The Nucleolus Stress Response is Coupled to an ATR-Chk1-Mediated G2 Arrest

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Abbreviations: Fucci: fluorescent ubiquitinylation-based cell cycle indicator; 5-EU: 5-ethynyluridine; ATR: ataxia telangiectasia and Rad3-related protein; Chk1: checkpoint kinase 1; DAPI: 4′,6-diamidino-2-phenylindole

ABSTRACT
We report experiments on the connection between nucleolar stress and cell cycle progression employing HeLa cells engineered with the fluorescent ubiquitinylation-based cell cycle indicator (Fucci). Nucleolar stress elicited by brief exposure of cells to a low concentration of actinomycin D that selectively inhibits ribosomal RNA synthesis had no effect on traverse of G1 or S, but stalled cells in very late interphase. Additional experiments revealed that a switch occurs during a specific temporal window during nucleolar stress, and that the subsequent cell cycle arrest is not triggered simply by the stress-induced decline in the synthesis of ribosomal RNA or by a ribosome starvation phenomenon. Further experiments revealed that this nucleolus stress-induced cell cycle arrest involves the action of a G2 checkpoint mediated by the ATR-Chk1 pathway. Based on analysis of the cell cycle stages at which this nucleolar stress effect is put into action, to become manifest later, our results demonstrate a feed-forward mechanism that leads to G2 arrest and identify ATR-Chk1 as molecular agents of the requisite checkpoint.

INTRODUCTION
Following the cytological recognition of the nucleolus in the mid-1800’s, another century passed before a function of this nuclear domain was defined: the synthesis of ribosomal RNA and the assembly of nascent ribosomes (Pederson, 2011). But before that breakthrough in the mid-1960’s, a number of cell biologists had presciently speculated that the nucleolus had something to do with progression of cells through interphase. One embodiment of this hypothesis was a study in ultraviolet light ablation of one of the two nucleoli in grasshopper neuroblasts resulted in a delay of progression into mitosis (Gaulden and Perry, 1958). More recently, other clues to a link between the nucleolus and the cell cycle have emerged including the presence of

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growth factors in nucleoli (Pederson, 1998a), the observation of numerous cell cycle-related proteins in proteomics studies of purified nucleoli (Andersen et al., 2002; Scherl et al., 2002) and the discovery of nucleostemin, a nucleolus-localized protein that drives the cell cycle by attenuating the tumor suppressor p53 (Tsai and McKay, 2002; Ma and Pederson, 2008b). While at first blush it could be imagined that these links between the nucleolus and the cell cycle might simply reflect a need for new ribosomes to advance progression through interphase, a number of considerations suggest that the situation is not that simple.

Beyond earlier interest (Pederson, 1998a; 1998b) we have been drawn to this issue more recently by our work with the aforementioned nucleostemin (Ma and Pederson, 2007; 2008a) and so we undertook a study in which we sought to investigate whether nucleolar homeostasis, aside from ongoing ribosome production, is related to cell cycle progression. We have deployed the experimental agency of an induced nucleolar stress and our results indicate that some yet to be defined normal nucleolar function (but not ribosome production) is critical for the ability of cells to progress through G2.

RESULTS

Nucleolar stress induces late interphase arrest
The Fucci system was ingeniously developed to visualize cell cycle progression by labeling G1 phase cells red, G1/S transition cells yellow, and S/G2/M phase cells green (Sakaue-Sawano et al., 2008). In the present investigation this system allowed us to precisely monitor cell cycle progression in response to nucleolar stress. Under our culture conditions, HeLa-Fucci cells displayed a G1 period of 11-12 hours, and a combined S-phase and G2 period of 8-9 hours (Figure S1). We treated HeLa-Fucci cells for 4 hours with a concentration of actinomycin D, 0.04 µg/ml, that has been previously established to selectively inhibit mammalian cell rRNA synthesis (Perry, 1962; Penman et al., 1968) and to induce an internal repositioning of nucleolar components (Schöfer et al., 1996; Dousset et al., 2000). The cells were then shifted to inhibitor-free medium and their cell cycle positions were assayed 20 hours later. As shown in Figure 1 (left panels), cells accumulated in S, G2 and M phases during the 20 hours after a 4 hour treatment with actinomycin. Flow cytometry revealed that green cells constituted 79.5% of the population 20 hours after actinomycin treatment, compared with a green fraction of 22.1% in untreated cells. Simultaneous flow cytometry of DAPI staining revealed that the majority of green cells had a 4C or near-4C DNA content and thus were in very late S, G2 or M. This is evident in the far right panels of Figure 1 in which the distributions of DNA contents among red (G1), yellow (the onset of S phase) and green (S/G2/M phase) cells are overlaid.

