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Nucleostemin stabilizes ARF by inhibiting the ubiquitin ligase ULF

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Abstract

Up-regulated expression of nucleolar GTPase Nucleostemin (NS) has been associated with increased cellular proliferation potential and tumor malignancy during cancer development. Recent reports attribute the growth regulatory effects of NS protein to its role in facilitating ribosome production. However, the oncogenic potential of NS remains unclear since imbalanced levels of NS have been reported to exert growth inhibitory effect by modulating p53 tumor suppressor activity. It also remains in questions if aberrant NS levels might play a p53-independent role in regulation of cell proliferation and growth. In this study, we performed affinity purification and mass spectrometry analysis to explore protein-protein interactions influencing NS growth regulatory properties independently of p53 tumor suppressor. We identified the Alternative Reading Frame (ARF) protein as a key protein associating with NS and further verified the interaction through *in vitro* and *in vivo* assays. We demonstrated that NS is able to regulate cell cycle progression by regulating the stability of the ARF tumor suppressor. Furthermore, overexpression of NS suppressed ARF polyubiquitination by its E3 ligase ULF and elongated its half-life, while knockdown of NS led to the decrease of ARF levels. Also, we found that NS can enhance NPM stabilization of ARF. Thus, we propose that in the absence of p53, ARF can be stabilized by NS and NPM to serve as an alternative tumor suppressor surveillance, preventing potential cellular transformation resulting from the growth inducing effects of NS overexpression.

Keywords

Nucleostmin; ARF; ULF; ubiquitination; proteolysis; cell proliferation; GTPase; NPM; p53; cell cycle

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Conflict of Interest

The authors declare no conflict of interest.

Introduction

Ribosomal biogenesis is a complex process responsible for the production of the cellular translational machinery required for protein synthesis and cell growth [1]. Perturbation in any key steps of the ribosomal assembly process can disrupt the delicate stoichiometric balance between ribosomal proteins and rRNA, resulting in numerous disease states, including growth abnormalities, anemia and cancer [2–4]. Nucleostemin (NS), a GTPase primarily residing in the nucleolus, was recently found to be essential in the late step of pre-RNA processing of the ribosomal assembly and overall protein synthesis [5–7]. NS has been frequently found up-regulated in various proliferating cell types including cancer cells and stem cells and thus designated as a cell proliferation marker as well as marker for poor prognosis in cancer patients [7–9]. Indeed, several lines of evidence support the oncogenic role of NS in cancer development. This includes the up-regulated expression of NS in cancer cells, but not primary cells [7, 8, 10], subdued cancer development and prolonged survival of lymphoma prone μ-myc transgenic mice in the absence of one copy of the NS gene and p53 null background [11], as well as the increased viability of human breast cancer cells lines, such as MCF-7 or p53 defective MDA-MB231 that overexpress NS protein, to initiate formation of tumorigenic atmospheres [12, 13]. Furthermore, neural cells displaying high NS promoter activity through the GFP reporter system in transgenic mouse were classified as potential tumor initiating cancer stem cells in the glioblastoma mouse model [12]. However, despite the association between NS levels and cellular proliferation potential, the influence of NS expression and activity on tumor development remains poorly understood and controversial. For instance, although NS protein levels are elevated in many cancer cell lines as well as rapidly proliferating stem cells, in p53 wild type U2OS cells, overexpression or depletion of NS triggered G1 cell cycle arrest [7, 14, 15].

Recent studies shed important insight into how NS may be regulated and potentially regulate cell proliferation and progression. NS possess two main GTP binding motifs, which allows NS to shuttle between the nucleolus and the nucleoplasm upon GTP hydrolysis [7, 16]. Inhibiting GTP hydrolysis by introducing point mutations to the GTP binding motif or depleting intracellular GTP pools by mycophenolic acid (MPA) treatment triggered NS re-localization from the nucleolus to the nucleoplasm as well as reduced protein stability. However, when treated with a proteasome inhibitor, such as MG132, NS protein levels were rescued. These observations suggest that NS undergoes proteasomal degradation in the nucleoplasm. Interestingly, whereas most proteins require polyubiquitination linkage to facilitate proteasome recognition, NS degradation does not require such a post-translational modification [17]. NS forms a complex with the E3 MDM2 in the nucleoplasm, but is not a substrate for MDM2. By binding the central acidic domain of MDM2, NS impaired its E3 ligase activity, consequently, leading to p53 stabilization and p53-dependent cell cycle arrest [14]. However, genetic and biochemical studies suggest that the ability of NS to regulate cell cycle progression is not confined to modulating p53 activity [18]. For instance, homozygous deletion of the NS alleles in mice caused embryonic lethality, which failed to be rescued by a p53 null background [18, 19]. Co-purification of NS containing molecular complexes from HeLa lysates demonstrates that NS forms a large multi-protein complex containing pre-RNA processing factors as well as RPs from the 60S ribosomal subunit, suggesting NS plays an

essential role in facilitating the late step of pre-RNA processing during ribosomal assembly. This notion was supported by the observation that knockdown of NS also significantly represses the formation of late step pre-RNA species as well as overall protein production [5]. In addition, depletion of NS in U2OS cells triggered ribosomal stress, during which free and unassembled RPs released into the nucleoplasm bind to MDM2 and inhibit its activity, thus enhancing p53 stability and p53 mediated cell cycle arrest [3, 14, 18]. These studies not only demonstrate that a delicate balance of NS protein levels is required for proper ribosome assembly, but also imply that the molecular interaction between NS and other proteins aside of p53 presence, may influence the activity and role of NS in tumor development [18].

To investigate the potentially novel proteins and factors influencing NS activity or role in tumor development independently of p53, we conducted a mass spectrometry analysis of flag-affinity purified proteins from lysates of 293 cells overexpressing flag alone or flag-NS. The top peptide candidate identified to interact with NS was the cyclin dependent kinase inhibitor 2A, more commonly known as the Alternative Reading Frame protein (ARF) [20] [21, 22]. The 14 kilodalton human or 19 kilodalton mouse ARF protein functions as a tumor suppressor to induce cell cycle arrest, apoptosis and senescence in the event of oncogene induction [23–27]. In the presence of oncogenic stimuli, ARF interacts with MDM2 in a similar manner as other nucleolar proteins, such as RPs or NS, to stabilize and induce p53 levels [3, 28, 29]. ARF has also been demonstrated to limit cell growth and proliferation through its regulation of the complicated processes in ribosomal biogenesis [28–32]. In this study, we reveal that NS not only interacts with ARF *in vitro* and *in vivo*, but also induces ARF protein stabilization, providing a potential regulatory mechanism preventing abnormal NS induced cellular activity and proliferation in the event of aberrant NS expression.

Results

NS is associated with ARF tumor suppressor

To identify NS associated proteins, lysates from 293 cells overexpressing Flag-tagged NS or Flag were subjected to affinity purification with the flag antibody. Isolated proteins were analyzed on SDS-PAGE and NS-associated proteins were subjected to mass spectrometry analysis. ARF was revealed as the top candidate and the ARF peptide sequences identified from the mass spectrometry are listed in Fig. 1A.

To confirm if the interaction between ARF and NS occurs in cells as suggested by the mass spectrometry results, we first examined whether endogenous NS and ARF also interact with each other. Lysates from H1299, HeLa or ARF null U2OS were subjected to co-immunoprecipitation (Co-IP) with appropriate antibodies. Results in Fig 1B showed that the antibody against either ARF or NS could co-immunoprecipitate NS or ARF, respectively, in H1299 and HeLa cells, but not in ARF-deficient U2OS cells, indicating that NS indeed interacts with ARF in cells. Next, we ectopically expressed ARF and NS or NS ^{G1dm}, the GTP binding mutant form of NS, which loses GTP-binding activity and only resides in the nucleoplasm instead of the nucleolus as does the wild type [17], to confirm their interaction in H1299 cells. As shown in Fig 1C, NS was co-immunoprecipitated with ARF using the anti-V5 antibody. Consistently, ARF was also co-immunoprecipitated with NS or NS ^{G1dm} by the anti-Flag antibody (Fig. 1C and D). To determine whether the binding between NS

and ARF is direct, GST-fusion protein-protein association assays were conducted using purified GST-ARF and His-NS or His-NS^{G1dm} protein from bacteria. As shown in Fig. 1D (upper panel), GST-ARF, but not GST alone, interacted directly with His-NS or His NS^{G1dm}, verifying that NS does directly bind to ARF and that this interaction occurs independently of the GTPase hydrolysis activity of NS.

