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ORIGINAL ARTICLE

# p28$^{GANK}$ inhibits endoplasmic reticulum stress-induced cell death via enhancement of the endoplasmic reticulum adaptive capacity

Rong-Yang Dai[1, 2, *], Yao Chen[2, *], Jing Fu[2], Li-Wei Dong[2], Yi-Bin Ren[2], Guang-Zhen Yang[2], You-Wen Qian[2], Jie Cao[2], Shan-Hua Tang[2], Sheng-Li Yang[1], Hong-Yang Wang[1, 2]

*[1]State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute and Cancer Institute of Shanghai Jiao Tong University, Shanghai 200032, China; [2]International Co-operation Laboratory on Signal Transduction, Eastern Hepatobiliary Surgery Institute, Shanghai 200438, China*

**It has been shown that oncoprotein p28$^{GANK}$, which is consistently overexpressed in human hepatocellular carcinoma (HCC), plays a critical role in tumorigenesis of HCC. However, the underlying mechanism remains unclear. Here, we demonstrated that p28$^{GANK}$ inhibits apoptosis in HCC cells induced by the endoplasmic reticulum (ER) stress. During ER stress, p28$^{GANK}$ enhances the unfolded protein response, promotes ER recovery from translational repression, and thereby facilitates cell's ability to cope with the stress conditions. Furthermore, p28$^{GANK}$ upregulates glucose-regulated protein 78 (GRP78), a key ER chaperone protein, which subsequently enhances the ER folding capacity and promotes recovery from ER stress. We also demonstrated that p28$^{GANK}$ increases p38 mitogen-activated protein kinase and Akt phosphorylation, and inhibits nuclear factor kappa B (NF-κB) activation under ER stress, which in turn contributes to GRP78 upregulation. Taken together, our results indicate that p28$^{GANK}$ inhibits ER stress-induced apoptosis in HCC cells, at least in part, by enhancing the adaptive response and GRP78 expression. We propose that p28$^{GANK}$ has potential implications for HCC progression under the ER stress conditions.**

***Keywords*:** p28$^{GANK}$, ER stress, UPR, GRP78, apoptosis

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## Introduction

The perturbation of endoplasmic reticulum (ER) homeostasis can result in the accumulation of unfolded or misfolded proteins that leads to ER stress [1, 2]. To cope with the accumulated unfolded or misfolded proteins, mammalian cells can trigger a specific response termed as the unfolded protein response (UPR) [3-5], which enables cells to reestablish normal ER function by attenuating translation, and promoting protein folding, secretion, and degradation [3, 6, 7]. These effects are mediated by three distinct components of the UPR signaling: PRK (RNA-dependent protein kinase)-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring gene 1 (IRE1) [8]. The UPR is an adaptive response that increases cell survival under ER stress conditions [9]. However, if unfolded protein aggregation is persistent and the stress cannot be resolved, the UPR signaling switches from pro-survival to pro-apoptotic mode and the cells are destroyed by apoptosis.

Glucose-regulated protein 78 (GRP78), a biomarker of ER stress, is one of the best-characterized ER chaperone proteins. GRP78 is involved in many cellular processes, including translocation of newly synthesized polypeptides across the ER membrane, their subsequent folding, maturation, and retrotranslocation, targeting misfolded proteins for proteasomal degradation, regulating calcium homeostasis, and serving as an upstream sensor for ER stress [2, 10, 11]. Under unstressed conditions, the luminal domains of ER stress sensors, PERK, ATF6, and IRE1, are occupied by GRP78, which represses the

*These two authors contributed equally to this work.
Correspondence: Hong-Yang Wang
Tel: +86-21-81875361; Fax: +86-21-65566851
E-mail: hywangk@vip.sina.com
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UPR signaling pathways. Upon ER stress, sequestration of GRP78 by unfolded proteins activates these sensors, inducing the UPR [12]. A recent report has revealed that GRP78 plays critical roles in cytoprotection and cancer development [13].

p28GANK, also named as gankyrin, was identified as an oncoprotein that is consistently overexpressed in human liver cancers [14, 15]. p28GANK was initially purified and characterized as the p28 component of the regulatory subunit in the 26S proteasome, which is an ATP-dependent protease responsible for the degradation of proteins [16]. p28GANK not only facilitates the phosphorylation and degradation of RB, but also binds MDM2, an E3 ubiquitin ligase that negatively regulates p53, and facilitates its degradation of p53 [14, 17]. Our recent study demonstrated that the expression of p28GANK siRNA in human hepatocellular carcinoma (HCC) cells caused apoptosis through the activation of caspase 8 and/or caspase 9 [18].

