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Depletion of the Nucleolar Protein Nucleostemin Causes G1 Cell Cycle Arrest via the p53 Pathway

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Nucleostemin (NS) is a nucleolar protein expressed in adult and embryo-derived stem cells, transformed cell lines, and tumors. NS decreases when proliferating cells exit the cell cycle, but it is unknown how NS is controlled, and how it participates in cell growth regulation. Here, we show that NS is down-regulated by the tumor suppressor p14^{ARF} and that NS knockdown elevates the level of tumor suppressor p53. NS knockdown led to G1 cell cycle arrest in p53-positive cells but not in cells in which p53 was genetically deficient or depleted by small interfering RNA knockdown. These results demonstrate that, in the cells investigated, the level of NS is regulated by p14^{ARF} and the control of the G1/S transition by NS operates in a p53-dependent manner.

INTRODUCTION

Destabilization or inactivation of the tumor suppressor p53 is typically required to maintain cell proliferation (Vogelstein *et al*., 2000). However, it is unclear which of many potential upstream regulators of p53 is responsible for cell cycle progression or exit. Nucleostemin (NS) is a nucleolar protein discovered in adult rat brain stem cells, and it is also preferentially expressed in embryo-derived stem cells, transformed cell lines, and human tumors (Tsai and McKay, 2002; Baddoo *et al*., 2003; Liu *et al*., 2004; Politz *et al*., 2005). NS dynamically shuttles between the nucleolus and nucleoplasm, and this exchange is based on its state of GTP binding (Tsai and McKay, 2005). Pull-down and coimmunoselection experiments reveal that NS interacts with p53 (Tsai and McKay, 2002), but whether NS controls cell proliferation in a p53-dependent manner remains unclear.

In a previous investigation, we found that the NS is concentrated in regions of the nucleolus that are relatively devoid of nascent ribosomes (Politz *et al*., 2005). We subsequently asked whether any other cell cycle progression-related proteins might occupy this same intranucleolar territory, and we found that the tumor suppressor p14^{ARF} (alternate reading frame [ARF]) precisely colocalizes with NS in these ribosome-sparse nucleolar regions (Supplemental Figure 1). Like NS, ARF is linked to p53, and yet NS and ARF play opposite roles in cell proliferation. These initial findings, therefore, motivated us to investigate whether ARF might regulate NS, and we found that it does. In turn, this led to the finding that NS regulates cell cycle progression via the p53 pathway.

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Abbreviations used: NS, nucleostemin; ARF, alternate reading frame.

MATERIALS AND METHODS

Cell Culture and Transfection

U2OS, Saos-2, and HeLa cells were cultured at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS). The NARF6 cell line (Stott *et al*., 1998) was maintained in DMEM containing 10% FBS, 150 μg/ml hygromycin, and 300 μg/ml Geneticin (G-418; Invitrogen, Carlsbad, CA). For transient transfections, cells were plated in 35-mm dishes, and plasmid DNA was introduced using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. ARF expression in NARF6 cells was induced by the addition of isopropyl-β-D-thiogalactopyranoside at 1 mM for 48 h. For analysis of cell cycle progression, U2OS and Saos-2 cells were exposed to 10 μM 5-bromodeoxyuridine (BrdU) for 24 h, and incorporation was determined by immunostaining (see below).

Plasmids

Human NS cDNA was cloned from HeLa cell total RNA (Stratagene, La Jolla, CA) by reverse transcription-polymerase chain reaction and cloned into the pmRFP-C1 vector (Campbell *et al*., 2002) at the XhoI and HindIII sites, resulting in the plasmid pmRFP-hNS-C1. Human ARF cDNA was cloned into the pEGFP-N1 vector (Clontech, Mountain View, CA) at the XhoI and HindIII sites, resulting in the plasmid pEGFP-ARF-N1. The plasmid pARF-N1 was generated by insertion of human ARF cDNA into pEGFP-N1 at the BamHI and NotI sites, resulting in excision of the enhanced green fluorescent protein (EGFP) coding sequence.

Small Interfering RNA (siRNA) Knockdown

The siRNA sequence used for depletion of human NS was that described by Tsai and McKay (2002). The other siRNA sequences used were human ARF: GCUUCCUAGAAGACCAGGUdTdT; human p53: AAGACUCCAGUGGUAAUCUACdTdT; and human retinoblastoma-associated protein (Rb): GAUACCAGAUCAUGUCAGAdTdT.

