



International Journal of Cancer & Cellular Biology Research

Short Communication

NF- κ B Prevents Oncogenic Ras-Induced β -Actin Cleavage in *p53*-Deficient Cells - a

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Submitted: 19 May 2017; Approved: 23 May 2017; Published: 24 May 2017

Citation this article: Sugimoto W, Itoh K, Kotari T, Guo AK, Ebata T, et al. NF- κ B Prevents Oncogenic Ras-Induced β -Actin Cleavage in *p53*-Deficient Cells. Int J Cancer Cell Biol Res. 2017; 2(1): 014-018.

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ABSTRACT

Tumor cell characteristics, including invasiveness, are influenced by the tumor suppressor *p53*. We recently reported that *p53* diminishes oncogenic Ras-driven cell invasion by promoting β -actin cleavage, which is mediated by the serine protease high-temperature requirement A2 (HtrA2/ Omi). In this study, we showed that nuclear factor (NF)- κ B is responsible for eliminating Ras - induced β -actin cleavage in *p53* - deficient cells. In *p53* knockout (*p53*^{-/-}) Mouse Embryonic Fibroblasts (MEFs), depletion of the NF- κ B component *p65/RelA* using its specific shRNA caused β -actin cleavage in response to expression of oncogenic Ras. Knockdown of *p65/RelA* enhanced the mitochondrial translocation of p38 MAPK, a critical step in HtrA2/Omi activation. Expression of mitochondria-targeting signal-tagged *p65/RelA* diminished β -actin cleavage in *p65/RelA*-knockdown cells. Our results provide novel insights into the mechanism underlying contribution of NF- κ B to malignancy of Ras-mutated cells with dysfunctional *p53*.

Keywords: *p53*; NF- κ B; Actin; Mitochondria; HtrA2/ Omi; Lamellipodia

INTRODUCTION

Ras controls fundamental cell behaviors, such as proliferation and survival, by regulating downstream signaling that includes the Raf-MEK-MAPK and PI3K-Akt pathways [1-3]. On the contrary, aberrant activation of Ras accompanied with its gene mutations is observed in several types of cancers. When oncogenic mutations occur in the RAS gene, the tumor suppressor *p53* plays an essential role in determining cell behaviors. Expression of oncogenic Ras in normal cells triggers DNA damage responses and induces apoptosis or senescence by induction of *p53*-dependent gene expression [4,5]. In contrast, oncogenic Ras induces cellular transformation when the ability of *p53* to prevent tumorigenesis is ablated as a consequence of its gene mutation or inactivation [6]. Furthermore, while oncogenic Ras promotes invasion and metastasis in transformed cells, these effects of Ras are also diminished by *p53* [7]. Thus, Ras-induced tumorigenesis and tumor progression are both dependent on the status of *p53*.

Recently, we have revealed a mechanism underlying suppression of Ras-driven cell invasion by *p53*. Oncogenic Ras stimulates cleavage of β -Actin by the mitochondrial protease, high-temperature requirement A2 (HtrA2; also known as Omi), which depends on the cytoplasmic *p53* [8]. The Ras-induced cleavage of β -Actin eliminates the formation of lamellipodia, actin-based membrane protrusions crucial for cancer cell invasion [9,10].

It has been well documented that the transcription factor NF- κ B plays an essential role in Ras-induced cellular transformation by *p53* dysfunction [6,11,12]. Oncogenic Ras promotes the metabolic shift from Oxidative Phosphorylation (OXPHO) to aerobic glycolysis, known as the Warburg effect [13], which is, at least in part, attributed to NF- κ B-induced expression of Glucose Transporter (GLUT) 3 [6]. Furthermore, NF- κ B-mediated gene expression is required for Epithelial-Mesenchymal Transition (EMT), which is involved in the early steps of metastasis, in Ras-transformed cells [14]. *p53* suppresses the NF- κ B-mediated gene expression by attenuating both I κ B kinase (IKK) activity and interaction of NF- κ B with its co-activator p300/CBP [6,15-17]. While NF- κ B translocates to the mitochondria to repress the expression of OXPHO-related mitochondrial genes, *p53* prevents the translocation of NF- κ B to the mitochondria by inhibiting the interaction of NF- κ B with the mitochondrial heat shock protein (mt-HSP70; also known as Mortalin) [18]. However, the functional role of mitochondrial NF- κ B in oncogenic Ras-expressing cells remains unclear.

