TUMORIGENESIS AND NEOPLASTIC PROGRESSION

MRPL35 Is Up-Regulated in Colorectal Cancer and Regulates Colorectal Cancer Cell Growth and Apoptosis

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Mitochondrial ribosome proteins (MRPs), which are encoded by the nuclear genomic DNA, are important for mitochondrial-encoded protein synthesis and mitochondrial function. Emerging evidence suggests that several MRPs also exhibit important extra-mitochondrial functions, such as involvement in apoptosis, protein biosynthesis, and signal transduction. In this study, we demonstrate a significant role of MRP L35 (MRPL35) in colorectal cancer (CRC). The expression of MRPL35 was higher in CRC tissues than in matched cancer-adjacent tissues and higher in CRC cells than in normal mucosal epithelial cells. Higher MRPL35 expression in CRC tissue correlated with shorter overall survival for CRC patients. *In vitro*, down-regulation of MRPL35 led to increased production of reactive oxygen species (ROS) together with DNA damage, loss of cell proliferation, G₂/M arrest, a decrease in mitochondrial membrane potential, apoptosis, and autophagy induction. MRPL35 knockdown inhibited tumor proliferation in a CRC xenograft nude mouse model. Furthermore, overexpression of MRPL35 or treatment of cells with the ROS scavenger, *N*-acetyl cysteine, abrogated ROS production, cell cycle arrest, and apoptosis *in vitro*. These findings suggest that MRPL35 plays an essential role in the development of CRC and may be a potential therapeutic target for CRC. (*Am J Pathol* 2019, 189: 1105–1120; <https://doi.org/10.1016/j.ajpath.2019.02.003>)

Colorectal cancer (CRC), characterized by fast progression and poor prognosis, is one of the most common malignancies in the world.¹ The mechanisms of CRC development are still poorly understood. Although surgery is the most effective therapy for CRC, chemotherapy and radiotherapy are still indispensable.² Side effects and drug resistance are common issues with most of these treatments reducing their efficacy. As a consequence, CRC is in urgent need of markers for early diagnosis and of intervention targets for effective therapy.

Mitochondria have a central role in cellular energy metabolism by oxidative phosphorylation, regulation of apoptosis, autophagy, and cell death.^{3–5} Defective mitochondrial functions have been thought to be possible underlying mechanisms for cancer.^{6,7} Mitochondria have their own DNA and

ribosomes (mitoribosomes), which produce 13 proteins essential for oxidative phosphorylation.⁸ Human mitoribosomes sediment as 55S particles, consisted of a 28S small subunit, formed by a 12S rRNA and 29 mitochondrial ribosome proteins (MRPs), and a 39S large subunit, formed by a 16S rRNA and 50 MRPs.^{9,10} The approximately 70 MRPs are encoded by nuclear genomic DNA, synthesized in the cytosol,

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imported into the mitochondrial matrix, and assembled into mitoribosomes.¹⁰

Studies indicate that the level of expression of many MRP genes is altered in different cancer types, and changes in their expression are correlated with various clinical features in some cancers.⁹ High expression of MRPS23 stimulates cell proliferation and correlates to poor prognosis in hepatocellular carcinoma.¹¹ MRPS29 (DAP3) and MRPS30 (PDCD9) are involved in promoting apoptosis in mammalian cells.¹² The MRPL33-L isoform can promote cancer cell growth and repress apoptosis in colorectal tumor development.¹³ MRPL41 is either expressed at reduced levels or absent in most tumor types and cell lines, and its expression suppresses tumor cell growth.¹⁴ Several studies suggested possible regulatory mechanisms of MRPs in cancer. As a potential mechanism for its role in cell growth control, MRPL10 regulates cyclin B1/Cdk1 activity in mammalian cells.¹⁵ Down-regulation of MRPS23 and overexpression of MRPS36 delay cell cycle progression in association with p53 modification and p21^{WAF1/CIP1} expression,^{16,17} whereas MRPL41 is involved in cell proliferation regulation through the p53 and p27^{kip1} pathway.¹⁴

Mitochondrial ribosomal protein L35 (MRPL35) is a 25-kDa protein encoded by the *MRPL35* gene located at 2p11.2. MRPL35 plays a key role in coordinating the synthesis and assembly of the cytochrome *c* oxidase complex in the yeast model *Saccharomyces cerevisiae* and thus serves as a link between the mitochondrial translation apparatus and the downstream oxidative phosphorylation assembly.¹⁸ Here, we investigate the expression and biological significance of MRPL35 in CRC and demonstrate that MRPL35 is overexpressed in CRC tissues and cells. MRPL35 knock-down inhibits cell proliferation and survival *in vivo* and *in vitro*. We also characterize the molecular mechanisms to show that the down-regulation of MRPL35 leads to increased reactive oxygen species (ROS) production which correlates with DNA damage to suppress the progression of CRC.

Materials and Methods

Reagents

QIaprep Spin Mini prep Kit (250) was purchased from Qiagen (Hilden, Germany) and jetPRIME DNA Transfection Reagent was purchased from Polyplus (Illkirch, France). DAPI Staining Solution was purchased from Boster (Wuhan, China). *N*-Acetyl-L-cysteine (NAC) was purchased from Solarbio (Beijing, China). Caspase Inhibitor Z-VAD-FMK was obtained from Beyotime (Shanghai, China).