Due to inadequate vascularization and rapid growth, tumor cells experience hypoxia and nutrient starvation. These can lead to the accumulation of unfolded proteins in ER, resulting in UPR [19]. The upregulation of GRP78, ATF6, and X-box binding protein 1 (XBP1) at mRNA levels has been found in HCC, indicating the potential involvement of ER stress in HCC progression [20]. In this study, we examined the potential association between p28GANK and the UPR. We demonstrated that p28GANK augments the adaptive capacity of ER by enhancing the UPR and upregulating GRP78 expression, thus inhibiting ER stress-induced apoptosis. Moreover, we also found that the p38 mitogen-activated protein kinase (p38 MAPK), phosphatidylinositol 3-kinase (PI3K)/Akt, and nuclear factor kappa B (NF-κB) pathways are involved in p28GANK-induced GRP78 upregulation under ER stress.

## Results

*Both p28GANK and the UPR biomarkers are overexpressed in HCC*

We investigated the expression of p28GANK and the UPR markers in HCC specimens. The mRNA levels of p28GANK and the ER stress markers, such as XBP1, GRP78, and GADD153, were evaluated by RT-PCR from 12 HCCs and the adjacent non-cancerous liver tissues. As shown in Figure 1A, the mRNA levels of XBP1, GRP78, GADD153, and p28GANK in many HCC tissues were significantly higher than that in the adjacent non-cancerous tissues. For quantitative analysis, the mRNA levels of XBP1, GRP78, GADD153, and p28GANK were normalized to the corresponding 18S rRNA levels (Figure 1B). The protein levels of GRP78, GADD153, and p28GANK were also examined from eight HCCs and their adjacent non-cancerous tissues by western blot. Consistent with the mRNA levels, the protein levels of GRP78, GADD153, and p28GANK were significantly increased in the HCC tissues (Figure 1C). These data suggest that there is a potential link between p28GANK and the UPR in HCCs.

*p28GANK protects HCC cells against ER stress-induced death*

It is known that the PERK pathway, a branch of ER stress response, inhibits global protein synthesis through eukaryotic translation initiation factor 2α (eIF2α) phosphorylation. We therefore studied whether ER stress regulates p28GANK expression in the HCC cells. We found that dithiothreitol (DTT) and thapsigargin (TG) can markedly elevate both the mRNA and protein levels of GRP78, and induce XBP1 mRNA splicing, an indicator of UPR activation, in SMMC-7721 and HepG2 cells (Figure 2A). The level of p28GANK protein was not altered by either treatment in SMMC-7721 and HepG2 cells (data not shown). Considering the activation of UPR in HCC, we examined ER stress-induced apoptosis in HCC cells. The data showed that SMMC-7721 and HepG2 cells were relatively resistant to ER stress-induced apoptosis triggered by DTT or TG treatment at relatively high doses (DTT 4 mM and TG 3 μM) and for a relatively long time (< 20% apoptotic cells at 72 h), which efficiently killed human liver cell lines L02 and QSG-7701 by induction of apoptosis (Figure 2B). Thus, these results suggest that HCC cells are more resistant to ER stress-induced death.

SMMC-7721 and HepG2 cells express higher levels of p28GANK than that of QSG-7701 and L02 cells (Figure 2B). To determine whether p28GANK is involved in protecting HCC cells against ER stress-induced death, we silenced p28GANK expression in SMMC-7721 and HepG2 cells using adenovirus-delivered p28GANK siRNA (Adsigank). The cells were infected with the virus for 24 h, then treated with DTT or TG for additional 48 h. The results showed that knockdown of p28GANK significantly sensitized SMMC-7721 and HepG2 cells to DTT- and TG-induced apoptosis (Figure 2C). p28GANK siRNA alone did not cause appreciable SMMC-7721 and HepG2 cell death (Figure 2C). Furthermore, L02 and QSG-7701 cells overexpressing p28GANK were more resistant to DTT- and TG-induced apoptosis in comparison to the control cells (Figure 2D). Taken together, these data support that p28GANK protects HCC cells from ER stress-induced apoptosis.

