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Inhibition of NF-κB stabilizes gadd45α mRNA

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Abstract

Growth arrest- and DNA damage-inducible protein α (gadd45α) is an important regulator for cell cycle, genomic stability, and cell apoptosis. In the present report, we demonstrated that NF-κB inhibition due to Ikkβ deficiency enhanced the stability of gadd45α mNRA. Using embryo fibroblast cells derived from wild type (wt) or Ikkβ gene knockout ($Ikk\beta^{-/-}$) mice, reverse transcription-polymerase chain reaction revealed a three- to fourfold increase of gadd45α mRNA in $Ikk\beta^{-/-}$ cells compared with wt cells. The deficiency in Ikkβ substantially decreased basal NF-κB activity and increased accumulation of reactive oxygen species (ROS). However, such deficiency had no effect on the basal expression or activity of Akt, FoxO3a, p53, and c-myc that regulate the transcription of gadd45α gene positively or negatively. Analysis of gadd45α mRNA stability showed a ROS-dependent increase in the half-life of gadd45α mRNA in $Ikk\beta^{-/-}$ cells. Immunoprecipitation experiments indicated an increased binding of a RNA stabilizing protein, nucleolin, to gadd45α mRNA in $Ikk\beta^{-/-}$ cells. The binding of nucleolin to gadd45α mRNA could be prevented by the antioxidant, *N*-acetyl-cysteine. Thus, these data are the first to suggest that inhibition of Ikkβ-NF-κB signaling up-regulates the expression of gadd45α mNRA through a post-transcriptional, rather than a transcriptional, mechanism.

Keywords: NF-κB; Ikkβ; Gadd45α; mRNA stability; Nucleolin

The nuclear factor-κB (NF-κB) is an important transcription factor involved in the regulation of cell apoptosis, cell cycle or carcinogenic transformation [1]. Several anti-apoptotic genes have been identified as NF-κB target genes, such as cIAP1, cIAP2, xiap, bclx, etc. NF-κB has also been implicated in the regulation of cell cycle transition as demonstrated by the fact that NF-κB stimulates the expression of cyclinD1 gene [2]. We have previously shown that inhibition of NF-κB enhanced arsenic-induced expression of gadd45α, a protein that delays the transition of G2/M phase of the cell cycle, in human bronchial epithelial cells [3]. Recently, studies by Zerbini et al. [4] provide direct evidence indicating that NF-κB is an inhibitory signal for the expression of gadd45α in human cell lines. Thus, these data clearly suggest that NF-κB is a negative regulator for gadd45α. However, it is unclear how NF-κB or its activation signal suppresses the expression of gadd45α.

The regulation of gadd45α gene expression has been extensively studied in the cellular response to DNA damage and a number of other genotoxic stress signals. The expression of gadd45α was originally believed to be p53-dependent. In response to ionizing radiation or other DNA damaging signals, there is an increased binding of p53 to the p53 consensus binding element in the third intron region of the gadd45α gene [5]. Intriguingly,

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a p53-independent regulation of gadd45α expression has also been demonstrated in the cellular response to UV irradiation [6]. Furthermore, the expression of gadd45α can be up-regulated by FoxO3a, an oxidative stress-inducible forkhead transcription factor. Three forkhead response elements (FHRE) were found in the promoter region of the gadd45α gene [7]. Certain DNA damage signals can stimulate the binding of FoxO3a to these FHREs and increase the expression of gadd45α. In addition to these transcriptional activators, two transcriptional repressors, c-myc and ZBRK1, have been demonstrated as negative regulators for the expression of gadd45α [8,9].

It is plausible to assume that the inhibitory effect of NF-κB on the gene expression of gadd45α is through these transcription factors that regulate the transcription of gadd45α either positively or negatively. A cross-talk between the signaling pathways of NF-κB and p53 [10], FoxO3a [11] or c-myc [10] has been previously established. However, the vast majority of these cross-talk studies were performed by using human cancer cell lines in which some key signal molecules were overexpressed. It is unclear, therefore, whether such cross-talk occurs in the cells close to the native condition. To solve this dilemma, we used fibroblast cells derived from wild type (wt) and Ikkβ gene knockout ($Ikk\beta^{-/-}$) mouse embryos, and demonstrated that deficiency in NF-κB signaling enhances expression of gadd45α by stabilizing gadd45α mRNA in an oxidative stress-dependent manner. Inhibition of NF-κB has no effect on the transcription of gadd45α gene.