A switch occurs during nucleolar stress
We reasoned that if the late S/G2/M phase arrest of actinomycin-treated cells were simply a consequence of inhibiting rRNA synthesis it should be evident after even a
briefer than 4 hours duration of inhibitor treatment, since it is known that only very brief exposures are required to substantially reduce rRNA synthesis. A 25 min. exposure of HeLa cells to actinomycin at 0.04 µg/ml, the concentration we have used, leads to a 85% reduction in rRNA synthesis, as determined by a subsequent 10 min. pulse label (Penman et al., 1968). Figure 2 confirms this specifically for the HeLa-Fucci cells we employed, where it can be seen that nucleolar RNA synthesis, measured by a click chemistry-based 5-ethynyluridine labeling method (Jao and Salic, 2008), is virtually undetectable following a 30 min. or 2 hour exposure of cells to actinomycin (Figure 2A). However, when we looked 20 hours later at RNA synthesis in cells that had been subjected to actinomycin for 30 min. or 2 hours, we had a surprise. As shown In Figure 2B, 20 hours after a 30 min. exposure to the inhibitor, nucleolar RNA synthesis had returned to the same levels as seen in untreated cells. This result was not unanticipated as actinomycin does not bind DNA covalently and thus a recovery of rRNA synthesis would be expected as actinomycin dissipates from the rDNA over the subsequent 20 hours of culturing the cells in inhibitor-free medium. But the striking result in Figure 2B is that in cells that were treated for 2 hours, the level of nucleolar RNA synthesis 20 hours later was still very depressed. Thus, it is clear that at some point between a 30 and 120 minute duration of nucleolar stress, a switch occurs as regards the ability of cells to resume normal rRNA synthesis levels. Yet, when we looked at the steady-state level of 28S rRNA in these cells, neither the brief (30 min.) nor the longer (2 or 4 hours) duration of nucleolar stress had any impact on the cell’s content of ribosomes (Figure 3A). This is expected because ribosomes are very stable in growing mammalian cells (e.g. Koldony, 1975). However, as can be seen in Figure 3B, after a 30 min. exposure to actinomycin, the level of pre-rRNA (indicative of resumed transcription of the rDNA) had returned to normal levels 20 hours later, whereas in cells treated with the inhibitor for 2 or 4 hours, the level of pre-rRNA remained very low 20 hours later. Clearly then, there is something very different about 30 min. vs. a longer actinomycin treatment and yet none of these treatments leads to a significant negative impact of the cells’ content of ribosomes (measured as total cell 28S rRNA). Furthermore, we found that protein synthesis was occurring at normal levels 20 hours after a 2 or 4 hour treatment with actinomycin D (Figure S1). So whatever the molecular basis of the cell cycle effect might be, it cannot be plausibly related to an impairment of translational capacity.

From these results we suspected that the situation might be more complex (and thus interesting) than initially contemplated. So we next used various durations of actinomycin treatment (0.5, 2 and 4 hrs) followed by culturing the cells in inhibitor-free medium for 20 hours to assess cell cycle progression (Figure 4). Following a 0.5 hour treatment there was only a slight increase in the percentage of late S, G2, and M cells, 27.2%, as compared to 19.1% in untreated cells. Thus, a brief but virtually complete inhibition of rRNA transcription 20 hours earlier did not trigger a subsequent late S/G2/M phase arrest. In contrast, when cells were treated for 2 or 4 hours, the conditions of nucleolar stress from which we had established cells cannot resume normal rRNA synthesis, 72.5% and 79.4% of the cells, respectively, became
arrested. (Figure 4, upper panels, 2 and 4 hours). The arrest of these cells in late S, G2 or M is further supported by the cytophotometry of DAPI-stained cells done in parallel (Figure 4, lower panels, 2 and 4 hours).

