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GADD45 family proteins suppress JNK signaling by targeting MKK7

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Abstract

Growth arrest and DNA damage-inducible 45 (GADD45) family genes encode related proteins, including GADD45α, GADD45β, and GADD45γ. In HeLa cells, expression of GADD45 members is differentially regulated under a variety of environmental conditions, but thermal and genotoxic stresses induce the expression of all genes. The heat shock response of GADD45β is mediated by the heat shock transcription factor 1 (HSF1), and GADD45β is necessary for heat stress survival. Heat and genotoxic stress-induced activation of c-Jun N-terminal kinase (JNK) is suppressed by the expression of GADD45 proteins. GADD45 proteins bind the JNK kinase mitogen-activated protein kinase kinase 7 (MKK7) and inhibit its activity, even under normal physiological conditions. Our findings indicate that GADD45 essentially suppresses the MKK7-JNK pathway and suggest that differentially expressed GADD45 family members fine-tune stress-inducible JNK activity.

Keywords: GADD45; heat shock; HSF1; JNK; MKK7.

1. **Introduction**

Stress responses integrate different cellular processes, including metabolic changes, cell cycle arrest, senescence, and apoptosis. The expression of GADD45 family genes *GADD45α*, *GADD45β*, and *GADD45γ* is induced in response to serum depletion, UV radiation, ionizing radiation, and alkylating agents. Tumor suppressors p53 and BRCA1 are transcriptional regulators of *GADD45α*, and nuclear factor-κB (NF-κB), which is involved in inflammatory and immune responses, is a regulator of *GADD45β* and *GADD45γ* [1-4]. GADD45 family genes encode small (approximately 18 kDa), highly homologous (approximately 55% similarity) proteins with similar molecular structures [5-7]. GADD45 proteins regulate cell cycle, cell survival or apoptosis, DNA repair, and genomic stability. These functions involve associations with proteins that include cyclin-dependent kinase CDK1, CDK inhibitor p21, mitogen-activated protein 3 kinase (MAP3K) MEKK4/MTK1, MKK7 MAP2K, p38 MAPK, proliferating cell nuclear antigen (PCNA), and ERCC excision repair 5 ERCC5/XPG [1, 8-10].

Higher temperatures cause denaturation and aggregation of numerous cellular proteins that trigger cell death pathways. Although various signal transduction pathways are activated upon heat shock, the MAPK pathway is crucial for the determination of cell fate [11, 12]. Extracellular signal-regulated kinase is important for cell survival, whereas JNK and p38 MAPK are more strongly tied to stress and thus involved in apoptosis. The JNK cascade plays a key role in the determination of cell survival or death induced by various environmental stressors, including heat shock, osmotic shock, oxidative stress, UV irradiation, inflammatory cytokines, and genotoxic agents [13-15]. In response to heat shock, JNK is activated by upstream MAP2Ks, MKK4 and MKK7 [16]. JNK phosphorylates and regulates the activity of mitochondrial pro- and anti-apoptotic proteins and transcription factors, such as c-Jun, activating transcription factor 2 (ATF-2), c-Myc, p53, and ELK1. Among these proteins, c-Jun

and ATF-2 are constituents of activating protein-1 (AP-1), which is a positive and negative modulator of apoptosis depending on the cellular context [14, 15]. Accumulation of denatured and aggregated proteins also triggers the activation of heat shock factor HSF1. HSF1 is the major transcriptional activator of heat shock protein (HSP) genes, whose products function as molecular chaperones, and genes that encode proteins involved in stress response and cell proliferation [17]. HSP has crucial roles not only in protein homeostasis but also in the control of apoptosis [18].

GADD45 proteins contribute to the control of cell survival and/or apoptosis, but their roles in the heat-stressed cells remain unknown. In the present study, we analyzed the expression of GADD45 family genes under various stress conditions. GADD45β is necessary for maintenance of cell viability following heat shock. We also demonstrate that all GADD45 proteins have the potential to suppress JNK signaling by targeting MKK7.

2. Materials and methods

2.1. Cell culture and transfection

HeLa cells were cultured as previously described [19]. The viable cell number was determined by trypan blue dye exclusion assay [19]. Plasmid DNA transfection was conducted using HilyMax (Dojindo Laboratories, Kumamoto, Japan) and Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) reagents, and siRNA transfection was conducted using RNAiMAX reagent (Invitrogen).

2.2. Gene silencing and constitutive gene expression

A plasmid containing a *HSF1* shRNA expression construct and a puromycin-resistance gene was introduced into HeLa cells [19, 20]. Cells were cultured in medium supplemented with 1 μg/ml puromycin to select *HSF1*-silenced cells. For silencing

GADD45β, cells were transfected with *GADD45β* siRNA [21]. The coding regions of *GADD45α*, *GADD45α1*, *GADD45β*, and *GADD45γ* were amplified by RT-PCR from HeLa total RNA. The influenza hemagglutinin (HA)-tagged *GADD45* family members were cloned into pEBMulti-Hyg (Wako Pure Chemicals, Osaka, Japan). Cells constitutively expressing HA-GADD45 proteins were selected in medium supplemented with 100~300 μg/ml hygromycin B.

