Nucleolar GTP-binding Protein-1 (NGP-1) Promotes G₁ to S Phase Transition by Activating Cyclin-dependent Kinase Inhibitor p21Clp1/Waf1

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**Background:** Breast cancer autoantigen nucleolar GTP-binding protein-1 (NGP-1) is overexpressed in cancers. Nucleolar GTP-binding protein (NGP-1) is overexpressed in various cancers and proliferating cells, but the functional significance remains unknown. In this study, we show that NGP-1 promotes G₁ to S phase transition of cells by enhancing CDK inhibitor p21Clp1/Waf1 expression through p53. In addition, our results suggest that activation of the cyclin D1-CDK4 complex by NGP-1 via maintaining the stoichiometry between cyclin D1-CDK4 complex and p21 resulted in hyperphosphorylation of retinoblastoma protein at serine 780 (p-RBSer-780) followed by stabilization upon Ngp-1 depletion. Furthermore, our data suggest that ribosomal protein RPL23A interacts with NGP-1 and abolishes NGP-1-induced p53 activity by enhancing Mdm2-mediated p53 polyubiquitination. Finally, reduction of p-RBSer-780 levels and expression upon ectopic expression of RPL23a resulted in arrest at the G₁ phase of the cell cycle. Collectively, this investigation provides evidence that NGP-1 promotes cell cycle progression through the activation of the p53/p21Clp1/Waf1 pathway.

**Results:** NGP-1 promotes G₁ to S phase transition by modulating the p53/p21 pathway.

**Conclusion:** CDK inhibitor, p21, enhances cell proliferation in certain situations.

**Significance:** This study provides evidence that p21 induces cell proliferation in addition to its traditional tumor suppressor function.

Nucleolar GTP-binding protein (NGP-1) is overexpressed in various cancers and proliferating cells, but the functional significance remains unknown. In this study, we show that NGP-1 promotes G₁ to S phase transition of cells by enhancing CDK inhibitor p21Clp1/Waf1 expression through p53. In addition, our results suggest that activation of the cyclin D1-CDK4 complex by NGP-1 via maintaining the stoichiometry between cyclin D1-CDK4 complex and p21 resulted in hyperphosphorylation of retinoblastoma protein at serine 780 (p-RBSer-780) followed by the up-regulation of E2F1 target genes required to promote G₁ to S phase transition. Furthermore, our data suggest that ribosomal protein RPL23A interacts with NGP-1 and abolishes NGP-1-induced p53 activity by enhancing Mdm2-mediated p53 polyubiquitination. Finally, reduction of p-RBSer-780 levels and expression upon ectopic expression of RPL23a resulted in arrest at the G₁ phase of the cell cycle. Collectively, this investigation provides evidence that NGP-1 promotes cell cycle progression through the activation of the p53/p21Clp1/Waf1 pathway.

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Complex formation and its activity as long as the p21 level is lower than the concentration required to inhibit the complex (51). This inhibitory activity of p21 in normal cells is a common phenomenon because CDK levels remain constant (52) and cyclin levels are tightly regulated (53). However, in cancers, both cyclin D1 and CDK4 levels are up-regulated (54–57) and lead to higher complex formation thus maintaining the stoichiometry with increased p21 levels. Interestingly, high levels of cyclin D1 and p21 were observed in breast cancers (28), and knockdown of cyclin D1 and p21 has been shown to inhibit proliferation by up-regulating p21 and possibly by maintaining some biogenesis by coupling ribosome biogenesis with cell proliferation (72). This role of RPs ensures that cell proliferation is halted in conditions of impaired ribosome biogenesis (59–61). Subsequently, E2F1 activates its own promoter (62) and its targets like cyclin A2, cyclin E1, and Myc, which are essential for cell proliferation (63–66).

It is well known that ribosomal proteins (RP) play a crucial role in modulating the p53-Mdm2 pathway (67) to regulate cell proliferation. Upon ribosomal stress, RPs like RPL5, RPL11, and RPL23 inhibit Mdm2-mediated p53 degradation (68–70), whereas RPL26 promotes p53 expression by binding to the 5′UTR of p53 mRNA (71). In contrast, RPL37 destabilizes p53 by repressing RPL11 expression (72). This role of RPs ensures that cell proliferation is halted in conditions of impaired ribosome biogenesis by coupling ribosome biogenesis with cell proliferation.

In this study, we demonstrated that NGP-1 promotes cell proliferation by up-regulating p21 and possibly by maintaining the stoichiometry between the cyclin D1-CDK4 complex and p21. Knockdown of p53 or p21 in NGP-1-overexpressed cells reduced G1 to S phase transition, suggesting that the activity of NGP-1 is p53/p21-dependent. Finally, our data provide evidence that NGP-1-mediated suppression of RPL23A activity is critical for cell cycle progression.

Experimental Procedures

Plasmid Construction—NGP-1-GFP and its deletion constructs (NGP-1(1–100), NGP-1(101–600), and NGP-1(601–731)) were generated as described elsewhere (16). Lyar and RPL23A were amplified from the HEK-293T cDNA library using appropriate primers (Table 1) and cloned as GST fusion constructs (NGP-1(1–100), NGP-1(101–600), and NGP-1(601–731)).

