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Balanced Production of Ribosome Components Is Required for Proper G_1/S Transition in *Saccharomyces cerevisiae*[*] [S]

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Fernando Gómez-Herreros[1], **Olga Rodríguez-Galán, Macarena Morillo-Huesca**[2], **Douglas Maya**[3], **María Arista-Romero, Jesús de la Cruz, Sebastián Chávez, and Mari Cruz Muñoz-Centeno**[4]

From the Departamento de Genética, Universidad de Sevilla and Instituto de Biomedicina de Sevilla, Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, 41012 Sevilla, Spain

Background: Ribosome biogenesis is tightly coupled to cell growth.
Results: The specific inhibition of RNA polymerases I or III leads to a G_1/S delay in yeast, which occurs through the accumulation of free ribosomal proteins.
Conclusion: The correct stoichiometry of ribosomal RNAs and proteins is necessary for accurate cell cycle progression.
Significance: From yeast to human, there are protective mechanisms that ensure cell cycle delay when ribosome biogenesis is impaired.

Cell cycle regulation is a very accurate process that ensures cell viability and the genomic integrity of daughter cells. A fundamental part of this regulation consists in the arrest of the cycle at particular points to ensure the completion of a previous event, to repair cellular damage, or to avoid progression in potentially risky situations. In this work, we demonstrate that a reduction in nucleotide levels or the depletion of RNA polymerase I or III subunits generates a cell cycle delay at the G_1/S transition in *Saccharomyces cerevisiae*. This delay is concomitant with an imbalance between ribosomal RNAs and proteins which, among others, provokes an accumulation of free ribosomal protein L5. Consistently with a direct impact of free L5 on the G_1/S transition, *rrs1* mutants, which weaken the assembly of L5 and L11 on pre-60S ribosomal particles, enhance both the G_1/S delay and the accumulation of free ribosomal protein L5. We propose the existence of a surveillance mechanism that couples the balanced production of yeast ribosomal components and cell cycle progression through the accumulation of free ribosomal proteins. This regulatory pathway resembles the p53-dependent nucleolar-stress checkpoint response described in human cells, which indicates that this is a general control strategy extended throughout eukaryotes.

Eukaryotic cells tightly regulate cell cycle transitions to ensure viability and the correct transmission of genetic information. A fundamental element of cell cycle regulation consists of arrests at particular steps to guarantee the completion of a previous cell cycle event, to repair cellular damage, or to resolve a challenge situation (1). Failures in these processes reduce cell survival and, in higher metazoans, lead to cancer and other diseases (2–4).

Ribosome biogenesis is a highly resource-consuming process and therefore involves the tight regulation and balanced synthesis of all its constituents (5–7). This complicated pathway requires the coordinated assembly of ribosomal RNAs (rRNAs), synthesized by RNA polymerases I and III (RNA pol I and III),[5] and ribosomal proteins (r-proteins), whose mRNAs are transcribed by RNA polymerase II (RNA pol II).

6-Azauracil (6AU) and mycophenolic acid (MPA) are well known nucleotide-depleting (NTP-depleting) drugs that interfere with transcription elongation *in vivo* by strongly inhibiting inosine monophosphate (IMP) dehydrogenase, a rate-limiting enzyme in the *novo* synthesis of guanine nucleotides (8, 9). Mycophenolate mofetil, a prodrug of MPA, is widely used as an immunosuppressive agent because it can effectively induce G_1 arrest in lymphocytes (10, 11). Moreover, type 1 IMP dehydrogenase has been identified as a MPA target in human cells (12). *Saccharomyces cerevisiae* contains four closely paralogous genes, *IMD1*, *IMD2* (formerly known as *PUR5*), *IMD3*, and *IMD4*, which encode IMP dehydrogenases. Interestingly, only *IMD2* provides resistance to NTP-depleting drugs. Two mechanisms are responsible for this resistance: (i) *IMD2* is normally repressed under guanine replete conditions, but is strongly induced when guanine nucleotides are low (8, 13); (ii) *Imd2* activity *in vivo* is intrinsically more resistant to MPA than that of *Imd3* or *Imd4* (14, 15).

In human cells, MPA treatment results in both a drastic reduction of pre-rRNA synthesis and the disruption of the

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[1] Present address: Genome Damage and Stability Centre, University of Sussex, Science Park Rd., Falmer, Brighton BN1 9RQ, United Kingdom.
[2] Recipient of a Formación de Personal Investigador (FPI) fellowship. Present address: Centro Andaluz de Biología Molecular y Medicina Regenerativa, CABIMER, Avda. Américo Vespucio s/n, Parque Científico y Tecnológico Cartuja 41092 Sevilla, Spain.
[3] Recipient of a fellowship from the Andalusian Government. Present address: Centre de Recherche en Cancérologie de Marseille (CRCM), INSERM UMR 1068, CNRS UMR 7258, Marseille, France.
[4] To whom correspondence should be addressed: Departamento de Genética, Facultad de Biología, Avda. Reina Mercedes 6, 41012 Sevilla, Spain. Tel.: 34-954550920; Fax: 34-954557104; E-mail: mcmunoz@us.es.

[5] The abbreviations used are: pol I, II, and III, polymerase I, II, and III, respectively; 6AU, 6-azauracil; MPA, mycophenolic acid; NTP, nucleotides triphosphate; r-protein, ribosomal protein; 1C, unreplicated DNA content value.

nucleolus, causing p53 activation, and consequently G_1 arrest through the inhibition of MDM2 by free r-proteins (16–22). R-proteins L5 and L11 have been reported to bind Mdm2, thus inducing p53 stabilization by inhibiting the Mdm2 E3 ubiquitin ligase function (23–27). Other r-proteins, such as S7, S27a, and L23, have also been described to provoke the induction of p53 and subsequent G_1 arrest (26, 28–30). However, recent evidence indicates that L5 and L11 are the r-proteins most directly required for p53 induction (31, 32).

In this work, we used the eukaryote *S. cerevisiae* model to investigate the effects of NTP-depleting drugs on ribosome biogenesis and their consequences on cell cycle progression. We show that these drugs also induce nucleolar stress and G_1 delay in yeast through the accumulation of free r-proteins. Yeast r-proteins L11 and L5 (orthologues of human L11 and L5, respectively) appear to play an important role in this phenomenon. Therefore, we postulate that the surveillance mechanism that links ribosome integrity to cell cycle control via the induction of p53 in human cells may have evolved from a process already present in lower eukaryotes.

EXPERIMENTAL PROCEDURES

Strains, Media, and Culture Reagents—All of the yeast strains used in this study are derivatives of the W303 and BY backgrounds. Genotypes are available in supplemental Table 1. For the experiments requiring NTP-depleting drug treatments, strains were first transformed with a centromeric plasmid that harbors the *URA3* gene and then grown in a complete minimal medium lacking uracil (SC-URA). MPA and 6AU (Sigma) were dissolved directly in growth media to the indicated concentrations. Doxycycline (Sigma) was dissolved in distilled water in a concentrated stock and was added at a final concentration of 5 μg/ml. To test growth, yeast cultures were diluted to the same OD_{600} and serial dilutions (1:10) were spotted onto plates. At least three independent experiments were carried out in all cases. Standard procedures were followed for synchronization at START and flow cytometry (33, 34).

Sucrose Gradient Centrifugation—Polysome and r-subunit preparations and analyses were performed as described previously (35) using an ISCO UA-6 system equipped to continuously monitor A_{254}.

Protein Extractions and Western Blotting Analyses—Total yeast protein extracts were prepared and analyzed by Western blotting according to standard procedures (36). The following primary antibodies were used: mouse monoclonal anti-Myc (Santa Cruz Biotechnology), rabbit polyclonal anti-L1 (a gift from F. Lacroute) (37), rabbit polyclonal anti-L5 (a gift from S. R. Valentini) (38), and rabbit polyclonal anti-S8 (a gift from G. Dieci) (39).

