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Essential role of ribosomal protein L11 in mediating growth inhibition-induced p53 activation

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The ribosomal protein L11 binds to and suppresses the E3 ligase function of HDM2, thus activating p53. Despite being abundant as a component of the 60S large ribosomal subunit, L11 does not induce p53 under normal growth conditions. In search of mechanisms controlling L11–HDM2 interaction, we found that the induction of p53 under growth inhibitory conditions, such as low dose of actinomycin D or serum depletion, can be significantly attenuated by knocking down L11, indicating the importance of L11 in mediating these growth inhibitory signals to p53. We show that L11 is not regulated by transcription or protein stability and its level remains relatively constant during serum starvation. However, serum starvation induces translocation of L11 from the nucleolus to the nucleoplasm, where it participates in a complex with HDM2. We propose that the nucleolus acts as a barrier to prevent L11 interacting with HDM2 during normal growth. Growth inhibition, presumably through suppression of rRNA production in the nucleolus, facilitates translocation of L11 to the nucleoplasm, thus activating p53 through inhibiting HDM2.

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Introduction

The tumor suppressor protein p53 plays a pivotal role in preventing damaged or abnormal cells from becoming malignant, and its loss of function is associated with a majority of human cancers (Vogelstein *et al*, 2000; Ryan *et al*, 2001). The activity of p53 is not required during normal cell proliferation, and the protein must be kept at low levels and inactive. This is accomplished by the proto-oncoprotein HDM2 either through ubiquitin-dependent p53 degradation in the cytoplasm (Haupt *et al*, 1997; Honda *et al*, 1997; Kubbutat *et al*, 1997) or repression of p53 transcriptional activity in the nucleus (Momand *et al*, 1992; Thut *et al*, 1997). The HDM2 gene, in turn, is transcriptionally activated by p53, constituting a feedback regulatory loop (Barak *et al*, 1993; Wu *et al*, 1993). Growing evidence has shown that p53 is regulated primarily by its protein stability (Ashcroft and Vousden, 1999). Major mechanisms of p53 stabilization and activation include the one that is triggered by DNA damage and induces p53 protein phosphorylation (Shieh *et al*, 1997; Giaccia and Kastan, 1998; Meek, 1999), or the one in response to aberrant growth signals, such as oncogenic Ras or Myc, which is mediated by $p14^{ARF}$ ($p19^{ARF}$ in mouse) (Sherr and Weber, 2000). In the past, a major focus of p53 research has been devoted to understanding p53 function and its regulation of DNA damage, and cell cycle progression; little is known about the connection between p53 function and ribosomal biogenesis, even though ribosomal biogenesis occupies a significant part of a cell's life. Whether and how p53 may be involved in checking ribosomal stress and the integrity of ribosomal biogenesis remains unclear (Ruggero and Pandolfi, 2003).

Recent studies have identified that the ribosomal protein L11 interacts with HDM2, stabilizes and activates p53, and induces a cell cycle arrest (Lohrum *et al*, 2003; Zhang *et al*, 2003). This is achieved, at least in part, through L11 inhibiting the E3 ligase activity of HDM2 (Zhang *et al*, 2003). To date, mechanisms of p53 activation mostly involve stress-mediated induction of signaling proteins, such as checkpoint kinases or ARF, which are either unexpressed or maintained at low levels under unstressed conditions. L11, however, as a component of the 60S large ribosomal subunit exists abundantly in normal growing cells, yet it does not induce p53. How does the abundant L11 not activate p53 under normal conditions and what physiological stimulus triggers the L11–HDM2 interaction are the focus of this study.

Results

L11 induces a p53-dependent cell cycle arrest in normal human fibroblast cells

Recently, it has been shown that overexpression of L11 induces a cell cycle arrest that is dependent on the presence of p53 (Lohrum *et al*, 2003; Zhang *et al*, 2003). These experiments were carried out in U2OS tumor cells transfected with L11 plasmid DNA. To recapitulate the L11-induced, p53-dependent cell cycle arrest in normal cells, we produced adenovirus expressing a myc-tagged L11 and used it to infect normal human fibroblasts WI-38 and the isogenic WI-38/E6, in which p53 is under negative regulation by a retroviral-mediated expression of the oncogene *E6* of the human papilloma virus (HPV) that promotes p53 degradation (Scheffner *et al*, 1993). Consistent with previous observations in U2OS cells, ad-myc-L11 infection in WI-38 cells increased p53 levels, as well as the levels of HDM2 and p21—two proteins whose genes can be transactivated by p53 (Figure 1A, lanes 1–4). The high levels of p21 and HDM2 in

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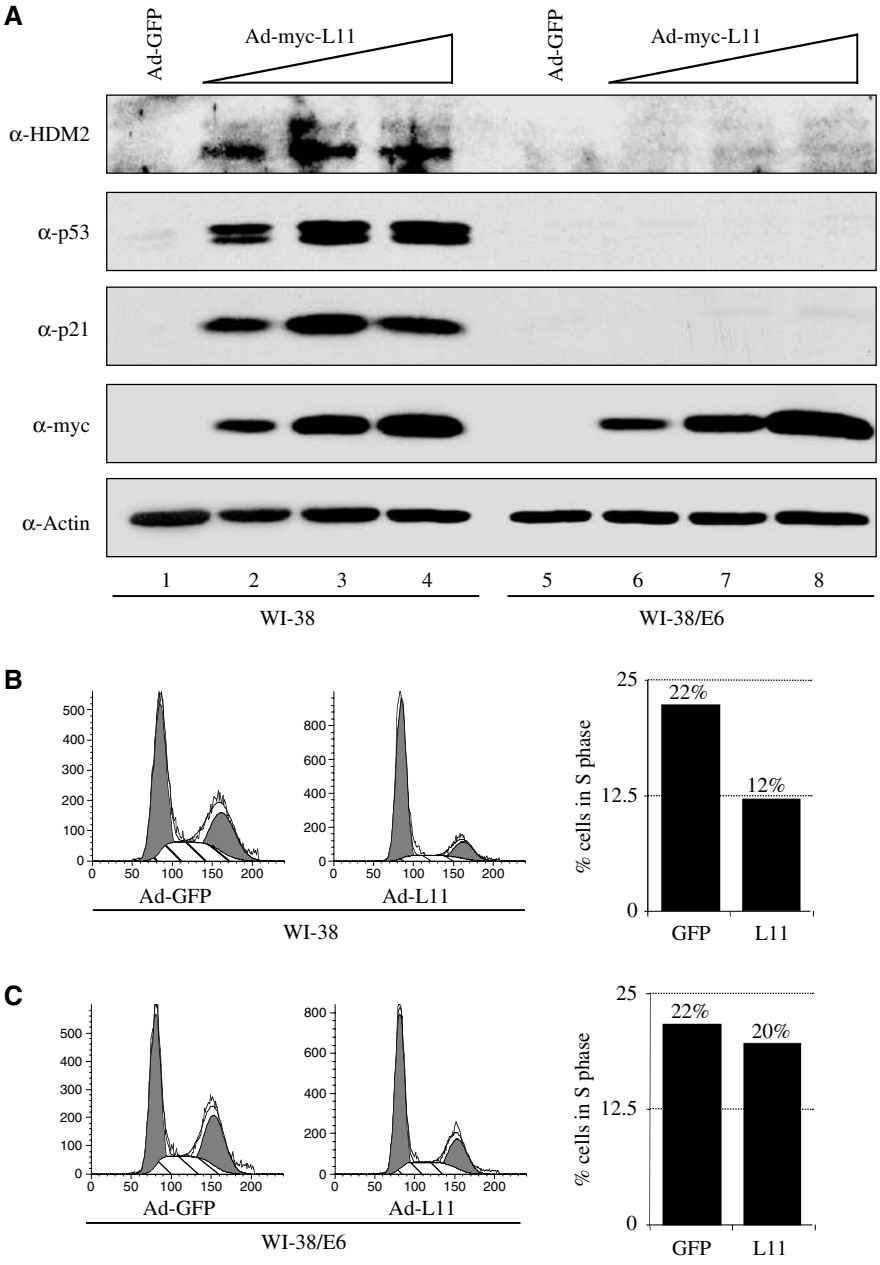