Mapping of protein-binding domains for ARF and NS

To further understand the significance of ARF and NS interaction, we mapped the binding regions between the two proteins. Based on our mapping studies by transiently introducing ectopic proteins into H1299 cells followed by IP-WB analyses, ARF bound to NS at its basic N terminus within the amino acids 1–268 (Fig. 2A), whereas NS bound to ARF at its N- terminal domain within the amino acids 1–65 (Fig. 2B). Despite ARF binds to the N-terminus region of NS, which is closely located to the previously reported MDM2 binding site, the interaction between ARF and NS appeared to be independent of MDM2 binding, as NS and ARF still interacted with each other in p53/MDM2 null cells (data not shown), which is consistent with our *in vitro* data that suggest NS and ARF directly interact with each other (Fig. 1D).

Previously, the N-terminal region of ARF was also shown to be critical for the binding to NPM [30], a nuclear chaperone that can sequester ARF in the nucleolus to prevent it from degradation [24]. Coincidently, the basic N-terminus of NS containing residues 1–46, which is required for the binding of ARF, is also essential for the binding to NPM [33]. Although both NS and NPM bind to ARF approximately at the same region, the two proteins did not appear to compete for ARF binding, since we were able to detect ternary complexes containing all three proteins by co-immunoprecipitating NS protein complexes from H1299 cell lysates (Data not shown). Since NS bound to ARF at a similar region as did NPM [30], which was previously demonstrated to protect ARF from proteasomal degradation, this raises the possibility that NS, like NPM [24, 34], may function to regulate ARF stability as well.

Knockdown of NS by siRNA leads to destabilization of ARF protein

To examine whether the alteration of NS protein levels in the cell would influence ARF protein levels, a cell-based assay was carried out by employing scramble siRNA or siRNA directed against NS mRNA sequence (si-NS) to knock down endogenous NS protein levels. Depletion of NS resulted in a drastic reduction in ARF half-life from 4 hours to 2.5 hours. Furthermore, although previous studies showed that NPM was required for ARF stabilization [24, 34], this phenomenon appeared to be independent of the NPM protein levels, as shown in Fig. 3A. Consistently, immunofluorescence staining with NS and ARF specific antibodies demonstrated that NS and ARF co-localized in the nucleolus, and the depletion of NS protein levels upon knockdown was accompanied by the reduction of ARF protein level as compared to the scramble treated control (Fig 3B). Altogether, these findings suggest a role for NS in regulating ARF protein stability.

Overexpression of NS leads to stabilization of ARF

To verify that the fluctuation in NS protein levels is involved in regulating ARF stability, we also performed overexpression experiments to examine whether NS could prolong the degradation rate of ARF and rescue ARF protein levels. Plasmids expressing Flag tag or Flag-NS were transfected into H1299 cells, and the degradation rate of ARF protein levels was evaluated based on WB. Overexpression of NS in H1299 cells mildly prolonged the stability of ARF when compared to the control (Fig 4A). We speculated that the enhanced stability of ARF by ectopically expressed NS might be overshadowed to some extent by the high levels of endogenous NS or NPM protein [24, 34] expressed in H1299 cells. To test this, we repeated the same experiment in the human lung fibroblast cell line WI-38, which, in contrast to rapidly growing cancer cells, expresses low levels of NS and NPM protein [24]. Due to the low transfection efficiency of WI-38, we opted to use a lentiviral infection system to overexpress a Flag tag alone or Flag-NS in this cell line. Results in Fig. 4A show that overexpression of NS in WI-38 cell line significantly prolonged the degradation rate and increased ARF protein levels compared to the control, which was much more apparent than that as observed in H1299 cells (compare upper panels with lower panels). This supported our assumption that endogenous ARF protein may already exist as stable molecular complexes in the presence of high NS and NPM protein levels in cancer cells, such as H1299. Interestingly, overexpression of the GTP binding mutant form of NS, NS^{G1dm}, was also capable of stabilizing ARF in the H1299 or WI-38 cell line (Fig. 4B), suggesting that either the localization of NS protein was not essential for the NS-mediated stabilization of ARF protein levels or it occurred both in the nucleoplasm and nucleolus. Again, the stabilization of ARF by this mutant NS was more apparent in WI-38 cells than that in H1299 cells (compare upper panels with lower panels of Fig. 4B). Together, these findings, supporting our knockdown studies, demonstrate that NS protein levels play a critical role in maintaining the stability of ARF by preventing it from degradation in the cells.

NS enhances ARF stability by stabilizing the NPM/ARF complex in the nucleolus

Next, we attempted to determine the mechanism underlying the NS regulation of ARF protein stability. Previous studies show that nucleolar protein NPM is able to form stable protein complexes with ARF in the nucleolus. This prevents ARF from interacting with its E3 ligase ULF in the nucleoplasm and subsequently inhibits ARF ubiqitination and proteasomal degradation [24]. Since NS binds to ARF at the N-terminus, the reported binding site of NPM [30], we examined the possibility that NS and NPM may regulate ARF stability in a co-dependent manner. For this purpose, we examined whether the overexpression of NPM would restore the stability of ARF protein levels when NS is knocked down. The half-life of ARF was examined after H1299 cells were co-transfected with control siRNA, NS siRNA together or without Flag-NPM. As shown in Fig. 5A, knockdown of NS dramatically induced ARF degradation while the overexpression of NPM was able to completely restore ARF stability triggered by NS knockdown. Next, we examined whether overexpression of NS would restore the instability of ARF caused by NPM knockdown. The depletion of NPM protein levels triggered ARF instability in manner similar as seen when NS was knocked down. However, overexpressing wild type NS under these conditions did not present any rescue-effects on NPM knockdown-caused ARF destabilization. Surprisingly, the nucleoplasmic NS mutant form, NS^{G1dm}, when

overexpressed, was able to partially rescue the NPM depletion induced ARF instability. This suggests that dissociated ARF from the NPM/ARF complex might be stabilized through a NS dependent mechanism in the nucleoplasm, because NS^{G1dm} resides only in the nucleoplasm. These results confirm the previous study showing that NPM is necessary to keep ARF in the nucleolus and stabilize ARF [24], and also suggest that NPM is more essential than NS in ARF stabilization in the nucleolus. To further examine the role of NS in stabilizing the nucleolar NPM/ARF complexes, we performed IP and immunofluorescence staining to examine any potential interactions between NS, ARF and NPM. For this purpose, we knocked down NS or NPM protein levels with siRNA and examined the influence on the localization of NS and NPM and their interaction with ARF. H1299 cells transfected with scramble and NS or NPM siRNA were treated with MG132 prior to immunofluorescence staining to avoid any degradation of NS and NPM that would potentially mask subtle nuclear translocation and interactions. As shown in Fig. 5B (left panel), endogenous ARF and NPM co-immunoprecipitated together by the anti-NPM antibody, but this association was reduced by NS knockdown (Fig. 5B left) possibly due to a mildly increased translocation of NPM into the nucleoplasm as indicated by the immunostaining (Fig. 5C). Knockdown of NPM likewise increased the translocation of NS from the nucleolus into the nucleoplasm, resulting in NS instability and reduced NS protein levels [17, 35] (Fig. 5D and Fig. S2). To examine if NS and ARF interaction was affected by NS translocation, Flag-NS and V5-ARF together with its E3 ligase ULF were exogenously expressed in H1299 cells and the lysates were subjected to co-immunoprecipitation with the anti-Flag antibody as shown in Fig. 5B (right panel). The co-IP data revealed that ARF and NS interaction remain unaffected by NPM knockdown, suggesting that ARF and NS perhaps bind to each other independently of NPM. Altogether, these results suggest that ARF, NPM and NS likely bind together in the nucleolus to form a stable complex. The depletion of either NPM or NS protein levels triggers dissociation of tightly bound formed molecular complexes in the nucleolus, and causes dispersion of these unassociated nucleolar proteins into the nucleoplasm. Thus, while NS is not required for the formation of NPM/ARF complex in the nucleolus (Fig. 4A), NS still plays an auxiliary role by reinforcing and maintaining the NPM/ARF complex in the nucleolus.