2.3. RT-PCR analysis

Total RNA was prepared from cells and analyzed by RT-PCR as described previously [19]. The primer sequences were as follows: *GADD45α*, 5'-TGGAGAGCAGAAGACCGA-3' and 5'-TTCCATTGAGATGAATGTGG-3'; *GADD45β*, 5'-GTTGATGAATGTGGACCCAG-3' and 5'-GTGAGGGTTCGTGACCAG-3'; *GADD45γ*, 5'-AGTCTTGAACGTGGACCC-3' and 5'-CCTCGTTGGGGTTCGAAA-3'; *HSP70*[19]; *GAPDH* [19].

2.4. Western blot analysis

Cell extracts were prepared and subjected to western blot analysis. The antibodies against HSF1 [19], cleaved caspase-3 (#9661, Cell Signaling Technology, Beverly, MA, USA), pan-JNK (#MAB1387, R&D Systems, Minneapolis, MN, USA), p38α (#612168, BD Biosciences, San Jose, CA, USA), phospho-JNK (Thr183/Tyr185) (#4668, Cell Signaling Technology; AF1205, R&D Systems), phospho-JNK (Thr183) (#ab124956, Abcam, Cambridge, UK), phospho-p38 (Thr180/Tyr182) (#612280, BD Biosciences), Myc-tag (#M192, MBL, Nagoya, Japan), HA-tag (#H9658, Sigma-Aldrich, St Louis, MO, USA), and GAPDH [19] were used.

2.5. Luciferase assay

The *GADD45β* promoter region was amplified by PCR from HeLa genomic DNA and cloned upstream of the firefly luciferase gene in the pGL3-Basic vector (Promega, Madison, WI, USA). Three copies of the AP-1 binding sequence were inserted upstream of the SV40 promoter of the pGL3-Promoter vector (Promega) [19]. The coding region of *MKK7* was amplified by RT-PCR. A constitutively active form of MKK7 (caMKK7) containing S287E, T291E, and S293E mutations was fused to the Myc and six-histidine (MycHis)-tag and cloned into pcDNA3.1(+) (Invitrogen). Firefly luciferase activity (arbitrary units) was calculated after the normalization to *Renilla* luciferase values as described previously [19, 20].

2.6. Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation analysis was performed as described previously [19, 20]. The primer sequences were as follows: *GADD45β*, 5'-TGGAAGGATTTCGCTGCTTC-3' and 5'-CGGCTACTTACTTCTGCGC-3'; *HSP70*, 5'-CCATGGAGACCAACACCCT-3' and 5'-CCCTGGGCTTTTATAAGTCG-3'.

2.7. His-tag pull-down assay

MycHis-tagged *MKK4*, *MKK7*, and *JNK1β1* were cloned into pcDNA3.1(+) (Invitrogen). Cells were transiently transfected with HA-GADD45 expression constructs and MAPK-MycHis expression constructs. Cells were lysed in IP buffer (20 mM Tris-Cl, pH 7.6, 0.1 M NaCl, 0.5% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Nacarai Tesque, Kyoto, Japan)). Extracts were adjusted to 7.5 mM imidazole and incubated with Ni-NTA agarose beads (Qiagen, Hilden, Germany) for 40 min. The agarose beads were washed with IP buffer containing 10 mM imidazole, and bound proteins were subjected to western blot analysis with antibodies recognizing Myc- and HA-tags.

2.8. In vitro kinase assay

The coding regions of *GADD45α*, *GADD45α1*, *GADD45β*, *GADD45γ*, and *JNK1β1* (containing a K55R amino acid substitution) were cloned into the glutathione *S*-transferase (GST) gene fusion vectors pGEX-2T and pGEX-6P-1 (GE Healthcare, Buckinghamshire, UK). GST-fusion proteins were purified by glutathione-Sepharose 4B (GE Healthcare) and were eluted by the addition of reduced glutathione. MKK7-MycHis was expressed in HeLa cells and immunoprecipitated using an anti-Myc antibody and protein A-Sepharose CL-4B beads (GE Healthcare). The immobilized kinases were mixed with GST-GADD45 in a buffer containing 20 mM Tris-Cl (pH 7.6) and 10 mM $MgCl_2$ at 30°C for 20 min. Then, GST-JNK1, ATP (final concentration of 0.5 mM), and dithiothreitol (final concentration of 1 mM) were added, and the reaction mixture was incubated for 20 min. The samples were subjected to western blot analysis with antibodies recognizing Thr183 phosphorylated JNK.

2.9. Statistical analysis

The data are representative of at least three independent experiments. Significant differences were determined by Student's *t*-test.

3. Results

3.1. Expression of GADD45 family genes is induced in response to various stresses

HeLa cells were used for this study because the heat shock response is conserved in many cell types and because HeLa cells are widely used for heat shock studies. We first analyzed the effect of heat stress on the expression of GADD45 family genes (Fig. 1A). RT-PCR results demonstrated that *GADD45β* and *GADD45γ* and the heat shock protein gene *HSP70* mRNA levels were robustly increased upon heat shock at 42.5°C for 1 h and

maintained at higher levels for at least 2 h recovery. Regarding *GADD45α*, longer PCR products that corresponded to the expected length were detected under both non-heat shock and heat shock conditions. The shorter PCR product *GADD45α1*, which lacked the second exon of *GADD45α*, possibly resulted from alternative splicing [22], and these products were observed in heat-shocked cells. Therefore, GADD45 family members are heat-inducible genes.