Materials and methods

Cell culture and preparation of protein extracts. Fibroblast cells derived from wild type (wt) or Ikkβ gene knockout ($Ikk\beta^{-/-}$) mouse embryos were grown in DMEM medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 4.5 g/L glucose, 10 mM Hepes, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C in 95% air, 5% CO_2 in a humidified incubator. Cells were seeded into six-well tissue culture plates and used for experiments when they reached 100% confluence. Fresh medium containing 0.1% FCS was placed on the cells 12 h before experiments. Whole cell extracts were prepared using SDS–PAGE sample buffer and subjected to SDS–PAGE in 8% or 16% gels. Antibodies against gadd45α, Akt, p53, c-myc, FoxO3a, phospho-Akt, and phospho-FoxO3a were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA) or Cell Signaling Technology (Beverly, MA).

RNA isolation and reverse transcription-polymerase chain reaction. Total RNA was extracted from wt and $Ikk\beta^{-/-}$ cells using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's instructions. For analysis of mRNA stability, cells were treated with 5 μg/ml actinomycin D for the indicated time in figure legends before collection of the cells. Immune complexes were used to determine the presence of gadd45α mRNA in the immunoprecipitation using anti-nucleolin antibody. Reverse transcription was conducted at 45 °C for 1 h in a 50-μl reaction with 100 ng of total RNA and the Promega Access reverse transcription-polymerase chain reaction (RT-PCR) system (Madison, WI). PCR products were resolved by 2% agarose gel electrophoresis and visualized by staining with ethidium bromide. PCR primers used for gadd45α and GAPDH were as follows: gadd45α sense, 5′-ATGG CATCCGAATGGAAATA-3′; antisense, 5′-TTCTCGCAGCTTCC TTCTTC-3′; GAPDH sense, 5′-CTGAACGGGAAGCTCACTGGC ATGGCCTTC-3′; antisense, 5′-CATGAGGTCCACCACCCTGT TGCTGTAGCC-3′.

Luciferase reporter gene activity assay. To determine the transcriptional activity of NF-κB, cells were transfected with 2× κB luciferase construct as reported previously [12]. The gadd45α promoter activity was determined by transfection of the cells with a gadd45α promoter luciferase vector in which the −994 to + 26 promoter region of gadd45α gene was inserted into the upstream of luciferase reporter gene.

Measurement of H_2O_2. H_2O_2 released from the cells in the cell culture medium was determined using a quantitative assay kit for H_2O_2 from OXIS (Portland, OR) according to the manufacturer's instructions. This assay is based on the principle that H_2O_2 oxidizes Fe^{2+} to Fe^{3+} that binds to and alters xylenol orange which changes the absorbance at 560 nm. H_2O_2 levels were estimated by comparison with values on a reference curve generated with known amounts of H_2O_2.

Results

Deficiency of Ikkβ impairs basal NF-κB activity and increases expression of gadd45α mRNA

To determine whether the expression of gadd45α was altered in the cells where NF-κB signaling pathway was deficient, we used fibroblast cells derived from wt and $Ikk\beta^{-/-}$ mouse embryos, respectively. Analysis of the expression of the IKK kinase subunit suggested an equal expression of IKKα in both wt and $Ikk\beta^{-/-}$ cells. As expected, the expression of IKKβ can be seen only in wt cells but not in $Ikk\beta^{-/-}$ cells (Fig. 1A). The activity of NF-κB in wt and $Ikk\beta^{-/-}$ cells was monitored by transient transfection of the cells with a κB-dependent luciferase reporter construct. A dramatic decrease of basal NF-κB activity was noted in $Ikk\beta^{-/-}$ cells, indicating that Ikkβ is required for the maintenance of the basal NF-κB activity (Fig. 1B). The expression level of gadd45α was determined by RT-PCR using total RNAs isolated from both wt and $Ikk\beta^{-/-}$ cells. As indicated in Figs. 1C and D, a substantial increase of gadd45α mRNA was observed in $Ikk\beta^{-/-}$ cells. Densitometry scanning indicated that the mRNA level of gadd45α was more than threefold higher in $Ikk\beta^{-/-}$ cells relative to wt cells. In agreement with the data of gadd45α mRNA expression, Western blotting experiment indicated an appreciable elevation of gadd45α protein in $Ikk\beta^{-/-}$ cells (Fig. 1E).