RNA Extractions and Northern Hybridization—RNA extraction and Northern hybridization analyses were carried out according to standard procedures (40, 41). In all of the experiments, RNA was extracted from exponentially growing cells. Equal amounts of total RNA were loaded onto gels. The oligonucleotides used for the hybridizations have been described previously (42). A phosphorimaging analysis was performed in an FLA-5100 imaging system (Fujifilm) at the Biology Service (CITIUS) of the University of Seville.

RESULTS

NTP-depleting Drugs Promote Transient Cell Cycle Delay at G_1—MPA and 6AU inhibit transcription elongation *in vivo* (8) and have been previously described to activate p53 and to induce p53-dependent G_1 arrest in certain human cell lines. To test whether MPA-induced G_1 arrest is a generally shared feature in eukaryotes, we studied the effect of NTP-depleting drugs on cell cycle progression in the model organism *S. cerevisiae*. First, we treated an asynchronously growing wild-type yeast culture with a sublethal dose of 6AU. As shown in Fig. 1*A*, this treatment led to delay in G_1/S transition, and most cells became unbudded with a 1C DNA content. This G_1 delay was clearly visible 2 h after adding the drug, and by 6 h after this addition, approximately 60% of cells remained unbudded with the 1C DNA content (Fig. 1*A*). We also tested whether 6AU promotes cell enlargement during G_1 delay. Both the visual observation of cells and the forward scattering measurements in a flow cytometer showed a clear increase in cell size in the presence of 6AU (Fig. 1*B*). The magnitude of this increase was comparable with that which *cdc* mutants undergo when shifted to restrictive conditions. Similar results were obtained when cells were treated with MPA (Fig. 1*A*; data not shown). These results suggest that the G_1 delay caused by these drugs must be mediated by specific mechanisms and cannot be simply explained as a passive consequence of a growth defect due to a general transcription impairment.

To further characterize the G_1 delay caused by NTP-depleting drugs in yeast, a classical order-of-function analysis was performed (43). Wild-type cells were arrested in START with α-factor. When cells were released from the pheromone, a significant delay took place in the presence of 6AU, indicating that the delay in G_1 produced by nucleotide depletion occurred in or after the α-factor-sensitive step (START) (Fig. 1*C*). In the reciprocal experiment (Fig. 1*D*), an accumulation of wild-type unbudded G_1 cells was first generated by treatment with 6AU. Further incubation of these cells in the absence of 6AU, but in the presence of α-factor, did not increase the proportion of budded cells, indicating that those cells arrested by 6AU were blocked in or before the α-factor-sensitive step. These reciprocal-shift experiments indicate that 6AU induces an accumulation of cells at START.

The Transient Delay in G_1 Correlates with the Reduction of Cellular NTP Pools—The G_1 delay described above was a transient phenomenon as wild-type cells started recovering normal cell cycle profiles at 6 h after drug addition (Fig. 1*A*). The reduction of intracellular NTP pools after 6AU or MPA treatment has been reported to also be transient as wild-type cells sense intracellular NTP depletion and compensate for it by inducing the transcription of *IMD2*. *IMD2* transcript levels dramatically increase in the presence of NTP-depleting drugs during a period in which the levels of GTP and total RNA synthesis are low. The most notable induction has been observed 2 h after the drugs challenge, and at this level, it declines to the base line by 10 h after treatment (8, 13).

We reasoned that if the transient G_1 delay correlates with the reduction in the cellular NTP pools, we could predict that the inactivation of *IMD2* would lead to a more enduring cell cycle

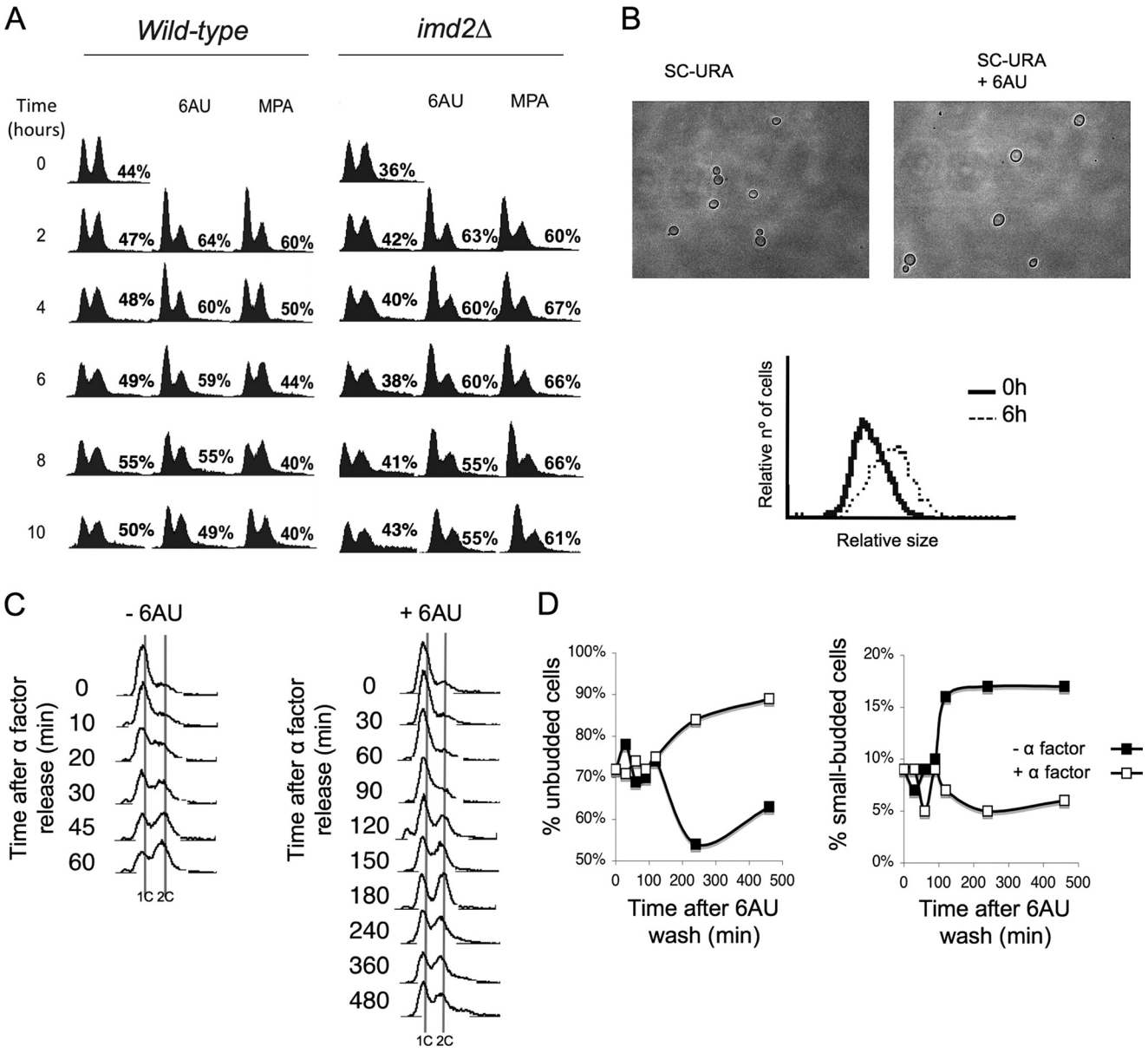
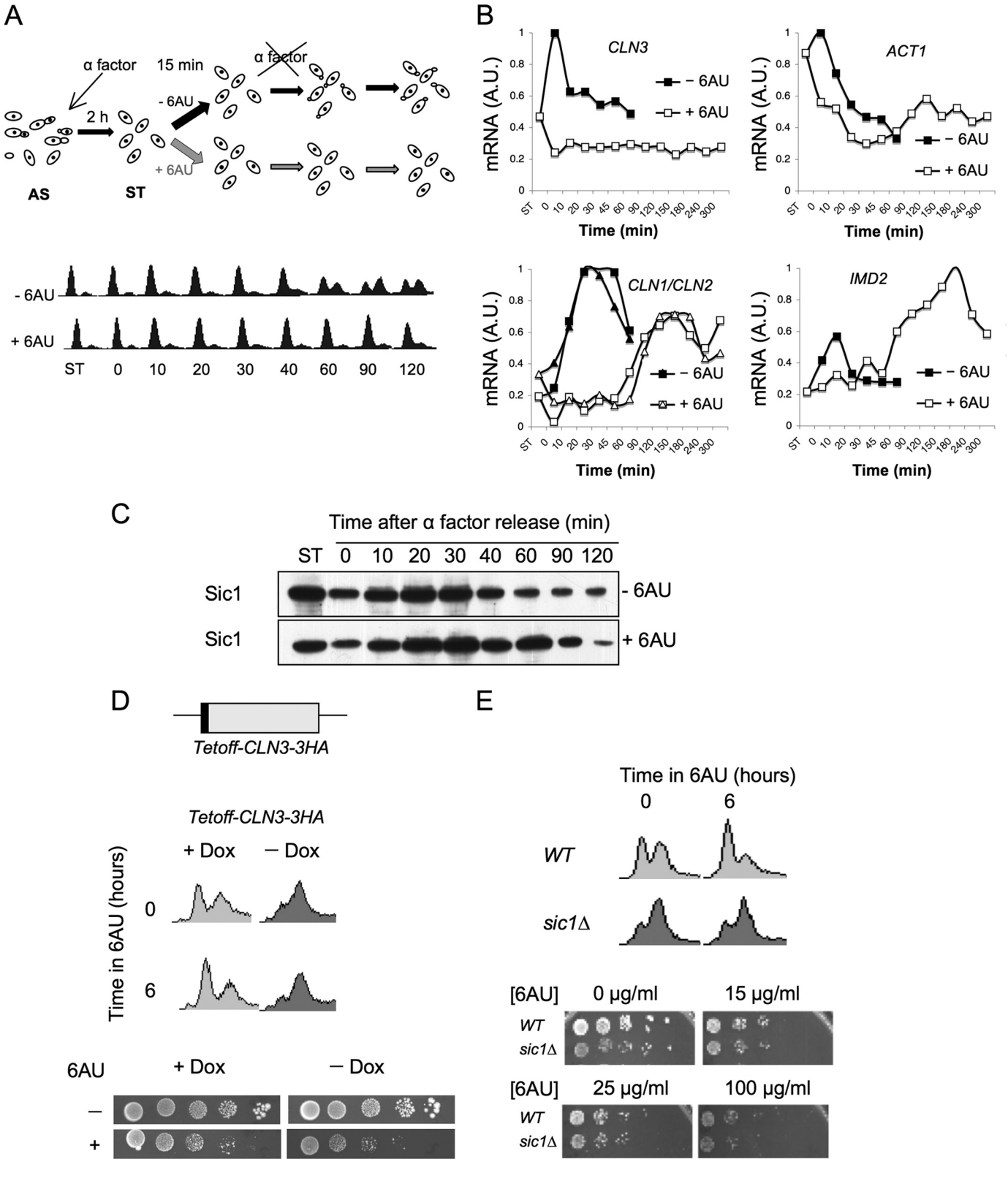