Figure 1 L11 induces a p53-dependent cell cycle arrest in normal human fibroblast cells. (**A**) WI-38 or WI-38/E6 cells were infected with adenovirus expressing either GFP or myc-L11 at a multiplicity of infection (m.o.i.) of approximately 5. At 48 h post-infection, the cells were lysed with a 0.5% NP-40 lysis buffer, and Western blotting was performed with antibodies as indicated. (**B, C**) Cells were infected as described in (A) and were subjected to FACS analysis. Bar graphs represent the percentage of the total number of cells in the S phase.

ad-myc-L11-infected WI-38 cells are likely the result of a stabilized and activated p53, since in WI-38/E6 cells, even though L11 was expressed to comparable levels, p21 and HDM2 were not induced (Figure 1A, lanes 5–8). We noticed an increased cell death when L11 was expressed to very high levels (our unpublished observation), which may have caused the decrease of p21 (lane 4) (Allan and Fried, 1999). The consequence of p53 activation by L11 was also determined by analysis of cell cycle distribution in WI-38 and WI-38/E6 cells. Consistent with the Western blot analysis, L11 induced cell cycle arrest only in WI-38 but not in WI-38/E6 cells, and the arrested cells accumulated mostly in the G_1 phase, indicating an activated function of p53 and p21 (Figure 1B and C). Together, our data show that the overexpression of L11 increases the protein level and the transactivation activity of p53, thus causing cell cycle arrest in normal fibroblast cells. In the absence of p53, however, L11 does not induce cell cycle arrest.

L11 and HDM2 are essential for actinomycin D-induced p53 accumulation and cell cycle arrest

In search of biological functions of the L11–HDM2 interaction, we have tested several cellular stress signals that might affect the expression of L11 or L11–HDM2 binding. Neither UV treatment nor overexpression of the oncogene E2F1 apparently affected the level of L11 or the interaction of L11

with HDM2 (Zhang *et al*, 2003 and data not shown), suggesting that L11 may not respond to DNA damage or abnormal oncogenic insult. It has been shown that actinomycin D treatment of cells augments the interaction of L11 with HDM2 (Lohrum *et al*, 2003; Zhang *et al*, 2003), and induces the expression of p53 (Ashcroft *et al*, 2000; Zhang *et al*, 2003). Actinomycin D has been used as a chemotherapeutic drug in the treatment of a variety of human cancers (da Rocha *et al*, 2001). At high concentrations (e.g. >30 nM) actinomycin D causes DNA damage and inhibits transcription from all three classes of RNA polymerase promoters, whereas at low concentrations (e.g. <10 nM) actinomycin D selectively inhibits RNA pol I-dependent transcription and thus ribosomal biogenesis (Perry and Kelley, 1970; Iapalucci-Espinoza and Franze-Fernandez, 1979).

Consistent with previous reports, we found that 5 nM actinomycin D causes cell cycle arrest in WI-38 but not in WI-38/E6 cells (data not shown), suggesting that at this concentration, actinomycin D arrests cells by activating p53. To investigate the role of L11 in mediating actinomycin D-induced p53 activation and cell cycle arrest, we carried out experiments using small interference RNA (siRNA) to knock down endogenous L11 in U2OS cells. Knocking down L11 by more than 50% (see Figure 2D for the level of L11 reduction) did not apparently affect cell growth in U2OS cells (Figure 2A). However, when the siRNA-transfected cells were followed by treatment with a 5 nM actinomycin D, knocking down L11 significantly attenuated actinomycin D-induced cell cycle arrest (Figure 2B), indicating that L11 is essential for mediating the actinomycin D effect. Consistent with the attenuated cell cycle arrest, knocking down L11 also attenuated actinomycin D-induced p53 stabilization and activation as shown in Figure 2D. To determine the specificity of L11 in mediating only actinomycin D-induced, but not DNA damage-induced, cell cycle arrest, we treated a parallel set of U2OS cells with 4 Gy of gamma irradiation followed by cell cycle analysis. Knocking down L11 had no effect on gamma irradiation-induced cell cycle arrest, nor did it affect p53 activation, indicating that L11 may not function in the DNA damage pathway to induce p53 (Figure 2C and data not shown).

It has been shown that L11 directly interacts with HDM2 but not p53 (Zhang *et al*, 2003). To establish the role of HDM2 in actinomycin D-induced p53, we utilized mouse embryo fibroblast (MEF) cells with defined genetic background. As shown in Figure 3, endogenous p53 in wild-type (WT) MEF cells can be induced by 5 nM actinomycin D (lanes 1 and 2). Similarly, ectopically expressed human p53 was stabilized by treating cells with 5 nM actinomycin D in $p53^{-/-}$ MEF cells, but not in $MDM2^{-/-}/p53^{-/-}$ MEF cells, strongly suggesting that MDM2 (the mouse counterpart of HDM2), like L11, is also required for actinomycin D-induced p53 activation (Figure 3, lanes 3–8). Taken together, our data demonstrate that a low level of actinomycin D-induced p53 activation and cell cycle arrest depend on both L11 and HDM2.

L11 is essential for serum starvation-induced p53 stabilization and cell cycle arrest

Three evidences led us to speculate that L11 might function in growth inhibition-induced p53 activation and cell cycle arrest. First, a low level of actinomycin D specifically inhibits rRNA transcription—an essential step in ribosomal biogenesis and cellular growth. Second, DNA damage-induced p53 does not require L11. Third, as a component of the 60S ribosomal subunit L11 should naturally be involved in ribosomal function and cell growth. However, actinomycin D is a pharmaceutical drug that does not exist physiologically in cells. To seek evidence of whether L11 might function in mediating growth inhibitory signals under normal physiological conditions, we examined the regulation of L11 in contact-inhibited and serum-starved WI-38 cells. It has been previously shown that serum withdrawal causes human cells to undergo a reversible G_0/G_1 cell cycle arrest in a p53-dependent manner (Itahana *et al*, 2002). When WI-38 cells grew to confluence in tissue culture dishes, p53 level was markedly increased as well as its downstream targets, HDM2 and p21, and cells were arrested in the G_1 and G_2 phases (Figure 4A). Interestingly, we observed a parallel increase of L11 in the confluent, contact-inhibited cells, which correlated with the increase of p53 and p21, suggesting that the increase of L11 might be related with the increase of p53. Similarly, WI-38 cells arrested by serum starvation (0.2% serum) not only had elevated levels of p53, HDM2, and p21, but also a parallel elevated level of L11 (Figure 4B), suggesting that serum starvation-induced p53 activation and cell cycle arrest, like that of contact inhibition, might also be related with the high level of L11. The increase of p53 by contact inhibition or serum starvation was also observed by immunofluorescence staining in WI-38 cells (Figure 4C). Adding serum back to the serum-starved cells resumed cell growth and decreased the levels of p53 and its downstream target HDM2, as well as the level of L11, further indicating that the varying levels of p53 and L11 may be related (Figure 4D).

