C-terminal region of GADD34 regulates eIF2 α dephosphorylation and cell proliferation in CHO-K1 cells

Ryo Otsuka¹ · Nagakatsu Harada² · Shouhei Aoki² · Kanna Shirai² · Kazuchika Nishitsuji³ · Ayane Nozaki² · Adzumi Hatakeyama² · Masayuki Shono⁴ · Noriko Mizusawa⁵ · Katsuhiko Yoshimoto⁵ · Yutaka Nakaya² · Hiroshi Kitahata¹ · Hiroshi Sakaue²

Received: 26 May 2015 / Revised: 5 August 2015 / Accepted: 10 August 2015 / Published online: 30 August 2015
© Cell Stress Society International 2015

Abstract GADD34 is a member of a growth arrest and DNA damage (GADD)-inducible gene family. Here, we established a novel Chinese hamster ovary (CHO)-K1-derived cell line, CHO-K1-G34M, which carries a nonsense mutation (termed the Q525X mutation) in the GADD34 gene. The Q525X mutant protein lacks the C-terminal 66 amino acids required for GADD34 to bind to and activate protein phosphatase 1 (PP1). We investigated the effects of GADD34 with or without the Q525X mutation on the phosphorylation status of PP1 target proteins, including the α subunit of eukaryotic initiation factor 2 (eIF2 α) and glycogen synthase kinase 3 β (GSK3 β). CHO-K1-G34M cells had higher levels of eIF2 α phosphorylation compared to the control CHO-K1-normal cells both in the presence and absence of endoplasmic reticulum stress. Overexpression of the wild-type GADD34 protein in CHO-K1-normal cells largely reduced eIF2 α phosphorylation,

while overexpression of the Q525X mutant did not produce similar reductions. Meanwhile, neither wild type nor Q525X mutation of GADD34 affected the GSK3 β phosphorylation status. GADD34 also did not affect the canonical Wnt signaling pathway downstream of GSK3 β . Cell proliferation rates were higher, while expression levels of the cyclin-dependent kinase inhibitor p21 were lower in CHO-K1-G34M cells compared to the CHO-K1-normal cells. The GADD34 Q525X mutant had a reduced ability to inhibit cell proliferation and enhance p21 expression of the CHO-K1-normal cells compared to the wild-type GADD34 protein. These results suggest that the GADD34 protein C-terminal plays important roles in regulating not only eIF2 α dephosphorylation but also cell proliferation in CHO-K1 cells.

Keywords CHO-K1 cell · GADD34 · eIF2 α · GSK3 β · Dephosphorylation · Proliferation

✉ Nagakatsu Harada
nharada@tokushima-u.ac.jp

¹ Department of Dental Anesthesiology, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15, Kuramoto-cho, Tokushima City 770-8504, Japan

² Department of Nutrition and Metabolism, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15, Kuramoto-cho, Tokushima City 770-8503, Japan

³ Department of Molecular Pathology, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15, Kuramoto-cho, Tokushima City 770-8503, Japan

⁴ Support Center for Advanced Medical Sciences, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15, Kuramoto-cho, Tokushima City 770-8503, Japan

⁵ Department of Medical Pharmacology, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15, Kuramoto-cho, Tokushima City 770-8504, Japan

Introduction

GADD34 is a member of a growth arrest and DNA damage (GADD)-inducible gene family (Fornace et al. 1989; Hollander et al. 1997). A mammalian GADD34 consisting of 590 amino acids was first cloned from UV-exposed Chinese hamster ovary (CHO) cells (Fornace et al. 1989). Subsequently identified human, rat, and mouse GADD34s were found to be composed of 674, 577, and 657 amino acids, respectively (Hollander et al. 1997; Su et al. 2005). GADD34 expression in mammals is known to be increased by DNA damage, nutrient deprivation, and other various cellular stresses, including endoplasmic reticulum (ER) stress (Fornace et al. 1989; Novoa et al. 2001, 2003). GADD34 can bind many different proteins, such as SNF5, HRX, binding factor of a type 1 collagen promoter (BFCOL1), translin, and inhibitor 1 to modulate

intracellular signaling in stressed cells (Hollander et al. 2003). Moreover, Hasegawa et al. found that GADD34 facilitates the transcription of p21, a cyclin-dependent kinase inhibitor, by inhibiting BFCOL1-dependent suppression of the p21 promoter (Hasegawa et al. 1999). On the other hand, cellular stresses such as ER stress or amino acid deprivation enhance Ser51 phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2 α) in mice to suppress protein synthesis (Brush et al. 2003; Novoa et al. 2001). A study by Novoa et al. revealed that the C-terminal region of mouse GADD34 could bind to the catalytic subunit of protein phosphatase 1 (PP1) (Novoa et al. 2001). The resulting GADD34-PP1 complex dephosphorylates eIF2 α , which, in turn, relieves the repression of protein synthesis (Kojima et al. 2003; Novoa et al. 2003). GADD34 expression has also been reported to induce apoptosis (Hollander et al. 1997), thus suggesting a role for GADD34 in both cellular proliferation suppression and cell senescence promotion (Minami et al. 2007).

In 1997, Su et al. cloned a GADD34-like protein, progression elevated gene-3 (PEG-3), from E11 cells, a mutant adenovirus type 5-transformed rat embryo cell clone (Su et al. 1997). The PEG-3 gene is identical to rat GADD34, but a single-base deletion just after the sequential exons that encode the first 415 amino acids produces a frameshift mutation that generates a premature termination codon, which leads to the production of a 457-amino acid GADD34 protein lacking the C-terminal 162 amino acids of the wild-type version (Su et al. 1997; Su et al. 2005). A PEG-3-like deletion of the GADD34 protein C-terminal region might frequently occur during the process of transformation and tumor progression in rat cells, since several transcripts with a single-base deletion or insertion in the open reading frame of the GADD34 gene, all of which produce a premature termination codon, were also obtained from several rat tumor cell lines and immortalized rat embryo fibroblasts (Su et al. 2005). Exogenously overexpressed PEG-3 indeed acts as a dominant negative of GADD34-induced growth inhibition (Su et al. 2005). However, another report showed that GADD34-mediated colony suppression was not inhibited by an exogenously overexpressed C-terminally truncated GADD34 protein (Hollander et al. 2003). Thus, loss of the GADD34 protein C-terminus itself might be closely associated with tumor progression. On the other hand, several other reports found that the GADD34 N-terminal region also contributes to tumor cell growth inhibition and apoptosis (Adler et al. 1999; Hollander et al. 2003).

