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**[Title] C-terminal region of GADD34 regulates eIF2 $\alpha$  dephosphorylation and cell proliferation in CHO-K1 cells**

[Authors]

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3 **Abstract**  
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6 GADD34 is a member of a growth arrest and DNA damage (GADD)-inducible gene family. Here we  
7 established a novel Chinese hamster ovary (CHO-K1)-derived cell line, CHO-K1-G34M, which carries a  
8 nonsense mutation (termed the Q525X mutation) in the GADD34 gene. The Q525X mutant protein lacks  
9 the C-terminal 66 amino acids required for GADD34 to bind to and activate protein phosphatase 1 (PP1).  
10 We investigated the effects of GADD34 with or without the Q525X mutation on the phosphorylation  
11 status of PP1 target proteins, including the  $\alpha$  subunit of eukaryotic initiation factor 2 (eIF2 $\alpha$ ) and  
12 glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ). CHO-K1-G34M cells had higher levels of eIF2 $\alpha$  phosphorylation  
13 compared to the control CHO-K1-normal cells both in the presence and absence of endoplasmic  
14 reticulum stress. Overexpression of wild type GADD34 protein in CHO-K1-normal cells largely reduced  
15 eIF2 $\alpha$  phosphorylation, while overexpression of the Q525X mutant did not produce similar reductions.  
16 Meanwhile, neither wild type nor the Q525X mutation of GADD34 affected the GSK3 $\beta$  phosphorylation  
17 status. GADD34 also did not affect the canonical Wnt signaling pathway downstream of GSK3 $\beta$ . Cell  
18 proliferation rates were higher, while expression levels of the cyclin dependent kinase inhibitor p21 were  
19 lower in CHO-K1-G34M cells compared to the CHO-K1-normal cells. The GADD34 Q525X mutant had  
20 a reduced ability to inhibit cell proliferation and enhance p21 expression of the CHO-K1-normal cells  
21 compared to the wild type GADD34 protein. These results suggest that the GADD34 protein C-terminal  
22 plays important roles in regulating not only eIF2 $\alpha$  dephosphorylation but also cell proliferation in  
23 CHO-K1 cells.  
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**Keywords:** CHO-K1 cell; GADD34; eIF2 $\alpha$ ; GSK3 $\beta$ ; dephosphorylation; proliferation

## 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; Novoa et al. 2003). GADD34 can bind many different proteins, such as SNF5, HRX, 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 eukaryotic initiation factor 2  $\alpha$  subunit (eIF2 $\alpha$ ) 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 $\alpha$ , 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 frame-shift 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

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2 growth inhibition and apoptosis (Adler et al. 1999; Hollander et al. 2003).  
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4 Here we cloned and established a novel CHO-K1-derived cell line, which we refer to as  
5 CHO-K1-G34M. A nonsense mutation in the GADD34 gene (a C to T mutation at Gln525 residue  
6 produces a premature termination codon, which is termed the Q525X mutation in this study) is present in  
7 the CHO-K1-G34M cells that resulted in the deletion of the GADD34 C-terminal 66 amino acids, which  
8 contain an RARA sequence that is a critical domain for binding of GADD34 to PP1 (Brush et al. 2003).  
9 Nearly 200 proteins, including eIF2 $\alpha$ , have been shown to interact with PP1 within cells (Bollen et al.  
10 2010). Another PP1-dependent protein that might contribute to cell growth is glycogen synthase kinase 3  
11 (GSK3), which is involved in various cellular signaling pathways, including the insulin signaling  
12 pathway and canonical Wnt signaling pathway (Beurel et al. 2015; Lee and Kim 2007). In some signaling  
13 pathways, GSK3 activity is inhibited by phosphorylation at its N-terminus (at Ser21 for GSK3 $\alpha$  and at  
14 Ser9 for GSK3 $\beta$ ), while dephosphorylation of these residues reverses this inhibition (Hernández et al.  
15 2010; Sutherland et al. 1993). In mammalian cells PP1 reportedly serves as a phosphatase for GSK3 $\beta$ , but  
16 not GSK3 $\alpha$  (Hernández et al. 2010). Thus, an interaction between GADD34 and PP1 might affect  
17 PP1-mediated dephosphorylation of GSK3 $\beta$ , although there are few reports concerning the role of  
18 GADD34 in GSK3 $\beta$  dephosphorylation. Here we examined the effects of GADD34 with or without its  
19 PP1-binding domain on phosphorylation of eIF2 $\alpha$  and GSK3 $\beta$ , as well as on cell proliferation, of the  
20 CHO-K1-derived cell line.  
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## Materials and methods

### Cell culture

CHO-K1 cells with (CHO-K1-G34M) or without (CHO-K1-normal) the Q525X mutation in the GADD34 gene were cultured in Nutrient Mixture F-12 Ham medium (Sigma, St. Louis, MO) 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 h 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'- AAGGCTGTGTTACCCCTGTTG-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). 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).

### Plasmids

cDNA fragments encoding the GADD34 gene open reading frame were amplified by RT-PCR using RNA isolated from normal CHO-K1 cells or CHO-K1-G34M cells. The gene-specific primers were as follows: forward, 5'-GGATCCAGACACATGGCCCCAAGCCC-3' (the *Bam*HI restriction site is underlined); reverse, 5'-ACGACTTGGGTTTAGCCACG-3'. The PCR products were subcloned into the pCR®-Blunt II TOPO® vector (Invitrogen, Carlsbad, CA) 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). A pGL4.49

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2 vector without the TCF-LEF response element (pGL4.49-empty) was generated by digesting the pGL4.49  
3 [luc2P/TCF-LEF RE/Hygro] vector with *Eco*53kI and *Eco*RV, followed by self-ligation of the resulting  
4 blunt-ended fragment.  
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#### 7 8 9 Quantitative real-time PCR

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11 Total RNA was extracted from CHO-K1 cells with TRIzol<sup>®</sup> reagent (Invitrogen). In some experiments,  
12 CHO-K1-normal cells grown in 35-mm dishes were transiently (24 h) transfected with 2.0 µg  
13 pBApo-CMV Pur plasmid with or without the GADD34 cDNA insert using Lipofectamine<sup>®</sup> 2000  
14 (Invitrogen) and used for RNA extraction. RNA was then reverse-transcribed using TaKaRa PrimeScript<sup>®</sup>  
15 RT reagent kits (TaKaRa). Quantitative real-time PCR was performed with the LightCycler<sup>®</sup> system  
16 (Roche Diagnostics, Tokyo, Japan) using TaKaRa SYBR<sup>®</sup> Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa)  
17 and the gene-specific primers listed in Table 1. Expression levels were normalized relative to the level of  
18 18S ribosomal RNA (the primer sequences are shown in Table 1) in each sample and are given in  
19 arbitrary units.  
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#### 27 28 Protein extraction and immunoblotting