The nontargeting siCONTROL siRNA (Dharmacon RNA Technologies, Lafayette, CO) was used as a negative control. siRNAs were purchased as duplexes from Dharmacon RNA Technologies and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols.

Immunofluorescence

Cells grown on coverslips were fixed for 12 min in phosphate-buffered saline (PBS) containing 4% formaldehyde, and washed twice in PBS, 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 for 1 h and washed three times with PBS before 1-h incubation with the appropriate secondary antibodies, and finally washed three times with PBS. All these

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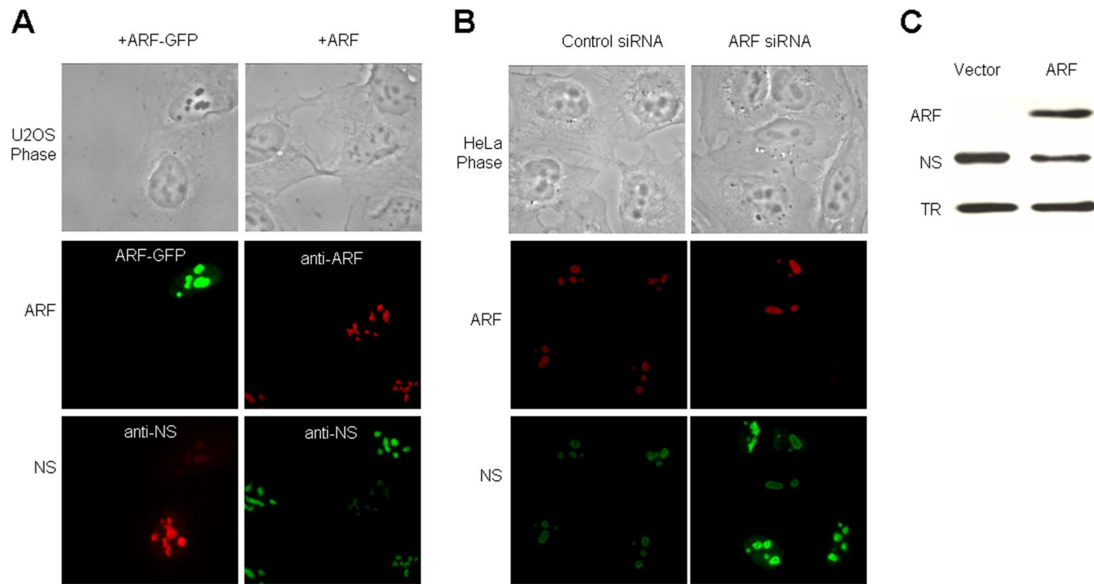

Figure 1. Down-regulation of nucleostemin by ARF expression. (A) U2OS cells were transfected with ARF-GFP or with ARF itself. After 24 h, NS was detected by immunostaining, and ARF was detected by immunostaining (right column) or by the fluorescence of ARF-GFP (left column). (B) Control or ARF siRNAs were transfected into HeLa cells, and ARF and NS were detected by immunostaining 24 h later. (C) Western blot performed 24 h after transfection of U2OS cells with vector DNA or the ARF-encoding plasmid. Transferrin receptor (TR) was used to assess equivalence of cell extract loading.

steps were carried out at room temperature. Coverslips were mounted in Prolong Antifade (Invitrogen), and two- or three-dimensional images were captured and in some cases subjected to deconvolution as described previously (Politz *et al*., 2005). The antibodies and dilutions used were as follows: rabbit anti-human NS polyclonal antibody (1:200; Chemicon International, Temecula, CA), mouse anti-human p14^{ARF} monoclonal antibody (mAb) 4C6/4 (1:400, Abcam, Cambridge, MA), mouse anti-human p53 mAb DO-1 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-human MDM2 mAb SMP-14 (1:50; Santa Cruz Biotechnology), and mouse anti-human Rb mAb G3-245 (1:50; BD Biosciences, San Jose, CA).

In the BrdU experiments, cells were fixed in ice-cold methanol for 15 min, and then they were immunostained for NS as described above. The coverslips were then refixed and incubated in 4 N HCl for 20 min at room temperature, and incorporation of BrdU was detected by immunostaining with mouse anti-BrdU mAb 3D4 (1:250; BD Biosciences). The percentage of BrdU-labeled nuclei was determined.

