RecQL4 regulates autophagy and apoptosis in U2OS cells

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Abstract

RecQL4, one of the five human RecQ helicases, is a key mediator of genomic stability and its deficiency can cause premature aging phenotypes. Here, by using CRISPR/Cas and RNAi technology, we demonstrated that autophagy level was elevated in both RecQL4 knockdown and knockout cells compared to those of control cells. Surprisingly, mitochondrial content was increased and LC3 co-localization with mitochondria was partially lost in RecQL4 knockout cells compared to control cells, suggesting that RecQL4 deficiency impaired mitophagic processes in U2OS cells. Furthermore, we found that knockout of RecQL4 de-stabilized PINK1. In addition, RecQL4 knockout cells were more susceptible to apoptosis under mitochondrial stress than control cells. In conclusion, our findings indicated a novel role of RecQL4 in the regulation of autophagy/mitophagy in U2OS cells.

Keywords: RecQL4; autophagy; mitophagy; apoptosis; mitochondrial stress

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Introduction

RecQ helicases play an important role in DNA metabolism. Mutations in RecQ helicases can consequently cause genomic instability (Chu et al. 2010). To date, five members of the RecQ helicase family have been identified in human cells. Mutations in three of these, BLM, WRN, and RecQL4, lead to severe human autosomal recessive disorders such as Bloom syndrome (BS), Werner syndrome (WS) and Rothmund-Thomson syndrome (RTS) (Kitao et al. 1999b; Monnat 2010; Singh et al. 2009). RecQL4 deficiency also causes two other autosomal recessive disorders, Rapadilino and Baller-Gerold syndromes (Kitao et al. 1999a). It has been reported that RecQL4 exerts ATPase and unwinding activity on various DNA substrates in vitro (Rossi et al. 2010; Suzuki et al. 2009; Xu and Liu 2009); can form initiation complex of DNA replication (Sangrithi et al. 2005); is involved in multiple DNA repair pathways, including mitochondrial DNA oxidative damage repair (Chi et al. 2012a; Schurman et al. 2009).

Autophagy is a housekeeping mechanism involved in the regulation and maintenance of cellular homeostasis (Galluzzi et al. 2014). During cellular stress, portions of the cytoplasm and organelles are engulfed in double-membrane vesicles termed “autophagosomes”. These autophagosomes fuse with lysosomes, and their contents are degraded (Abada and Elazar 2014; Yee et al. 2009). Three basic types of autophagy have been identified: micro-autophagy, chaperon-mediated autophagy, and macro-autophagy (Kuriyama and Nochi 2015). Generally, autophagy is considered to be nonspecific. However, specific organelles such as mitochondria, ribosomes, or peroxisomes are selectively degraded under certain circumstances. The selective degradation of mitochondria by the autophagic machinery is called mitophagy (Kim et al. 2007) and contributes to cell survival by removing damaged mitochondria (Colell et al. 2007; Zhang et al. 2008). Both autophagy and mitophagy protect cells against stress and play an important role in various physiological processes (Levine and Kroemer 2008). WRN and BLM, two members of RecQ helicase family, have been found to be associated with autophagy (Khan et al. 2014; Maity et al. 2014). However, involving of RecQL4 in autophagy and mitophagy has so far not been reported. In this study, we aimed to elucidate the effects of CRISPR/Cas nuclease-induced RecQL4 loss-of-function on autophagy and mitophagy-mediated protection against stress, to gain insights into the relationship between RecQL4, autophagy/mitophagy, and aging.

Materials and methods

Cell culture

Human osteosarcoma cell line U2OS (American Type Culture Collection, USA) was cultured in Dulbecco's modified Eagle's medium (Thermo Scientific, Waltham, Mass., USA) supplemented with 10% fetal bovine serum (Hyclone, Los Angeles, USA), 100 IU/ml penicillin, and 100 µg/ml streptomycin.

Antibodies and Immunoblotting

Primary antibodies used for western blot analysis were as follows: mouse anti-GAPDH (1:1,000; Millipore, Massachusetts, USA); rabbit anti-β-actin (1:1,000; Cell Signaling Technology, Boston, Mass., USA); rabbit anti-LC3 (1:1,000; Novus, Littleton, USA); mouse anti-PARP-1 (1:1,000; Millipore, Massachusetts, USA); rabbit anti-p-p53 (1:1,000; Novus, Littleton, USA), and rabbit anti-mTOR (1:1,000; Cell Signaling Technology, Boston, Mass., USA). Horseradish peroxidase conjugated anti-mouse IgG (Cell Signaling) or anti-rabbit IgG (Thermo Scientific, Waltham, Mass., USA) were used as secondary antibodies at dilutions of 1:2000. Signals were detected with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Waltham, Mass., USA).