FIGURE 1. **Asynchronous cultures accumulate in G_1 (START) under NTP-depleting drug treatment.** *A*, asynchronously growing wild-type and *imd2*Δ cells were treated with 100 μg/ml 6AU or MPA. Samples were taken at different time points to analyze DNA content by flow cytometry and the proportion of unbudded cells by microscopy. *B*, a representative microscopic (100×) image shows wild-type cultured cells before and after a 6-h 6AU treatment. Average cell size was also measured by flow cytometry. *C*, an α-factor-synchronized culture at START was incubated for a 15-min period with or without 6AU. Cells were then released, and samples were taken at the indicated times for the DNA content analysis by flow cytometry. *D*, an exponentially growing asynchronous culture was treated with 6AU (100 μg/ml) for 2 h. After three washes, half the culture was incubated with the α-factor for synchronization at START. The percentages of unbudded and small-budded cells were scored.

delay as loss of *IMD2* suffices to manifest NTP-depleting drug sensitivity (9). As shown in Fig. 1*A*, the cells lacking *IMD2* presented a continued G_1 delay after 6AU or MPA treatment, which caused an increase in the 1C unbudded cells, which was still observed by 10 h after treatment. This result indicates that the G_1/S transient impairment induced by these drugs correlates with the reduction in the NTP pools.

Cell Cycle Regulatory Elements Are Involved in This G_1 Delay—To understand the cell cycle regulatory mechanisms mediating the observed G_1 delay, we investigated the effect of NTP-depleting drugs on the different regulatory elements involved in the G_1/S transition. First, we analyzed the consequences of NTP depletion on the expression of G_1 cyclins *CLN1*, *CLN2*, and *CNL3*. Cells were treated for 2 h with α-factor to arrest cells at START, and then for 15 min in either the presence or absence of 6AU, but in the continuous presence of the pheromone, as schematized in Fig. 2*A*. Next, we released the cells from the arrest and analyzed the mRNAs levels of *CLN1*, *CLN2*, and *CNL3* by Northern blotting. The *CLN3* mRNA levels were clearly lower in the pheromone-treated cells in the presence of 6AU than in its absence (Fig. 2*B*). It should be noted that 6AU-treated cells remained arrested at G_1 after removing the α-factor, which reveals that this treatment impaired G_1/S transition (Fig. 2*A*). In contrast, the mRNA levels of *ACT1*, a constitutive noncycling gene, were not significantly affected after the 6AU treatment. The START-specific induction of *CLN1* and *CLN2*

was strongly delayed until *IMD2* induction overcame NTP scarcity (Fig. 2*B*). We also analyzed the levels of the Sic1 protein, the inhibitor of the Cdc28-Clb complexes, which regulates entry into S phase (44). As expected, an abnormal accumulation of Sic1 was observed in the 6AU-treated cells (Fig. 2*C*). These results suggest the existence of a control mechanism causing the down-regulation of *CLN3* expression and a subsequent delay in *CLN1* and *CLN2* induction. This mechanism might protect cells from the deleterious effect of replicating the genome under NTP depletion conditions. To test this scenario,



we forced the entry into the S phase of NTP-depleted cells. To do so, we overexpressed *CLN3* with a *TET-off::CLN3* construct (negatively regulated by doxycycline and kindly provided by M. Aldea), which suppressed the accumulation of the treated cells in G_1 and slightly increased 6AU sensitivity (Fig. 2*D*). Similar results were obtained in cells lacking Sic1 (Fig. 2*E*). These data indicate that the forced progression of the 6AU-treated cells into the S phase is deleterious, and, therefore, the G_1 delay triggered by NTP depletion is cell-protective.

NTP Depletion Differentially Impacts the RNA Products of the Three RNA Polymerases—As the described transient G_1delay correlates with the reduction in the cellular nucleotide pools, two different scenarios can be hypothesized: (i) the low NTP levels are *per se* the signal for G_1 delay, or (ii) a subsequent consequence of nucleotide reduction triggers the effect on cell cycle progression. To distinguish between these possibilities, we investigated the effect of 6AU on the RNA products specified by RNA pol I, II, and III. In a wild-type strain, cells were collected at different times after 6AU addition; total RNA was extracted and analyzed by Northern blotting (Fig. 3). RNA pol I transcripts were followed by studying the steady-state levels of all pre- and mature rRNAs by Northern hybridization (Fig. 3*A*). A strong and early reduction in the levels of pre-rRNA 35S was observed in the cells after 15 min of 6AU treatment. The pre-rRNA 35S is the first detected precursor of rRNAs (41); therefore, this result indicates that 6AU strongly inhibits RNA pol I transcription. Consistent with this result, a similar reduction pattern was presented by other pre-rRNA species transcribed by RNA pol I; *i.e.* 32S, 27SA, 27SB and 20S. The levels of 27S and 20S pre-rRNAs, however, decreased more slowly than those of 35S pre-rRNA, reflecting that these are downstream precursors of the pre-rRNA processing pathway. No drop in the levels of mature 18S and 25S rRNA was observed (Fig. 3*A*), which most likely corresponds to pre-existing stable ribosomes in this 2-h time course of 6AU treatment.