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30 CHO-K1 cells grown in 35-mm dishes were transiently transfected with 2.0 µg pBApo-CMV Pur plasmid  
31 with or without the GADD34 cDNA insert using Lipofectamine<sup>®</sup> 2000 (Invitrogen). After 24 h, cells were  
32 treated with thapsigargin (300 nM) or vehicle (DMSO) as described above. In some experiments,  
33 untransfected CHO-K1 cells were used. Cells were lysed in RIPA buffer containing 20 mM Tris-HCl (pH  
34 7.4), 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Nonidet-P40, 2 mM EDTA, 50 mM NaF,  
35 50 mM β-glycerophosphate, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were then passed  
36 through a 21-gauge needle followed by centrifugation at 4 °C for 30 min at 13,500 g. The supernatant was  
37 used as a cell protein sample. The protein concentration of the samples was measured with a  
38 bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Equal amounts of sample proteins were  
39 denatured by boiling in SDS sample buffer containing 1% β-mercaptoethanol. The proteins were then  
40 electrophoresed on an SDS-polyacrylamide gel and transferred onto an Immobilon-P membrane  
41 (Millipore, Bedford, MA). The membrane was blocked in 5% skim milk in Tris-buffered saline  
42 containing 0.05% Tween 20 for 1 h and incubated with either rabbit anti-phospho eIF2α (Ser51)  
43 polyclonal antibody (#9721, Cell Signaling Technology, Inc., Danvers, MA), rabbit anti-eIF2α polyclonal  
44 antibody (#9722, Cell Signaling Technology), rabbit anti-GAPDH polyclonal antibody (#sc-25778, Santa  
45 Cruz Biotechnology, Inc., Dallas, TX), rabbit anti-ATF4 (CREB-2) polyclonal antibody (#sc-200, Santa  
46 Cruz Biotechnology), rabbit anti-calnexin polyclonal antibody (#sc-11397, Santa Cruz Biotechnology),  
47 rabbit anti-phospho GSK3β (Ser 9) polyclonal antibody (#5558, Cell Signaling Technology), mouse  
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2 anti-GSK3 $\beta$  monoclonal antibody (#9832, Cell Signaling Technology), mouse anti-cyclin D1 monoclonal  
3 antibody (#14561A, Pharmingen, San Diego, CA), rabbit anti-cyclin B1 polyclonal antibody (#4138, Cell  
4 Signaling Technology), rabbit anti-p21 polyclonal antibody (#sc-397, Santa Cruz Biotechnology), or  
5 mouse anti-DYKDDDDK tag (FLAG tag) monoclonal antibody (018-22381, Wako, Osaka, Japan).  
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7 Proteins were then visualized with an anti-rabbit (#458) or anti-mouse (#330) IgG horseradish  
8 peroxidase-conjugated secondary antibody (Medical & Biological Laboratories Co., LTD., Aichi, Japan)  
9 using a Clarity<sup>TM</sup> western ECL substrate (Bio-Rad Laboratories, Inc., Hercules, CA).  
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#### 15 Luciferase assay

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18 CHO-K1 cells grown on 24-well plates were transiently transfected with 0.30  $\mu$ g of plasmid DNAs  
19 containing 0.05  $\mu$ g pGL4.49 luciferase reporter plasmid, 0.20  $\mu$ g pBApo-CMV Pur plasmid with or  
20 without a GADD34 cDNA insert, and 0.05  $\mu$ g of the  $\beta$ -galactosidase ( $\beta$ -gal) expression vector pCMV- $\beta$   
21 (Clontech, Palo Alto, CA) using Lipofectamine<sup>®</sup> 2000 (Invitrogen). After 24 h, cells were harvested in the  
22 lysis buffer supplied with the luciferase assay kit (Promega), and the lysates were assayed for luciferase  
23 activity as recommended in the manufacturer's protocol.  $\beta$ -gal activity was assayed and used for  
24 normalization of transfection efficiency as described previously (Harada et al. 2008).  
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#### 31 Proliferation assay

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

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

### Cloning of the CHO-K1-G34M cell line

GADD34 gene cDNA was cloned by a 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 eIF2 $\alpha$  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 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, 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 the 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

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2 antibodies and an original anti-GADD34 antibody that was designed and produced in our laboratory were  
3 used in preliminary experiments. However, these antibodies failed to detect clearly either the wild type or  
4 Q525X GADD34 protein both at endogenously expressed levels (using protein samples derived from  
5 CHO-K1-normal and CHO-K1-G34M cells with or without thapsigargin treatment) and at exogenously  
6 overexpressed levels (using protein samples derived from CHO-K1-normal cells that had been transfected  
7 with a GADD34 expression plasmid) (data not shown).  
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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 $\alpha$ dephosphorylation

As described above, the GADD34 Q525X mutant protein lacks the RARA motif that was previously  
predicted to be necessary for eIF2 $\alpha$  dephosphorylation (Fig. 1b). To confirm that this mutation affects  
eIF2 $\alpha$  phosphorylation levels, we first compared the phosphorylation status of eIF2 $\alpha$  between  
CHO-K1-normal and CHO-K1-G34M cells. As shown in Fig. 3a, the amount of phosphorylated eIF2 $\alpha$   
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 $\alpha$  (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 $\alpha$  phosphorylation and ATF4 protein levels. The levels of phosphorylated  
eIF2 $\alpha$  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. Fig. 3b shows the effect of exogenous overexpression of the wild  
type GADD34 protein on the phosphorylation status of eIF2 $\alpha$  in each CHO-K1 strain. Overexpression of  
wild type GADD34 decreased eIF2 $\alpha$  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 $\alpha$  dephosphorylation relative to wild type protein. Overexpression of wild type GADD34  
largely decreased eIF2 $\alpha$  phosphorylation levels even in the presence of thapsigargin (Fig. 3c). While  
GADD34 Q525X mutant overexpression slightly decreased eIF2 $\alpha$  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