Here, we show that in the absence of *p53*, knock down of the NF- κ B component, *p65/RelA*, increases β -Actin cleavage and

decreases lamellipodia formation in cells expressing oncogenic Ras. Ectopic expression of a mitochondria-targeting form of *p65/RelA*, in turn, reduces cleavage of β -Actin. Our results suggest that NF- κ B may promote oncogenic Ras-induced invasion of *p53*-deficient cells by facilitating the formation of lamellipodia through reduction of β -Actin cleavage.

MATERIALS AND METHODS

Cell culture and retroviral infection

p53^{-/-} MEFs were prepared as previously described [6] and infected following 3 passages. Cells were cultured in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% FBS. We performed all experiments within two weeks after selection to avoid genetic abnormalities acquired during prolonged culture. Retroviral infection was performed as previously described [19]. Infected cells were selected using hygromycin (300 μ g/ml) and puromycin (1.5 μ g/ml) for 3 days.

Plasmids

pSuper retro *p65* puro was cloned *p65* target sequence [6], 5'-GAAGAAGAGTCCTTTCAAT-3' into a pSuper retro puro (Oligoengine, Seattle, WA). pBabe Ha-RasV12 with a hygromycin selection marker were used [6]. The Mito-DsRed expression vector was obtained from Clontech Laboratories Inc. To generate a pcDNA3-Mito-Flag-*p65* vector, the mitochondrial targeting sequence from subunit VIII of human cytochrome c oxidase was fused to the 5'-end of the Flag-tagged *p65*.

Antibodies and materials

Anti-p38 rabbit polyclonal (C-20, Santa Cruz Biotechnology), p38 rabbit polyclonal (Cell Signaling Technology), anti-HtrA2/ Omi goat polyclonal (V-17, Santa Cruz Biotechnology), anti- β -Actin mouse monoclonal (ACTBD11B7, Santa Cruz Biotechnology), anti-VDAC rabbit polyclonal (Cell Signaling), and anti- α -tubulin mouse monoclonal (DM1A, Sigma) were used for immunoblot analysis. Anti-Flag mouse monoclonal (M2, Sigma) antibodies were used for immunoblot and immunofluorescence analyses. Rabbit (H-191, Santa Cruz Biotechnology) was used for immunofluorescence analysis.

Immunoblot analysis

To obtain total cell lysates, cells were solubilized with ice-cold lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, 10 mM EDTA, 1 mM Na₃VO₄, 10 mM NaF, protease inhibitor cocktail [Nakarai Tesque]). Lysates were sonicated and centrifuged at 20,000 x g for 15 min. The supernatants were used as total cell lysates. To obtain mitochondria and cytosol fractions,



a mitochondria isolation kit (QIAGEN) was used according to the manufacturer's protocol. The isolated mitochondrial fraction was solubilized with ice-cold lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, 10 mM EDTA, 1 mM Na_3VO_4 , 10 mM NaF, protease inhibitor cocktail) and then centrifuged at 20,000 $\times g$ for 15 min. The supernatants were used as mitochondrial proteins. For immunoblotting against β -Actin, cells were solubilized with an SDS sample buffer. The lysates were subjected to Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Immunofluorescence

Cells were fixed with 4% PFA, permeabilized with 0.2% Triton X-100, and then blocked with 2% BSA in PBS. Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes) and Alexa Fluor 546-conjugated goat anti-mouse IgG were used as secondary antibodies. Alexa Fluor 594-conjugated phalloidin (Molecular Probes) were used to stain F-actin. Images were acquired using a confocal microscope (LSM700; Zeiss) and then analyzed with ImageJ software (NIH).

Monitoring mitochondrial membrane potential

Monitoring mitochondrial membrane potential was performed as previously described [8]. Acquired images using a confocal microscope (LSM700; Zeiss) were cropped and fluorescence intensities were quantified using Image J software (version 1.45f).

Statistical analysis

Data were analyzed by unpaired Student's *t*-test.