Here, we cloned and established a novel CHO-K1-derived cell line, which we refer to as CHO-K1-G34M. A nonsense mutation in the GADD34 gene (a C to T mutation at Gln525 residue produces a premature termination codon, which is termed the Q525X mutation in this study) is present in the CHO-K1-G34M cells that resulted in the deletion of the GADD34 C-terminal 66 amino acids, which contain an

RARA sequence that is a critical domain for binding of GADD34 to PP1 (Brush et al. 2003). Nearly 200 proteins, including eIF2 α , have been shown to interact with PP1 within cells (Bollen et al. 2010). Another PP1-dependent protein that might contribute to cell growth is glycogen synthase kinase 3 (GSK3), which is involved in various cellular signaling pathways, including the insulin signaling pathway and canonical Wnt signaling pathway (Beurel et al. 2015; Lee and Kim 2007). In some signaling pathways, GSK3 activity is inhibited by phosphorylation at its N-terminus (at Ser21 for GSK3 α and at Ser9 for GSK3 β), while dephosphorylation of these residues reverses this inhibition (Hernández et al. 2010; Sutherland et al. 1993). In mammalian cells, PP1 reportedly serves as a phosphatase for GSK3 β , but not GSK3 α (Hernández et al. 2010). Thus, an interaction between GADD34 and PP1 might affect the PP1-mediated dephosphorylation of GSK3 β , although there are few reports concerning the role of GADD34 in GSK3 β dephosphorylation. Here, we examined the effects of GADD34 with or without its PP1 binding domain on phosphorylation of eIF2 α and GSK3 β , as well as on cell proliferation, of the CHO-K1-derived cell line.

Materials and methods

Cell culture

CHO-K1 cells with (CHO-K1-G34M) or without (CHO-K1-normal cells) the Q525X mutation in the GADD34 gene were cultured in Nutrient Mixture F-12 Ham medium (Sigma, St. Louis, MO, USA) containing 10 % fetal bovine serum (FBS) with 1 % penicillin and streptomycin. In some experiments, cells were washed once with phosphate-buffered saline (PBS) and incubated in F-12 Ham medium without FBS and antibiotics but with 300 nM thapsigargin or vehicle for 6 or 24 h. Thapsigargin was dissolved in dimethyl sulfoxide (DMSO; final DMSO concentration in F-12 Ham medium was 0.01 %).

CHO-K1 cell genotyping

Genomic DNA was isolated from the CHO-K1 cells using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma). A DNA fragment encoding a part of the GADD34 gene was amplified by PCR using the PrimeSTAR® GXL DNA polymerase (TaKaRa, Kyoto, Japan). The gene-specific primers used for PCR were as follows: forward, 5'-AAGGCTGTGTTCCACCCTGTTG-3' (complementary to intron 2), and reverse 5'-ACGACTTGGGTTTAGCCACG-3' (complementary to exon 3). PCR products were electrophoresed and purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). DNA sequencing of

the PCR products around the nucleotides encoding amino acid 525 was conducted using a BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA).

Plasmids

Complementary DNA (cDNA) fragments encoding the GADD34 gene open reading frame were amplified by reverse transcription PCR (RT-PCR) using RNA isolated from normal CHO-K1 cells or CHO-K1-G34M cells. The gene-specific primers were as follows: forward, 5'-GGATCC-AGACACATGGCCCCAAGCCC-3' (the *Bam*HI restriction site is underlined), and reverse, 5'-ACGACTTGGGTTTAGCCACG-3'. The PCR products were subcloned into the pCR[®]-Blunt II TOPO[®] vector (Invitrogen, Carlsbad, CA, USA), and sequence-confirmed cDNA fragments were isolated by restriction enzyme digestion and inserted into the multicloning site of the pBApo-CMV Pur vector (TaKaRa). The expression plasmid encoding the GADD34 protein (wild type or Q525X mutant) with a FLAG tag at the N-terminus was constructed as follows. The DNA sequence for the FLAG peptide was obtained by annealing two oligonucleotides (the FLAG-1 set; Nakagawa et al. 2012). The FLAG sequence was then inserted in-frame into a *Bam*HI site of each GADD34-encoding plasmid. The pGL4.49 [luc2P/TCF-LEF RE/Hygro] plasmid, which contains eight copies of a TCF-LEF response element upstream of a firefly luciferase reporter gene, was purchased from Promega (Madison, WI, USA). A pGL4.49 vector without the TCF-LEF response element (pGL4.49-empty) was generated by digesting the pGL4.49 [luc2P/TCF-LEF RE/Hygro] vector with *Eco*53kI and *Eco*RV, followed by self-ligation of the resulting blunt-ended fragment.

Quantitative real-time PCR

Total RNA was extracted from CHO-K1 cells with TRIzol[®] reagent (Invitrogen). In some experiments, CHO-K1-normal cells grown in 35-mm dishes were transiently (24 h) transfected with 2.0 μ g pBApo-CMV Pur plasmid with or without the GADD34 cDNA insert using Lipofectamine[®] 2000 (Invitrogen) and used for RNA extraction. RNA was then reversely transcribed using TaKaRa PrimeScript[®] RT reagent kits (TaKaRa). Quantitative real-time PCR was performed with the LightCycler[®] system (Roche Diagnostics, Tokyo, Japan) using TaKaRa SYBR[®] Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa), and the gene-specific primers are listed in Table 1. Expression levels were normalized relative to the level of 18S ribosomal RNA (the primer sequences are shown in Table 1) in each sample and are given in arbitrary units.

Table 1 Primer sets used for PCR

Target	Primer sequence
GADD34	Forward: 5'-GAGAGAAGCCAGCACCACCT-3' Reverse: 5'-CCCAGACAGCAAGGAAATGG-3'
p21	Forward: 5'-CTGTCGCTGCTCTGCACTCT-3' Reverse: 5'-GGCACATTAGGGCTTCCTCTT-3'
Cyclin D1	Forward: 5'-AGTGCCTGCAGAGGGAGATT-3' Reverse: 5'-TAGGGGCTCCAGAGACAGGA-3'
18S Ribosomal RNA	Forward: 5'-AAACGGCTACCACATCCAAG-3' Reverse: 5'-GGCCTCGAAAGAGTCTCTGTA-3'