Primers used for cloning and RT-qPCR analysis

| Primer used for cloning | Primer used for qPCR |
|------------------------|----------------------|
| NGP-1 Xhol +           | AAGCTCTGACGCTAGGATCTGAC |
| NGP-1 FLAG Kpn1 −      | GCTGCTTTAGACGCTGGATTT |
| LYAR EcoRI +           | TTAAGAGAGAGACCTCAATCCT TC |
| LYAR Xhol −            | CTTCAACTTACGAGTTGAGGAG |
| RPI23a BamHI +         | GGCGATCCATCCGAGGAG |
| RPI23a EcoRI −         | GCGGATCCATCCGAGGAG |
| RPI23a XhoI +          | GCTGCTTTAGACGCTGGATTT |
| RPI23a EcoRI −         | TTAAGAGAGAGACCTCAATCCT TC |

Antibodies

- Actin, HA, and FLAG (Sigma);
- NPG-1 and RPL23A (Abcam);
- Mdm2 (Calbiochem);
- p21, total cyclin D1 (BD Biosciences).

Chemicals—Cycloheximide (Sigma) and thymidine (Sigma) were dissolved in water and used at a final concentration of 50 µg/ml and 2 mM, respectively. MG132 (Calbiochem) was dissolved in DMSO and used at a final concentration of 20 µM.

Expression and Purification of RPL23A and LYAR Fusion Proteins—Full-length LYAR and RPL23A expression vectors were transformed into Escherichia coli BL21-DE3 and grown at 37 °C. Protein expression was induced for 4 h at 37 °C with 1 mM isopropyl 1-thio-β-D-galactopyranoside. Cells were lysed in bacterial lysis buffer (150 mM NaCl, 10 mM Tris, pH 8, 1 mM EDTA, pH 8, 1 mM DTT, 2 µg/ml lysozyme) and mixed with glutathione-Sepharose beads (GE Healthcare). Bound proteins were eluted in elution buffer (10 mM reduced glutathione in 50 mM Tris-Cl, pH 8), and the integrity of fusion proteins was

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|---------|---------------------------------------------|
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| NGP-1 FLAG Kpn1 −      | GCTGCTTTAGACGCTGGATTT |
| LYAR EcoRI +           | TTAAGAGAGAGACCTCAATCCT TC |
| LYAR Xhol −            | CTTCAACTTACGAGTTGAGGAG |
| RPI23a BamHI +         | GGCGATCCATCCGAGGAG |
| RPI23a EcoRI −         | GCGGATCCATCCGAGGAG |
| RPI23a XhoI +          | GCTGCTTTAGACGCTGGATTT |
| RPI23a EcoRI −         | TTAAGAGAGAGACCTCAATCCT TC |
FIGURE 1. NGP-1 promotes cell proliferation. NGP-1-FLAG or the control vector was transfected in MCF-7 or ZR75.1 cells. Cell proliferation was determined by MTT assay (A), BrdU incorporation assay (B), CFSE proliferation assay (C, panel i), and cell growth assay (C, panel ii).

FIGURE 2. NGP-1 promotes cell cycle progression. NGP-1-FLAG or the control vector was transfected in MCF-7 or ZR75.1 cells and harvested 48 or 72 h after transfection, respectively. A, panel i, expression of NGP-1-FLAG was checked by Western blot analysis using anti-FLAG antibody. A, panel ii, cell cycle analysis was performed with unsynchronized MCF-7 cells. A, panel iii, representative histograms of cell cycle profile for NGP-1-FLAG and vector-transfected cells. NGP-1 up-regulates p53 and its downstream targets. B, panel i, expression levels of NGP-1-FLAG and indicated proteins were analyzed by Western blot analysis using anti-FLAG, -p53, -p21, and -Mdm2 antibodies. β-Actin was used as loading control. B, panel ii, densitometry analyses of the corresponding Western blots were carried out by normalizing the expression levels of the indicated endogenous proteins to β-actin. The bar diagram represents fold difference of the normalized proteins in NGP-1-transfected cells with respect to vector-transfected cells. The error bars represent the standard deviation between three independent experiments. NGP-1 promotes G1 to S phase transition during cell cycle. C, MCF-7 cells were transfected with NGP-1-FLAG or the corresponding vector and treated with 2 mM thymidine. After 24 h of treatment, cells were harvested at 0 and 4 h, and cell cycle patterns were analyzed by flow cytometry.
FIGURE 3. NGP-1 induces G1 to S phase transition through up-regulation of p53. NGP-1 increases p53 transcriptional activity. A, MCF-7 cells were co-transfected with NGP-1-FLAG or control vector with PG13-luciferase (p53 reporter plasmid) and pRL-TK-Renilla luciferase plasmid (internal control). After 48 h of transfection, luciferase assay was performed to check p53-dependent promoter activity. RLU, relative light unit. B, MCF-7 cells were transfected with NGP-1-FLAG or vector control, and after 48 h, levels of NGP-1, p53, p21, and Mdm2 mRNA were determined by RT-qPCR. NGP-1 induced G1 to S phase transition is p53-dependent. C, MCF-7 cells were co-transfected with NGP-1-FLAG or corresponding vector and 200 pmol of p53-specific or control siRNA. C, panel i, transfected cells were treated with 2 mM thymidine for 24 h and were harvested at 0 and 4 h followed by cell cycle analysis. C, panel ii, expression of NGP-1-FLAG and endogenous levels of p53 and p21 was confirmed by Western blot analysis using anti-FLAG, -p53, and -p21 antibodies, respectively. β-Actin was used as loading control. C, panel iii, densitometry analyses of the Western blots representing p53 and p21 levels.

FIGURE 4. Cell proliferation induced by NGP-1 is p21-dependent. MCF-7 cells were co-transfected with NGP-1-FLAG or the corresponding vector and p21-specific or control shRNA. Forty eight hours after transfection, cell proliferation was checked by MTT assay (A) and BrdU incorporation assays (B). C, 24 h after transfection, cells were treated with 2 mM thymidine for 24 h and were harvested at 0 and 4 h followed by cell cycle analysis. D, panel i, expression of NGP-1-FLAG and p21 (endogenous) was confirmed by Western blot analysis using anti-FLAG and -p21 antibodies, respectively. β-Actin was used as loading control. D, panel ii, densitometry analyses of the Western blot representing endogenous p21 level.
checked on SDS-12% PAGE followed by staining with Cooma-
sie Blue.