*p28GANK promotes the UPR*

The UPR is the major protective mechanism utilized

by cells to cope with ER stress and inhibit the ER stress-induced apoptosis. To evaluate whether p28$^{GANK}$ can regulate this adaptive response, we treated stable p28$^{GANK}$-expressing NIH3T3 cells and their control cells with DTT or TG. The activities of PERK and IRE1α, and the mRNA levels of typical UPR biomarkers (XBP1, GRP78, and GADD153) were measured. As shown in Figure 3A and 3B, p28$^{GANK}$ promoted the phosphorylation of PERK and IRE1α, and upregulated the mRNA levels of XBP1, GRP78 and GADD153 under ER stress conditions. The data also showed that p28$^{GANK}$ also increased the stability of GADD153 transcripts (Supplementary information,

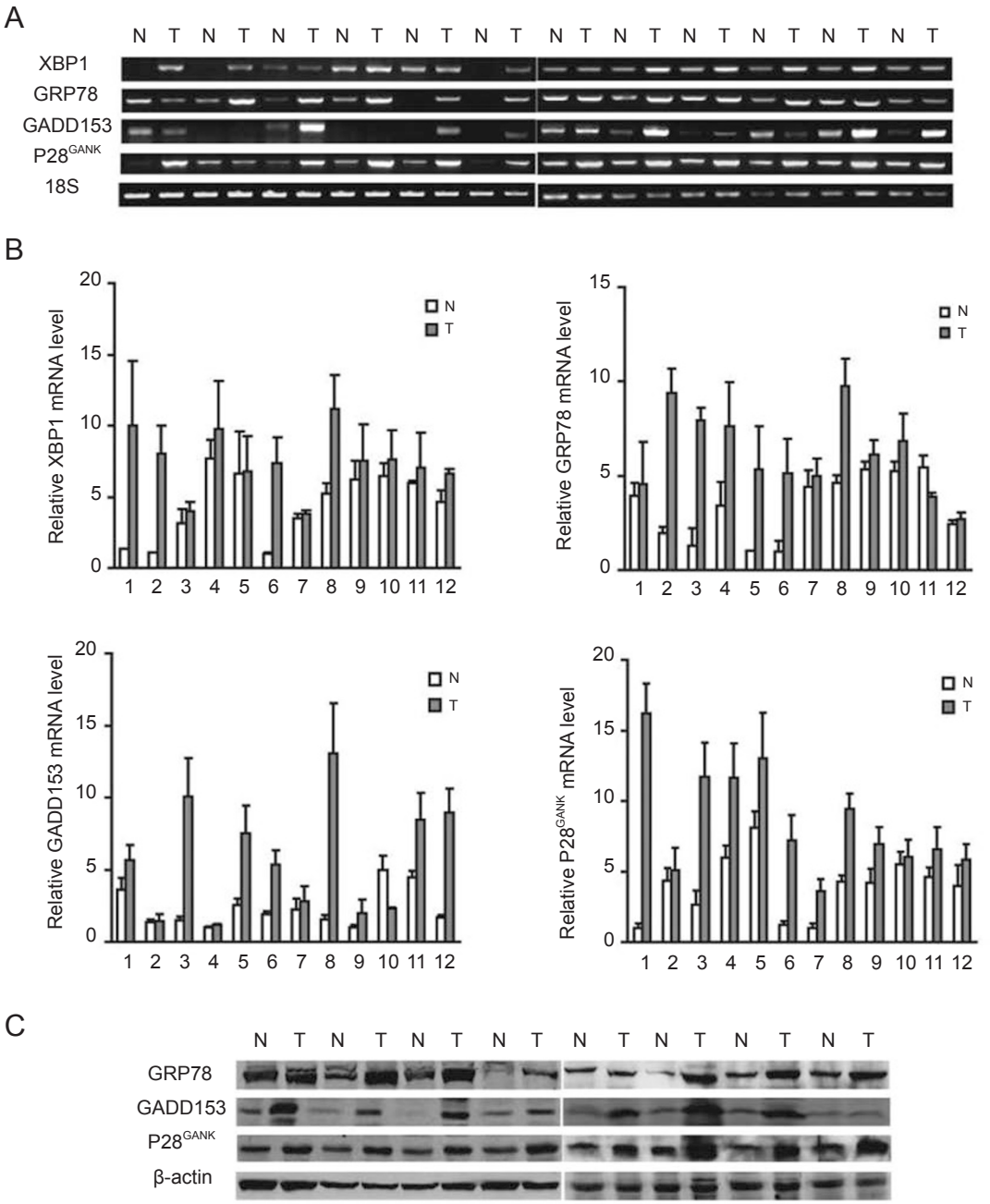


**Figure 1** Both p28$^{GANK}$ and the UPR biomarkers overexpressed in HCC. **(A)** Total RNA extracted from human hepatocellular carcinoma (T) and adjacent non-cancerous (N) liver tissues were analyzed by RT-PCR. **(B)** The mRNA values were normalized to 18S rRNA content. Columns, mean of three individual experiments; bars, SE. **(C)** Tissue lysates from human hepatocellular carcinoma (T) and adjacent non-cancerous (N) liver were analyzed by western blot.