RecQL4 knockout (RecQL4 KO)

RecQL4 KO in U2OS cells was achieved with the CRISPR/Cas9 system. sgRNA, 25-bases in length (5'-CACCGAAGCAGAAACACTACGTGCG-3'), was designed to target the RecQL4 gene. U2OS cells were transfected with the PX330-U6-gRNA-Cas9 expression vector aided by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 24 h of transfection, a single cell expressing green fluorescent protein (GFP) was selected with flow cytometry sorters (BD Biosciences, CA, USA) and cultured in 96-well plate. Targeted cleavage was Sanger sequenced in pMD-19T vector (Takara Bio, Beijing, China) with the PCR product of RecQL4-deficient colonies (forward primer: 5'-GAGAAGCGGAGATGGAACGA-3'; reverse primer: 5'-CCAGGGAGTAGAGTGGCAGC-3').

Long-range quantitative PCR (QPCR)

Expression of mitochondrial NADH dehydrogenase 1 (mtND1) was quantified by real-time PCR. Nuclear 18S rRNA served as the internal control. A full-length (16261 bp) mtDNA fragment was amplified using LA Taq (Takara) according to the manufacturer's protocol. The input of mtDNA

template was adjusted based on mtND1 to normalize the mtDNA template in each sample. Primers used were as follows: Nu18S-F, 5'-TAGAGGGACAAGTGGCGTTC and Nu18SR, 5'-CGCTGAGCCAGTCAGTGT; mtND1-3212F, 5'-CACCCAAGAACAGGGTTTGT and mtND1-3319R, 5'-TGGCCATGGGTATGTTGTAA; hmt15149F, 5'-TGAGGCCAAATATCATTCTGAGGGGC and hmt14841R, 5'-TTTCATCATGCGGA GATGTTGGATGG. PCRs were performed at 94 °C for 1 min followed by 25 cycles at 98 °C for 10 s, 60 °C for 40 s, 68 °C for 16 min and a final elongation for 10 min. The products were separated by electrophoresis on a 0.8% agarose gel and visualized by ethidium bromide staining.

RecQL4 knockdown using adenovirus

HEK293 cells were transfected with pAdEasy-1 construct containing RecQL4 shRNA aided by Lipofectamine 2000 (Invitrogen). After 48 h, supernatants containing viral particles were used to infect U2OS cells. After 3 days of infection, cells were collected for analysis. The control and shRNA sequences for RecQL4 were GAAGAGGACACGCCTTAGACT and GCTCAAGGCCAATCTGAAAGG, respectively.

Construct of stable cell line expressing EGFP-LC3B

The full-length sequence of human LC3B coding region was amplified with the following primers: forward: 5'-ATGCCGTCGGAGAAGACCT-3', reverse: 5'-TTACACTGACAATTTTCATCCCGA-3'. PCR products were cloned into pEGFP-C1 vector (Clontech, Palo Alto, Calif., USA). U2OS cells were transfected with 1 µg of plasmid DNA aided by Lipofectamine 2000 (Invitrogen). Stable cell lines were selected by means of 400 µg/ml G418 (Corning, NY, USA).

Immunofluorescence staining

U2OS cells expressing EGFP-LC3B were seeded onto chamber slides for 24 h. Cells were then transfected with control and RecQL4 shRNA adenovirus for 3 days. Cells were rinsed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde. After washing thrice with PBS, cells were incubated with DAPI for 2 min. EGFP-LC3B foci were detected with a Leica confocal laser scanning microscope TCS SP (Nikon, Tokyo, Japan).

To investigate the localization of RecQL4, wild type (WT) and RecQL4 KO U2OS cells were seeded onto chamber slides and 1 µM rotenone was added for 24 h. The cells were rinsed with PBS

and incubated with 0.5 M MitoTracker Red CM-H2XRos (Molecular Probes, USA) for 30 min. The cells were rinsed with PBS and fixed with 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 for 10 min, and then blocked with 5% non-fat milk. Cells were incubated overnight with anti-LC3 antibody at 4 °C. After washing three times with PBS, cells were incubated at room temperature with fluorescence-conjugated Texas Green Goat anti-rabbit IgG antibody for 2 h, and co-stained with DAPI. The localization of mitochondria versus LC3 protein was analyzed with a Leica confocal laser scanning microscope TCS SP.