RNA pol III activity was followed by analyzing the short-life primary transcript of *SUP56*, which encodes a $tRNA^{Leu}$. The *SUP56* pre-tRNA levels lowered drastically after 15 min of 6AU treatment (Fig. 3*B*). No drop, however, was observed in the levels of 5S rRNA which, as above, is consistent with the stability of mature ribosomes through the 2-h time course of the experiment.

RNA pol II-dependent gene expression was followed by assessing the mRNA levels of r-protein genes *RPL5* and *RPS3* and the ribosome-unrelated *ADH1* gene. In contrast to the previous results, the mRNA levels showed a very slight reduction at early time points after 6AU addition (Fig. 3*C*). These results demonstrate that, even though NTPs are essential substrates for all three RNA polymerases, the effect of NTP-depleting drugs is faster and stronger in the RNA pol I and RNA pol III transcript levels than on the RNA pol II ones (see Fig. 3*D*). Therefore, NTP-depleting drugs generate a clear imbalance between pre-rRNAs and mRNAs.

We speculate that this imbalance might trigger the transient G_1 delay observed after NTP depletion. To further explore this possibility, we reproduced the imbalance between pre-rRNAs and mRNAs through the selective depletion of essential subunits of RNA pol I or III. We first analyzed a conditional allele of *RPA43* (encoding an RNA pol I subunit), which harbors this gene under the control of a *TET-off* promoter. In the absence of doxycycline, this strain behaves similarly to the wild type in terms of its doubling time, levels of 35S pre-rRNA, and cell cycle profile (Fig. 4, *A–C*). However, depletion of *RPA43* clearly diminished the 35S pre-rRNA, whereas the levels of the RNA pol II *RPL5* and *ADH1* transcripts were not affected (Fig. 4, *A* and *B*). Strikingly, depletion of *RPA43* also led to a delay in the G_1 phase of the cell cycle (Fig. 4*C*).

We performed similar experiments with a conditional allele for *RPC17* (encoding an RNA pol III subunit), which also harbors this gene under the control of the *TET-off* promoter. In the absence of doxycycline, this strain behaved similarly to the wild type in terms of its doubling time, levels of *SUP56* pre-tRNA, and cell cycle profile (Fig. 4, *D–F*). The Northern blot analysis of the *SUP56* tRNAs from *RPC17*-depleted cells showed normal stationary levels for the RNA pol I and RNA pol II products (35S pre-rRNA, and *RPL5* and *ADH1* mRNAs), but sharply lowered the levels of *SUP56* pre-tRNA, thus reflecting a deficit of RNA pol III products (Fig. 4, *D* and *E*). As above, a pronounced G_1 cells accumulation correlated with RNA pol III inactivation (Fig. 4*F*). In conclusion, the deficit of newly synthesized RNA pol I and III products affects G_1/S transition similarly to the way NTP depletion does.

Deficit of Pre-rRNAs Leads to Accumulation of Free r-Proteins—The synthesis of rRNAs and r-proteins are two parallel, coordinated pathways that lead to ribosome biosynthesis (for review, see Refs. 5, 6). In mammalian cells, it has been previously demonstrated that MPA treatment results in the drastic reduction of pre-rRNA synthesis and the disruption of the nucleolus; this situation brings about the accumulation of free r-proteins, including L5 and L11 (16). Above, we describe that both NTP-depleting drug treatments and RNA pol I and III inactivation in *S. cerevisiae* cause an imbalance between pre-rRNAs and r-protein mRNAs. This situation led us to test whether free r-proteins are accumulated in yeast under NTP depletion.

To do so, wild-type and *imd2*Δ cells were treated with 6AU for 15 min; extracts were performed and subjected to centrifu-

FIGURE 2. **Cell cycle regulators actively mediate the G_1 delay promoted by NTP-depleting drugs.** *A*, experimental design and flow cytometry analysis of the yeast cultures used in *B*. Cells were synchronized with α-factor for 135 min. For those treated with 6AU, the drug was added during the last 15 min of synchronization and was maintained after the α-factor wash and release. Samples were taken after 120 min of synchronization (*ST*) and after the α-factor release at the indicated times. *B*, the mRNA levels of the RNA pol II-transcribed genes *ACT1*, *IMD2*, and the G_1 cyclins *CLN1* (*empty*, +6AU, and *filled*, −6AU, *triangles*), *CLN2* (*empty*, +6AU, and *filled*, −6AU, *squares*), and *CLN3* are shown. *A.U.*, arbitrary units. *C*, a *SIC1::MYC* strain was synchronized, treated with 6AU, and released as described above. Extracts from equal amounts of cells were loaded in SDS-polyacrylamide gels and analyzed by Western blotting with an anti-Myc antibody. *D*, effect of the *CLN3* overexpression on cell cycle progression and cell viability during 6AU treatment. Wild-type cells containing a *TET-off:CLN3*-expressing plasmid were incubated for 6 h in the presence of 6AU for the flow cytometry analysis. The same strain was incubated in SC-URA with or without doxycycline (*Dox*) and with or without 6AU for the growth test by serial dilutions. *E*, effect of *SIC1* deletion on cell cycle progression and cell viability in the presence of 6AU. A *sic1::KAN* strain was incubated for 6 h in 6AU and cells analyzed by flow cytometry. The same strain was assayed in a growth test in the presence of increasing concentrations of 6AU.