Immunoblotting

Cells were lysed on ice in 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 7.4 containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) as specified by the manufacturer. Protein concentration was determined using the BCA assay (Pierce Chemical, Rockford, IL). Samples were boiled with 2× Laemmli buffer and electrophoresed on 10% polyacrylamide gels containing 0.1% SDS, followed by transfer to Immobilon-P membranes (Millipore, Billerica, MA), and then they were incubated with specific primary antibodies. Proteins of interest were detected with appropriate horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence substrate (Pierce Chemical). The antibodies used were rabbit anti-human nucleostemin (1:5000; Chemicon International), mouse anti-human p14^{ARF} (4C6/4, 1:500; Abcam), mouse anti-human p53 (DO-1, 1:500), and mouse anti-human transferrin receptor (clone H68.4, 1:1000; Zymed, South San Francisco, CA). Reactive bands of interest were quantified by densitometry.

RESULTS AND DISCUSSION

U2OS is a human osteosarcoma cell line that does not express ARF. When either ARF-GFP or ARF itself was expressed in U2OS cells, the level of NS significantly declined (Figure 1A). Western blotting showed that ARF expression decreased the level of NS by ~50% in the cell population (Figure 1C). Conversely, the level of NS became elevated when endogenous ARF was knocked down in HeLa cells (Figure 1B). Thus, the level of nucleostemin responds to either an increase or decrease in ARF. Because knockdown of NS did not affect the level of ARF in HeLa cells (data not shown), it would seem that ARF is an upstream regulator of NS.

NS is thought to interact with p53 (Tsai and McKay, 2002) whereas ARF is known to stabilize p53 by binding MDM2, a ubiquitin ligase that normally degrades p53 (Haupt *et al*., 1997; Kubbutat *et al*., 1997; Stott *et al*., 1998). To examine the possible links among ARF, NS, and p53, we used the U2OS-derived cell line NARF6, in which ARF expression is inducible (Stott *et al*., 1998). Expression of ARF in NARF6 cells resulted in a pronounced increase of p53 and a significant decline of NS (Figure 2A). This result does not distinguish between a direct action of ARF that destabilizes NS versus an indirect effect in which ARF stabilizes p53 (*vide supra*), which causes NS to be destabilized. To determine whether the level of NS is related to that of p53, U2OS cells were subjected to UV-induced DNA damage under conditions known to elevate p53. As can be seen in Figure 2B, this resulted in a significant decrease of NS. This result is consistent with the interpretation that experimental elevation of ARF (Figure 2A) may negatively impact NS via a stabilization of p53.

It was next logical to ask whether NS modulates p53. As shown in Figure 3A, knockdown of NS led to an elevation of p53. Immunoblot analysis of these cells (Figure 3C) revealed an ~60% knockdown of NS and an approximately threefold elevation of p53. To ask whether the increased p53 is functional, we examined the level of MDM2 after NS knockdown. Functional p53 acts as a transcriptional activator of MDM2 gene expression, so an elevation of MDM2 protein would imply functionality of the elevated p53. As can be seen in Figure 3B, the level of MDM2 was indeed increased in cells in which NS was knocked down, in support of the hypothesis that the p53 induced by NS knockdown is functional. We also investigated whether knockdown of p53