NF-κB inhibition has no effect on the transcriptional regulation of gadd45α

A number of DNA damaging or stress signals induce the expression of gadd45α gene through the activation of FoxO3a or p53 transcription factor [7,13]. The activity of FoxO3a is negatively modulated by Akt kinase

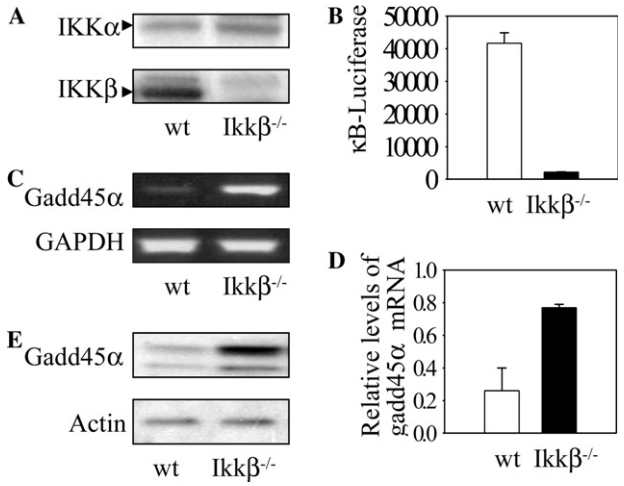

Fig. 1. Ikkβ deficiency reduces NF-κB activation and increases gadd45α mRNA. (A) Immunoblotting for IKKα and IKKβ in wt and Ikkβ$^{-/-}$ cells as indicated. Data are representative of at least five experiments. (B) NF-κB-dependent luciferase activity assay in both wt and Ikkβ$^{-/-}$ cells transfected with a κB-dependent luciferase reporter construct. The luciferase activity was determined 36 h post-transfection. Data are the average of three experiments. The error bar for the luciferase activity in Ikkβ$^{-/-}$ cells is too small to be shown. (C) RT-PCR analysis of gadd45α mRNA in both wt and Ikkβ$^{-/-}$ cells. (D) Quantification of gadd45α mRNA in three RT-PCR experiments by densitometry scanning. (E) Western blotting of Gadd45α (upper panel) and actin (lower panel) protein expression in wt and Ikkβ$^{-/-}$ cells.

that can phosphorylate and sequester FoxO3a in cytoplasm [14]. Thus, it is possible that NF-κB inhibition alters the activity of Akt or FoxO3a, leading to the elevation of gadd45α mRNA in Ikkβ$^{-/-}$ cells. To test this possibility, the activity of Akt and FoxO3a was determined in both wt and Ikkβ$^{-/-}$ cells. A roughly equal phosphorylation status of Akt, an index of Akt activation, was noted between wt and Ikkβ$^{-/-}$ cells (Fig. 2A). This basal activation of Akt might reflect a

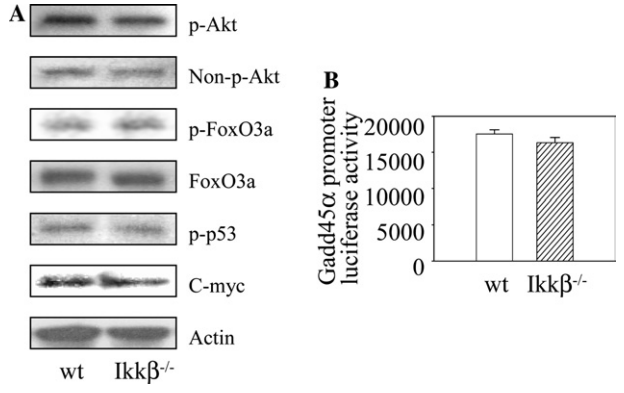

Fig. 2. Ikkβ deficiency does not affect the transcription of gadd45α gene. (A) Total cell lysates were prepared from both wt and Ikkβ$^{-/-}$ cells, and subjected to immunoblotting for phospho-Akt (p-Akt), total Akt, phospho-FoxO3a (p-FoxO3a), total FoxO3a, phospho-p53 (p-p53), c-myc, and actin. Data are representative of three experiments. (B) Promoter activity assay of the cells transfected with a gadd45α promoter luciferase reporter construct ($n = 4$).