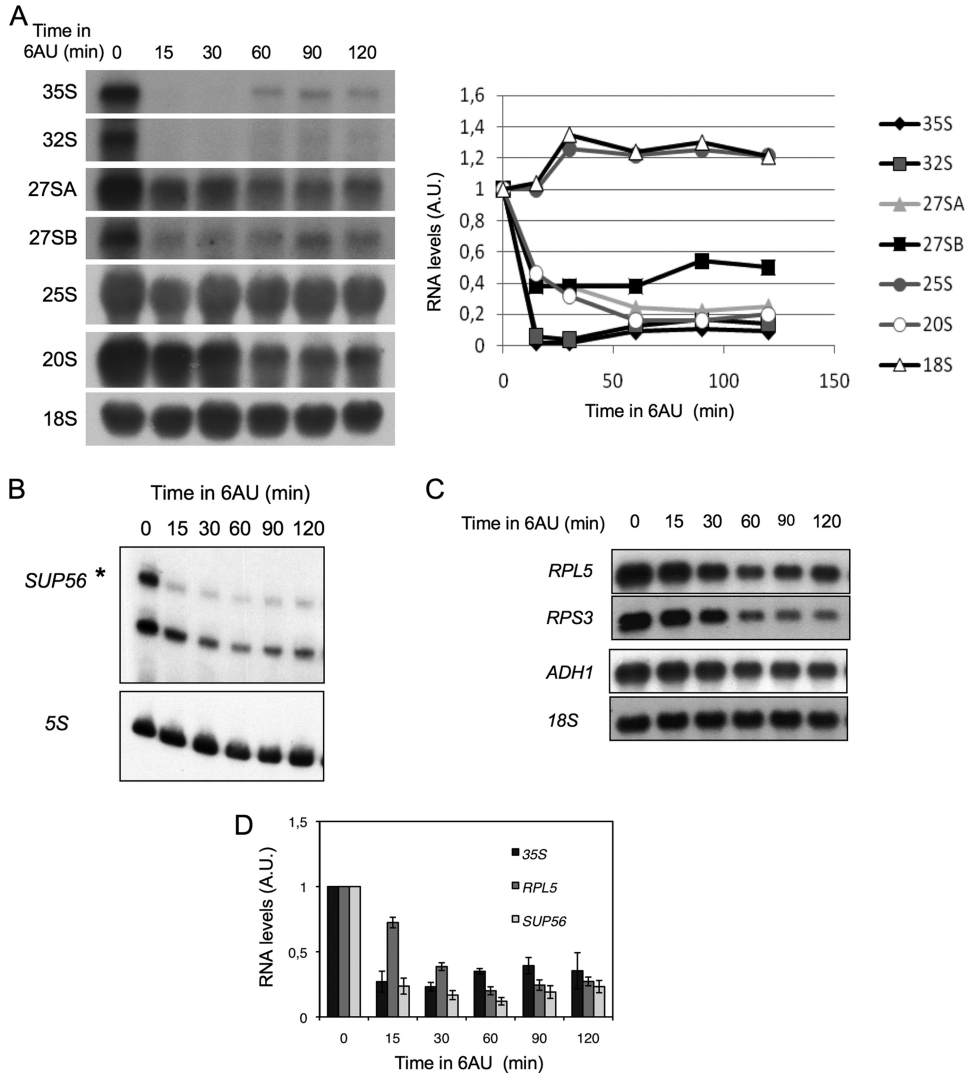


FIGURE 3. **NTP-depleting drugs differentially affect the levels of the three RNA polymerase transcripts.** Exponentially growing cells were treated with 100 μg/ml 6AU. Samples were taken at the indicated times, and the RNA levels of RNA pol I, pol II, and pol III transcribed genes were analyzed. *A*, Northern blot analysis of high molecular mass pre- and mature rRNAs. Specific probes were used to reveal the different pre- and mature rRNAs shown in each *panel*. Mature rRNA 18S was used as the loading control. Signal intensities of Northern blot analysis of the pre- and mature rRNAs presented in *A* were measured by phosphorimaging; values were normalized to those obtained for the wild-type control before 6AU addition and arbitrarily set at 1.0. *B*, Northern blot analysis of *SUP56* pre-tRNA and mature 5S rRNA. The first precursor transcript of *SUP56* is indicated (*). *C*, Northern blot analysis of RNA pol II transcribed *RPL5*, *RPS3*, and *ADH1* mRNAs. Mature rRNA 18S was used as the loading control. *D*, quantification of the relevant RNA species shown in *A*, *B*, and *C*. 35S pre-rRNA and *RPL5* mRNA were normalized against the 18S rRNA signal, whereas the first precursor of *SUP56* (*) was normalized against 5S rRNA. All the data have been obtained in relation to the untreated cells levels (time 0 h) and are expressed as the average of at least three independent experiments ± S.D. (*error bars*).

gation through polysome sucrose gradients. Proteins were extracted from each fraction and analyzed by Western blotting with specific antibodies against r-proteins L1, L5, and S8 (yeast S8 is the orthologue of human S8). However, no free r-proteins were detected (data not shown). Because efficient degradation of unassembled r-proteins has been described in both yeast and mammalian cells when either r-protein genes are amplified (45, 46) or rRNA synthesis is inhibited (47–49), we reasoned that free r-proteins can be detected only under conditions where protein degradation is partially impaired. Therefore, we repeated the experiments in a *cim3-1* mutant (kindly provided by C. Mann) because *CIM3* encodes a regulatory subunit of the 26S proteasome involved in the degradation of ubiquitinated substrates (50). Experiments were carried out at the semipermissive temperature of 30 °C and in G_1-synchronized cells to avoid any influence of the Cim3 function on G_2/M transition. In this genetic background, the clear presence of L1 and L5, but not of S8, was detected in the low molecular weight fractions of the polysome profile after 6AU treatment (Fig. 5*A*). This free r-protein accumulation was observed at 15 min after treatment in parallel to a drastic reduction in the rRNA levels. Importantly, wild-type polysome profile was observed for this strain at this time point, indicating that translation is still not affected (Fig. 5*A*). Taken together, these results demonstrate that the imbalance between pre-rRNAs and r-protein mRNAs, caused by 6AU treatment, generates an accumulation of free r-proteins, including L5.

In mammalian cells, it has been demonstrated that only free L11 and L5 r-proteins are directly required for p53 induction (31, 32). To investigate whether yeast L5 and L11 also play a key role in the G_1 delay caused by NTP depletion, we forced the accumulation of free r-protein L5 in the absence of L11 and

