

# Aberrant, differential and bidirectional regulation of the unfolded protein response towards cell survival by 3'-deoxyadenosine

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The unfolded protein response (UPR) is involved in a diverse range of pathologies triggered by endoplasmic reticulum (ER) stress. Endeavor to seek selective regulators of the UPR is a promising challenge towards therapeutic intervention in ER stress-related disorders. In the present report, we describe aberrant, differential and bidirectional regulation of the UPR by 3'-deoxyadenosine (cordycepin) towards cell survival. 3'-Deoxyadenosine blocked ER stress-induced apoptosis via inhibiting the IRE1–JNK pro-apoptotic pathway. 3'-Deoxyadenosine also inhibited apoptosis through reinforcement of the pro-survival eIF2 $\alpha$  signaling without affecting PERK activity. It was associated with depression of GADD34 that dephosphorylates eIF2 $\alpha$ , and dephosphorylation of eIF2 $\alpha$  by salubrinal mimicked the anti-apoptotic effect of 3'-deoxyadenosine. Unexpectedly, although 3'-deoxyadenosine caused activation of eIF2 $\alpha$ , it inhibited downstream pro-apoptotic events including induction of ATF4 and expression of CHOP. Cooperation of adenosine transporter and A3 adenosine receptor, but not A1/A2 receptors, mediated the pluripotent effects of 3'-deoxyadenosine. In mice, ER stress caused activation of JNK, expression of CHOP and induction of apoptosis in renal tubules. The apoptosis was significantly attenuated by administration with 3'-deoxyadenosine, and it was correlated with blunted induction of JNK and CHOP in the kidney. These results disclosed atypical pro-survival regulation of the UPR by 3'-deoxyadenosine, which may be advantageous for the treatment of intractable, ER stress-related disorders.

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Endoplasmic reticulum (ER) stress-mediated tissue injury is implicated in a wide range of pathologies including cancers, infection, atherosclerosis, ischemia, neurodegenerative disorders and metabolic diseases such as diabetes mellitus.<sup>1</sup> Seeking selective regulators for the unfolded protein response (UPR) is a promising challenge towards control of ER stress and ER stress-related disorders. To date, however, little is known about biological substances that allow for selective manipulation of individual UPR pathways.

A number of pathophysiological insults lead to accumulation of unfolded proteins in the ER, that is, ER stress. Three major transducers for ER stress are located on the membrane of the ER; activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) and PKR-like ER kinase (PERK), all of which contribute to attenuation of stress conditions.<sup>2</sup> In response to ER stress, p90ATF6 transits to the Golgi where it is cleaved by the proteases S1P and S2P, yielding a free cytoplasmic domain (p50ATF6) that functions as a transcription factor. Similarly, activated IRE1 catalyzes removal of a small intron from X-box-

binding protein 1 (*XBP1*) mRNA. This splicing event creates a translational frameshift in *XBP1* to produce an active transcription factor. p50ATF6 and XBP1 subsequently bind to the ER stress response element (ERSE) and the UPR element (UPRE), leading to expression of target genes including (i) ER chaperones such as 78 kDa glucose-regulated protein (GRP78), GRP94 and 150 kDa oxygen-regulated protein (ORP150), and (ii) ER-associated degradation (ERAD) factors such as ER degradation enhancing  $\alpha$ -mannosidase-like protein 1 (Edem1). Activation of PERK leads to phosphorylation of eukaryotic translation initiation factor 2 $\alpha$  (eIF2 $\alpha$ ), which causes general inhibition of protein synthesis. This molecular event contributes to attenuation of ER stress, and salubrinal, an inhibitor of eIF2 $\alpha$  dephosphorylation, is considered as a potential therapeutic agent for the treatment of ER stress-related pathologies including viral infection.<sup>3,4</sup>

During the UPR, however, death signals, as well as survival signals, may also be transduced via PERK and IRE1.<sup>5</sup> For example, the PERK–eIF2 $\alpha$  pathway induces expression of

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**Abbreviations:** ER, endoplasmic reticulum; UPR, unfolded protein response; ATF6, activating transcription factor 6; IRE1, inositol-requiring enzyme 1; PERK, PKR-like ER kinase; XBP1, X-box-binding protein 1; ERSE, ER stress response element; UPRE, UPR element; GRP78, 78 kDa glucose-regulated protein; ORP150, 150 kDa oxygen-regulated protein; ERAD, ER-associated degradation; Edem1, ER degradation enhancing  $\alpha$ -mannosidase-like protein 1; eIF2 $\alpha$ , eukaryotic translation initiation factor 2 $\alpha$ ; CHOP, CCAAT/enhancer-binding protein-homologous protein; GADD34, growth arrest and DNA damage gene 34; ASK1, apoptosis signal-regulating kinase 1; JNK, c-Jun N-terminal kinase; DTT, dithiothreitol; SubAB, subtilase cytotoxin; DPSPX, 1,3-dipropyl-8-(*p*-sulfophenyl)xanthine; NBT1, S-(4-nitrobenzyl)-6-thioinosine; SEAP, secreted alkaline phosphatase; SV40, simian virus 40; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EF-1 $\alpha$ , elongation factor-1 $\alpha$ ; CMV, cytomegalovirus; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; FBS, fetal bovine serum; DAPI, 4',6-diamidino-2-phenylindole; XBP1(U), unspliced form of *XBP1*; XBP1(S), spliced form of *XBP1*

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pro-apoptotic CCAAT/enhancer-binding protein-homologous protein (CHOP) through the ATF4—the amino acid response element pathway.<sup>6</sup> The PERK—eIF2 $\alpha$ —ATF4 pathway also induces expression of another pro-apoptotic molecule, growth arrest and DNA damage gene 34 (GADD34).<sup>7</sup> On the other hand, activation of IRE1 results in recruitment and activation of apoptosis signal-regulating kinase 1 (ASK1) and downstream c-Jun N-terminal kinase (JNK), both of which are involved in a wide range of pro-apoptotic processes.<sup>8</sup> These signaling pathways offset the effects of the pro-survival UPR. Based on this current concept, development of agents that selectively reinforce the pro-survival UPR but suppress the pro-apoptotic UPR is an important challenge towards effective treatments for ER stress-related diseases.

3'-Deoxyadenosine, also called cordycepin, has been identified as a major bioactive metabolite in *Cordyceps militaris*.<sup>8,9</sup> It possesses a wide range of biological effects including anti-thrombotic, anti-cancer, anti-viral, anti-fungal and anti-inflammatory activities. In mice, administration of 3'-deoxyadenosine reduces tumor formation in a model of metastasis<sup>10</sup> and has been proposed as an anti-cancer drug. The anti-cancer activity of 3'-deoxyadenosine is, in part, ascribed to its anti-mitogenic effect<sup>10,11</sup>, but many recent studies suggested that induction of apoptosis is one of major mechanisms involved in the potential of 3'-deoxyadenosine against malignant tumors.<sup>12–15</sup> In contrast, anti-apoptotic properties of this agent have not been reported to date. Recently, we occasionally found that 3'-deoxyadenosine has the potential to modulate the UPR against ER stress-induced cell injury. In the present report, we provide evidence for aberrant, pro-survival regulation of the UPR by 3'-deoxyadenosine.

## Results

**Selective suppression of ER stress-induced apoptosis by 3'-deoxyadenosine.** Previous reports suggested that induction of apoptosis is one of major mechanisms underlying the anti-tumorigenic potential of 3'-deoxyadenosine at high concentrations.<sup>12–15</sup> However, we occasionally found that non-toxic doses of 3'-deoxyadenosine (~5  $\mu$ g/ml) suppress apoptosis triggered by ER stress. When NRK-52E rat renal tubular cells were treated with tunicamycin, cytoplasmic vacuolation was induced (Figure 1a, top middle). Further incubation caused shrinkage of the cytoplasm and membrane blebbing typical of apoptosis (Figure 1a, bottom middle). Concomitant treatment with 3'-deoxyadenosine suppressed these morphological changes (Figure 1a, right). Quantitative analysis showed that cellular death triggered by tunicamycin was significantly suppressed by 3'-deoxyadenosine (Figure 1b). The cell damage was reversed by 3'-deoxyadenosine in a dose-dependent manner (Figures 1c and d). The pro-survival effect was similarly observed in cells treated with thapsigargin, a different inducer of ER stress (Figures 1e and f). Hoechst staining (Sigma-Aldrich Japan, Tokyo, Japan) showed that nuclear fragmentation caused by tunicamycin or thapsigargin was suppressed by 3'-deoxyadenosine (Figures 1g and h), confirming inhibition of apoptotic cell death. Western blot analysis also exhibited that activation of caspase-3 was blocked by the treatment with 3'-deoxyadenosine (Figure 1i).

The similar cytoprotective effect was observed in other cell types including human lung epithelial cells BEAS-2B (Supplementary Figures S1A and S1B).

We examined selectivity of the anti-apoptotic potential of 3'-deoxyadenosine. Under TNF- $\alpha$ -stimulated condition, 3'-deoxyadenosine markedly enhanced TNF- $\alpha$ -induced cell injury (Figures 1j and k). Similarly, treatment with 3'-deoxyadenosine promoted cellular damage caused by cadmium and cigarette smoke extract (Figure 1j). Staurosporine-induced cellular damage was unaffected by 3'-deoxyadenosine. In contrast, like tunicamycin- and thapsigargin-induced apoptosis, cellular death triggered by other ER stress inducers (dithiothreitol (DTT) and subtilase cytotoxin (SubAB)) was suppressed by 3'-deoxyadenosine (Figures 1j and l). Of note, even after the full induction of the UPR, post-treatment with 3'-deoxyadenosine similarly suppressed morphological damage (Supplementary Figure S2A) and cellular death (Supplementary Figure S2B).

**Involvement of extracellular and intracellular actions in anti-apoptotic effect of 3'-deoxyadenosine.** Extracellular adenosine and adenine nucleotides control a variety of cell function.<sup>16–18</sup> Microscopic analysis showed that, in contrast to 3'-deoxyadenosine, ATP, ADP and adenosine did not inhibit thapsigargin-induced cell injury (Figure 2a). Quantitative analysis confirmed that ER stress-induced cellular death was not affected by the treatment with these adenosine-related substances (Figure 2b).

A previous report suggested that 3'-deoxyadenosine inhibits growth of tumor cells through binding to the A3 adenosine receptor.<sup>10</sup> We tested an effect of A3 receptor antagonist, MRS1523, on the pro-survival action of 3'-deoxyadenosine. As shown in Figures 2c–f, the anti-apoptotic effect of 3'-deoxyadenosine was abolished in the presence of MRS1523. In contrast, treatment with A1/A2 receptor antagonist 1,3-dipropyl-8-(*p*-sulfophenyl)xanthine (DPSPX) did not reverse the effect of 3'-deoxyadenosine even at high concentrations (Supplementary Figures S3A and S3B).