highly proliferative nature of both wt and Ikkβ$^{-/-}$ mouse embryo fibroblast cells. In addition, both wt and Ikkβ$^{-/-}$ cells exhibited similar levels of FoxO3a phosphorylation and expression (Fig. 2A). Thus, these data ruled out the possibility that the increased expression of gadd45α in Ikkβ$^{-/-}$ cells is due to the altered Akt signaling that regulates FoxO3a activity.

The tumor suppressor p53 was considered to be a key regulator for the expression of gadd45α [5]. In addition, cross-talk between NF-κB and p53 has been well established previously [10]. Thus, deficiency in Ikkβ-NF-κB may change the expression or activation of p53 that affects the expression of gadd45α. However, our immunoblotting data indicated a similar level of p53 phosphorylation between wt and Ikkβ$^{-/-}$ cells (Fig. 2A). No difference in p53 activity as determined by a p53-based luciferase reporter gene activity assay was observed between wt and Ikkβ$^{-/-}$ cells (data not shown).

C-myc has been previously demonstrated to be a transcriptional repressor for gadd45α gene [8]. Studies by Zerbini et al. [4] suggested that NF-κB inhibition impaired expression of c-myc, leading to the loss of inhibitory signal for gadd45α and the increase of gadd45α gene expression. We have also observed a substantial decrease of arsenic-induced c-myc expression in Ikkβ$^{-/-}$ cells or other NF-κB-inhibited cells (Zhang et al., unpublished). However, there is no appreciable difference in the expression of c-myc under the basal condition between Ikkβ$^{-/-}$ cells and the wt cells (Fig. 2A). Therefore, we conclude that the increased mRNA level of gadd45α is not a result of decrease in c-myc expression in Ikkβ$^{-/-}$ cells.

Finally, we investigated the gadd45α promoter activity by transfection of a gadd45α promoter luciferase construct into both wt and Ikkβ$^{-/-}$ cells. The promoter activity is a reliable indicator for the transcriptional regulation. Again, no significant difference was observed in the promoter activity of gadd45α gene between wt and Ikkβ$^{-/-}$ cells (Fig. 2B).

NF-κB inhibition stabilizes gadd45α mRNA through reactive oxygen species

We have observed an elevation of gadd45α mRNA in Ikkβ$^{-/-}$ cells (Fig. 1) but failed to demonstrate any significant difference in the activation or activity of several transcriptional regulators responsible for the expression of gadd45α between wt and Ikkβ$^{-/-}$ cells (Fig. 2). Thus, these data suggest that NF-κB inhibition elevates gadd45α mRNA through a post-transcriptional mechanism, most notably, alteration of the stability of gadd45α mRNA. To determine whether the rate of gadd45α mRNA turnover was altered in Ikkβ$^{-/-}$ cells, both wt and Ikkβ$^{-/-}$ cells were treated with 5 μg/ml actinomycin D for 0, 1, 2, or 4 h to block further synthesis of mRNA. RT-PCR analysis of the RNA samples collected at different times post-actinomycin D treatment revealed an