RESULTS AND DISCUSSION

Lamellipodia formation has been shown to be suppressed upon expression of oncogenic Ras in cells bearing wild-type *p53*, but not in *p53*-deficient cells [8]. We found that *p65/RelA* knock down diminished lamellipodia formation in *p53*^{-/-} MEFs expressing

oncogenic Ras (Ha-RasV12) (Figure 1A). Since oncogenic Ras-induced β -Actin cleavage by HtrA2/Omi impairs lamellipodia formation, we further examined whether *p65/RelA* knock down stimulates β -Actin cleavage in *p53*^{-/-} MEFs. Immunoblotting showed the fragment of β -Actin in cells expressing both Ha-RasV12 and *p65/RelA*-shRNA, while Ha-RasV12 expression or *p65/RelA* knock down alone was not sufficient for induction of β -Actin cleavage (Figure 1B). These results suggest that NF- κ B contributes to lamellipodia formation in Ras-transformed cells by reducing the actin proteolysis.

A decrease in the inner mitochondrial membrane potential ($\Delta\Psi_m$) is involved in the β -Actin cleavage [8]. We, therefore, examined the effect of *p65/RelA* knockdown on $\Delta\Psi_m$ in *p53*^{-/-} MEFs expressing Ha-RasV12, and found that $\Delta\Psi_m$ was significantly decreased in a subset of mitochondria (Figure 2A and B). A decrease in $\Delta\Psi_m$ causes mitochondrial translocation of p38 MAPK, which leads to HtrA2/Omi activation to cleave β -Actin [8]. Consistently, the amount of p38 MAPK in the mitochondrial fraction was higher in *p65/RelA* knock down cells than in control cells, even though the amount of HtrA2/Omi in cytosol was not significantly different between these cells (Figure 2C). Taken together, NF- κ B is likely to suppress HtrA2/Omi-mediated β -Actin cleavage by maintaining $\Delta\Psi_m$ to eliminate mitochondrial translocation of p38 MAPK.

While *p53* reportedly suppresses mitochondrial translocation of NF- κ B [18], NF- κ B was indeed found in the mitochondrial fraction in *p53*^{-/-} MEFs expressing Ha-RasV12 (Figure 2C). We, therefore, hypothesized that mitochondrial NF- κ B might prevent p38 MAPK translocation into mitochondria and concomitant β -Actin cleavage in oncogenic Ras-expressing *p53*-deficient cells. To test this hypothesis, the mitochondrial targeting peptide-fused flag-tagged *p65* (Mito-Flag-*p65*) was expressed in *p65/RelA*-knockdown *p53*^{-/-} MEFs expressing Ha-RasV12. While Mito-Flag-*p65* was localized at the mitochondria (Figure 3A), the amount of p38 MAPK in the mitochondrial fraction was unexpectedly not