Adenosine and other adenine nucleosides are transported across the cell membrane via adenosine transporters. To investigate whether intracellular action also contributes to the pro-survival effect of 3'-deoxyadenosine, we tested an effect of adenosine transport inhibitor S-(4-nitrobenzyl)-6-thioinosine (NBTI). As shown in Figures 2g–j, the anti-apoptotic effect of 3'-deoxyadenosine was inhibited by NBTI. Treatment with NBTI alone did not induce any cellular injury.

**Effects of 3'-deoxyadenosine on the expression of endogenous ER stress markers.** To elucidate whether or not 3'-deoxyadenosine attenuates ER stress, its effects on the induction of endogenous ER stress markers were examined. Northern blot analysis revealed that 3'-deoxyadenosine markedly suppressed induction of *CHOP* and *GRP78* in a dose-dependent manner (Figure 3a). Similar suppressive effects were observed on the expression of other ER stress markers, *GRP94* and *ORP150* (Figure 3b). The suppressive effect of 3'-deoxyadenosine was also observed in cells exposed to SubAB, a more selective inducer of ER stress<sup>19</sup> (Figure 3c). This phenomenon was observed in different cell types including rat mesangial cells (Figures 3d and e) and mouse

3T3-L1 preadipocytes (our unpublished data). Consistent with the results shown in Supplementary Figures S2A and S2B, post-treatment with 3'-deoxyadenosine similarly downregulated the levels of *CHOP* and *GRP78* mRNAs (Supplementary Figure S4). The reduction in *CHOP* and *GRP78* was caused by transcriptional suppression, because degradation rate of these mRNAs was not influenced by 3'-deoxyadenosine (Figure 3f). Of note, 3'-deoxyadenosine did not affect expression levels of secreted alkaline phosphatase (*SEAP*) driven by the simian virus 40 (SV40) promoter (Supplementary Figure S5). Expression levels of endogenous housekeeping genes elongation factor-1 $\alpha$  (*EF-1 $\alpha$* ) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were not suppressed by 3'-deoxyadenosine (Supplementary Figure S6).

In contrast to 3'-deoxyadenosine, ATP, ADP and adenosine did not suppress the expression of *CHOP* and *GRP78* triggered by ER stress inducers (Figure 3g). Consistent with the results shown in Figure 2c–f, MRS1523 partially reversed the suppressive effects of 3'-deoxyadenosine on *CHOP* and *GRP78* (Figure 3h). DPSPX did not affect the effect of 3'-deoxyadenosine (Figure 3i). Furthermore, the suppressive effects of 3'-deoxyadenosine on *CHOP* and *GRP78* were also reversed partially by NBTI (Figure 3j). When cells were co-treated with MRS1523 and NBTI, the inhibitory effect of 3'-deoxyadenosine was abrogated (Figure 3k).

**Suppression of the IRE1 pathway by 3'-deoxyadenosine.** ER stress causes the UPR that involves

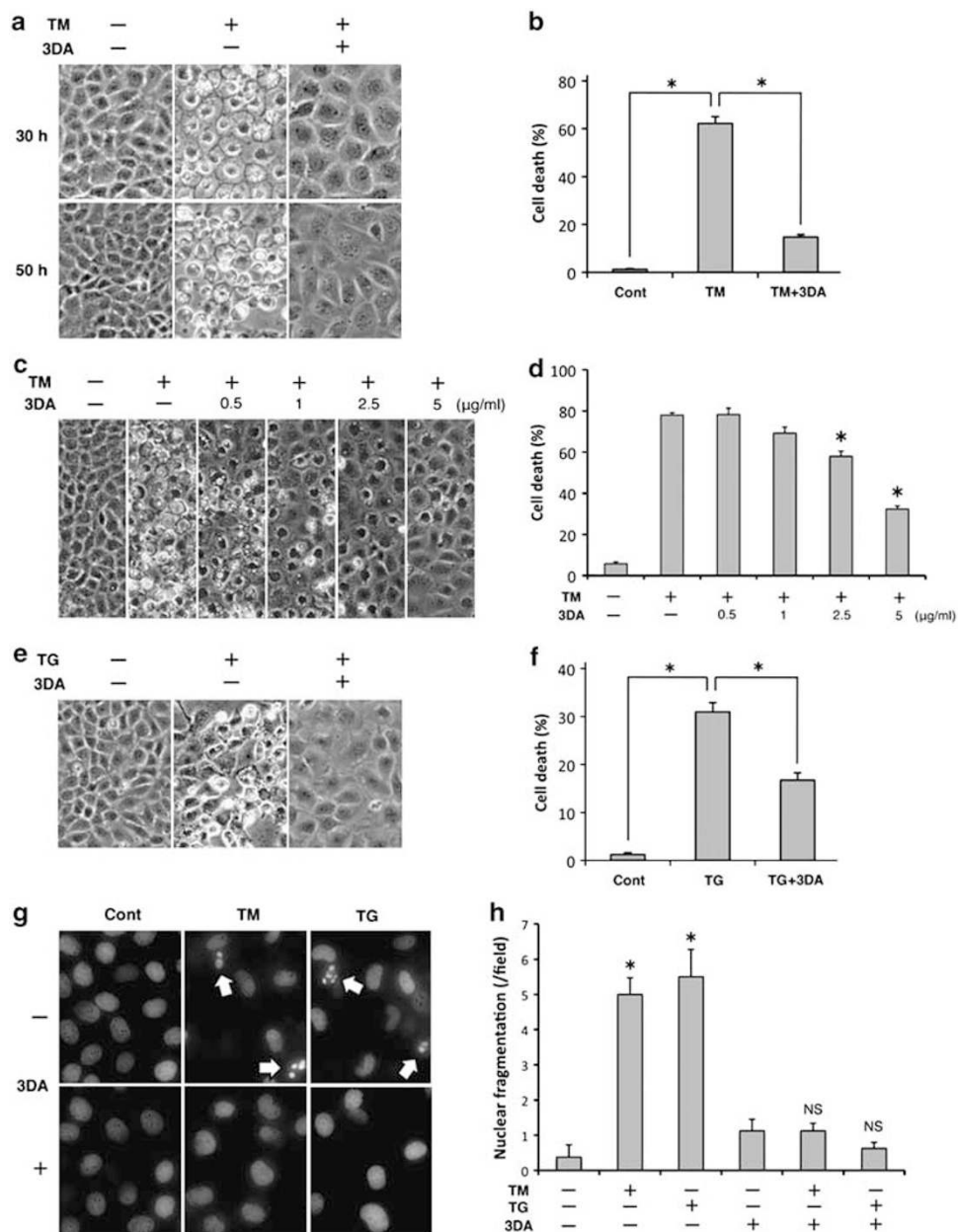
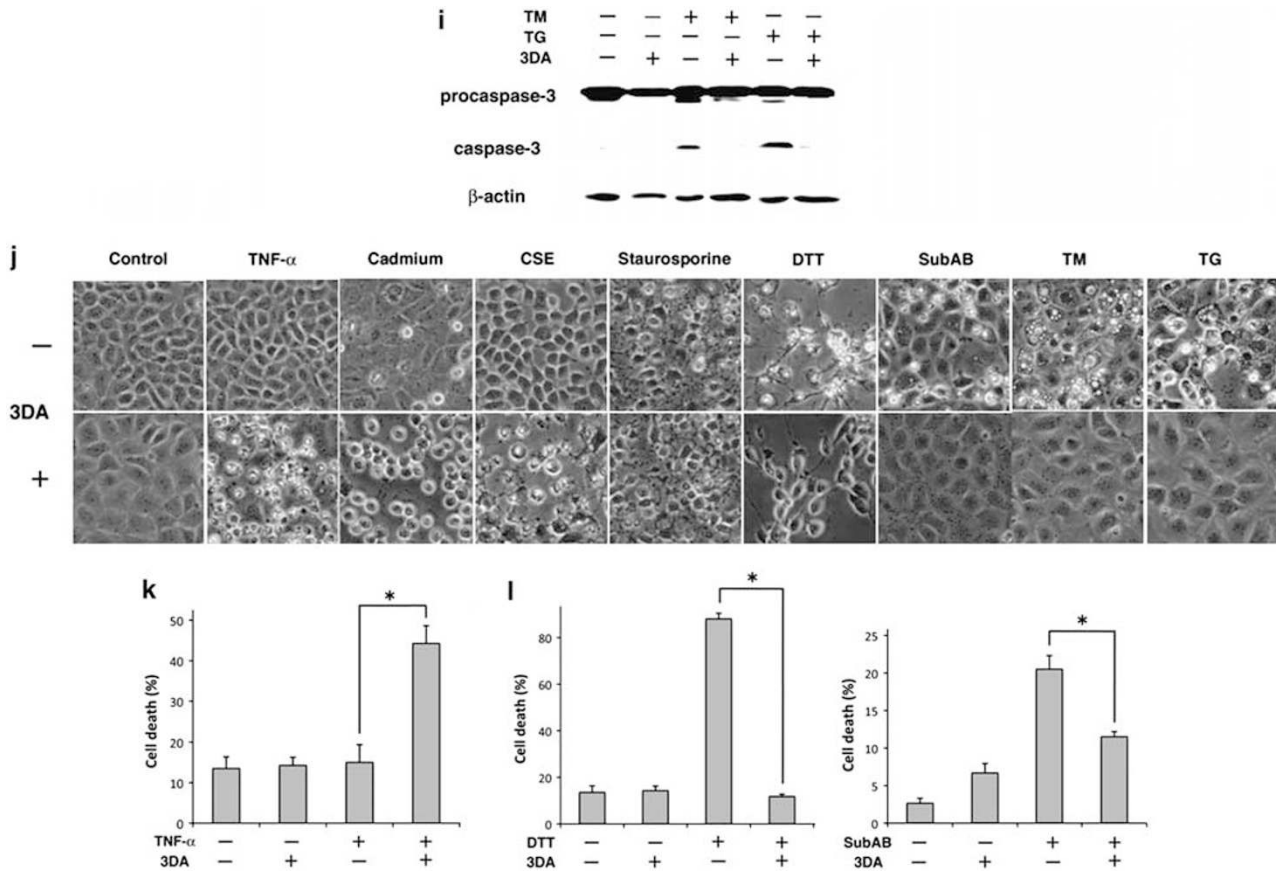


Figure 1 (Continued)