Cell Culture, Transfection, and Western Blot Analysis—
MCF-7 or ZR75.1 cells were maintained in Dulbecco’s modified
Eagle’s medium (DMEM) or RPMI 1640 medium, respectively,
supplemented with 10% fetal bovine serum and 1% antibiotic/
antimycotic (Life Technologies, Inc.). Transfection with vari-
ous plasmids was carried out using Lipofectamine2000 (Life
Technologies, Inc.) according to the manufacturer’s protocol.
Wild-type or deletion constructs of NGP-1-GFP were trans-
fected using the T7-RNA polymerase vaccinia virus expression
system as described elsewhere (73). For Western blotting, cells
were solubilized in 1× cell lysis buffer (20 mM HEPES, pH 7.9,
150 mM NaCl, 1 mM EDTA, pH 8, 1 mM EGTA, pH 8, 1%Non-
idet P-40, 1 mM Na_3P_2O_7, 1 mM PMSF, 1 mM DTT, 1 mM NaF,
1 mM Na_3VO_4 and 1× protease inhibitor mixture (PIC)). For
phosphoprotein analysis, cells were lysed by sonication in 1×
cell lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA,
pH 8, 1 mM EGTA, 1% Nonidet P-40, 1× PIC, 1 mM PMSF, 2
mM Na_3VO_4, 100 mM okadaic acid, 0.5% sodium deoxycholate,
50 mM NaF, 10 mM Na_3P_2O_7, and 0.1% SDS). Equal quantities of
protein were resolved on SDS-PAGE and transferred to
Hybond-P membrane (GE Healthcare) and probed with the
indicated antibodies. Protein-bound antibodies were probed
with HRP-conjugated specific secondary antibodies and devel-
oped using enhanced chemiluminescence-prime detection sys-
tem (GE Healthcare). Densitometry analyses of the corre-
sponding Western blots were carried out by normalizing the
expression levels of the mentioned endogenous proteins to
β-actin. The bar diagram represents the fold difference of the
normalized proteins in transfected cells with respect to vector-
transfected cells. The intensities of the Western blots were
determined by ImageJ software and plotted as bar diagrams
using Graph Pad Prism 5 platform.

FIGURE 5. NGP-1 regulates cell cycle by maintaining the relative stoichiometry between p21 and cyclin D1-CDK4 complex. MCF-7 cells were transfected
with NGP-1-FLAG or the corresponding vector, and cell lysates were prepared after 48 h of transfection. A, panel i, co-immunoprecipitation was performed with
anti-cyclin D1 antibody followed by Western blot analysis using anti-CDK4, -cyclin D1, and -p21 antibodies. The expression of NGP-1-FLAG and the indicated
endogenous proteins was confirmed by Western blot analysis using anti-FLAG, -cyclin D1, -CDK4, and -p21 antibodies. β-actin is used as loading control. A,
panel ii, densitometry analyses of the corresponding Western blots. NGP-1-FLAG or the corresponding vector-transfected MCF-7 cells were treated with 2 mM
thymidine for 24 h. B, panel i, expression levels of total and phosphorylated RB protein were determined by Western blot analysis using anti-p-RBSer-780 and
-p-total RB antibodies, respectively. B, panel ii, densitometry analyses of the Western blots representing p-RB/Total RB. C, expression levels of NGP-1, cyclin A2,
cyclin E1, E2F1, and Myc were determined by RT-qPCR.
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Luciferase Assay—NGP-1-FLAG or Rpl23-HA expression plasmids were co-transfected with PG13-luciferase-p53 reporter and pRL-TK-Renilla luciferase plasmids (internal control). After 48 h of transfection, cells were washed with phosphate-buffered saline (PBS) and incubated with luciferase lysis buffer (0.1% Triton X-100, 9 mM KH₂PO₄, pH 5.1, 90 mM K₂HPO₄, pH 8, and 1 mM DTT) for 10 min with vigorous shaking. Cell lysates were transferred to Optiplate, and assay was performed with luciferase/Renilla assay buffer as described elsewhere (74) using a Luminometer (PerkinElmer Life Sciences).

In Vivo Ubiquitination Assay—HA-ubiquitin and RPL23A-HA-transfected cells were treated with 20 μM MG132 for 5 h, 43 h after transfection. Cell extracts were subjected to immunoprecipitation using anti-p53 antibody followed by Western blot analysis using anti-HA antibody.

RNA Interference—Cells were transfected with NGP-1 or p53 or control esiRNA 1 (Sigma) by using Lipofectamine2000 according to the manufacturer’s instructions. For the p21 knockdown experiment, cells were co-transfected with p21 shRNA (TRCN0000040126, Sigma) or the control plasmid (SHC016, Sigma) together with NGP-1-FLAG or NGP-1 esiRNA or control esiRNA 1 using Lipofectamine2000 according to the manufacturer’s instructions. After 72 h of transfection, cells were harvested, and endogenous NGP-1, p21, or p53 levels were determined by Western blot analysis using the respective antibodies.

Cell Synchronization—Twenty four hours post-transfection, cells were treated with 2 μM thymidine as described elsewhere (75). After 24 h, cells were washed three times with PBS, replaced with DMEM, and harvested at 0 and 4 h post-thymidine release to monitor G₁ to S phase transition using a flow cytometer.