GADD45α and *GADD45α1* mRNA levels were significantly increased when cells were exposed to various stress inducers, including hydrogen peroxide (an oxidative stress inducer), tunicamycin (an endoplasmic reticulum stress inducer), and methyl methanesulfonate (MMS, a DNA damage inducer) (Fig. 1B). Exposure to hydrogen peroxide and tunicamycin resulted in a slight induction of *GADD45β* and *GADD45γ*, whereas the expression of both genes was substantially induced by MMS treatment. These results indicate that GADD45 expression is upregulated under DNA damage conditions and is differentially upregulated under diverse proteotoxic conditions.

Heat shock factor HSF1 is a heat-inducible transcription factor for various HSP and non-HSP genes [17]. It has been shown that HSF1 regulates the expression of *GADD45α* thorough interaction with p53 [23]. In heat-shocked cells, mRNA accumulation of *GADD45β* but not *GADD45γ* was inhibited when HSF1 was silenced by shRNA, suggesting that HSF1 is a transcriptional regulator of *GADD45β* (Fig. 1C). The HSF1-binding sequence, termed heat shock element (HSE), is inverted repeats of the 5-bp sequence NGAAN, and these sequences are found downstream of the transcription initiation site of *GADD45β* (Fig. 1D). The luciferase activity of a reporter construct containing the *GADD45β* promoter region from -564 to +216 was increased 2.5-fold in heat-shocked cells; however, the nucleotide substitution mutations in the putative HSE caused a significant decrease in the activity without changing basal/uninduced levels (Fig. 1E). The HSE-dependent heat shock response was also observed

using reporter constructs containing the *GADD45β* core promoter from -65 to +216. The binding of HSF1 to the *GADD45β* promoter was examined by chromatin immunoprecipitation analysis. As shown in Fig. 1F, the promoter fragment spanning the *GADD45β* HSE was occupied by HSF1 in heat-shocked cells. These results demonstrate that heat-induced expression of *GADD45β* is mediated by HSF1 through binding to the HSE.

3.2. GADD45β is involve in cell survival under heat shock conditions

To elucidate the function of heat-induced GADD45β, we analyzed the ability of *GADD45β* knockdown cells to survive heat stress. Transfection of *GADD45β* siRNA caused partial *GADD45β* silencing as assessed by RT-PCR (Fig. 2A). Silencing GADD45β did not affect cell proliferation under normal physiological conditions (data not shown). Cells were subjected to heat shock at 42.5°C for 90 min, and the number of viable cells was determined after recovery at 37°C (Fig. 2B). The number of control cells was unchanged after a 1-day period, suggesting the arrest of cell proliferation. In contrast, the number of *GADD45β*-silenced cells was reduced to ~70% of the initial cell number. From days 1 to 2, the numbers of control and silenced cells were increased 1.8- and 1.9-fold, respectively, which correspond to the normal growth rate of HeLa cells. These results indicate that GADD45β is necessary for maintaining cell viability following heat shock.

Heat stress results in induction of cellular apoptosis and necrosis [11, 12]. We examined the activation of caspase-3 by western blot analysis (Fig. 2C). A small amount of activated, cleaved caspase-3 was detected in heat-shocked control cells, and the levels were notably elevated in *GADD45β*-silenced cells. This result implicates GADD45β in the inhibition of heat activation of caspase-3.

3.3. GADD45 suppresses heat- and MMS-induced JNK activation

GADD45 regulates the MAPK pathway, and stress-induced activation of JNK and p38 is involved in caspase activation and cell death [1, 2, 14, 15]. JNK was activated upon heat shock at 42.5°C for 60 min, as judged by the increase in the dually phosphorylated forms of JNK as assessed by western blot analysis (Fig. 3A). The levels of phosphorylated JNK decreased during the 1- and 2-h recovery periods at 37°C. In heat-shocked cells, siRNA-mediated knockdown of *GADD45β* increased the phosphorylated forms of JNK and sustained phosphorylation without changing the total amounts of JNK. In contrast, the levels of phosphorylated JNK were decreased by the expression of HA-tagged GADD45β compared with control cells (Fig. 3B). Overexpression of HA-GADD45α, HA-GADD45α1, and HA-GADD45γ also caused low-level activation of JNK. JNK activity was induced by treatment of cells with MMS (Fig. 3C). Similarly, GADD45 proteins suppressed MMS-induced phosphorylation of JNK. These results demonstrate that the GADD45 family members negatively regulate JNK under heat and MMS stress conditions. Of note, in heat-shocked cells, the levels of phosphorylated and activated p38 were slightly elevated in *GADD45β*-silenced cells and were slightly reduced in cells overexpressing HA-GADD45 proteins; however, the effects were very minimal (Fig. 3A and B).