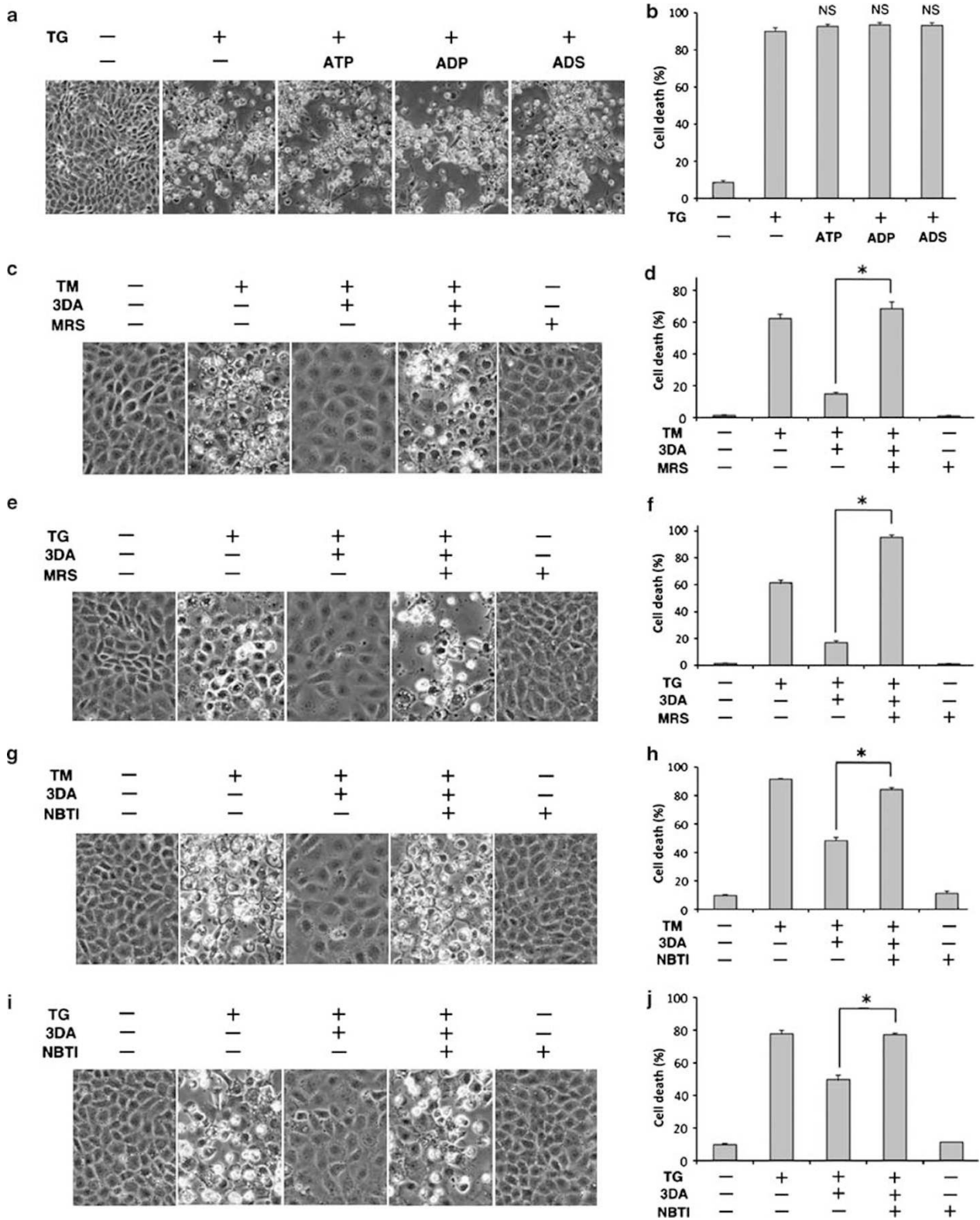
**Figure 1** Suppression of ER stress-induced apoptosis by 3'-deoxyadenosine. NRK-52E cells were treated with 2.5  $\mu$ g/ml tunicamycin (TM) (a–d) or 500 nM thapsigargin (TG) (e and f) in the absence (–) or presence (+) of 3'-deoxyadenosine (3DA; 5  $\mu$ g/ml) and subjected to phase-contrast microscopy (a, c and e). Both attached cells and detached cells were harvested separately and subjected to trypan blue analysis. The percentages of dead cells against total cells were evaluated (b, d and f). Assays were performed in quadruplicate, and data are presented as means  $\pm$  S.E. Asterisks indicate statistically significant differences ( $P < 0.05$ ). (g and h) Cells were exposed to TM or TG with or without 3DA and subjected to Hoechst staining. (g) fluorescent microscopy. Nuclear fragmentation is indicated by arrows. Cont, untreated control. (h) quantitative analysis of nuclear fragmentation. NS, not statistically significant. (i) Cells were treated with TM, TG and 3DA as indicated, and western blot analysis was performed to evaluate activation of caspase-3. The level of  $\beta$ -actin is shown at the bottom as a loading control. (j–l) NRK-52E cells were exposed to pro-apoptotic stimuli (10 ng/ml TNF- $\alpha$ , 20  $\mu$ M cadmium, 5% cigarette smoke extract (CSE), 200 nM staurosporine, 2 mM DTT, 50 ng/ml subtilase cytotoxin (SubAB), TM and TG) in the absence or presence of 3DA and subjected to phase-contrast microscopy (j) and assessment of cell death (k and l)

activation of IRE1, ATF6 and PERK–eIF2 $\alpha$  pathways. We first examined effects of 3'-deoxyadenosine on the activation of the IRE1 pathway. Splicing of *XBP1* mRNA was used as an indicator for IRE1 activation. As shown in Figure 4a, thapsigargin induced splicing of *XBP1* time-dependently, which was suppressed by the treatment with 3'-deoxyadenosine. Consistently, RT–PCR analysis showed that splicing of endogenous *XBP1* mRNA caused by tunicamycin and thapsigargin was inhibited by 3'-deoxyadenosine (Figure 4b). The suppression of IRE1 was further confirmed by blunted activation of UPRE and ERSE (Figures 4c and d). This inhibitory effect was not due to non-specific effects on the reporter system, because luciferase activity driven by the EF-1 $\alpha$  promoter and the cytomegalovirus (CMV) promoter/enhancer was not depressed by 3'-deoxyadenosine (Supplementary Figure S7). Similarly, induction of *Edem1*, a molecule downstream of the IRE1–XBP1 pathway,<sup>20</sup> was inhibited by 3'-deoxyadenosine (Figure 4e). Phosphorylation of JNK, another downstream indicator for IRE1 activation, was also suppressed by the treatment with 3'-deoxyadenosine (Figure 4f).

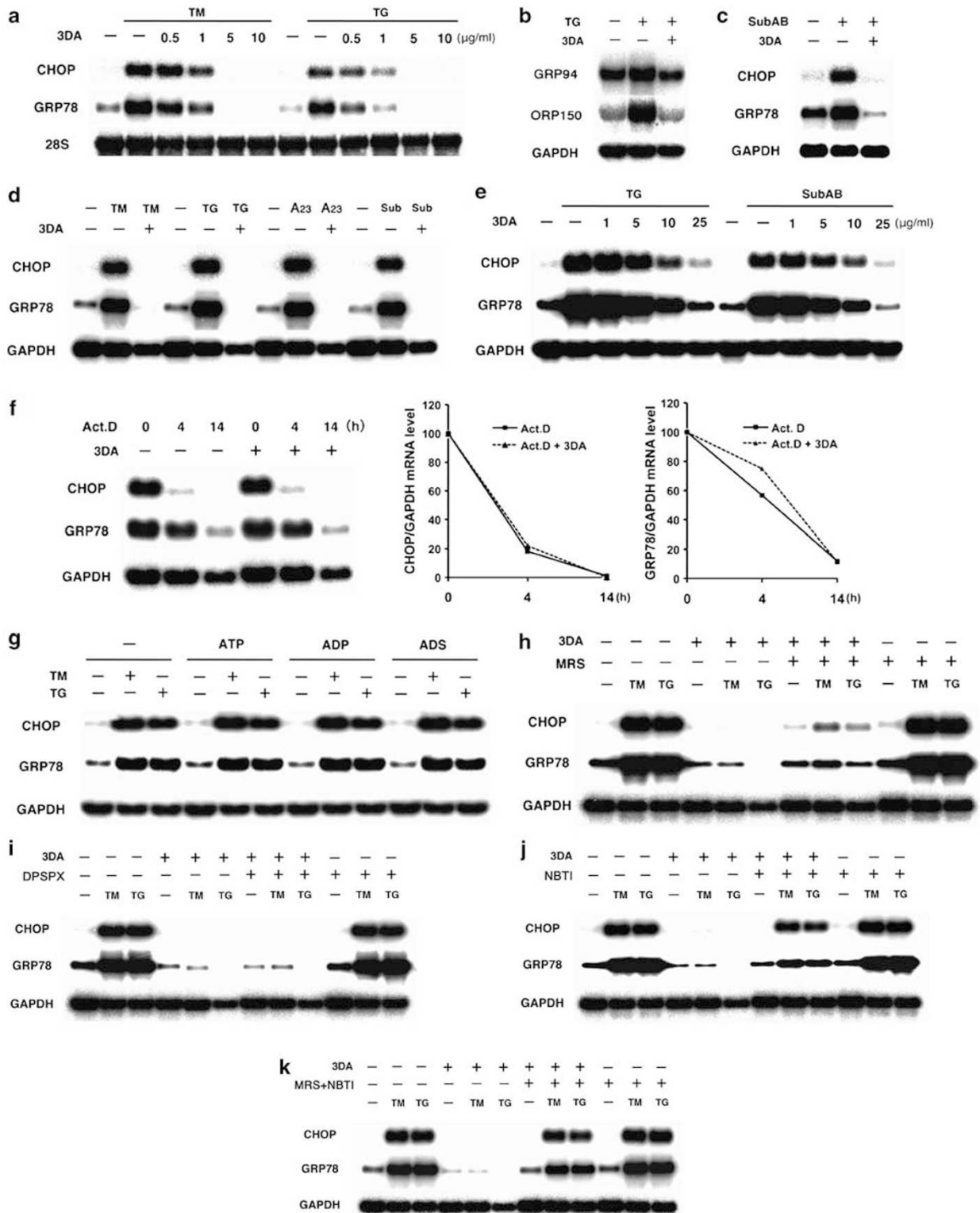
ERSE is activated not only by XBP1 but also by ATF6. Activation of the ATF6–ERSE pathway is the major mechanism underlying the induction of ER chaperones by ER stress.<sup>21</sup> Our data showed that 3'-deoxyadenosine suppressed ER stress-induced activation of ERSE and induction of *GRP78*, *GRP94* and *ORP150* (Figures 3a–e, 4d). These results indicate a possibility that the ATF6 pathway is also blocked by 3'-deoxyadenosine.

Consistent with the results of northern blot analysis, MRS1523 modestly and NBTI substantially reversed the suppressive effects of 3'-deoxyadenosine on the splicing of *XBP1* mRNA and activation of UPRE and ERSE (Figures 4g–i). In contrast, treatment with DPSPX had no impacts on the effects of 3'-deoxyadenosine.