Protein extraction and immunoblotting

CHO-K1 cells grown in 35-mm dishes were transiently transfected with 2.0 μ g pBApo-CMV Pur plasmid with or without the GADD34 cDNA insert using Lipofectamine[®] 2000 (Invitrogen). After 24 h, the cells were treated with thapsigargin (300 nM) or vehicle (DMSO) as described above. In some experiments, untransfected CHO-K1 cells were used. Cells were lysed in RIPA buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 1 % Nonidet P40, 2 mM EDTA, 50 mM NaF, 50 mM β -glycerophosphate, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were then passed through a 21-gauge needle followed by centrifugation at 4 °C for 30 min at 13,500g. The supernatant was used as a cell protein sample. The protein concentration of the samples was measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of sample proteins were denatured by boiling in SDS sample buffer containing 1 % β -mercaptoethanol. The proteins were then electrophoresed on an SDS-polyacrylamide gel and transferred onto an Immobilon-P membrane (Millipore, Bedford, MA, USA). The membrane was blocked in 5 % skim milk in Tris-buffered saline containing 0.05 % Tween 20 for 1 h and incubated with either rabbit anti-phospho eIF2 α (Ser51) polyclonal antibody (#9721; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-eIF2 α polyclonal antibody (#9722; Cell Signaling Technology), rabbit anti-GAPDH polyclonal antibody (#sc-25778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-ATF4 (CREB-2) polyclonal antibody (#sc-200; Santa Cruz Biotechnology), rabbit anti-calnexin polyclonal antibody (#sc-11397; Santa Cruz Biotechnology), rabbit anti-phospho GSK3 β (Ser9) polyclonal antibody (#5558; Cell Signaling Technology), mouse anti-GSK3 β monoclonal antibody (#9832; Cell Signaling Technology), mouse anti-cyclin D1 monoclonal

antibody (#14561A; Pharmingen, San Diego, CA, USA), rabbit anti-cyclin B1 polyclonal antibody (#4138; Cell Signaling Technology), rabbit anti-p21 polyclonal antibody (#sc-397; Santa Cruz Biotechnology), or mouse anti-DYKDDDDK tag (FLAG tag) monoclonal antibody (018-22381; Wako, Osaka, Japan). Proteins were then visualized with an anti-rabbit (#458) or anti-mouse (#330) IgG horseradish peroxidase-conjugated secondary antibody (Medical & Biological Laboratories Co., Ltd., Aichi, Japan) using a Clarity™ western ECL substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Luciferase assay

CHO-K1 cells grown on 24-well plates were transiently transfected with 0.30 µg of plasmid DNAs containing 0.05 µg pGL4.49 luciferase reporter plasmid, 0.20 µg pBapo-CMV Pur plasmid with or without a GADD34 cDNA insert, and 0.05 µg of the β-galactosidase (β-gal) expression vector pCMV-β (Clontech, Palo Alto, CA, USA) using Lipofectamine® 2000 (Invitrogen). After 24 h, cells were harvested in the lysis buffer supplied with the luciferase assay kit (Promega) and the lysates were assayed for luciferase activity as recommended in the manufacturer's protocol. β-Gal activity was assayed and used for normalization of transfection efficiency as described previously (Harada et al. 2008).

Proliferation assay

CHO-K1 cells were seeded at 5×10^4 cells/60-mm dish. After 24, 48, or 72 h, cells ($n=3$ for each time point) were trypsinized, precipitated, and resuspended in PBS containing 0.2 % trypan blue. The number of live cells in suspension was counted in triplicate using a Luna™ automated cell counter (Logos Biosystems, Annandale, VA, USA), and the results were used to calculate the absolute cell number in the dish.

Statistical analysis

Data are expressed as the means±SD and were analyzed by Student's *t* test (with Holm's corrections for multiple comparisons). A *P* value <0.05 was considered to be statistically significant.

Results

Cloning of the CHO-K1-G34M cell line

GADD34 gene cDNA was cloned using an RT-PCR method using RNA from CHO-K1 cells that had been frozen in a single vial for decades in our laboratory. DNA sequencing of the cloned cDNA revealed that all resulting clones had a

nonsense mutation at Gln525 (from CAG in wild type to TAG in the mutant at this residue produces a premature termination codon, which is termed the Q525X mutation in this study). Sequencing of a PCR fragment pool derived from genomic DNA from this cell population also showed the C to T mutation (i.e., the Q525X mutation) without any doubled peaks at each nucleotide position (Fig. 1a). Single-cell cloning from this cell population, termed CHO-K1-G34M, was carried out using 96-well plates, and two lines (line 1 and line 2) of the CHO-K1-G34M cells were obtained. For both lines, DNA sequencing of a pool of PCR fragments derived from genomic DNA again showed the C to T mutation (the Q525X mutation) without any doubled peaks as described above. The *normal* CHO-K1 cells (termed CHO-K1-normal in this study) without the GADD34 Q525X mutation were derived from another frozen stock and used here as a control (Fig. 1a).

Protein structures of the wild-type and mutant GADD34

The wild-type GADD34 protein in hamster has 590 amino acids (Novoa et al. 2001), with a region containing repetitive (3.5 repeats) amino acid sequences located between residues 279 to 415 (Fig. 1b). The KVHF motif and RARA sequence, which are both reportedly essential for PP1 binding and eIF2α dephosphorylation (Brush et al. 2003), are located between residues 505 and 508, and 562 and 565, respectively. The predicted Q525X mutant of the GADD34 protein derived from CHO-K1-G34M cells lacks the C-terminal 66 amino acids that contain the RARA sequence (Fig. 1b).

GADD34 expression in normal and mutant CHO-K1 cells

GADD34 messenger RNA (mRNA) expression levels were compared between CHO-K1-normal and CHO-K1-G34M cells. The mRNA level was significantly lower in CHO-K1-G34M line 2 cells relative to CHO-K1-normal cells in the absence of thapsigargin treatment (Fig. 2a). However, in CHO-K1-G34M line 1 cells, no such significant reduction in GADD34 mRNA levels was seen. ER stress induced by chemical inducers such as thapsigargin has been previously shown to enhance GADD34 mRNA levels in mammalian cells (Kojima et al. 2003). Consistent with this finding, thapsigargin increased GADD34 mRNA levels in the CHO-K1-normal and two CHO-K1-G34M cell lines to similar levels (Fig. 2a). We next detected GADD34 at the protein level by immunoblotting. A series of commercially available anti-GADD34 antibodies and an original anti-GADD34 antibody that was designed and produced in our laboratory were used in preliminary experiments. However, these antibodies failed to detect clearly either the wild-type or Q525X GADD34 protein both at endogenously expressed levels (using protein samples derived from CHO-K1-normal and CHO-K1-G34M cells with or without thapsigargin treatment)

