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ORIGINAL ARTICLE

Downregulation of NOP53 Ribosome Biogenesis Factor Leads to Abnormal Nuclear Division and Chromosomal Instability in Human Cervical Cancer Cells

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Abstract
NOP53 ribosome biogenesis factor (NOP53) is a nucleolar protein involved in oncogenesis/tumor suppression, cell cycle regulation, and cell death. Here, we investigated the role of NOP53 in the maintenance of normal nuclear shape and chromosomal stability. Depletion of NOP53 by shRNA caused abnormal nuclear morphology, including large nucleus, irregular nucleus, and multinucleated cells, and chromosomal instability resulting in micronucleus or nuclear bud formation. The abnormal nuclear shape and chromosomal instability were restored by re-expression of NOP53. We further showed that NOP53 was involved in chromosome congression in metaphase. Downregulation of NOP53 induced aberrant chromosome congression and spindle checkpoint activation, resulting in delayed mitosis and mitotic arrest. Thus, our findings demonstrated that the nucleolar protein NOP53 participated in mitotic progression and that dysregulated NOP53 expression caused chromosomal instability in cancer cells.

Keywords NOP53 · Mitosis · Abnormal nucleus · Chromosomal instability

Introduction

The nucleolus is a highly dynamic organelle receiving and responding to signaling events by regulating critical cellular processes, such as ribosome biogenesis, apoptosis, cell cycle progression, and cell growth [1]. In addition, some nucleolar proteins participate in maintaining mitotic integrity and nuclear morphology [2, 3]. Depletion of nucleophosmin (NPM) or fibrillarin by RNAi, for example, leads to distortion of nuclear morphology with the formation of multiple micronuclei and reduction of cell growth [2, 3].

NOP53 ribosome biogenesis factor (NOP53/GLTSCR2/Pict-1) is a nucleolus-localized protein [4] that translocates to the nucleoplasm in response to ribosomal stress and regulates the stability of stress-responsive proteins [5]. Specifically, nucleoplasm-mobilized NOP53 physically interacts with p53 and increases p53 stability by inhibiting the mouse double minute 2 homolog (MDM2)-mediated polyubiquitination pathway [6]. Moreover, NOP53 is involved in DNA damage response induced by ultraviolet light or neocarzinostatin [5]. NOP53 depletion reduces the formation of nuclear foci of histone γH2AX and abolishes the G2/M checkpoint [5]. These findings strongly suggest that NOP53 may play an important role in cell cycle regulation and maintenance of DNA integrity under stress conditions. However, the roles and molecular mechanisms of NOP53 in the regulation of mitotic integrity have not yet been elucidated.

In this report, we investigated the participation of NOP53 in the maintenance of nuclear morphology and mitotic integrity during nuclear division. Given that chromosomal instability plays an essential role in cancer development, our findings suggested that tight regulation of NOP53 expression may be a critical event in carcinogenesis.

Materials and Methods

Cell Culture, Antibody, and Reagents

HeLa cells were purchased from the Korean Cell Line Bank (Seoul, Korea) and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum

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(FBS) at 37 °C. The anti-NOP53 antibody was purified from immunized rabbit serum (polyclonal antibody) as described previously [7]. Anti-α-tubulin and anti-SS18L1 antibodies were obtained from Abcam (Cambridge, UK) and Novus Biologicals (Colorado, USA), respectively. All other reagents were purchased from Sigma-Aldrich, Inc. (MO, USA).

Construction and Transduction of Plasmids and Knockdown of NOP53 and Aurora B

A plasmid and adenovirus expressing NOP53 were generated using standard cloning techniques, and plasmid transfection was carried out using Lipofectamine 2000 (Invitrogen, CA, USA), as described previously [7]. The expression of NOP53 was knocked down by transducing cells with a lentivirus harboring NOP53 shRNA (Santa Cruz, CA, USA). Downregulation of NOP53 was confirmed by western blotting. Knockdown of Aurora B was performed by transducing cells with small interfering RNA (siRNA) targeting Aurora B (Cell Signaling Technology, MA, USA).

Immunofluorescence Staining and Western Blotting

HeLa cells grown on coverslips were fixed with cold fixative (4% formaldehyde in phosphate-buffered saline [PBS]), and immunocytochemical staining was performed as previously described [7]. DNA staining was performed using 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI), and images were visualized using confocal microscopy (META 710; Zeiss, Jena, Germany). Western blotting was performed as described previously [7].