We next tracked individual cells to precisely observe the above effects in situations where the cell cycle position of a given cell at the time of treatment can be known, thanks to the Fucci staging colors. Figure 5A shows a series of single cell tracking observations of cells that were in mitosis at the time of actinomycin treatment. Compared with an untreated mitotic cell (upper row), cells treated with actinomycin for 0.5, 2 or 4 hours - the treatment commencing in mitosis in all cases - were able in all three cases, to exit mitosis and progress through G1 and S with unperturbed kinetics (Figure 5A, lower three rows), meaning that the synthesis of new ribosomes during the first 2 or 4 hours of G1 (prior to placing the cells in inhibitor-free medium) is not required for G1 traverse and progression into S. However, the cells that were treated with actinomycin commencing at mitosis displayed a prolonged S period and G2 phase, as can be seen by that fact that even by 24 hours these cells had not yet reached mitosis (Figure 5A, lower three rows - compare with the arrival in mitosis at 20 hours in the case of the untreated cell tracked in the upper row). Figure 5B shows a similar set of single cell tracking observations but in which the cells were at the onset of S phase at the time actinomycin treatment commenced. As shown in the upper row of Figure 4B it took 8 hrs for an untreated S-phase cell to reach mitosis and from the known cell cycle parameters of these cells (Figure S2) it can be deduced that this cell was in early S at 0 hours in the tracking images. In contrast, S-phase cells treated with actinomycin for 0.5 hours displayed a delay in reaching mitosis (see images with asterisks in middle two rows of Figure 5B) but once this delayed mitosis was completed the daughter cells progressed into G1 and into S. Moreover, the S-phase cells treated for two or four hours failed to reach mitosis in even 24 hours (Figure 5B, two lower row), in keeping with the late S/G2/M phase arrest demonstrated in the whole population analysis (Figures 1 and 4).

The nucleolar stress-induced cell cycle arrest involves the ATR-Chk1 pathway

The cell cycle arrest we have observed after actinomycin treatment most plausibly involves a G2 checkpoint. Although the drug intercalates, rather than breaks, DNA we needed to consider the possibility that a DNA damage response was being induced. As we shown in Figure 6, there was no elevation of DNA damage 20 hours after a 30 or 120 minute treatment with actinomycin, based on immunostaining for phosphorylated histone H2AX. In contrast, 20 hours after a 120 minute exposure to doxorubicin there was the expected increase in DNA damage (Fornari et al., 1994). Caffeine is an inhibitor of phosphatidylinositol-3-kinase family members such as ATM and ATR which are essential for G2 checkpoint activation (Sarkaria et al, 1999). As can seen in the left panels of Figure 7A, caffeine itself had little effect on cell cycle progression as indicated by the similar presence of red and green cells in the two populations. As in the previous experiments, actinomycin D treatment led to an accumulation of S/G2 cells (upper panel in second column) whereas caffeine
treatment abrogated this effect (lower panel, second column). This result suggested 
ATM and/or ATR are involved in the nucleolar stress-induced arrest and, if so, this 
would define the arrest as occurring at specifically G2 as opposed to very late S or in 
mitosis. To further clarify this, we used UCN-01, a specific inhibitor of the ATR 
target checkpoint kinase Chk1 (Busby et al., 2000). As shown in the middle right 
column of Figure 7A, UCN-01 had no effect on cell cycle progression in cells not 
subjected to nucleolar stress. However UCN-01 treatment abrogated the nucleolar 
stress-induced cell cycle arrest (Figure 7A, rightmost panels). Flow cytometry (Figure 
7B) confirmed the abrogation of the cell cycle arrest by caffeine. Compared to 25.5% 
untreated cells with a 4C DNA content, 71.4% of the actinomycin-treated cells had a 
4C DNA content (lower panel) compatible with the accumulation of green cells in the 
mCherry vs. Venus FACS plots (upper panel). No appreciable effect of caffeine itself 
can be seen in both the Fucci dual color FACS plot (upper panel) and in the DAPI 
plot (lower panel). In contrast, in the cells that underwent actinomycin-induced 
nucleolar stress in the presence of caffeine, the cell cycle distribution of cells (right 
panels) was very similar to that observed in unstressed cells both in the Fucci dual 
color FACS and the DAPI plots. Figure 7C shows the results of combined nucleolar 
stress and caffeine or UCN-01 treatments in cells imaged at various times. The 
now-familiar, progressive accumulation of green cells after actinomycin treatment 
alone (second row) contrasts with the control-like patterns observed in the caffeine or 
UCN-01 treated cells. Based on the known mode of action of Chk1 (Wiksker et al. 
2008), its level of phosphorylation should correlate with the conditions that elicit G2 
arrest. As shown in Figure 8, phosphorylation of Chk1 was elevated 20 hours after a 2 
hour (second row, middle panel) or 4 hour (second row, middle right) actinomycin 
treatment, while no elevation was seen 20 hours after a 30 minute treatment (second 
row, middle left). Thus, the results in Figures 7 and 8 define the cell cycle arrest as 
ilying within G2 and reveal ATR/Chk1 as molecular elements in this regulatory 
circuit.