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2 eIF2 $\alpha$  dephosphorylation in CHO-K1 cells, and that GADD34 Q525X expressed in the CHO-K1-G34M  
3 cells has a lowered ability to affect eIF2 $\alpha$  dephosphorylation compared to the wild type GADD34 protein.  
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7 GADD34 did not affect the phosphorylation status of GSK3 $\beta$   
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10 Since GADD34 participates in regulating cell proliferation, the effect of GADD34 on the phosphorylation  
11 status of GSK3 $\beta$  (Ser9) was examined. As shown in Fig. 4a, the basal levels of GSK3 $\beta$  phosphorylation  
12 were similar for the CHO-K1-normal and the two CHO-K1-G34M cell lines. In addition, overexpression  
13 of wild type GADD34 did not affect GSK3 $\beta$  phosphorylation in all three cell strains examined (Fig. 4b, c).  
14 Overexpression of the Q525X mutant protein also had little effect on the phosphorylation status of  
15 GSK3 $\beta$  in CHO-K1-normal cells (Fig. 4c). We also examined the effect of GADD34 on the Wnt signaling  
16 pathway. In the canonical Wnt signaling pathway, GSK3 phosphorylates  $\beta$ -catenin, which promotes its  
17 ubiquitin-dependent degradation (Lee and Kim 2007). When GSK3 activity is inhibited by stimuli such as  
18 Wnt,  $\beta$ -catenin can evade phosphorylation and be translocated to the nucleus to form a complex with  
19 TCF/LEF transcription factors that accelerates expression of cell cycle-driving genes such as cyclin D1  
20 (Lee and Kim 2007; Shtutman et al. 1999). In the Wnt signaling pathway GSK3 $\beta$  is thought to remain in  
21 an unphosphorylated state regardless of whether Wnt stimulation occurs (Ding et al. 2000). On the other  
22 hand, Ser phosphorylation is required for the regulation of several downstream Wnt signaling pathway  
23 proteins, including APC, Axin, and  $\beta$ -catenin (Lee and Kim 2007). Here we transfected CHO-K1 cells  
24 with the Wnt signaling reporter plasmid, pGL4.49 [luc2P/TCF-LEF RE/Hygro], which encodes TCF-LEF  
25 response elements upstream of a firefly luciferase reporter gene. For the CHO-K1-normal and two  
26 CHO-K1-G34M cell lines luciferase activities were similar (Fig. 4d). Furthermore, overexpression of  
27 wild type GADD34 had no significant effect on the luciferase activity in CHO-K1-normal cells (Fig. 4e).  
28 These results suggest that GADD34 does not function as a regulator of GSK3 $\beta$  and Wnt signaling in  
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44 CHO-K1-G34M cells proliferated more rapidly than CHO-K1-normal cells  
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47 Cell proliferation rates for CHO-K1-normal and the two CHO-K1-G34M cells over 72 hours were next  
48 examined. We found that both CHO-K1-G34M cell lines proliferated more rapidly than control  
49 CHO-K1-normal cells (Fig. 5a). Moreover, expression levels of the cyclin dependent kinase inhibitor p21  
50 were decreased in the CHO-K1-G34M cells compared to CHO-K1-normal cells (Fig. 5b, d). The  
51 expression levels of cyclin D1, an important regulator of cell cycle progression, were unchanged (in line  
52 1) or decreased (in line 2) in the CHO-K1-G34M cells compared to CHO-K1-normal cells (Fig. 5c, d).  
53 The expression levels of cyclin B1 were also not increased in the CHO-K1-G34M cells (Fig. 5d).  
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3 GADD34 protein C-terminal regulates cell proliferation in CHO-K1 cells  
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6 Overexpression of wild type GADD34 largely inhibited cell proliferation (~50% inhibition) and  
7 significantly increased p21 expression levels (Fig. 6a, b, d). Treatment of the cells with thapsigargin,  
8 which increased GADD34 levels (Fig. 2a), also inhibited cell proliferation (>77% inhibition) and  
9 increased p21 levels (Fig. 6a, b, d). Meanwhile, relative to wild type GADD34, the Q525X mutant had a  
10 lowered ability to inhibit cell proliferation and enhance p21 levels in CHO-K1-normal cells (Fig. 6a, b, d).  
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12 These results may indicate that the C-terminal 66 amino acids of GADD34 regulate cellular proliferation  
13 through a p21-dependent mechanism. Although neither wild type nor the Q525X mutant of GADD34  
14 affected the cyclin D1 levels in CHO-K1-normal cells (Fig. 6c, d), treating the cells with thapsigargin  
15 largely suppressed cyclin D1 expression (Fig. 6c, d), which suggests that thapsigargin-induced reductions  
16 in cyclin D1 levels occurred through a GADD34-independent pathway.  
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## 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 $\alpha$  (Brush et al. 2003; Novoa et al. 2001). These domains include a KVHF motif (KVRF in humans) and a RARA sequence, both of which are necessary for PP1 binding and formation of an eIF2 $\alpha$ -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 $\alpha$  (Brush et al. 2003; Novoa et al. 2001) and GSK3 $\beta$  (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 $\alpha$  phosphorylation relative to CHO-K1-normal cells both in the presence and absence of thapsigargin (Fig. 3). Furthermore, overexpression of wild type GADD34 protein in CHO-K1-normal cells could largely reduce eIF2 $\alpha$  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 $\alpha$  in mammalian cells. Brush et al. demonstrated that loss of the RARA motif almost completely abolished the ability of human GADD34 to facilitate eIF2 $\alpha$  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 KVRF 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 $\alpha$  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; McGlinchy and Smith 2008) or produces a long 3'-untranslated region (UTR) (one study suggested that the length may exceed 420