CRC Tissue Microarray and IHC Analysis

The tissue microarray contained 151 CRC samples and matched cancer-adjacent tissues, obtained from the Department of Pathology of the First Affiliated Hospital of Shanxi

Medical University (Shanxi, China), from 2005 to 2010. Each tissue was selected to be 0.2 cm in diameter and 4 µm in thickness. To determine the prognostic value of MRPL35, all samples were analyzed in relation to detailed survival data. The sections were dewaxed with xylene solution and gradient ethanol and were gradually hydrated. H₂O₂ (3%) was used to inactivate endogenous peroxidases. The sections were boiled in 0.01 mol/L citrate buffer (pH 6) for 3 minutes for antigen retrieval. Then, the sections were incubated with a specific rabbit antibody diluted at 1:200 (MRPL35; Abnova, Taipei, Taiwan) for 1 hour at 37°C and 30 minutes at room temperature, and incubated with biotinylated anti-rabbit secondary antibody at 37°C. Finally, the sections were visualized with diaminobenzidine of the DAB-0031 Kit (MXB, Fuzhou, China), and hematoxylin was used to counterstain the nuclei. Finally, the tissue microarray was dehydrated in gradient ethanol and xylene solution and then was sealed with neutral gum and observed under a microscope. The same method of immunohistochemistry (IHC) was used for the mouse tissues after sectioning.

Analysis and Quantification of Staining

Two pathologists (Y.X. and J.X.) examined the immunohistochemical staining using a double-blind method. The staining intensity (SI) was scored as follows: negative = 0, weak = 1 (I), moderate = 2 (II), and strong = 3 (III). The percentage of positively (PP) stained cells was scored as follows: 0 (negative), 1 (1% to 25%), 2 (26% to 50%), 3 (51% to 75%), and 4 (76% to 100%). The immunoreactive score (IRS) was calculated using the following equation: IRS = SI × PP.

An IRS >6 was defined as high expression and an IRS of ≤6 was defined as low expression.

Cell Lines and Tumor Tissues

Cell lines (HCT116, HCT15, SW620, LS174T, Lovo, SW480, HT29) used in these experiments were purchased from Shanghai Institutes for Biological Sciences (Shanghai, China). Fresh tumor tissues and paired adjacent tissues (seven pairs of ascending colon, nine pairs of descending colon, six pairs of sigmoid colon, and four pairs of rectum) were obtained from 26 CRC patients who underwent surgery at the First Affiliated Hospital of Shanxi Medical University and the Shanxi Provincial Cancer Hospital (Shanxi, China).

Lentiviral Infection and Plasmid Transfection

Lentiviral particles of MRPL35 (shMRPL35) were designed and produced by GeneChem (Shanghai, China). The shRNA sequence targeting MRPL35 was as follows: 5'-GTCAA-GAATGCCTCTCTTA-3'. For overexpression studies, plasmid DNA (GV147/MRPL35) with the restriction enzyme cutting site XhoI/KpnI was produced by GeneChem.

Lentiviral infection and the specific steps for plasmid transfection were performed according to the supplier's protocol.

RNA Extraction and RT-qPCR

Total RNA was isolated using Trizol reagent (Thermo Fisher Scientific, Shanghai, China), according to the manufacturer's instructions. The mRNA levels were determined by TransStart Tip Green qPCR SuperMix (TransGen Biotech, Beijing, China) on a StepOne RT-qPCR instrument (ABI, Waltham, MA). All measurements were performed three times. The following primers were used: MRPL35, forward 5'-TTGGCATCTTCAACCTACCGC-3' and reverse 5'-GGAGGAAACAACCTGGTGTCTGA-3'; β -actin, forward 5'-CTCACCATGGATGATGATATCGC-3' and reverse 5'-AGGAATCCTTCTGACCCATGC-3'.

Western Blot Analysis

Total protein extracts were isolated from cells and fresh tumor tissues in RIPA lysis buffer (Beyotime) containing a protease inhibitor (Bioscience, Beijing, China) and a phosphatase inhibitor (Boster). Mitochondria isolation and protein extraction were performed by the Cell Mitochondria Isolation Kit (Beyotime). Nuclear and cytoplasmic proteins were extracted by the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime). Protein concentrations were measured using the BCA Protein Assay Kit (Beyotime). Proteins were separated by 6% to 12% SDS-PAGE and transferred to nitrocellulose membranes. Primary antibodies were diluted using Primary Antibody Dilution Buffer (Beyotime), whereas anti-mouse or anti-rabbit secondary antibodies were diluted using tris-buffered saline and Tween 20. Primary antibodies included MRPL35 (dilution 1:200) from LSBio (Seattle, WA); and Phospho-H2A.X (Ser139; 20E3; dilution 1:1000), Phospho-ataxia-telangiectasia mutated (ATM; Ser1981; D6H9; dilution 1:500), caspase-3 (dilution 1:400), cleaved-caspase-9 (dilution 1:500), cleaved-poly (ADP-ribose) polymerase (PARP; D64E10; dilution 1:500), Bcl-1-associated X protein (Bax; dilution 1:500), cytochrome *c* (136F3; dilution 1:500), phospho-p53 (Ser15) (16G8; dilution 1:500), P21 Waf1/Cip1 (12D1; dilution 1:500), cyclin B1 (D5C10; dilution 1:500), phospho-cdc2 (Tyr15; 10A11; dilution 1:500), phospho-Wee1 (Ser642) (D47G5; dilution 1:1000), histone H3 (dilution 1:1000), phospho-Chk1 (Ser345) (133D3; dilution 1:500), phospho-Chk2 (Thr68; C13C1; dilution 1:500), Atg5 (D5F5U; dilution 1:1000) from Cell Signaling Technology (Danvers, MA); DNA damage regulated autophagy modulator 1 (DRAM1; dilution 1:1000) from Novus Biologicals (Shanghai, China); glyceraldehyde-3-phosphate dehydrogenase (dilution 1:1000) from Sangon (Shanghai, China). All these antibodies were specific rabbit monoclonal antibodies, except that phospho-p53 was the specific mouse monoclonal antibody.