**DISCUSSION**

The most significant step in the modern era of the nucleolus was the perception that 
this nuclear domain does more than build ribosomes. This idea was born in a 
speculative synthesis (Pederson, 1998b) but soon got traction from numerous quarters, 
including the discovery that the nucleolus is the site of assembly of the signal 
recognition particle (Jacobson and Pederson, 1998; Ciufo and Brown, 2000; Politz 
and Pederson, 2000; Politz et al., 2000; Grosshans et al., 2001; Politz et al., 2002; 
Sommerville et al., 2005) and the findings that purified nucleoli harbor many proteins 
unrelated to ribosome biosynthesis but which have roles in cell cycle progression 
(Andersen et al., 2002; Pederson, 2002; Scherl et al., 2002). A prime exemplification 
of this has been work in budding (Shou et al., 1999; Visintin et al., 1999) and fission 
yeast (Trautmann et al., 2001) showing that Cdc14 phosphatase or Cdc14-like 
phosphatase trigger mitotic exit by release from the nucleolus (reviewed by Amon, 
2008). Increasing cases of nucleolar protein:cell cycle connections have been as well
in mammalian cells (Pederson and Tsai, 2009). The investigation reported here adds to the evidence that the nucleolus monitors cell cycle progression and that it does so outside of its role in ribosome synthesis. The notion that an interference with the non-ribosome production functions of the nucleolus could impact the cell cycle was previously raised in the context of a human disease - Diamond-Blackfan anemia. This is a neonatal-pediatric bone marrow deficiency caused by mutations in certain ribosomal proteins. A plausible case can be made that the pathogeneic trigger is an effect of these mutations on nucleolar homeostasis, rather than the production of functionally impaired ribosomes per se (Pederson, 2007).

There are two caveats in this study that warrant mention. The G2 arrest we have defined leads eventually to apoptosis (data not shown) so it is not likely to be a situation that would be tolerated in an intact organism unless the cues for apoptosis were overridden in certain cells or tissues. G2-arrested cells normally exist in many healthy mammalian tissues and are activated to enter mitosis promptly after a stimulus (Pederson and Gelfant, 1970). We of course cannot readily extrapolate the present finding with a cultured human cell line to how nucleolar stress might affect the cell cycle in an organism. The second caveat is that the mode of nucleolar stress we have chosen, viz. the selective inhibition of ribosomal RNA synthesis by a low concentration of actinomycin, presumes that the ribosomal RNA genes are the only target. The selectivity of actinomycin for these genes at such low concentrations is due to their very high (~70%) G+C content and the preference of actinomycin to intercalate at G-C base pairs. But there could be other sites in the genome with high concentrations of G-C pairs and we cannot rule out that the observed cell cycle effects might reflect transcriptional inhibition of these putative regions.