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2 nucleotides) (Brojna and Wen 2009; Singh et al. 2008), the nonsense-mediated mRNA decay (NMD)  
3 system could reduce the level of such PTC-containing nonsense mRNA to 5 to 25% of normal mRNA  
4 levels (Kuzmiak and Maquat 2006). Our results show that only line 2 of the two CHO-K1-G34M lines  
5 characterized here had significantly lower levels (about 50 % reduction) of GADD34 mRNA under basal  
6 conditions (Fig. 2a). Since the Q525X mutation is located in the last exon (exon 3) of the GADD34  
7 mRNA and this nonsense mutation elongates the 3'-UTR by only 198 bases, the observed reduction in  
8 GADD34 mRNA in CHO-K1-G34M line 2 likely did not depend on the NMD-mediated mRNA  
9 breakdown. However, enhanced phosphorylation of eIF2 $\alpha$  was also reported to inhibit NMD signaling  
10 pathways (Gardner 2008). Thus, the increased eIF2 $\alpha$  phosphorylation observed in the CHO-K1-G34M  
11 cells might in turn increase levels of nonsense mRNA that would result in the apparently unchanged  
12 mRNA levels that were observed in line 1 of the mutant cells. As such, whether the regulation of  
13 GADD34 mRNA in the CHO-K1-G34M cells involves NMD awaits further study.  
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22 Here we hypothesized a role for GADD34 in regulating GSK3 activity. Mammalian GSK3 consists  
23 of two isoforms: GSK3 $\alpha$  and GSK3 $\beta$  and acts in many intracellular signaling pathways, including  
24 glycogen synthesis, insulin signaling, protein synthesis, and the canonical Wnt signaling pathway (Beurel  
25 et al. 2015; Hernández et al. 2010; Lee and Kim 2007). The GSK3 $\alpha$  and GSK3 $\beta$  catalytic domains share  
26 97% similarity and both are inactivated by phosphorylation at N-terminal Ser residues (Ser21 in GSK3 $\alpha$   
27 and Ser9 in GSK3 $\beta$ ) (Hernández et al. 2010; Sutherland et al. 1993). However, the actions and substrate  
28 specificities of the two isoforms are differentially regulated (Beurel et al. 2015). In this regard, several  
29 reports have described and demonstrated that GSK3 $\beta$  activation induced by dephosphorylation at Ser9  
30 was mediated by PP1, while activation of GSK3 $\alpha$  following dephosphorylation of Ser21 was induced by  
31 PP2A (Hernández et al. 2010; Morfini et al. 2004). The prediction that GSK3 $\beta$  targets more substrates  
32 (possibly over 500) than other kinases (Beurel et al. 2015) supports the expectation that PP1 play central  
33 roles as regulators of many cellular functions, including those that involve GADD34. However, in the  
34 present study we failed to observe any significant effect of GADD34 on the phosphorylation status of  
35 GSK3 $\beta$  regardless of whether the Q525X mutation was present (Fig. 4), suggesting that interaction of  
36 GADD34 with PP1 does not function as a regulator of GSK3 $\beta$  in CHO-K1 cells. While the reason for the  
37 lack of GADD34 involvement in GSK3 $\beta$  dephosphorylation is unclear, the subcellular localization of  
38 GADD34 could explain these mechanisms. In keeping with this line of thinking, Brush et al. reported that  
39 GADD34 binds to PP1 to target it to the endoplasmic reticulum, where PP1 can dephosphorylate eIF2 $\alpha$   
40 and promote protein translation (Brush et al. 2003). On the other hand, activation and inactivation of  
41 GSK3 $\beta$  likely occurs in the cytosol (Lee and Kim 2007) such that the population of PP1 enzymes that  
42 participates in GSK3 $\beta$  dephosphorylation may bind to other regulator(s) and in turn occupy GADD34  
43 binding domains. Further investigation will be needed to explore these possibilities.  
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56 GADD34 gene knockout has been reported to decrease the expression of p21, a cyclin dependent  
57 kinase inhibitor, and enhance cell proliferation of mouse embryonic fibroblasts (Minami et al. 2007).  
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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 (see Fig. 2a and Fig. 5b). However, CHO-K1-G34M cell line 1 also had lower p21 levels without a significant reduction in the GADD34 mRNA level (see Fig. 2a and Fig. 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 $\alpha$ -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 $\alpha$  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  $\beta$ -catenin/LEF1 pathway (the canonical Wnt signaling pathway) could transactivate the cyclin D1 promoter (Shtutman et al. 1999), while GSK3 $\beta$  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 are 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 $\alpha$  dephosphorylation and cell proliferation, whereas GADD34 itself may not be involved in GSK3 $\beta$  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.

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Table 1 Primer sets used for PCR

Target	Primer sequence
GADD34	Forward: 5'-GAGAGAAGCCAGCACCCACCT-3'
	Reverse: 5'-CCCAGACAGCAAGGAAATGG-3'
p21	Forward: 5'-CTGTCGCTGTCCTGCACTCT-3'
	Reverse: 5'-GGCACATTAGGGCTTCCTCTT-3'
Cyclin D1	Forward: 5'-AGTGCGTGCAGAGGGAGATT-3'
	Reverse: 5'-TAGGGGCTCCAGAGACAGGA-3'
18S ribosomal RNA	Forward: 5'-AAACGGCTACCACATCCAAG-3'
	Reverse: 5'-GGCCTCGAAAGAGTCCTGTA-3'

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3 **Figure legends**  
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6 **Fig. 1**  
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9 **(a)** Structure of hamster GADD34 cDNA. Boxes indicate exons and the locations of the translational  
10 initiation (ATG) and termination (Stop) codons are indicated. Part of the original sequencing data for  
11 exon 3 in genomic DNA is shown for CHO-K1-normal and CHO-K1-G34M cells. Gln525 in  
12 CHO-K1-normal cells is indicated as Q<sup>525</sup>. In CHO-K1-G34M cells a C to T mutation at this residue  
13 produces a premature termination codon, which is indicated as X (termed Q525X mutation in this study).  
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15 **(b)** Structure of hamster GADD34 proteins. The amino acid (AA) numbers indicate the locations of a  
16 repetitive region (3.5 repeats), KVHF motif, and RARA sequence. The Q525X mutant GADD34 protein  
17 in CHO-K1-G34M cells lacks the C-terminal 66 amino acids that contain the RARA sequence.  
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23 **Fig. 2**  
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26 **(a)** GADD34 mRNA expression levels in CHO-K1-normal cells (indicated as normal) and the two (line 1  
27 and line 2) lines of CHO-K1-G34M (indicated as G34M). Cells were incubated in F-12 Ham medium  
28 including 300 nM thapsigargin (TG) or vehicle (DMSO, indicated as D) for 24 h. Total RNA was then  
29 isolated, reverse-transcribed and subjected to quantitative real-time PCR as described in “Materials and  
30 methods”. Data are expressed as the means  $\pm$  SD of 5 culture dishes (35-mm dishes) for each group. Each  
31 mRNA expression level was normalized relative to the expression of 18S ribosomal RNA (rRNA) in each  
32 group. The mean value for the CHO-K1-normal cells with DMSO treatment is designated as 1. \*P < 0.05.  
33 †P < 0.05, when compared with CHO-K1-normal cells with DMSO treatment. **(b)** Detection of  
34 FLAG-tagged GADD34 protein in CHO-K1-normal cells. Cells were transfected with a plasmid  
35 expressing either N-terminal FLAG-tagged wild type (WT) or Q525X mutant GADD34, or an empty  
36 vector (vec). After 24 h, protein samples were prepared from each group for immunoblotting.  
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45 **Fig. 3**  
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48 Effect of GADD34 on eIF2 $\alpha$  phosphorylation status  
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50 **(a)** CHO-K1-normal (indicated as normal) and the two (line 1 and line 2) lines of CHO-K1-G34M  
51 (indicated as G34M) cells were treated for 6 h with 300 nM thapsigargin (TG) or vehicle (DMSO,  
52 indicated as D). Protein was then extracted and subjected to immunoblotting. **(b)** CHO-K1-normal,  
53 CHO-K1-G34M line 1, and line 2 cells were transfected with a wild type (WT) GADD34-encoding  
54 expression plasmid or an empty vector (vec). After 24 h, protein samples were prepared from each group  
55 for immunoblotting. **(c)** CHO-K1-normal cells were transfected with an empty vector (vector) or an  
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2 expression plasmid encoding a wild type (WT) or Q525X mutant GADD34 protein. After 24 h, cells were  
3 treated with TG (300 nM) or D for 6 h as described above. Protein samples were subjected to  
4 immunoblotting. P-eIF2 $\alpha$ ; Phosphorylated eIF2 $\alpha$ .  
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9 **Fig. 4**