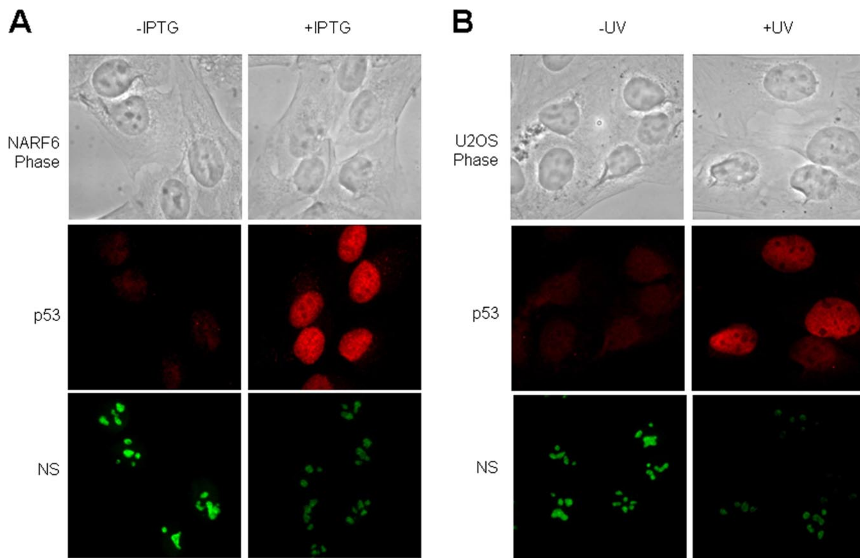

Figure 2. Down-regulation of NS coincides with up-regulation of p53. (A) NARF6 cells (a U2OS-derived ARF-inducible cell line) were immunostained for p53 and NS after 48-h treatment with 1 mM isopropyl-β-D-thiogalactopyranoside. (B) U2OS cells were subjected to 254-nm irradiation (10 J/cm^2) to activate p53, and then they were immunostained 16 h later for p53 and NS.

affects the level of NS and found that it does not (Supplemental Figure 2), indicating that NS acts as an upstream regulator of p53.

A role of NS in cell cycle progression is indicated by the fact that its depletion in U2OS cells reduces S phase entry, that blastocysts or fibroblasts from NS +/− embryos display haploinsufficiency with respect to growth rate, and that NS −/− blastocysts fail to enter S phase (Tsai and McKay, 2002; Beekman *et al.*, 2006; Zhu *et al.*, 2006). Based on our finding that depletion of NS up-regulates p53, which plays a key role in the surveillance of cell cycle progression, we hypothesized that the p53 pathway might be involved in cell cycle arrest in NS-deficient cells. We therefore investigated cell cycle progression in U2OS (p53 positive, Rb positive, and ARF negative) and Saos-2 cells (p53 negative, Rb negative, and ARF negative) as a function of NS and p53 expression levels. Compared with cells transfected with a control siRNA, knockdown of NS caused a significant decrease in the percentage of cells entering S phase in U2OS cells but not in Saos-2 cells (Figure 4, A and B). These results thus suggest that NS depletion-induced G1 cell cycle arrest may require p53. To address this issue, p53 was knocked down in U2OS cells (Supplemental Figure 2). As expected, this did not affect the percentage of cells traversing S as these cells are already cycling at a high rate (Figures 4C, left two columns, and D). NS knockdown again reduced cell cycle progression (Figure 4D). However, when p53 was knocked down in addition to NS, the percentage of cycling cells returned to the same high level as seen in control cells (Figure 4D), providing direct evidence that cell cycle arrest induced by depletion of NS is mediated by the p53 pathway.