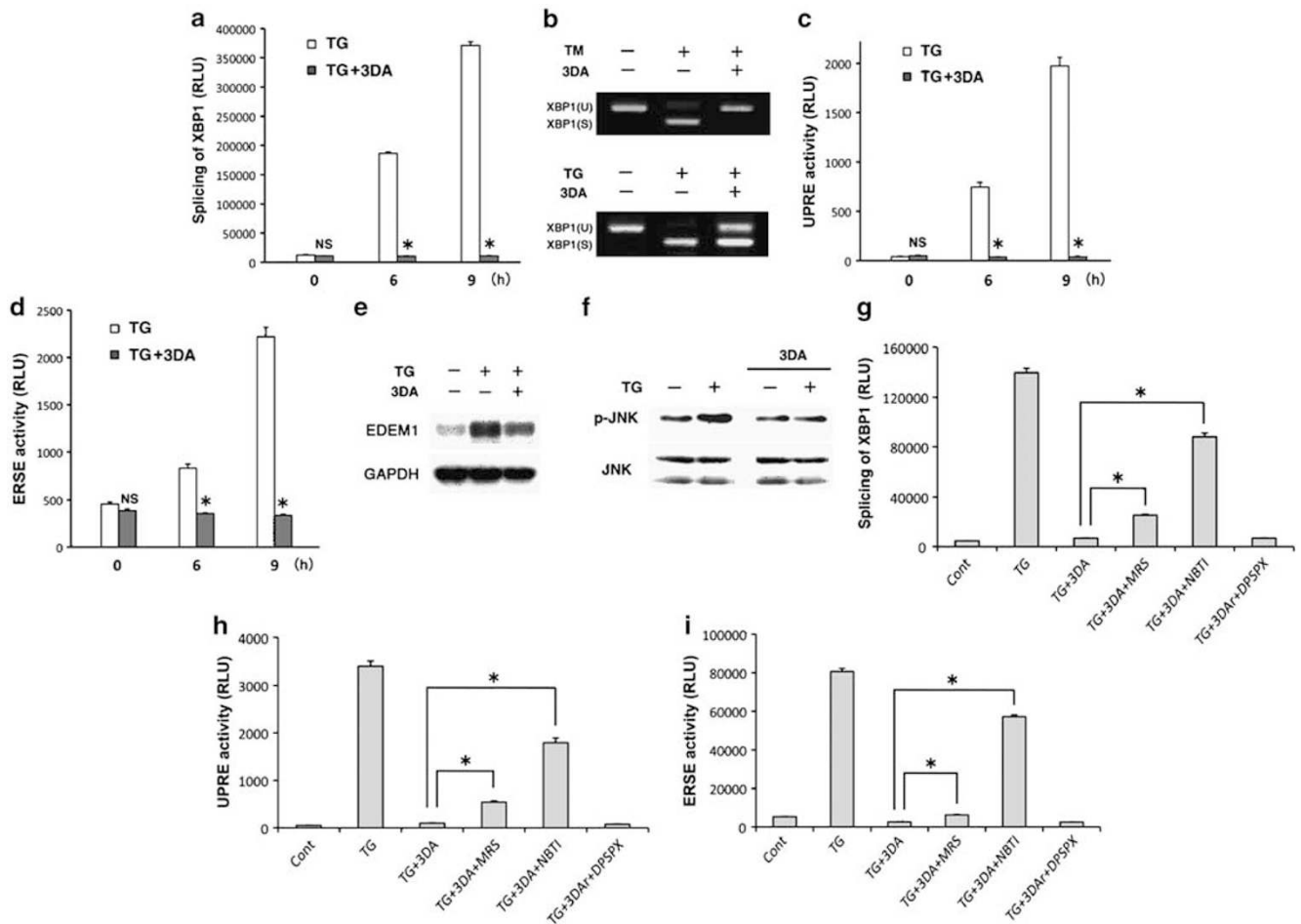
**Atypical regulation of the PERK–eIF2 $\alpha$  pathway by 3'-deoxyadenosine.** We further examined effects of 3'-deoxyadenosine on the activation of the PERK–eIF2 $\alpha$  pathway by ER stress. Western blot analysis showed that, unexpectedly, treatment with 3'-deoxyadenosine did not



**Figure 2** Involvement of extracellular and intracellular actions in the anti-apoptotic effect of 3'-deoxyadenosine. (a and b) NRK-52E cells were exposed to TG in the presence of ATP, ADP or adenosine (ADS) at 100  $\mu$ M and subjected to phase-contrast microscopy (a) and assessment of cell death (b). (c-f) Cells were treated with TM, TG and 3DA in the absence or presence of 5  $\mu$ M MRS1523 (MRS), and subjected to phase-contrast microscopy (c and e) and assessment of cell death (d and f). (g-j) Cells were exposed to TM or TG with or without 10  $\mu$ M NBTI and subjected to phase-contrast microscopy (g and i) and cell death analysis (h and j). \* $P$  < 0.05



**Figure 3** Effects of 3'-deoxyadenosine on the induction of ER stress markers. (a–c) NRK-52E cells were stimulated for 8 h with TM or TG (a and b) or 25 ng/ml SubAB (c) in the presence of 3DA, and expression of *CHOP* and *GRP78* (a and c) and *GRP94* and *ORP150* (b) was evaluated by northern blot analysis. Levels of 28S ribosomal RNA and *GAPDH* are shown at the bottom as loading controls. (a and e) SM43 mesangial cells were exposed to various inducers of ER stress (TM, TG, 500 nM A23187 (A23), SubAB) in the presence of 100  $\mu$ g/ml 3DA (d) or its serial concentrations (e), and induction of *CHOP* and *GRP78* was assessed. (f) Cells were treated with 5  $\mu$ g/ml actinomycin D (Act.D) in the absence or presence of 3DA for 4 and 14 h, and northern blot analysis was performed. The levels of *CHOP* and *GRP78* were normalized by the level of *GAPDH*, and relative levels of *CHOP* and *GRP78* mRNAs are shown in graphs. (g) Cells were exposed to TM or TG for 8 h in the absence or presence of ATP, ADP or ADS, and northern blot analysis was performed. (h–k) Cell were treated with TM, TG and 3DA in the presence of MRS (h), 10 nM DPSPX (i), NBTI (j) or MRS + NBTI (k) and subjected to northern blot analysis



**Figure 4** Suppression of the IRE1 pathway by 3'-deoxyadenosine. (a, c and d) NRK-52E cells transiently transfected with a XBP1 reporter plasmid (a), NRK/UPRE-Luc cells (c) and NRK/ERSE-Luc cells (d) were treated with TG for 6 h and 9 h in the absence or presence of 3DA and subjected to chemiluminescent assay. RLU, relative light unit. (b) Cells were treated with TM or TG for 4 h and subjected to RT-PCR analysis of *XBP1* mRNA. XBP1(U), unspliced form of *XBP1*; XBP1(S), spliced form of *XBP1*. (e) Cells were stimulated for 8 h with TG in the absence or presence of 3DA, and expression of *Edem1* was evaluated by northern blot analysis. (f) Cells were stimulated with TG for 15 min in the absence or presence of 3DA, and phosphorylation of JNK (p-JNK) was evaluated by western blot analysis. Total JNK protein is shown as a loading control. (g–i) NRK-52E cells transiently transfected with the XBP1 reporter plasmid (g), NRK/UPRE-Luc cells (h) and NRK/ERSE-Luc cells (i) were treated with indicated agents for 12 h and subjected to chemiluminescent assay. \* $P < 0.05$

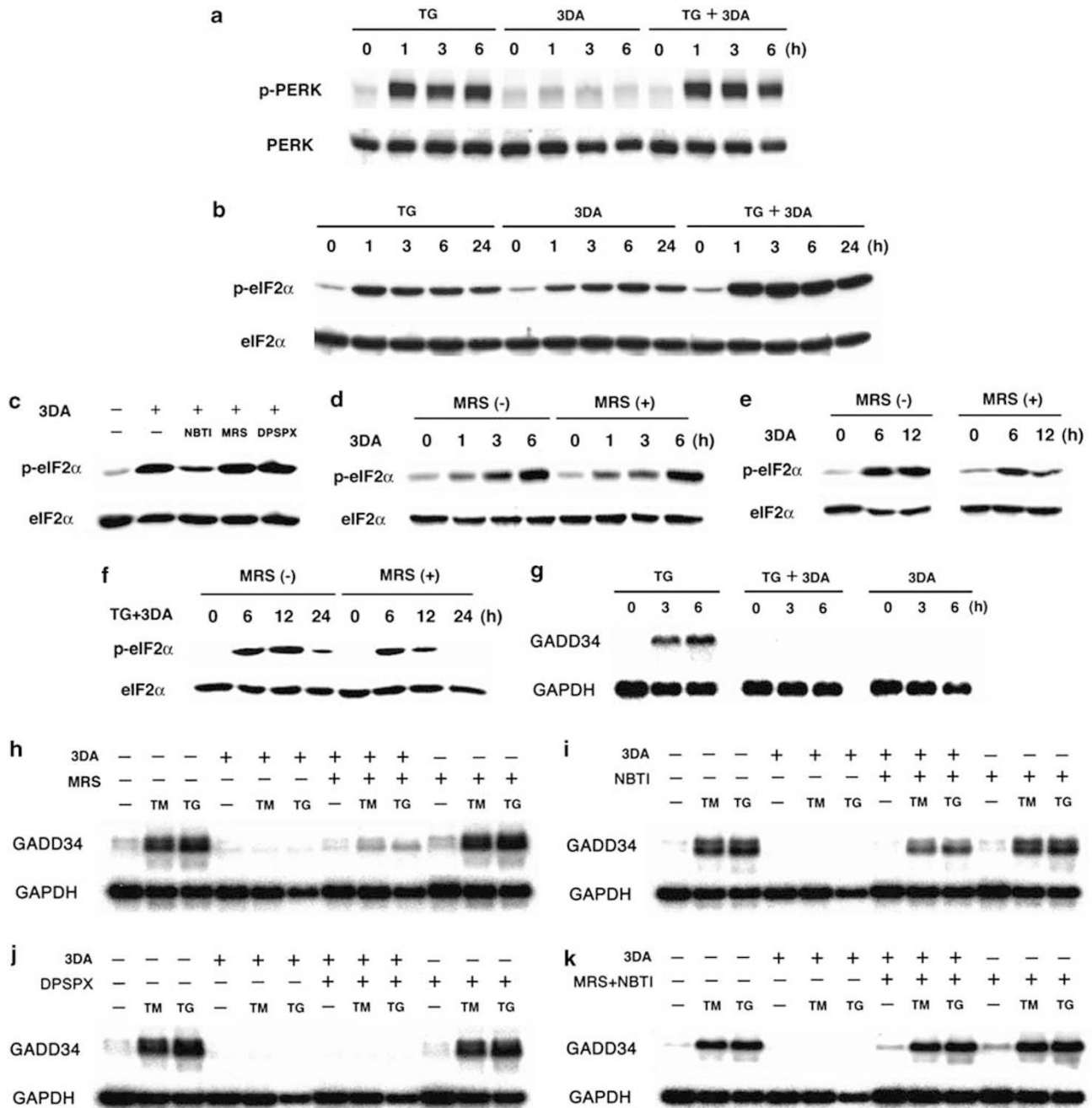
suppress the early phosphorylation of PERK by ER stress (Figure 5a). Furthermore, 3'-deoxyadenosine induced substantial phosphorylation of eIF2 $\alpha$  and rather enhanced thapsigargin-triggered eIF2 $\alpha$  phosphorylation (Figure 5b).