Fig. 1 **a** Structure of hamster GADD34 cDNA. Boxes indicate exons, and the locations of the translational initiation (ATG) and termination (stop) codons are indicated. Part of the original sequencing data for exon 3 in genomic DNA is shown for CHO-K1-normal and CHO-K1-G34M cells. Gln525 in CHO-K1-normal cells is indicated as Q⁵²⁵. In CHO-K1-G34M cells, a C to T mutation at this residue produces a

premature termination codon, which is indicated as X (termed Q525X mutation in this study). **b** Structure of hamster GADD34 proteins. The amino acid (AA) numbers indicate the locations of a repetitive region (3.5 repeats), KVHF motif, and RARA sequence. The Q525X mutant GADD34 protein in CHO-K1-G34M cells lacks the C-terminal 66 amino acids that contain the RARA sequence

and at exogenously overexpressed levels (using protein samples derived from CHO-K1-normal cells that had been transfected with a GADD34 expression plasmid) (data not shown).

Detection of FLAG-tagged GADD34 protein in CHO-K1 cells

CHO-K1-normal cells were transfected with an expression plasmid encoding N-terminally FLAG-tagged wild-type or Q525X mutant GADD34 protein. As shown in Fig. 2b, immunoblotting with an anti-FLAG tag antibody successfully detected the exogenously expressed wild-type and Q525X mutant GADD34 proteins.

GADD34 is required for eIF2 α dephosphorylation

As described above, the Q525X mutant GADD34 protein lacks the RARA motif that was previously predicted to be necessary for eIF2 α dephosphorylation (Fig. 1b). To confirm that this mutation affects eIF2 α phosphorylation levels, we first compared the phosphorylation status of eIF2 α between CHO-K1-normal and CHO-K1-G34M cells. As shown in Fig. 3a, the amount of phosphorylated eIF2 α was higher in the two CHO-K1-G34M lines relative to CHO-K1-normal cells under vehicle (DMSO)-treated conditions. The levels of ATF4 protein, the expression of which is post-translationally upregulated by phosphorylated eIF2 α

(Harding et al. 2000), increased in a similar fashion in the CHO-K1-G34M cells (Fig. 3a). Meanwhile, treatment of the CHO-K1-normal cells with thapsigargin largely increased both eIF2 α phosphorylation and ATF4 protein levels. The levels of phosphorylated eIF2 α in the presence of thapsigargin were higher in the CHO-K1-G34M cells compared to the CHO-K1-normal cells (Fig. 3a). Expression levels of other proteins such as calnexin and GAPDH were not increased in CHO-K1-G34M cells. Figure 3b shows the effect of exogenous overexpression of the wild-type GADD34 protein on the phosphorylation status of eIF2 α in each CHO-K1 strain. Overexpression of wild-type GADD34 decreased eIF2 α phosphorylation levels in CHO-K1-normal cells as well as CHO-K1-G34M cells (Fig. 3b). Next, we compared the functional ability of the Q525X GADD34 protein to promote eIF2 α dephosphorylation relative to wild-type protein. Overexpression of wild-type GADD34 largely decreased eIF2 α phosphorylation levels even in the presence of thapsigargin (Fig. 3c). While GADD34 Q525X mutant overexpression slightly decreased eIF2 α phosphorylation both in the presence and absence of thapsigargin treatment, the phosphorylation levels were still higher than those seen in cells transfected with wild-type GADD34 (Fig. 3c). Together, these results suggest that GADD34 is required for eIF2 α dephosphorylation in CHO-K1 cells and that GADD34 Q525X expressed in the CHO-K1-G34M cells has a reduced ability to affect eIF2 α dephosphorylation compared to the wild-type GADD34 protein.

Fig. 2 **a** GADD34 mRNA expression levels in CHO-K1-normal cells (indicated as *normal*) and the two (*line 1* and *line 2*) lines of CHO-K1-G34M (indicated as *G34M*). Cells were incubated in F-12 Ham medium including 300 nM thapsigargin (*TG*) or vehicle (DMSO, indicated as *D*) for 24 h. Total RNA was then isolated, reversely transcribed, and subjected to quantitative real-time PCR as described in the “Materials and methods” section. Data are expressed as the means $\pm$ SD of five culture dishes (35-mm dishes) for each group. Each mRNA expression level was normalized relative to the expression of 18S ribosomal RNA (*rRNA*) in each group. The mean value for the CHO-K1-normal cells with DMSO treatment is designated as 1. * P <0.05; † P <0.05, when compared with CHO-K1-normal cells with DMSO treatment. **b** Detection of FLAG-tagged GADD34 protein in CHO-K1-normal cells. Cells were transfected with a plasmid expressing either N-terminal FLAG-tagged wild-type (*WT*) or Q525X mutant GADD34 or an empty vector (*vec*). After 24 h, protein samples were prepared from each group for immunoblotting

GADD34 did not affect the phosphorylation status of GSK3 β

Since GADD34 participates in regulating cell proliferation, the effect of GADD34 on the phosphorylation status of GSK3 β (Ser9) was examined. As shown in Fig. 4a, the basal levels of GSK3 β phosphorylation were similar for the CHO-K1-normal and two CHO-K1-G34M cell lines. In addition, overexpression of wild-type GADD34 did not affect GSK3 β phosphorylation in all three cell strains examined (Fig. 4b, c). Overexpression of the Q525X mutant protein also had little effect on the phosphorylation status of GSK3 β in CHO-K1-normal cells (Fig. 4c). We also examined the effect of

GADD34 on the Wnt signaling pathway. In the canonical Wnt signaling pathway, GSK3 phosphorylates β -catenin, which promotes its ubiquitin-dependent degradation (Lee and Kim 2007). When GSK3 activity is inhibited by stimuli such as Wnt, β -catenin can evade phosphorylation and be translocated to the nucleus to form a complex with TCF-LEF transcription factors that accelerates expression of cell cycle-driving genes such as cyclin D1 (Lee and Kim 2007; Shtutman et al. 1999). In the Wnt signaling pathway, GSK3 β is thought to remain in an unphosphorylated state regardless of whether Wnt stimulation occurs (Ding et al. 2000). On the other hand, Ser phosphorylation is required for the regulation of several downstream Wnt signaling pathway proteins, including APC, axin, and β -catenin (Lee and Kim 2007). Here, we transfected CHO-K1 cells with the Wnt signaling reporter plasmid, pGL4.49 [luc2P/TCF-LEF RE/Hygro], which encodes TCF-LEF response elements upstream of a firefly luciferase reporter gene. For the CHO-K1-normal and two CHO-K1-G34M cell lines, luciferase activities were similar (Fig. 4d). Furthermore, overexpression of wild-type GADD34 had no significant effect on the luciferase activity in CHO-K1-normal cells (Fig. 4e). These results suggest that GADD34 does not function as a regulator of GSK3 β and Wnt signaling in CHO-K1 cells.