Cell Cycle Analysis—Cells were transfected with various plasmids in triplicate using Lipofectamine2000. Unsynchronized or synchronized cells were washed with PBS and treated with hypotonic buffer (0.1% sodium citrate, 0.1% Nonidet P-40, 45 μg/ml propidium iodide, 50 μg/ml ribonuclease A, pH 7.4) for 5 min at room temperature. The cell cycle pattern was analyzed by a flow cytometer.

MTT and BrdU Incorporation Assay—Cells were transfected with NGP-1-FLAG or RPL23A-HA and processed after 48 h of transfection. For MTT assay, 12 mM MTT solution (Vybrant MTT Cell Proliferation assay kit, Life Technologies, Inc.) was added and incubated for 2 h at 37 °C as per manufacturer’s protocol, and the absorbance was measured at 540 nm using Enspire Multimode Reader (PerkinElmer Life Sciences). For BrdU incorporation assay, 1X BrdU (BrdU cell proliferation assay kit, Cell Signaling Technology) was added and incubated for 6 h as per manufacturer’s protocol. Cells were fixed, and incubation with anti-BrdU primary antibody was followed by HRP-conjugated secondary antibodies. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad).

RT-qPCR Analysis—Total RNA was isolated from cells transfected with various plasmids using TRIzol reagent (Life Technologies, Inc.). Integrity of RNA was checked before being used for reverse transcription (RT). RT (1 μg of total RNA) was carried out using ImProm-II kit (Promega Corp.) according to the

All the densitometric analyses are represented as bar diagrams along with the Western blots in the following figures: 2B, panel ii; 3C, panel iii; 4D, panel ii; 5, A, panel ii, and B, panel ii; 8, C, panel ii, D, panel ii, and E, panel ii; 9, G, panel ii, and D, panel ii; 10B, panel ii; 11A, panel ii; 13C, panel ii; and 14D, panel ii.

Carboxyfluorescein Succinimidyl Ester (CFSE) Proliferation Assay—MCF-7 cells were transfected with NGP-1-FLAG or vector, and after 24 h of transfection, cells were stained with the CFSE dye (CellTrace™ CFSE staining kit, Life Technologies, Inc.) as per the manufacturer’s protocol. Forty eight hours later, cells were analyzed using FACSDairy II flow cytometer (BD Biosciences).

Cell Proliferation Assay—MCF-7 cells were transfected with NGP-1-FLAG or vector, and 24 h after transfection, cells were seeded at 0.05 million cells/well in a 24-well plate. Cell numbers were counted every alternate day for a period of 6 days.

Protein-Protein Interaction Assays—For GST pulldown assay, cell lysates with equal amounts of protein were incubated with glutathione-Sepharose beads pre-bound with GST-RPL23A or GST-LYAR as described elsewhere (16). For co-immunoprecipitation, equal amounts of cell lysates were incubated with appropriate antibody, and the bound protein complexes were eluted and resolved on SDS-PAGE followed by Western blotting with appropriate antibodies.

Half-life Determination—RPL23A-HA-transfected cells were treated with 50 μg/ml CHX after 48 h of transfection and harvested at different time periods followed by Western blot analysis.
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Quantitative PCR was carried out in RealPlex cycler (Eppendorf) using SYBR Green mix (Roche Applied Science). All reactions were carried out in triplicate, and the gene expressions relative to \( H_9252 \)-actin levels were analyzed according to the manufacturer’s instructions. The primers used for RT-qPCR analysis are shown in Table 1.

Statistical Analysis—All the statistical analyses were carried out using GraphPad Prism 5 platform. All experiments were repeated three times with three biological replicates. Representative values were used to calculate the mean, standard deviation, and the significance by an unpaired Student’s t test and are represented in the corresponding bar diagrams shown in the following figures: 1, A–C; 2, A, panel ii, and C; 3, A–C, panels i; 4, A–C, panels i; 5, C; 6A; 9E; 10A; 11, B–E; 12, A and B; 13, A and B; and 14, A–C.

Results

NGP-1 Promotes Cell Proliferation—Previous studies suggested an association of NGP-1 with proliferating cells (15, 16), but the mechanism(s) remains poorly understood. Because NGP-1 was identified as a breast cancer autoantigen and is overexpressed in breast cancer (15), in this study we used MCF-7 (breast cancer cell line) cells to understand the role of NGP-1 in cell cycle regulation. NGP-1 was overexpressed in MCF-7 cells, and cell proliferation was analyzed by MTT and BrdU incorporation assays. MTT and BrdU incorporation assays indicate that ectopic expression of NGP-1 increases cell viability and DNA replication (Fig. 1, A and B). The overall growth of NGP-1-transfected cells was also monitored by CFSE dye-based cell proliferation assay (Fig. 1, C, panel i) and by direct cell counting (Fig. 1, C, panel ii). CFSE dye-based cell proliferation assay covalently labels the intracellular molecules with the fluorescent dye, carboxyfluorescein, and the percentage of cells progressing to the next generation (generation 2) from parental generation (generation 1) can be assessed by measuring the decrease in cell fluorescence due to dilution of the dye as represented in Fig. 1, C, panel i. Cells transfected with NGP-1 progressed much faster into the new generation compared with the vector-transfected cells indicating a higher growth rate (Fig. 1, C, panel i). To further confirm this, the growth of NGP-1-expressing cells was monitored by direct counting of cell numbers at periodic time intervals up to 6 days after 24 h of transfection, and the result in Fig. 1, C, panel ii, clearly shows that NGP-1 expression significantly increased the cell number compared with the vector-transfected cell lines.

