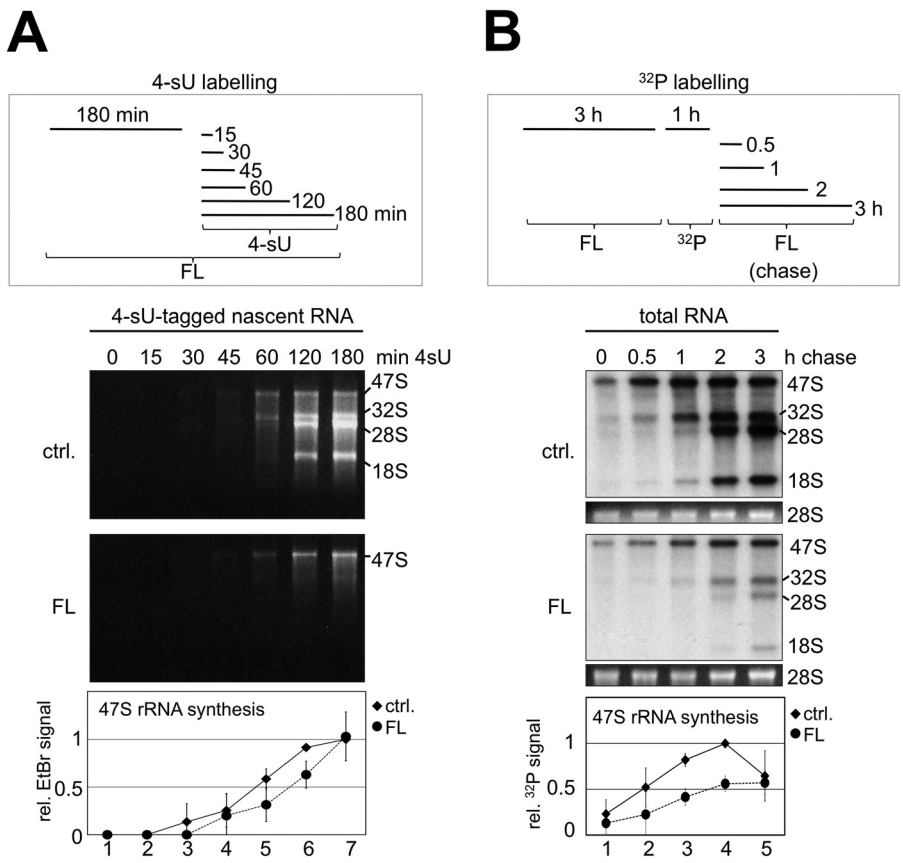

FIGURE 6. **Inhibition of 47 S rRNA processing feeds back to RNAPI transcription.** *A*, shown is analysis of 47 S rRNA transcription measured by 4-sU labeling after flavopiridol. Cells were treated with FL (0.8 μM) and 4-sU (10 μM) as indicated. 4-sU-tagged RNA was separated from total RNA by biotin-streptavidin, separated by gel electrophoresis, and stained with EtBr (see "Experimental Procedures"). EtBr signals for 47 S rRNA were plotted. Signal intensity in control cells after 180 min (*lane 7*) were used for normalization. *B*, flavopiridol diminishes 47 S rRNA transcription. U2OS cells were treated with FL (0.8 μM) for 6 h and labeled with [^{32}P]orthophosphate as indicated. Relative ^{32}P signals for 47 S rRNA were plotted over time. The 47 S rRNA signal intensity for control cells after 2 h (*lane 4*) were used for normalization.

RNAPII transcription is highly sensitive to α-amanitin, whereas transcription by RNAPI and RNAPIII is resistant to low concentrations of this drug. In fact, inhibition of RNAPII transcription induced the same defect in rRNA processing as observed for the knockdown of Cdk9 before. Thus, the activity of RNAPII is directly linked to processing of rRNA.