We examined roles of adenosine transporters and adenosine receptors in the phosphorylation of eIF2 $\alpha$ . Western blot analysis showed that treatment with NBTI, but not MRS1523 and DPSPX, suppressed activation of eIF2 $\alpha$  by 3'-deoxyadenosine (Figure 5c). The lack of involvement of the adenosine A3 receptor was further confirmed by a time-lapse experiment (Figure 5d). However, in the later phase (12–24 h), blockade of the A3 receptor reduced the phosphorylation level of eIF2 $\alpha$  (Figure 5e). This phenomenon was also observed when cells were treated with 3'-deoxyadenosine together with thapsigargin (Figure 5f).

GADD34 forms a functional complex with protein phosphatase 1 and causes dephosphorylation of eIF2 $\alpha$ .<sup>7,22</sup> To identify molecular events involved in the sustained phosphorylation of eIF2 $\alpha$  by 3'-deoxyadenosine, expression of *GADD34* was evaluated. Northern blot analysis showed that 3'-deoxyade-

nosine did not induce *GADD34* and rather suppressed induction of *GADD34* by ER stress (Figure 5g). Consistent with its regulation for *GRP78* and *CHOP*, suppression of *GADD34* required the A3 receptor and adenosine transporters, but not A1/A2 receptors (Figures 5h–j). Concomitant blockade of the A3 receptor and adenosine transporters completely reversed the suppressive effect of 3'-deoxyadenosine (Figure 5k). It is worthwhile to note that basal expression of *GADD34* was detectable in unstimulated cells, and it was suppressed by 3'-deoxyadenosine.

To examine whether the enhanced phosphorylation of eIF2 $\alpha$  is responsible for the pro-survival effect of 3'-deoxyadenosine, cells were exposed to thapsigargin in the absence or presence of salubrinal, a selective blocker of eIF2 $\alpha$  dephosphorylation.<sup>3</sup> Treatment with salubrinal caused rapid phosphorylation of eIF2 $\alpha$  (Supplementary Figure S8), and like 3'-deoxyadenosine, salubrinal substantially suppressed ER stress-induced apoptosis (Figures 6a and b). The pro-survival property of eIF2 $\alpha$  signaling was further confirmed using cells stably transfected with a dominant-negative mutant of PERK (Supplementary



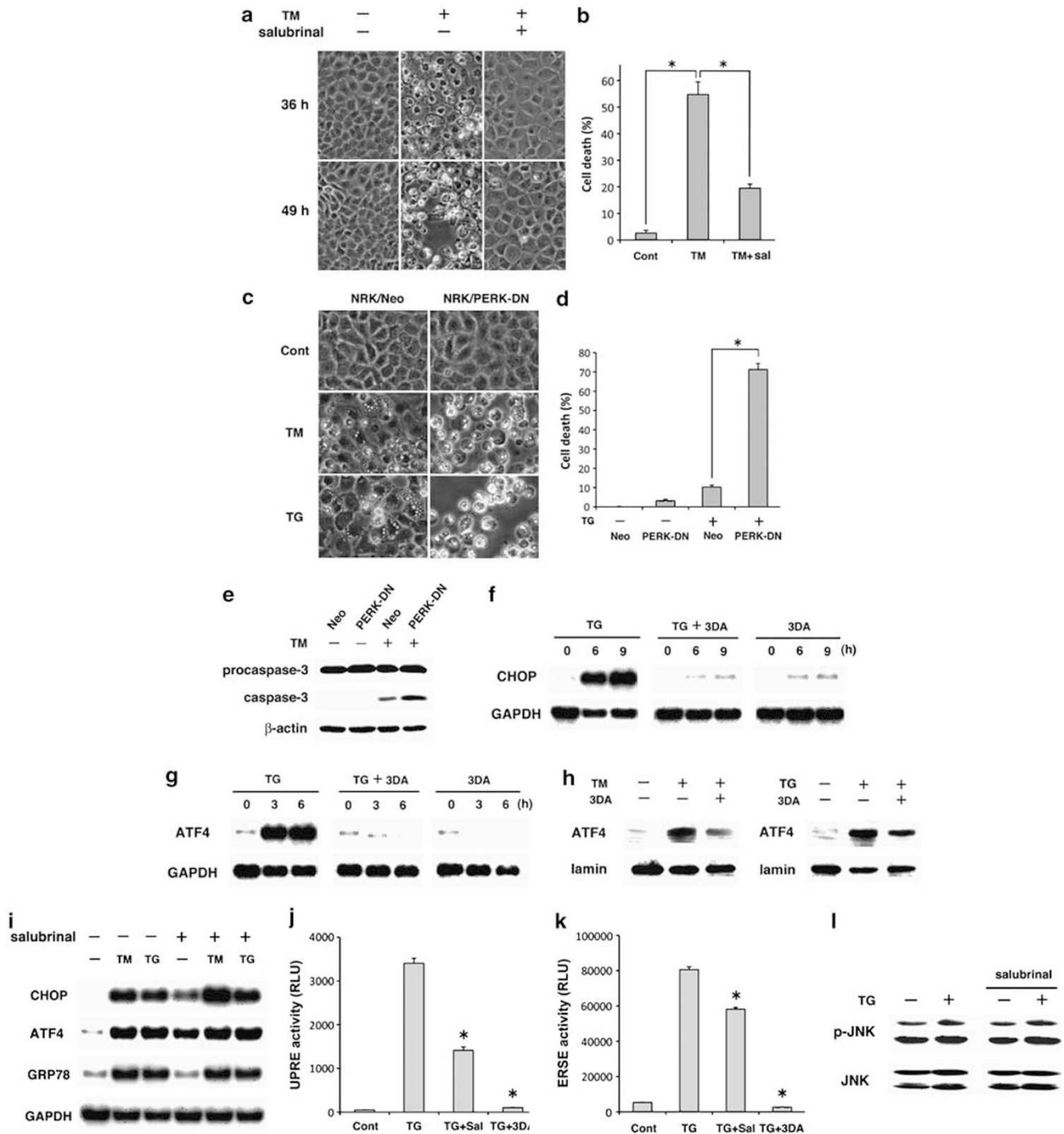
**Figure 5** Atypical pro-survival regulation of the PERK–eIF2 $\alpha$  pathway by 3'-deoxyadenosine. (a and b) NRK-52E cells were treated with TG, 3DA or TG + 3DA for indicated time periods and subjected to western blot analysis of phosphorylated PERK (a) and phosphorylated eIF2 $\alpha$  (b). Total proteins of PERK and eIF2 $\alpha$  are shown at the bottom as loading controls. (c) Cells were treated with 3DA in the presence of NBTI, MRS or DPSPX for 6 h, and phosphorylation of eIF2 $\alpha$  was evaluated. (d–f) Cells were treated with 3DA (d and e) or TG + 3DA (f) for indicated time periods in the absence or presence of MRS and subjected to western blot analysis. (g) Cells were treated with TG, TG + 3DA or 3DA alone for 3–6 h and subjected to northern blot analysis of *GADD34*. (h–k) Cells were treated with indicated agents for 8 h and subjected to northern blot analysis. \* $P < 0.05$

Figures S9A, S9B and S9C). When these cells were exposed to tunicamycin and thapsigargin, apoptosis was significantly enhanced when compared with mock-transfected cells (Figures 6c and d). It was associated with increased activation of caspase-3 (Figure 6e).

In general, activation of eIF2 $\alpha$  causes induction of *CHOP* via upregulation of ATF4.<sup>23</sup> The induction of ATF4 may be regulated at both transcriptional and translational levels.<sup>24,25</sup> However,

treatment with 3'-deoxyadenosine did not trigger substantial expression of *CHOP* and *ATF4*, and rather suppressed induction of these genes by ER stress (Figures 6f and g). The suppressive effect of 3'-deoxyadenosine on ATF4 was also confirmed by western blot analysis of nuclear ATF4 (Figure 6h). This effect was contrastive to salubrinal that induced expression of *CHOP* and *ATF4*, and did not suppress induction of these pro-apoptotic genes by ER stress inducers (Figure 6i).