Reverse transcription and quantitative PCR (qPCR)

The relative expression levels of poly (ADP-ribose) polymerase 1 (PARP-1), AMP-activated protein kinase (AMPK), and mechanistic target of rapamycin (m-TOR) mRNAs in WT and RecQL4 KO U2OS cells were determined by quantitative real-time PCR (Applied Biosystems 7300, Calif., USA) using SYBR Green PCR master mix (TOYOBO, Tokyo, Japan). Total RNA was isolated from cells with the TRIzol reagent (Invitrogen). cDNA was synthesized with the First-Strand Synthesis System (Thermo Scientific, Waltham, Mass., USA). Primers used for qPCR were as follows: 5'-CCCTAAAGGCTCAGAACGACC-3' (sense) and 5'-TGACGTCCCCAGTGCAGTAAT-3' (antisense) for PARP-1; 5'-TCAGTTCCTGGAGAAAGATG-3' (sense) and 5'-CTACTTTATGCCCAGTCAAT-3' (antisense) for AMPK; 5'-TGTAGCCGCCCTTCGTG-3' (sense) and 5'-TTCTCTCAGACGCTCTCCCTC-3' (antisense) for mTOR; 5'-AGCGAGCATCCCCAAAGTT-3' (sense) and 5'-GGGCACGAAGGCTCATCAT-3' (antisense) for β -actin. The expression levels of mRNA were normalized to β -actin, and real-time PCR was performed as described above. Data obtained with RT-qPCR were analyzed by the $\Delta\Delta$ CT method.

Measurement of mitochondrial content with the MitoTracker Red

WT and RecQL4 KO cells were plated in 35-mm dishes and treated with rotenone (Sigma, Silicon Valley, USA) at a concentration of 1 μ M for 24 h. Cells were harvested by trypsinization, and then re-suspended in DMEM without phenol indicator. Half of the cells were stained with 0.5 M MitoTracker Red CM-H2XRos (Molecular Probes, USA) for 30 min at 37 °C. Cells were washed twice, re-suspended in pre-warmed PBS, and fluorescence was measured with a flow cytometer (Becton, Dickinson and Company, New Jersey, USA). Unstained cells were used to gate for stained

cells.

Measurement of cellular ROS

WT and RecQL4 KO U2OS cells were harvested by trypsinization, washed, and re-suspended in pre-warmed DMEM without phenol indicator, and incubated with 5 μ M DHE probe (VARSAL, Beijing, China) for 30 min at 37 °C. The probe was removed and cells were washed twice with PBS. Cells were re-suspended in BD Falcon tubes at a concentration of 1×10^7 cells/ml. Fluorescence was measured at 510 nm excitation and 580 nm emission with a flow cytometer. Data analysis was performed with WinMDI28 software. Relative ROS production was calculated by mean values of mean fluorescence intensity (PE-A) from three independent experiments.

Apoptosis assay

Apoptosis levels were measured using the Annexin V-FITC apoptosis detection kit (Beyotime, Beijing, China). In brief, WT and RecQL4 KO U2OS cells were treated with or without 1 μ M rotenone for 24 h. The cells were washed twice with cold PBS and re-suspended in FITC binding buffer. Approximately 1×10^5 cells were transferred into a BD Falcon tube, and 5 μ l FITC and 9 μ l PI were added. Cells were incubated at room temperature in the dark for 15 min, followed by gentle vortexing, and analyzed by flow cytometry. Data analysis was performed with FlowJo7.6 software from three independent experiments.

Western blot analysis

Cell lysates were prepared and protein concentrations were determined with the BCA kit (Becton, Dickinson and Company, New Jersey, USA) according to the manufacturer's instructions. An appropriate amount of protein was resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, Billerica, Mass., USA). The membrane was incubated overnight with primary antibodies at 4 °C. The HRP-conjugated goat anti-rabbit secondary antibodies (ZSGB-BIO, Beijing, China) were used at 1:2000 dilution. Immunodetection was performed with the enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Band densities were measured with Image J software.