Cell Cycle Analysis and Cytokinesis-Block Micronucleus (CBMN) Assays

Cells transduced with lentivirus were fixed with cold 70% ethanol for 1 h, treated with RNase (20 mg/mL) for 15 min at 4 °C, and stained with propidium iodide (50 mg/mL) in PBS at room temperature for 20 min. The cells were examined in a Cytomics FC500 (Beckman Coulter, CA, USA), and the results were analyzed using CXP software (Beckman Coulter). CBMN assays were performed using cytokinesis-block induced by cytochalasin B as described previously [8].

Determination of Nuclear Abnormalities and Statistical Analysis

Cells with a nuclear diameter larger than 50% of the average diameter were considered cell with large nuclei. Cells with irregular nuclear contours, such as abnormal budding, invagination, and deep clefting of the nuclear membrane, were counted as having irregular nuclei. SPSS software 12.0 (SPSS, IL, USA) was used for statistical analyses. Fisher exact tests or Pearson $\chi 2$ tests were used. Differences were considered statistically significant when the P value was less than 0.05.

Live Cell Imaging

HeLa cells were stably transduced with Histone 2B-red fluorescent protein (RFP) plasmid and cultured in an LCI live cell instrument (Seoul, Korea). Fluorescent images were collected on a confocal microscope (Zeiss), and DIC images were also obtained with a transmitted light detector.

Results

Localization of NOP53 during Mitosis

Previous studies have reported that NOP53 is colocalized with NPM/B23 within the interphase cell nucleolus [9]. However, its dynamic localization and distribution during the cell cycle have not yet been studied. Accordingly, we then performed immunofluorescence staining for NOP53 in HeLa cells in the mitotic phase. During interphase, NOP53 was mainly localized in the nucleolus and was weakly detected in the nucleoplasm (Fig. 1). During prophase, most NOP53 was already dispersed throughout the cytoplasm but was more condensed around the chromosomes. When the nuclear membrane was disrupted during prometaphase and metaphase, the majority of NOP53 was concentrated along with the chromosomes and was slightly distributed in the cytoplasm. During anaphase, NOP53 moved with the chromosome and became concentrated at the chromosome periphery. During telophase, NOP53 still associated with the chromosome. Finally, during cytokinesis, NOP53 was densely packed into the coarse bodies in the nucleoplasm. These findings indicated that most NOP53 moved with the chromosome during mitosis, although some NOP53 was also detected in the cytoplasm. This chromosome-associated distribution of NOP53 during mitosis was similar to other nucleolar proteins, including NPM and nucleolin [2, 3, 10]; however, NOP53 was more closely associated with the chromosome than NPM. In addition, the perichromosomal concentration of NOP53 suggested that NOP53 may play a role in maintaining chromosomal stability or nuclear integrity during mitosis.

Depletion of NOP53 Led to Abnormal Nuclear Shape

To investigate whether NOP53 was involved in maintaining nuclear integrity, we generated HeLa cells with stable knockdown of NOP53 expression by transducing cells with lentivirus expressing NOP53 shRNA (shNOP53) and observed morphological changes in the nucleus as compared with scrambled shRNA-expressing (shSCR) control cells. NOP53-knockdown did not significantly affect the expression levels