Inhibition of RNAPII Activity Causes Defective rRNA 3′ Processing—Inhibition of RNAPII activity by Cdk9 inactivation or α-amanitin treatment induces a global rRNA processing defect and the occurrence of an extended 47 S rRNA, which contains sequences downstream of the T-site. This phenotype could be caused either by a defective termination of RNAPI at terminator sequence 1 (T1) or by an rRNA processing defect. In the first case RNAPI would terminate immediately downstream of T1 in the presence of Cdk9 activity without transcribing sequences further downstream. Inhibition of RNAPII activity prevents standard termination, and default termination probably occurs at the first poly-T-stretch further downstream. Alternatively, RNAPI transcription is not affected, but processing of rRNA at the T-site is impaired. Several pieces of evidence argue for the second scenario. First of all, the presence of additional sequences in 47 S rRNA 3′ of the T-site is accompanied with a severe general processing defect of 47 S rRNA. Not only sequences 3′ of the T-site remain associated with the 47 S precursor but also the 3′ETS, which usually is rapidly removed from 47 S rRNA. We have further no evidence for removal of 5′ETS leader sequences from the primary transcript, nor do we see processing of ITS-1 and ITS-2 sequences. Instead, we observe a constant accumulation of unprocessed 47 S rRNA with continuous FL treatment. Importantly, in the absence of Cdk9 inhibitors no signals downstream of the T-site and specific for an extended 47 S rRNA were detected, ruling out the possibility that significant read-through transcription is present in control cells. Therefore, we propose a general rRNA processing block of 3′ sequences and processing steps downstream thereof. However, we can not exclude a termination defect of RNAPI as primary defect. Retention of the nascent transcript to RNAPI might reduce its accessibility to processing factors, prevent its degradation, and contribute to its stabilization.

Data from yeast suggest that correct splicing and processing of pre-mRNA precursors may be critical for 47 S rRNA processing, particularly of ribosomal protein encoding mRNAs (46–48). In addition, many microRNAs and snoRNAs are transcribed by RNAPII as intronic sequences of pre-mRNA transcripts, and pre-mRNA splicing is a prerequisite for the biogenesis of many human microRNAs and snoRNAs (49). Based on our data, reduced production of snoRNAs such as U8 and probably others may be causative for inhibition of 47 S rRNA processing. U8 is present in higher eukaryotes but absent in yeast.

U8 is the only known snoRNA required for proper 3′ processing of 3′ETS sequences and forms a stable hybrid with 3′ sequences of 47 S rRNA (38). Depletion of U8 and interference with its displacement from the primary ribosomal transcript by oligonucleotides were shown to impair 3′ processing of 28 S rRNA and induce the accumulation of pre-rRNA with unprocessed 3′ETS sequences (39, 50). In line with that, we find U8 snoRNA levels strongly reduced upon inhibition of Cdk9 activity. Recent studies of Pestov and co-workers (40) showed that removal of U8 snoRNA by the helicase Ddx51 is a crucial step for the successful processing of 47 S rRNA 3′ sequences. U8 snoRNA remains associated with unprocessed rRNA after knockdown of Ddx51. Thus, rapid loss of U8 snoRNA after knockdown of Cdk9 may be one possible explanation for the observed rRNA processing defect and stabilization of the entire 47 S rRNA precursor. The inhibition of 47 S rRNA processing also reduces the synthesis rate of 47 S rRNA (see below).

Does CTD Phosphorylation Link RNAPII Transcription to rRNA 3′ Processing?—Cdk9 is a major kinase of RNAPII and phosphorylates serine 2 and serine 5 residues of the CTD heptad-repeat sequences. Phosphorylation of the CTD is a prerequisite for the recruitment of various cellular factors to the transcription machinery, which enables transcription of chromatin templates but is also essential for correct capping, splicing, termination, and export of mRNAs (51–54). Recent evidence suggests that specific CTD modifications are required for the control of mammalian snoRNA synthesis. RNAPII CTD is methylated at a single arginine residue (Arg-1810) by the arginine methyltransferase 1 (CARM1). Mutation of Arg-1810 results in the overexpression of a variety of snRNAs and snoRNAs, an effect that is also observed in CARM1$^{-/-}$ cells (17). The result suggests that the production rate of snoRNAs is well controlled and that not each snoRNA sequence is subject to maturation after transcription. Evidence for a tightly regulated production of snoRNAs comes also from recently published ultrashort and progressive 4-sU labeling experiments, which describe key characteristics of RNA processing at nucleotide resolution. The authors show that processing of most, but not all snoRNA-containing introns is remarkably inefficient with the majority of introns being spliced and degraded rather than processed into mature snoRNAs (18).