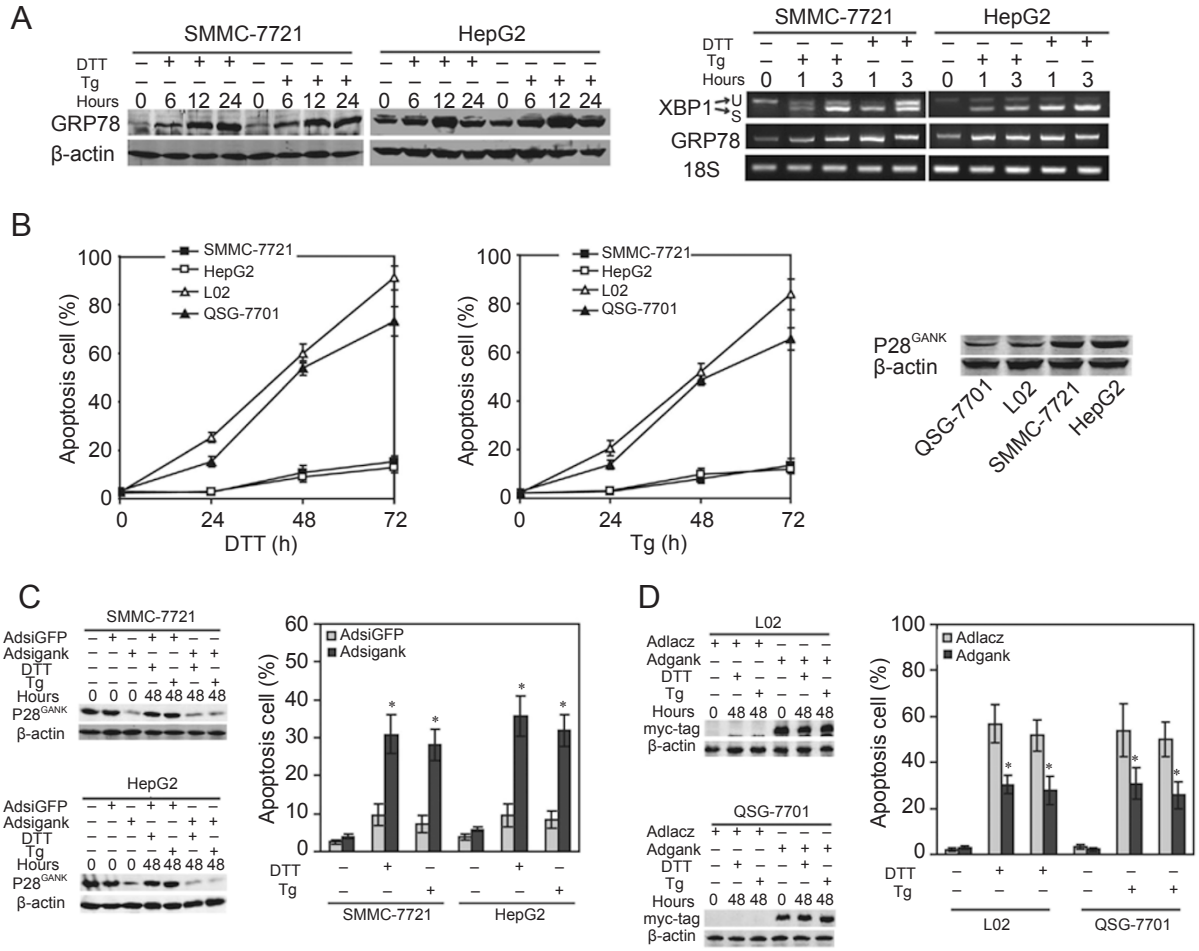


**Figure 2** p28$^{GANK}$ protects HCC cells against ER stress-induced death. **(A)** Dithiothreitol and thapsigargin induce the UPR in HCC cells. Dithiothreitol (2.5 mM) or thapsigargin (1 μM) treated SMMC-7721 and HepG2 cells were analyzed by western blot and RT-PCR. The shorter band (S) and longer band (U) indicated the spliced form (active form) and un-spliced form (inactive form) of XBP1 mRNA, respectively. **(B)** HCC cells are relatively resistant to ER stress-induced apoptosis. Dithiothreitol (4 mM) or thapsigargin (3 μM) treated L02, QSG-7701, SMMC-7721, and HepG2 cells were subjected to apoptosis analysis. Points, mean of three individual experiments; bars, SE. p28$^{GANK}$ protein levels in QSG-7701, L02, SMMC-7721, and HepG2 cells were analyzed by western blot. **(C)** p28$^{GANK}$ protects HCC cells against ER stress-induced apoptosis. After transfected with adenovirus-delivered p28$^{GANK}$ siRNA (Adsigank) for 24 h, SMMC-7721 and HepG2 cells were treated with or without dithiothreitol (2.5 mM) and thapsigargin (1 μM) for another 48 h, then subjected to apoptosis analysis. Columns, mean of three individual experiments; bars, SE. *$P$ < 0.05. **(D)** p28$^{GANK}$ protects L02 and QSG-7701 cells against ER stress-induced apoptosis. After transfected with adenovirus-delivered p28$^{GANK}$ expression vector (Adgank) and the control vector (Adlacz) for 24 h, L02 and QSG-7701 cells were treated with or without dithiothreitol (4 mM) and thapsigargin (3 μM) for another 48 h, then subjected to apoptosis analysis. Columns, mean of three individual experiments; bars, SE. *$P$ < 0.05.

Figure S1). Furthermore, the protein level of GADD153, which acts as a convergence point for the UPR signaling, was increased by p28$^{GANK}$ in DTT- or TG-treated NIH3T3 and L02 cells (Supplementary information, Figure S2). Taken together, these data indicate that p28$^{GANK}$ promotes the UPR.

*p28$^{GANK}$ increases the intracellular pool of GRP78*

GRP78, a central regulator of ER homeostasis, plays a pivotal role in protecting cells from ER stress-induced apoptosis. We therefore examined whether p28$^{GANK}$ can inhibit ER stress-induced apoptosis through increasing the intracellular pool of GRP78. As shown in Figure 4A, when p28$^{GANK}$ expression was suppressed, DTT- and TG-induced GRP78 protein levels were decreased significantly in SMMC-7721 and HepG2 cells at the indicated time points. Furthermore, overexpression of p28$^{GANK}$ resulted in increased accumulation of GRP78 proteins in