Cell Cycle and Apoptosis Analysis

Cell cycle analysis by flow cytometry was performed using the Cell Cycle Kit (KeyGEN BioTECH, Nanjing, China). Apoptosis detection by flow cytometry [FACSCalibur; Becton Dickinson Biosciences (BD Biosciences), San Jose, CA] was performed using the Annexin V-allophycocyanin/7-aminoactinomycin D Cell Apoptosis Kit (KeyGEN BioTECH).

Cell Proliferation and Colony Formation Assay

Cell proliferation was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Solarbio) method in the different groups on days 1 through 5. Each experiment was performed in triplicate and repeated three times. For the colony formation assay, 200 cells per well were seeded in 6-well plates and cultured for 14 days in medium that contained 10% fetal bovine serum. The colonies were fixed with methyl alcohol for cell migration and invasion assays.

To assess cell migration, wound healing assays were performed. Transfected cells were cultured in 6-well plates until confluent monolayers were formed. A 200- μ L pipette tip was used to scratch the monolayer to create the wound. After the debris were washed away with phosphate-buffered saline, cells were cultured for 48 hours to allow wound healing. The cell invasion assay was performed using Transwell chambers (8- μ m pore size; Millipore, Burlington, MA) with Matrigel (BD Biosciences). The detailed procedure was performed according to the manufacturer's instruction. The number of invaded cells was counted in five randomly selected microscopic fields and photographed.

Xenograft Tumor Growth Analysis

HCT116 cells stably infected with shControl or shMRPL35 were injected subcutaneously into the dorsal surface of each animal [6-week-old male nude mice obtained from the Animal Center of the Chinese Academy of Sciences (Shanghai, China); $n = 3$]. Tumor size was measured at the indicated times using Vernier calipers. Tumors were excised after 21 days. The tumor tissues were formalin-fixed and paraffin-embedded for hematoxylin and eosin staining and IHC staining of Ki67. The NIH's *Guide for the Care and Use of Laboratory Animals*¹⁹ was followed for all animal experiments.

Measurement of Mitochondrial Membrane Potential and ROS

The mitochondrial membrane potential ($\Delta\Psi_m$) and ROS of cells were detected by flow cytometry and fluorescence microscopy according to the manufacturer's instructions (Cell Signaling Technology, KeyGEN BioTECH, Nanjing, China). Cells were untreated or treated with carbonyl cyanide 3-chlorophenylhydrazone (400 μ mol/L, 20 minutes), followed by labeling with tetramethylrhodamine ethyl ester

(200 nmol/L, 30 minutes). For ROS assay, cells were incubated with dihydroethidium probe (100 μ mol/L) for 1 hour, and fluorescence of the cells was examined and quantified with fluorescence microscopy (Olympus, Tokyo, Japan) and flow cytometry (FACSCalibur, BD Biosciences).

Statistical Analysis

The statistical software SPSS 19.0 (IBM, White Plains, NY) and GraphPad Prism 6 (GraphPad Software, San Diego, CA) were used to perform the statistical analyses. The data are generally expressed as the means $\pm$ SD. The *P* values for the Kaplan–Meier survival analysis were generated by the log-rank test. *P* < 0.05 was considered statistically significant, using *t*-test or one-way analysis of variance.

Results

MRPL35 mRNA and Protein Levels Are Highly Expressed in CRC Tissues and Cells

Analysis of The Cancer Genome Atlas (TCGA) data showed that MRPL35 was expressed differentially in tumor versus normal tissues, especially showing higher expression status in CRC tissue (Supplemental Figure S1A). To confirm this result, the expression of MRPL35 was compared in paired human CRC tissues by Western blot analysis and quantitative RT-PCR. CRC tissues displayed higher levels of MRPL35 protein than the paired adjacent normal tissues in 70% of cases (7 of 10) (Figure 1A). Consistent with elevated protein overexpression, MRPL35 mRNA was also overexpressed in 73.08% of CRC tissue samples (19 of 26) (Figure 1B). The expression of MRPL35 was next studied in CRC cells. MRPL35 was overexpressed in most CRC cells except for SW620 cells (Figure 1, C and D), in comparison with that in the normal mucosal epithelial cell, FHC.

IHC was used to confirm MRPL35 expression in the CRC tissues and matched cancer-adjacent tissues. MRPL35 was distributed mainly in the cytoplasm, and its expression was significantly up-regulated in CRC tissues (Figure 1E). Moreover, the patients with high expression of MRPL35 (*n* = 80) had shorter overall survival than patients with low expression of MRPL35 (*n* = 71; *P* < 0.01) (Figure 1F), indicating that the level of MRPL35 expression was correlated with clinical outcome of CRC. Surprisingly, it was contrary to the result of survival analysis on TCGA (Supplemental Figure S1, B and C). The discrepancy may be attributed to differences in the sample population. Overall, elevated expression of MRPL35 correlated to CRC progression and may be a biomarker for early stage CRC.

Down-Regulation of MRPL35 Attenuates CRC Cell Proliferation and Induces Apoptosis *in Vitro*

To further examine the physiological role of MRPL35 in CRC progression, MRPL35 expression was down-regulated

by infecting the CRC cell lines HCT116, LS174T, SW480, and HT29 with lentiviral particles expressing shMRPL35 or shControl (Supplemental Figure S2). The green fluorescence intensity represented the efficiency of infection, which was consistently >80%. On transfection, the expression of MRPL35 was significantly down-regulated in CRC cells (HCT116, LS174T, SW480, and HT29), indicated by RT-qPCR and Western blot assays (Supplemental Figure S2, B and C).