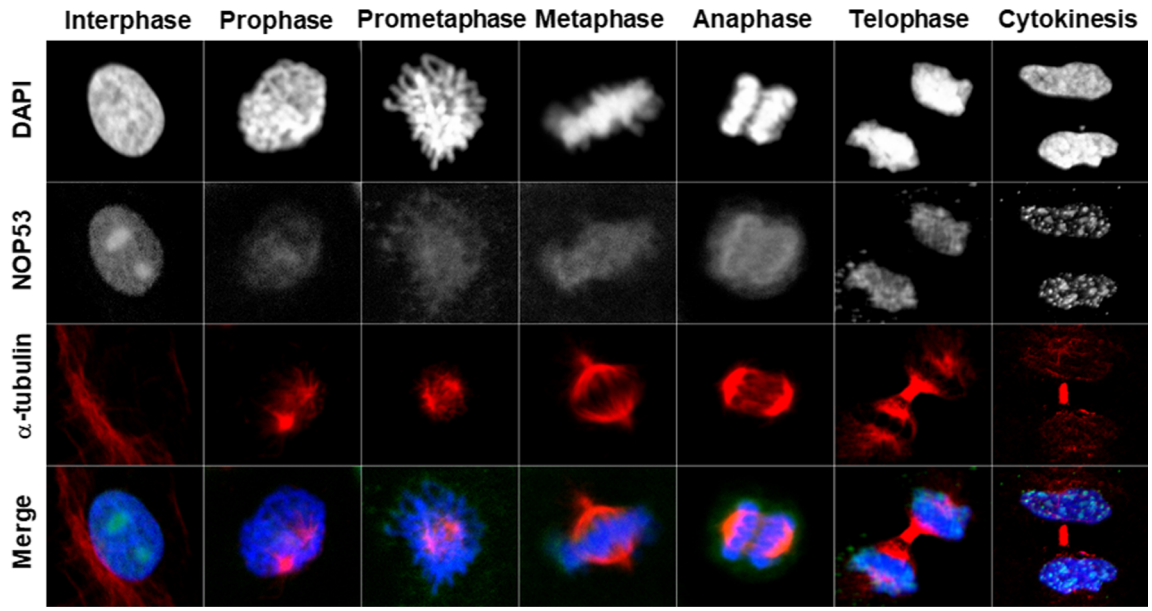

Fig. 1 Dynamic localization of NOP53 during the cell cycle. HeLa cells in the respective cell cycle phase were immunostained for NOP53. DNA was counterstained with DAPI. DNA (blue), NOP53 (green), and α-tubulin (red) images were merged

of nucleolin or fibrillarin (Fig. 2a). As shown in Fig. 2b, 29% of NOP53-knockdown cells displayed nuclear morphological abnormalities, including multinucleated cells, irregular nucleus, and large nucleus, whereas 4-5% of control cells had abnormal nuclear shapes.

We then investigated whether recovery of NOP53 expression in cells with nuclear abnormalities induced by NOP53-knockdown restored nuclear morphology. Ectopic expression of NOP53 by transduction with an NOP53- expressing plasmid (pNOP53) restored the nuclear shape distorted by NOP53-knockdown (Fig. 2b). Next, we carried out CBMN assays to determine whether depletion of NOP53 was associated with chromosomal instability. As shown in Fig. 2c, NOP53-knockdown induced a significant increase in micronucleus formation, nuclear buds, and nucleocytoplasmic bridges compared with control cells. Interestingly, these biomarkers of chromosomal instability were also restored by NOP53 expression (Fig. 2c). Taken together, our results demonstrated that NOP53 was involved in the maintenance of nuclear architecture and chromosomal stability.

Depletion of NOP53 Led to Mitotic Delay

Abnormal nuclear configuration caused by dysregulated gene expression leads to failure of mitotic progression [11]. To obtain insights into the effects of NOP53 depletion on mitotic progression, we initially analyzed the cell cycle profiles by flow cytometry. Cell cycle progression of NOP53-knockdown cells was delayed in the G2/M phase compared with control cells (Fig. 3a). Moreover, the cells with DNA contents greater than 4N were significantly high in NOP53-knockdown cells compared to control cells (28% vs. 10%), suggesting that NOP53-knockdown cells underwent another round of DNA replication in the absence of cell division.

To evaluate the mitotic delay due to NOP53 depletion, we measured the time from nuclear envelop breakdown (NEB) to centrosome segregation at telophase (prometaphase-telophase time). HeLa cells were stably transduced with an RFP-tagged histone 2B-expressing plasmid, and NOP53 expression was then knocked down by transduction with NOP53 shRNA. As depicted in Fig. 3b, about 80% of control cells had a prometaphase- telophase time of less than 60 min, whereas more than 50% of NOP53-knockdown cells had a prometaphase- telophase time of more than 120 min, indicating a longer mitotic progression period in NOP53-knockdown cells. In addition, about 12% of NOP53-depleted cells died after the prolonged mitotic period (data not shown). To define the underlying mechanism responsible for the mitotic delay in NOP53-depleted cells, we further determined whether NOP53 depletion affected the mitotic index. The mitotic index was measured by counting mitotic cells after α-tubulin staining. As shown in Fig. 3c, the mitotic index was significantly increased in NOP53- depleted cells. Taken together, our results demonstrated that NOP53 depletion caused mitotic delay by inhibiting mitotic progression from prometaphase to telophase.