NS interacts with ULF, the ARF E3 ligase, and inhibits ULF mediated ARF degradation

A previous study demonstrated that ARF E3 ligase ULF is localized in the nucleoplasm and mediates ARF polyubiquitination and proteasomal degradation [24]. Since our results suggest that NS binds and stabilizes ARF more efficiently in the nucleoplasm (Fig. 4B and Fig. 5A), we examined whether NS may enhance ARF stability by inhibiting ARF polyubiquitination by ULF. We first investigated whether NS and ULF could interact with each other. Flag-HA tagged ULF and GFP-NS expression plasmids alone or together were introduced into human embryonic kidney HEK293 cells. Lysates from the transfected cells were subjected to immunoprecipitation to pull down ULF and NS protein complexes with the anti-flag antibody. As shown in Fig. 6A, GFP-NS co-immunoprecipitated with Flag-HA ULF, suggesting the two proteins interact with one another. To verify the interaction between endogenous NS and ULF, we utilized an antibody specific to ULF to co-immunoprecipitate ULF and NS molecular complexes. ARF and NPM, which were previously reported to interact with ULF [24], were also probed as positive controls. As

shown in Fig. 6B, NS, ARF and NPM co-immunoprecipitated with ULF by the ULF specific antibody, but not with the control rabbit IgG antibody, consistent with the result of Fig. 5B (right). These results indicate that NS interacts with ULF as well.

To examine the significance of the interaction between NS and ULF, we conducted an *in vivo* polyubiquitination assay to determine whether overexpressing NS would interfere with ULF E3 ligase activity. A combination of plasmids was transfected into H1299 as indicated in Fig. 6C. Lysates from harvested cells were subjected to Ni^{2+} precipitation to isolate His-ubiquitinated proteins and to evaluate the levels of polyubiquitinated ARF. As expected, expression of the E3 ligase ULF increased ARF polyubiquitination. However, in the presence of increasing levels of NS, ARF polyubiquitination by ULF was hindered. These results suggest that NS stabilizes ARF protein levels by inhibiting ULF mediated ARF polyubiquitination and proteasomal degradation. In Figs. 4B and 5A, we showed that overexpression of Flag-NS^{G1dm} was also able to reduce the degradation rate of ARF. However, it was unclear whether this NS GTP binding mutant was capable of interacting with ULF as the wild type and stabilizing ARF in the similar manner. To confirm the interaction of the two proteins, we overexpressed Flag-NS^{G1dm} together with V5-ULF or V5-ULF alone in H1299 cells, and conducted co-IP with the lysates utilizing a control rabbit IgG or an antibody specific to ULF. As shown in Fig. 6E, despite the mutations that impair NS GTP hydrolysis activity, NS^{G1dm} was still able to bind to ULF. We next examined the ability of the mutant nucleoplasmic NS^{G1dm} to negate ARF polyubiquitination, in comparison to the NS deletion fragments 47–270 and 268–549. Our mapping of ARF-NS interaction sites showed that ARF binds NS 47–270 (Fig. 2A) that did not appear to bind to NPM as shown previously (Ma & Pederson, 2008), whereas, the 268–549 fragment of NS failed to interact with ARF (Fig. 2A). Consistently, overexpression of either Flag NS 47–270 or Flag NS^{G1dm}, but not Flag NS 268–549, hindered ULF mediated ARF polyubiquitination. This suggests that the binding to ARF, but not to NPM, is more essential for NS to inhibit ULF ubiquitin ligase activity and stabilize ARF. This inhibition can occur in the nucleoplasm because this mutant NS, NS^{G1dm}, which resides in the nucleus only, is able to bind to ARF and ULF and inhibit ULF-mediated ARF ubiquitination as well.

To further determine whether the binding of NS to ULF could disrupt the ULF-ARF interaction and therefore block ULF E3 ligase activity toward ARF, we performed a co-immunoprecipitation assay. Since Flag-NS^{G1dm} was shown to co-localize with ULF in the nucleoplasm [17, 24] (left panel of Fig 6E), we transfected H1299 cells with the plasmid encoding Flag-NS^{G1dm} to avoid any unknown cell compartment-related influence and focused on the interaction of NS, ULF and ARF in the nucleoplasm. The right panel of Fig. 6E showed that ULF readily co-precipitated together with ARF using the V5 antibody, but lessened when NS^{G1dm} was introduced. Together, these results suggest that NS physically might disrupt the interaction between ULF-ARF in the nucleoplasm and thus prevent ARF from undergoing ULF mediated polyubiquitination and proteasomal degradation.

NS induces ARF-dependent G1 arrest in p53 null cells

Having established the role of NS in prolonging ARF stability, we next examined whether the subsequent elevation of ARF levels caused by NS induction would alter the cell growth

and proliferation. Since imbalanced levels of NS trigger p53 activation [14], we opted to perform cell cycle analysis in MEF cells containing p53 and MDM2 gene double deletions (DKO), and those with p53, MDM2, ARF triple deletions (TKO) under the condition of enforced NS expression. Utilizing these cell lines, we attempted to rule out the involvement of p53 in cell cycle regulation and primarily focus on the cell growth regulatory activity of ARF only. NS WT, NS^{G1dm} or GFP expression plasmids were first transfected into DKO or TKO MEF cells and the transfection efficiency or the expression of exogenous proteins was confirmed by WB. Results in Fig. 6A demonstrated that enforced expression of either wild type or mutant NS in $p53^{-/-}$ $MDM2^{-/-}$ MEF cells significantly increases the G1-phase population from 35% to 50%, while decreasing the S-phase population from 55% to around 35%. However, in the $p53^{-/-}$ $MDM2^{-/-}$ $ARF^{-/-}$ MEF cells, either NS-WT or NS^{G1dm} overexpression caused little change on the cell cycle distribution. These results demonstrate that the ARF tumor suppressor pathway potentially antagonizes the growth stimulating properties caused by elevation in NS protein levels, by primarily inducing G1 cell cycle arrest independently of p53.

Discussion

In this study, we reveal ARF as a cell cycle checkpoint to ensure that cells express NS at a physiologically optimal level. We demonstrate that ARF is a direct binding partner of NS and the fluctuation in NS protein levels alters ARF protein stability. While the depletion of NS protein by siRNA significantly reduces ARF protein levels and shortens its stability, ectopic expression of NS stabilizes ARF protein levels. We further show that NS binds to the E3 ligase of ARF, ULF. When expressed in excess, NS enters the nucleoplasm and blocks ULF mediated ARF polyubiquitination and degradation. The elevation of ARF caused by NS overexpression in turn triggers G1 cell cycle arrest as illustrated in Fig 6B. Although the physiological significance of this phenomenon needs to be further investigated, this could be interpreted as one additional cellular surveillance mechanism independently of p53 to make sure that cells would not over grow or over proliferate if NS is highly expressed as further explained below. This mechanism is reminiscent to other oncogenes c-Myc or NPM, whereas the stabilization of ARF occurs in the presence of abnormally high NPM protein levels to prevent potential oncogenic transformation by inducing p53 activation [24]. ARF tumor suppressor activities have been largely attributed to its ability to induce p53 activity by hindering MDM2 E3 ligase activity [36, 37]. A previous mapping of NS and ARF binding site to MDM2 suggests that both proteins bind to the central region domain of MDM2, but does not compete in terms of binding. Our current results from sequential co-IP assay also demonstrate a molecular complex that contains NS, ARF and MDM2 (Fig. S1). In fact, in the presence of ARF, the binding between NS and MDM2 appeared to be enhanced [14]. The close proximity between the binding sites of ARF and NS to MDM2, as well as the direct interaction between NS and ARF shown in this study, raises the possibility that ARF may serve as an adaptor protein between NS and MDM2 to induce cell cycle arrest more efficiently in a p53 dependent manner.

Since mutation or loss of p53 frequently occurs during cancer development [38], how the relationship between NS and ARF influences the course of cancer development remains elusive. Our data suggests that under such a condition, ARF would act as an alternative

tumor suppressor to respond to oncogenic challenge. Induction of ARF upon ectopic expression of NS in MEF cells leads to G1 cell cycle arrest in spite the absence of p53 and MDM2. This observation supports the growing evidence that ARF is capable of regulating the cell cycle independently of p53 [30, 39–41].