3.4. GADD45 inhibits MKK7 activity

To explore how GADD45 suppresses stress-induced phosphorylation of JNK, we examined physical interactions of GADD45β with JNK1 and upstream MAP2Ks, MKK4 and MKK7. When HA-GADD45β was expressed in cells alone or in combination with MycHis-tagged kinases, HA-GADD45β was pulled down by Ni-NTA beads only in the presence of MKK7-MycHis (Fig. 4A). Similar experiments were conducted using HA-tagged GADD45 family proteins, and binding of MKK7 to GADD45α, GADD45α1, and GADD45γ was observed (Fig. 4B).

The above findings implied that the interaction of MKK7 with GADD45 proteins inhibited the kinase activity toward JNK. We then tested whether GADD45 inhibits MKK7 activity under normal physiological conditions. JNK phosphorylation was induced when constitutively active MKK7-MycHis was expressed in cells (Fig. 4C). Co-expression of HA-GADD45β resulted in a substantial decrease in phosphorylated JNK. JNK phosphorylates c-Jun, and activated c-Jun induces gene expression through binding to the AP-1 regulatory site [15]. Using reporter gene assays, constitutively active MKK7-MycHis increased the luciferase activity of the construct containing the AP-1 site presumably through the MKK7->JNK->c-Jun->reporter gene expression pathway (Fig. 4D). Co-expression of HA-GADD45 proteins reduced MKK7-induced luciferase activity, whereas HA-GADD45β failed to affect the reporter gene expression in the absence of the AP-1 site. These results suggest that GADD45 inhibits MKK7 kinase activity toward JNK.

We conducted in vitro kinase assays to assess the effect of GADD45 on phosphorylation of JNK at threonine 183, which is preferentially phosphorylated by MKK7 [16]. The purified substrate GST-JNK1 was phosphorylated by MKK7-MycHis, which was immunoprecipitated from cells only in the presence of ATP (Fig. 4E). In the kinase reaction, purified GST-GADD45β inhibited GST-JNK1 phosphorylation in a dose-dependent manner. GST-tagged GADD45α, GADD45α1, and GADD45γ also blocked JNK1 phosphorylation by MKK7 (Fig. 4F). Thus, GADD45 binds to MKK7 and inhibits JNK1 phosphorylation.

4. Discussion

Expression of all GADD45 family members is induced in response to heat shock and genotoxic stress caused by the DNA alkylating agent MMS. GADD45α expression is also induced under oxidative stress and endoplasmic reticulum stress conditions. Heat-induced expression of GADD45β is mediated by HSF1, and GADD45β is necessary for inhibition of

caspase activation and heat stress survival. All GADD45 members suppress JNK activation induced by heat and MMS. GADD45 proteins bind MKK7 and inhibit the kinase activity toward JNK, also under normal physiological conditions. These observations indicate that GADD45 essentially inhibits the MKK7-JNK pathway. In the stress response, we suggest that differentially expressed GADD45 family proteins fine-tune JNK activity to determine cell fate.

Evidence suggests that GADD45β physically binds to the JNK kinase MKK7 and inhibits its activity [24]. However, whether other GADD45 family members also modulate the MKK7 activity is not known. We have demonstrated that all four GADD45 proteins bind to MKK7 and function as a potent inhibitor of the kinase. The central region of GADD45β is necessary for binding and inactivation of MKK7 [25, 26]. This region is well conserved among GADD45 family proteins, including GADD45α1. It is possible that GADD45α, GADD45α1, and GADD45γ inhibit MKK7 via a similar mechanism as GADD45β. In this context, it is noteworthy that all GADD45α, GADD45β, and GADD45γ interact with various proteins, including CDK1, p21, MEKK4, and PCNA, and the central region mediating dimerization of GADD45 proteins is involved in the binding to these proteins [7, 27-29]. Three members are inhibitors of CDK1/Cyclin B1; however, the regulatory mechanisms are slightly different [28]. GADD45α1 also interacts with CDK1 but more weakly than GADD45α [22]. GADD45 proteins therefore serve similar but not identical functions. Four members essentially suppress the MKK7-JNK pathway through MKK7 inactivation not only under stress conditions but also under physiological conditions. In embryonic development, the gene expression patterns of GADD45 family members differ [30]. GADD45 expression is deregulated in various types of cancer [1, 10]. The balance of GADD45 proteins and the interactions with MKK7 have a pivotal role in cell fate determination.

Heat stress leads to cell cycle arrest and apoptosis, where the JNK and p38 pathways

are activated. GADD45β knockdown resulted in sustained activation of JNK, caspase-3 activation, and cell death, suggesting that GADD45β is necessary for suppression of JNK activation and heat stress survival. Sustained activation of JNK is critical for the induction of apoptosis [31, 32]. Long exposure to heat stress but not short exposure induces persistent activation of JNK and causes apoptotic cell death [33]. In the tumor necrosis factor α (TNFα) signaling pathway, GADD45β inhibits continuous activation of JNK and thus antagonizes TNFα-induced cell killing [24, 34]. Heat shock activates MKK4 and MKK7, which phosphorylate the tyrosine and threonine residues of JNK, respectively. Both kinases contribute to synergistic activation of JNK, suggesting that inactivation of MKK7 by GADD45 proteins is sufficient to suppress the JNK activation [16, 35]. One may argue that interactions with other JNK cascade kinases, such as MEKK4, are relevant to GADD45-mediated regulation of JNK activation [5]. However, in mouse cells, MEKK4 is not the major MAP3K of the heat shock response [36]. In HeLa cells, silencing MEKK4 by siRNA did not affect heat-induced phosphorylation of JNK (data not shown). Heat and MMS stresses induce the expression of all GADD45 family members, and overexpression of these proteins downregulates the JNK pathway. In the heat shock response, the regulation of JNK activity duration by GADD45 proteins is critical for cell death and survival.

