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Senescence-related functional nuclear barrier by down-regulation of nucleo-cytoplasmic trafficking gene expression

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ABSTRACT

One of the characteristic natures of senescent cells is the hypo- or irresponsiveness not only to growth factors but also to apoptotic stress. In the present study, we confirmed the inhibition of nuclear translocation of activated p-ERK1/2 and NF-kB p50 in response to growth stimuli or LPS in the senescent human diploid fibroblasts. In order to elucidate the underlying mechanism for the senescence-associated hypo-responsiveness, we carried out the comparison study for gene expression profiles through microarray analysis. In consequence, we observed the vast reduction in expression of nucleo-cytoplasmic trafficking genes in senescent cells, when compared with those in young cells. Expression levels of several nucleoporins, karyopherin α, karyopherin β, Ran, and Ran-regulating factors were confirmed to be down-regulated in senescent HDFs by using RT-PCR and Western blot methods. Taken together, these data suggest the operation of certain senescence-associated functional nuclear barriers by down-regulation of the nucleo-cytoplasmic trafficking genes in the senescent cells.

Introduction

Senescent human diploid fibroblasts (HDFs) show several characteristic features when compared to young HDFs such as the distinct flat and enlarged morphology [1,2]; appearance of senescence-associated β-galactosidase activity [3]; hypo-responsiveness to growth factors [4,5]; resistance to apoptosis induced by various stimuli [1,6] and broad changes in gene expression [7–9].

Hypo-responsiveness to growth factors is one of the fundamental features of the cellular senescence. In the case of HDFs, senescent cells do not proliferate upon epidermal growth factor (EGF) stimulation, while they have normal levels of EGF receptors and downstream signaling molecules [10]. Intriguingly, we have previously found that the nuclear translocation of p-ERK1/2 in response to EGF stimulation is significantly inhibited in senescent HDFs, though MEK activities increase in the senescent cells [11]. Recent findings show that Erk can bind nuclear carrier proteins to translocate into the nucleus [12]. These findings suggested that the senescence-associated hypo-responsiveness to growth factors could be a result from failure of the nucleo-cytoplasmic trafficking of signaling molecules.

Moreover, in search of the senescence-associated resistant nature to apoptosis *in vitro* and *in vivo* [13,14], we have reported that senescence-dependent nuclear localization of gelsolin [15], overexpression of major vault protein (MVP) [16] and failure of stress-induced down-regulation of Bcl-2 [17], which would be tightly related with the certain defect in regulation of stress signal molecules for apoptosis. Thereby, we conjectured that these phenomena might be ultimately related with senescence-dependent defects in intracellular signal trafficking, especially in the nucleo-cytoplasmic trafficking.

In eukaryotes, many signaling molecules and transcriptional factors shuttle between the cytoplasm and the nucleus to exert their function. This nucleo-cytoplasmic trafficking is a highly organized process carried out by the specialized transport machinery consisting of nuclear pore complexes (NPCs), transport receptors, and Ran, a member of Ras-related GTPase superfamily. The NPC is a large multiprotein complex composed of ~30 different proteins called nucleoporins. It penetrates the nuclear envelope to form a channel, through which molecules are able to shuttle between the cytoplasm and the nucleus [18,19]. Large molecules (usually >40 kDa) are first bound to transport receptors and then actively transported through the NPCs. The transport receptors are called importins and exportins depending on their direction of transport, or collectively called karyopherins. Ran proteins regulate the assembly and disassembly between cargo proteins and karyopherins. After a cargo-importin α-importin β complex enters into the nucleus, the GTP-bound form of Ran (RanGTP) binds to importin β and thereby induces conformational changes in the complex, resulting in the dissociation of cargo proteins from importin α. On the other hand, RanGTP binds exportin β to facilitate the

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assembly between a cargo protein and exportin β [20]. Ran GTP-GDP exchange factor (RanGEF) and Ran GTPase-activating protein (RanGAP) generate a concentration gradient of RanGTP across the nuclear envelope with a high concentration in the nucleus and a low concentration in the cytoplasm.