But what could be the specific role of CTD phosphorylation by Cdk9 for snoRNA production. We cannot rule out a general transcription block of certain genes after knockdown of Cdk9, which probably also has a severe impact on splicing and maturation of pre-mRNAs. Several reports have described a specific processing defect of small RNAs upon inhibition of Cdk9 activity. For example, transcription of U2 snRNA and histone H2b genes does not depend on Cdk9; however, the activity of the kinase is required for recognition of the 3′ box of U2 snRNA by processing factors (55) and for prevention of the artificial 3′-polyadenylation of H2b mRNAs (56). Likewise, flavopiridol has been reported to reduce the accumulation of hsp70 mRNAs in *Drosophila* cells without affecting the density of RNAPII on the gene. Instead, a major defect at the level of 3′ end processing was observed (57). U8 snoRNA is embedded in the 3′-UTR of TMEM107 mRNA (58). Thus, 3′ processing of U8 pre-snoRNA might also be inhibited in analogy to hsp70 pre-mRNA. Given that the synthesis and maturation of RNAPII-dependent pre-snoRNAs requires functional splicing and 3′ processing of pre-mRNA, it is tempting to speculate that Cdk9 regulates maturation of U8 pre-snoRNA by posttranscriptional mechanisms, most likely by regulation of the CTD modification pattern. The importance of RNAPII CTD to facilitate pre-snoRNA maturation is best described in yeast but remains to be established in mammalian cells.

Defective 3′-rRNA Processing Negatively Feeds Back on RNAPI Transcription—The rRNA 3′ processing block observed in cells after treatment with FL or knockdown of Cdk9 did not result in a pronounced accumulation of 47 S rRNA in metabolic labeling experiments. The reason for the absent accumulation was unclear, because 47 S rRNA was labeled after a 3-h chase to a similar extent in treated and control cells (Figs. 1 and 3). Two labeling kinetics, with ^{32}P and 4-sU, helped to solve this enigma. In the ^{32}P labeling kinetics, the label was incorporated in 47 S rRNA more slowly in FL-treated cells and reached a maximum after 3 h. Maximum label in control cells was reached already between 1 and 2 h and thereafter declined due to the onset of 47 S RNA processing. From these results we conclude that the pool of 47 S rRNA in FL-treated cells is filled more slowly as in control cells and in addition has a lower turnover rate due to the 3′ processing defect. But how can a 3′ processing defect in 47 S rRNA feed back on RNAPI transcription? An answer may give a model for the topology of active ribosomal genes, which was recently proposed as the result of a collaborative work of several laboratories (59, 60). By using a combination of chromatin immunoprecipitation and chromosome conformation capture techniques, the authors showed that the promoter upstream region and the terminator R3 of active rRNA genes are held together spatially throughout the cell cycle, forming a stable core around which the transcribed region is organized. In this core-helix model the interaction of a so-called SL1 complex with the promoter and the terminator region is of central importance and keeps these two elements connected. If RNAPI fails to terminate at the end of the gene due to a 3′ processing block, this failure may have direct impact on initiation of the next RNAPI molecule at the adjacent promoter, and as a consequence the transcription rate is slowed down.

Taken together, we conclude that inhibition of Cdk9 activity blocks 47 S rRNA processing. Defective 3′ processing negatively feeds back on RNAPI transcription and stabilizes a 3′-extended 47 S primary transcript. Linkage of RNAPII transcription and rRNA processing by facilitating the production of critical, RNAPII-dependent snoRNAs such as U8 is a major contribution of Cdk9 to ribosome biogenesis.

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