10 Effect of GADD34 on GSK3 $\beta$  phosphorylation and the canonical Wnt signaling pathway

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12 **(a)** Protein samples from CHO-K1-normal (indicated as N) and the two CHO-K1-G34M lines (line 1 and  
13 line 2, which are indicated as L1 and L2, respectively) were subjected to immunoblotting. **(b)** Cells were  
14 transfected with a wild type (WT) GADD34-encoding expression plasmid or an empty vector (vec). After  
15 24 h, protein samples were prepared from each group for immunoblotting. **(c)** The CHO-K1-normal cells  
16 were transfected with an empty vector (vec) or an expression plasmid encoding wild type (WT) or Q525X  
17 mutant GADD34 protein. After 24 h, protein samples were extracted and subjected to immunoblotting. In  
18 **a**, **b**, and **c**, P-GSK3 $\beta$  indicates phosphorylated GSK3 $\beta$ . **(d)** N, L1 and L2 cells were transfected with a  
19 plasmid DNA mixture containing a pGL4.49 luciferase reporter (pGL4.49 [luc2P/TCF-LEF RE/Hygro] or  
20 pGL4.49-empty), pCMV $\beta$  and pBApo-CMV Pur plasmids. After 24 h, luciferase activity in the cell  
21 lysates was measured. **(e)** CHO-K1-normal cells were transfected with plasmid DNAs containing a  
22 pGL4.49 luciferase reporter plasmid (pGL4.49 [luc2P/TCF-LEF RE/Hygro] or pGL4.49-empty), a  
23 pCMV $\beta$ , and a pBApo-CMV Pur plasmid with (WT) or without (vec) a wild type GADD34 cDNA insert.  
24 After 24 h, luciferase activity of the cell lysates was measured. Luciferase activity in **d** and **e** was  
25 normalized for  $\beta$ -gal activity and the data (means  $\pm$  SD, n=4) for pGL4.49 [luc2P/TCF-LEF  
26 RE/Hygro]-transfected cells are shown as the fold-increase from values for cells transfected with empty  
27 pGL4.49. A mean value for N cells (in **d**) or vector-transfected cells (in **e**) is designated as 1.  
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41 **Fig. 5**

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43 **(a)** Cell proliferation curve for each CHO-K1 cell line. Cells were seeded at  $5 \times 10^4$  cells/60-mm dish at  
44 time 0. After 24, 48, or 72 h, cells were trypsinized and resuspended in PBS containing 0.2% trypan blue.  
45 The number of live cells in suspension was counted as described in “Materials and methods”, and the  
46 results were used to calculate the absolute cell number in the dish (the y-axis). Data are expressed as the  
47 means  $\pm$  SD of 3 culture dishes for each time point. \*P < 0.05, when compared with CHO-K1-normal  
48 cells at the same time point. **(b, c)** p21 **(b)** and cyclin D1 **(c)** mRNA expression levels in CHO-K1-normal  
49 (indicated as N) and the two CHO-K1-G34M cell lines (line 1 and line 2, which are indicated as L1 and  
50 L2, respectively). Total RNA isolated from each cell group was reverse-transcribed and subjected to  
51 quantitative real-time PCR as described in “Materials and methods”. Data are expressed as the means  $\pm$   
52 SD of 5 culture dishes (35-mm dishes) in each group. Each mRNA expression level was normalized  
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2 relative to the expression of 18S ribosomal RNA (rRNA) in each group. The mean value for the  
3 CHO-K1-normal cells is designated as 1. †P < 0.05, when compared with CHO-K1-normal cells. **(d)**  
4 Protein samples from CHO-K1-normal (indicated as N) and the two CHO-K1-G34M lines (L1 and L2)  
5 were subjected to immunoblotting.  
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10 **Fig. 6**

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12 Effect of GADD34 on cell proliferation