The effects of decreased MRPL35 expression were assessed on these cells. First, the effect on proliferation of the CRC cells was determined with the use of the MTT assay. Down-regulation of MRPL35 inhibited CRC cell proliferation by 62.3% in HCT116 cells, 65.5% in SW480 cells, 54.5% in LS174T cells, and 62.2% in HT29 cells (Figure 2A). Colony formation assays confirmed the above result, because down-regulation of MRPL35 reduced the number of colonies compared with the controls (Figure 2, B and C). Then, it was determined whether MRPL35 knock-down induced apoptosis in CRC cells. The percentage of apoptotic cells was lower in the control group than those infected with shMRPL35 in HCT116 cells (5.31% versus 9.13%), as well as in other CRC cells (3.63% versus 9.33% in SW480 cells, 7.36% versus 19.52% in LS174T cells, and 6.99% versus 21.07% in HT29 cells) (Figure 2, D and E). However, down-regulation of MRPL35 did not significantly affect the migration and invasion of HCT116 cells (Supplemental Figure S3, A–D). Consistent with this result, proteins related to epithelial–mesenchymal transition, such as Vimentin, β -catenin, and matrix metalloproteinase 9 showed no significant changes (Supplemental Figure S3E). Epithelial–mesenchymal transition has been shown to play a critical role in cancer cell migration and invasion.^{20,21}

MRPL35 Knockdown Inhibits CRC Tumor Growth *in Vivo*

Having demonstrated an effect of MRPL35 knock-down on CRC cell proliferation *in vitro*, the effect of MRPL35 knockdown was investigated on CRC cell growth in nude mice. HCT116 cells were selected for establishing the xenograft model. HCT116 cells have the most efficient infection by lentivirus. Besides, down-regulation of MRPL35 can significantly inhibit the proliferation of HCT116 cells. HCT116 cells stably infected with shMRPL35 lentivirus or shControl were injected subcutaneously into the dorsal surface of each nude mouse (Figure 3A). Compared with the control groups, MRPL35 knockdown significantly suppressed CRC growth in nude mice (Figure 3B). After 21 days, the tumor volumes of the shMRPL35 group were significantly smaller than those generated from the control group (*P* < 0.05) (Figure 3C). However, there was not much difference between the groups until day 7, suggesting that there was no effect of MRPL35 during the initiation of tumor but only its progression. To determine whether the reduction in tumor growth was associated with an antiproliferative effect, immunohistochemical analysis for the proliferation marker

Figure 1 Mitochondrial ribosomal protein L35 (MRPL35) expression is up-regulated in colorectal cancer (CRC) patients and cell lines. **A:** Total proteins extracted from paired tissues of the CRC patients (selected on the basis of MRPL35 mRNA level) were analyzed by Western blot analysis with MRPL35-specific antibody. The protein levels were quantified by using ImageJ software version 1.50i (NIH, Bethesda MD). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. **B:** The mRNA levels of MRPL35 of CRC tissues were detected by quantitative RT-PCR (RT-qPCR). **C** and **D:** Western blot analysis and RT-qPCR analysis of the expression of MRPL35 in CRC cells (HCT116, HCT15, SW620, LS174T, Lovo, SW480, HT29) and normal mucosal epithelial cell FHC. **E:** Representative immunohistochemical staining detection of MRPL35, revealing higher staining in malignant CRC tissue than in paired cancer-adjacent normal tissue. The areas indicated by **black arrows** are shown in higher magnification to the right. **F:** Survival analysis of CRC patients based on MRPL35 expression by the Kaplan–Meier method. **Dashed line** indicates the median survival percent. Data are expressed as means $\pm$ SD. $n = 10$ paired tissues (**A**); $n = 26$ CRC tissues (**B**); $n = 3$ or more experiments (**C** and **D**). $^{**}P < 0.01$. Original magnification: $\times 40$ (**E**, left column); $\times 100$ (**E**, middle column); $\times 400$ (**E**, right column). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; N, matched cancer-adjacent normal tissues; T, colorectal cancer tissues.

Figure 2 Down-regulation of mitochondrial ribosomal protein L35 (MRPL35) attenuated colorectal cancer (CRC) cell proliferation and induced apoptosis *in vitro*. **A**: Reduction of MPRL35 inhibited the growth rate of CRC cells (HCT116, LS174T, SW480, HT29) detected by 5-diphenyltetrazolium bromide (MTT) assay. **B** and **C**: Colony formation assays for CRC cells were infected as in **A**. **D** and **E**: Representative flow cytometric analysis of apoptosis stained with Annexin V-allophycocyanin (APC) and 7-aminoactinomycin D (7-AAD) in CRC cells infected with shMRPL35 or shControl. Data are expressed as means $\pm$ SD. $n = 3$ independent experiments repeated three times with similar results. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus shControl.

Ki-67 was performed on excised engrafted tumor tissues of each experimental group. A striking decrease in the number of nuclei positively stained for the Ki-67 protein was observed in the shMRPL35 group compared with the control group

(Figure 3D). Figure 3D shows the representative hematoxylin and eosin staining as histologic identification of neoplasms. Taken together, these data suggested a suppressive role of shMRPL35 in CRC proliferation *in vivo*.

Figure 3 Knockdown of mitochondrial ribosomal protein L35 (MRPL35) suppressed colorectal cancer (CRC) cell growth *in vivo*. HCT116 cells stably transfected with shMRPL35 or shControl were injected subcutaneously into nude mice. The mice were euthanized, and the tumors were excised. **A:** Image of mice bearing shControl (left arrows) or shMRPL35 (right arrows). HCT116 cell xenografts at 21 days after injection. **B:** Excised tumors from shControl (upper) or shMRPL35 (lower) HCT116 cells. **C:** At the indicated times, tumors were measured with Vernier calipers. **D:** Representative hematoxylin and eosin (HE) staining of neoplasms and immunohistochemical staining of Ki67 in tumors. Data are expressed as means $\pm$ SD. $n = 3$. $*P < 0.05$. Original magnification, $\times 40$ (**D**).