HSF1 is the major transcriptional activator of heat-inducible chaperone genes. HSF1 also activates the expression of the non-chaperone protein GADD45β, which functions as a negative regulator of MKK7. These results do not exclude the possibility that GADD45 proteins suppress cell death that is not dependent on the JNK pathway. GADD45α protects hematopoietic cells from UV-induced apoptosis through activation of p38 and the survival transcription factor NF-κB [37]. Upon oxidative stress induced by arsenite exposure, GADD45β protects cells from apoptosis via promoting degradation of the key apoptosis regulator p53 [38]. Rather, our observations connect stress-regulated gene expression and

signal transduction pathways.

Conflict of interest

Authors declare no conflict of interest.

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Figure Legends

Fig. 1. Effect of various stresses on the expression of GADD45 family genes.

(A) Heat-induced expression of *GADD45*. Cells cultured at 37°C (C) were heat-shocked at 42.5°C for 1 h and allowed to recover (R) at 37°C for 0, 1, 2, and 4 h. Total RNA prepared from the cells was subjected to RT-PCR. The closed (α) and open (α1) triangles indicate the *GADD45α* transcripts with or without the second exon. A representative data of five independent experiments is shown.

(B) Expression of *GADD45* under various stress conditions. Cells cultured under physiological conditions (C) were treated with 0.2 mM hydrogen peroxide (HP), 2.5 μg/ml tunicamycin, or 100 μg/ml MMS for 4 h. Total RNA prepared from the cells were subjected to RT-PCR. A representative data of five independent experiments is shown.

(C) Heat-induced expression of *GADD45β* and *GADD45γ* in HSF1-silenced cells. Cells were stably transfected with HSF1 shRNA expression plasmid (Kd) or scrambled shRNA expression plasmid (Scr). The left panel presents western blot analysis of HSF1. The relative levels of HSF1 are indicated. In HSF1-silenced cells, the levels of HSF1 were reduced to 18±6% of control cells in three independent experiments ($P < 0.05$). The right panel presents the results of RT-PCR analysis of *GADD45* mRNA. Cells cultured at 37°C (C) were heat-shocked at 42.5°C for 40 min (HS). A representative data of three independent experiments is shown.

(D) Heat shock element in the *GADD45β* promoter. Nucleotide sequence of the *GADD45β* promoter from +73 to +102 is shown. HSE is indicated by bold upper case letters. Nucleotide substitution mutations are also presented.

(E) *GADD45β* promoter analysis. Thick lines with numbers indicate the *GADD45β* promoter and nucleotide positions. Squares represent the HSE, and nucleotide substitution mutations in the HSE are presented as 'X'. Cells transfected with the reporter constructs were cultured at 37°C (control), exposed to 42.5°C for 60 min and allowed to recover at 37 °C for 4.5 h (heat shock). Firefly luciferase activities (arbitrary units) are expressed as the mean ± SE of three independent experiments (*$P < 0.05$).

(F) HSF1 binding to the *GADD45β* promoter. Cells cultured at 37°C (C) were heat-shocked at 42.5°C for 40 min (HS). Chromatin samples were immunoprecipitated with control (α-Cont) and HSF1 (α-HSF1) antibodies, and DNA in the input and immunoprecipitated samples were analyzed by PCR. A representative data of three independent experiments is shown.

Fig. 2. Effect of *GADD45β* silencing on cell survival under heat shock conditions.

(A) Silencing of *GADD45β.* Cells transfected with *GADD45β* siRNA (Kd) or scrambled siRNA (Scr) were heat-shocked at 42.5°C for 60 min and allowed to recover at 37°C for 1.5 h (R1.5). Total RNA prepared from the cells was subjected to RT-PCR. The relative levels of *GADD45β* mRNA are indicated. In *GADD45β*-silenced cells, the levels of *GADD45β* mRNA were reduced to 43±4% of control cells in three independent experiments ($P < 0.05$).

(B) Proliferation of *GADD45β*-silenced cells under heat shock conditions. Control (Scr) and *GADD45β*-silenced (Kd) cells were exposed to 42.5°C for 90 min and recovered at 37°C for 1 and 2 days. The number of viable cells was counted, and the relative cell number was determined by normalization to the initial cell number. Data are expressed as the mean ± SE of four independent experiments ($*P < 0.01$).