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14 **(a)** The CHO-K1-normal cells (indicated as normal) were transfected with an empty vector (vec) or an  
15 expression plasmid encoding wild type (WT) or Q525X mutant GADD34 protein. After 24 h, cells were  
16 seeded at  $5 \times 10^4$  cells/60-mm dish. In some experiments, CHO-K1-normal cells without transfection  
17 were seeded at  $5 \times 10^4$  cells/60-mm dish in the presence of 300 nM thapsigargin (TG) or vehicle (DMSO,  
18 indicated as D). After 48 h, cells were trypsinized and resuspended in PBS containing 0.2% trypan blue.  
19 The number of live cells in suspension was counted as described in “Materials and methods”, and the  
20 results were used to calculate the absolute cell number in the dish (the y-axis). **(b, c)** p21 **(b)** and cyclin  
21 D1 **(c)** mRNA expression levels in CHO-K1-normal cells that had been transfected with an empty vector  
22 (vec) or an expression plasmid encoding wild type (WT) or Q525X mutant GADD34 protein. In some  
23 experiments, CHO-K1-normal cells treated with 300 nM TG or vehicle (D) for 24 h were used. Data in **a**,  
24 **b**, and **c** are expressed as the means  $\pm$  SD (n=3). A mean value for the vector (vec)-transfected cells or  
25 vehicle (D)-treated cells is designated as 1 in **b** and **c**. \*P < 0.05, when compared with vector  
26 (vec)-transfected cells. †P < 0.05, relative to wild type GADD34 (WT)-transfected cells. #P < 0.05,  
27 relative to vehicle (D)-treated cells. **(d)** CHO-K1-normal cells were transfected with an empty vector  
28 (vec) or an expression plasmid encoding either wild type (WT) or Q525X mutant GADD34 protein. After  
29 24 h, cells were subjected to immunoblotting. In some experiments, CHO-K1-normal cells without  
30 transfection were cultured in the presence of 300 nM TG or vehicle (D) for 6 h prior to protein extraction.  
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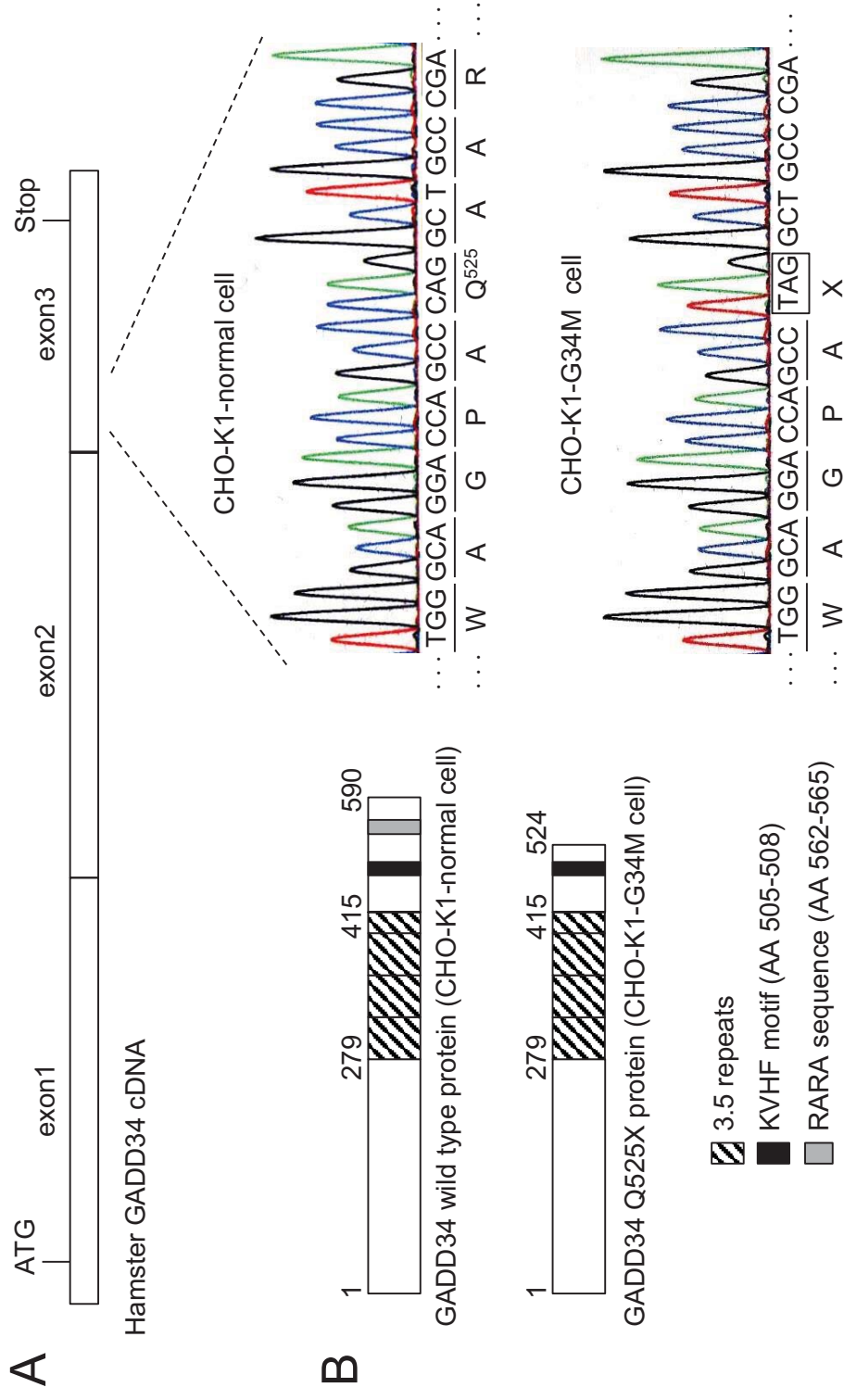


Fig. 1

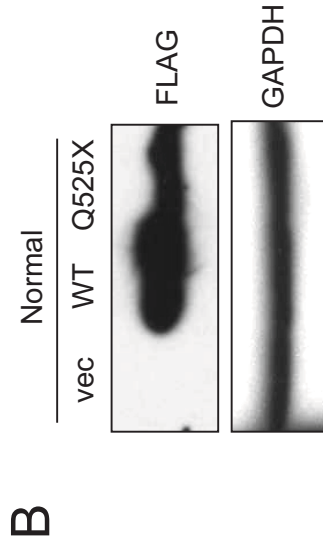
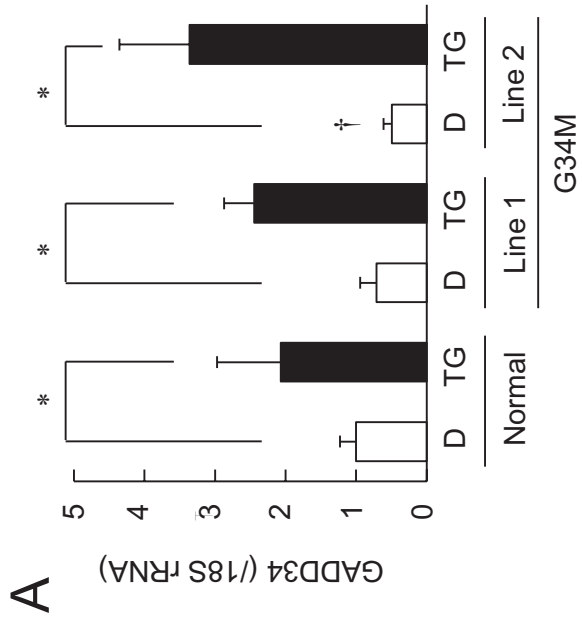