The ARF tumor suppression activities have been also closely integrated into the regulation of the complicated steps of ribosomal biogenesis [41]. For example, ARF has been shown to suppress rRNA transcription by interacting with the transcription factor TTF-1 or to prevent chaperoning of ribosomal subunits and processing of rRNA by interacting with nuclear chaperone NPM [30, 42–44]. It is speculated that by binding to NS, ARF might interfere with its biological activity during ribosomal biogenesis to restrict cellular growth and proliferation in a similar manner since NS is involved in this process [5, 6]. Furthermore, NS and NPM can not only interact with each other [33], but also bind to ARF at the same N-terminus region [30, 33]. These studies suggest that NPM and NS may work together by perhaps forming a hetero-oligomeric complex to modulate ARF stability and tumor suppressor activities. Previous reports showed that NPM-containing protein and NS-coming complexes display a similar native molecular weight of over 2 MDa [5, 45]. This coincidence supports the possibility of the existence of a hetero-oligomeric protein complex that contains all of the three proteins. However, from our current study, NS is not required for the formation of the nucleolar NPM/ARF complex, but perhaps reinforces the complex by promoting NPM nucleolar retention instead. Our assumptions stem from the observations that NS depletion does not significantly alter NPM protein levels, but induces a mild re-localization of NPM into the nucleoplasm. Indeed, a recent study also revealed that in the absence of NS, small ribonuclear proteins (snoRNPs) that facilitate the processing of rRNAs as well as telomerase RNA exhibit a more subtly scattered distribution in the nucleolus and fail to interact with other key members in the snoRNP complex, including dyskerin and fibrillarin [6]. These observations suggest that the absence of NS may alter the interaction between nucleolar proteins and subsequently molecular complexes.

Together with previous studies, our findings suggest that whether or not NS plays an oncogenic or tumor suppression role may depend on the genetic background of cells. Crosstalk between the ribosomal biogenesis and the p53-ARF signaling pathway normally serve as an important internal surveillance mechanism to halt cell growth or induce cell death when cells are growing under aberrant ribosome synthesis conditions [3, 28]. However, accumulation of genetic instability during the course of cancer development often leads to the loss of p53 or ARF tumor suppressor functions [38]. While overexpression of NS may lead to cell cycle arrest in the presence of p53 or ARF, the loss of p53 or ARF tumor suppressor activity may allow cells overexpressing NS drive ribosomal biogenesis to promote rapid proliferation and cell growth. Therefore, further investigation of the functional interplay between ARF and NS in cell growth and proliferation will provide more insight into our better understanding of the molecular events underlying these cellular activities and also useful information for our future anti-cancer drug discovery.

Material and methods

Cell lines

Human non-small lung carcinoma H1299 cells (p53-null), Human embryonic kidney 293 cells (p53 mutant) and human lung fibroblast cell line WI-38, $p53^{-/-}$ $MDM2^{-/-}$ double-knockout MEF and $p53^{-/-}$ $MDM2^{-/-}$ $ARF^{-/-}$ triple-knockout MEF cells were cultured in DMEM supplemented with 10% FBS and 100U/ml penicillin/ streptomycin at 37°C in a 5% CO2 humidified atmosphere.

Plasmids

Flag-NS, Flag-NSG261VG266V double mutant (NS^{G1dm}), His-Ubiquitin, V5-ARF plasmids utilized in this study were previously described[14]. Flag-HA-ULF construct was generously provided by Dr. Wei Gu from Columbia University. Flag-NS PCR was derived from previous pcDNA3-Flag-NS reported in using: forward primer 5′ ctaGCTAGCtggtaccgatccaccatggac 3′; reverse primer 5′ ctaGCTAGCtggtaccgatccaccatggac 3′. The Flag-NS PCR product was digested with NheI and Not I and ligated into the linearized lentivirus vector CDH-CMV-copGFP digested with the same restriction enzymes. Ligated products were transformed into bacteria and miniprep DNA was sequenced to confirm the sequence is correct. To generate an empty control for lentivirus production, the NheI/NotI linearized CDH-CMV-copGFP vector was subjected to digestion with Klenow Polymerase to create blunt ends, followed by self-ligation with T4 ligase.

Antibodies

Antibodies used for immunoprecipitation or WB include monoclonal anti-flag, anti-tubulin, anti-beta-actin (Sigma), monoclonal V5 (Invitrogen), polyclonal rabbit NS (H-270) and NPM (C19) (Santa Cruz Biotechnology), monoclonal anti-ARF (4C64, Cell Signaling) and anti-ARF (P1402, Neomarker), polyclonal ARF (200-111, Novus Biological), ULF (A301-814A, Bethyl laboratories) antibodies.

Transfection and Western Blot

Cells were transfected with the Metafectene Pro reagent along with plasmids as indicated in figure legends according to manufactor's protocol. Cells were harvested at 36–48 h after transfection on ice, and lysed in cell lysis buffer consisting of 50mM Tris pH8.0, 1mM EDTA, 150mM NaCl, 0.5% NP-40, 2mM PMSF and 1 mM DTT or RIPA buffer containing 50mM Tris, 150mM NaCl, SDS 0.1%, Na Deoxycholate, NP-40 1% with appropriate protease inhibitors. An equal amount of proteins in lysates was subjected to analysis by SDS-PAGE and Western blot (WB) with the antibodies indicated in the figure legends.

In vivo ubiquitination Assays

Cells were transfected with the indicated plasmids in figure legends. Thirty-six hours after transfection, cells were treated with 10uM of MG132 for 6–8 h and harvested on ice. The collected pellets were split into two portions. One portion was used for the in vivo ubiquitination assay as described previously with some modifications [46]. The cell pellets were lysed in buffer B containing (8M urea, 0.1M TrisCl, 0.1M Na2HPO4/NaH2PO4,

10mM B-Mercaptoethanol, pH 8.0) and incubated with Ni beads for 4 h. Beads were washed 2x with Buffer B and then 1x with Buffer C containing (8M urea, 0.1M Tris-Cl, 0.1M Na2HPO4/NaH2PO4, 10mM B-Mercaptoethanol, pH 6.3). The second aliquot was subjected to WB as mentioned previously to determine protein expression.

Immunoprecipitation

Harvested cell pellets were lysed in 0.5% NP-40 lysis buffer. An equal amount of lysates was incubated Dyna beads pre-conjugated with appropriate antibody for 10 minutes at room temperature. Incubated beads were washed 3 times in PBS and co-immunoprecipitated proteins were loaded onto SDS PAGE along with 10% input lysate for WB analysis.

GST binding assays

Protein-protein interaction assays were conducted as previously described (28). Bacterial purified His-tagged NS or His-tagged NS^{G1dm} was incubated with Sepharose 4B beads (Sigma) conjugated to glutathione (GST) alone or GST-ARF for 30 minutes at room temperature. After washing, bound proteins were analyzed by gel electrophoresis and detected by coomasie staining or WB with the anti-NS antibody.

Protein half-life assays

Cells transfected with the indicated plasmids as shown in the figure legends were treated with 50ug/ml cycloheximide (CHX) after 24–36 hours after transfection. Cells were harvested at different time points in ice cold PBS, followed by lysis with NP-40 or RIPA buffer. Equal amounts of lysates were loaded onto SDS-PAGE gel, followed by WB with appropriate antibodies.

Lentivirus production

Low passage 293 T packaging cells and packaging plasmid pCMVΔ8.2 were kindly provided by Dr. Yan Dong from the Department of Structural and Cellular Biology, Tulane University School of Medicine. The 293T cells at 90% confluency cells were split 1: 2.5 into poly-lysine coated 10 cm plates in antibiotic free media. The following day, 293T cells were transfected with 3.3 ug CDH-CMV-Flag-NS-copGFP or CDH-CMV empty vector together with packaging vectors 8.4 ug pCMVΔ8.2 and 4.2 ug pMD2.G using Metafectene Pro transfection reagent. Virus supernatant was collected 48 hours after transfection. Virus titer was titrated in WI-38 cells by evaluating fluorescence intensity after 72 hours post lentivirus infection. Actual experiments were conducted in 10 cm plates using the same ratio of cells to virus supernatant and the method of infection.