Actinomycin D (Waksman and Woodruff, 1940), first manufactured by Merck, Sharp and Dohme, was soon the subject of hopeful studies on tumors and cancer cell lines, yet the drug’s sites of action were completely unknown. We now know, in hindsight, that the concentrations used in almost of those early studies were ones that inhibited all three RNA polymerases. The key discovery (Perry, 1962) that a much lower concentration of the drug than had been used before selectively inhibits ribosomal RNA synthesis was a significant advance for the study of mammalian cell RNA biosynthesis. The use of low actinomycin to induce a nucleolar stress response, as we have done in this study, has brought new insight on the control of the cell cycle. One might ponder the extent to which the rare and paradoxical successes of this drug in cancer chemotherapy (e.g. it is the front-line standard for Wilm’s tumor) could have a nucleolar stress response as an underlying factor at the patient drug dosing employed. Recently, this notion has received some support from the development of a small molecule inhibitor of RNA polymerase I transcription that displays a significant cytostatic selectivity for human B-cell lymphoma and leukemia cells lines relative to normal lymphocytes (Bywater et al., 2012).
While we have implicated ATR-Chk1 in the G2 arrest induced by low actinomycin, it is obvious that we have not defined the entire pathway or interactome of this revealed circuit. There may be many other players in the overall regulatory link, amongst which may be one or more of the many cell cycle regulatory proteins that are known to constantly shuttle between the nucleolus and extra-nucleolar sites in the nucleus (Pederson and Tsai, 2009; Pederson, 2011). The fact that there is a major switch in the execution of this G2 arrest pathway depending on the duration of nucleolar stress points to the existence of unknown events that occur during the stress response which, either by the schedule of their execution or, alternatively, by the accumulated sum of their impacts, reach forward to have an effect many hours later, in G2. The study reported here thus reveals an important link between nucleolar stress and cell cycle progression, but also opens many questions for future investigation.

MATERIALS AND METHODS

Cell culture and induction of nucleolar stress
HeLa-Fucci cells (Sakaue-Sawano et al., 2008) were cultured at 37°C in Dulbecco-modified Eagle’s Minimum Essential Medium (DMEM, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). For induction of nucleolar stress, cells were exposed to actinomycin D (Sigma-Aldrich, St. Louis, MO) for various times. In the experiments to examine the signaling pathway responsible for the induced cell cycle arrest, cells were exposed to the ATM/ATR inhibitor caffeine at 2 mM or the Chk1 inhibitor UCN-01 at 200 nM (both inhibitors purchased from Sigma-Aldrich). For single cell tracking studies, cells were grown on Lab-Tek 2-well coverglasses in HEPES-buffered DMEM (Life Technologies 21063) containing 10% FBS, penicillin (100 units/ml) and streptomycin (100 µg/ml) and then overlaid with mineral oil. The microscope stage incubation chamber was maintained at 37°C as described previously (Jacobson and Pederson, 1977). Phase-contrast and fluorescence microscopy was performed with a Leica DM-IRB inverted microscope equipped a halogen lamp, a 10- position filter wheel (Sutter Instrument, Novato, CA), CFP/YFP/HcRed filter set (Semrock, Rochester, NY), a CCD camera (Photometrics, Tuscon, AZ) and MetaMorph acquisition software (Molecular Devices, San Jose, CA).