FIGURE 7. NGP-1 interacts with LYAR and RPL23A. MCF-7 cells were transfected with NGP-1-GFP or the corresponding control vectors using T7-RNA polymerase vaccinia virus expression system (vTF7-3). After 48 h of transfection, cell lysates were prepared, and GST pulldown assay was performed with GST-LYAR (A) or GST-RPL23A (C) followed by Western blot analysis using anti-GFP antibody. GST was used as negative control. The expression of NGP-1-GFP was checked by Western blot analysis using anti-GFP antibody. IP, immunoprecipitation; IB, immunoblot. B, interaction of endogenous RPL23A with GST-LYAR was determined by GST pulldown assay with MCF-7 lysates followed by Western blot analysis using anti-RPL23A antibody. D, MCF-7 cell lysates containing NGP-1-FLAG were subjected to co-immunoprecipitation using anti-FLAG antibody. Complexes were eluted and separated on SDS-12% PAGE followed by Western blot analysis with anti-RPL23A antibody.
with vector-transfected cells suggesting an increase in the growth rate. To check whether NGP-1 modulates cell cycle profile, flow cytometry analysis was performed with NGP-1-transfected cells. A lower percentage of NGP-1-overexpressing cells accumulated in G1 and S phases in contrast to the G2/M phase of the cell cycle (Fig. 2A). Together, these results suggest that NGP-1 promotes cell cycle progression and thereby enhances cell proliferation in MCF-7 cells. To test whether NGP-1-mediated cell proliferation is conserved in breast cancer cell lines, cell proliferation was determined in another breast cancer cell line, ZR75.1 (harboring wild-type p53), and similar patterns of cell proliferation were observed (Fig. 1, A–C, panel i). Interestingly, other members of the HSR1-MMR1 family, NS and GNL3L, have been shown to modulate p53 to regulate the cell cycle (3, 8). Because p53 is an important regulator of the cell cycle, we determined the status of p53 and its targets upon NGP-1 expression. Results in Fig. 2B indicate an increase in p53 levels upon ectopic expression of NGP-1 (lane 2). Furthermore, the levels of p53 target genes, p21 and Mdm2, were also elevated upon NGP-1 overexpression (Fig. 2B, lane 2). NGP-1 expression in ZR75.1 cells also increased the expression levels of p53 and p21 (Fig. 2B). Because p53 is known to modulate the G1/S phase of cell cycle, the effect of NGP-1 on G1 to S phase transition was determined by synchronizing the transfected cells at the G1 phase by thymidine block, as described under “Experimental Procedures,” followed by cell cycle analysis at 4 h after release from thymidine block (S phase lasts for 6 h in MCF-7). Results in Fig. 2C indicate that a significantly higher number of NGP-1-expressing cells progressed to S phase suggesting that NGP-1 promotes faster G1 to S phase transition.

NGP-1 Regulates Cell Cycle through Cyclin-dependent Kinase Inhibitor p21—NGP-1 promoting cell proliferation despite enhanced p53 expression was an unexpected observation. Therefore, it was worthwhile to test whether NGP-1-induced p53 was functional during cell proliferation. Toward this end, NGP-1 was co-expressed with p53 reporter plasmid (PG13-luciferase), and the transcriptional activity of p53 was determined. Results in Fig. 3A indicate that ectopic expression of NGP-1 significantly increased p53-dependent luciferase activity. Additionally, RT-qPCR analysis indicates an increased expression of p53 target genes, p21 and Mdm2, upon NGP-1 overexpression (Fig. 3B). These data confirm that p53 synthe-
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sized during NGP-1 expression was transcriptionally active. Furthermore, to validate whether NGP-1-induced G₁ to S phase transition is p53-dependent, NGP-1 was expressed with or without p53 knockdown (using p53-specific siRNA) followed by cell synchronization at the G₁ phase by thymidine block. Cell cycle analysis after 4 h of release from thymidine block indicates that p53 knockdown during NGP-1 expression did not accelerate the progression of cells, which is in contrast to cells overexpressing NGP-1 alone (Fig. 3C, panel i). Knockdown of p53 was confirmed by Western blot analysis (Fig. 3C, panel ii). The knockdown of p53 in the NGP-1-overexpressed condition was expected to promote faster progression of cells into the S phase as observed in cells with p53 deletion alone. Interestingly, our observations appear to contradict the current model of p53 action, but the results provided evidence that promotion of G₁ to S phase transition by NGP-1 is p53-dependent. Additionally, we also confirmed reduced levels of p21 in cells overexpressing NGP-1 with p53 knockdown (Fig. 3C, panel ii, lane 4) suggesting that NGP-1 up-regulates p21 in a p53-dependent manner. Collectively, these results suggest that up-regulation of p21 during NGP-1 expression may be critical for faster G₁ to S phase transition during the cell cycle. To further explore the role of p21 in NGP-1 function during cell proliferation, NGP-1 was transfected alone or in combination with p21 shRNA-expressing plasmid. Results from MTT (Fig. 4A) and BrdU (Fig. 4B) incorporation assays reveal that NGP-1 expression with p21 alleviation failed to induce cell proliferation, which is in contrast with cells overexpressing NGP-1. The depletion of p21 and expression of NGP-1 were confirmed by Western blot analysis (Fig. 4C). To further understand the role of p21 in NGP-1 function during cell proliferation, Western blot analysis was performed with cells expressing NGP-1 with or without p21 knockdown. Results in Fig. 4C suggest that p21 knockdown in NGP-1-overexpressing cells failed to accelerate G₁ to S phase transition that was otherwise observed in cells with p21 knockdown or NGP-1 overexpression. Together,
these results strongly suggest the critical requirement of p21 in NGP-1-induced G1 to S phase transition.