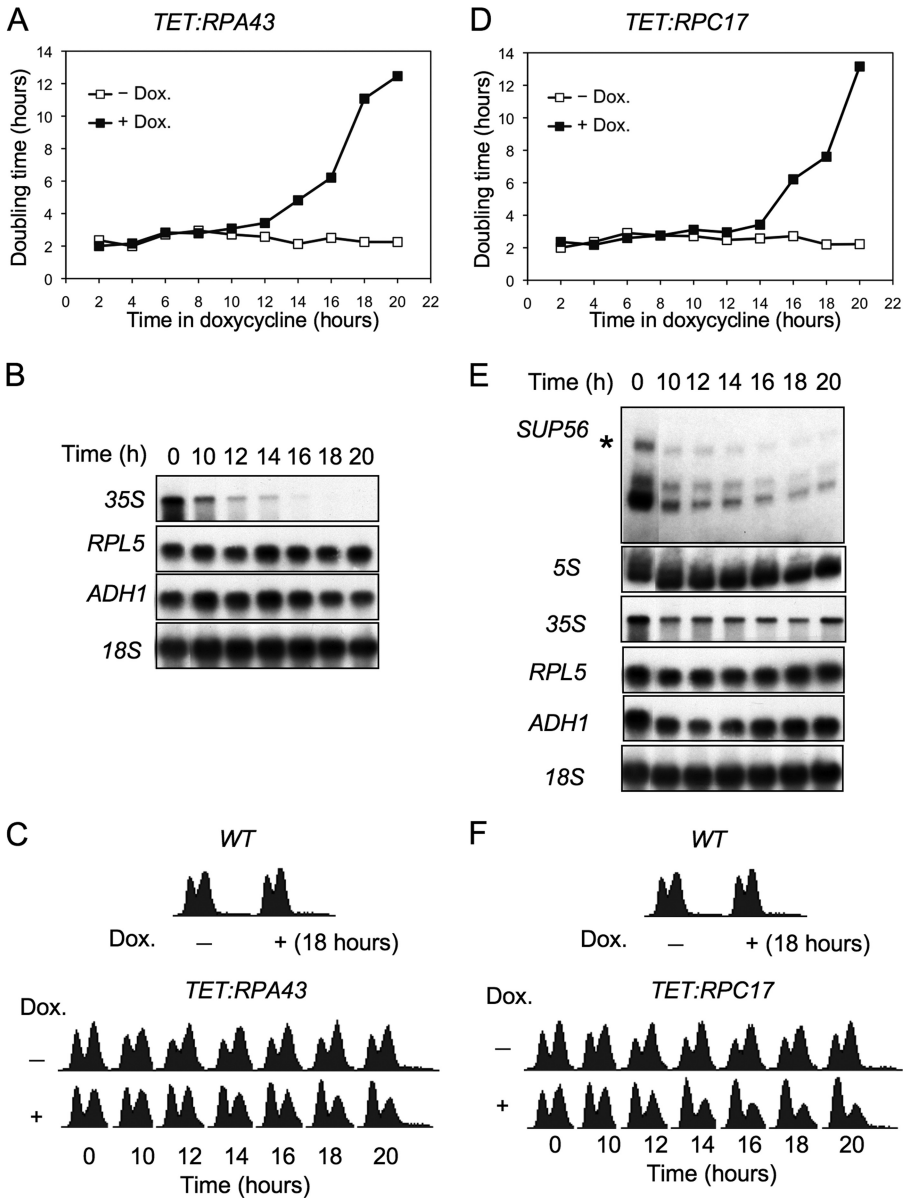


FIGURE 4. **Depletion of selected RNA pol I or RNA pol III subunits reproduces NTP-depleting drug-mediated RNA imbalance and induces G_1 cell accumulation.** *A*, doubling time progression of a strain with the gene of the RNA pol I subunit *RPA43* under the control of a *TET-off* promoter plus/minus doxycycline (*Dox.*). *B*, Northern blot analysis of RNA pol I-transcribed 35S pre-rRNA and of the mRNAs of RNA pol II-transcribed genes *ADH1* and *RPL5* during Rpa43 depletion. *C*, flow cytometry analysis, at the indicated times, of cell cycle progression during Rpa43 depletion after treatment with 100 μg/ml 6AU. *D*, doubling time progression of a strain with the gene of the RNA pol III subunit *RPC17* under the control of a *TET-off* promoter with or without doxycycline. *E*, Northern blot analysis, during Rpc17 depletion, of the RNA pol III-transcribed pre-tRNA *SUP56* (the first precursor is indicated *) and 5S rRNA, the mRNAs of the RNA pol II-transcribed genes *RPL5* and *ADH1*, and the RNA pol I-transcribed 35S pre-rRNA. *F*, flow cytometry analysis, at the indicated times, of cell cycle progression during Rpc17 depletion in cells treated with 100 μg/ml 6AU.

assessed the G_1/S delay response of the 6AU-treated cells. It has been previously described that the amount of L5 not assembled into ribosomes increases upon depletion of L11 (51). Thus, we performed flow cytometry analyses with the strains carrying either *rpl11A*Δ or *rpl11B*Δ deletions. The asynchronous cultures of cells lacking r-protein L11A or L11B exhibited a longer accumulation of G_1 cells compared with the wild type after adding NTP-depleting drugs (Fig. 5*B*). This enhancement became especially clear after 4 or 6 h of treatment. The same result was obtained when cells were treated with MPA (data not shown).

We anticipate that those conditions impairing the expression of r-protein genes, particularly *RPL5*, should suppress the G_1 delay caused by NTP depletion. We have previously described that the transcription of r-protein genes under NTP-depleting conditions requires the transcription factor TFIIS (52). Moreover, in a *dst1*Δ strain, a mutant lacking TFIIS, we showed that 35S pre-rRNA accumulates after 60 min in the presence of 6AU, whereas the mRNA levels of L5 drops (52). According to our model, this deficit of L5 mRNA should impede the cell cycle response of *dst1*Δ cells to NTP depletion. The FACS analysis confirmed this prediction as the *dst1*Δ cells treated with 6AU exhibited no accumulation of cells in G_1 for as long as 4 h in the presence of 6AU (Fig. 5*C*).

We also analyzed two mutant alleles affected in the Rrs1 protein, *rrs1-84* and *rrs1-1* (kindly provided by Keiko Mizuta). Rrs1

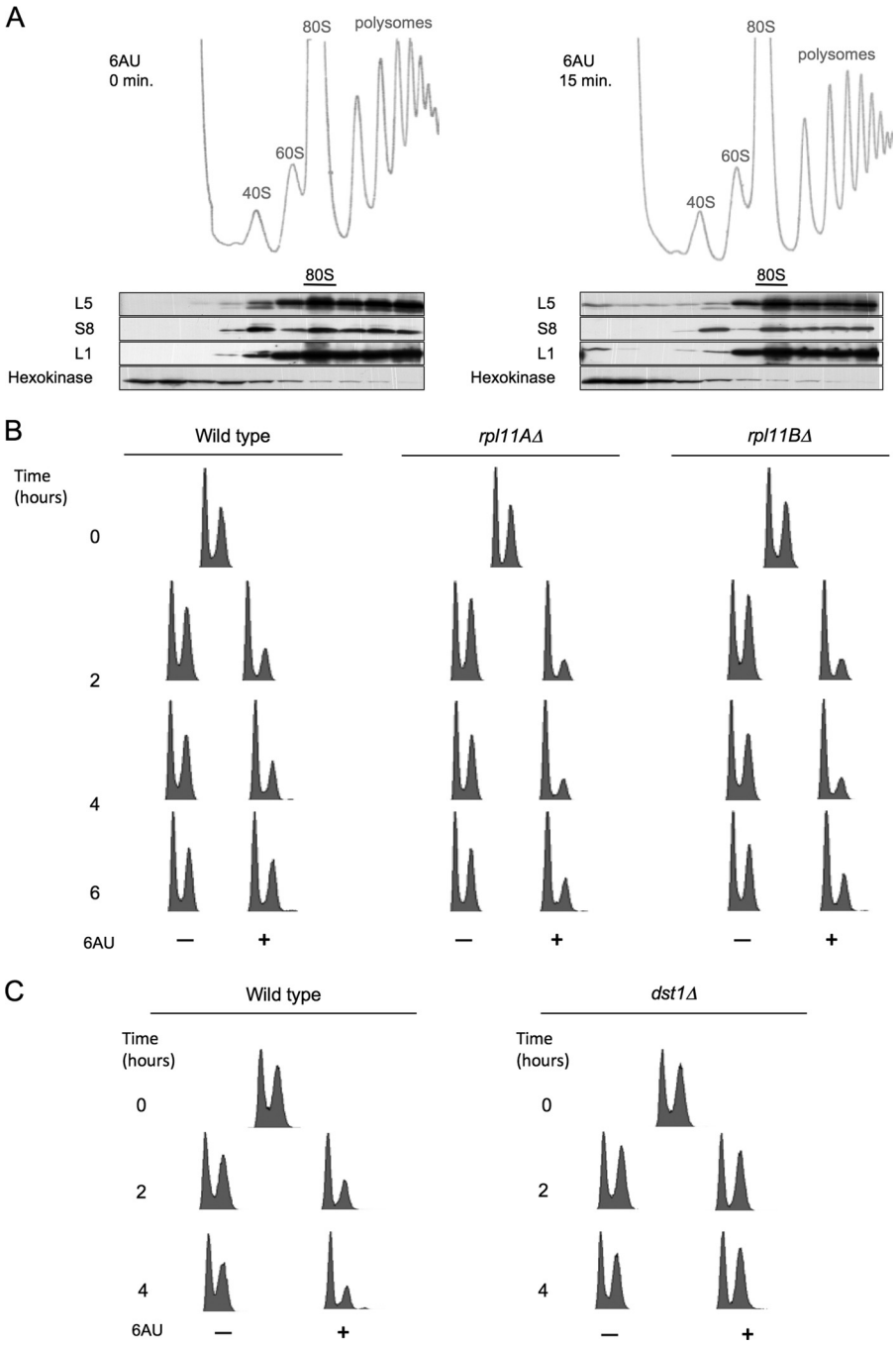


FIGURE 5. **NTP-depleting drugs induce the appearance of free L5 r-protein which correlates with the G_1 response.** *A*, a proteasome-deficient *cim3-1* strain was grown in SC-URA at 30 °C (permissive temperature) and harvested at an A_{600} of 0.5 after α-factor synchronization and with or without 15 min of 100 μg/ml 6AU treatment. Cell extracts were prepared, and 10 A_{260} of each extract was resolved in 7–50% sucrose gradients. A_{254} was continuously measured. Sedimentation is from *left* to *right*; 40S, 60S, 80S, and polysome peaks are indicated. Fractions were collected from the gradients, and proteins were extracted from an equal volume of each fraction and analyzed by Western blotting with the indicated specific antibodies. *B*, asynchronously growing wild-type, *rpl11A*Δ, and *rpl11B*Δ cells were treated with or without 100 μg/ml 6AU. Samples were taken at different time points, and the DNA content was analyzed by flow cytometry. *C*, wild-type and *dst1*Δ asynchronous cultures were treated, or not, with 100 μg/ml 6AU. Samples were taken at different time points and the DNA content analyzed by flow cytometry.