NOP53 Was Involved in Chromosome Congression

To further investigate the mechanisms of mitotic delay caused by NOP53 depletion, we tested whether NOP53 was involved in chromosome congression. NOP53-knockdown cells were arrested at the metaphase-anaphase transition using MG132

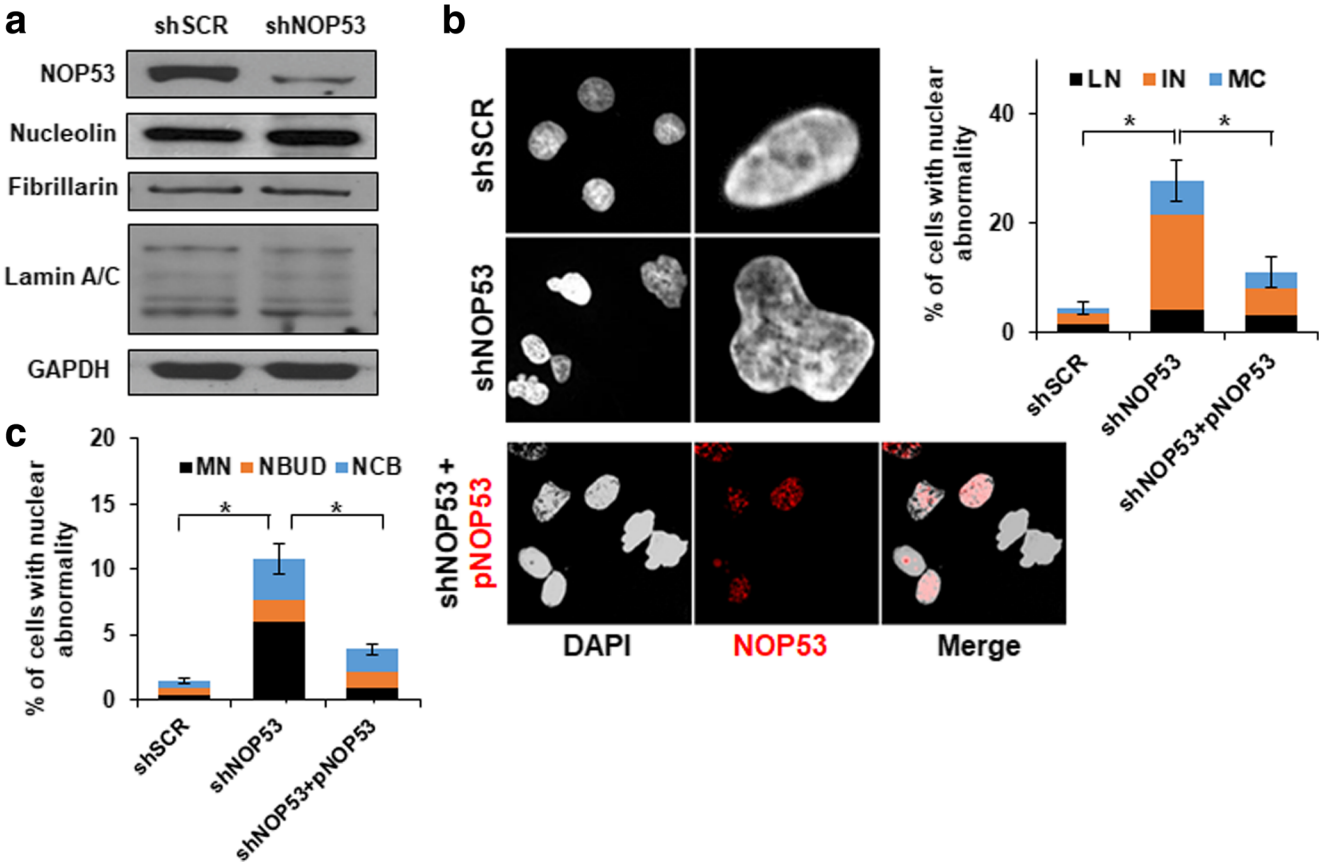

Fig. 2 NOP53 depletion led to abnormal nuclear morphology and chromosomal instability. a HeLa cells were transduced with shRNA targeting NOP53 (shNOP53) or scrambled shRNA (shSCR). Western blotting was performed using the indicated antibodies. **b** shSCR, shNOP53, and NOP53- expressing shNOP53 cells (shNOP53 + pNOP53) were stained using DAPI, and the irregular nuclear architecture was determined under a fluorescence microscope. NOP53 restoration was performed by transducing cells with an NOP53-expressing plasmid tagged with HA (pNOP53), and exogenous NOP53 expression was determined by immunostaining for HA (red). Representative images of nuclear architecture are shown in the left panel. A histogram representing the percentage of cells with nuclear abnormalities is shown. Data are expressed as the mean ± SD of triplicate determinations. $*p < 0.01$. LN, large nucleus; IN, irregular nucleus; MC, multinucleated cells. **c** Cytokinesis-block micronucleus (CBMN) assays using cytochalasin B were performed for shSCR, shNOP53, or shNOP53 + pNOP53 cells, and cells with chromosomal aberrations were counted after Giemsa staining. Data are expressed as the mean ± SD of triplicate determinations (left panel). $*p < 0.01$. Representative images of chromosomal aberrations are shown in the right panel