In the present study, it was attempted to elucidate the mechanism underlying the senescence-associated hypo-responsiveness either to growth factors or to apoptotic stress under the assumption that senescence-associated hypo-responsiveness would be dependent on the reduced efficacy of nucleo-cytoplasmic trafficking.

Materials and methods

Cell culture. Human diploid fibroblasts (HDFs) were isolated from the foreskin of a 4-year-old boy and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a 5% CO_2 incubator. Cells were subcultured serially at a ratio of 1:4. We defined young cells as those resulting from <25 population doublings, and old cells were from >66 population doublings. Cellular senescence of all of the old cells was confirmed by their delayed population doubling times and by a senescence-associated β-galactosidase activity assay (Supplementary Fig. 1A) as described by Dimri et al. [3]. After being grown in a semi-confluent state, senescence-associated β-galactosidase, pH 6.0, activity was examined. Cells were washed with phosphate-buffered saline and fixed with 2% paraformaldehyde containing 0.2% glutaraldehyde in phosphate-buffered saline for 5 min at room temperature. After washing with phosphate-buffered saline, cells were incubated with β-galactosidase reagent (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 40 mm citric acid/sodium phosphate buffer, pH 6.0, 5 mm potassium ferrocyanide/potassium ferricyanide, 150 mm NaCl, 2 mm $MgCl_2$) at 37 °C.

RNA isolation and gene expression profiling. Total RNA was extracted from cultured young and old HDFs using TRIZOL reagent (Invitrogen) and the microarray analysis was performed according to the manufacturer's instruction. Briefly, 300 ng of total RNA for each sample was converted to cDNAs, from which cRNAs were generated by *in vitro* transcription reactions. cDNAs were regenerated and then fragmented by the treatment with uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease (APE 1). The fragmented cDNAs were end-labeled with biotinylated dideoxynucleotides and then hybridized to the GeneChip® Human Gene 1.0 ST arrays (Affymetrix) for 16 h at 45 °C. The chips were stained and washed in a Genechip Fluidics Station 450, and scanned with a Genechip Array scanner 3000 7G (Affymetrix).

Semi-quantitative RT-PCR. Total RNA was extracted and reverse transcribed to cDNAs by Superscript II reverse transcriptase (Invitrogen). Appropriate dilutions of each single-stranded cDNA were prepared for subsequent PCR amplification by monitoring the GAPDH gene as a quantitative control. Then, the cDNAs were subjected to PCR amplification with a specific primer set for each gene (Supplementary Table 1). RT-PCR was performed in the following condition: 42 °C for 1 h, 95 °C for 15 min, 32 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 1 min, and then 72 °C for 10 min. Samples were analyzed by gel electrophoresis and bands were revealed by staining gels with ethidium bromide.

Western blotting. Cells were lysed in a lysis buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, pH 8.0, 1 mM protease inhibitor cocktail (Roche), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM NaF, and 1 mM sodium orthovanadate. Protein samples were resolved by SDS–PAGE and transferred onto nitrocellulose membranes (Schleicher & Schuell Bioscience). The membranes were incubated with primary antibodies for 16 h at 4 °C and then with secondary antibodies for 1 h at RT. The antigen–antibody complexes were detected by enhanced chemiluminescence (Pierce). Antibodies for actin (A5441), Nup153 (sc-101545) and Nup155 (sc-133858) were purchased from Santa Cruz Biotechnology. Polyclonal antibodies against Nup50 (A301–782A) and Nup107 (A301-9579) were purchased from Bethyl Laboratories. Monoclonal antibodies for karyopherin α (61485), karyopherin β (610559), and Ran (610340) were obtained from BD Biosciences.

Immunofluorescence analysis. Cells cultured on glass coverslips were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, and permeabilized with 0.5% Triton X-100 in PBS for 10 min. Nonspecific protein binding sites were then saturated with 2% bovine serum albumin in PBS for 30 min. The cells were then washed with PBS and incubated with FITC conjugated monoclonal antibodies against p-Erk (sc-7383) or Alexa Fluor 488 conjugated recombinant full-length human antibodies against NF-kB p50 (616704, Biolegend). Nuclei were then fluorescently labeled with 4′,6-diamidino-2-phenylindole (DAPI, Sigma). The coverslips were then washed and mounted on glass slides. Fluorescent images were obtained using a confocal microscope.