is necessary for the assembly of L11 and the L5–5S rRNA complex on pre-60S ribosomal particles (Fig. 6*A*) (53, 54). Higher levels of free L5 and L11 have been predicted when compromising the Rrs1 function (54). Interestingly, and as expected, the extracts prepared from *rrs1-84* cells showed the presence of free r-protein L5, even in the absence of the drug, a fact that is not observed when extracts from isogenic wild-type cells are prepared (Fig. 6*B*). The presence of free r-protein L5 in the mutant strain was exacerbated in the presence of NTP-depleting drugs (Fig. 6*B*); free L1, but not free S8 r-proteins, was also detected in this mutant in response to 6AU (data not shown). In this genetic background, the extracts prepared from wild-type cells after 15 min of 6AU treatment also showed free L5 (Fig. 6*B*). Accordingly, we found an enhanced accumulation of G_1

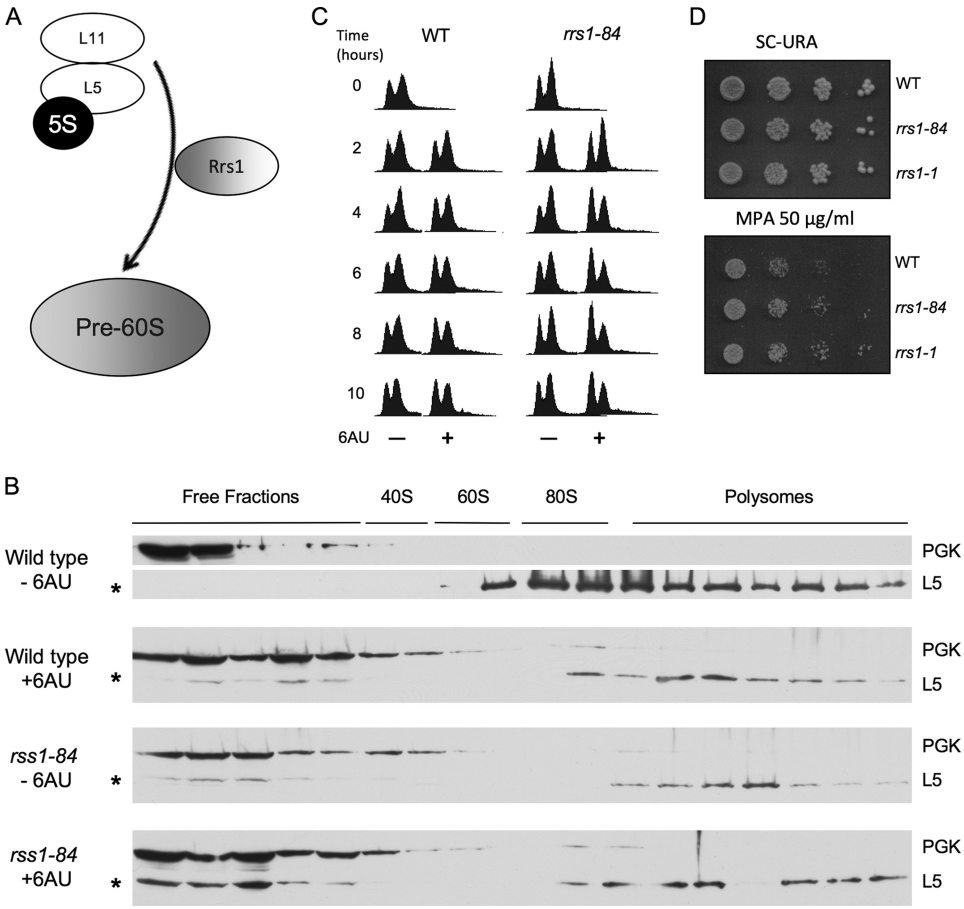


FIGURE 6. **Mutations in Rrs1 exacerbate both free L5 accumulation and G_1 delay in the presence of NTP-depleting drugs.** *A*, Rrs1 is required for the incorporation of the L11 r-protein and the L5–5S r-protein-rRNA complex into pre-60S ribosomal particles. *B*, isogenic wild-type and *rrs1-84* strains were synchronized as described in Fig. 5*A* in the absence or presence of 100 μg/ml 6AU. Cell extracts were prepared, and a 10 A_{260} of each extract was resolved in 7–50% sucrose gradients. A_{254} was continuously measured. Sedimentation is from *left* to *right*. Free, 40S, 60S, 80S, and polysome fractions are indicated. Fractions were collected from the gradients, and proteins were extracted from equal volume of each fraction and analyzed by Western blotting with anti-L5 and anti-PGK antibodies. *C*, asynchronous growing cells were treated with 100 μg/ml 6AU, and samples were taken every 2 h to analyze DNA content by flow cytometry. *D*, growth test of mutant alleles *rss1-1*, *rss1-84*, and their isogenic wild-type strain is shown. Serial dilutions were performed on SC-URA plates with or without MPA.

cells in the *rrs1-84* mutant when treated with NTP-depleting drugs (Fig. 6*C*).

The strong accumulation of free L5 in *rrs1-84* in response to 6AU predicts a protective effect of this mutation for the cell. We analyzed the relative sensitivity of *rrs1* mutants to 6AU and found that both *rrs1-84* and *rrs1-1* were more resistant to this drug than the wild type (Fig. 6*D*). In summary, the transient G_1 delay triggered by NTP-depleting drugs in yeast is a cell-protective mechanism that seems to be mediated by the accumulation of free L5 r-protein.

DISCUSSION

Yeast Cells Delay the Cell Cycle in G_1 in Response to NTP Depletion—In this work, we dissected the process leading to cell cycle delay after adding NTP-depleting drugs in the yeast *S. cerevisiae*. We show that these drugs provoke a transient deficit of pre-rRNAs that originates the accumulation of free r-proteins, including L5, which should act as a specific stress signal to trigger this delay of the cell cycle in G_1.

S. cerevisiae wild-type cells transiently accumulate at G_1 after NTP-depleting drug treatment (Fig. 1, *A* and *B*). This G_1 delay suggests that NTP depletion inhibits the performance of START. This issue has been confirmed by reciprocal-shift experiments, demonstrating that this arrest occurs at START (Fig. 1, *C* and *D*), the equivalent to the restriction point in metazoan cells (55).

To coincide with this fact, we found that the regulatory elements controlling the G_1/S transition participate in the delay provoked by MPA and 6AU. We detected that NTP depletion causes *CLN3* mRNA down-regulation which, in turn, avoids *CLN1* and *CLN2* induction at START and, by stabilizing Sic1, prevents cell entrance into the S phase. In agreement with this prediction, NTP-depleted cells underwent decreased viability when forced to enter the S phase by either overexpressing *CLN3* or deleting *SIC1*, demonstrating that the G_1 arrest provoked by NTP depletion is cell-protective (Fig. 2).

In *S. cerevisiae*, both NTP depletion and the observed G_1 arrest are transient phenomena (8, 13). We observed that the time course of NTP recovery fits well with G_1 transient arrest. Accordingly, both NTP depletion and G_1 arrest become permanent in the absence of *IMD2* (Fig. 1*A*), indicating a correlation between these two events.