and immunostained with anti-SS18L1 (green) and anti-α-tubulin (red) antibodies. NOP53-knockdown cells in metaphase showed a significant increase in cells with uncongressed kinetochores, including misalignment or nonalignment, compared with control cells (Fig. 4a). As shown in Fig. 4b, measurement of the misaligned chromosome in metaphase also showed the broader DAPI staining in NOP53- depleted cells than in control cells (Fig. 4b). Next, to define whether re-expression of NOP53 restored uncongressed chromosomes, we transduced NOP53-depleted cells with adenovirus expressing NOP53 and observed the metaphase chromosome. The transduction efficiency of adenovirus was more than 90% based on counting of cells expressing green fluorescent protein. As shown in Fig. 4a and b, ectopic NOP53 expression decreased cell proportion with abnormal metaphase chromosome congression and reduced the broad DAPI staining. Our results demonstrated that NOP53 was clearly involved in chromosome congression. Accumulation of mitotic cells and prolonged division times from prometaphase to anaphase due to abnormal chromosome congression indicated that the spindle checkpoint was activated in NOP53-knockdown cells.

Finally, to test whether NOP53 knockdown activated the spindle checkpoint, we knocked down NOP53 or Aurora-B expression alone or in combination transducing cells with shRNAs targeting NOP53 and Aurora-B (Fig. 4c). The mitotic index in NOP53-knockdown cells increased to 10%, whereas that in double knockdown cells decreased to the level of the control cells, suggesting that the spindle checkpoint was activated in NOP53- knockdown cells. Taken together, our findings indicated that abnormal chromosome congression due to NOP53 depletion led to spindle checkpoint activation and mitotic arrest.