CHO-K1-G34M cells proliferated more rapidly than CHO-K1-normal cells

Cell proliferation rates for the CHO-K1-normal and two CHO-K1-G34M cells over 72 h were next examined. We found that both CHO-K1-G34M cell lines proliferated more rapidly than control CHO-K1-normal cells (Fig. 5a). Moreover, expression levels of the cyclin-dependent kinase inhibitor p21 were decreased in the CHO-K1-G34M cells compared to CHO-K1-normal cells (Fig. 5b, d). The expression levels of cyclin D1, an important regulator of cell cycle progression, were unchanged (in line 1) or decreased (in line 2) in the CHO-K1-G34M cells compared to CHO-K1-normal cells (Fig. 5c, d). The expression levels of cyclin B1 were also not increased in the CHO-K1-G34M cells (Fig. 5d).

GADD34 protein C-terminal regulates cell proliferation in CHO-K1 cells

Overexpression of wild-type GADD34 largely inhibited cell proliferation (~50 % inhibition) and significantly increased p21 expression levels (Fig. 6a, b, d). Treatment of the cells with thapsigargin, which increased GADD34 levels (Fig. 2a), also inhibited cell proliferation (>77 % inhibition) and increased p21 levels (Fig. 6a, b, d). Meanwhile, relative to wild-type GADD34, the Q525X mutant had a reduced ability to inhibit cell proliferation and enhance p21 levels in CHO-K1-normal cells (Fig. 6a, b, d). These results may indicate that

Fig. 3 Effect of GADD34 on eIF2 α phosphorylation status. **a** CHO-K1-normal (indicated as *normal*) and the two (*line 1* and *line 2*) lines of CHO-K1-G34M (indicated as *G34M*) cells were treated for 6 h with 300 nM thapsigargin (*TG*) or vehicle (DMSO, indicated as *D*). Protein was then extracted and subjected to immunoblotting. **b** CHO-K1-normal and CHO-K1-G34M line 1 and line 2 cells were transfected with a wild-type (*WT*) GADD34-encoding expression plasmid or an empty vector

(*vec*). After 24 h, protein samples were prepared from each group for immunoblotting. **c** CHO-K1-normal cells were transfected with an empty vector (*vector*) or an expression plasmid encoding a WT or Q525X mutant GADD34 protein. After 24 h, cells were treated with TG (300 nM) or D for 6 h as described above. Protein samples were subjected to immunoblotting. *P-eIF2 α* indicates phosphorylated eIF2 α

Fig. 4 Effect of GADD34 on GSK3 β phosphorylation and the canonical Wnt signaling pathway. **a** Protein samples from CHO-K1-normal (indicated as *N*) and the two CHO-K1-G34M lines (*line 1* and *line 2*, which are indicated as *L1* and *L2*, respectively) were subjected to immunoblotting. **b** Cells were transfected with a wild-type (*WT*) GADD34-encoding expression plasmid or an empty vector (*vec*). After 24 h, protein samples were prepared from each group for immunoblotting. **c** The CHO-K1-normal cells were transfected with an empty *vec* or an expression plasmid encoding WT or Q525X mutant GADD34 protein. After 24 h, protein samples were extracted and subjected to immunoblotting. **a–c** *P-GSK3 β* indicates phosphorylated GSK3 β . **d** *N*, *L1*, and *L2* cells were transfected with a plasmid DNA mixture containing a pGL4.49 luciferase reporter (pGL4.49 [luc2P/TCF-

LEF RE/Hygro] or pGL4.49-empty), pCMV β , and pBAPo-CMV Pur plasmids. After 24 h, luciferase activity in the cell lysates was measured. **e** CHO-K1-normal cells were transfected with plasmid DNAs containing a pGL4.49 luciferase reporter plasmid (pGL4.49 [luc2P/TCF-LEF RE/Hygro] or pGL4.49-empty), pCMV β , and a pBAPo-CMV Pur plasmid with (WT) or without (*vec*) a wild-type GADD34 cDNA insert. After 24 h, luciferase activity of the cell lysates was measured. Luciferase activity in **d**, **e** was normalized for β -gal activity and the data (means $\pm$ SD, $n=4$) for pGL4.49 [luc2P/TCF-LEF RE/Hygro]-transfected cells are shown as the fold-increase from values for cells transfected with empty pGL4.49. A mean value for *N* cells (in **d**) or vector-transfected cells (in **e**) is designated as 1

Fig. 5 **a** Cell proliferation curve for each CHO-K1 cell line. Cells were seeded at 5×10^4 cells/60-mm dish at time 0. After 24, 48, or 72 h, cells were trypsinized and resuspended in PBS containing 0.2 % trypan blue. The number of live cells in suspension was counted as described in the “Materials and methods” section, and the results were used to calculate the absolute cell number in the dish (the *y*-axis). Data are expressed as the means $\pm$ SD of three culture dishes for each time point. * $P < 0.05$, when compared with CHO-K1-normal cells at the same time point. **b** p21 (**b**) and cyclin D1 (**c**) mRNA expression levels in CHO-K1-normal (indicated as *N*) and the two CHO-K1-G34M cell lines (line 1 and line 2, which are

indicated as *L1* and *L2*, respectively). Total RNA isolated from each cell group was reversely transcribed and subjected to quantitative real-time PCR as described in the “Materials and methods” section. Data are expressed as the means $\pm$ SD of five culture dishes (35-mm dishes) in each group. Each mRNA expression level was normalized relative to the expression of 18S ribosomal RNA (*rRNA*) in each group. The mean value for the CHO-K1-normal cells is designated as 1. † $P < 0.05$, when compared with CHO-K1-normal cells. **d** Protein samples from CHO-K1-normal (indicated as *N*) and the two CHO-K1-G34M lines (*L1* and *L2*) were subjected to immunoblotting

the C-terminal 66 amino acids of GADD34 regulate cellular proliferation through a p21-dependent mechanism. Although neither wild type nor Q525X mutant of GADD34 affected the cyclin D1 levels in CHO-K1-normal cells (Fig. 6c, d), treating the cells with thapsigargin largely suppressed cyclin D1 expression (Fig. 6c, d), which suggests that thapsigargin-induced reductions in cyclin D1 levels occurred through a GADD34-independent pathway.