Fig. 2

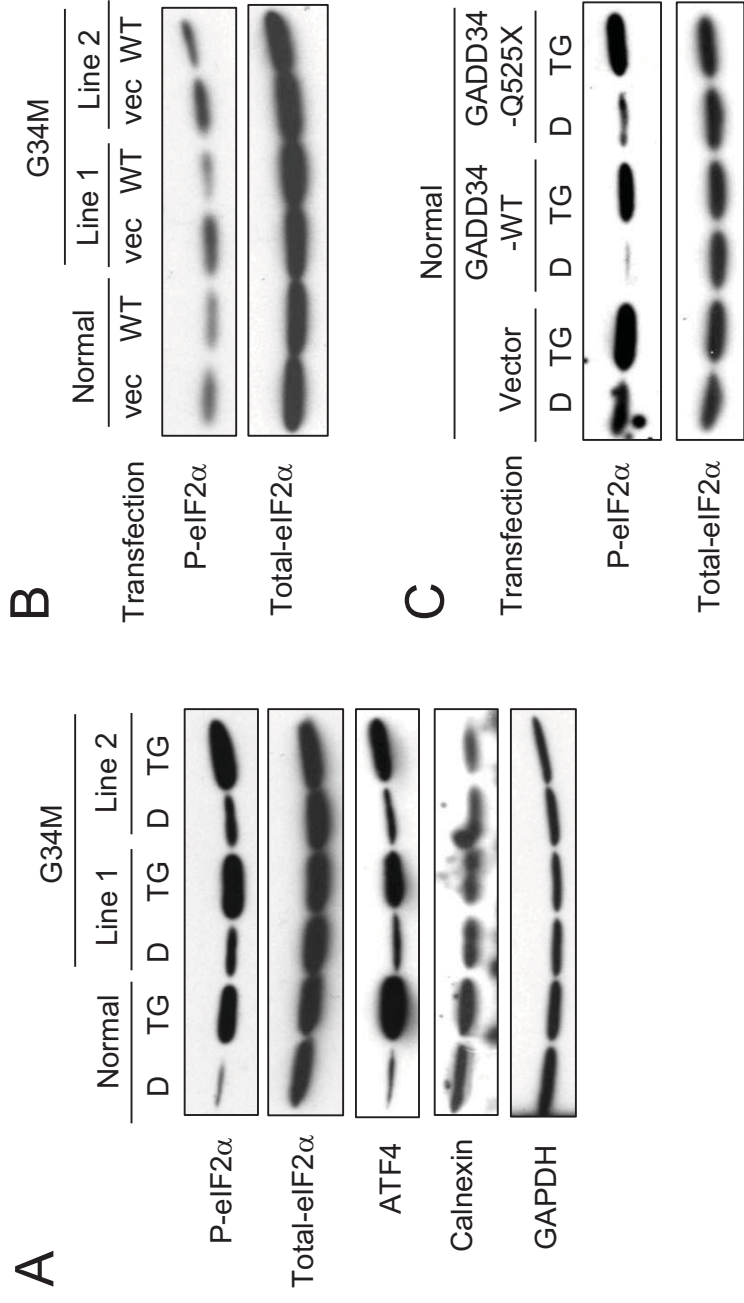


Fig. 3

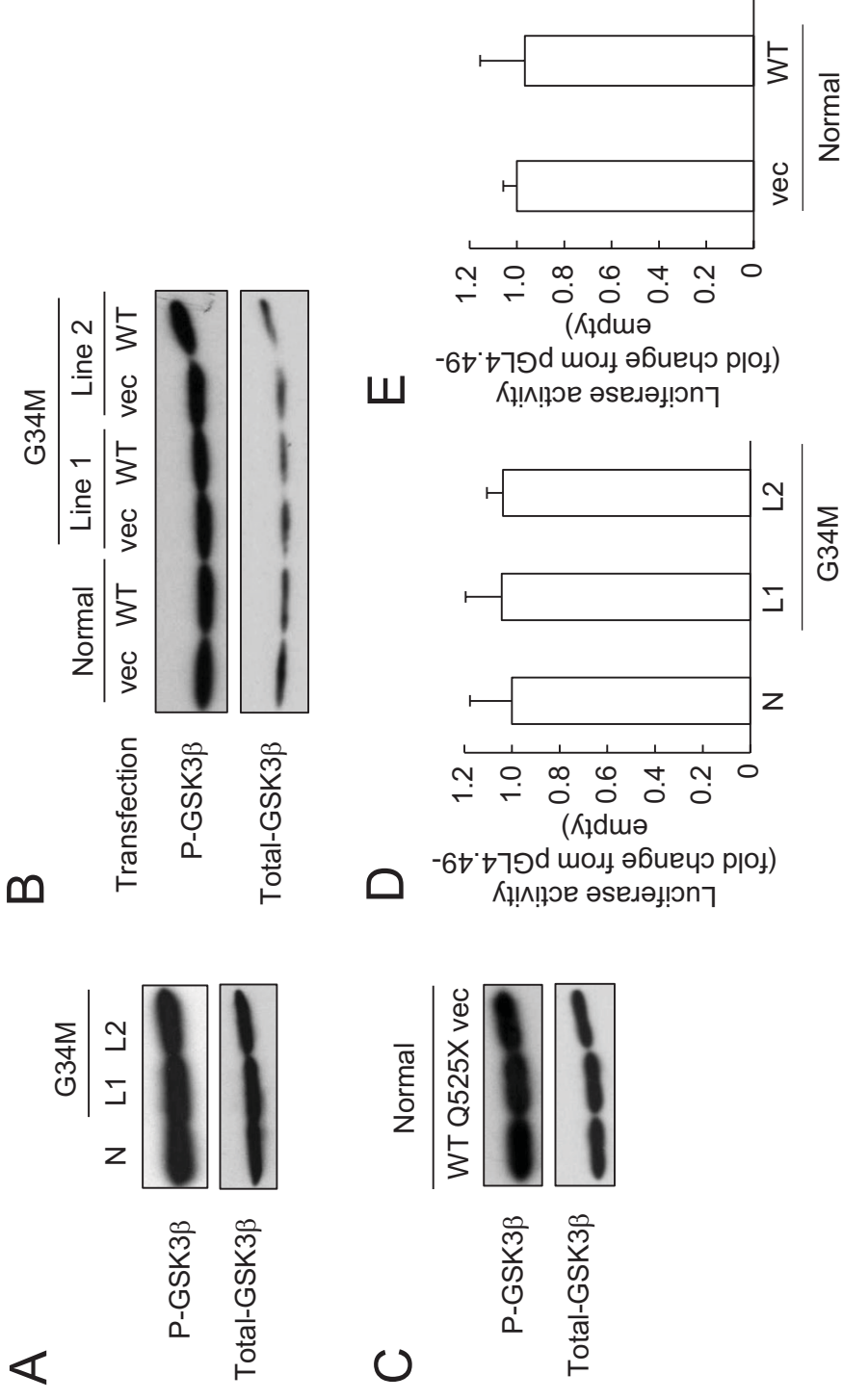


Fig. 4