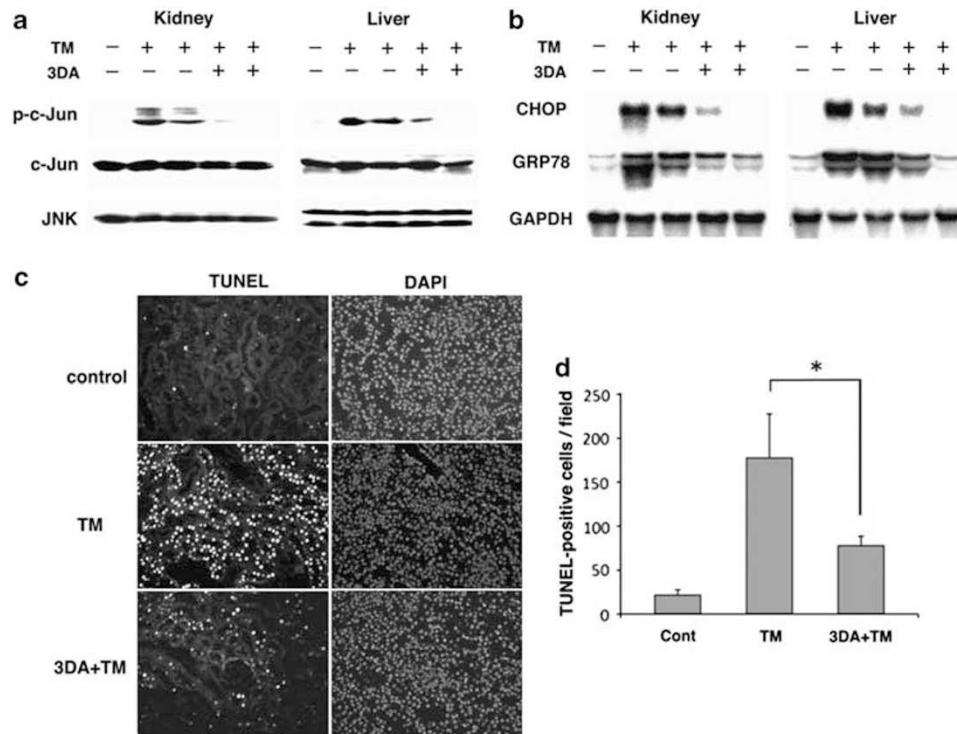




**Figure 6** Suppression of apoptosis by 3'-deoxyadenosine through activation of eIF2 $\alpha$  without induction of the ATF4-CHOP pathway. (a and b) NRK-52E cells were exposed to TM in the presence of 50  $\mu$ M salubrial (sal) and subjected to phase-contrast microscopy (a) and assessment of cell death (b). (c-e) NRK/Neo cells and NRK/PERK-DN cells were treated with TM or TG and subjected to phase-contrast microscopy (c), assessment of cell death (d) and evaluation of caspase-3 activation (e). (f and g) NRK-52E cells were treated with TG, TG + 3DA or 3DA alone for indicated time periods and subjected to northern blot analysis of CHOP (f) and ATF4 (g). (h) Cells were treated with TG for 6-12 h in the absence or presence of 3DA, and nuclear protein was subjected to western blot analysis of ATF4. The level of lamin B1 is shown at the bottom as a loading control. (i) Cells were treated with TM or TG in the absence or presence of salubrial, and expression of CHOP, ATF4 and GRP78 was examined. (j and k) NRK/UPRE-Luc cells (j) and NRK/ERSE-Luc cells (k) were treated with TG in the absence or presence of salubrial or 3DA for 12 h and subjected to luciferase assay. (l) Cells were stimulated with TG in the absence or presence of salubrial, and phosphorylation of JNK was evaluated by western blot analysis. \* $P < 0.05$

We examined a link between the eIF2 $\alpha$  pathway and the IRE1 pathway in 3'-deoxyadenosine-treated cells. As shown in Figures 6j and k, activation of eIF2 $\alpha$  by salubrial partially

suppressed induction of the IRE1 pathway by ER stress. It was contrastive to strong suppression of UPRE and ERSE activation by 3'-deoxyadenosine. These results indicate that



**Figure 7** *In vivo* suppression of ER stress-induced apoptosis by 3'-deoxyadenosine. Mice were injected with PBS or 3DA (3 mg/kg) intraperitoneally on day 1, 2, 3 and 4. On day 2, mice were administered with TM (1.5 mg/kg). After 3 days (day 5), kidneys (and livers) were sampled and subjected to western blot analysis of phosphorylated c-Jun, total c-Jun and total JNK protein (a), northern blot analysis of *CHOP* and *GRP78* (b), and TUNEL assay (c). The number of TUNEL-positive cells per field in the renal cortex was evaluated quantitatively (d). Data are expressed as means  $\pm$  S.E., and an asterisk indicates a statistically significant difference ( $P < 0.05$ ). DAPI, 4'-6-diamidino-2-phenylindole staining

suppression of the IRE1 pathway by 3'-deoxyadenosine may be mediated, in part, by the activation of eIF2 $\alpha$ , whereas other mechanisms are also involved in the suppressive effect of 3'-deoxyadenosine. Indeed, we found that early phosphorylation of JNK by ER stress was inhibited by 3'-deoxyadenosine (Figure 4f), but not by salubrinal (Figure 6l).

***In vivo* suppression of ER stress-induced apoptosis by 3'-deoxyadenosine.** We evaluated *in vivo* effects of 3'-deoxyadenosine on ER stress-induced apoptosis in mice. As shown in Figure 7a, activation of JNK was induced by tunicamycin in kidneys and livers, and it was suppressed by the administration with 3'-deoxyadenosine. Northern blot analysis also showed that *in vivo* induction of *CHOP* and *GRP78* was attenuated by 3'-deoxyadenosine (Figure 7b).

Tunicamycin induces renal tubular injury and apoptosis via ER stress.<sup>26</sup> We evaluated an *in vivo* effect of 3'-deoxyadenosine on tunicamycin-induced apoptosis in renal tubules. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) exhibited numerous TUNEL-positive apoptotic cells in kidneys of ER stress-induced mice, and it was substantially attenuated by the treatment with 3'-deoxyadenosine (Figure 7c). The number of TUNEL-positive cells was significantly reduced by 3'-deoxyadenosine from  $178 \pm 34$  cells/field to  $78 \pm 11$  cells/field (means  $\pm$  S.E.;  $P < 0.05$ ) (Figure 7d). Consistent with the *in vitro* data, these results provide *in vivo* evidence for the pro-survival potential of 3'-deoxyadenosine to inhibit ER stress-induced apoptosis.

## Discussion

ER stress-initiated apoptosis is regulated by three major branches of the UPR. We found that 3'-deoxyadenosine inhibits activation of the IRE1 pathway. Attenuation of ER stress *per se* was not the primary mechanism, because phosphorylation of PERK by ER stress was unaffected by 3'-deoxyadenosine. The fact that thapsigargin-induced down-regulation of ER stress-responsive alkaline phosphatase, an exogenous indicator for ER stress,<sup>27</sup> was not reversed by the treatment with 3'-deoxyadenosine (Supplementary Figure S10) further supports this conclusion. Under ER stress conditions, the IRE1-ASK1-JNK pathway contributes to the induction of apoptosis.<sup>5</sup> The suppression of this pro-apoptotic pathway may be one mechanism by which 3'-deoxyadenosine exerts its pro-survival effect.

In this report, we disclosed that 3'-deoxyadenosine induces phosphorylation of eIF2 $\alpha$ . Salubrinal mimicked the anti-apoptotic effect of 3'-deoxyadenosine, and blockade of the PERK-eIF2 $\alpha$  pathway rendered the cells susceptible to ER stress-induced apoptosis. These results indicate that translational suppression via eIF2 $\alpha$  may play a role in the pro-survival effect of 3'-deoxyadenosine. Indeed, a recent report showed that 3'-deoxyadenosine strongly inhibited total protein synthesis.<sup>28</sup> This phenomenon was observed in NIH3T3 cells treated by high concentrations of 3'-deoxyadenosine (50–200  $\mu$ M). However, we found that in NRK-52E cells, 3'-deoxyadenosine (5  $\mu$ g/ml, approximately 20  $\mu$ M)

rather upregulated phosphorylation of p70S6 kinase that facilitates general translation. Using connexin 43 as an indicator, we also found that treatment with salubrinal for 24–48 h downregulated the level of this protein, whereas 3'-deoxyadenosine did not (our unpublished data). Although 3'-deoxyadenosine may have the potential to inhibit protein synthesis via interfering with transcriptional and translational events,<sup>28–30</sup> further investigation will be required to determine the importance of translational suppression in the anti-apoptotic effect of 3'-deoxyadenosine.

Currently, it is unclear how 3'-deoxyadenosine activates eIF2 $\alpha$ . One possibility is its suppressive effect on GADD34 that is involved in dephosphorylation of eIF2 $\alpha$ .<sup>7</sup> As shown in this report, GADD34 was expressed in unstimulated cells, and it was suppressed by the treatment with 3'-deoxyadenosine. The downregulation of basal GADD34 might be a possible mechanism underlying phosphorylation of eIF2 $\alpha$  by 3'-deoxyadenosine.

In general, phosphorylation of eIF2 $\alpha$  causes induction of ATF4, resulting in expression of pro-apoptotic CHOP. The induction of ATF4 may be regulated at both transcriptional and translational level.<sup>24,25</sup> Indeed, treatment with salubrinal resulted in expression of ATF4 and CHOP in NRK-52E cells. However, in contrast to salubrinal, 3'-deoxyadenosine did not induce and rather attenuated expression of ATF4 and CHOP. This unusual effect may, at least in part, contribute to the anti-apoptotic effect of 3'-deoxyadenosine. Previous reports suggested that 3'-deoxyadenosine inhibits transcriptional events through multiple mechanisms.<sup>29,30</sup> It inhibits mRNA polyadenylation presumably by acting as a chain terminator.<sup>30</sup> At high doses, 3'-deoxyadenosine inhibits incorporation of uridine into mRNA, but not into its precursor hnRNA, indicating that export, processing or stability of transcribed mRNA is affected.<sup>29</sup> The suppression of ATF4 and CHOP by 3'-deoxyadenosine, observed in this report, may be through the similar mechanisms on mRNAs. Of note, however, individual mRNAs have different susceptibility to 3'-deoxyadenosine. In our experimental setting, some mRNAs were unaffected by the treatment with 3'-deoxyadenosine. For example, the transcriptional level of luciferase driven by viral and housekeeping gene promoters was not depressed by 3'-deoxyadenosine. Although basal expression of  $\beta$ -actin mRNA was reduced by 3'-deoxyadenosine (our unpublished data), expression of *EF-1 $\alpha$*  and *GAPDH* mRNAs was unaffected by the treatment with this substance. Furthermore, we recently found that 3'-deoxyadenosine rather upregulated expression of monocyte chemoattractant protein 1 in differentiating preadipocytes (Supplementary Figure S11). These results are consistent with a recent report showing different sensitivity of different mRNAs to 3'-deoxyadenosine.<sup>31</sup> Thus, the effect of 3'-deoxyadenosine on transcriptional events may not be so simple as was previously proposed.

Based on our current results, we propose a model underlying the anti-apoptotic effect of 3'-deoxyadenosine against ER stress (Figure 8a). 3'-Deoxyadenosine suppresses the IRE–JNK pro-apoptotic UPR at the proximal site. On the other hand, 3'-deoxyadenosine triggers phosphorylation of eIF2 $\alpha$  and enhances ER stress-induced eIF2 $\alpha$  activation, resulting in translational suppression and consequent attenuation of ER stress in the later phase. 3'-Deoxyadenosine also suppresses induction of ATF4 and GADD34, leading to sustained

activation of eIF2 $\alpha$ . Although 3'-deoxyadenosine causes phosphorylation of eIF2 $\alpha$ , it does not induce and rather suppresses induction of the ATF4–CHOP pro-apoptotic pathway. It is contrastive to the effect of salubrinal that leads to induction of pro-apoptotic CHOP and GADD34. In addition, 3'-deoxyadenosine may attenuate ER stress via transcriptional suppression that preferentially targets particular mRNAs.