(C) Caspase-3 activation in heat-shocked cells. Control (Scr) and *GADD45β*-silenced (Kd) cells were exposed to 42.5°C for 90 min and recovered at 37°C for 6 h, and cleaved caspase-3 was analyzed by western blotting. The relative levels of cleaved caspase-3 are expressed as the mean ± SE of three independent experiments ($*P < 0.05$).

Fig. 3. Effect of GADD45 family members on JNK activation.

(A) Heat-induced phosphorylation of JNK in *GADD45β*-silenced cells. Control (Scr) and *GADD45β* -silenced (Kd) cells were cultured at 37°C (C), exposed to 42.5°C for 60 min, and allowed to recover (R) at 37°C for 0, 1, and 2 h. Dually phosphorylated forms (p-JNK and p-p38) and total proteins (T-JNK and T-p38) were analyzed by western blotting. The positions of p54 and p46 JNK are presented. The relative levels of phosphorylated JNK (both p54 and p46) are expressed as the mean ± SE of three independent experiments ($*P < 0.05$).

(B) Heat-induced phosphorylation of JNK in GADD45-overexpressing cells. Cells were

transfected with pEBMulti-Hyg (-, empty vector), pEBMulti-HA-45α, pEBMulti-HA-45α1, pEBMulti-HA-45β, and pEBMulti-HA-45γ. Stably transfected cells were selected and exposed to 42.5°C for 60 min (R0), and cell extracts were subjected to western blot analysis.
(C) Methyl methanesulfonate-induced phosphorylation of JNK in GADD45-overexpressing cells. Cells cultured as in (B) were treated with 100 μg/ml MMS for 1 h, and extracts were subjected to western blot analysis.

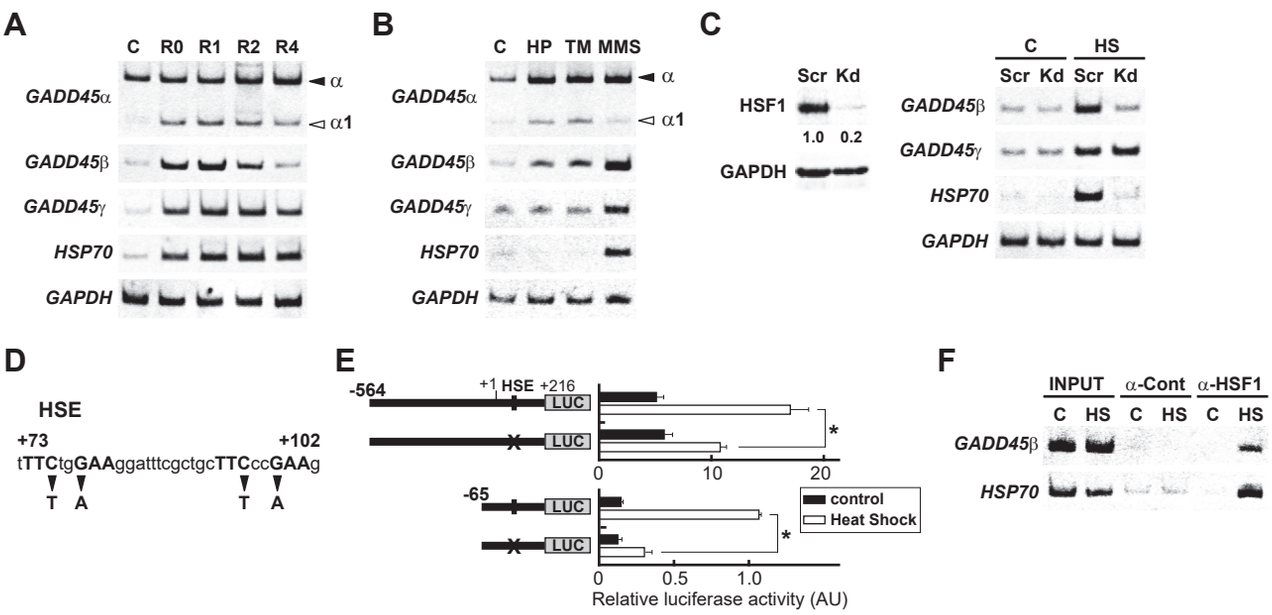
Fig. 4. Effect of GADD45 on JNK phosphorylation by MKK7.
(A) Binding of GADD45β to JNK cascade kinases. HA-GADD45β expression construct was transiently transfected along with MKK4-MycHis, MKK7-MycHis, or JNK1β1-MycHis expression constructs. Cell extracts were incubated with Ni-NTA agarose, and input and bound proteins were subjected to western blot analysis using anti-HA and anti-Myc antibodies. A representative data of three independent experiments is shown.
(B) Binding of GADD45 family proteins to MKK7. HA-GADD45 family expression constructs were transfected along with MKK7-MycHis expression construct. Cell extracts were incubated with Ni-NTA agarose, and input and bound proteins were subjected to western blot analysis. A representative data of three independent experiments is shown.
(C) JNK phosphorylation in cells expressing constitutively active MKK7 and GADD45β. Cells were transiently transfected with expression constructs of constitutively active caMKK7-MycHis and HA-GADD45β. Dually phosphorylated forms (p-JNK) and total proteins (T-JNK) were analyzed by western blotting. The positions of p54 and p46 JNK are presented. In cells expressing caMKK7-MycHis, the levels of phosphorylated JNK were increased 2.8±0.6-fold relative to control cells and reduced to 58±2% by co-expression of HA-GADD45β in three independent experiments ($P < 0.05$).
(D) Reporter gene expression in cells expressing constitutively active MKK7 and GADD45.