Discussion

A novel CHO-K1-based cell line, CHO-K1-G34M, was established in this study. These cells carry a nonsense mutation—Q525X—in the GADD34 gene, which encodes a 524-amino acid GADD34 protein that lacks the C-terminal 66 residues of the 590 amino acid wild-type protein. Several

domains in the GADD34 protein play roles in PP1-mediated dephosphorylation of eIF2 α (Brush et al. 2003; Novoa et al. 2001). These domains include a KVHF motif (KVRF in humans) and an RARA sequence, both of which are necessary for PP1 binding and formation of an eIF2 α -phosphatase complex (Brush et al. 2003). The predicted amino acid sequence of the GADD34 Q525X protein lacks the C-terminal RARA motif, suggesting that this mutant protein may have a reduced capacity for PP1 binding compared to the wild-type protein (Fig. 1). Thus, the effects of GADD34 with or without the Q525X mutation on the phosphorylation status of PP1 target proteins, including eIF2 α (Brush et al. 2003; Novoa et al. 2001) and GSK3 β (Hernández et al. 2010), were investigated in this study.

As expected, CHO-K1-G34M cells with the GADD34 Q525X mutation had higher levels of eIF2 α phosphorylation relative to CHO-K1-normal cells both in the presence and

Fig. 6 Effect of GADD34 on cell proliferation. **a** The CHO-K1-normal cells (indicated as normal) were transfected with an empty vector (*vec*) or an expression plasmid encoding wild-type (*WT*) or Q525X mutant GADD34 protein. After 24 h, cells were seeded at 5×10^4 cells/60-mm dish. In some experiments, CHO-K1-normal cells without transfection were seeded at 5×10^4 cells/60-mm dish in the presence of 300 nM thapsigargin (*TG*) or vehicle (DMSO, indicated as *D*). After 48 h, cells were trypsinized and resuspended in PBS containing 0.2 % trypan blue. The number of live cells in suspension was counted as described in the “Materials and methods” section, and the results were used to calculate the absolute cell number in the dish (the *y*-axis). p21 (**b**) and cyclin D1 (**c**) mRNA expression levels in CHO-K1-normal cells that had been transfected with an empty *vec* or an expression plasmid encoding WT

or Q525X mutant GADD34 protein. In some experiments, CHO-K1-normal cells treated with 300 nM *TG* or vehicle (*D*) for 24 h were used. Data in **a–c** are expressed as the means $\pm$ SD ($n=3$). A mean value for the *vec*-transfected cells or vehicle (*D*)-treated cells is designated as 1 in **b**, **c**. * $P<0.05$, when compared with *vec*-transfected cells. † $P<0.05$, relative to wild-type GADD34 (*WT*)-transfected cells. # $P<0.05$, relative to vehicle (*D*)-treated cells. **d** CHO-K1-normal cells were transfected with an empty *vec* or an expression plasmid encoding either WT or Q525X mutant GADD34 protein. After 24 h, cells were subjected to immunoblotting. In some experiments, CHO-K1-normal cells without transfection were cultured in the presence of 300 nM *TG* or vehicle (*D*) for 6 h prior to protein extraction

absence of thapsigargin (Fig. 3). Furthermore, overexpression of wild-type GADD34 protein in CHO-K1-normal cells could largely reduce eIF2 α phosphorylation, while Q525X mutant overexpression had reduced ability to lower phosphorylation levels. These results suggest that the C-terminal region of GADD34 plays a central role in regulating dephosphorylation of eIF2 α in mammalian cells. Brush et al. demonstrated that loss of the RARA motif almost completely abolished the ability of human GADD34 to facilitate eIF2 α dephosphorylation (Brush et al. 2003). However, the Q525X mutant in hamster GADD34 in this study retained a slight dephosphorylation-

facilitating activity (Fig. 3c). Such a difference in protein activity between human and hamster GADD34 might be due to differences in protein structure that may arise from the longer amino acid sequence in humans (674 residues in humans vs. 590 residues in hamsters). In addition, the number of repeats in the repetitive amino acid sequences is 4 in human GADD34 (Novoa et al. 2001) as opposed to 3.5 in hamster GADD34 (Fig. 1b) and the KVRV sequence in human GADD34, which is considered to be necessary but not sufficient for PP1 binding (Brush et al. 2003), is replaced with KVHF in hamster GADD34 (Fig. 1b), which may explain the results of our

preliminary experiments using an anti-GADD34 polyclonal antibody (raised against amino acids 483–674 of human GADD34) that detected both human and mouse GADD34 protein but failed to detect hamster GADD34 protein (data not shown). However, the amino acid sequences in hamster GADD34 Q525X protein that contribute to eIF2 α dephosphorylation have not been determined and await characterization.

Whether the GADD34 Q525X mutation affects GADD34 mRNA levels remains unclear. If the mutation generates a premature termination codon (PTC) at least 50–55 nucleotides upstream from the last exon-exon junction on the mature RNA (Kuzmiak and Maquat 2006; McGlinchey and Smith 2008) or produces a long 3'-untranslated region (UTR) (one study suggested that the length may exceed 420 nucleotides) (Brogna and Wen 2009; Singh et al. 2008), the nonsense-mediated mRNA decay (NMD) system could reduce the level of such PTC-containing nonsense mRNA to 5 to 25 % of normal mRNA levels (Kuzmiak and Maquat 2006). Our results show that only line 2 of the two CHO-K1-G34M lines characterized here had significantly lower levels (about 50 % reduction) of GADD34 mRNA under basal conditions (Fig. 2a). Since the Q525X mutation is located in the last exon (exon 3) of the GADD34 mRNA and this nonsense mutation elongates the 3'-UTR by only 198 bases, the observed reduction in GADD34 mRNA in CHO-K1-G34M line 2 cells likely did not depend on the NMD-mediated mRNA breakdown. However, enhanced phosphorylation of eIF2 α was also reported to inhibit NMD signaling pathways (Gardner 2008). Thus, the increased eIF2 α phosphorylation observed in the CHO-K1-G34M cells might, in turn, increase levels of nonsense mRNA that would result in the apparently unchanged mRNA levels that were observed in line 1 of the mutant cells. As such, whether the regulation of GADD34 mRNA in the CHO-K1-G34M cells involves NMD awaits further study.