Statistical analysis

Data from at least three independent experiments were analyzed by one-way analysis of variance (ANOVA) and Duncan's test by means of SAS software (SAS Institute, Cary, NC, USA).

Differences were considered statistically significant when $P < 0.05$.

Results

Generation of RecQL4 gene knockout U2OS cells using the CRISPR/Cas9 system

To investigate the effect of RecQL4 depletion on autophagy, a CRISPR/Cas9 strategy was used to induce a haploid deletion mutation, and this resulted in a cell line with haploid knockout of RecQL4. The mutation was validated by Sanger sequencing. The altered UO2S cell line had a 31-bp deletion and a 1-bp insertion mutation in the RecQL4 gene sequence (Fig. 1A). To double check for the knockout of RecQL4 in mutated cells (KO), western blotting was performed with wild type (WT) and mutant U2OS cells. As predicted, the protein of RecQL4 was lost in mutated U2OS cells (Fig. 1B).

To investigate the effects of DNA damage in RecQL4 KO U2OS cells, we performed long range qPCR of full length mitochondrial DNA (mt-FL) in WT and KO U2OS cells after treating the cells with menadione for 0, 5, 24, and 48 h. In the initial experiment, cells were co-cultured with 12.5 μ M menadione for 60 min. Mitochondrial DNA fragmentation was observed in both WT and RecQL4 KO U2OS cells. After removal of menadione, full-length mitochondrial DNA content in WT U2OS was significantly higher than that in RecQL4 KO cells.

RecQL4 KO increases autophagy in U2OS cells

To determine the effects of RecQL4 deletion on autophagy in U2OS cells, we examined the expression levels of LC3 protein by western blot analysis. As shown in Fig. 2A and B, both knockdown and knockout of RecQL4 in U2OS cells led to an increase in the abundance of LC3-II. The formation of autophagosomes was then tracked by means of EGFP-LC3. As shown in Fig. 2C, the number of EGFP-LC3 puncta was significantly increased in RecQL4 knockdown U2OS cells compared to that in control cells. To further characterize the status of autophagic flux in RecQL4 KO U2OS cells, we determined the levels of LC3-II and p62 by treating the cells with chloroquine. As shown in Fig. 2D, after the treatment with chloroquine, the levels of LC3-II were still significantly higher in RecQL4 KO U2OS cells than those in control U2OS cells. p62 expression also increased in RecQL4 KO cells after treatment with chloroquine (Fig. 2D). Notably, knockout of RecQL4 did not affect the level of autophagy after nutrition deprivation for 6 h (Fig. 2E), which indicates that RecQL4 is not a metabolic intermediate in mitochondria. Our results indicate that

RecQL4 deficiency in U2OS cells results in increased autophagy.

Effects of RecQL4 KO on the expression of autophagy-associated proteins

Autophagy has been implicated in ROS-mediated biological processes. Our previous studies have shown that RecQL4 deficiency leads to raised mitochondrial ROS levels in U2OS cells (Chi et al. 2012b). In light of these results, the levels of cellular ROS in KO cells were measured by means of dihydroethidium (DHE), an indicator of superoxide radicals. As shown in Fig. 3A, RecQL4 deletion resulted in a 3.5 fold increase of the ROS level in U2OS cells, which is close to the ROS level induced by 1 mM H₂O₂ in WT cells (4 fold), indicating that ROS generation in cells lacking RecQL4 is extremely high. To investigate the dependence of RecQL4 deficiency induced autophagy on ROS, we treated WT and RecQL4 KO cells with *N*-acetyl Cysteine (NAC), a ROS scavenger. Levels of LC3-II were reduced in RecQL4 knockout cells after treatment with NAC (Fig. 3B). Furthermore, the RecQL4 KO cells treated with NAC showed an increased amount of p62 (Fig. 3B), suggesting that RecQL4 deficiency induces autophagy via ROS accumulation.