MRPL35 Knockdown Induces ROS Leading to DNA Damage

ROS are known to play a major role in the development of CRC,^{22–24} and excessive ROS could enter the nucleus to cause DNA damage.^{25–27} To determine whether ROS level played a role in the inhibition of proliferation caused by MRPL35 knockdown, it was examined whether MRPL35 knockdown affected intracellular ROS generation in CRC. Red fluorescence representing ROS production was significantly increased by two times after down-regulation of MRPL35 in HCT116 cells (Figure 4A). It was then tested whether generation of ROS resulted in DNA damage by measuring ATM S1981 phosphorylation as a DNA damage marker.^{28,29} ATM S1981 phosphorylation was increased (Figure 4D), accompanied by increased phosphorylation of H2A.X on Ser139 (γ -H2AX), another hallmark of DNA damage response³⁰ (Figure 4D). Consistent with the above-mentioned observations, overexpressing or rescuing MRPL35 expression by plasmid GV147/MRPL35 in HCT116 cells reduced the phosphorylation of ATM and H2A.X (Supplemental Figure S4, Figure 4E).

To further verify that the DNA damage caused by MRPL35 was mediated by increased production of ROS, it

was tested whether the ROS scavenger NAC could decouple DNA damage from MRPL35 knockdown (Supplemental Figure S5A). Consistent with the hypothesis, NAC treatment abolished the induction of γ -H2AX and ATM phosphorylation (Figure 4D), further demonstrating that induction of DNA damage was mediated by ROS. Dihydroethidium fluorescence intensity by flow cytometry further supported this conclusion (Figure 4, B and C, and Supplemental Figure S5, B and C). Overall, these data demonstrated that MRPL35 knockdown could induce ROS accumulation to cause DNA damage.

DNA Damage Arrests the Cell Cycle at the G₂/M Phase

Cell-cycle checkpoints are downstream effectors in response to DNA damage, which can cause cell cycle arrest at the G₁ or G₂ phase. Because MRPL35 knockdown resulted in DNA damage, it was next determined how the cell cycle was affected. After treatment with shMRPL35 or shControl, DNA content in CRC cells was measured by FACS. SW480 cells pretreated with MRPL35 down-regulation displayed a clearly increased G₂/M phase population (17.4%) compared with the control cells (7.8%) (Figure 5A) ($P < 0.05$). The same pattern was observed in HT29 cells (14.7% versus

Figure 4 Mitochondrial ribosomal protein L35 (MRPL35) knockdown induced reactive oxygen species (ROS) and resulted in DNA damage. **A:** Detection of ROS by incubation with 100 μ mol/L dihydroethidium (DHE) in both shMRPL35 and shControl groups in HCT116 cells. Cells were observed using a fluorescence microscope. **Boxed areas** are shown at higher magnification in the **right column**. **B:** HCT116 cells were treated as in **A** and analyzed by flow cytometry. The x axis represents the red fluorescence intensity, the vertical axis represents cells analyzed by flow cytometry. **C:** Quantifications of relative mean red fluorescence intensity of shMRPL35 and shControl groups in HCT116 cells. **D:** Western blot analysis of P-H2A.X (Ser139) and P-ataxia-telangiectasia mutated (ATM; Ser1981) protein levels in HCT116 cells infected with shMRPL35 or shControl, and with or without 10 mmol/L antioxidant *N*-acetyl cysteine (NAC). **E:** Western blot analysis of P-H2A.X (Ser139) and P-ATM (Ser1981) protein levels in HCT116 cells infected with shMRPL35 or shControl and transfected with GV147/MRPL35 or GV147/Vector. *n* = 3 independent experiments performed with similar results. **P* < 0.05 versus shControl. Scale bars = 100 μ m. Original magnification: $\times 100$ (**A**, **left** and **middle columns**); $\times 400$ (**A**, **right column**).

9.3%) and HCT116 cells (12.4% versus 9.2%). Although there was no statistically significant difference in HT29 and HCT116 cells, the cell cycle retardation could still be seen after MRPL35 down-regulation. The cell lines may have different reaction time on MRPL35 knockdown. In this experiment, all three cell lines were collected at the same time point for the sake of consistency.

DNA damage accumulation induced ATM phosphorylation and activation (Figure 4D). On ATM activation, downstream effectors, such as Chk2 and p53, were phosphorylated and activated,^{31,32} which in turn prevented the activation of the mitotic cyclin-dependent kinase (CDK)1-cyclinB complex by blocking the dephosphorylation of threonine 14/tyrosine 15 CDK1.^{33,34} Consequently, the cell goes to G₂ arrest. To investigate if this pathway was activated by MRPL35 knockdown, the Western blot assay was used to examine the expression level and phosphorylation status of key regulators of the G₂/M checkpoint, including Cdk1/cdc2, cyclin B1, p21^{Waf1/Cip1}, Wee1, Chk2, and p53 in MRPL35 knockdown and control cells.^{35,36} Significant increases in p53 phosphorylation at Ser15 and Chk2

phosphorylation at Thr68 (Figure 5B) were observed after down-regulation of MRPL35 in HCT116 cells. Similarly, MRPL35 knockdown significantly increased expressions of p21 and cdc2 phosphorylation at Tyr15 and decreased the expression of cyclin B1 (Figure 5B). DNA damage is known to cause the phosphorylation and activation of Wee1, which leads to the inhibition of Cdk1 and cell cycle arrest at the G₂ phase.^{37,38} HCT116 cells with low expression of MRPL35 showed that the phosphorylation of Wee1 was indeed elevated (Figure 5B). These results suggested that the ATM-Chk2/P53-Cdk1/cyclin B1 cascade was involved in the G₂/M arrest in response to MRPL35 knockdown.