Here, we hypothesized a role for GADD34 in regulating GSK3 activity. Mammalian GSK3 consists of two isoforms, GSK3 α and GSK3 β , and acts in many intracellular signaling pathways, including glycogen synthesis, insulin signaling, protein synthesis, and the canonical Wnt signaling pathway (Beurel et al. 2015; Hernández et al. 2010; Lee and Kim 2007). The GSK3 α and GSK3 β catalytic domains share 97 % similarity, and both are inactivated by phosphorylation at N-terminal Ser residues (Ser21 in GSK3 α and Ser9 in GSK3 β) (Hernández et al. 2010; Sutherland et al. 1993). However, the actions and substrate specificities of the two isoforms are differentially regulated (Beurel et al. 2015). In this regard, several reports have described and demonstrated that GSK3 β activation induced by dephosphorylation at Ser9 was mediated by PP1 while activation of GSK3 α following dephosphorylation of Ser21 was induced by PP2A (Hernández et al. 2010; Morfini et al. 2004). The prediction that GSK3 β targets more substrates (possibly over 500) than

other kinases (Beurel et al. 2015) supports the expectation that PP1 plays central roles as regulators of many cellular functions, including those that involve GADD34. However, in the present study, we failed to observe any significant effect of GADD34 on the phosphorylation status of GSK3 β regardless of whether the Q525X mutation was present (Fig. 4), suggesting that an interaction of GADD34 with PP1 does not function as a regulator of GSK3 β in CHO-K1 cells. While the reason for the lack of GADD34 involvement in GSK3 β dephosphorylation is unclear, the subcellular localization of GADD34 could explain these mechanisms. In keeping with this line of thinking, Brush et al. reported that GADD34 binds to PP1 to target it to the endoplasmic reticulum, where PP1 can dephosphorylate eIF2 α and promote protein translation (Brush et al. 2003). On the other hand, activation and inactivation of GSK3 β likely occurs in the cytosol (Lee and Kim 2007) such that the population of PP1 enzymes that participates in GSK3 β dephosphorylation may bind to other regulator(s) and, in turn, occupy GADD34 binding domains. Further investigation will be needed to explore these possibilities.

GADD34 gene knockout has been reported to decrease the expression of p21, a cyclin-dependent kinase inhibitor, and enhance the cell proliferation of mouse embryonic fibroblasts (Minami et al. 2007). These findings suggest that GADD34 might promote cellular senescence (Minami et al. 2007). In the present study, the proliferation of CHO-K1-G34M cells was actually higher than the control CHO-K1-normal cells (Fig. 5). The accelerated proliferation of CHO-K1-G34M cells might be ascribed in part to the reduction in p21 expression levels (Fig. 5). Indeed, a significant reduction of GADD34 mRNA levels paralleled a relatively larger reduction in p21 mRNA expression levels (44 % reduction) in CHO-K1-G34M line 2 cells (see Figs. 2a and 5b). However, CHO-K1-G34M cell line 1 also had lower p21 levels without a significant reduction in the GADD34 mRNA level (see Figs. 2a and 5b, d). The GADD34 Q525X mutant had a reduced ability to inhibit cell proliferation and enhance p21 expression of the CHO-K1-normal cells compared to the wild-type GADD34 protein (Fig. 6), suggesting a role for the C-terminal 66 amino acids of GADD34 in promoting p21 expression and suppressing cellular proliferation. The inhibitory interaction of GADD34 with BFCOL1, which can transactivate the p21 minimal promoter (Hasegawa et al. 1999), would thus not require the C-terminal region of the GADD34 protein (Hollander et al. 2003). Whether other phenomena such as a stabilization of p53, an inducer of p21 expression, by GADD34 (Haneda et al. 2004; Yagi et al. 2003) involve the GADD34 C-terminal region remains to be clarified. We have speculated that activation of the eIF2 α -ATF4 axis promoted by GADD34 Q525X, which lacks the C-terminus (Fig. 3a), did not cause the reduced p21 expression seen in CHO-K1-G34M cells (Fig. 5b) since ATF4 could enhance p21 expression levels in several mammalian cell

types (Bagheri-Yarmand et al. 2003; Ebert et al. 2010). For this reason, a GADD34-PP1 interaction might regulate p21 expression and cell proliferation through eIF2 α dephosphorylation-independent mechanisms. On the other hand, expression levels of cyclin D1, a key regulator of G1-to-S phase progression in the cell cycle, were not increased in the CHO-K1-G34M cells (Fig. 5c, d). Thus, cyclin D1 appeared not to play a pivotal role in the accelerated proliferation of the CHO-K1-G34M cells. The β -catenin/LEF1 pathway (the canonical Wnt signaling pathway) could transactivate the cyclin D1 promoter (Shtutman et al. 1999), while GSK3 β phosphorylates cyclin D1, which promotes its degradation by the ubiquitin-proteasome system (Diehl et al. 1998). In the present study, the lack of enhanced canonical Wnt signaling pathway activity (Fig. 4) might reflect the absence of elevated cyclin D1 levels in CHO-K1-G34M cells (Fig. 5c, d). Furthermore, cyclin B1 expression levels also remained constant in CHO-K1-G34M cells (Fig. 5d), but whether the expression of other cyclins or regulators of cell cycle progression is involved in the accelerated proliferation of CHO-K1-G34M cells remains to be determined.

In conclusion, our results suggest that the GADD34 C-terminus plays important roles in regulating eIF2 α dephosphorylation and cell proliferation, whereas GADD34 itself may not be involved in GSK3 β dephosphorylation and Wnt signaling in CHO-K1 cells. PP1-dependent cellular functions would be differentially targeted by GADD34 through mechanisms that have yet to be determined.

Acknowledgments We thank Dr. Kenji Uchimura (Department of Biochemistry, Nagoya University Graduate School of Medicine) for kindly providing an ancestor of the CHO-K1-normal cells. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, to Nagakatsu Harada (23700906, 25350887).