Electron microscopy. Subconfluent cells were pelletized and fixed with 3% glutaraldehyde/phosphate-buffered saline at pH 7.4. After washing with 0.2 M sodium cacodylate buffer, pH 7.4, cell pellets were treated with 1% osmium tetroxide in a cacodylate buffer for 1 h. The cells were then dehydrated in graded ethanol steps through propylene oxide and embedded in epoxy resin (Polyscience Co.). Ultrathin sections were cut and stained with uranyl acetate and lead citrate. Sections were observed with a transmission electron microscope (JEM1400, JEO USA, Inc.).

Results

Impaired nuclear translocation in senescent human diploid fibroblasts

The nuclear translocation of activated p-ERK1/2 and NF-kB p50 in response to growth stimuli or LPS were inhibited significantly in senescent fibroblasts following accumulation in the cytoplasm (Fig. 1A and B). However, the activation steps of these enzymes were not impaired, because the phosphorylation and activation of ERK1/2 occurs as efficiently in senescent cells as in pre-senescent cells (Supplementary Fig. 1B). The above findings suggested that ERK and NF-kB might be unable to redistribute properly to the nucleus upon activation in senescent conditions.

Microarray analysis of senescence-related repression of nucleo-cytoplasmic trafficking genes

To investigate the mechanism by which senescent cells show hypo-responsiveness, we analyzed gene expression profiles of young and senescent human diploid fibroblasts (HDFs) by using Affymetrix GeneChip® Human Gene 1.0 ST oligonucleotide arrays.

The microarray analysis on 28,869 genes showed that expression of most nucleo-cytoplasmic trafficking genes were down-regulated in senescent HDFs as compared to young HDFs. First, expression levels of most nucleoporin genes decreased in senescent HDFs, including *Nup107*, *Nup155*, *Nup205*, *Nup43*, and *Nup85* (Supplementary Fig. 2, upper panel). Second, expression levels of transport receptor genes decreased in senescent HDFs, including *IPO11* (importin 11), *KPNA2* (karyopherin α2 or importin α1), *KPNB1* (karyopherin β1 or importin β1) and *XPO1* (exportin 1) (Supplementary Fig. 2, middle panel). Third, expression levels of Ran and Ran-regulating factors decreased in senescent HDFs, including *RAN*, *RANBP1* (Ran-binding protein 1), and *RANGAP1* (Ran GTPase-activating protein 1) (Supplementary Fig. 2, lower panel).