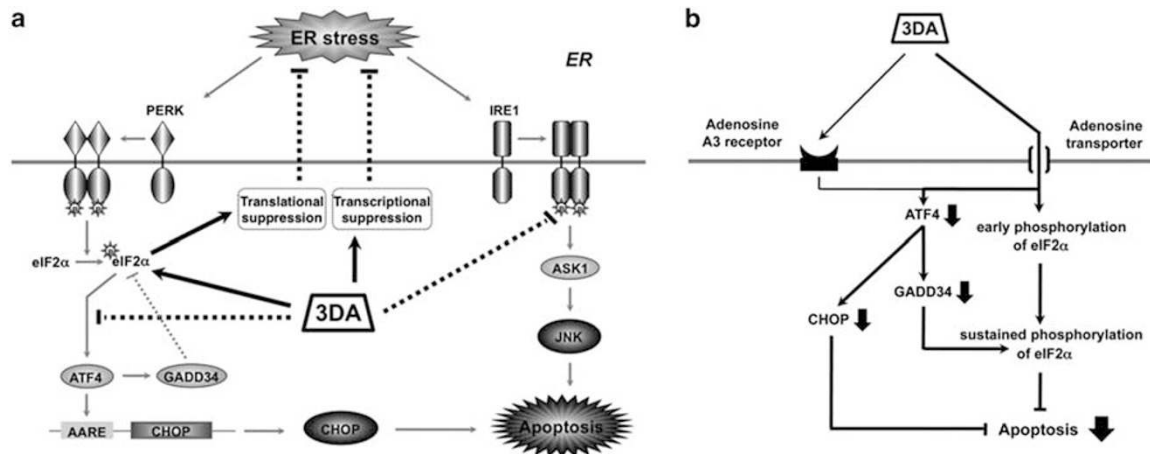
In the present report, we showed that the anti-apoptotic effect of 3'-deoxyadenosine is through adenosine receptor-dependent and -independent mechanisms, the former of which requires the A3 adenosine receptor, but not A1/A2 receptors. Contribution of the A3 receptor is consistent with a previous report showing that 3'-deoxyadenosine inhibited growth of tumor cells through the A3 receptor.<sup>10</sup> In concert with this extracellular action, the transporter-dependent intracellular action of 3'-deoxyadenosine also plays a significant role for its effects against ER stress; for example, suppression of ATF4, GADD34 and CHOP. Regarding the activation of eIF2 $\alpha$  by 3'-deoxyadenosine, however, the extracellular action and the intracellular action play distinct roles. We demonstrated that adenosine transporters, but not the A3 receptor, contributed to early phosphorylation of eIF2 $\alpha$  by 3'-deoxyadenosine. In contrast, the A3 receptor was involved in the maintenance of phosphorylated eIF2 $\alpha$  in the later phase. Based on our current results, we propose a hypothetical model shown in Figure 8b.

Individual UPR pathways possess both pro-apoptotic and anti-apoptotic properties. For example, the PERK pathway causes translational suppression that attenuates ER stress, whereas it also induces pro-apoptotic molecules including CHOP and GADD34. The IRE1 pathway triggers apoptosis via activation of ASK1 and JNK, but activation of IRE1 also induces ER chaperones and ERAD factors, both of which relieve ER stress.<sup>32</sup> In general, however, the PERK pathway is considered pro-survival, whereas the IRE1 pathway is regarded pro-apoptotic.<sup>3,4,33</sup> From this viewpoint, the pharmacological of 3'-deoxyadenosine should be advantageous for blockade of ER stress-induced cell injury. Indeed, we demonstrated in this report that *in vivo* administration with 3'-deoxyadenosine suppressed ER stress-induced activation of the IRE1–JNK pathway, induction of CHOP and consequent apoptosis in the kidney.

Taken together, the current results elucidated involvement of multiple targets for the anti-apoptotic action of 3'-deoxyadenosine against ER stress. This aberrant, differential and bidirectional regulation of the UPR may be advantageous for cell survival. Our results raise a possibility that 3'-deoxyadenosine could be useful for therapeutic intervention in an array of ER stress-related pathologies including infections, diabetes, malignant diseases, ischemic injury and neurodegenerative disorders.

#### Materials and Methods

**Reagents.** 3'-Deoxyadenosine, tunicamycin, thapsigargin, A23187, staurosporine, DTT, adenosine, MRS1523, NBTI, DPSPX, actinomycin D and Hoechst33258 were purchased from Sigma-Aldrich Japan. Cadmium chloride, ATP and ADP were obtained from Wako Pure Chemical (Osaka, Japan). Salubrinal was purchased from Tocris Bioscience (Ellisville, MO, USA), and human recombinant TNF- $\alpha$  was from R&D Systems (Minneapolis, MN, USA). Cigarette smoke extract was prepared, as described previously.<sup>34</sup> SubAB was kindly provided by Dr. James Paton (University of Adelaide). Throughout the study,



**Figure 8** Molecular mechanisms involved in the suppression of ER stress-induced apoptosis by 3'-deoxyadenosine. (a) 3DA suppresses the IRE–JNK pro-apoptotic UPR at the proximal site. On the other hand, 3DA triggers phosphorylation of eIF2 $\alpha$  and enhances ER stress-induced activation of eIF2 $\alpha$ , resulting in translational suppression and consequent attenuation of ER stress. 3DA also suppresses induction of ATF4 and GADD34, leading to sustained activation of eIF2 $\alpha$ . Although 3DA causes sustained phosphorylation of eIF2 $\alpha$ , it does not induce and rather suppresses induction of the ATF4–CHOP pro-apoptotic pathway. 3DA may attenuate ER stress via transcriptional suppression that targets particular mRNAs. (b) 3DA is transported into the cells through adenosine transporters and causes early phosphorylation of eIF2 $\alpha$ . 3DA suppresses expression of ATF4 and downstream GADD34 and CHOP by its intracellular action (major action) and extracellular action via the adenosine A3 receptor (minor action). These effects allow for sustained phosphorylation of eIF2 $\alpha$  and blunted expression of pro-apoptotic genes, leading to attenuation of apoptosis

3'-deoxyadenosine, tunicamycin and thapsigargin were used at concentrations 5  $\mu$ g/ml, 2.5  $\mu$ g/ml and 500 nM, respectively.

**Cells.** The rat renal tubular epithelial cell line NRK-52E and the human bronchial epithelial cell line BEAS-2B were purchased from American Type Culture Collection (Manassas, VA, USA), and the murine 3T3-L1 preadipocytes were obtained from Health Science Research Resources Bank (Osaka, Japan). The rat mesangial cell line SM43 was established and identified, as described previously.<sup>35</sup> Cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 5–10% fetal bovine serum (FBS). Assays were performed in the presence of 1% FBS.

**Assessment of cell death.** Cells were seeded in 24-well plates and treated with ER stress inducers for 48–72 h. Both attached cells and detached cells were harvested separately and subjected to trypan blue analysis. The percentages of dead cells against total cells were evaluated in individual wells. Assays were performed in quadruplicate.

**Hoechst staining.** Cells were fixed in 2% formaldehyde for 10 min, stained by Hoechst33258 (10  $\mu$ g/ml) for 1 h and subjected to fluorescence microscopy.

**Establishment of stable transfectants.** NRK-52E cells were stably transfected with pUPRE-Luc or pERSE-Luc (provided by Dr. Laurie H Glimcher, Harvard Medical School)<sup>36</sup> that introduces a luciferase gene under the control of UPRE or ERSE respectively, and NRK/UPRE-Luc cells and NRK/ERSE-Luc cells were established. NRK/PERK-DN cells were established by stable transfection with pcDNA3-hPERK.K621M (provided by Dr. Ronald C Wek, Indiana University School of Medicine).<sup>37</sup> NRK/Neo cells transfected with pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) were used as a control. SM/SV-SEAP cells were established by stable transfection of SM43 cells with pSEAP2-Control (BD Biosciences, Palo Alto, CA, USA) that introduces a SEAP gene under the control of the SV40 promoter.

**Transient transfection.** Using electroporation, NRK-52E cells were transiently transfected with pEF-1 $\alpha$ -Luc, pCMV-Luc (provided by Dr. Takahiro Taira, University of Yamanashi) or pSV40-Luc (pGL3-Control; Promega, Madison, WI, USA), treated with 3'-deoxyadenosine and subjected to chemiluminescent assay. pEF-1 $\alpha$ -Luc, pCMV-Luc and pSV40-Luc introduce a luciferase gene under the control of the EF-1 $\alpha$  promoter, the CMV promoter/enhancer and the SV40 promoter/enhancer, respectively. To evaluate cleavage of XBP1 mRNA, cells were transfected with pCAX-F-XBP1 $\Delta$ DBD-Luc (provided by Dr. Takao Iwawaki, RIKEN),<sup>38</sup> treated with thapsigargin together with 3'-deoxyadenosine and

subjected to analysis. pCHOP-Luc (provided by Dr. Pierre Faourmoux, INRA de Theix) that introduces a luciferase gene under the control of the CHOP gene promoter (originally designated as pCHOP(–954  $\pm$  91)-Luc)<sup>39</sup> was also used to evaluate activity of the eIF2 $\alpha$  pathway.

**SEAP assay.** Activity of SEAP was evaluated by a chemiluminescent method using Great EscAPe SEAP Detection Kit (BD Biosciences) according to the manufacturer's protocol.<sup>27</sup>

**Luciferase assay.** Activity of luciferase was evaluated by Luciferase Assay System (Promega) according to the manufacturer's protocol.<sup>27</sup>

**Northern blot analysis.** Total RNA was extracted by a single-step method, and northern blot analysis was performed as described before.<sup>40</sup> cDNAs for GRP78 (provided by Dr. Kazunori Imaizumi, University of Miyazaki), CHOP (provided by Dr. David Ron, New York University School of Medicine), PERK (provided by Dr. Ronald C Wek), GADD34 (provided by Dr. Takeshi Omasa, Osaka University), ATF4 (provided by Dr. David Ron), GRP94 (provided by Dr. Amy S Lee, University of South California), ORP150 (provided by Dr. Satoshi Ogawa, Kanazawa University), Edem1 (provided by Dr. Nobuko Hosokawa, Kyoto University), EF-1 $\alpha$  (provided by Dr. Jianxin Sun, New Jersey Medical School) and  $\beta$ -actin were used for preparation of radio-labeled probes. Expression of GAPDH was used as a loading control. Densitometric analysis was performed using Scion Image (Scion Corporation, Frederick, MO, USA).

**Western blot analysis.** Western blot analysis was performed as described before.<sup>33</sup> Primary antibodies used were anti-caspase-3 antibody, anti-phospho eIF2 $\alpha$  (Ser51) antibody, anti-c-Jun antibody and anti-phospho c-Jun (Ser63) antibody from Cell Signaling (Beverly, MA, USA); and anti-PERK antibody, anti-phospho PERK (Thr981) antibody, anti-eIF2 $\alpha$  antibody and anti-ATF4 (CREB-2) antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). As a loading control, levels of  $\beta$ -actin and lamin B1 were evaluated using anti- $\beta$ -actin antibody (Sigma-Aldrich Japan) and anti-lamin B1 antibody (Invitrogen). Phosphorylation of JNK was assessed by using PhosphoPlus SAPK/JNK (Thr183/Tyr185) Antibody Kit (Cell Signaling) following the protocol provided by the manufacturer. Nuclear protein was extracted using ProteoExtract Subcellular Proteome Extraction Kit (Merck KGaA, Darmstadt, Germany).