To test whether the level of PARP-1, a single-stranded DNA damage repair ribozymes, was altered in cells without RecQL4, and whether mTOR expression was reduced in cells with increased autophagy (Tripathi et al. 2013; Zhang et al. 2014). We used RT-PCR and western blotting to determine PARP-1 and mTOR expression and protein levels in cells with and without RecQL4. As shown in Fig. 3C and D, expression of PARP-1 was increased in terms of both mRNA and protein levels in RecQL4 KO cells. At the same time, expression of mTOR mRNA and protein were down-regulated in RecQL4 KO cells (Fig. 3C and D). In addition, phosphorylation of p70S6K, a key downstream target of mTOR, was inhibited in RecQL4 KO cells (Fig. 3C and D). Furthermore, we found that AMPK, a negative regulator of mTOR signaling, was activated and its mRNA expression was increased in RecQL4 KO cells (Fig. 3C and D), suggesting that multiple autophagy-related signals were activated in cells that lack RecQL4.

RecQL4 knockout impairs mitophagy in U2OS cells

Our previous studies have shown that RecQL4 plays a role in mitochondrial stability and function (Chi et al. 2012b). To investigate whether mitochondrial content was changed in RecQL4 KO cells, cells were stained with MitoTracker Red. As shown in Fig. 4A, mitochondrial content was increased in RecQL4 KO U2OS cells compared to that in control cells. These findings were confirmed by

treating the cells with rotenone, which inhibits mitochondrial complex I and induces mitophagy (Fig. 4A). The results of this experiment led us to believe that mitochondrial changes in RecQL4 KO cells could be attributed to specific defects in mitophagy. Next, two mitochondrial toxins, rotenone and the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), were used to induce mitophagy. As shown in Fig. 4B, the toxins produced a significantly increased level of LC3-II in WT cells but not in RecQL4 KO U2OS cells, indicating that the mitophagic process was impaired in cells without RecQL4 protein. Furthermore, we found that rotenone enhanced the co-localization of LC3 and mitochondria in RecQL4 WT cells as measured by Pearson's coefficient, whereas RecQL4 KO cells showed a loss of co-localization of LC3 and mitochondria (Fig. 4C).

Most mitophagy processes are associated with PINK1 overexpression in the outer mitochondrial membrane (Narendra et al. 2010). Our research has shown that RecQL4 is partially located in the mitochondria and participates in the repair of oxidative damage in HEK293 and U2OS cells (Chi et al. 2012a). We assessed the stability of full-length PINK1 (F-PINK) in control and RecQL4 KO cells after rotenone treatment. As shown in Fig. 4D, we found that cleaved PINK1 (C-PINK) was elevated and full-length PINK1 was depressed in RecQL4 KO cells compared to those levels in control cells. Taken together, our results indicate that RecQL4 KO in U2OS cells increased the level of cleaved PINK1, which may affect the ability of the cell to clear damaged mitochondria.

RecQL4 KO in U2OS cells promotes apoptosis in response to mitochondrial toxin

It has been reported that autophagy/mitophagy may play a role opposite to that of apoptosis in cell viability (Green et al. 2011). To investigate whether RecQL4 KO cells were susceptible to apoptosis when exposed to a mitochondrial toxin, RecQL4 KO cells and WT cells were treated with or without 0.1 μ M rotenone for 24 h, and then stained with Annexin V/PI for apoptosis analysis by flow cytometry. We found that apoptosis levels were significantly increased after rotenone treatment in RecQL4 KO/KD cells, while WT cells were not affected (Fig. 5A). Concordant with the observed apoptosis levels, the apoptosis markers, cleaved caspase-3 and phosphorylated p53 (Ser 46), had increased significantly in RecQL4 KO cells that were treated with or without rotenone (Fig. 5B). Taken together, our findings indicated that RecQL4 KO cells were more susceptible to

mitochondrial stress induced apoptosis.

Discussion

We demonstrated that RecQL4 deficiency induces autophagy and inhibits mitophagy in U2OS cells. In addition, the RecQL4-deficient cells exhibited a higher rate of apoptosis under mitochondrial stress than control cells.

General or selective autophagy is an important process in the maintenance of cellular metabolism and allows cells to survive under conditions of hypoxia, starvation, or nutrient deprivation. Several DNA repair-associated proteins, including XPA, CSA, and CSB, are located in the mitochondria and are involved in autophagy (Fang et al. 2014; Kim et al. 2014; Scheibye-Knudsen et al. 2012). Specifically, XPA is involved in mitophagy via PARP-1 hyper activation and NAD⁺/SIRT1 reduction (Fang et al. 2014). Our previous study demonstrated that RecQL4 was partially localized in the mitochondria and participated in both nuclear and mitochondrial DNA oxidative damage repair (Chi et al. 2012a).