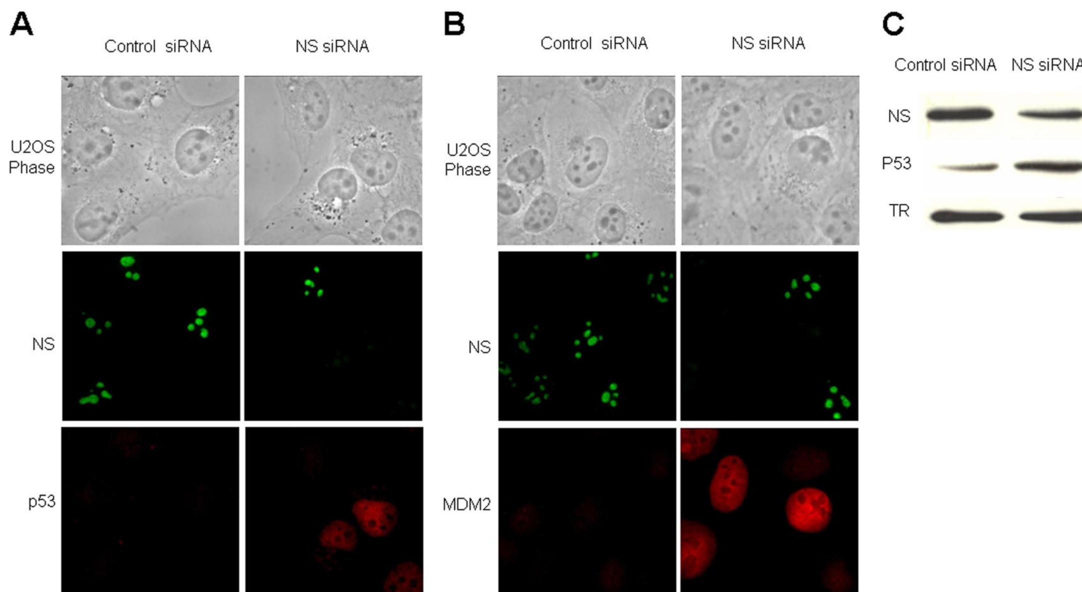

Figure 3. Knockdown of NS leads to elevation of p53 and MDM2. Control or NS siRNAs were transfected into U2OS cells. (A) NS and p53 were detected by immunostaining after 48 h. (B) NS and MDM2 were detected after 48 h. (C) Western blot performed 48 h after transfection with control or NS siRNAs.