Immunofluorescence staining

H1299 cells were transfected with scramble DNA, NS-siRNA or NPM-siRNA. 48 hours after transfection, cells were fixed in 4% PFA for 25 minutes, followed by treatment in 0.3% Triton X-100 for 20 minutes. The fixed cells were blocked with 5% BSA for 30 minutes, the cells were incubated with a mouse anti-ARF antibody (Cell signaling, CA) and a rabbit anti-NS antibody (Santa Cruz) at 4°C overnight. FITC-conjutated anti-mouse and Rhodamine-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) were used for ARF and

NS detection, and DAPI for nuclear staining. The cellular localization of NS/ARF was examined under a fluorescence microscope and double-negative cells were counted. At least six randomly chosen fields of view were examined with a minimum number of 800 cells scored for each condition.

Cell cycle analysis

$p53^{-/-}$ $MDM2^{-/-}$ MEF and $p53^{-/-}$ $MDM2^{-/-}$ $ARF^{-/-}$ MEF cells were plated into 60-mm plates, and incubated at 37°C overnight. After transfection with copGFP-Flag-NS WT, copGFP-Flag-NS ^{G1dm} MT or copGFP- empty vector for 48 hours, cells were harvested, fixed in pre-cooled 70% Ethanol overnight at −20°C, resuspended in propidium iodide-solution (50 μg/ml PI, 0.1 mg/ml RNase A, 0.05% Tritin X-100 in PBS) for 40min at 37°C, then the GFP-positive cells were sorted and analyzed for DNA content using a flow cytometer (FACS Calibur, Becton Dickinson) and proprietary software (FlowJo software and ModFit LT, Verity Software House, Topsham, ME, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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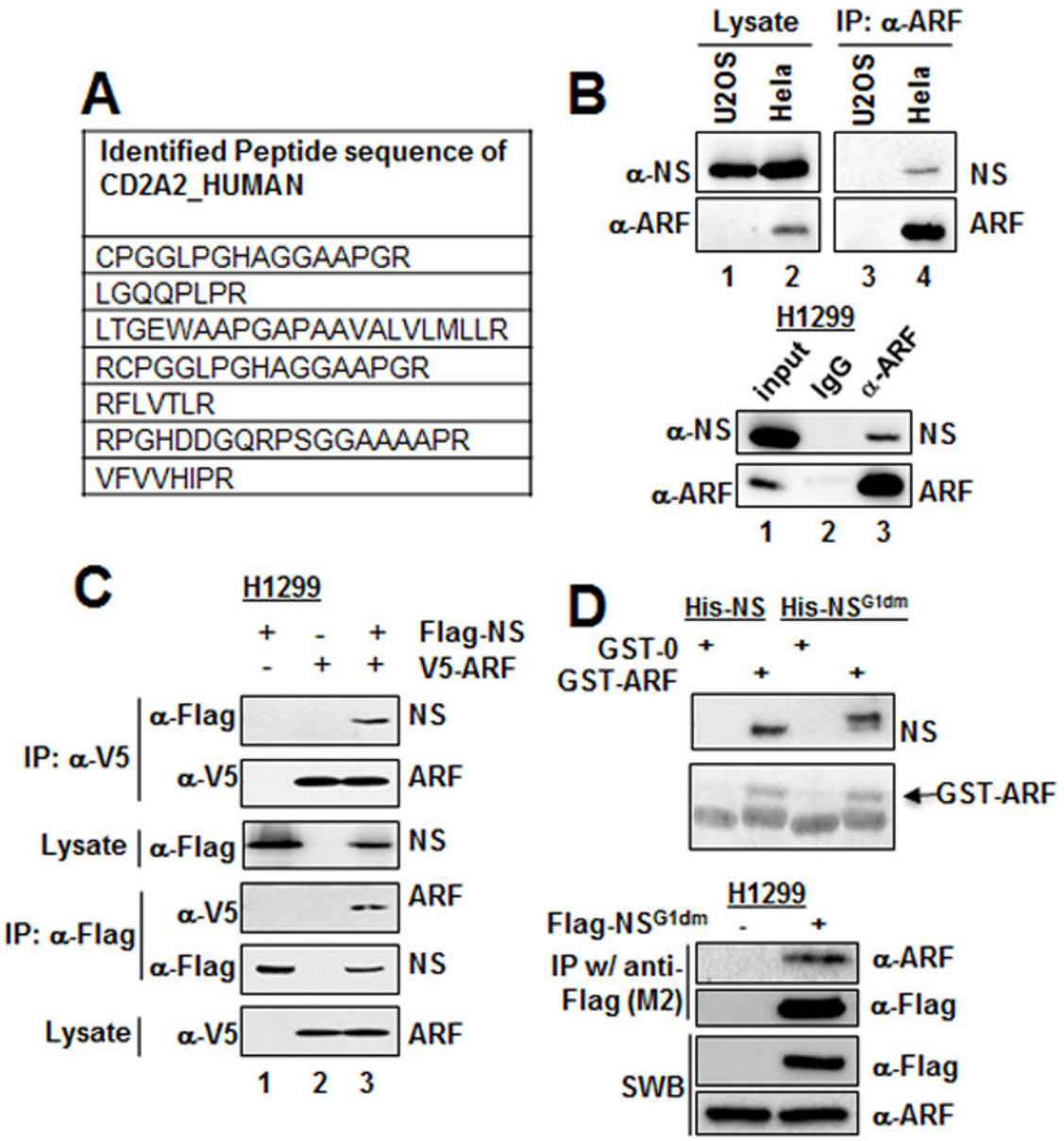

Figure 1. NS and ARF interact with each other *in vivo* and *in vitro*

(A) List of the sequences of the top candidate protein $p14^{ARF}$ identified from the NS flag affinity purification and mass spectrometry analysis. (**B**) NS can interact with ARF *in H1299* cells and *HELA* cells. Lysates from H1299, U2OS and HELA were subjected to immunoprecipitation (IP) by ARF antibody or control IgG antibody. Precipitated proteins were analyzed by SDS-PAGE and western blot with the indicated antibodies. (**C**) Exogenously expressed NS and ARF can interact with each other. H1299 cells were transiently transfected with a combination of plasmids expressing Flag-NS or V5-ARF. Transfected cells were lysed 48 hours later. Lysates were subjected to IP using either the flag antibody or the V5 antibody. The immunoprecipitated protein and input lysate were analyzed by SDS-PAGE and WB with the indicated antibodies. (**D**) **Upper:** GST-ARF and

His-NS or His-NS ^{G1dm} can interact. GST alone or GST-ARF purified protein was incubated with either His-NS or His-NS ^{G1dm}. Following incubation, the GST beads were washed and beads were loaded onto SDS-PAGE. Pulled down protein was detected by western blot with the rabbit NS antibody and Coomasie staining. **Lower:** Ectopically expressed ARF and NS GTP binding mutant interact with each other. H1299 cells were transiently transfected with a combination of plasmids expressing Flag-NS^{G1dm} or V5-ARF. Transfected cells were lysed 48 hours later. Lysates were subjected to IP using the flag antibody (anti-Flag beads M2). Immunoprecipitated proteins and input lysates were analyzed by SDS-PAGE and WB with the indicated antibodies.