DNA damage in response to MRPL35 knockdown was mediated by the increased production of ROS (Figure 4D). This conclusion would predict that the activation of the ATM-Chk2/P53-Cdk1/cyclin B1 cascade should also be dependent on the overproduction of ROS. This was indeed true as treatment with ROS scavenger NAC inhibited ATM, P53, and Chk2 (Figure 5B). These experiments together demonstrated that MRPL35 down-regulation resulted in

Figure 5 Mitochondrial ribosomal protein L35 (MRPL35) knockdown caused G₂/M phase arrest in colorectal cancer (CRC) cells. **A:** Flow cytometric analysis of the cell cycle in HCT116 cells when MRPL35 was down-regulated. Representative fluorescence histograms show the effect on the cell cycle distribution of HCT116, SW480, and HT29 cells. Bar graphs show the percentage of CRC cells in G₀/G₁, S, and G₂/M phases. **B** and **C:** Representative Western blot analyses for p-Chk2, P-p53, p21 Waf1/Cip1, P-Wee1, P-cdc2, and cyclin B1 proteins in MRPL35 knockdown of HCT116 cells. Data are expressed as the means $\pm$ SD. $n = 3$ independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 6 Reactive oxygen species (ROS) decreased mitochondrial membrane potential ($\Delta\Psi_m$) to induce HCT116 cell apoptosis. **A:** Detection of $\Delta\Psi_m$ by incubation with 200 nmol/L tetramethylrhodamine ethyl ester perchlorate (TMRE) in mitochondrial ribosome protein L35 (MRPL35) knockdown of HCT116 cells with or without 400 μ mol/L mitochondrial membrane disrupter carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Cells were observed using a fluorescence microscope. **Boxed areas** are shown at higher magnification in the **right column**. **B and C:** HCT116 cells treated as in **A** were analyzed by flow cytometry, and quantification of relative mean red fluorescence intensity was analyzed. **D and E:** Representative Western blot analyses for Bcl-1-associated X protein (Bax), cytochrome *c*, cleaved-caspase 9, cleaved-caspase 3, and cleaved-proline-rich acidic protein 1 (PRAP) proteins in MRPL35 knockdown of HCT116 cells. Data are expressed as means $\pm$ SD. $n = 3$ independent experiments. *** $P < 0.001$. Scale bars = 100 μ m. Original magnification: $\times 100$ (**A, middle three columns**); $\times 400$ (**A, right column**). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; NAC, *N*-acetyl cysteine.

Figure 7 DNA damage and p53-induced autophagy. **A:** HCT116 cells in which mitochondrial ribosomal protein L35 (MRPL35) was knocked down were treated with and without 10 mmol/L antioxidant *N*-acetyl cysteine (NAC) and analyzed by Western blot analysis. **B:** HCT116 cells, in which MRPL35 was down-regulated, were fractionated into nuclear and cytosolic extracts and analyzed by Western blot analysis. Histone H3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were both used as controls. **C:** HCT116 cells were transfected with sip53 or siControl and analyzed by Western blot analysis. **D:** HCT116 cells were transfected with GV147/MRPL35 or GV147/Vector and analyzed by Western blot analysis. **E** and **F:** HCT116 cells were treated with and without 20 μ mol/L pan-caspase inhibitor Z-VAD-FMK. Cells stained with Annexin V-allophycocyanin (APC) and 7-aminoactinomycin D (7-AAD) were analyzed for viability by representative flow cytometry. **G:** Western blot analysis of cleaved-caspase3 and cleaved-caspase9 in HCT116 cells after shControl and shMRPL35 infection and with or without Z-VAD-FMK. GAPDH was used as a loading control. Data are expressed as means $\pm$ SD of percentage of cells in various phases of the process. $n = 3$ or more independent experiments. $**P < 0.01$. DRAM1, DNA damage regulated autophagy modulator 1.

ROS accumulation, which led to DNA damage and G₂/M arrest. To further confirm this conclusion, it was tested if the overexpression of MRPL35 could rescue the cell cycle arrest caused by MRPL35 knockdown. MRPL35 was first knocked down with shMRPL35 in HCT116 cells, and then

the expression of MRPL35 was restored using plasmid GV147/MRPL35. The normal cell cycle was recovered (Figure 5C). These results collectively showed that G₂/M phase cell cycle arrest was caused by DNA damage induced by MRPL35 knockdown.

ROS Induces CRC Apoptosis by Decreasing $\Delta\Psi_m$

Excessive ROS can also affect $\Delta\Psi_m$ and damage mitochondria, which then leads to cellular apoptosis.^{39,40} Down-regulation of MRPL35 significantly increased CRC cell apoptosis (Figure 2D).

The molecular mechanisms of apoptosis were further explored in HCT116 cells because of MRPL35 knockdown. To confirm the ROS involvement, both groups of HCT116 cells were first exposed to 400 $\mu\text{mol/L}$ mitochondrial membrane disrupter, carbonyl cyanide 3-chlorophenylhydrazone, and then treated with or without 10 mmol/L NAC. The cells were then incubated with 200 nmol/L tetramethylrhodamine ethyl ester perchlorate, and $\Delta\Psi_m$ was measured by flow cytometry (Figure 6, A and B). The mitochondrial membrane integrity, indicated by the $\Delta\Psi_m$, was disrupted in the MRPL35 knockdown group, and pretreatment with NAC attenuated the decrease in $\Delta\Psi_m$ (Figure 6, A–C, and Supplemental Figure S6), indicating that ROS was a link between MRPL35 knockdown and the decrease in $\Delta\Psi_m$. The mechanism of MRPL35 down-regulation–induced apoptosis was further explored. The proteins related to apoptosis were measured by Western blot analysis. Down-regulation of MRPL35 enhanced the expression of Bax, stimulated the release of cytochrome *c*, and activated caspase-9 and caspase-3 (Figure 6D). Activated caspase-3 could cause proteolytic cleavage of PARP, a key characteristic of apoptosis.⁴¹ Indeed, a proteolytic cleavage of PARP to an 89-kDa fragment (Figure 6D) was also increased in MRPL35 knockdown cells. Activation of both caspases 3 and 9, cytochrome *c*, and PARP by MRPL35 knockdown revealed the induction of apoptosis via a mitochondrial-dependent mechanism in HCT116 cells. In reverse, when ROS production was blocked by NAC or the expression of MRPL35 was rescued or overexpressed, all of the observed changes to the above proteins were reversed (Figure 6, D and E). These results further