Flow cytometry
Cells were trypsinized and centrifuged at 200 g for 5 min., washed with phosphate-buffered saline (PBS) and then fixed with 4% formaldehyde in PBS for 10 min at room temperature, followed by one wash with PBS and permeabilization in 0.2% Triton X-100 in PBS for 10 min at room temperature and then another wash with PBS. The cells were resuspended at 1 x 10^6/ml in PBS containing DAPI at 2 µg/ml (Sigma-Aldrich) and incubated for 5 min. Fluorescence-activated cell sorting was performed in the UMass Medical School FACS Core Facility with a SLR II flow cytometer (BD Biosciences, San Jose, CA) using FACSDiva software (BD Biosciences. mVenus was excited by a 488 nm laser line and its emission was
collected using a 530/30 bandpass filter; mCherry was excited by a 561 nm laser line and its emission was collected using a 610/20 bandpass filter; Pacific Blue (the violet fluorescent tag on the reaction products in the RNA synthesis assay using 5-EU and click chemistry) was excited by a 405 nm laser line and its emission collected using a 450/50 bandpass filter. FACS measurements of DAPI fluorescence were done using the same 405 nm excitation and 450/50 filtered-emission as for Pacific Blue. FACS data were analyzed using FlowJo software (Tree Star, Ashland, OR). Sorting of cells for green fluorescence was done with a FACSARia II instrument (BD Biosciences).

**Real-time quantitative PCR**

A reverse transcription primer for 28S rRNA and pre-rRNA (5′-AGTTTACCACCCGCTTTGG-3′) was combined with total cell RNA and first-strand cDNAs were synthesized using SuperScript III reverse transcriptase (Invitrogen, Grand Island, NY). The resulting cDNAs were used as templates for real-time quantitative PCR with primer sets for either 28S rRNA (forward and reverse primers 5′-AGTAACGCCGAAGTGACCG-3′ and 5′-GCCTCGATCAAGAAGGACTTG-3′, respectively) or pre-rRNA (forward and reverse primers 5′-TCTCTCCGGTCGCTCT-3′ and 5′-TCTGATCTGAGGCGAGCC-3′, respectively), using the StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA) and the QuantiTect SYBR Green PCR kit (Qiagen).

**Imaging newly synthesized RNA or protein by click chemistry**

To detect RNA synthesis cells were treated with actinomycin for various times and then, either immediately or 20 hours later, were incubated for one hour with 5-ethynyluridine (5-EU) at 500 µM. The cells were rinsed with PBS, fixed in 4% formaldehyde in PBS, rinsed once with PBS and permeabilized with 0.5% Triton X-100 in PBS for 5 min. To image the sites of 5-EU incorporation, the coverslips were incubated for 30 min. at room temperature in a reaction cocktail (Invitrogen) containing Pacific Blue azide and the components for a copper (I)-catalyzed cycloaddition (“click” chemistry) of the ethynyl groups with the azide dye (Jao and Salic, 2008). The cells were then washed twice with PBS and imaged. The images were scaled the same using MetaMorph acquisition software (Molecular Devices, Sunnyvale, CA). For the detection of protein synthesis cells were treated with actinomycin for various times and then incubated for one hour with L-homopropargylglycine (HPG) at 100 µM (Beatty and Tirrel, 2008). The cells were rinsed with PBS, fixed in 4% formaldehyde in PBS, rinsed once with PBS and permeabilized with 0.2% Triton X-100 in PBS for 10 min. To image the sites of HPG incorporation, the coverslips were processed and imaged as above for detection of 5-EU incorporation.

**Immunofluorescence**

Cells grown on coverslips were fixed for 12 min in phosphate buffered saline (PBS) containing 4% formaldehyde, followed by permeabilization with 0.5% Triton X-100
for 5 min. Coverslips were then incubated with primary antibodies in PBS-1% bovine serum albumin (BSA) for 1 hour before washing and incubation with the pacific blue labeled secondary antibodies (Invitrogen). All these steps were carried out at room temperature. Coverslips were mounted in Prolong Antifade (Invitrogen) and imaged. The primary antibodies, dilutions and suppliers were: phospho-histone H2AX (S139) (JBW301) mouse monoclonal antibody (1:200, Millipore) and phospho-Chk1 (Ser317) (D12H3) Rabbit monoclonal antibody (1:200, Cell Signaling Technology).