Free Ribosomal Proteins and Cell Cycle in Yeast—As we have demonstrated that progression through the S phase seems to be dependent on NTP levels, the first objective of this work was to elucidate whether the yeast cell cycle responds to NTP levels

per se or to the consequence of their depletion. In higher eukaryotes, G_1 arrest has been previously described to be a consequence of nucleolar stress, which can be elicited by different external or internal stimuli, such as the inhibition of RNA pol I activity by actinomycin D (56), genetic disruption of RNA pol I transcription initiation factor TIF-IA (57), inhibition of rRNA processing by the loss-of-function mutations of rRNA processing factor Bop1 (58), treatment of cells with 5-fluorouracil (18), inhibition of overall ribosomal biogenesis by serum starvation (25), and genetic inactivation of r-protein S6 (59). In all cases, p53 can be induced and activated, leading to cell cycle arrest at the G_1 phase. As NTP depletion in yeast affects the three RNA polymerases, it was intriguing to compare the impact on them all. Fig. 3 clearly shows that MPA treatment results in a drastic, faster reduction of the pre-rRNAs transcribed by RNA pol I and the pre-tRNAs transcribed by RNA pol III, whereas mRNAs, transcribed by RNA pol II, undergo a slower decline, likely due to their higher stability. Having genetically achieved the decrease in the levels of RNA pol I- and III-dependent RNA products, by inactivating polymerase subunits, the consequences on the cell cycle were similar, indicating that NTP depletion itself is not the signal for G_1 delay (Fig. 4).

Ribosomal biogenesis requires the coordinated function of RNA pol I, II, and III to produce rRNAs and r-proteins. Cells manage a balanced production of r-proteins as they are required in equimolar quantities. Indeed, regulation exists at all gene expression levels, from transcription to protein turnover (for review, see Ref. 60). Both treatment with NTP-depleting drugs and inactivation of RNA pol I or RNA pol III essential subunits led to the accumulation of r-protein mRNAs compared with pre-rRNAs. In this situation, an excess of r-proteins is expected. We detected such free r-proteins in a specific yeast background (Fig. 6), although in the background mostly used in this work, it was necessary to impair the proteasome to stabilize the free r-proteins accumulated (Fig. 5*A*).

The key role of mammalian r-proteins L5 and L11 for this essential response has been demonstrated very well (31, 32). Thus, we focused on the role of these two r-proteins in yeast. Our results show a clear accumulation of free L5 after NTP depletion treatment (Figs. 5 and 6).

The free r-protein accumulation shown in yeast might act as a signal for modulating cell cycle progression through the G_1/S transition, which is precisely the case for L5 and L11 in mammalian cells (31, 32). According to this model, if free signaling protein levels increased, we would expect a more drastic G_1 arrest after the NTP-depleting drug challenge. We have obtained evidence for free yeast L5 as a key signaling protein for the G_1 delay using L11-depleted strains. It has been previously described that depletion of L11A brings about an increase of free L5 r-protein (51). Interestingly, our data demonstrate that deletion of L11 genes (*rpl11A*Δ and *rpl1B1*Δ) extends the length of the G_1 response to NTP-depleting drugs (Fig. 5*B*). On the contrary, impairment of *RPL5* transcription by deleting the gene encoding transcription factor TFIIS (52) suppresses G_1 delay in response to NTP depletion (Fig. 5*C*). All of these results are consistent with a major role of free L5 in signaling G_1 arrest in response to NTP depletion. Clearly, further genetic and biochemical experiments will be required to unravel the precise function of free L5 and L11 r-proteins during progression of yeast cell cycle.

The analysis of *rrs1* mutants further confirmed this model. The *rrs1* mutant cells treated with NTP-depleting drugs at a semipermissive temperature showed a marked rise in free L5, accompanied by a strongly enhanced G_1 response and cell viability (Fig. 6). To summarize, G_1 delay is enhanced only for conditions under which the levels of free L5 increase. Taken together, these results reveal that changes in the levels of free L5 alter the G_1/S response. In all the cases tested, the viability of NTP-depleted cells behaves in accordance with the protective role for this G_1 delay.

The accumulation of free r-proteins under ribosomal stress may result after the release from intact ribosomes, which has been proposed in mammalian cells (18, 25), or may be the result of defects in ribosomal biogenesis after a drastic reduction in the pre-rRNAs supply. The high r-protein mRNA/35S pre-rRNA ratio detected in yeast cells after NTP depletion and the fact that free r-proteins are detected quite early after 6AU addition without translation being affected, support the second scenario.

The results of this study emphasize the fine coordination of cell cycle progression with ribosome biogenesis and suggest that free r-proteins negatively regulate G_1/S transition in yeast. We propose a model where the proper coordination of pre-rRNA and r-proteins levels is required for accurate cell cycle progression. This coordination is critical for an effective utilization of cell resources and requires a balanced function of the RNA pol I, II, and III transcription activities. Our group has recently described a new cell cycle phenomenon that allows cells to respond to the free histones evicted from transcription by arresting cells at START before starting DNA replication (61). Thus, the detection of abnormal locations of relevant cell elements (such as histones evicted from nucleosomes or r-proteins from ribosomes) seems a general cellular strategy to ensure the progression of division with optimal viability prospects.

Finally, it is important to stress that this work indicates the parallelism between the mechanisms responding to nucleolar stress in yeast and metazoa, suggesting that it is a general control strategy extended throughout eukaryotes. Considering the advantage of using *S. cerevisiae* as a model organism, this study opens up new perspectives to examine the antiproliferative effects of a diverse group of metabolic inhibitors, including many clinically important anticancer drugs which strongly inhibit rRNA synthesis or processing (62–66). Likewise, nucleolar stress and ribosome biogenesis are the base of a group of human diseases called ribosomopathies (67, 68). Yeast may also help the molecular study of these pathologies.

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SUPPLEMENTAL DATA (Gómez-Herreros *et al*.)

Yeast strain	Relevant genotype	Reference
BY4741	MAT a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
FGY41.41	BY4741 *imd2::hphMX4*	Gomez-Herreros, F. *et al.* (2012) Nucleic Acids Res 40, 6508-6519
MMY9.2	BY4741 *dst1::kanMX4*	Gomez-Herreros, F. *et al.* (2012) Nucleic Acids Res 40, 6508-6519
CMY763	*cim3-1 ura3-52 leu2Δ1*	Ghislain, M. et al. (1993) Nature 366, 358-362
YPH499	MAT a *ade2-101 lys2-80 ura3-52 trp1Δ63 his3Δ200 leu2Δ1*	Sikorski, R. S., and Hieter, P. (1989) Genetics 122, 19-27
YMLF2	MAT α *ade2-11 lys2-801 ura3-52 trp1Δ63 his3Δ200 leu2Δ1 rpc17::HIS3 pCM185::RPC17*	Siaut, M. *et al* (2003) Mol Cell Biol 23, 195-205
YFN25	MAT a *ade2-1 lys2-801 ura3-52 trp1Δ63 his3Δ200 leu2Δ1 rpa43::LEU2 pCM185::RPA43*	Zaros, C. *et al.* (2007) Nucleic Acids Res 35, 634-647
W303-1A	MAT a *ade2-1 can1-100 ura3-1 leu2-3,112 his3-11,15 trp1-1*	Thomas, B. J. *et al.* (1989) Cell 56, 619-630
KM370	W303-1A *rrs1::LEU2 RRS1::TRP1*	Miyoshi, K et al. (2004) FEBS Lett 565, 106-110
KM921	W303-1A *1 rrs1::LEU2 rrs1-84::TRP1*	Miyoshi, K et al. (2004) FEBS Lett 565, 106-110
KM923	W303-1A *rrs1::LEU2 rrs1-124::TRP1*	Miyoshi, K et al. (2004) FEBS Lett 565, 106-110
FGY70	BY4741 *ydr007w(trp1)::kanMX4 SIC1::MYC::TRP1*	This work
Y02435	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sic1::KAN*	Euroscarf
Y04715	BY4741 *ygr085c(rpl11B)::kanMX4*	Euroscarf
FGY73	BY4741 *ypr102c(rpl11A)::hphMX4*	This work

Balanced Production of Ribosome Components Is Required for Proper G_1/S Transition in *Saccharomyces cerevisiae*

Fernando Gómez-Herreros, Olga Rodríguez-Galán, Macarena Morillo-Huesca, Douglas Maya, María Arista-Romero, Jesús de la Cruz, Sebastián Chávez and Mari Cruz Muñoz-Centeno

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