Luciferase reporter constructs with or without AP-1 binding sequences were transiently transfected along with caMKK7-MycHis and HA-GADD45 expression constructs. Firefly luciferase activities (arbitrary units) are expressed as the mean ± SE of five independent experiments. Asterisks indicate significant downregulation by GADD45 proteins (*$P < 0.05$).

(E) Inhibition of JNK phosphorylation by GADD45β in vitro. MKK7-MycHis kinase was mixed with GST-JNK1β1 substrate and the indicated amounts of GST or GST-GADD45β. Kinase reaction was initiated by the addition of ATP. JNK phosphorylation at Thr183 was analyzed by western blotting. A representative data of three independent experiments is shown.

(F) Inhibition of JNK phosphorylation by GADD45 proteins in vitro. The kinase reaction mixture contained MKK7-MycHis kinase, GST-JNK1β1 substrate, 100 ng of GST or GST-GADD45 (α, α1, β, and γ) fusion proteins, and ATP. JNK phosphorylation at Thr183 was analyzed by western blotting. A representative data of three independent experiments is shown.

Figure 1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α1
GADD45β
GADD45γ
HSP70
GAPDH
B
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α
α1
GADD45β
GADD45γ
HSP70
GAPDH
C
Scr Kd
HSF1
1.0 0.2
GAPDH
C HS
Scr Kd Scr Kd
GADD45β
GADD45γ
HSP70
GAPDH
D
HSE
+73
+102
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T A T A
E
-564
+1 HSE +216
LUC
LUC
*
0 10 20
-65
LUC
LUC
*
control
Heat Shock
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Relative luciferase activity (AU)
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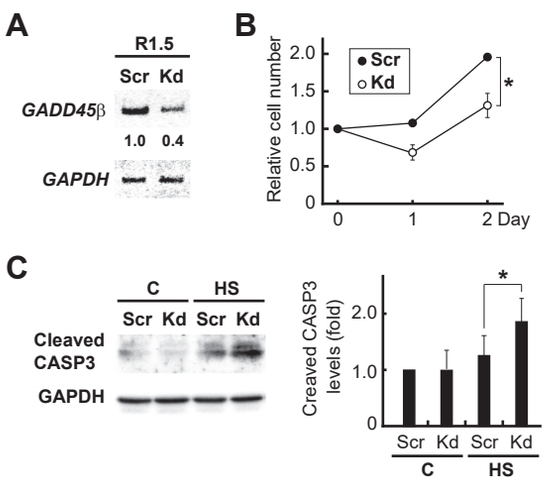
C HS C HS C HS
GADD45β
HSP70

Figure 2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GADD45β
1.0 0.4
GAPDH
B
Relative cell number
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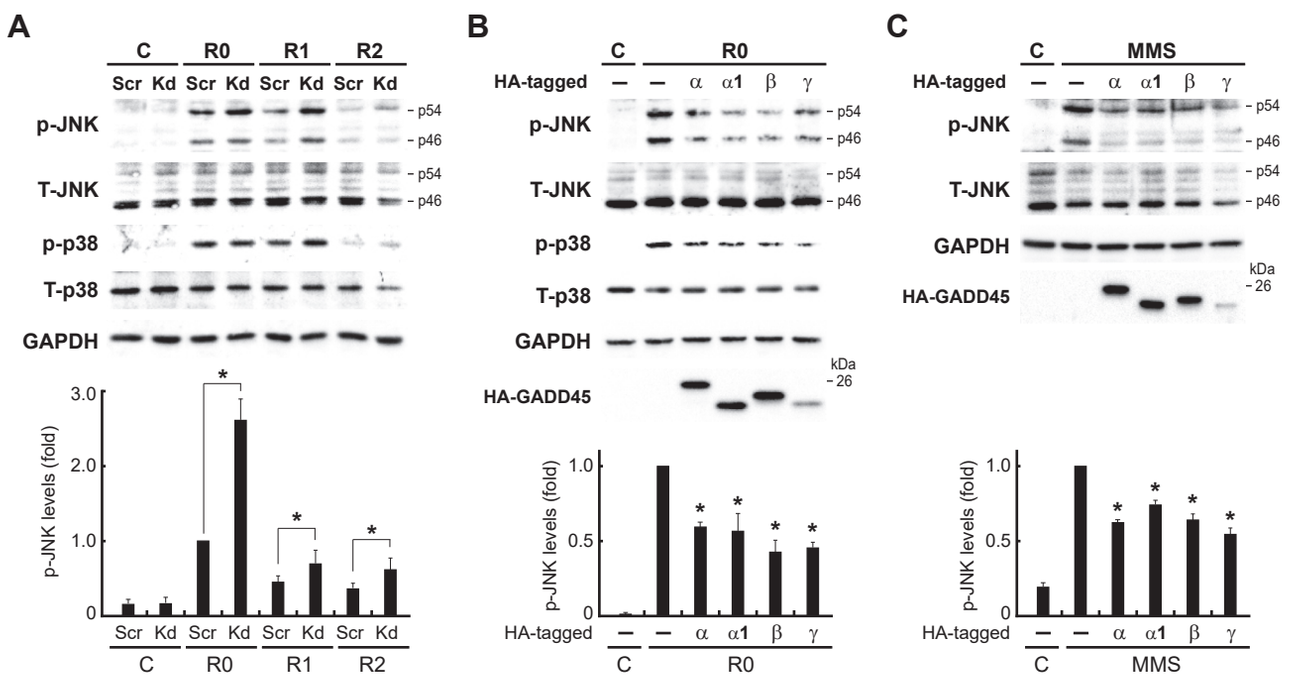
1.5
1.0
0.5
0
Scr
Kd
*
0 1 2 Day
C
C HS
Scr Kd Scr Kd
Cleaved CASP3
GAPDH
Creaved CASP3 levels (fold)
2.0
1.0
0
*
Scr Kd Scr Kd
C HS