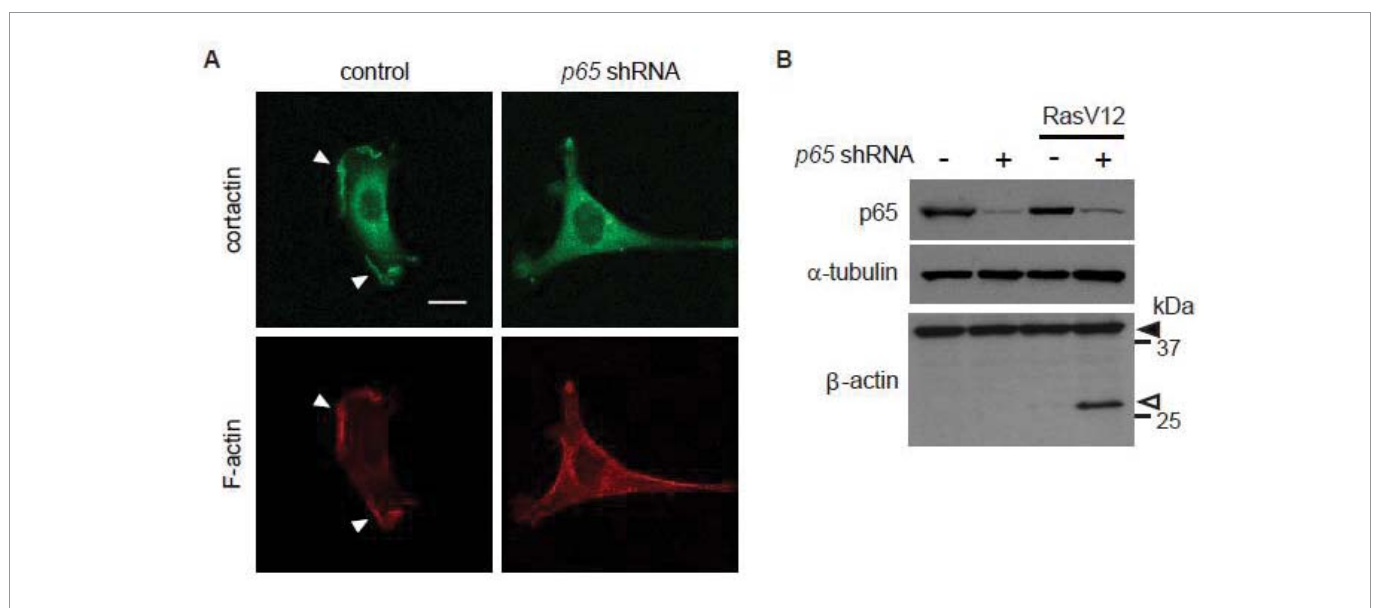


Figure 1: Oncogenic Ras induces β -actin cleavage in *p53*^{-/-} MEFs expressing *p65/RelA* shRNA.

Cells were infected with a control or Ha-RasV12-expressing retrovirus together with or without a *p65/RelA* shRNA-expressing retrovirus. (A) Confocal images of the cells stained for cortactin (green) as an indicator of lamellipodia or F-actin (red) are shown. Scale bars, 20 μ m. Z-stack images with an interval of 1.0 μ m were obtained using a confocal microscope, and projected images are shown. The white arrowheads point to the edge of the lamellipodia. (B) The levels of *p65/RelA* and the cleavage of β -actin were evaluated by immunoblot analysis. Black arrowhead indicate full-length β -actin, and white arrowhead indicate cleaved fragment.

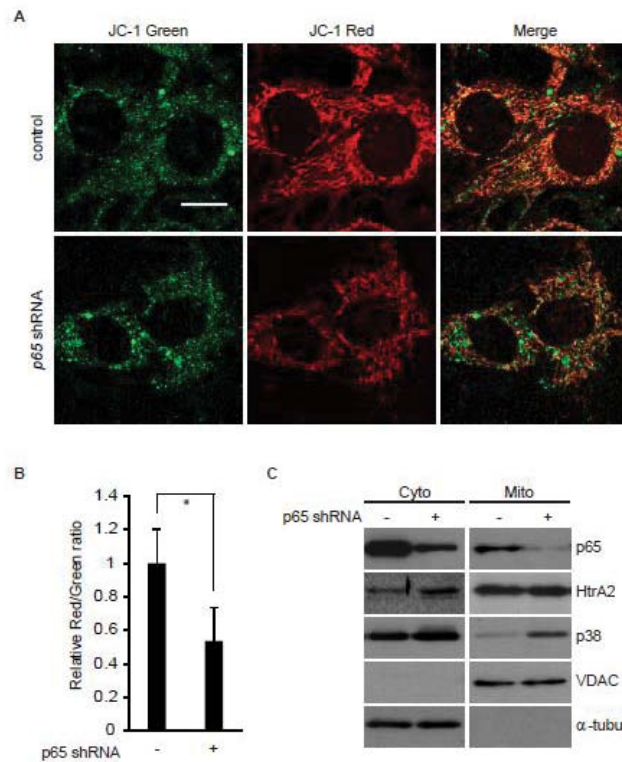


Figure 2: *p65/ RelA* knockdown enhances mitochondrial translocation of p38 MAPK in *p53*^{-/-} MEFs expressing Ha-RasV12. Cells were infected with Ha-RasV12-expressing retrovirus together with or without a *p65/ RelA* shRNA-expressing retrovirus. (A) Confocal images of cells stained with JC-1. Red fluorescence (J-aggregate) and green fluorescence (monomer) are shown. Scale bars, 20 μ m. (B) The ratio of red fluorescence to green fluorescence, which is correlated with mitochondrial membrane potential, at peripheral mitochondria was quantified from images in A. (C) Following subcellular fractionation of the cytosol (Cyto) and mitochondria (Mito), the distribution of *p65/ RelA*, p38 MAPK, and HtrA2/Omi was evaluated by immunoblot analysis. VDAC and α -tubulin were used as mitochondrial and cytosolic markers, respectively.