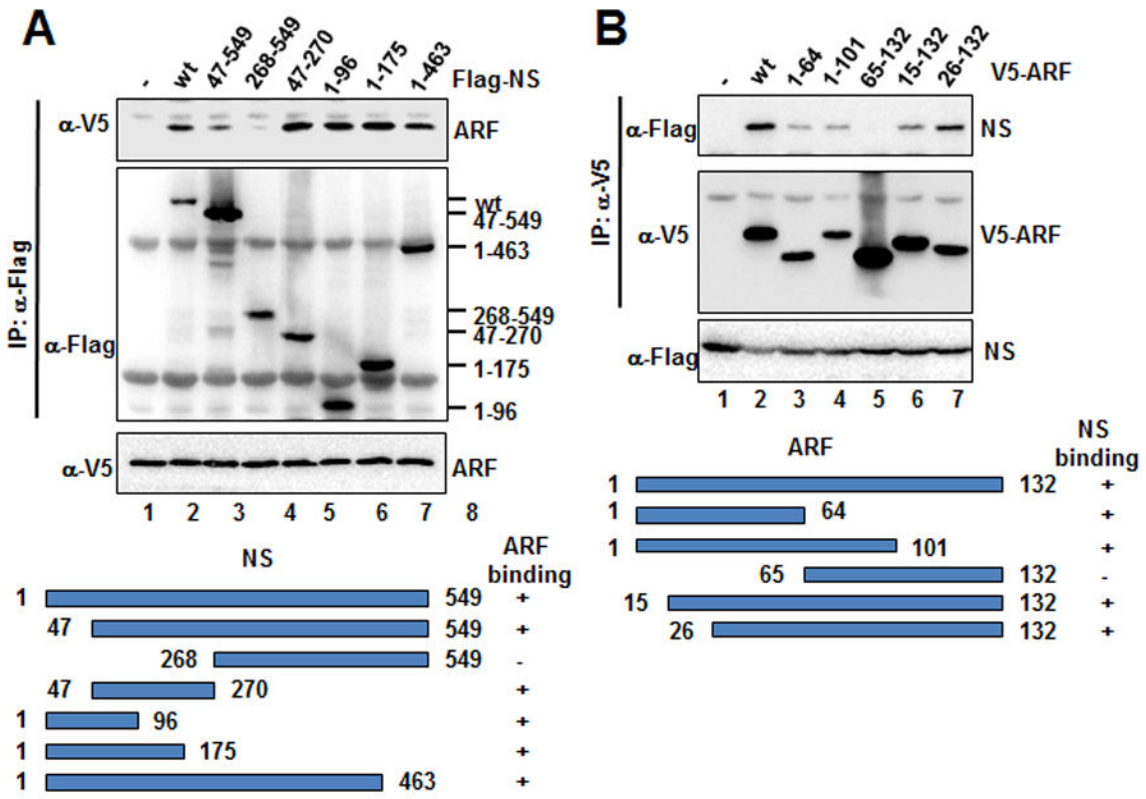

Figure 2. Mapping of ARF and NS binding domains

(**A**) ARF interacts with the N terminal basic domain of NS. H1299 cells are transfected with Flag-NS and Flag-NS deletion fragments together with V5-ARF. Cells were harvested 48 hours after transfection and lysates were subjected to IP with the Flag antibody. Immunoprecipitates were analyzed by SDS-PAGE and WB with indicated with the V5 or Flag antibody. (**B**) NS interacts with N terminal basic domain of ARF. H1299 cells were transfected with V5-Arf and V5-ARF deletion fragments together with Flag-NS. Cells were harvested 48 hours after transfection and lysates were subjected to IP with V5 antibody. Co-immunoprecipitad proteins were analyzed by SDS-PAGE and WB with the Flag or V5 antibody.

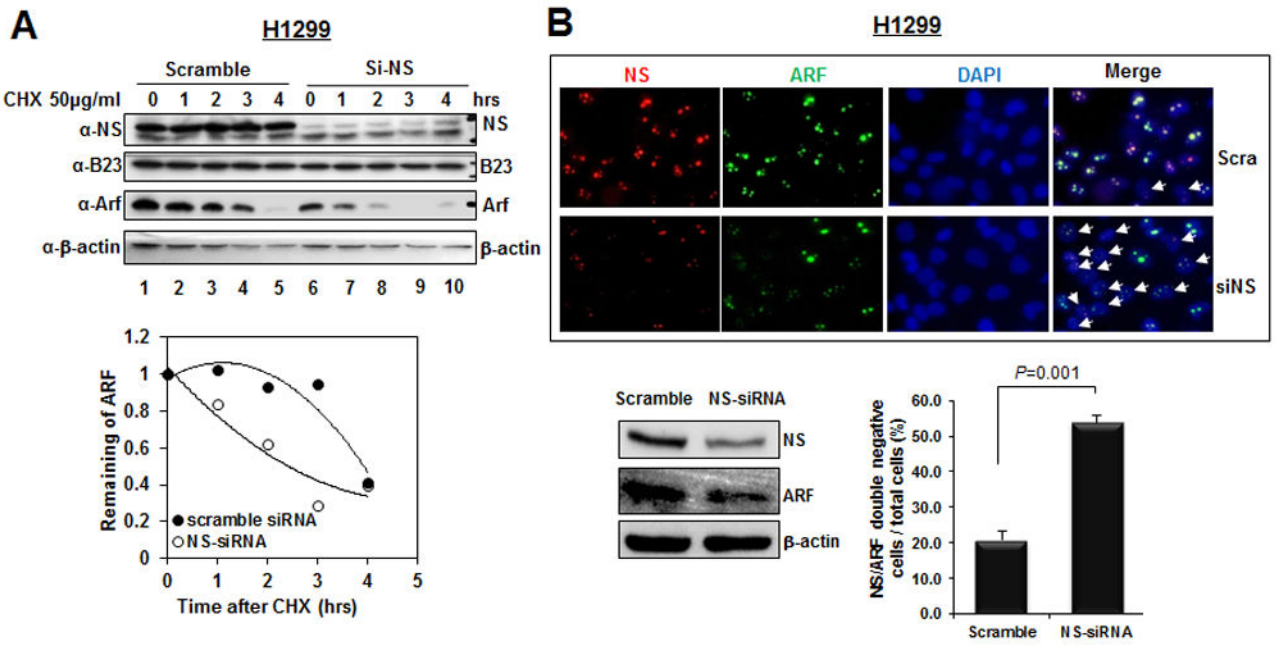

Figure 3. NS knockdown reduces ARF stability

(A) Depletion of NS destabilizes ARF. H1299 cells were transfected with either scramble siRNA or NS siRNA. 48 hours after transfection, transfected cells were split to 60mM plates at 50% confluency. Cells were treated with CHX the following day and harvested at the indicated time points. Equal amounts of proteins from cell lysate were used for WB to compare the level of ARF protein from each time point. WB bands were quantified and plotted on a graph as mentioned above. (**B**) NS knockdown reduces ARF protein levels. H1299 cells were transfected with scramble siRNA or NS siRNA. 72 hours after transfection, cells were fixed in 4% paraformaldehyde and stained with the indicated antibodies.

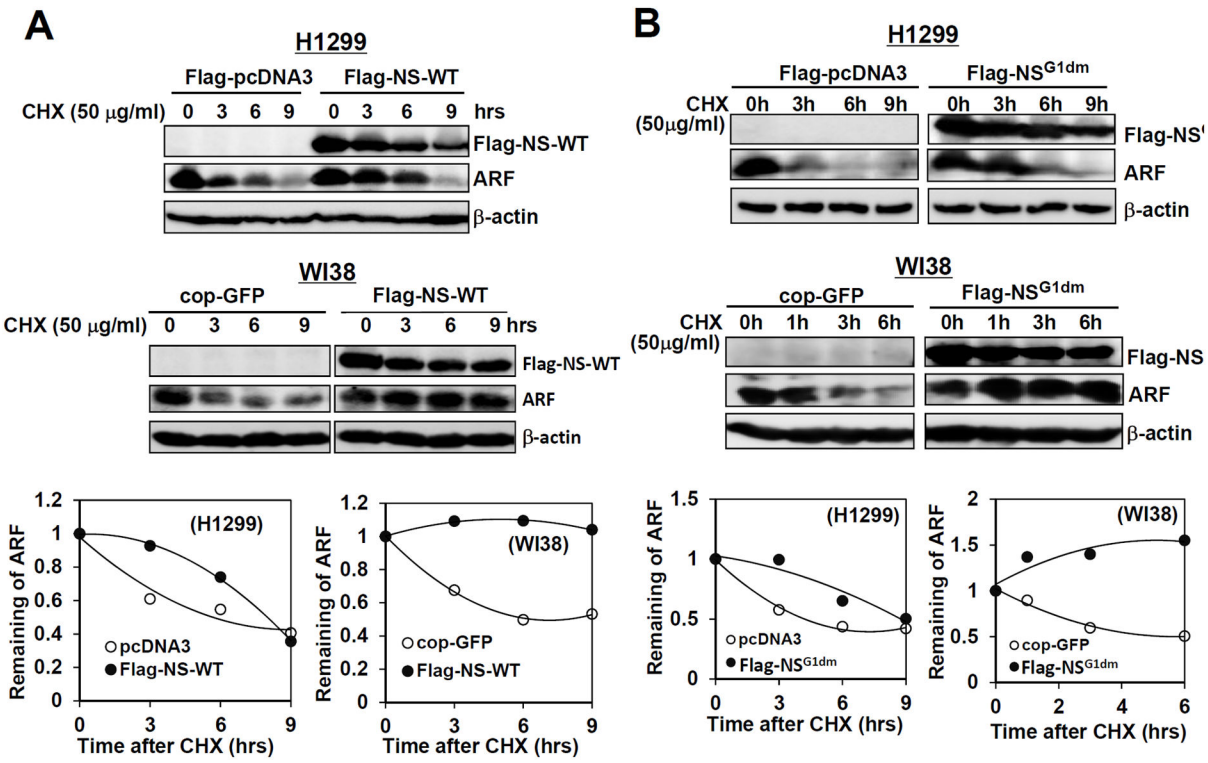

Figure 4. Overexpression of NS or NS GTP binding mutant leads to the stabilization of ARF protein in WI-38 and H1299 cells

H1299 or WI-38 cells were subjected to overexpression of (**A**) flag or flag-NS; (**B**) flag or flag-NS ^{G1dm} by transfection or lentivirus infection. 24 hours after transfection or 72 hours after lentivirus infection, cells were subjected to CHX treatment and harvested at the indicated time points. Equal amounts of protein from harvested cell lysates at each time point were analyzed by SDS-PAGE and WB to compare the level of ARF protein from each time point. WB Bands were quantified and plotted on a graph as mentioned above.