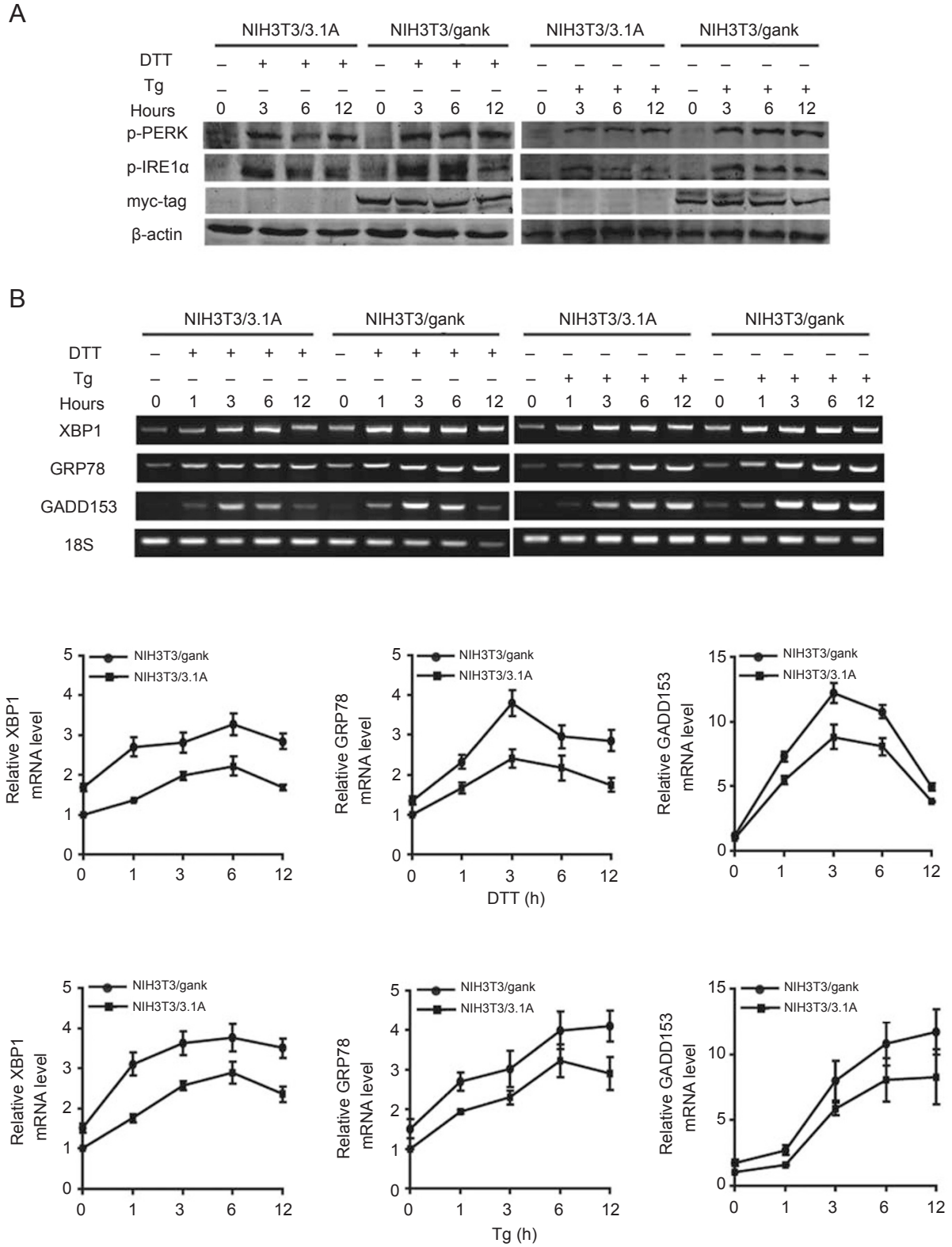


**Figure 3** p28$^{GANK}$ promotes the UPR. **(A)** p28$^{GANK}$ increases PERK and IRE1α phosphorylation. Whole cell lysates from dithiothreitol (2.5 mM) or thapsigargin (1 μM) treated NIH3T3 cells were analyzed by western blot. **(B)** p28$^{GANK}$ elevates mRNA levels of the UPR typical signatures. Total RNA extracted from dithiothreitol (2.5 mM) or thapsigargin (1 μM) treated NIH3T3 cells were analyzed by RT-PCR. The mRNA values were normalized to 18S rRNA content. Points, mean of three individual experiments; bars, SE.