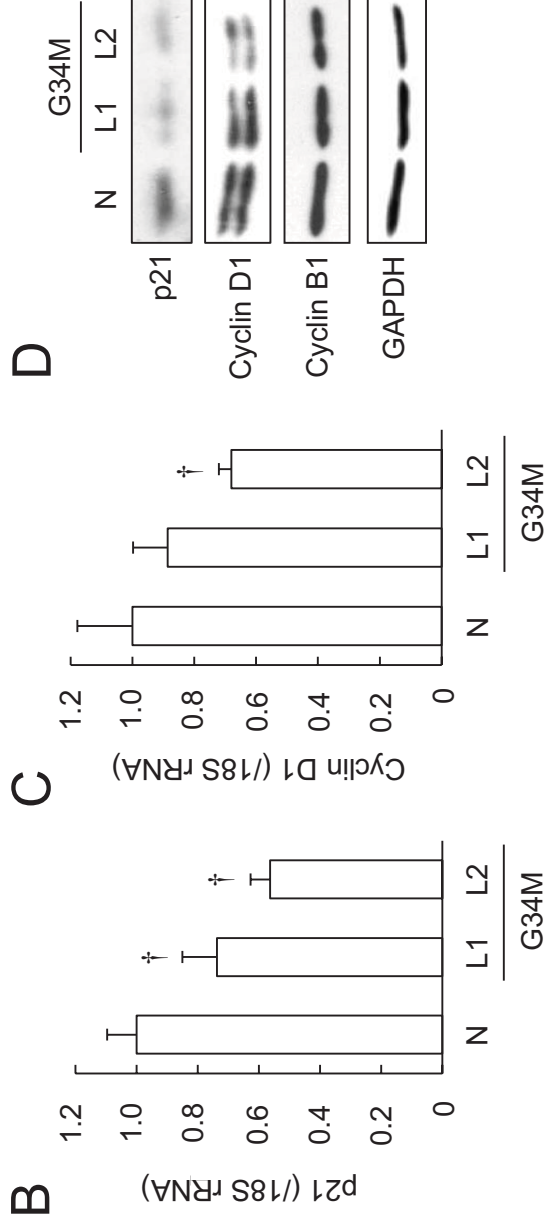
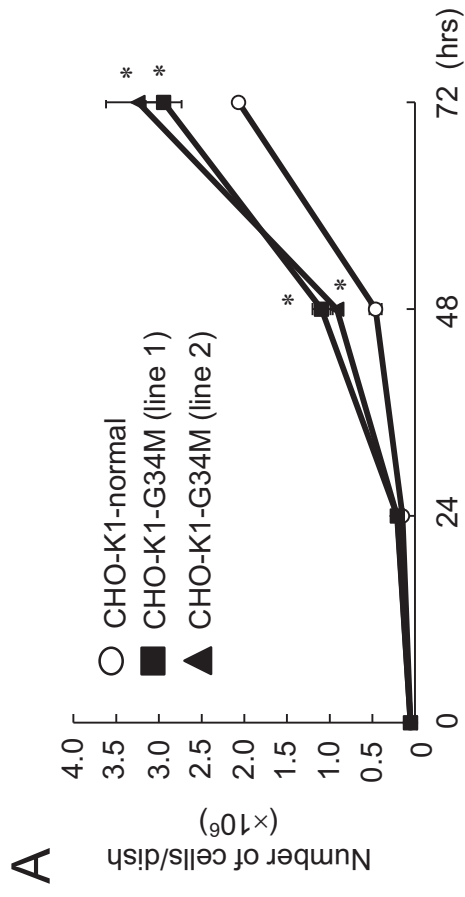


Fig. 5

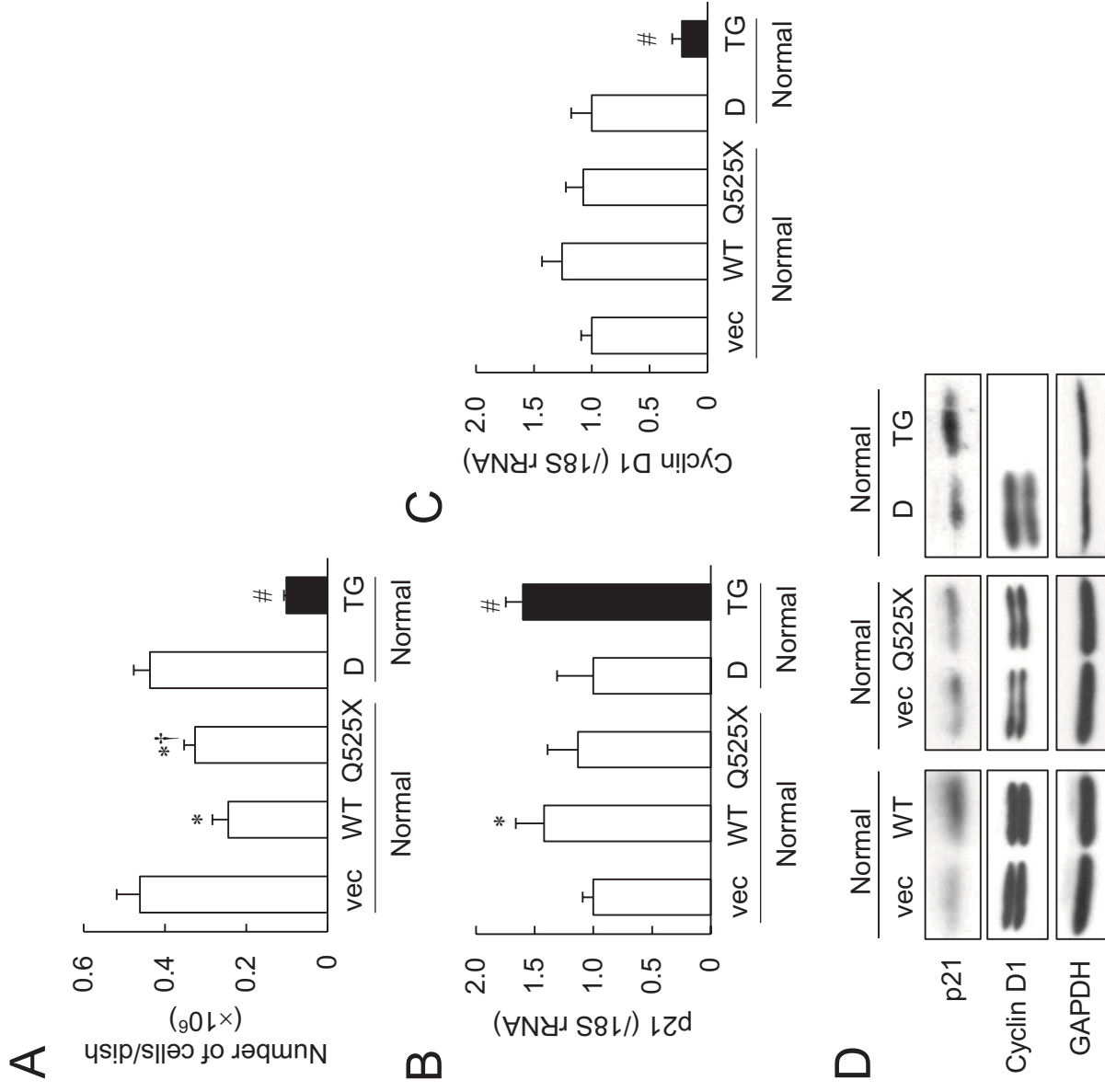


Fig. 6