NGP-1 Regulates Cell Cycle Possibly by Maintaining the Stoichiometry between CyclinD1-CDK4 Complex and p21—It is well known that p21 inhibits activity of cyclin D1-CDK4 complex that results in decreased phosphorylation of RB at serine 780 (p-RB Ser-780) (59–61). However, evidence from the existing literature also suggests that p21 enhances the activity of cyclin D1-CDK4 during NGP-1 expression by promoting the complex formation between them, when p21 is stoichiometrically lower than the inhibitory concentration (51). To understand whether the increased p21 levels during NGP-1 overexpression promote the cyclin D1-CDK4 complex formation, we performed co-immunoprecipitation assay using anti-cyclin D1 antibody followed by Western blot analysis using anti-HA, -p53, -Mdm2, and p21 antibodies, respectively. β-Actin was used as loading control. B, panel i; densitometry analyses of corresponding Western blots.

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NGP-1 Down-regulates Ribosomal Protein, RPL23A—Mass spectroscopy analysis identified NGP-1 as part of a complex containing the nucleolar protein, LYAR, and ribosomal protein, RPL23A (76). To define the significance of this complex with relevance to NGP-1 function, we first examined whether NGP-1 interacts with LYAR. GST pulldown assay revealed that NGP-1 physically interacts with LYAR (Fig. 7A, lane 1). We then checked the interaction between LYAR and RPL23A by GST pulldown assay. Results in Fig. 7B indicate that Lyar also physically interacts with RPL23A (lane 1). Next, we determined the interaction between NGP-1 and RPL23A. Results in Fig. 7C reveal a specific interaction between NGP-1 and RPL23A (lane 1). To further understand whether NGP-1 and RPL23A interact in vivo, we performed a co-immunoprecipitation assay with NGP-1-overexpressing cells. Results in Fig. 7D indicate an interaction between NGP-1 and RPL23A (lane 2). We next transfected various NGP-1 deletion constructs (Fig. 8A) to identify the domain(s) responsible for its interaction with RPL23A. Results from GST pulldown assay suggest that domains between amino acids 1–100 and 601–731 of NGP-1 independently interact with RPL23A (Fig. 8B, lanes 2 and 4). To understand the functional significance of NGP-1 and RPL23A interaction, we first checked whether NGP-1 modulates RPL23A levels. Interestingly, ectopic expression of NGP-1 in MCF-7 cells resulted in decreased RPL23A levels (Fig. 8C, lane 2). A similar pattern of RPL23A levels was also observed in ZR75.1 cells (data not shown). Furthermore, NGP-1 depletion up-regulated RPL23A levels (Fig. 8D, lane 2), which suggests that NGP-1 suppresses RPL23A expression. However, ectopic expression of RPL23A did not alter NGP-1 levels (Fig. 8E, lane 2). Together, these data suggest that RPL23A may be a downstream target of NGP-1.

FIGURE 10. RPL23A suppresses the expression of p53 and its target genes. MCF-7 cells were transfected with RPL23A-HA or the control vectors. A, transcript levels of RPL23A, p53, Mdm2, and p21 were determined by RT-qPCR. B, panel i, expression of RPL23A, p53, Mdm2, and p21 was determined by Western blot analysis using anti-HA, -p53, -Mdm2, and p21 antibodies, respectively. β-Actin was used as loading control. B, panel ii, densitometry analyses of corresponding Western blots.
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**FIGURE 11.** RPL23A inhibits cell proliferation. MCF-7 cells were transfected with RPL23A-HA. A, panel i, expression and phosphorylation status of RB were determined by Western blot analysis using anti-p-RB(Ser-780) and -total RB antibodies, respectively. A, panel ii, densitometric analyses of corresponding Western blots. B, levels of NGP-1, cyclin A2, cyclin E1, E2F1, and Myc were determined by RT-qPCR. C, cell cycle profiles were analyzed by flow cytometry. The rate of proliferation was measured by MTT assay (D) and BrdU incorporation assay (E) simultaneously with cell cycle analysis.

**FIGURE 12.** NGP-1 relieves RPL23A-mediated inhibition on cell cycle progression. A, MCF-7 cells were transfected with indicated plasmids. pRL-TK-Renilla luciferase plasmid was used as internal control. After 48 h of transfection, luciferase assay was performed to check p53-dependent promoter activity. B, NGP-1-FLAG was co-transfected with RPL23A-HA in MCF-7 cells. After 48 h of transfection, cell cycle profiles were analyzed by flow cytometry. Expression of NGP-1-FLAG and RPL23A-HA was determined by Western blot analysis using anti-FLAG and -HA antibodies, respectively.
RPL23A Promotes p53 Degradation—Earlier studies suggested that various ribosomal proteins regulate the p53-Mdm2 pathway (67), but the role of RPL23A in the p53 pathway remained unexplored. Consequently, we performed co-immunoprecipitation assay to determine the interaction between RPL23A and p53. Results clearly indicate that RPL23A readily interacts with Mdm2 (Fig. 9B, lane 2) but not with p53 (Fig. 9A, lane 2). To determine whether the interaction between RPL23A and Mdm2 alters p53 stability, in vivo ubiquitination assay was performed. Data in Fig. 9C suggest that ectopic expression of RPL23A increased p53 polyubiquitination (lane 2). To confirm further, p53 steady-state levels were determined upon RPL23A overexpression by cycloheximide (CHX) chase assay. Interestingly, degradation of p53 was observed within 15 min in cells expressing RPL23A in contrast to 120 min in vector-transfected cells (Fig. 9D). To understand the effect of RPL23A on p53 function, luciferase assay was performed in cells co-transfected with p53-dependent reporter plasmid (PG13-luciferase) and RPL23A. Results in Fig. 9E suggest that RPL23A reduced p53-dependent promoter activity. In addition, expression levels of known p53 target genes, p21 and Mdm2, were also reduced (Fig. 10, A and B) upon ectopic expression of RPL23A. Collectively, these data provide evidence that RPL23A promotes p53 degradation possibly through Mdm2.