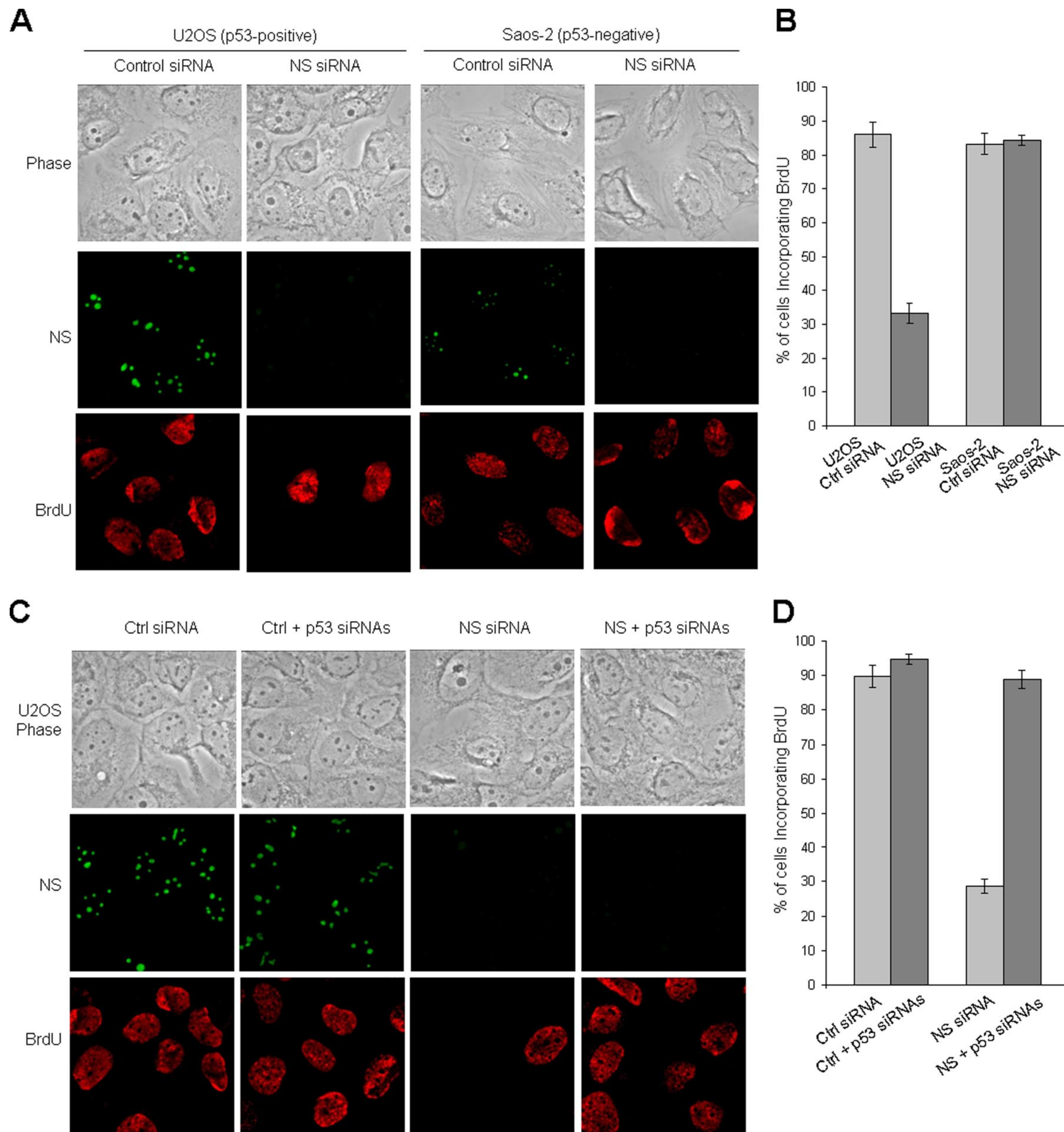

Figure 4. Loss of p53 restores cell cycle progression in NS knocked down cells. (A) U2OS cells (p53 positive) and Saos-2 (p53 null) were transfected with control or NS siRNAs, respectively, and 10 μM BrdU was added 72 h later. After 24 h, the cells were sequentially immunostained for NS and BrdU. (B) The percentages of cells that had incorporated BrdU were determined. Each bar indicates the mean ± SD of data from three independent experiments. (C) U2OS cells were transfected with control siRNA, control + p53 siRNAs, NS siRNA, or NS + p53 siRNAs. BrdU (10 μM) was added 72 h later and after another 24 h, the cells were immunostained for NS and BrdU. (D) Each bar indicates the mean ± SD of data from three independent experiments.

We next asked whether NS might also control cell cycle through the Rb pathway. In contrast to our finding with p53, we found that NS depletion-induced G1 arrest was not rescued by Rb depletion (Figure 5, A and B, and Supplemental Figure 3), thus indicating that NS does not operate via the Rb pathway, at least in U2OS cells.

p53 is a pivotal regulatory protein that evokes cell cycle arrest in response to numerous stress signals including hypoxia, nutrient depletion, heat shock, and DNA damage (Vogelstein *et al.*, 2000; Bensaad and Vousden, 2005; Vousden, 2006). Because these stimuli often trigger nucleolar disorganization and p53 activation, it has been suggested that the nucleolus plays a role in modulating the cell cycle's response to stress (Rubbi and Milner, 2003; Horn and Vousden, 2004; Raska *et al.*, 2006). This idea has received additional support from the finding that under such conditions the level of p53 can be coordinated by a variety of nucleolar proteins, including the ribosomal proteins S6, L5, L11, L23, and L26 as well as TIF-IA, nucleolin, Bop1, B23 (nucleophosmin), ARF, PML, and WRN (Blander *et al.*, 1999; Pestov *et al.*, 2001; Colombo *et al.*, 2002; Daniely *et al.*, 2002; Lohrum *et al.*, 2003; Bernardi *et al.*, 2004; Dai and Lu, 2004; Jin *et al.*, 2004; Sulic *et al.*, 2005; Takagi *et al.*, 2005; Yuan *et al.*, 2005; Raska *et al.*, 2006). It is also of interest to recall that the nucleolus contains mitogenic growth factors such as fibroblast growth factor and angiogenin (Pederson, 1999), an observation that now might be productively revisited given the