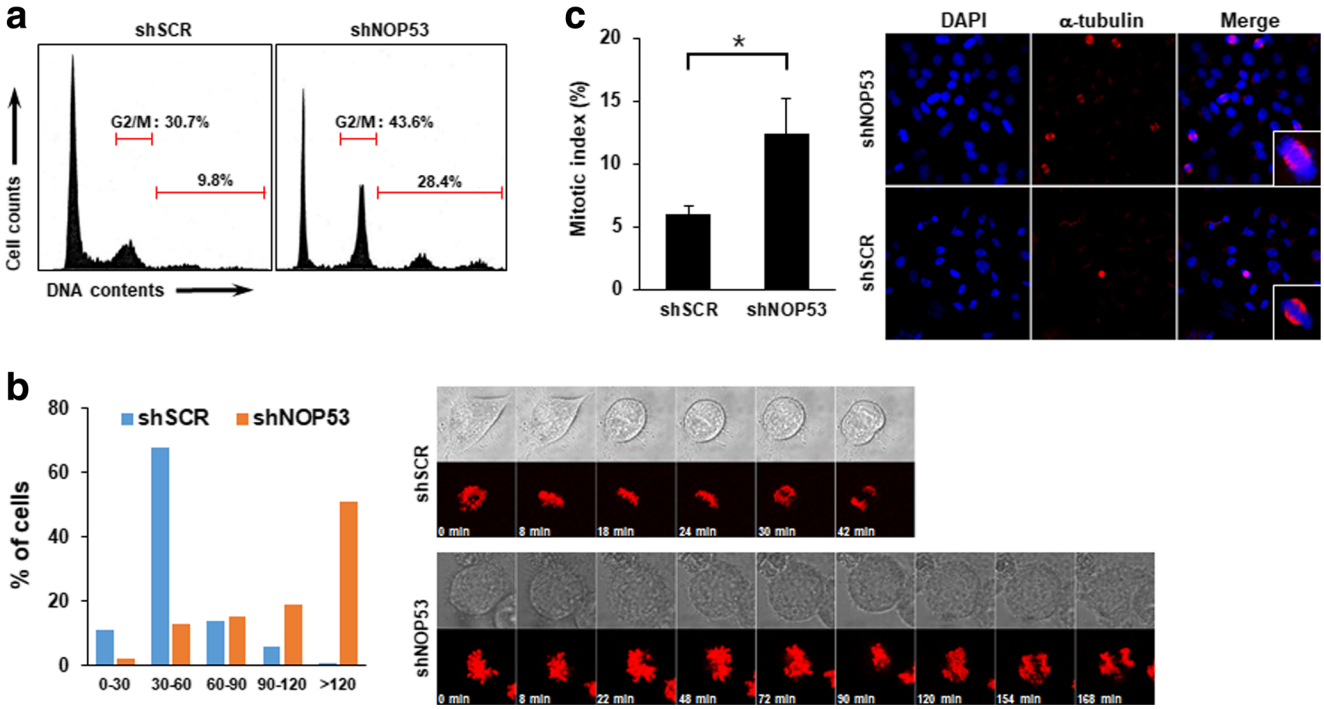

Fig. 3 **NOP53 depletion caused mitotic delay. a** shSCR and shNOP53 cells were stained with propidium iodide, and cell cycle profiles were determined by flow cytometry. **b** Time-lapse analysis of shSCR-H2B and shNOP53-H2B cells. shSCR-H2B or shNOP53-H2B cells, obtained from shSCR and shNOP53 cells, respectively, by stably transducing histone 2B-RFP plasmid, were cultured in a live cell instrument, and successive frames of fluorescent images were acquired. The graph displays the percentage of cells according to time distribution consumed from prometaphase to telophase. At least 30 cells per sample were observed. Representative live images are shown in the right panel. **c** shSCR and shNOP53 cells were immunostained with anti-α-tubulin antibodies, and the mitotic index was determined by the appearance of duplicated chromatid pairs aligned in the center of dividing cells. At least 300 cells were counted on three slides. The histogram represents the mean ± SD of triplicate determinations (left panel). $*p < 0.01$. Representative images are shown in the right panel

Discussion

Accumulating evidence has indicated that the nucleolus plays important roles in regulating the cell cycle and maintaining chromosomal stability. Depletion of nucleolar proteins induces abnormal cell growth caused by defective mitotic progression, whereas overexpression of these proteins enhances rapid cell proliferation [12]. Thus, dysregulation of nucleolar proteins is closely associated with various human pathological conditions, including cancers [13].

Previously, we reported that the nucleolar protein NOP53 is involved in modulating DNA damage responses through regulating DNA damage repair proteins and p53 expression through the MDM2-mediated polyubiquitination pathway [6]. These findings strongly suggested that NOP53 was involved in maintaining genomic stability, possibly during mitotic division. However, the exact roles of NOP53 in cell division have not yet been determined.

We initially showed that the localization of NOP53 during cell division was dynamic. The distribution pattern of NOP53 from interphase to metaphase was similar to that of NPM [2, 14], a binding partner of NOP53, but more closely associated with condensed chromosomes in anaphase and telophase. This perichromosomal condensation of NOP53 implied a putative role of NOP53 in maintenance of chromosomal stability during nuclear division. In order to elucidate the role of NOP53 in mitotic division, we stably knocked down NOP53 by transducing cells with shRNA and found that downregulation of NOP53 expression increased the number of cells with abnormal morphology, such as irregularly shaped or dumbbell-shaped cells. Together with the higher DNA contents in NOP53-knockdown cells, these findings strongly suggested that NOP53 played a critical role in nuclear division and that knockdown of NOP53 induced defects in mitosis and cytokinesis. However, these nuclear changes were not due to defects in nuclear envelop assembly because we did not detect any alterations in lamin A/C, a major nuclear envelop protein [15], as compared with that in control cells (data not shown). A previous report by Amin et al. showed similar nuclear changes without defects in postmitotic nuclear envelop assembly in fibrillarin-knockdown cells [3]. In NOP53-knockdown cells, cell cycle progression was delayed as the mitotic index increased. Based on time-lapse microscopic observation, this delayed cell division was