NGP-1 Relieves RPL23A-mediated Inhibition on Cell Cycle Progression—Results from the above experiments indicate that RPL23A down-regulates p53 and its downstream target, p21, suggesting the possibility that RPL23A might modulate the p53-p21 axis. To understand this, we first checked the levels of p-RBSer-780 in RPL23A-overexpressing cells, and the results in Fig. 11A indicate that ectopic expression of RPL23A reduced the p-RBSer-780 level without altering total RB expression (lane 2). In addition, RT-qPCR analysis shows reduced expression levels of E2F1 and its target genes, cyclin A2, cyclin E1, and c-Myc, upon expression of RPL23A (Fig. 11B). To further explore RPL23A function during cell proliferation, we performed flow cytometry analysis in cells expressing RPL23A. Results indicate that more numbers of RPL23A-overexpressing cells accumulated in G1 phase in contrast to G2/M phase, whereas no change in the S phase profile was observed (Fig. 11C). To further define the consequence of the observed change in the cell cycle pattern upon RPL23A overexpression, we performed MTT (Fig. 11D) and BrdU incorporation (Fig. 11E) assays in parallel. Results clearly indicate that ectopic expression of RPL23A decreased the rate of cell proliferation. Collectively, these results suggest that RPL23A down-regulates cell cycle progression possibly by altering p53 function.

This investigation clearly suggests the following: 1) NGP-1 suppresses RPL23A expression and up-regulates expression of p53 and its target gene, p21; 2) enhanced p-RBSer-780 levels and expression of E2F1 target genes upon NGP-1 expression resulted in faster G1 to S phase transition; and 3) RPL23A arrests the cells at G1 phase by modulating the p53 and RB pathway. Collectively, results from this study lead to the hypothesis that NGP-1 may modulate p53 function during cell cycle progression by down-regulating RPL23A activity. To test this hypothesis, we co-expressed NGP-1 with RPL23A in
MCF-7 cells and checked NGP-1 function during cell cycle progression. Interestingly, NGP-1 overcomes RPL23A-mediated suppression on p53-dependent promoter activity (Fig. 12A) and cell cycle progression (Fig. 12B). Together, these data suggest the possibility that NGP-1 promotes cell cycle progression via the p53-p21 axis by suppressing RPL23A activity.

**Depletion of NGP-1 Leads to G1 Arrest**—Overall, the above results suggest that NGP-1 promotes cell cycle progression. To define the specificity of NGP-1 function during cell proliferation, it was worthwhile to explore the cell cycle pattern under NGP-1 knockdown conditions. Toward this end, we depleted NGP-1 (knockdown confirmed by Western blot analysis and data represented in Fig. 13A) and observed that NGP-1-ablated cells accumulated more in G1 phase compared with S and G2/M phases of the cell cycle (Fig. 13A). We next performed BrdU incorporation assay in NGP-1-alleviated cells, and the results in Fig. 13B indicate that NGP-1 depletion reduced cell proliferation as indicated by the low BrdU incorporation. Results from BrdU incorporation assay together with cell cycle pattern suggest that NGP-1 knockdown caused G1 arrest. Western blot analysis indicates that expression of p53 and its target genes, p21 and Mdm2, was increased in NGP-1-ablated cells (Fig. 13C, lane 2). Interestingly, reduction of p-RBSer-780 levels in NGP-1-depleted cells was observed (Fig. 13C, lane 2). Expression levels of p53 and p21 together with cell cycle patterns in NGP-1 overexpression and depletion conditions suggest that NGP-1 might modulate multiple pathways to regulate cell proliferation. These data suggest the possibility that increased levels of p21 in NGP-1-depleted cells may negatively regulate cell proliferation. This leads to the hypothesis that the knockdown of p21 in NGP-1-depleted cells probably would rescue the cells from G1 arrest.

To test this, NGP-1 siRNA and p21 shRNA expression plasmids were co-transfected in MCF7 cells, and the status of cell proliferation was determined by MTT (Fig. 14A) and BrdU incorporation (Fig. 14B) assays. The levels of both NGP-1 and p21 depletion were determined by Western blot analysis using respective antibodies (Fig 14D). Results clearly indicate that the depletion of p21 indeed rescued the inhibition on cell proliferation in NGP-1 knockdown cells (Fig. 14, A and B). Furthermore, analysis of cell cycle profile 4 h after thymidine release also indicates that ablation of p21 reversed the G1 arrest from NGP-1 depletion in MCF-7 cells (Fig. 14C). Collectively, these results suggest that NGP-1 modulates p21 function during cell proliferation.
In this study, we made an attempt to gain insight into the function of NGP-1 during cell proliferation. Our results suggest for the first time that NGP-1 promotes cell proliferation by inducing faster G1 to S phase transition of cells via activation of the p53-p21 axis. This observation was unanticipated because p53 and p21 are suppressors of cell proliferation. Nevertheless, mutant p53 (nonfunctional or oncogenic) is often up-regulated in cancers. In this study, the MCF-7 cell line harboring wild-type p53 was used to understand the interplay between NGP-1 and p53-p21 pathway. Our data suggest that NGP-1-induced p53 was functional (Fig. 3A), which is supported by up-regulation of p53 target genes. Furthermore, we observed that NGP-1-expressing cells moved faster from G1 to S phase and resulted in increased cell proliferation as supported by results from MTT, BrdU incorporation, CFSE proliferation, and cell growth assays. However, NGP-1 failed to up-regulate cell cycle progression in the absence of p53 or p21 in MCF-7 cells. Collectively, these data provide evidence that NGP-1-mediated cell cycle progression requires functional p53 and its downstream target p21.