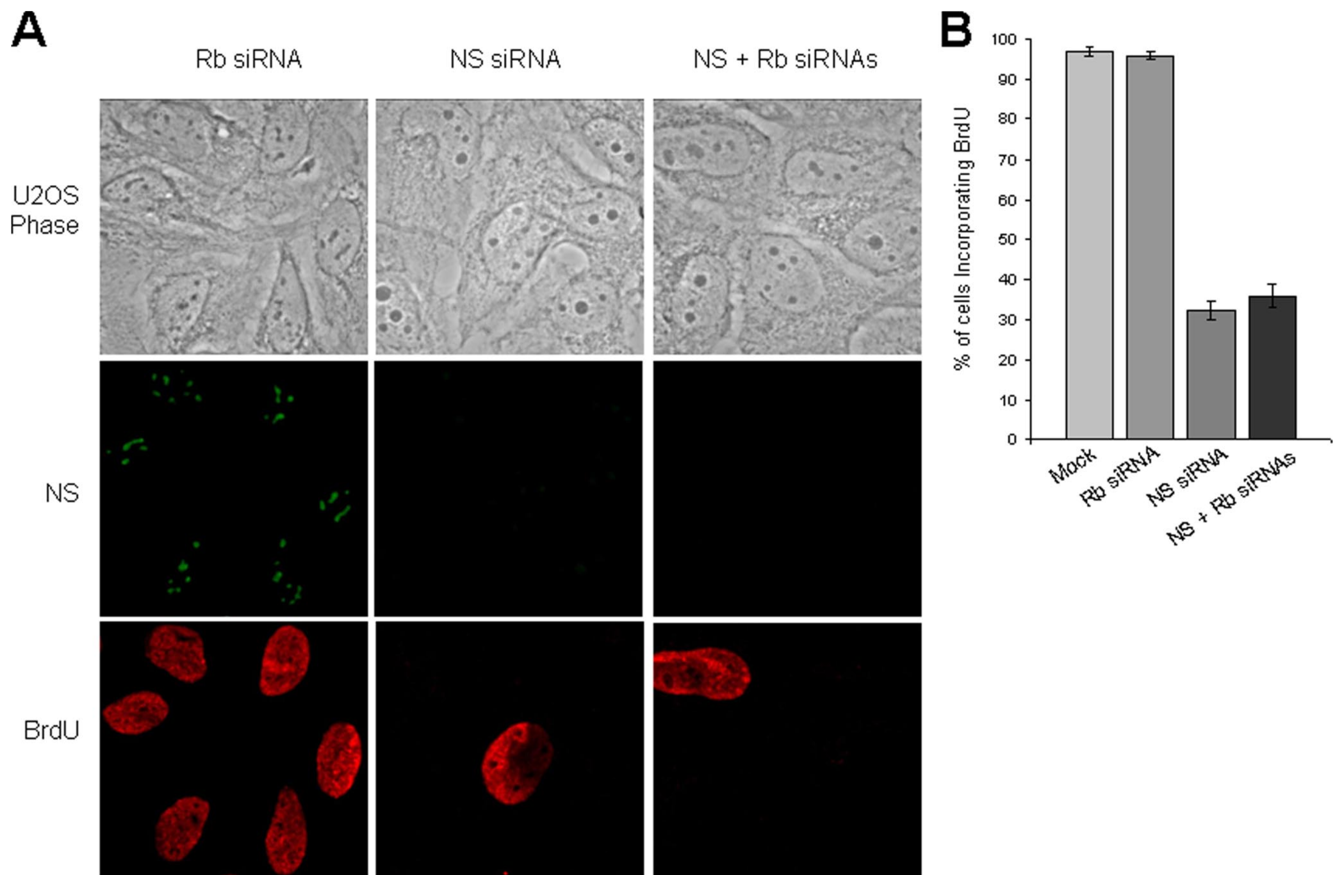

Figure 5. Depletion of Rb does not restore cell cycle progression in NS knocked down cells. (A) Rb siRNA, NS siRNA, or NS + Rb siRNAs were transfected into U2OS cells. Seventy-two hours later, BrdU was added, and after another 24 h, NS and BrdU were detected. (B) Each bar indicates the mean ± SD of data from three independent experiments.

connections that have been made among p53, Rb, and myc in the nucleolus (Raska *et al.*, 2006). In our experiments, NS depletion did not lead to nucleolar disruption as judged either by phase-contrast microscopy (see figures) or immunostaining for the nucleolar marker proteins UBF, fibrillarin, and B23 (data not shown). This suggests that NS depletion per se does not constitute a nucleolar stress signal, notwithstanding the fact that p53 is elevated in response to NS depletion as it is in other situations in which nucleolar effects are observed. This consideration supports the initial idea that NS and p53 operate primarily through a nucleoplasmic interaction (Tsai and McKay, 2002, 2005). Nonetheless, it would now be all the more relevant to track the dynamics and molecular interactions of NS, p53 and related proteins within the nucleus of living cells.

NS −/− mice abort before blastula and NS +/− fibroblasts display reduced NS levels and slower growth, but normal levels of p53 (Zhu *et al.*, 2006). In NS +/− fibroblasts, it is possible that the haploinsufficiency of NS as regards optimal growth rate nevertheless does not result in a depletion of NS sufficient to evoke the stabilization of p53. Alternatively, NS may be operating in a p53-independent manner. The possibility that NS can function via a p53-independent pathway during embryogenesis is also indicated by finding that p53 loss in NS −/− blastocysts does not rescue embryonic lethality (Beekman *et al.*, 2006). Based on previous studies and the current investigation, it seems plausible at present that there are p53-dependent and -independent roles of NS in cell proliferation.

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