demonstrated that MRPL35 knockdown induced ROS accumulation, caused a loss of $\Delta\Psi_m$, and resulted in apoptosis in HCT116 cells.

DNA Damage and P53 Induce Autophagy in a DRAM-Dependent Manner

Results in the previous sections indicated that excessive ROS production resulted in DNA damage and a decrease in the $\Delta\Psi_m$, which then led to apoptosis. It has been reported that these events could also induce autophagy.^{40,42} To determine whether ROS-induced DNA damage resulted in autophagy, the expression of the damage-regulated autophagy modulator, DRAM1, was examined by Western blot analysis. DRAM1 expression was dramatically increased in HCT116 cells when MRPL35 was down-regulated (Figure 7A), and its expression was restored in the presence of NAC (Figure 7A). Consistent with this result, higher expression of ATG5 (Figure 7A), an effector of autophagy required for responses to all autophagic stimuli,⁴³ was also detected. DRAM1 is a sensor, signaling autophagy in certain situations perhaps to target selective autophagic cargos.⁴⁴ These results confirmed that autophagy was induced by MRPL35 knockdown.

A number of studies indicated that p53 plays dual roles in the regulation of autophagy.⁴⁵ Nuclear p53 was shown to promote autophagy by transcriptionally regulating genes in the autophagic pathway, such as AMPK- β 1/2, phosphatase, and tensin homolog and DRAM,^{46,47} whereas cytoplasmic p53 inhibited autophagy.⁴⁵ To establish whether p53-mediated DRAM1 induced autophagy, the nuclear localization of p53 was characterized in response to MRPL35 knockdown. P53 was located in the nucleus, and it showed increased accumulation in MRPL35 knockdown of HCT116 cells compared with the control group (Figure 7B). MRPL35 knockdown of HCT116 cells were then transfected with sip53 or siControl to knockdown p53 protein levels. Inhibition of p53 expression resulted in attenuation

Figure 8 Proposed model of the mechanisms of mitochondrial ribosomal protein L35 (MRPL35) down-regulation–induced reactive oxygen species (ROS)-mediated DNA damage, which induced G₂/M arrest and cell death of colorectal cancer (CRC) cells. Bars indicate inhibition, and arrows indicate activation. ATM, ataxia-telangiectasia mutated; DRAM1, DNA damage regulated autophagy modulator 1; NAC, *N*-acetyl cysteine; PARP, proline-rich acidic protein 1; $\Delta\Psi_m$, mitochondrial membrane potential.

of the expression of DRAM1 and Atg5 (Figure 7C), and we observed similar DRAM1 and Atg5 expression signature in MRPL35-rescued cells (Figure 7D). These results indicated that DRAM1 was induced by DNA damage via endogenous p53.

To gain mechanistic insights into autophagy in cell death, it was next determined whether autophagy caused by MRPL35 knockdown in HCT116 cells was sensitive to the pan-caspase inhibitor Z-VAD-FMK by flow cytometry. Although Z-VAD-FMK dramatically blocked the cleavage of caspase-3 and caspase-9 (Figure 7G), it failed to block the cell death induced by DRAM1 (Figure 7, E and F), indicating that the cell death due to MRPL35 knockdown was not mediated by a caspase-3- and caspase-9-dependent apoptosis. These results indicated that down-regulation of MRPL35 activated an autophagy-dependent cell death pathway that did not strictly rely on caspase activity for cell death execution.

Discussion

The development of CRC, like many other cancers, is a multistep process based on the accumulation of genetic and epigenetic alterations.⁴⁸ Mitochondria are important mediators of tumorigenesis, but the relationship between mitochondria and tumorigenesis is still poorly understood.^{49,50} MRPs encoded by nuclear genes are important for mitochondrial-encoded protein synthesis and mitochondrial function. In addition to this primary function, MRPs may be directly involved in the process of cancer cell proliferation, apoptosis, and metastasis.^{12,51} Overexpression of MRPL41 contributes to p53 stability and induces apoptosis and causes cell cycle arrest at the G₁ phase.¹⁴ MRPS36 overexpression is also found to delay cell cycle progression and to retard cell proliferation in association with p53 modification and p21^{WAF1/CIP1} expression.¹⁶ However, the function of most MRPs has not been investigated individually.

This study demonstrates that MRPL35 is up-regulated during progression of CRC, which is associated with poor prognosis for CRC patients. However, TCGA data indicate that the CRC patients with low expression of MRPL35 had shorter overall survival. The discrepancy may be attributed to differences in the population, ethnic and geographic, from which the samples are obtained. Clinical samples from other cities of China need to be selected for further validation. At the same time, the clinical sample size must be expanded to get more convincing data support. Knockdown of MRPL35 decreased the proliferation of colorectal carcinoma *in vitro* and *in vivo*, but it had no effect on migration or invasion. In addition, MRPL35 may only play a major role in the development of CRC rather than the stage of cancer initiation. MRPL35 knockdown induced ROS accumulation, which caused DNA damage and cell cycle arrest at the G₂/M phase, disrupted the $\Delta\Psi_m$ to induce apoptosis, and

activated the autophagy-dependent cell death pathway (Figure 8).