To determine whether L11 is indeed responsible for serum starvation-induced p53 stabilization and activation, we transfected WI-38 cells with L11 siRNA to knock down endogenous L11 followed by culturing the cells in a 0.2% serum medium. By knocking down L11, serum starvation-induced p53 stabilization and activation were evidently attenuated, demonstrating that L11 is indispensable for efficient p53 induction under such growth inhibitory conditions (Figure 5A). Consistent with the attenuated p53 induction by serum starvation after knocking down L11, serum starvation-induced cell cycle arrest was also attenuated (Figure 5B). We propose that the attenuated p53 activation by knocking down L11 is due to the disruption of a specific signaling pathway mediated by L11, and not due to protein synthesis shutdown, because both the nascent protein synthesis (Figure 5C) and the level of 60S ribosomal subunits (Figure 5D) appeared unaffected by knocking down L11. However, we have noticed that prolonged (more than 3 days) knockdown of L11 could eventually induce cell cycle arrest in WI-38 cells. This is consistent with studies showing that in normal cells there exists a stringent surveillance system to monitor the integrity of ribosomal biogenesis that is compromised in tumor cells (Rotenberg *et al*, 1988; Volarevic *et al*, 2000; Du and Stillman, 2002). Thus, our data demonstrate that under certain physiological growth inhibitory conditions, such as serum starvation, L11 is induced in parallel with p53, and the induction of L11 is the likely cause of induction of p53. Taken together with the data showing requirement of L11 and HDM2 in mediating actinomycin D-induced p53 activation and cell cycle arrest, we

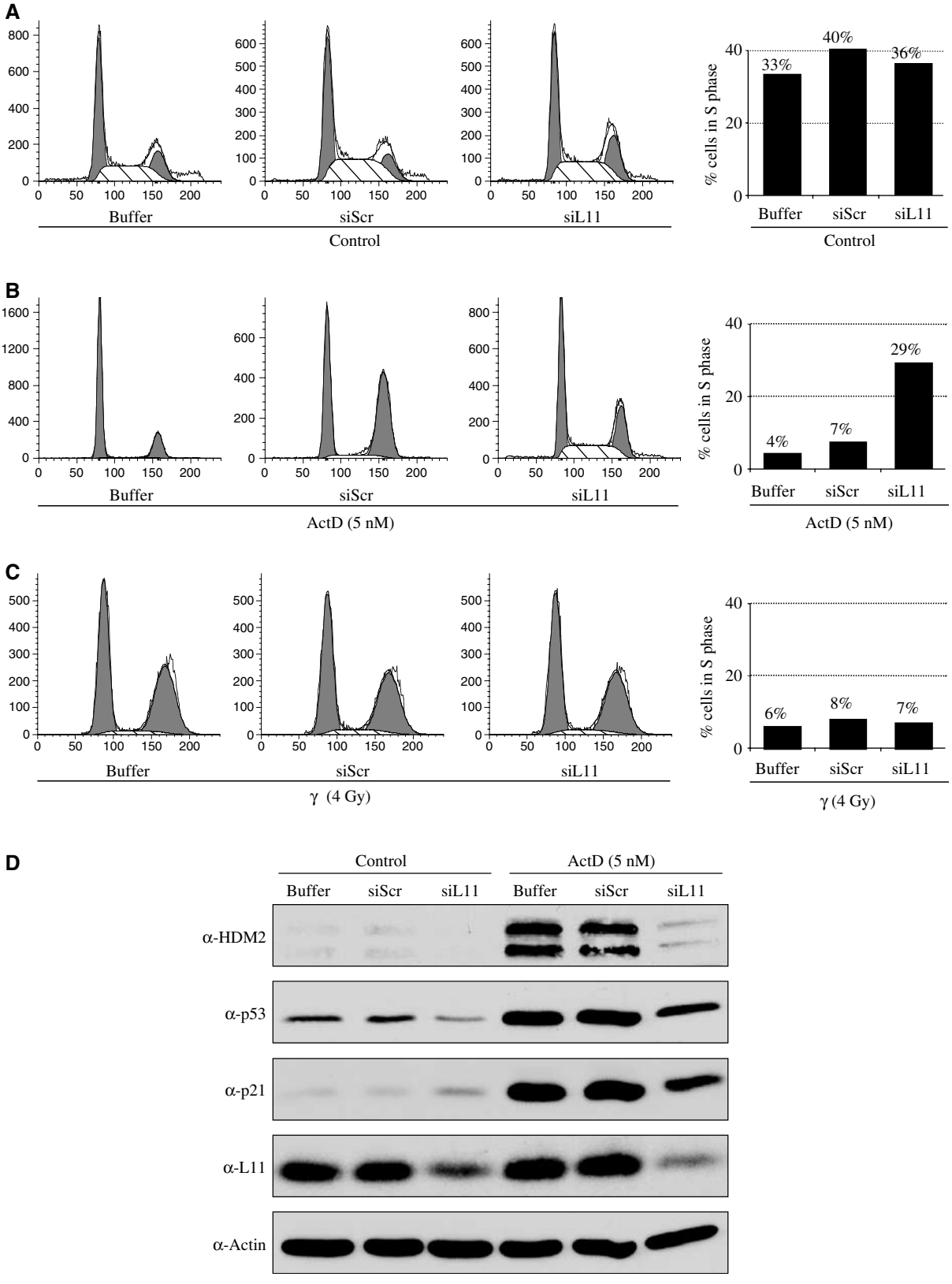

Figure 2 L11 is essential for actinomycin D-induced p53 accumulation and cell cycle arrest. U2OS cells were transfected with a 21-bp RNA duplex targeting L11 (siL11) or with a control 21-bp scrambled RNA duplex (siScr). At 12 h after transfection, cells were untreated (**A**), treated with 5 nM of actinomycin D (**B**), or exposed to 4 Gy of gamma irradiation (**C**) for an additional 16 h and were collected for cell cycle distribution analysis by FACS assay. In parallel, untreated or actinomycin D-treated cells were collected and the expression of various proteins was determined by Western blotting with indicated antibodies (**D**).