p21 is observed to be up-regulated in cancers (27–34) and is also perceived to be oncogenic under certain conditions (29, 77). Mutation in p21 is a rare phenomenon (44, 45), and a non-conventional search into the biology of tumor cells led to unraveling of the “stoichiometric” model suggesting that p21 promotes cyclin D1-CDK4 complex formation as long as its level is lower than the inhibitory concentration (51). CDK4 or cyclin D1 up-regulation in breast cancer (54, 56, 57) enhances the probability of complex formation and hence increases the inhibitory threshold. This investigation shows that NGP-1 up-regulates p21 thereby enhancing the complex formation between cyclin D1 and CDK4 without altering their levels (Fig. 5A). However, the concentration of the active cyclin D1-CDK4 complex may be maintained not only by increasing the overall pool of the proteins but also by increasing the active form of CDK4 (78–80). Moreover, the existing literature suggests the involvement of other adaptor proteins that might enhance cyclin D1-CDK4 complex formation (81). Therefore, NGP-1 may modulate additional unknown factors that increase the probability of active cyclin D1-CDK4 complex formation without changing their levels. Additionally, p21 induced by NGP-1 was lower than the inhibitory concentration, thus promoting activity of the complex as supported by the increased level of p-RB\textsuperscript{Ser-780} (substrate for cyclin D1-CDK4 complex) and the expression of E2F1 target genes. These results lead to the hypothesis that NGP-1 might maintain the stoichiometry between the cyclin D1-CDK4 complex and p21 and thus increase the inhibitory threshold (Fig. 15). In support of this hypothesis, we observed that higher concentration of p21 in cells co-expressing NGP-1 and p21 inhibited the cell cycle progression because the overall pool of p21 reached the inhibitory threshold. Interestingly, ablation of NGP-1 also resulted in higher p21 expression but showed decreased p-RB\textsuperscript{Ser-780} levels and caused G1 arrest. These results suggest that only an increase in p21 level might not be sufficient to promote cell cycle progression, but the presence of NGP-1 is necessary to maintain the stoichiometry between the cyclin D1-CDK4 complex and p21 to decide the fate of the cell.

FIGURE 15. Proposed model for NGP-1 function during cell proliferation. NGP-1 up-regulates p21 in a p53-dependent manner by inhibiting RPL23A. Ectopic expression of NGP-1 elevates p21 and activates the cyclin D1-CDK4 complex by maintaining the stoichiometric balance between cyclin D1-CDK4 and p21. Increased phosphorylation of RB at Ser\textsuperscript{780} upon NGP-1 expression resulted in release of E2F1 from the RB-E2F1 inhibitory complex followed by enhanced expression of E2F1 target genes and faster G1 to S phase transition.
In this study, we have unexpectedly observed an increase in p53 levels upon overexpression as well as knockdown of NGP-1. Previous reports have suggested that both overexpression and alleviation of NS, a closely related member of the HSR1-MMR1 family of GTPases, increased p53 levels (82) by inhibiting Mdm2-mediated p53 polyubiquitination (3). However, NS is also involved in rRNA processing (9); ablation of NS resulted in ribosomal stress and reduced p53 polyubiquitination (82). These data suggest that NS modulates p53 levels through multiple mechanisms. Similarly, NGP-1 may regulate p53 protein levels and activity based on the requirement to coordinate the cell cycle. Recent reports suggested the function of NGP-1 in ribosome biogenesis in zebrafish (18). In addition, Nog2p, a yeast homolog of NGP-1, is a critical component in ribosome biogenesis (83). However, the role of NGP-1 in ribosome biogenesis in the mammalian system remains unexplored. Hence, further experiments are needed to understand whether the observed up-regulation of p53 and cell cycle arrest in NGP-1 knockdown condition are due to ribosomal stress induced by NGP-1 depletion. Various studies have suggested the importance of ribosomal proteins in p53 function during the cell cycle. Here, for the first time, we have demonstrated that the ribosomal protein, RPL23A, causes cell cycle arrest (Fig. 11C) despite down-regulating p53 activity (Fig. 10B). Surprisingly, elevated levels of RPL23A during NGP-1 knockdown did not alter the p53/p21 level, which is in contrast to the RPL23A-overexpressed condition. However, further studies are required to understand the role of RPL23A on cell proliferation during ribosomal stress or NGP-1 knockdown condition.

Collectively, this investigation demonstrates that NGP-1 promotes cell cycle progression in a p53/p21-dependent manner possibly by balancing the stoichiometry between cyclin D1-CDK4 complex and p21 and also by down-modulating RPL23A function (Fig. 15). The observed high level expression of NGP-1 and p21 in primary tumors together with the results from this study on the activation of p21 by NGP-1 suggest the possibility that NGP-1 may favor faster cell proliferation in tumor conditions by up-regulating p21. Because NGP-1 is overexpressed in various cancers, utilization of NGP-1 in a diagnostic/therapeutic perspective in cancers overexpressing p21 could be further explored.

Author Contributions—D. D. designed and performed experiments, analyzed the data, and wrote the paper. K. A. performed experiments shown in Figs. 1B, 4B, 5A, 11B, 11F, 13B, and 14B and also contributed to designing the experiments. S. R. performed experiments shown in Fig. 9, C and D. R. S. P. performed the experiment shown in Fig. 1C, panel ii, and contributed to the preparation of manuscript. P. D. performed the experiment shown in Fig. 5C. S. M. conceived, coordinated the study, designed the experiments, analyzed the data, and wrote the paper.

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