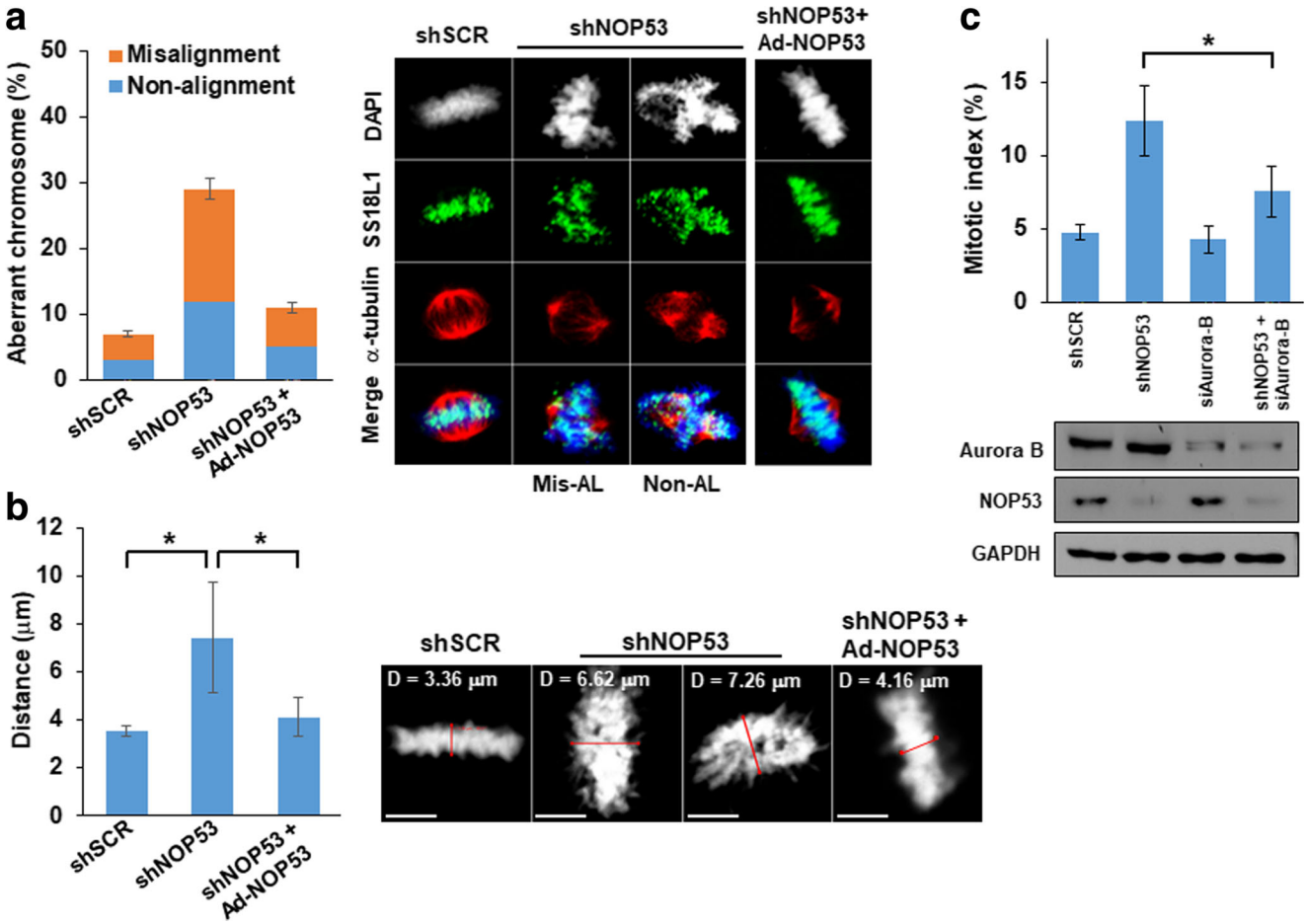

Fig. 4 NOP53 depletion caused mitotic defects through aberrant chromosome congression in HeLa cells. **a** The percentages of cells showing mitotic cells with aberrant chromosomes and disrupted mitotic spindles in shSCR, shNOP53, and NOP53-restored shNOP53 cells. Cells were arrested in metaphase using MG132 prior to immunostaining with α-tubulin (red) and anti-SS18L1 (green) antibodies and staining with a DNA dye, DAPI (blue). Data are the mean ± SD of three independent experiments (n = at least 100 mitotic cells). Representative immunostaining images are shown in the right panel. Mis-AL, misalignment; Non-AL, nonalignment **b** The graph displays the average width of congressed chromosomes in metaphase. Data were obtained from at least 60 cells per sample. *$p < 0.01$. Representative images showing the width distance of congressed chromosomes. The distance was measured by ZEN software (Zeiss). Scale bar, 5 μm. **c** shSCR and shNOP53 cells were transduced with siRNA targeting Aurora B, and the mitotic index was then determined. Histogram represents the mean ± SD of triplicate determinations (upper panel). *$p < 0.01$. The expression levels of NOP53 and Aurora B are shown in the lower panel

mainly due to the longer time lapse between prophase and metaphase. The prophase-to-metaphase delay could be caused by spindle checkpoint activation due to improper chromosomal congression at the spindle equator [16]. DAPI images after immunostaining using anti-SS18L1 and anti-α-tubulin antibodies revealed that NOP53 knockdown induced abnormal chromosome congression, including misalignment and nonalignment. In addition, mitotic arrest was abrogated by double knockdown of NOP53 and Aurora-B, indicating that the spindle checkpoint was activated in cells with NOP53 depletion.