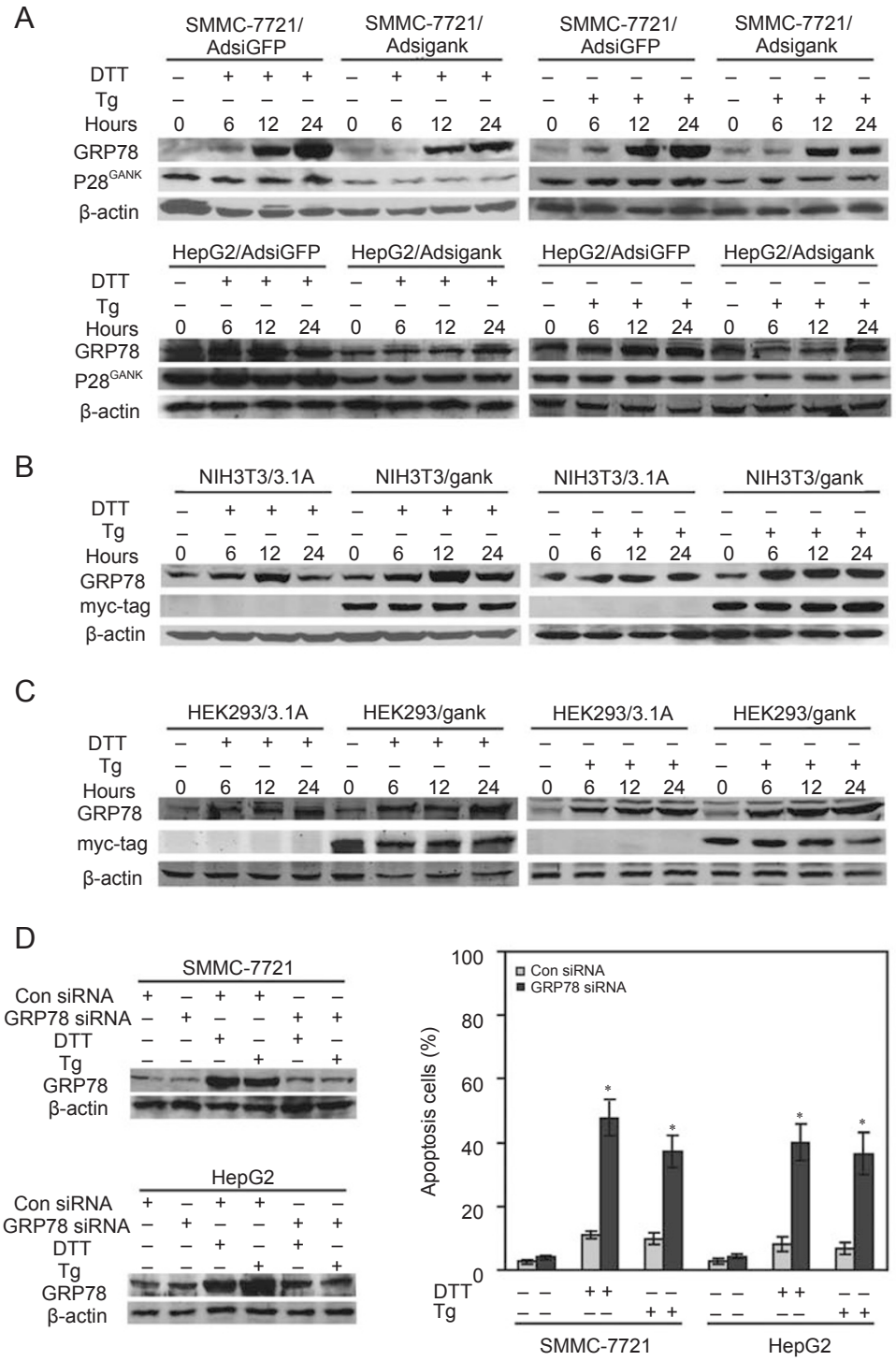


**Figure 4** p28GANK increases the intracellular pool of GRP78. **(A)** p28GANK knockdown inhibits GRP78 induction. After transfected with p28GANK siRNA for 24 h, SMMC-7721 and HepG2 cells were treated with or without dithiothreitol (2.5 mM) and thapsigargin (1 μM) for indicated time periods, then subjected to western blot. **(B-C)** p28GANK elevates GRP78 induction. p28GANK-stably overexpressed NIH3T3 cell **(B)** and p28GANK-transiently transfected HEK293 cell **(C)** were treated with dithiothreitol (2.5 mM) or thapsigargin (1 μM). Whole cell lysates were analyzed by western blot. **(D)** GRP78 knockdown sensitizes HCC cells to ER stress-induced apoptosis. After transfected with GRP78 siRNA for 24 h, SMMC-7721 and HepG2 cells were treated with or without dithiothreitol (2.5 mM) and thapsigargin (1 μM) for another 48 h. Apoptosis was measured using flow cytometry. Columns, mean of three individual experiments; bars, SE. *$P < 0.05$.

DTT- or TG-treated NIH3T3 and HEK293 cells (Figure 4B and 4C). Together, these data indicate that p28$^{GANK}$ promotes GRP78 protein expression under ER stress conditions.