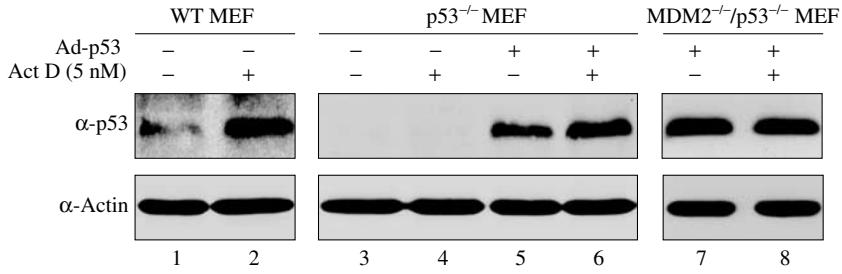

Figure 3 Actinomycin D induces MDM2-dependent p53 activation. MEF cells were either mock-infected (WT MEFs) or infected with adenoviruses expressing human p53 ($p53^{-/-}$ and $p53^{-/-}$/$MDM2^{-/-}$ MEFs) for 24 h followed by actinomycin D treatment as indicated for an additional 24 h. Endogenous mouse p53 (WT MEFs) and overexpressed human p53 levels were detected by Western blotting analysis with Ab-3 antibody (Calbiochem).

propose an L11–HDM2–p53 pathway that represents a checkpoint in surveillance of abnormal ribosomal functions.

L11 is not regulated by transcription or protein stability

The accumulation of L11 upon growth inhibition (Figures 4A and B) can be explained by two possible mechanisms: either an increase in transcription or an increase in protein stability. To determine whether L11 is transcriptionally activated by growth inhibition, WI-38 cells were serum-starved and the total L11 transcripts were determined. As shown in Figure 6A, the level of L11 mRNA remained constant up to 1 day after serum starvation. In contrast to an obvious increase of L11 protein levels after 1-day serum starvation (Figure 4B), these results indicate that the increase of L11 by serum starvation may not be through transcriptional activation. We then examined the protein stability of L11 by a half-life assay. WI38 cells were treated with 10 μg/ml puromycin to block protein synthesis (Pestka, 1974), followed by Western blotting to determine the protein levels of L11 and p53. p53, being a fast turnover protein, had a half-life of about 30 min (Figure 6B). Under the same experimental conditions, however, L11 appeared to be very stable and its level was without any discernible change after 180 min of chasing (Figure 6B). Thus, the increased protein levels of L11 observed during growth inhibition may not be due to an altered L11 protein stability.

If L11 is neither regulated by transcription nor by protein stability, then how does its level increase upon growth inhibition? In search of a mechanism regulating the changes of L11, we considered its subcellular localization. As a ribosomal protein, L11 was found to localize in the nucleolus where it participates in ribosomal precursor assembly, and in the cytoplasm where it is a part of the mature, membrane-bound ribosome/polysome, with very little found in the nucleoplasm (our unpublished observations). The distinct localization of L11 (in the nucleolus and the cytoplasm) and HDM2 (predominantly in the nucleoplasm) under unstressed conditions may explain why the abundant L11 does not normally interact with HDM2 and activate p53. The NP-40 lysis buffer used in our Western blot analysis (Figures 4A, B, and D) penetrates the cytoplasmic and nuclear membranes and extracts soluble proteins (Harlow and Lane, 1988), but does not completely dissolve nucleolus or nuclear/cytoplasmic membranes, especially under conditions where cells are only incubated in the lysis buffer for a limited time (e.g. 20 min in our experiments). The undissolved nucleoli and membranes, a rich source of L11, are precipitated as cell debris and are excluded from the estimation of L11 protein levels. Therefore, L11 in the NP-40 lysis buffer may represent only the soluble fraction, but not the total amount of the protein. To determine whether the total L11, including those in the nucleolus and the membrane, are also increased upon serum starvation, we lysed cells in a 2% SDS–dithiothreitol (DTT) loading buffer and analyzed the level of L11 by Western blotting. Surprisingly, total L11 analyzed by the SDS lysis did not change after serum starvation, whereas p53 levels increased in the SDS lysate similar to that observed with the NP-40 lysate (Figure 6C). Thus, our data show that the total L11 in cells remain relatively constant under normal and growth inhibitory conditions, whereas the NP-40-soluble L11 fluctuates. The data also imply that the conventional NP-40 lysis cannot completely extract nucleolar- or membrane-bound proteins, and caution must be exercised when estimating the level of ribosomal proteins.

L11 translocates from the nucleolus to the nucleoplasm upon serum starvation

The above results led us to hypothesize that the higher levels of L11 in the NP-40 lysate upon growth inhibition might reflect translocation of L11 protein from the nucleolus/membrane-bound state to a free, nucleoplasm/cytoplasm-localized state. To prove directly that L11 is regulated by translocation, we carried out a fractionation assay to separate cell lysates into nucleolar and nucleoplasmic fractions before and after serum starvation. As expected, under normal growing conditions, L11 levels were higher in the nucleolus compared to the nucleoplasm in WI-38 cells (Figure 7A, lanes 1 and 2). Upon serum starvation, the nucleolar L11 was evidently decreased and the nucleoplasmic L11 accumulated (Figure 7A, lanes 3 and 4). As controls, the distribution of the nucleolar protein B23 (also known as nucleophosmin, numatrin, or NO38), which is localized predominantly in the nucleolus with some accumulation in the nucleoplasm (Spector *et al*, 1984), and the nuclear protein PCNA were not dramatically altered by serum starvation (Figure 7A). To further distinguish whether the accumulated L11 in the nucleoplasm upon growth inhibition is free, ribosome-unbound L11, or the 60S ribosomal subunit-incorporated L11, we carried out a chromatography fractionation assay to separate soluble proteins from ribosomal monosomes/polysomes in normal growing and serum-starved WI-38 cells. Cell lysates were fractionated on a 9–45% sucrose gradient, and