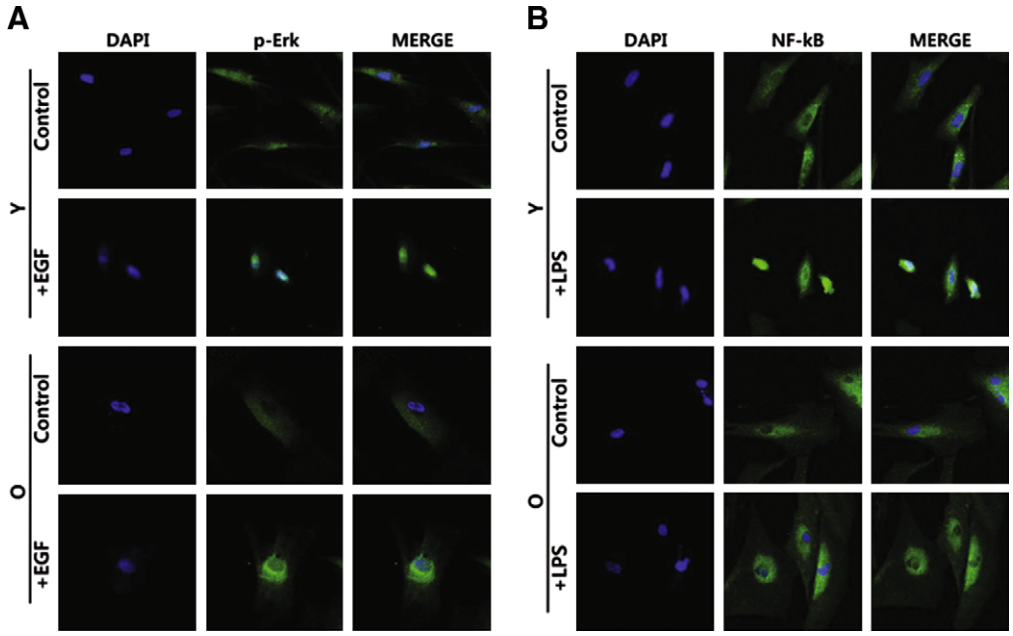

Fig. 1. Impaired nuclear translocation of p-Erk and NF-kB p50 in senescent human diploid fibroblasts. Confocal laser microscopy was used to analyze the subcellular distribution of p-Erk (A) and NF-kB p50 (B). Young (Y) and Old (Y) HDFs were starved of fetal bovine serum and treated with EGF at 10 ng/ml for 15 min (A). Young and senescent HDFs were starved of fetal bovine serum and treated with LPS 10 μmol/L for 30 min (B).

Confirmation of senescence-related repression of nucleo-cytoplasmic trafficking genes

To verify the microarray data, we analyzed expression levels of some selected genes by using semi-quantitative reverse transcription polymerase chain reaction (RT–PCR) method. As shown in Fig. 2A, expression levels of 10 selected nucleoporin genes remarkably decreased in senescent HDFs when compared to young HDFs. We also performed the RT-PCR analysis on several importin genes including *KPNA2* (karyopherin 2 or importin 1) and *KPNB1* (karyopherin 2 or importin 2). The data showed that expression levels of all 6 selected importin genes prominently decreased in senescent HDFs (Fig. 2B). In addition, expression levels of Ran, RanBP1 (Ran-binding protein 1), RanBP5, RanGAP1 (Ran GTPase-activating protein 1), and RanGRF (Ran guanine nucleotide release factor) genes were observed to be markedly decreased in senescent HDFs (Fig. 2C).

We further analyzed expression levels of Nup50, Nup88, Nup107, Nup155, karyopherin α, karyopherin β, and Ran by western blotting with available antibodies. As shown in Fig. 4, protein levels of 7 tested genes prominently decreased in senescent HDFs when compared to young HDFs (Fig. 3).

Taken together, these results demonstrate that expression of nucleo-cytoplasmic trafficking genes is vastly repressed in

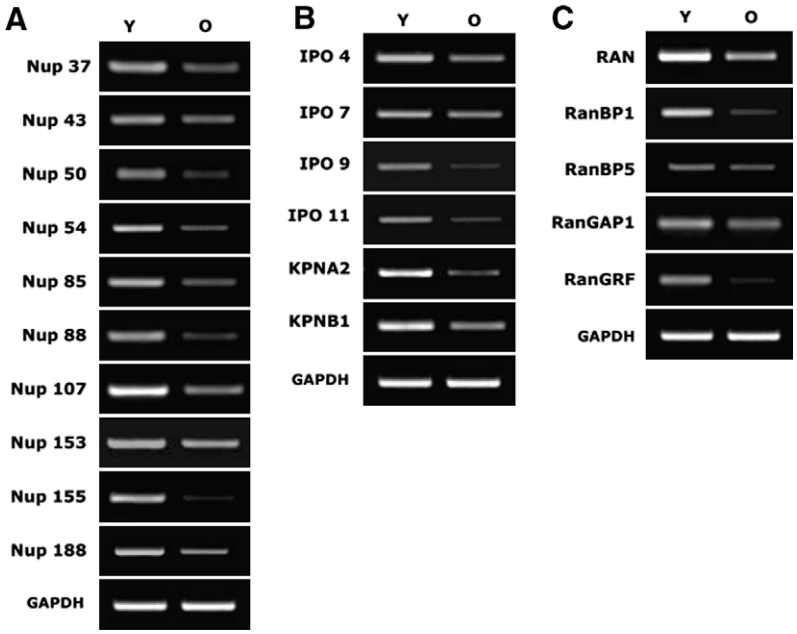

Fig. 2. Decreased mRNA levels of nucleocytoplasmic trafficking genes in senescent HDFs. Total RNA was extracted from young (Y) and senescent (O) fibroblasts, and RT-PCRs were performed for several nucleoporin genes (A), for several transport receptor genes(B), and for Ran and Ran-regulating factor genes.(C). GAPDH was used as a quantitative loading control.