References

- Adler HT, Chinery R, Wu DY, Kussick SJ, Payne JM, Fornace AJ Jr, Tkachuk DC (1999) Leukemic HRX fusion proteins inhibit GADD34-induced apoptosis and associate with the GADD34 and hSNF5/INI1 proteins. *Mol Cell Biol* 19:7050–7060
- Bagheri-Yarmand R, Vadlamudi RK, Kumar R (2003) Activating transcription factor 4 overexpression inhibits proliferation and differentiation of mammary epithelium resulting in impaired lactation and accelerated involution. *J Biol Chem* 278:17421–17429
- Beurel E, Grieco SF, Jope RS (2015) Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. *Pharmacol Ther* 148C: 114–131
- Bollen M, Peti W, Ragusa MJ, Beullens M (2010) The extended PP1 toolkit: designed to create specificity. *Trends Biochem Sci* 35:450–458
- Brogna S, Wen J (2009) Nonsense-mediated mRNA decay (NMD) mechanisms. *Nat Struct Mol Biol* 16:107–113
- Brush MH, Weiser DC, Shenolikar S (2003) Growth arrest and DNA damage-inducible protein GADD34 targets protein phosphatase 1 alpha to the endoplasmic reticulum and promotes dephosphorylation of the alpha subunit of eukaryotic translation initiation factor 2. *Mol Cell Biol* 23:1292–1303
- Diehl JA, Cheng M, Roussel MF, Sherr CJ (1998) Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* 12:3499–3511
- Ding VW, Chen RH, McCormick F (2000) Differential regulation of glycogen synthase kinase 3beta by insulin and Wnt signaling. *J Biol Chem* 275:32475–32481
- Ebert SM, Monteys AM, Fox DK, Bongers KS, Shields BE, Malmberg SE, Davidson BL, Suneja M, Adams CM (2010) The transcription factor ATF4 promotes skeletal myofiber atrophy during fasting. *Mol Endocrinol* 24:790–799
- Fornace AJ Jr, Nebert DW, Hollander MC, Luethy JD, Papathanasiou M, Fargnoli J, Holbrook NJ (1989) Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. *Mol Cell Biol* 9:4196–4203
- Gardner LB (2008) Hypoxic inhibition of nonsense-mediated RNA decay regulates gene expression and the integrated stress response. *Mol Cell Biol* 28:3729–3741
- Haneda M, Kojima E, Nishikimi A, Hasegawa T, Nakashima I, Isobe K (2004) Protein phosphatase 1, but not protein phosphatase 2A, dephosphorylates DNA-damaging stress-induced phospho-serine 15 of p53. *FEBS Lett* 567:171–174
- Harada N, Yonemoto H, Yoshida M, Yamamoto H, Yin Y, Miyamoto A, Hattori A, Wu Q, Nakagawa T, Nakano M, Teshigawara K, Mawatari K, Hosaka T, Takahashi A, Nakaya Y (2008) Alternative splicing produces a constitutively active form of human SREBP-1. *Biochem Biophys Res Commun* 368:820–826
- Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, Ron D (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol Cell* 6:1099–1108
- Hasegawa T, Xiao H, Isobe K (1999) Cloning of a GADD34-like gene that interacts with the zinc-finger transcription factor which binds to the p21(WAF) promoter. *Biochem Biophys Res Commun* 256:249–254
- Hernández F, Langa E, Cuadros R, Avila J, Villanueva N (2010) Regulation of GSK3 isoforms by phosphatases PP1 and PP2A. *Mol Cell Biochem* 344:211–215
- Hollander MC, Zhan Q, Bae I, Fornace AJ Jr (1997) Mammalian GADD34, an apoptosis- and DNA damage-inducible gene. *J Biol Chem* 272:13731–13737
- Hollander MC, Poola-Kella S, Fornace AJ Jr (2003) Gadd34 functional domains involved in growth suppression and apoptosis. *Oncogene* 22:3827–3832
- Kojima E, Takeuchi A, Haneda M, Yagi A, Hasegawa T, Yamaki K, Takeda K, Akira S, Shimokata K, Isobe K (2003) The function of GADD34 is a recovery from a shutoff of protein synthesis induced by ER stress: elucidation by GADD34-deficient mice. *FASEB J* 17: 1573–1575
- Kuzmiak HA, Maquat LE (2006) Applying nonsense-mediated mRNA decay research to the clinic: progress and challenges. *Trends Mol Med* 12:306–316
- Lee J, Kim MS (2007) The role of GSK3 in glucose homeostasis and the development of insulin resistance. *Diabetes Res Clin Pract* 77(Suppl 1):S49–S57
- McGlinchey NJ, Smith CW (2008) Alternative splicing resulting in nonsense-mediated mRNA decay: what is the meaning of nonsense? *Trends Biochem Sci* 33:385–393
- Minami K, Inoue H, Terashita T, Kawakami T, Watanabe R, Haneda M, Isobe K, Okabe H, Chano T (2007) GADD34 induces p21 expression and cellular senescence. *Oncol Rep* 17:1481–1485
- Morfini G, Szebenyi G, Brown H, Pant HC, Pigino G, DeBoer S, Beffert U, Brady ST (2004) A novel CDK5-dependent pathway for regulating GSK3 activity and kinesin-driven motility in neurons. *EMBO J* 23:2235–2245

- Nakagawa T, Harada N, Miyamoto A, Kawanishi Y, Yoshida M, Shono M, Mawatari K, Takahashi A, Sakaue H, Nakaya Y (2012) Membrane topology of murine glycerol-3-phosphate acyltransferase 2. *Biochem Biophys Res Commun* 418:506–511
- Novoa I, Zeng H, Harding HP, Ron D (2001) Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2 α . *J Cell Biol* 153:1011–1022
- Novoa I, Zhang Y, Zeng H, Jungreis R, Harding HP, Ron D (2003) Stress-induced gene expression requires programmed recovery from translational repression. *EMBO J* 22:1180–1187
- Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A (1999) The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci U S A* 96:5522–5527
- Singh G, Rebbapragada I, Lykke-Andersen J (2008) A competition between stimulators and antagonists of Upf complex recruitment governs human nonsense-mediated mRNA decay. *PLoS Biol* 6:e111
- Su ZZ, Shi Y, Fisher PB (1997) Subtraction hybridization identifies a transformation progression-associated gene PEG-3 with sequence homology to a growth arrest and DNA damage-inducible gene. *Proc Natl Acad Sci U S A* 94:9125–9130
- Su ZZ, Emdad L, Sarkar D, Randolph A, Valerie K, Yacoub A, Dent P, Fisher PB (2005) Potential molecular mechanism for rodent tumorigenesis: mutational generation of progression elevated gene-3 (PEG-3). *Oncogene* 24:2247–2255
- Sutherland C, Leighton IA, Cohen P (1993) Inactivation of glycogen synthase kinase-3 β by phosphorylation: new kinase connections in insulin and growth-factor signaling. *Biochem J* 296(Pt 1):15–19
- Yagi A, Hasegawa Y, Xiao H, Haneda M, Kojima E, Nishikimi A, Hasegawa T, Shimokata K, Isobe K (2003) GADD34 induces p53 phosphorylation and p21/WAF1 transcription. *J Cell Biochem* 90:1242–1249