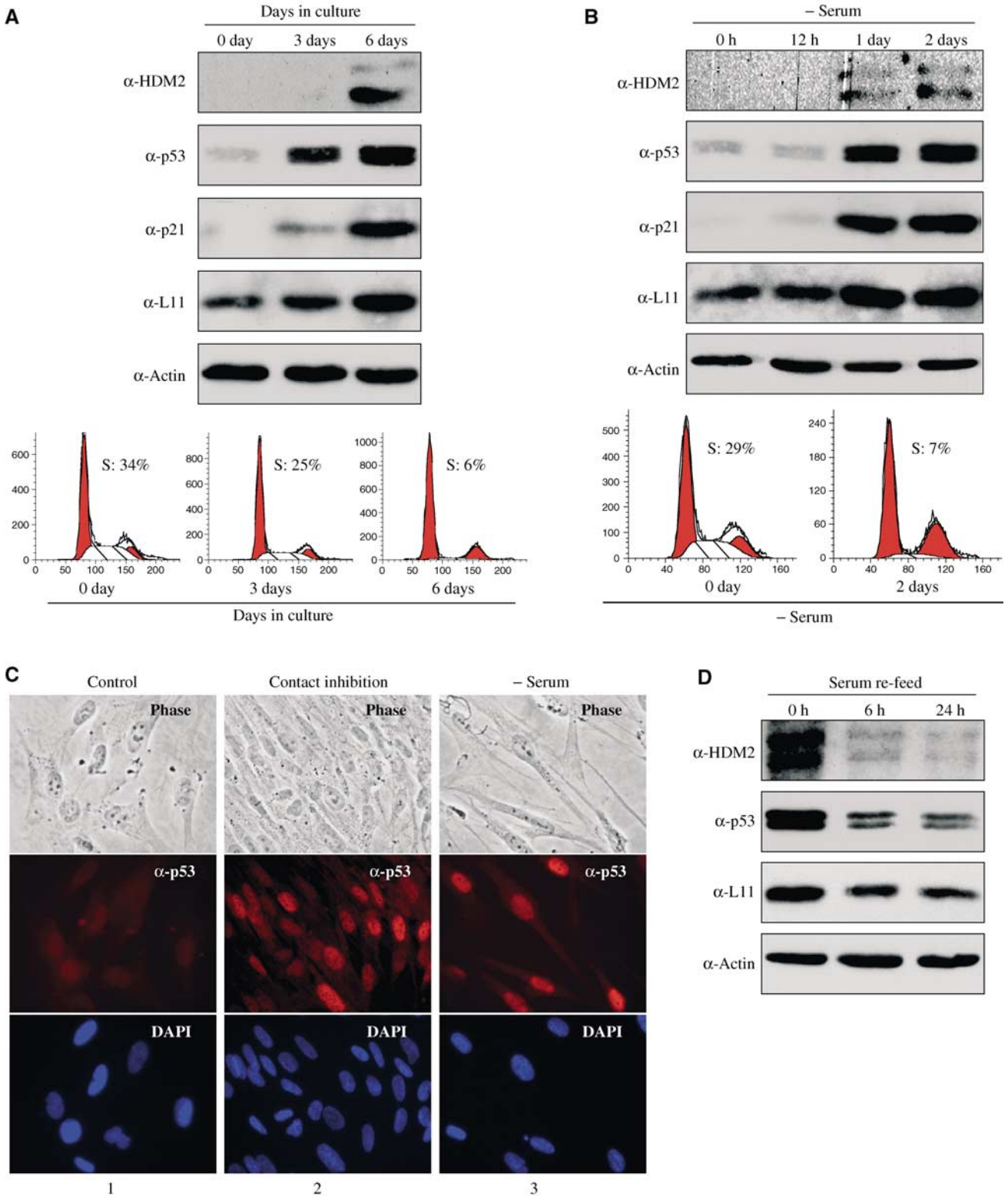

Figure 4 Fluctuation of L11 protein levels correlates with p53 levels under physiological growth inhibitory conditions. (**A**) For contact inhibition studies, WI-38 cells seeded at a density of 3×10^5 cells per 60 mm dishes were allowed to grow in the presence of DMEM + 10% FBS and cells were collected on days as indicated. (**B**) Serum starvation was carried out in DMEM media containing 0.2% FBS for the indicated time points. Duplicate plates of contact-inhibited or serum-starved WI-38 cells were either lysed after the indicated time points for Western analysis (top panels) or subjected to FACS analysis (bottom panels). (**C**) Control, contact-inhibited (5 days in culture), or serum-starved WI-38 cells were fixed with paraformaldehyde and immunostained with p53 antibody as shown. Nuclei were visualized by DAPI staining. Cells were photographed with equal exposure for each set. (**D**) Serum-starved (24 h) WI-38 cells were re-fed with DMEM media containing 10% FBS cells for the indicated time points, and the respective proteins were detected by Western blotting.

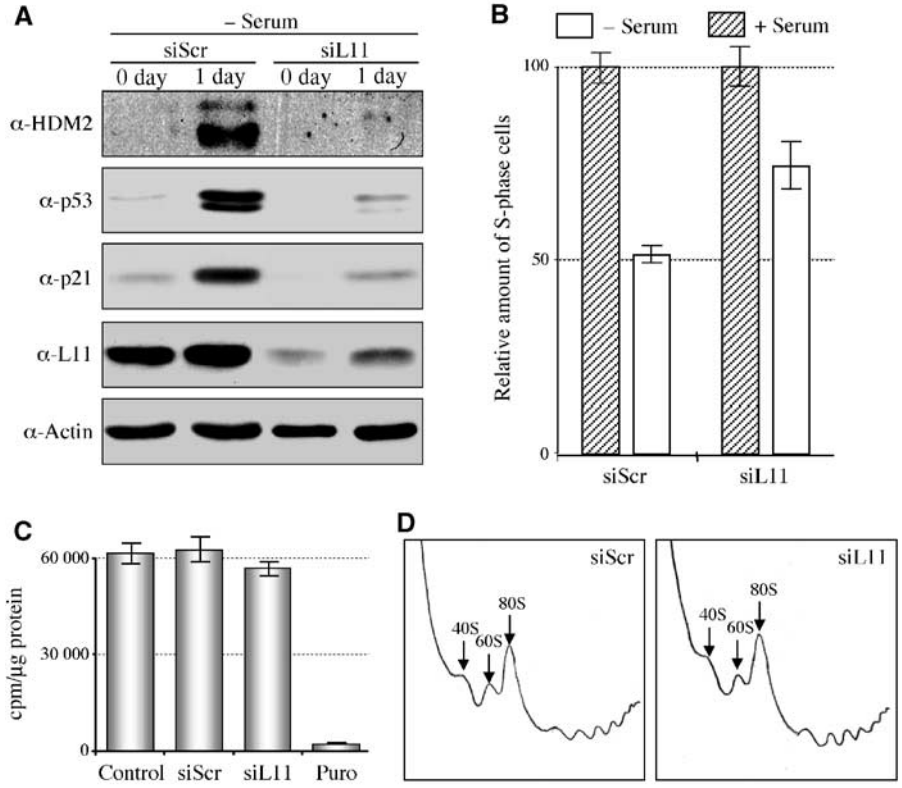

Figure 5 L11 is essential for p53 activation upon serum starvation. Duplicate sets of WI-38 cells were transfected with siRNA duplexes targeting L11 (siL11) or with a control scrambled RNA duplex (siScr) for 12 h followed by serum starvation for an additional 24 h. (**A**) One set of the cells were lysed with 0.5% NP-40 lysis buffer and protein expression was determined by Western blotting with indicated antibodies. (**B**) Another set of the cells was collected by trypsinization and was subject to FACS analysis. The bar graph in (B) represents the relative amount of the S-phase population compared by setting the control cells (+serum) as 100% and is the average of three measurements. (**C**) WI-38 cells were transfected with siRNA as described above for 48 h and the total [^{35}S]methionine incorporation over 2 h, as a measure of nascent protein synthesis, was analyzed by a scintillation counter. The bar graphs represent the average of triplicate experiments. Puromycin was used as a positive control to inhibit protein synthesis. (**D**) Polysome profiles of WI-38 cells after transfected with siRNA for 48 h. The positions of the individual ribosome subunits are indicated.

the positions of ribosomal subunits (40S, 60S), ribosomal monomers (80S), and polysomes in the fractions were continuously measured by a UV monitor (chromatograms in Figure 7B). Consistent with a previous report (Duncan and McConkey, 1982), serum starvation caused a decrease of polysomes and an increase of 80S monosomes. The collected fractions were then analyzed for indicated proteins by Western blotting (three bottom panels in Figure 7B). Notably, the levels of L11 in the soluble, ribosome-unbound fractions were evidently increased after serum starvation, indicating that L11 was released from the ribosomal subunits as a free, soluble protein in the cell lysate (Figure 7B). As a control of the fractionation, the level of p53 in the serum-starved cells was increased and was detected only in the soluble but not in the ribosomal fractions.