To confirm the protective role of GRP78 in preventing HCC cells from ER stress-induced apoptosis, expression of GRP78 was suppressed by the GRP78-specific siRNA in SMMC-7721 and HepG2 cells. Figure 4D shows that suppression of GRP78 can substantially increase the sensitivity of HCC cells to DTT- and TG-induced apoptosis. These results suggest that the cytoprotective function of p28$^{GANK}$ under ER stress is dependent, at least in part, on enhanced GRP78 induction.

*Reduced NF-κB activation is involved in p28$^{GANK}$-induced GRP78 upregulation*

Since our previous study demonstrated that p28$^{GANK}$ suppresses NF-κB nuclear localization through direct binding to RelA/p50 [21], it is possible that p28$^{GANK}$ may inhibit the ER stress-induced NF-κB activation. To test this possibility, we determined whether p28$^{GANK}$ can affect p65 activity under ER stress in HEK293 cells. The results showed that p28$^{GANK}$ reduced DTT- and TG-induced p65 nuclear localization (Figure 5A), and inhibited the p65 DNA-binding activity (Figure 5B). Importantly, overexpression of p65 resulted in decreased GRP78 induction under ER stress in SMMC-7721 cells (Figure 5C), suggesting that p28$^{GANK}$ may upregulate the expression of GRP78 through reducing NF-κB activation. As expected, p28$^{GANK}$ overexpression dose-dependently attenuated the inhibitory effect of p65 on GRP78 expression in SMMC-7721 cells (Figure 5D). These results suggest that reduced NF-κB activation is involved in p28$^{GANK}$-induced GRP78 upregulation.