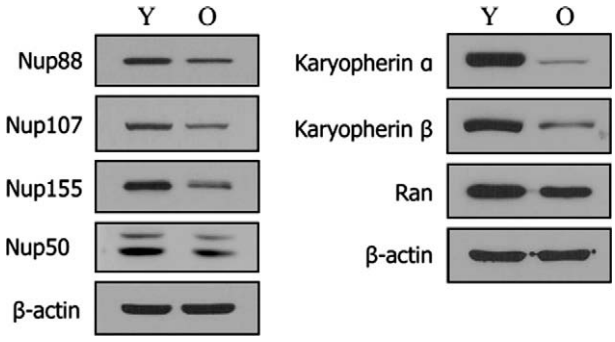

Fig. 3. Decreased protein levels of nucleocytoplasmic trafficking genes in senescent HDFs. Cell lysates were prepared from young (Y) and senescent (O) fibroblasts. Western blotting was performed using antibodies against Nup50, Nup88, Nup107, Nup155, karyopherin a, karyopherin b and Ran. b-actin was used as a loading control.

senescent HDFs, and strongly suggest that the nucleo-cytoplasmic trafficking would be defective in senescent HDFs.

The number of nuclear pore complexes decreases in senescent human diploid fibroblasts

Nucleoporins are essential components of nuclear pore complexes (NPCs). It is assumed that about 500–1000 nucleoporins present per NPC [18,21]. Since our data showed that expression of most nucleoporin genes decreased in senescent HDFs, we checked whether the number of NPCs was down-regulated in senescent fibroblasts.

Electron microscopic analysis was performed and the data showed that the number of NPCs present in the nuclear envelope apparently decreased in senescent HDFs as compared to young HDFs (Fig. 4). This result again confirms that the nucleo-cytoplasmic trafficking is severely impaired in senescent HDFs.

Discussion

Many signaling molecules as well as transcription factors should be imported to the nucleus to exert their own function. For example, upon epidermal growth factor (EGF) stimulation, p-ERK should be imported to the nucleus to activate AP1 transcription factor and thereby to trigger cell cycle progression [22]. Therefore, if the nucleo-cytoplasmic trafficking systems were defective, cells could not properly respond to growth factors. Actually, we have previously found that senescent HDFs might accumulate p-ERK1/2 in the cytoplasm and could not translocate them to the nucleus in response to EGF stimulation [11]. In addition, we have reported the senescence-dependent nuclear accumulation of actin and gelsolin [15].

In addition to the hypo-responsiveness to growth factors, senescent HDFs are resistant to a variety of apoptotic stimuli [1]. Apoptotic response of cells is a well-programmed process requiring intracellular signaling pathways. In this context, we previously illustrated the high levels of major vault protein [16] and the failure of stress-induced down-regulation of Bcl-2 in senescent HDFs [17]. These data also suggested the operation of a certain barrier between the nucleus and the cytoplasm in the senescent cells. Therefore, the senescence-dependent hypo-responsiveness to apoptotic stress as well as to growth factors could be presumed to the hypothetical functional nuclear barrier, operating in the senescent cells. Intriguingly, in the present study, we could confirm some evidences supporting our assumptive senescence-dependent nuclear barriers. The nuclear translocation of activated p-ERK1/2 and NF-kB p50 in response to growth stimuli or LPS was shown to be inhibited significantly in the senescent fibroblasts (Fig. 1A and B), resulting in decrease of the growth factor response as well as the inflammatory response. These findings strongly implicate the possibility of the presence of aging dependent functional nuclear barriers.