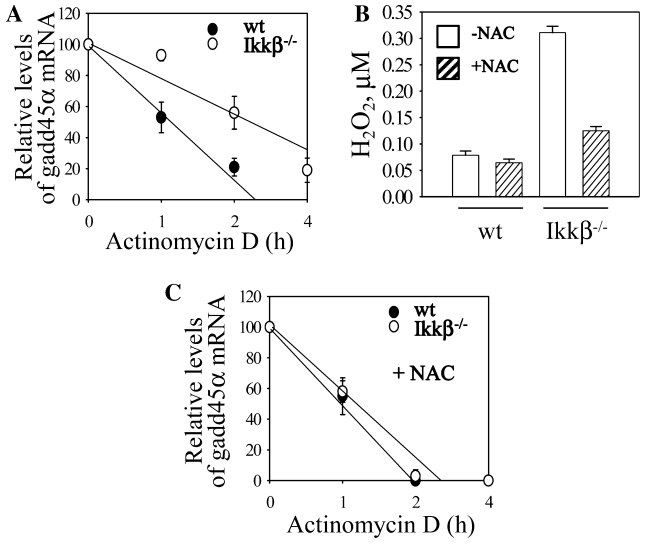

Fig. 3. Ikkβ deficiency stabilizes gadd45α mRNA. (A) Both wt and Ikkβ$^{-/-}$ cells were treated with 5 μg/ml actinomycin D for the indicated time (h). Total RNA was isolated at each time point. The level of gadd45α mRNA was determined by RT-PCR. (B) Colorimetric analysis of H_2O_2 accumulation in wt and Ikkβ$^{-/-}$ cells cultured in the absence or presence of 10 mM NAC for 12 h. The H_2O_2 value in the cell culture medium was determined by measuring the absorbance at 560 nm ($n = 6$). (C) Both wt and Ikkβ$^{-/-}$ cells were pre-treated with 10 mM NAC for 12 h and then treated with actinomycin D for the determination of gadd45α mRNA stability as described in (A).

increase in gadd45α mRNA stability in Ikkβ$^{-/-}$ cells compared with wt cells (Fig. 3A). The half-life of gadd45α mRNA was ~1 h in wt cells and ~3 h in Ikkβ$^{-/-}$ cells.

The next question was why gadd45α mRNA was stabilized in the cells where the basal NF-κB was inhibited due to Ikkβ deficiency. We have previously shown a dramatic increase in basal fluorescent signal in Ikkβ$^{-/-}$ cells after staining of wt and Ikkβ$^{-/-}$ cells with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H(2)DCFDA), a compound that can generate a fluorescent signal after oxidation by H_2O_2, a common reactive oxygen species (ROS) [15]. Oxidative stress induced by ROS has been previously shown to be able to stabilize several mRNAs in either fission yeast or mammalian cells [16–18]. Based on these considerations, we investigated the connection between oxidative stress and the stability of gadd45α mRNA in both wt and Ikkβ$^{-/-}$ cells. The level of H_2O_2 in the cell culture medium was measured directly by a biochemical approach. In agreement with our previous report, a significant level of H_2O_2 was present in the cell culture medium of Ikkβ$^{-/-}$ cells (Fig. 3B). Treatment of the cells with 10 mM *N*-actyl-cysteine (NAC) reduced the level of H_2O_2 as well as the half-life of gadd45α mRNA in Ikkβ$^{-/-}$ cells (Figs. 3B and C).

Increased binding of nucleolin to gadd45α mRNA in Ikkβ$^{-/-}$ cells

In mammalian cells, the stability of mRNA is regulated by a number of RNA binding proteins that either stabilize or destabilize mRNA. Nucleolin, also known as C23, is a RNA binding protein that stabilizes several mRNAs, including IL-2 [19], β-amyloid precursor protein (APP) [20], bcl-2 [21], renin [22], and CD154 [23], by binding to the 5′- or 3′-untranslated region (UTR). Nucleolin can also bind to pre-rRNA that contains the so-called nucleolin recognition element (NRE) with a consensus sequence, (U/G)CCCG(A/G), in a loop of variable size (7–14 nucleotides) and at least a four base-pair stem [24]. The possible binding of nucleolin to gadd45α mRNA in Ikkβ$^{-/-}$ cells was determined by immunoprecipitation of nucleolin from lysates of wt and Ikkβ$^{-/-}$ cells. The presence of gadd45α mRNA in the immune complex was examined by RT-PCR. In wt cells, a residual amount of gadd45α mRNA was detected in the control immunoprecipitation in which the nucleolin antibody was omitted (Fig. 4, lane 1). Inclusion of nucleolin antibody did not increase gadd45α mRNA in the immune complex (Fig. 4, lane 2). In contrast, a threefold increase of gadd45α mRNA was observed in the nucleolin immune complex from Ikkβ$^{-/-}$ cells (Fig. 4, lane 6). Treatment of the cells with 10 or 20 mM NAC decreased the binding of nucleolin to gadd45α mRNA in the Ikkβ$^{-/-}$ cells (Fig. 4, lanes 7