In summary, in this report, we showed that NOP53 was required for proper chromosomal alignment during mitosis and that depletion of NPO53 caused chromosomal instability, leading to abnormal nuclear shapes and mitotic arrest through activation of spindle checkpoint proteins. Our results demonstrated that dysregulation of NOP53 expression may be involved in various pathological conditions caused by abnormal mitosis as well as cancers.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

References

1. Boulon S, Westman BJ, Hutten S, Boisvert FM, Lamond AI (2010) The nucleolus under stress. Mol Cell 40:216–227
2. Amin MA, Matsunaga S, Uchiyama S, Fukui K (2008) Depletion of nucleophosmin leads to distortion of nucleolar and nuclear structures in HeLa cells. Biochem J 415:345–351
3. Amin MA, Matsunaga S, Ma N, Takata H, Yokoyama M, Uchiyama S, Fukui K (2007) Fibrillarin, a nucleolar protein, is required for normal nuclear morphology and cellular growth in HeLa cells. Biochem Biophys Res Commun 360:320–326
4. Kalt I, Levy A, Borodianskiy-Shteinberg T, Sarid R (2012) Nucleolar localization of GLTSCR2/PICT-1 is mediated by multiple unique nucleolar localization sequences. PLoS One 7:e30825
5. Kim JY, Seok KO, Kim YJ, Bae WK, Lee S, Park JH (2011) Involvement of GLTSCR2 in the DNA damage response. Am J Pathol 179:1257–1264
6. Lee S, Kim JY, Kim YJ, Seok KO, Kim JH, Chang YJ, Kang HY, Park JH (2012) Nucleolar protein GLTSCR2 stabilizes p53 in response to ribosomal stresses. Cell Death Differ 19:1613–1622
7. Yim JH, Kim YJ, Ko JH, Cho YE, Kim SM, Kim JY, Lee S, Park JH (2007) The putative tumor suppressor gene GLTSCR2 induces PTEN-modulated cell death. Cell Death Differ 14:1872–1879
8. Shibamoto Y, Streffer C, Fuhrmann C, Budach V (1991) Tumor radiosensitivity prediction by the cytokinesis-block micronucleus assay. Radiat Res 128:293–300
9. Kim JY, Cho YE, Park JH (2015) The nucleolar protein GLTSCR2 is an upstream negative regulator of the oncogenic nucleophosmin-MYC axis. Am J Pathol 185:2061–2068
10. Ugrinova I, Monier K, Ivaldi C, Thiry M, Storck S, Mongelard F, Bouvet P (2007) Inactivation of nucleolin leads to nucleolar disruption, cell cycle arrest and defects in centrosome duplication. BMC Mol Biol 8:66
11. Ma N, Matsunaga S, Takata H, Ono-Maniwa R, Uchiyama S, Fukui K (2007) Nucleolin functions in nucleolus formation and chromosome congression. J Cell Sci 120:2091–2105
12. Tsai RY, Pederson T (2014) Connecting the nucleolus to the cell cycle and human disease. FASEB J 28:3290–3296
13. Takada H, Kurisaki A (2015) Emerging roles of nucleolar and ribosomal proteins in cancer, development, and aging. Cell Mol Life Sci 72:4015–4025
14. Amin MA, Matsunaga S, Uchiyama S, Fukui K (2008) Nucleophosmin is required for chromosome congression, proper mitotic spindle formation, and kinetochore-microtubule attachment in HeLa cells. FEBS Lett 582:3839–3844
15. Maraldi NM, Lattanzi G, Capanni C, Columbaro M, Merlini L, Mattioli E, Sabatelli P, Squarzoni S, Manzoli FA (2006) Nuclear envelope proteins and chromatin arrangement: a pathogenic mechanism for laminopathies. Eur J Histochem 50:1–8
16. Vogt E, Kirsch-Volders M, Parry J, Eichenlaub-Ritter U (2008) Spindle formation, chromosome segregation and the spindle checkpoint in mammalian oocytes and susceptibility to meiotic error. Mutat Res 651:14–29