Figure 3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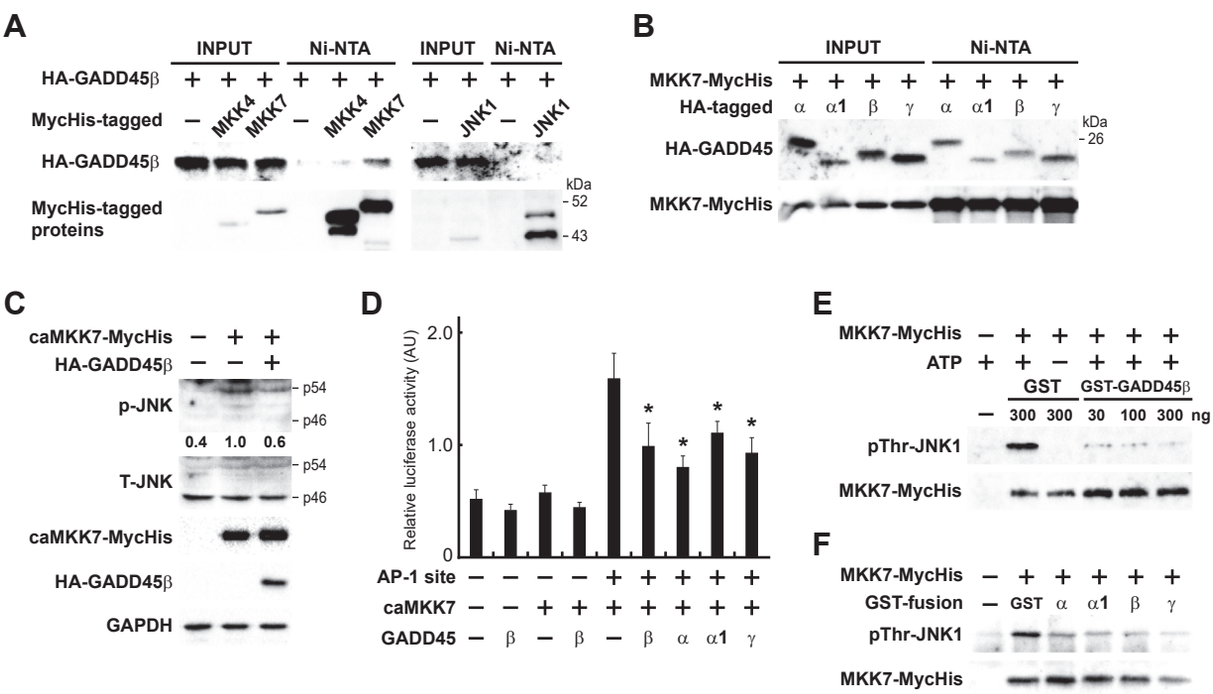
C
R0
R1
R2
Scr Kd Scr Kd Scr Kd Scr Kd
p-JNK
T-JNK
p-p38
T-p38
GAPDH
p54
p46
p-JNK levels (fold)
3.0
2.0
1.0
0
B
C
R0
HA-tagged
— — α α1 β γ
HA-GADD45
kDa
26
C
MMS

Figure 4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MKK7
MKK4
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JNK1
JNK1
HA-GADD45β
MycHis-tagged proteins
kDa
52
43
B
INPUT
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MKK7-MycHis
HA-tagged
α
α1
β
γ
α
α1
β
γ
HA-GADD45
MKK7-MycHis
kDa
26
C
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HA-GADD45β
p-JNK
p54
p46
0.4
1.0
0.6
T-JNK
p54
p46
caMKK7-MycHis
HA-GADD45β
GAPDH
D
Relative luciferase activity (AU)
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1.0
0
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α
α1
γ
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MKK7-MycHis
ATP
GST
GST-GADD45β
300
300
30
100
300
ng
pThr-JNK1
MKK7-MycHis
F
MKK7-MycHis
GST-fusion
GST
α
α1
β
γ
pThr-JNK1
MKK7-MycHis