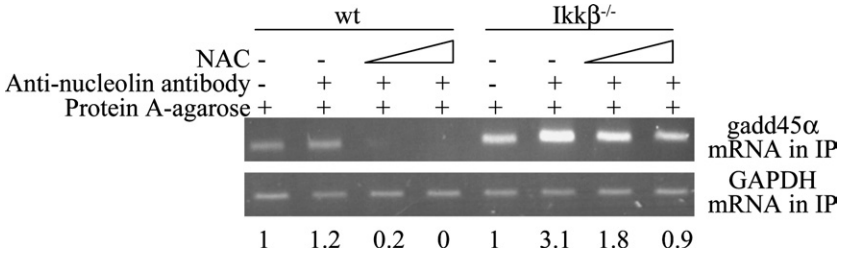

Fig. 4. Ikkβ deficiency enhances the binding of nucleolin to gadd45α mRNA. Both wt and Ikkβ$^{-/-}$ cells were cultured in the absence or presence of 10 (lanes 3 and 7) or 20 mM (lanes 4 and 8) NAC for 12 h. Cell lysates were prepared and subjected to immunoprecipitation using anti-nucleolin antibody. The gadd45α mRNA in the immune complex was determined by RT-PCR. The nucleolin antibody was omitted in the control immunoprecipitation. The numbers at the bottom indicate fold change of gadd45α mRNA compared with the control immunoprecipitation in lanes 1 or 5.

and 8). The presence of gadd45α mRNA in the immunoprecipitation without nucleolin reflected a non-specific association of residual gadd45α mRNA with the protein A–agarose. Such non-specific association could also be observed for the GAPDH mRNA (Fig. 4, bottom panel).

Discussion

In this report, we have observed an elevation of gadd45α mRNA in cells where the basal NF-κB was inhibited due to a deficiency of the Ikkβ gene. The activity or activation of several transcriptional regulators for the gadd45α gene does not appear to be altered by the inhibition of NF-κB. Stability analysis of gadd45α mRNA suggests that inhibition of NF-κB up-regulates gadd45α through a post-transcriptional mechanism which stabilizes gadd45α mRNA. The stabilization of gadd45α mRNA in Ikkβ$^{-/-}$ cells is prevented by the treatment of the cells with NAC that decreases H_2O_2 levels in these cells. A further novel finding is that inhibition of NF-κB appears to be able to increase the binding of the mRNA stabilization protein, nucleolin, to the gadd45α mRNA. The increased binding of nucleolin to gadd45α mRNA is possibly due to oxidative stress, since the antioxidant NAC can prevent such binding.

Nucleolin can stabilize interleukin-2 mRNA in a fashion which is c-jun N-terminal kinase (JNK)-dependent [19]. In human bronchial epithelial cells, we have previously shown that inhibition of NF-κB due to overexpression of a kinase-mutated IKKβ enhanced arsenic-induced JNK activation and gadd45α [3]. It is unclear whether the increased binding of nucleolin to gadd45α mRNA in Ikkβ$^{-/-}$ cells is also dependent on the activation of JNK. Although a prolonged JNK activation induced by arsenic was observed in Ikkβ$^{-/-}$ cells, neither wt cells nor Ikkβ$^{-/-}$ cells showed detectable activation of JNK under basal conditions [15]. Thus, it is unlikely that the binding of nucleolin to gadd45α mRNA is a result of altered basal JNK activation in Ikkβ$^{-/-}$ cells.

The binding sequence of nucleolin to the 5′- or 3′-UTR of IL-2, APP, bcl-2, or CD154 mRNA has been identified [19–21,23]. However, there is little sequence similarity between the UTR region of gadd45α and the UTR regions of IL-2, APP, bcl-2, or CD154. Interestingly, prediction of the secondary structure of the 5′-UTR of mouse gadd45α pre-mRNA revealed a potential stem–loop with a sequence, GCCCGG, in the loop that is similar to the nucleolin recognition element, (T/G)CCCG(A/G), in pre-rRNA [24]. Such a structural characteristic can also be found in both 5′- and 3′-UTR of human gadd45α mRNA. We are currently investigating whether nucleolin binding to gadd45α mRNA is through this region or via interaction with other RNA binding proteins.

Acknowledgments

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