It has been suggested that ROS such as superoxide, hydrogen peroxide, and hydroxyl radical, may have a causative role in the progression of carcinogenesis.^{52,53} Under physiological conditions, ROS are constantly generated in the mitochondria through the respiratory chain.⁵³ At normal cellular levels, ROS plays a role in normal cell signaling in the cell. However, when in excess, ROS may cause lipid, protein, and DNA damage and result in cell apoptosis.⁵⁴

The data indicated that MRPL35 knockdown induced the generation of ROS, which then resulted in DNA damage. In response to the DNA damage, the phosphorylation of H2A.X on Ser139 (γ -H2A.X) and ATM on Ser1981 increased, and the ATM-Chk2/P53-Cdk1/cyclin B1 cascade was activated, ultimately resulting in G₂/M checkpoint arrest. The protein kinase ATM is best known for its role as a primary mediator of the DNA damage response.⁵⁵ H2A.X is phosphorylated by ATM immediately after DNA damage.^{30,56} Although this DNA damage response pathway is well established, these data for the first time established that MRPL35 knockdown can cause overproduction of ROS to initiate the activation of this pathway.

As a protein kinase, ATM can also phosphorylate numerous proteins involved in cell cycle regulation and DNA repair, including p53, NBS1, 53BP1, BRCA1, Smc1, and Chk2.^{57,58} Chk2 was phosphorylated on Thr68, and p53 was phosphorylated at Ser15 by ATM. Their phosphorylation by ATM causes cell-cycle arrest at G₁/S, intraS, and G₂/M checkpoints. Cell cycle progression is partly controlled by CDKs and their activating partners, the cyclins.⁵⁹ The activation of Cdk1 kinase needs its interacting protein cyclin B to form the maturation-promoting factor, which phosphorylates their respective substrates and controls their functions.⁶⁰ The p53 protein is a key tumor suppressor which plays a pivotal role in anticancer defense.⁶¹ Under normal conditions, p53 is kept at low levels through ubiquitination and proteasomal degradation, which is mediated mainly by the negative regulators MDM2 and MDM4.⁶² MRPL41 inhibits MDM2-mediated p53 ubiquitination and degradation and induces cell cycle arrest at G₁.¹⁴ However, a number of cellular stresses, such as DNA damage, can also stabilize p53,⁶³ which regulates gene expression as a transcription factor, leading to apoptosis, senescence, and cell cycle arrest.^{64,65} As demonstrated in this study, the activation of the ATM-Chk2/P53-Cdk1/cyclin B1 cascade is likely one of a suite of complex cellular responses.

Higher than normal levels of ROS cause cell death; apoptosis, autophagy, and necrosis are the three main mechanisms that result in cell death.⁶⁶ Apoptosis can be triggered extrinsically by receptor-mediated pathways or intrinsically by mitochondria-mediated mechanisms.⁶⁷ Both intrinsic and extrinsic mechanisms are integrated to regulate cell apoptosis through mitochondria.⁶⁸ Here, we showed

that down-regulation of MRPL35 enhanced ROS-induced loss of $\Delta\Psi_m$, which then facilitated the release of cytochrome *c* into the cytoplasm, the activation of the caspases, and the initiation of apoptosis. These results support the hypothesis that the loss of $\Delta\Psi_m$ is an early cellular response to apoptotic stimuli and plays a crucial role in mitochondria-mediated apoptosis.⁶⁹ Furthermore, NAC, an ROS scavenger, significantly elevated $\Delta\Psi_m$ and alleviated HCT116 cell growth inhibition caused by MRPL35 knockdown, which further supported the important role of ROS in cell apoptosis.

Autophagy is a cell self-cannibalization process. ROS may also regulate autophagy through multiple signaling pathways, such as c-Jun N-terminal kinases (JNK), Akt-mTOR (mammalian target of rapamycin), and AMP-activated protein kinase (AMPK).^{70,71} In the nucleus, ROS sequentially activate the HIF-1 transcription factor, p53, FOXO3, and NRF2, and they in turn stimulate the transcription of BNIP3 and NIX, TIGAR, LC3, and BNIP3 and p62 to regulate autophagy.⁷² DRAM1 is a TP53 target gene coding a lysosomal membrane protein and has an essential role in TP53-mediated autophagy activation and apoptosis.^{73–75} The expression of both DRAM1 and ATG5 is up-regulated because of MRPL35 knockdown in the induction of autophagy. Both DRAM1 and ATG5 are required for autophagy.^{43,76}

It is also known that autophagy is a cell survival pathway under starvation conditions, and when cells are put under stresses such as treatment by arsenic trioxide,⁴² sodium selenite,⁷⁷ or hypoxia,⁷⁸ autophagic cell death is induced. Mounting evidence demonstrates that increase in production of ROS eventually distresses the $\Delta\Psi_m$ and provokes autophagic-cell death.^{42,79} In this study, although Z-VAD-FMK blocked the cleavage of caspase-9 and caspase-3, it failed to block the cell death induced by MRPL35 knockdown to a substantial extent. This finding indicates that autophagy-dependent cell death is involved in this process. DRAM1 may also influence apoptosis through the up-regulation of BAX and BAX lysosomal translocation.⁸⁰ Thus, the relationship between autophagy and classic apoptosis is likely complex, and the exact mechanisms of this relationship require further investigation.

Conclusion

This work reveals an important role of MRPL35 in the development of CRC and may be a novel molecular marker for malignancy and as a prognostic indicator in CRC. However, many questions about this role await further exploration and validation.

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Supplemental Data

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