To determine whether the accumulated L11 in the nucleus and in the soluble fractions after serum starvation was in a complex with HDM2, we carried out IP–Western assay to assess the level of HDM2-bound L11 in WI-38 cells growing under normal and serum-starved conditions. As shown in Figure 7C, serum starvation not only induced the expression of HDM2 in WI-38 cells but also augmented the level of L11 that bound with HDM2 (compare lanes 1 and 2, lanes 5 and 6). To discriminate whether the increase of HDM2-bound L11 was simply due to an increase in the level of HDM2 or was serum starvation-specific, we either treated cells with MG-132 or infected cells with ad-HDM2 to bring up HDM2 to a similar level as it was seen by serum starvation. Interestingly, HDM2 that was stabilized by MG-132 or was accumulated by ad-HDM2 infection, even though expressed at a higher level than that with serum starvation, precipitated less L11 (Figure 7C, compare lanes 2 and 4, lanes 6 and 8). Together with the data in Figures 7A and C, this increase in the amount of L11 co-precipitating with HDM2 in serum-starved samples compared to those treated with MG-132 or ad-HDM2 strongly argues that serum starvation specifically mediates translocation of L11 from the nucleolus to the nucleoplasm, thus enhancing the L11–HDM2 interaction.

Finally, in search for an explanation that justifies the translocation of L11 upon serum starvation, we considered the inhibition of ribosomal RNA synthesis, which involves addition of ribosomal proteins onto an rRNA backbone in the nucleoli, as a possibility (Scheer and Hock, 1999). Inhibition of rRNA synthesis could lead to disassembly of ribosomal precursors and release ribosomal-unbound ribosomal proteins from the nucleolus. To test this hypothesis, we pulse-labeled WI38 cells with [^{3}H]methionine and followed by isolation of total RNA. The amount of radioactivity, which represents only the newly synthesized rRNA precursor, was determined by a scintillation counter. As shown in Figure 7D,

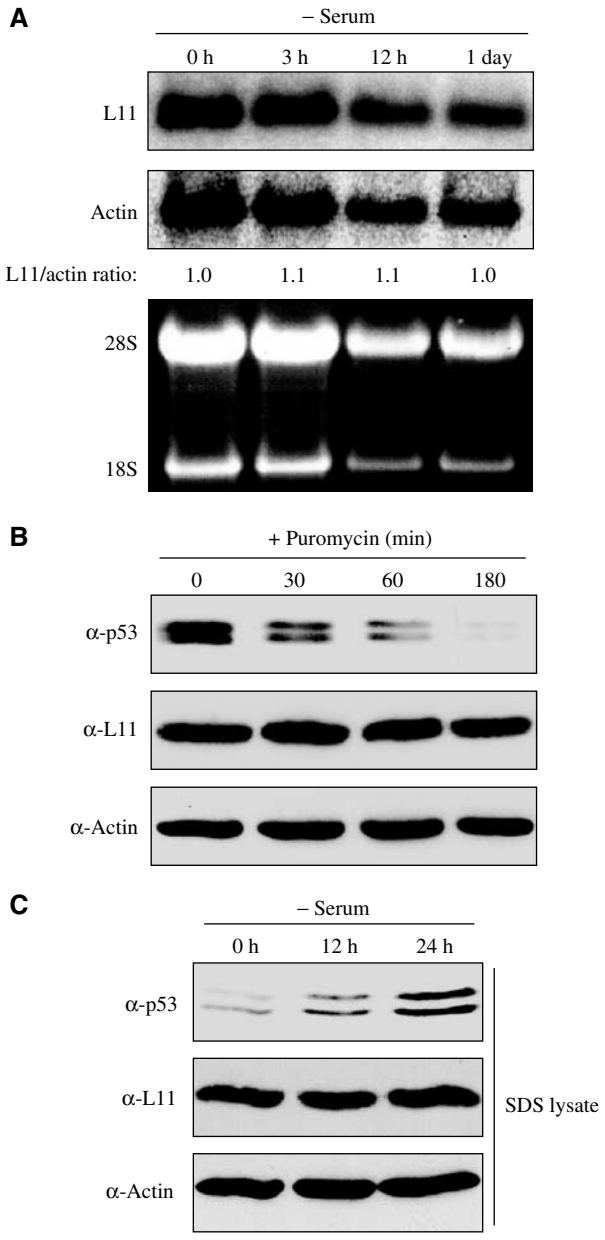

Figure 6 L11 is not regulated by transcription or protein stability and the total level of L11 does not change under growth inhibitory conditions. (**A**) WI-38 cells were serum-starved for the indicated time points and the L11 mRNA levels were analyzed by Northern blotting with a DNA probe containing the entire L11- or actin-coding sequences. (**B**) WI-38 cells were treated with 10 μg/ml puromycin for 60 min followed by collecting cells at indicated time points and analyzed by Western blotting. (**C**) WI-38 Cells were collected by trypsinization and lysed directly with 2% SDS–DTT protein gel loading buffer and the cell lysates were separated by SDS–PAGE followed by Western blotting.

rRNA synthesis was reduced by approximately four times after cells were serum-starved for 24 h. Hence, growth inhibition, by serum starvation, causes a reduction of rRNA synthesis and this is a probable cause for the translocation of L11 from the nucleolus. Consistent with this notion, ribosomal protein S6, which is part of the 40S ribosomal subunit, also translocated from the nucleolus to the nucleoplasm and accumulated in the soluble, ribosome-unbound fractions after serum starvation (Figures 7A and B). In conclusion, we have found that growth inhibition, presumably through inhibition of rRNA production, releases L11 from the nucleolus and the free L11 enters nucleoplasm to interact with HDM2, thus activating p53.

Discussion

Previous studies have shown that perturbation of ribosome function leads to cell cycle arrest. For example, deletion of the ribosomal protein gene S6 in mouse or L16 in yeast, or a gene involved in ribosomal biogenesis, such as Yph1p in yeast, is sufficient to block cell proliferation (Rotenberg *et al*, 1988; Volarevic *et al*, 2000; Du and Stillman, 2002), indicating that in cells there exists a signaling pathway to communicate perturbations of ribosomal biogenesis to cell cycle control. The involvement of p53 in monitoring the status of ribosomal biogenesis has been shown in a study in which depletion of ribonucleotide pool induces a p53-dependent cell cycle arrest that does not involve DNA damage (Linke *et al*, 1996). Recently, it has been shown that functional inactivation of Bop1, a nucleolar protein involved in rRNA processing and ribosomal assembly, leads to a p53-dependent cell cycle arrest (Pestov *et al*, 2001). These studies, although unclear about the molecular mechanisms, demonstrate that in multicellular organisms the ribosome status is constantly monitored, and at least some of the monitoring system involves the function of p53.