The type II CRISPR/Cas system, a prokaryotic adaptive immunity system, has proved to be an efficient genome editing technology (Deveau et al. 2008; Horvath and Barrangou 2010; Wiedenheft et al. 2012). To our knowledge, we are the first to use CRISPR/Cas9 endonuclease to establish a RecQL4 KO cell line. In the present study, the level of LC3-II protein was significantly increased in RecQL4 KO cells, which was confirmed in shRNA-mediated RecQL4 knockdown cells. In addition, our data showed that the formation of autophagosomes and LC3-II levels were significantly increased in RecQL4 KO cells, indicating that RecQL4 deficiency contributed to enhanced autophagy. However, it was unclear how RecQL4 deficiency induced autophagy in U2OS cells. Further studies are needed to elucidate the mechanism.

ROS accumulation has been implicated in age-related cardiovascular diseases and vascular lesion formation in atherosclerosis (Tavakoli and Asmis 2012). Autophagy is activated to prevent the accumulation of DNA mutations due to ROS production (LyifVasquez-Trincado et al. 2015). In our study, the increased autophagy in RecQL4 deficient U2OS cells was probably induced by ROS accumulation in the cells. Prolonged stress due to ROS could result in cell death. In the present study, RecQL4 KO results in the phosphorylation and activation of AMPK α at the Thr172 site. The mTOR signaling pathway is the key mechanism of autophagy, and AMPK is among the major

upstream regulators of mTOR (Tripathi et al. 2013). The activation of AMPK can inhibit mTOR activation by reducing the phosphorylation of p70S6K and 4EBP1 and trigger autophagy (Li et al. 2015). In our study, the level of mTOR mRNA and protein both decreased in RecQL4 KO U2OS cells. Thus, RecQL4 deficiency-induced autophagy in U2OS cells may be partially activated via the AMPK-mTOR pathway. PARP-1 is activated in response to oxidative stress and is closely associated with autophagy (Zhang et al. 2011). We reproduced this result in the present study; the expression of PARP-1 protein increased significantly in RecQL4 KO cells. Autophagy can be induced via a ROS-dependent PARP-1-LKB1-AMPK-mTOR pathway (Li et al. 2015). AMPK activation could be an adaptive mechanism related to PARP-1 activation (Fang et al. 2014). However, the effect of PARP-1 and AMPK activation in autophagy induced by RecQL4 deficiency remains to be elucidated.

It has been suggested that more selective forms of autophagy that result in the engulfment and degradation of only mitochondria play an important role in the removal of damaged mitochondria (Yee et al. 2009). Although we observed an induction of autophagy, mitochondrial content in RecQL4-deficient U2OS cells was increased compared to that in controls. RecQL4 deficiency thus appears to have a negative effect on mitophagy. Moreover, we showed that rotenone induced a significant increase in LC3-II levels in WT cells, whereas LC3-II levels decreased in RecQL4 KO U2OS cells, which points to an impaired mitophagic process in the absence of RecQL4 protein. It is generally known that mitochondria with depolarized matrix metalloproteinase (MMP) are more easily degraded by mitophagy via a PINK1/Parkin-dependent pathway (Fang et al. 2014). We observed that loss of RecQL4, led to the cleavage and removal of the kinase PINK1. We therefore supposed that the impaired mitophagy process may be due to the instability of PINK1. Therefore, the mechanism by which RecQL4 mutation impairs the mitophagy process requires further investigation.

It is possible that autophagy contributes to the induction of other cell death responses, such as apoptosis. The role of selective mitochondrial autophagy in the regulation of apoptosis is complex (Yee et al. 2009). Mutations in RecQL4 that drive apoptosis have been reported in a previous study (Fang et al. 2013). To verify the relationship between mitophagy inhibition and apoptotic signals produced by RecQL4 deficiency, we examined whether autophagy could affect the apoptotic

response in U2OS cells. In our study, RecQL4-deficient cells treated with mitochondrial toxin were more likely to undergo apoptosis than control cells. This leads us to speculate that diminished mitophagy accelerates apoptosis in RecQL4-deficient U2OS cells.