The nucleo-cytoplasmic trafficking is a highly sophisticated process involving many specific proteins. Nucleoporins are major components of nuclear pore complexes (NPCs) [23]. NPCs can allow passive diffusion of ions and small molecules, and may facilitate active transport of macromolecules. The cargo molecules are usually equipped with the short sequence elements, such as nuclear localization sequences (NLSs) and nuclear export sequences (NESs). Karyopherin α can bind to NLSs of cargo molecules, while karyopherin β can bind to both karyopherin α and nucleoporins. Thereby, karyopherin β provides the link between cargo-karyopherin α complex and NPCs [20]. Ran plays a critical role in importing and exporting cargoes. The nucleotide state of Ran is regulated by several factors such as Ran GTP–GDP exchange factor (RanGEF or RCC1) and Ran GTPase-activating protein (RanGAP). In the nucleus, RanGEF associates with Ran to maintain its state as RanGTP. In the cytoplasm, RanGAP stimulates the GTPase activity of Ran to maintain its state as RanGDP [24,25]. Therefore, the reduction in expression of those genes related with nucleo-cytoplasmic trafficking might be assumed that the nuclear translocation of the signaling molecules would be impaired.

We here found that expression of the nucleo-cytoplasmic trafficking genes was vastly repressed in senescent HDFs through microarray analysis (Supplementary Fig. 2). In addition, expression levels of various nucleoporins, nuclear receptors, Ran, and Ran-regulating factors were confirmed to be down-regulated in senescent HDFs in RT-PCR and western blot analysis (Figs. 2 and 3). Moreover, the number of nuclear pore complexes on the nuclear membrane was apparently down-regulated in senescent HDFs by electron microscopic analysis (Fig. 4), suggesting the aging depen-

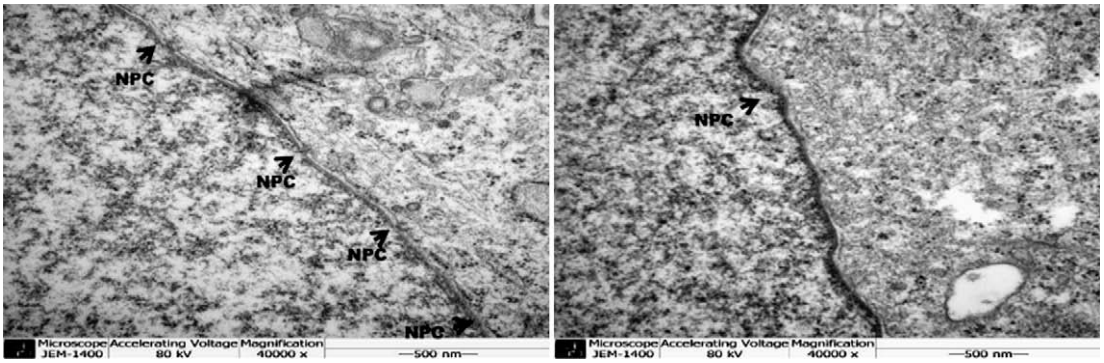

Fig. 4. Decreased number of nuclear pore complexes in senescent HDFs. Scanning electron microscopy reveals nuclear pore complexes (NPCs, arrows) on the nuclear envelope in young (left panel) and senescent fibroblasts (right panel).

dent reduction in formation of nuclear pore complexes. These data strongly support our hypothesis of the functional nuclear barriers for the nucleo-cytoplasmic trafficking and also implicate that the physical structures for nuclear pore complexes could be directly affected by aging dependent down-regulation of those related genes.

Taken together, we have shown here that genes for nucleo-cytoplasmic trafficking of the signaling molecules are vastly down-regulated in the senescent HDFs at the transcriptional level. Our study strongly implicates that the senescence related hypo-responsiveness either to growth factors or to apoptotic stress might be due to the defective nucleo-cytoplasmic trafficking of signaling molecules in the senescent cells. For this defective nucleo-cytoplasmic trafficking, the presence of the hypothetical functional nuclear barrier in the senescent cells could be assumed. Furthermore, the biological significance of this nuclear barrier could be evaluated for survival nature of the senescent cells against the toxic stimuli in sacrifice of growth response. These data might provide the new insight into the mechanism of aging especially for growth arrest and apoptosis resistance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.10.154.

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