Our study demonstrates that L11 plays an important role in mediating ribosomal stress/growth inhibition to induce a p53-dependent cell cycle arrest. This effect is probably mediated by L11 binding to and inhibiting the E3 ligase function of HDM2, and consequently stabilizing and activating p53 (Lohrum *et al*, 2003; Zhang *et al*, 2003). Unlike DNA damage- or aberrant oncogene-induced p53 activation, which requires transactivation or induction of proteins (such as checkpoint kinases or ARF) that are usually maintained at basal levels in unstressed cells, L11 is always abundant in cells regardless of the growing conditions. Then, how is the L11–HDM2 interaction prevented under normal, favorable growth conditions, and promoted during growth inhibitory conditions? As shown in this study, nucleolar L11, in response to growth inhibitory signals, translocates to the nucleoplasm, where it interacts with HDM2 and induces p53. Through translocation and switching binding partners (from an rRNA backbone to HDM2), L11 employs a distinct mechanism in p53 activation.

It is conceivable that L11 plays a dual role as either a component of the 60S ribosomal subunit for protein synthesis under growth favorable conditions, or a component of the HDM2–p53 pathway in regulating cell cycle progression under growth inhibitory conditions. We therefore propose a 'swing' model to explain the dual physiological roles of L11, in which growth inhibition by suppression of ribosomal biogenesis (e.g. by serum starvation), or a general perturbation of nucleolar function (e.g. by actinomycin D), would release L11 from the nucleolus (Figure 8). A recent study showing that the disruption of the nucleolus mediates a stabilization of p53 provides support to our model (Rubbi and Milner, 2003). L11 may not act alone in mediating ribosomal stress to the HDM2–p53 pathway. Other ribosomal proteins, including L5 (Marechal *et al*, 1994) and L23 (our unpublished data), have been found to interact with HDM2. It is tempting to propose that the interaction of ribosomal

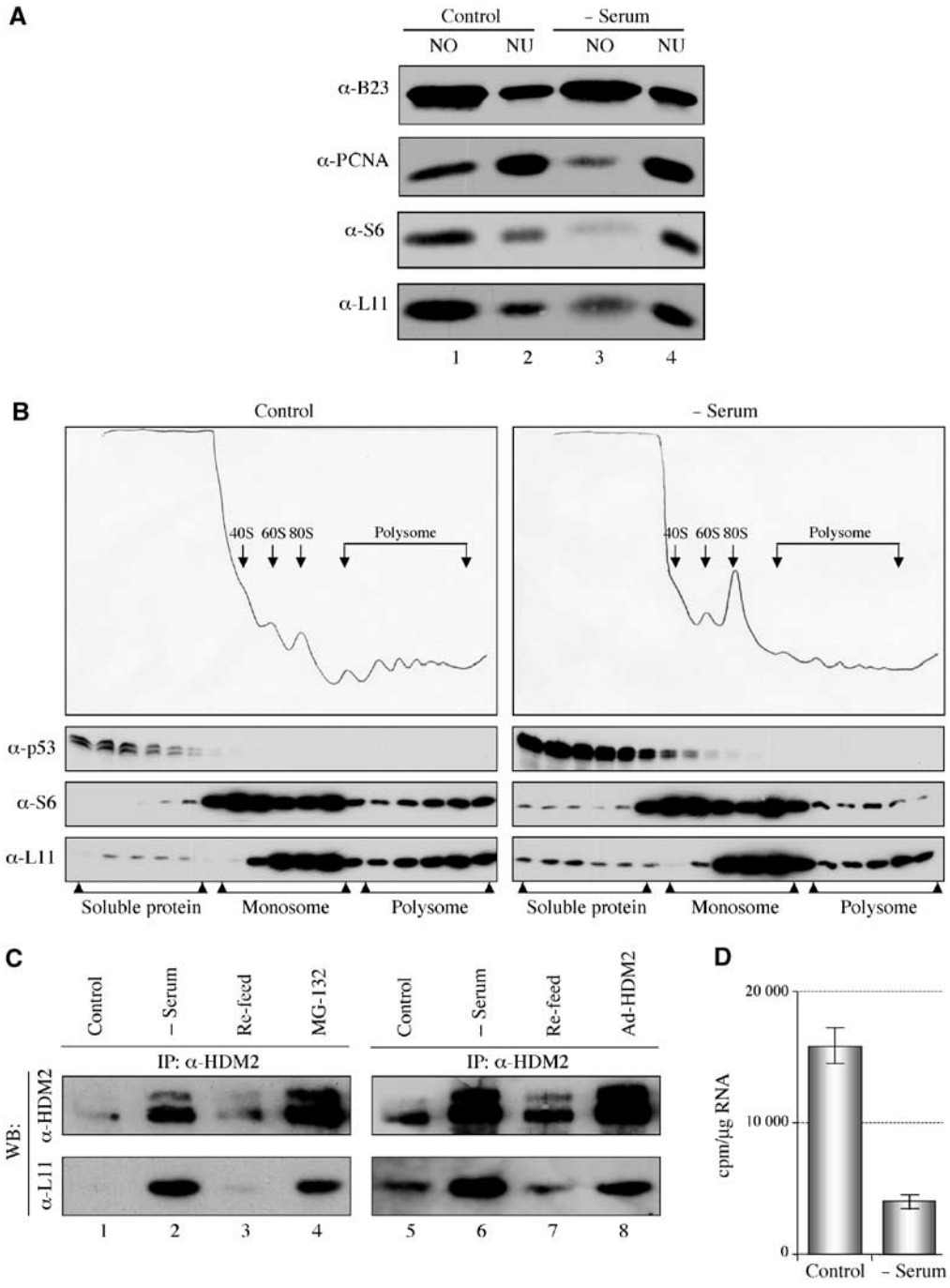

Figure 7 L11 translocates from the nucleolus to the nucleoplasm upon growth inhibition. (**A**) WI-38 cells were serum-starved for 24 h and equal number of cells were subjected to cell fractionation as described in Materials and methods. The nucleolar (NO) and nucleoplasmic (NU) fractions were isolated and analyzed for protein expression with antibodies as indicated. (**B**) Polysome profiles from control (left panel) or serum-starved (right panel) WI-38 cells are shown. The fractions were precipitated with 20% TCA, and the precipitated proteins were analyzed by Western blotting. (**C**) Control, serum-starved, serum re-fed, or treated (MG-132, 10 μM, 8 h; or Ad-MDM2 infection, 24 h) WI-38 cells were lysed with 0.5% NP-40 lysis buffer and immunoprecipitated with HDM2 (4B11) antibody followed by Western blotting to detect HDM2 and L11. (**D**) WI-38 cells were pulse-labeled with [methyl-^{3}H]methionine for 30 min to label the newly synthesized ribosomal RNA precursors, followed by isolation of total RNA. The radioactivity of the RNA was measured by a scintillation counter as described in Materials and methods. The bar graphs represent the average of duplicate experiments.

proteins with the HDM2–p53 pathway may represent a surveillance system to safeguard the integrity of ribosomal biogenesis in higher order eukaryotic cells, and to coordinate growth with proliferation. In this regard, the idea that mutations either in *L11* or *HDM2* that inhibit their interaction could have developed in certain human cancers seems not too far-fetched and needs to be explored.