In conclusion, our results show that RecQL4 KO has an effect on autophagy/mitophagy in U2OS cells. RecQL4 deficiency impaired mitophagy and drove apoptosis in consequence of mitochondrial stress. We therefore inferred that RecQL4 has a novel role in maintaining mitochondrial homeostasis via autophagy. These findings will facilitate further studies of the mechanisms of mitochondrial removal in RecQL4-deficient cells, with implications for the development of novel therapeutic strategies for premature aging phenotypes in humans.

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Figure legends

Figure 1. RecQL4 knockout by CRISPR/Cas9 in U2OS cell line. (A) Sanger sequencing of RecQL4 in normal U2OS (WT) and mutant U2OS cells (KO). Arrowhead indicates mutations consisting of a 31 bp deletion and a 1 bp insertion, respectively. (B) Western blot analysis of RecQL4 level in WT and RecQL4 KO U2OS cells. GAPDH served as internal control. (C) Long-range QPCR of full-length mitochondrial DNA (mt-FL) in WT and RecQL4 KO U2OS cells.

Figure 2. RecQL4 knockout promotes autophagy. (A) Western blot analysis of LC3 in whole cell lysates of WT and RecQL4 KO U2OS cells. β -actin served as internal control. (B) Western blot analysis in whole cell lysates of LC3-II in U2OS cells with knockdown (KD) of the RecQL4 level. (C) Subcellular distribution of exogenous EGFP-LC3 after transfection for 3 days was determined by confocal microscopy as the foci per cell. Error bars, $\pm$ SD. ** $P < 0.01$. (D) WT and RecQL4 KO U2OS cells were treated with chloroquine (40 μ M) for 9 h and then subjected to western blot analysis with anti-LC3 and p62 antibodies. Levels of LC3-II and p62 were normalized to GAPDH (mean $\pm$ SD; $n = 3$). (E) WT and RecQL4 KO U2OS cells were starved for 6 h and then subjected to western blotting with anti-LC3 and p62 antibodies. Quantification was performed as described in (D) (mean $\pm$ SD; $n = 3$).

Figure 3. Effects of RecQL4 knockout on some autophagy-associated proteins in U2OS cells

(A) Cellular ROS level was measured using DHE dye with Flow cytometry in WT and RecQL4 KO U2OS cells. WT cells treated with H₂O₂ (1mM) for 2 h were used as a positive control. ** $P < 0.01$. (B) After treatment with or without NAC (5 μ M) for 1.5 h, LC3 protein was analyzed by western blot in WT and RecQL4 KO cells. GAPDH served as internal control (mean $\pm$ SD; n = 3). (C) PARP-1, AMPK, and mTOR mRNA levels in WT and RecQL4 KO cells were measured by qRT-PCR and normalized to β -actin. Data are presented as mean $\pm$ SEM from 3 independent experiments.* $P < 0.05$. (D) Western blot analysis of PARP-1, p-AMPK, mTOR and p-p70S6K proteins in WT and RecQL4 KO cells. GAPDH served as a loading control.

Figure 4. RecQL4 knockout suppresses mitophagy and induces instability of PINK1. (A) Mitochondrial content in WT and RecQL4 KO cells was measured by flow cytometry with or without rotenone (mean $\pm$ SD; n = 3). (B) Cells were treated with mitochondrial toxins for 24 h, and LC3 was detected by western blot from total cell lysates. Rotenone (Rot.) is a mitochondrial complex I inhibitor and FCCP is a mitochondrial uncoupler. Actin served as internal control. (mean $\pm$ SD; n = 3). (C) Representative images of WT and RecQL4 KO U2OS cells stained with MitoTracker Red and LC3 after treatment with vehicle or rotenone(1 μ M) for 24 h. Mitochondria and LC3 were quantified with Pearson's coefficient (n = 4; data are represented as mean $\pm$ SEM). (D) Changes in PINK1 were monitored by Western blot in WT and RecQL4 KO cells treated with or without rotenone for 24 h. GAPDH served as internal control.

Figure 5. RecQL4 knockout sensitizes U2OS cells to apoptosis under mitochondrial stress. (A) WT and RecQL4 KO/KD cells were treated with or without rotenone for 24 h. Apoptosis was detected using Annexin V/PI with flow cytometry (mean $\pm$ SD; n = 3). (B) After treatment with rotenone for 24 h, expression levels of cleaved caspase-3 and phosphorylated p53 were determined by western blot. GAPDH served as a internal control.

Figure 1

A

B

C

Figure 2

Figure 3

A

B

C

D

Figure 4

Figure 5

A

B