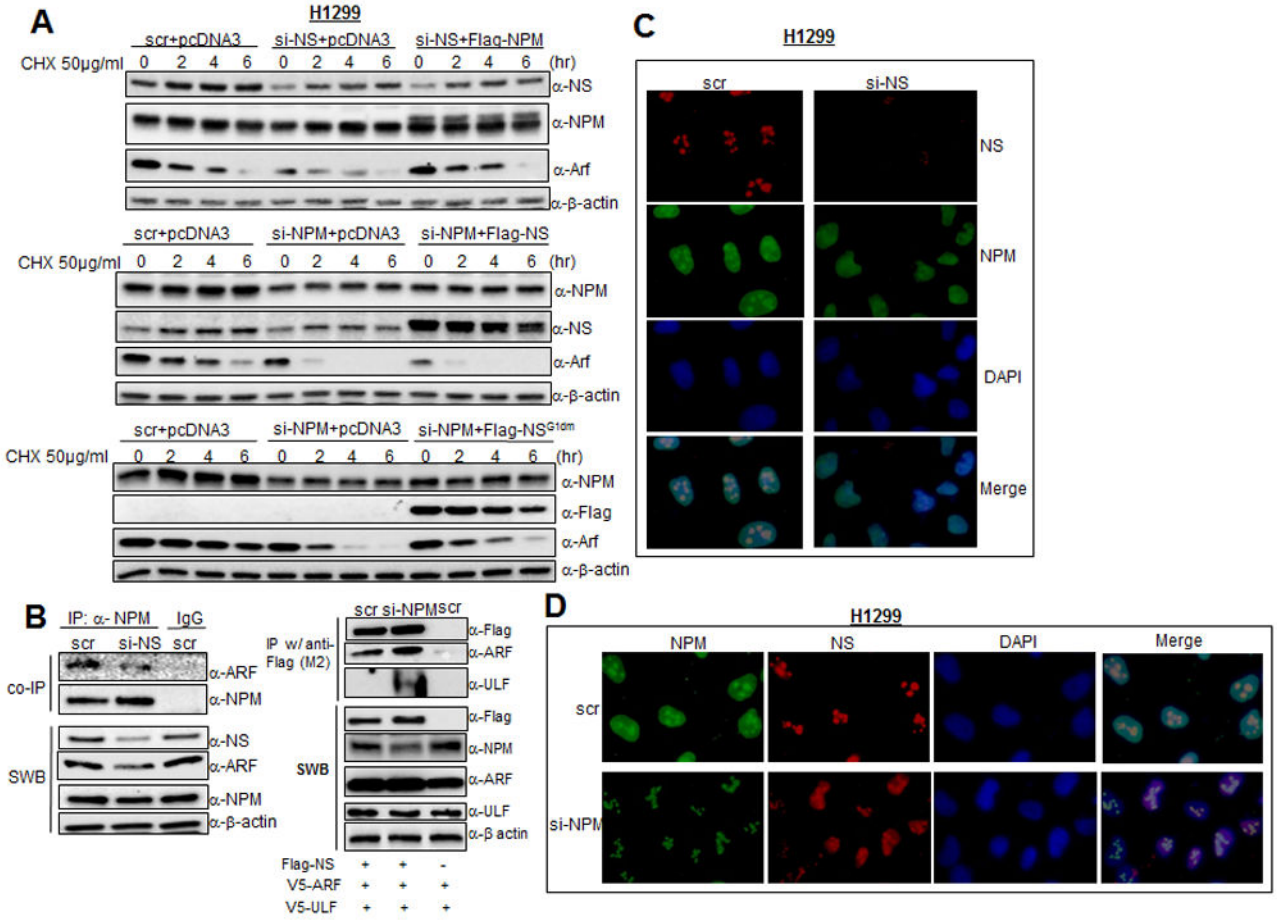

Figure 5. NS enhances the formation of NPM/ARF complex in the nucleolus

(A) NS cannot rescue NPM downregulation-caused ARF degradation. H1299 cells were co-transfected with the indicated siRNAs and plasmids. 48 hours after transfection, cells were treated with CHX and harvested at the indicated time points. Equal amounts of proteins from cell lysate were used for WB to compare the level of ARF protein from each time point. **(B) Left:** NS depletion reduces the NPM-ARF interaction. H1299 cells were transfected with either scramble siRNA or NS siRNA. Transfected cells were lysed 72 hours later. Lysates were subjected to IP using the anti-NPM antibody. Immunoprecipitated proteins and input lysates were analyzed by SDS-PAGE and WB with the indicated antibodies. **Right:** NS-ARF interaction is not affected upon knockdown of NPM. H1299 cells were co-transfected with the indicated siRNAs and plasmids. Transfected cells were lysed 48 hours later. Lysates were subjected to IP using the anti-Flag antibody conjugated beads (M2). Immunoprecipitated proteins and input lysates were analyzed by SDS-PAGE and WB with the indicated antibodies. **(C)** NS depletion causes the re-localization of NPM into the nucleoplasm. H1299 cells were transfected with scramble siRNA or NS siRNA for 72hr, and incubated with MG132 (20μM) for 6hr before fixation. Cells were fixed in 4% paraformaldehyde and stained with the indicated antibodies. **(D)** NPM knockdown causes the re-localization of NS from the nucleolus to the nucleoplasm. H1299 cells were transfected with scramble siRNA or NPM siRNA for 72hr, and incubated with MG132

(20μM) for 6hr before fixation. Cells were fixed in 4% paraformaldehyde and stained with the indicated antibodies.