Materials and methods

Cell culture, vectors, and FACS analysis

U2OS cells from ATCC (Manassas, VA), WT, p53$^{-/-}$, and p53$^{-/-}$/MDM2$^{-/-}$ MEF cells from Dr G Lozano (UTMD Anderson Cancer Center, Houston, TX), and WI-38 cells from Dr J Campisi (Lawrence Berkeley National Laboratory, Berkeley, CA) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing

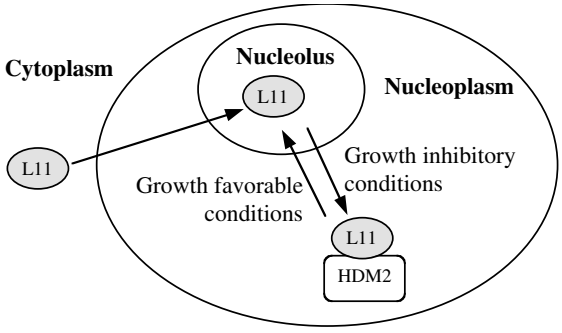

Figure 8 A 'swing' model for the dual physiological roles of L11. See text for details.

10% FBS in a 37°C incubator with 5% CO_2. Actinomycin D was dissolved in 100% ethanol as a 5 μM stock and a final concentration of 5 nM was used in all treatments. For serum starvation experiments, WI-38 cells were plated at 5×10^5 cells/100 mm dish. After 24 h, cells were washed three times with PBS and DMEM media containing 0.2% serum was added for the indicated time points. Adenoviruses expressing p53 and myc-tagged L11 were generated by contract with a Core Facility (Baylor College of Medicine, Houston, TX). For flow cytometric analysis, cells were collected after the indicated treatments by trypsinization, fixed with 70% ethanol, stained with propidium iodide, and analyzed by FACS as described previously (Zhang *et al*, 1998).

Protein and mRNA analysis

For Western blotting, cultured cells were lysed using standard 0.5% NP-40 lysis buffer unless otherwise indicated. mRNA analysis was performed as described elsewhere (Itahana *et al*, 2002). Immunofluorescence and immunoprecipitation experiments were carried out as described previously (Zhang and Xiong, 1999). p53 (D01, Lab Vision-Neomarkers; Ab421, Calbiochem; FL393, Santa Cruz Biotechnology; Ab-3, Calbiochem), p21 (HZ52, Lab Vision-Neomarkers), HDM2 (IF-2 and 4B11, Calbiochem), B23 (Zymed Laboratories), Myc (9E10, Lab Vision-Neomarkers), actin (MAB1501, Chemicon International), S6 (Cell Signaling Technology), and PCNA (RB9055, Lab Vision-Neomarkers) monoclonal or polyclonal antibodies were all purchased commercially. Rhodamine red-, fluorescein isothiocyanate (FITC)-conjugated donkey secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. Rabbit polyclonal anti-human L11 antibody was produced using a synthetic peptide (CIGAKHRISKEEAMRWFQQK) corresponding to amino-acid residues 149–168 of human L11.

RNA interference

Purified and annealed duplex siRNA oligonucleotides targeting nucleotides 363–383 relative to the translation initiation codon of human L11, and a control scrambled siRNA oligonucleotide were commercially synthesized at Dharmacon (Lafayette, CO). Transfection (20–30 nM of RNA) was performed using Lipofectamine and Plus reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions for 12–14 h followed by serum starvation or actinomycin D treatment.

Cell lysate fractionation

Nucleoli and nucleoplasmic fractions were isolated from WI-38 cells as described previously with slight modifications (Muramatsu *et al*, 1974). Control or serum-starved WI-38 cells grown in five 100 mm Petri dishes were trypsinized, pelleted, washed with PBS, and centrifuged at 1500 rpm for 5 min. The cell pellet was then resuspended in 1 ml of RSB buffer (0.01 M Tris–HCl, 0.01 M NaCl, 1.5 mM $MgCl_2$, pH 7.4) and placed on ice for 10 min. Swollen cells were then collected by centrifugation at 1500 rpm for 8 min, and resuspended in 1 ml of RSB buffer containing 0.5% NP-40. Cell membranes were then broken using a Dounce homogenizer (tight pestle) by 10 up and down strokes. Nuclei (pellet) were collected by centrifugation at 1500 rpm for 8 min and resuspended in 0.75 ml of 0.25 M sucrose, 10 mM $MgCl_2$ and overlaid onto 1 ml of 0.88 M sucrose, 0.05 mM $MgCl_2$ and purified by centrifugation again at 2500 rpm for 10 min. The pelleted nuclei were resuspended in 1 ml of 0.34 M sucrose, 0.05 mM $MgCl_2$ and sonicated for 60 s with 10 s intervals. The sonicated cell lysate was then overlaid onto 1 ml of 0.88 M sucrose, 0.05 mM $MgCl_2$ and centrifuged at 13 000 rpm for 20 min. The pellet contained the purified nucleoli and the supernatant represented the nucleoplasm. All buffers contained 1 × protease inhibitors cocktail added freshly before use.

Ribosome fractionation

For sucrose density gradient fractionation of ribosomes, cells were scraped and collected after adding cycloheximide (50 μg/ml) in culture for 5 min. Cells were then lysed using 0.5% NP-40 lysis buffer containing 130 mM KCl, 10 mM $MgCl_2$, 10 μg/ml cycloheximide, 0.2 mg/ml heparin, 200 U/ml Rnasin, and 2.5 mM DTT for 4 h at 4°C. The lysates were centrifuged at 8000 *g* for 5 min and the supernatants were collected and measured for RNA concentration at OD_{260}. The lysates containing 150 μg of RNA were loaded on a 9–45% sucrose gradient. The gradients were centrifuged at 36 000 rpm for 3 h at 4°C using a Beckman SW40Ti rotor and fractionated by detecting absorbance at 254 nm using an EM-1 UV monitor (Bio-Rad, Hercules, CA). The fractions were collected and the proteins were concentrated using 20% trichloroacetic acid (TCA) and analyzed by Western blotting.

Analysis of rRNA and nascent protein synthesis

For examining ribosomal RNA synthesis, pulse-chase experiments using [methyl-^{3}H]methionine were carried out (Strezoska *et al*, 2000). Cells were starved of methionine for 30 min on 60 mm plates with methionine-free medium and were labeled with 50 μCi of L-[methyl-^{3}H]methionine (Amersham-Pharmacia) for 30 min. After washing with PBS, total RNA was purified by an RNA isolation system (Promega) and the incorporated radioactivity was measured by a liquid scintillation counter. For measuring nascent protein synthesis, WI-38 cells were washed twice with PBS and starved in methionine (Met)-free DMEM media supplemented with dialyzed 10% FBS for 30 min and then labeled at 37°C for 2 h by adding 10 μCi [^{35}S]Met (Amersham) per 100 mm plate. Cells were then washed thoroughly with PBS, lysed with 0.5% NP-40 buffer, total proteins were precipitated with 20% ice-cold TCA, pelleted and dissolved in 0.1 N NaOH. Equal amounts of protein were then counted in a scintillation counter for methionine incorporation.

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