**RT–PCR.** Reverse transcription was performed using Omniscript Reverse Transcriptase (Qiagen, Valencia, CA, USA). Splicing of XBP1 mRNA was examined

using following primers: 5'-ACACGCTTGGGGATGATGC-3' and 5'-CCATGGG AAGATGTTCTGGG-3' (Sigma-Aldrich Japan).

**Animal experiments.** C57BL/6 mice (20–25 g body weight; 12 male mice) were intraperitoneally injected (i.p.) with PBS or 3'-deoxyadenosine (3 mg/kg) on day 1, 2, 3 and 4. On day 2, mice were exposed to ER stress by single i.p. of tunicamycin (1.5 mg/kg). After 3 days (day 5), kidneys were removed and processed for tissue sectioning for histopathological analysis and TUNEL assay. Renal cortex and liver were also used for western and northern blot analyses. 4'-6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich Japan) was used for nuclear staining.

**Histopathological analysis and TUNEL assay.** Kidneys were fixed in 4% phosphate-buffered paraformaldehyde overnight at 4 °C and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin. TUNEL assay was performed using Apoptosis Detection System, Fluorescein (Promega), as described previously.<sup>33</sup> The number of TUNEL-positive cells per field (renal cortex) was counted, and the data were compared in different groups.

**Statistical analysis.** Data were expressed as means ± S.E. Statistical analysis was performed using the non-parametric Mann–Whitney *U*-test to compare data in different groups. A *P* value <0.05 was considered to indicate a statistically significant difference.

### Conflict of interest

The authors declare no conflict of interest.

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- Aridor M, Balch WE. Integration of endoplasmic reticulum signaling in health and disease. *Nat Med* 1999; **5**: 745–751.
- Wu J, Kaufman RJ. From acute ER stress to physiological roles of the unfolded protein response. *Cell Death Differ* 2006; **13**: 374–384.
- Boyce M, Bryant KF, Jousse C, Long K, Harding HP, Scheuner D *et al*. A selective inhibitor of eIF2 $\alpha$  dephosphorylation protects cells from ER stress. *Science* 2005; **307**: 935–939.
- Wiseman RL, Balch WE. A new pharmacology—drugging stressed folding pathways. *Trends Mol Med* 2005; **11**: 347–350.
- Kim R, Emi M, Tanabe K, Murakami S. Role of the unfolded protein response in cell death. *Apoptosis* 2006; **11**: 5–13.
- Ma Y, Brewer JW, Diehl JA, Hendershot LM. Two distinct stress signaling pathways converge upon the CHOP promoter during the mammalian unfolded protein response. *J Mol Biol* 2002; **318**: 1351–1365.
- Novoa I, Zeng H, Harding HP, Ron D. Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2 $\alpha$ . *J Cell Biol* 2001; **153**: 1011–1022.
- Cunningham KG, Manson W, Spring FS, Hutchinson SA. Cordycepin, a metabolic product isolated from cultures of *Cordyceps militaris* (Linn). *Nature* 1950; **166**: 949.
- Kaczka EA, Trenner NR, Anson B, Walker RW, Folkers K. Identification of cordycepin, a metabolite of *Cordyceps militaris*, as 3'-deoxyadenosine. *Biochem Biophys Res Commun* 1964; **14**: 456–457.
- Nakamura K, Yoshikawa N, Yamaguchi Y, Kagota S, Shinozuka K, Kunitomo M. Antitumor effect of cordycepin (3'-deoxyadenosine) on mouse melanoma and lung carcinoma cells involves adenosine A3 receptor stimulation. *Anticancer Res* 2006; **26**: 43–47.
- Chang W, Lim S, Song H, Song BW, Kim HJ, Cha MJ *et al*. Cordycepin inhibits vascular smooth muscle cell proliferation. *Eur J Pharmacol* 2008; **597**: 64–69.
- Lallas GC, Courtis N, Havredaki M. K562 cell sensitization to 5-fluorouracil- or interferon- $\alpha$ -induced apoptosis via cordycepin (3'-deoxyadenosine): fine control of cell apoptosis via poly(A) polymerase upregulation. *Int J Biol Markers* 2004; **19**: 58–66.

- Wu WC, Hsiao JR, Lian YY, Lin CY, Huang BM. The apoptotic effect of cordycepin on human OEC-M1 oral cancer cell line. *Cancer Chemother Pharmacol* 2007; **60**: 103–111.
- Chen LS, Stellrecht CM, Gandhi V. RNA-directed agent, cordycepin, induces cell death in multiple myeloma cells. *Br J Haematol* 2008; **140**: 682–691.
- Shi P, Huang Z, Tan X, Chen G. Proteomic detection of changes in protein expression induced by cordycepin in human hepatocellular carcinoma BEL-7402 cells. *Methods Find Exp Clin Pharmacol* 2008; **30**: 347–353.
- Erlinge D. Extracellular ATP: a growth factor for vascular smooth muscle cells. *Gen Pharmacol* 1998; **31**: 1–8.
- Kumar V, Sharma A. Adenosine: an endogenous modulator of innate immune system with therapeutic potential. *Eur J Pharmacol* 2009; **616**: 7–15.
- Czajkowski R, Barańska J. Cross-talk between the ATP and ADP nucleotide receptor signalling pathways in glioma C6 cells. *Acta Biochim Pol* 2002; **49**: 877–889.
- Paton AW, Beddoe T, Thorpe CM, Whisstock JC, Wilce MC, Rossjohn J *et al*. AB5 subtilase cytotoxin inactivates the endoplasmic reticulum chaperone BiP. *Nature* 2006; **443**: 548–552.
- Yoshida H, Matsui T, Hosokawa N, Kaufman RJ, Nagata K, Mori K. A time-dependent phase shift in the mammalian unfolded protein response. *Dev Cell* 2003; **4**: 265–271.
- Lee AS. The glucose-regulated proteins: stress induction and clinical applications. *Trends Biochem Sci* 2001; **26**: 504–510.
- Novoa I, Zhang Y, Zeng H, Jungreis R, Harding HP, Ron D. Stress-induced gene expression requires programmed recovery from translational repression. *EMBO J* 2003; **22**: 1180–1187.
- Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M *et al*. Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol Cell* 2000; **6**: 1099–1108.
- Armstrong JL, Flockhart R, Veal GJ, Lovat PE, Redfern CP. Regulation of endoplasmic reticulum stress-induced cell death by ATF4 in neuroectodermal tumor cells. *J Biol Chem* 2010; **285**: 6091–6100.
- Blais JD, Filipenko V, Bi M, Harding HP, Ron D, Koumenis C *et al*. Activating transcription factor 4 is translationally regulated by hypoxic stress. *Mol Cell Biol* 2004; **24**: 7469–7482.
- Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA *et al*. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid- $\beta$ . *Nature* 2000; **403**: 98–103.
- Hiramatsu N, Kasai A, Hayakawa K, Yao J, Kitamura M. Real-time detection and continuous monitoring of ER stress *in vitro* and *in vivo* by ES-TRAP: evidence for systemic, transient ER stress during endotoxemia. *Nucleic Acids Res* 2006; **34**: e93.
- Wong YY, Moon A, Duffin R, Barthelet-Barateig A, Meijer HA, Clemens MJ *et al*. Cordycepin inhibits protein synthesis and cell adhesion through effects on signal transduction. *J Biol Chem* 2010; **285**: 2610–2621.
- Penman S, Rosbash M, Penman M. Messenger and heterogeneous nuclear RNA in HeLa cells: differential inhibition by cordycepin. *Proc Natl Acad Sci USA* 1970; **67**: 1878–1885.
- Müller WE, Seibert G, Beyer R, Breter HJ, Maidhof A, Zahn RK. Effect of cordycepin on nucleic acid metabolism in L5178Y cells and on nucleic acid-synthesizing enzyme systems. *Cancer Res* 1977; **37**: 3824–3833.
- Wong YY, Moon A, Duffin R, Barthelet-Barateig A, Meijer HA, Clemens MJ *et al*. Cordycepin inhibits protein synthesis and cell adhesion through effects on signal transduction. *J Biol Chem* 2010; **285**: 2610–2621.
- Rutkowski DT, Kaufman RJ. That which does not kill me makes me stronger: adapting to chronic ER stress. *Trends Biochem Sci* 2007; **32**: 469–476.
- Yokouchi M, Hiramatsu N, Hayakawa K, Kasai A, Takano Y, Yao J *et al*. Atypical, bidirectional regulation of cadmium-induced apoptosis via distinct signaling of unfolded protein response. *Cell Death Differ* 2007; **14**: 1467–1474.
- Tagawa Y, Hiramatsu N, Kasai A, Hayakawa K, Okamura M, Yao J *et al*. Induction of apoptosis by cigarette smoke via ROS-dependent endoplasmic reticulum stress and CCAAT/enhancer-binding protein-homologous protein (CHOP). *Free Radic Biol Med* 2008; **45**: 50–59.
- Kitamura M, Taylor S, Unwin R, Burton S, Shimizu F, Fine LG. Gene transfer into the rat renal glomerulus via a mesangial cell vector: site-specific delivery, *in situ* amplification, and sustained expression of an exogenous gene *in vivo*. *J Clin Invest* 1994; **94**: 497–505.
- Lee AH, Iwakoshi NN, Glimcher LH. XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol Cell Biol* 2003; **23**: 7448–7459.
- Fernandez J, Yaman I, Sarnow P, Snider MD, Hatzoglou M. Regulation of internal ribosomal entry site-mediated translation by phosphorylation of the translation initiation factor eIF2 $\alpha$ . *J Biol Chem* 2002; **277**: 19198–19205.
- Iwakaki T, Akai R. Analysis of the XBP1 splicing mechanism using endoplasmic reticulum stress-inducers. *Biochem Biophys Res Commun* 2006; **350**: 709–715.
- Bruhat A, Jousse C, Carraro V, Reimold AM, Ferrara M, Fafournoux P. Amino acids control mammalian gene transcription: activating transcription factor 2 is essential for the amino acid responsiveness of the CHOP promoter. *Mol Cell Biol* 2000; **19**: 7192–7204.
- Kitamura M, Suto T, Yokoo T, Shimizu F, Fine LG. Transforming growth factor- $\beta$ 1 is the predominant paracrine inhibitor of macrophage cytokine synthesis produced by glomerular mesangial cells. *J Immunol* 1996; **156**: 2964–2971.

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