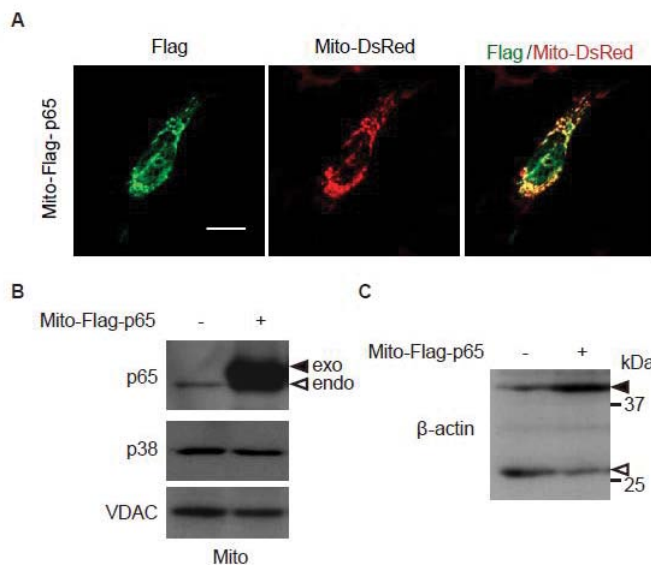


Figure 3: Expression of *p65/ RelA*, localized in the mitochondria, attenuates β -actin cleavage in *p65/ RelA*-knockdown *p53*^{-/-} MEFs expressing Ha-RasV12. Cells were infected with Ha-RasV12-expressing retrovirus together with *p65/ RelA* shRNA-expressing retrovirus. (A) The cells were transfected with Mito-Flag-*p65* expression vector together with Mito-DsRed expression vector to visualize mitochondria. Confocal images of cells stained with anti-Flag antibody for Mito-Flag-*p65* (green) and Mito-DsRed (red) are shown. Z-stack images with an interval of 1.0 μ m were obtained using a confocal microscope, and projected images are shown. Scale bars, 20 μ m. (B) and (C) The cells were transfected with control or Mito-Flag-*p65* expression vector. (B) Following subcellular fractionation of the mitochondria (Mito), the distribution of p38 MAPK was evaluated by immunoblot analysis. VDAC was used as a mitochondrial marker. Black arrows indicate exogenous *p65/ RelA*, and white arrow indicates endogenous *p65/ RelA*. (C) The cleavage of β -actin was evaluated by immunoblot analysis. Black arrowhead indicate full-length β -actin, and white arrowhead indicate the cleaved fragment.



affected by Mito-Flag-*p65* expression (Figure 3B). However, Mito-Flag-*p65* expression decreased β -Actin cleavage (Figure 3C). These results suggest that elimination of oncogenic Ras-induced β -Actin cleavage by mitochondrial NF- κ B is not mediated by the regulation of p38 MAPK translocation into mitochondria. Thus, in addition to its well-established role as a transcriptional factor, NF- κ B regulates proteolysis of the cytoskeletal element under the Ras-transformed condition.

NF- κ B upregulates the expression of integrins α v and β 3 [20-22], which are also involved in lamellipodia formation in *p53*-depleted cells [19]. This together with the results of this study on NF- κ B-dependent repression of β -Actin cleavage, indicates that NF- κ B is likely to play a prominent role in increasing oncogenic Ras-driven tumor cell malignancy by promoting lamellipodia formation.

ACKNOWLEDGEMENTS

We thank Dr. Shota Yamauchi for discussion. This work was supported by JSPS KAKENHI Grant Number 26890024 and Grant for Collaborative Research from Nagoya University (2614Dj-02b).

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