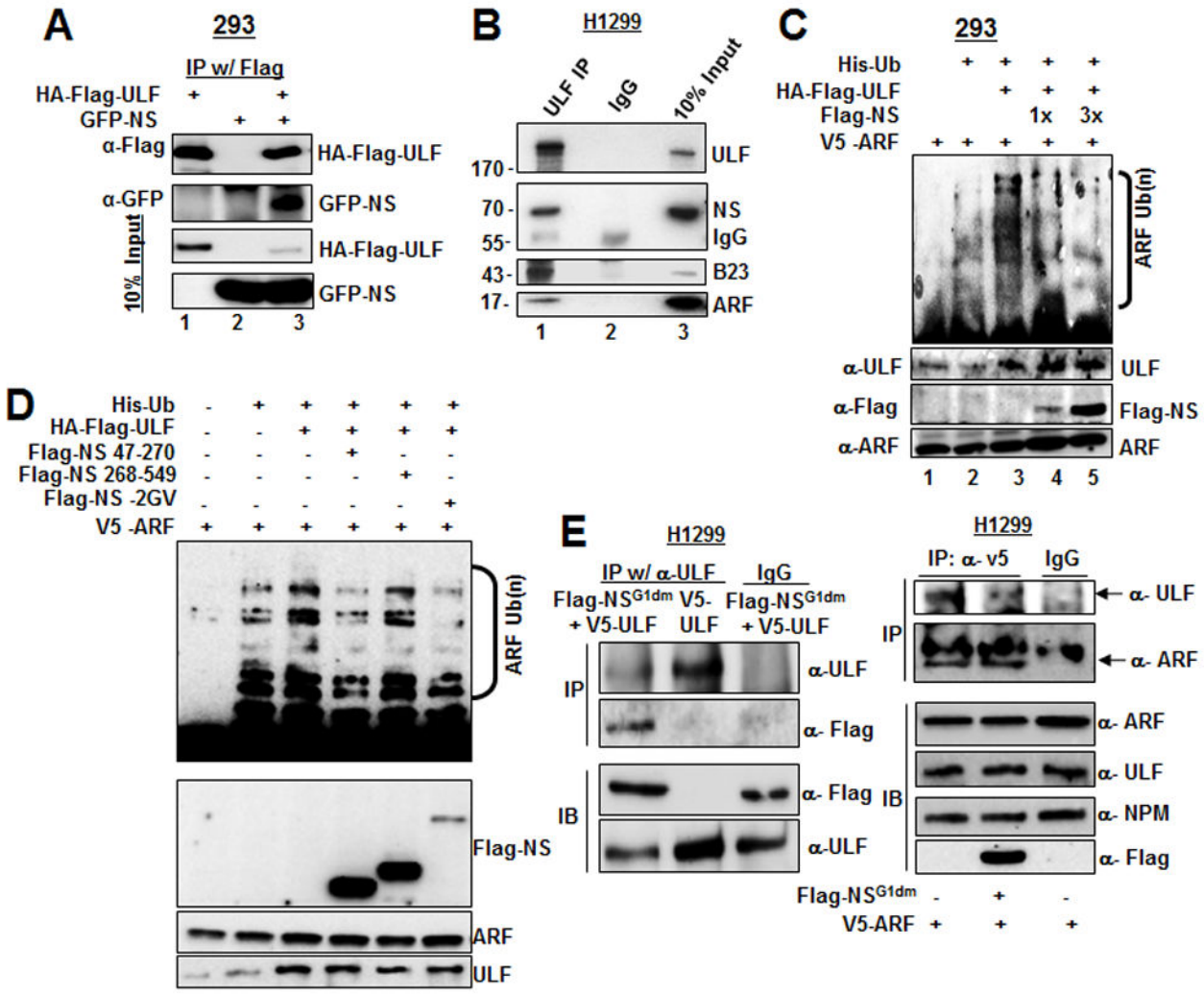

Figure 6. NS inhibits ARF polyubiquitination

(A) Exogenously expressed NS interacts with ULF. H1299 cells were transfected with plasmids expressing GFP-NS, Flag-HA-ULF alone or together. Lysates were subjected to IP with Flag antibody. Precipitated proteins were resolved by SDS-PAGE and detected by WB with the indicated antibodies. (**B**) Endogenously expressed NS interacts with ULF in H1299 cells. Lysates from H1299 cells were subjected to IP using ULF antibody or control IgG rabbit antibody. Precipitated proteins were resolved by SDS-PAGE and detected by western blot with the indicated antibodies. (**C**) NS inhibits ULF mediated ARF polyubiquitination. H1299 cells were transfected with the indicated plasmids. 42 hours after transfection, cells were treated with 20μM MG132 and harvested 6 hours later. Polyubiquitinated proteins from lysates were isolated using Ni2+ beads and analyzed by western blot along with input protein. (**D**) Both NS^{G1dm} and NS fragment 47–270 inhibit ULF mediated ARF polyubiquitination. H1299 cells were transfected with the indicated plasmids. 42 hours after transfection, cells were treated with 20μM MG132 and harvested 6 hours later. Polyubiquitinated proteins from lysates were isolated using Ni2+ beads and analyzed by western blot along with input protein. **(E) Left:** Exogenously expressed NS GTP binding

mutant and ULF interact in H1299 cells. H1299 cells were transfected with plasmids expressing Flag-NS^{G1dm} with V5-ULF together or alone. Lysates were subjected to IP with ULF antibody. Precipitated proteins are resolved by SDS-PAGE and detected by WB with the indicated antibodies. **Right:** NS^{G1dm} reduces ARF-ULF interaction. H1299 cells were transfected with plasmids expressing V5-ARF with Flag-NS^{G1dm} together or alone. Lysates were subjected to IP with anti-V5 antibody. Precipitated proteins were resolved by SDS-PAGE and detected by WB with the indicated antibodies.

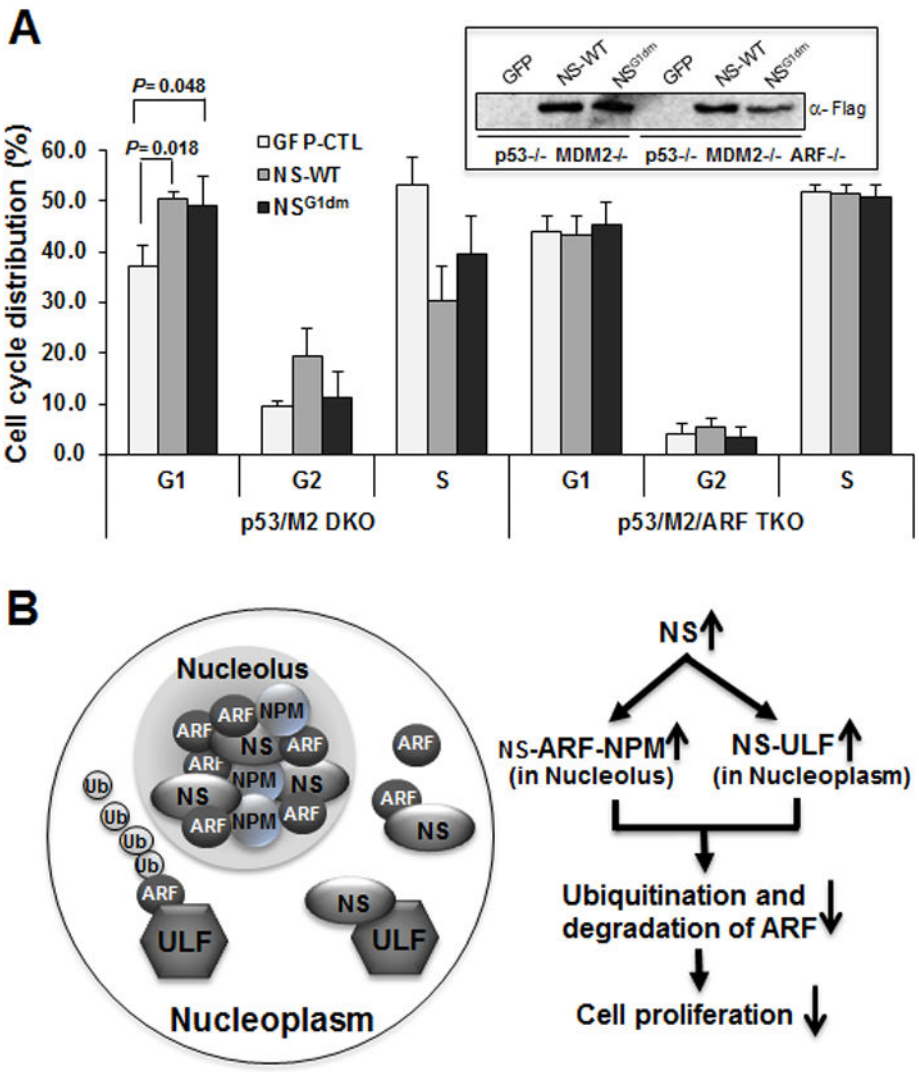

Figure 7. NS or NS GTP mutant induces ARF-dependent G1-phase arrest

(A) Overexpression of NS-WT or NS-^{G1dm} induces G1 arrest in the p53$^{-/-}$ MDM2$^{-/-}$ MEF but not in the p53$^{-/-}$ MDM2$^{-/-}$ ARF$^{-/-}$ MEF cells. MEF cells were transfected with the plasmids expressing NS-WT or NS-^{G1dm} for 48hr, and fixed in 70% ethanol for FACS analysis. The expression of the proteins was determined by WB as shown in the insert panel. (**B**) Model illustrating the potential mechanisms through NS for stabilizing the ARF protein. NS overexpression induces ARF stabilization by directly interacting with ARF, stabilizing the NPM/ARF complex within the nucleolus. Excess NS released in the nucleoplasm binds to ULF, the E3 ligase of ARF, interferes between ARF and ULF interaction, thus preventing ULF mediated polyubiquitination and proteasomal degradation of ARF. Stabilization of ARF induces G1 cell cycle arrest and prevents cellular proliferation.