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Figure 1. Actinomycin induced cell cycle arrest.
HeLa-Fucci cells were treated with 0.04 µg/ml actinomycin D for 4 hrs and fluorescence imaging and FACS analysis were carried out after 20 hrs of culture in drug-free medium. Left column: representative fields showing G1 cells (red) and late S/G2/M cells (green), with occasional yellow cells; scale bar, 20 µm. Scatter plots in the next column are FACS-based intensity distributions of the two cell cycle indicator proteins tagged with mCherry and Venus (Sakaue-Sawano el al., 2008) and the next two panels are standard DNA content profiles after DAPI staining. The right-most panels are DAPI plots broken down into three distributions for each cell cycle population: mCherry (red), mCherry + Venus (yellow), Venus (green) cells.
Figure 2. Actinomycin inhibition of nucleolar RNA synthesis.
HeLa-Fucci cells treated with actinomycin for the times shown, released into inhibitor-free medium for 0 or 20 hours, and then pulse labeled for one hour with 5-EU. The levels of newly synthesized nucleolar and nucleoplasmic were detected by a click chemistry-based fluorescence tagging procedure as detailed in the Material and Methods. A: RNA synthesis immediately after actinomycin treatment. B: RNA synthesis 20 hours after actinomycin treatment. Scale bar, 20 µm.
Figure 3. Cell cycle arrest is not due to a cell depletion of mature ribosomes.

Cells were treated with actinomycin for various times (0, 0.5, 2 and 4 hrs) and then cultured in inhibitor-free medium for 20 hours, followed by FACS selection of Venus-only cells, RNA isolation and RT-qPCR for either mature 28S rRNA (A) or pre-rRNA (B), using the primer sets indicated by the red and black arrows (see Materials and Methods for primer and RT-qPCR details). Three replicate PCR runs were performed for each pair of primers and the bar graphs shown are from the average values.
Figure 4. Cell cycle arrest is more pronounced after 2-4 hours of nucleolar stress. Cells were exposed to actinomycin for 30 min, 2 or 4 hours and the same multi-color FACS analyses of red, yellow, green and DAPI-stained (blue) cells were conducted as in Figure 1.
Figure 5. Single cell tracking.
The progression of selected cells was tracked after various times of actinomycin treatment. A: Release of the inhibitor was at mitosis. B: Release of inhibitor was at the G1/S transition. Scale bars, 10 µm.
Figure 6. Actinomycin D treatment does not lead to DNA damage.
Cells were treated with either actinomycin D or doxorubicin for the various times indicated. Phospho-H2AX was detected by immunofluorescence 20 hrs after treatment. Images were captured along with Fucci colors. Scale bar, 50 µm.
Figure 7. The arrest is ATR-Chk1 pathway dependent.
Cells were pretreated with either caffeine or UNC-01, subjected to nucleolar stress in the presence or absence of either inhibitor, and then cultured an additional 20 hours in medium lacking actinomycin but with either inhibitor. A: Images after a four hour nucleolar stress. Left four panels: caffeine; right four panels: UCN-01. Scale bar = 100 µm. B: Multi-color FACS analyses of the cells shown in A. C: Cells were exposed to ATR-Chk1 inhibitors for 2 hours, then actinomycin (with continued inhibitors) for 4 hours, and then examined at 0, 8, 16 or 24 hours. Scale bar, 50 µm.
Figure 8. Elevation of Chk1 phosphorylation after 2-4 hours of nucleolar stress. Cells were treated with either actinomycin D or doxorubicin for the various times indicated. Phospho-Chk1 was detected by immunofluorescence 20 hrs after treatment. Images were captured along with Fucci colors. Scale bar, 50 µm.
**Figure S1.** Protein synthesis after actinomycin treatment.
HeLa-Fucci cells were treated with actinomycin for the times shown, released into inhibitor-free medium for 20 hours, and then pulse labeled for one hour with L-homoproparglyglycine. The levels of newly synthesized protein were detected by a click chemistry-based fluorescence tagging procedure as detailed in the Material and Methods. Scale bar, 50 μm.

**Figure S2.** Timing cell cycle progression in control HeLa-Fucci cells.
HeLa-Fucci cells were cultured in Lab-Tek chambered coverglasses and single cells were tracked and imaged. Scale bar, 10 μm.
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**Fucci**

**Protein Labeling**