*Elevated p38 MAPK activation is involved in p28$^{GANK}$-induced GRP78 upregulation*

As induction of endogenous GRP78 by ER stress requires the p38 MAPK pathway [22], and upregulation of GRP78 by p38 MAPK protects cells from drug-induced apoptosis [23], we addressed whether p38 MAPK participates in the p28$^{GANK}$-induced GRP78 upregulation.

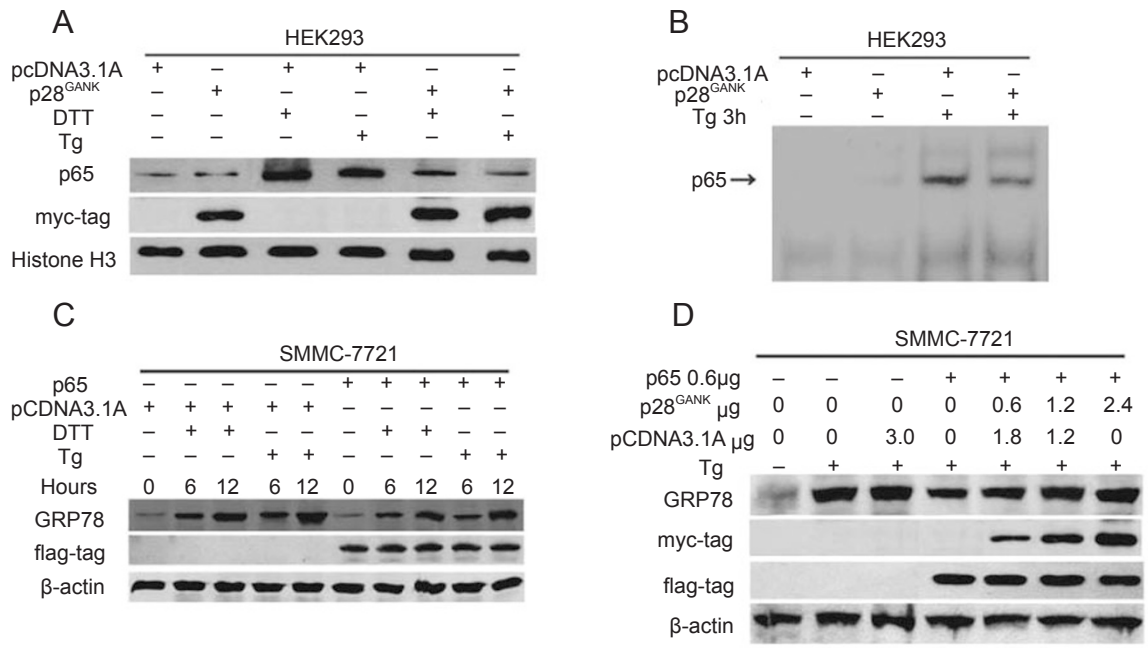


**Figure 5** NF-κB is involved in p28$^{GANK}$-induced GRP78 upregulation. **(A)** p28$^{GANK}$ inhibits dithiothreitol- and thapsigargin-induced p65 nucleus location. After transient transfection with p28$^{GANK}$ or the control vector for 24 h, HEK293 cells were treated with or without dithiothreitol (2.5 mM) or thapsigargin (1 μM) for another 6 h, nuclear extracts were analyzed by western blot. **(B)** p28$^{GANK}$ inhibits thapsigargin-mediated p65 DNA-binding activity. p28$^{GANK}$- or empty vector-transiently transfected HEK293 cells were treated with thapsigargin (1 μM) for 6 h, nuclear extracts were analyzed by EMSA. **(C)** p65 inhibits dithiothreitol- and thapsigargin-induced GRP78 expression. After transient transfection with p65 construct for 24 h, SMMC-7721 cells were treated with dithiothreitol (2.5 mM) or thapsigargin (1 μM) for indicated time periods, then subjected to western blot analysis. **(D)** p28$^{GANK}$ attenuates p65-mediated GRP78 downregulation. SMMC-7721 cells were co-transfected with plasmids expressing p65 and p28$^{GANK}$. After 24 h, the cells were treated with dithiothreitol (2.5 mM) or thapsigargin (1 μM) for another 12 h, then subjected to western blot analysis.

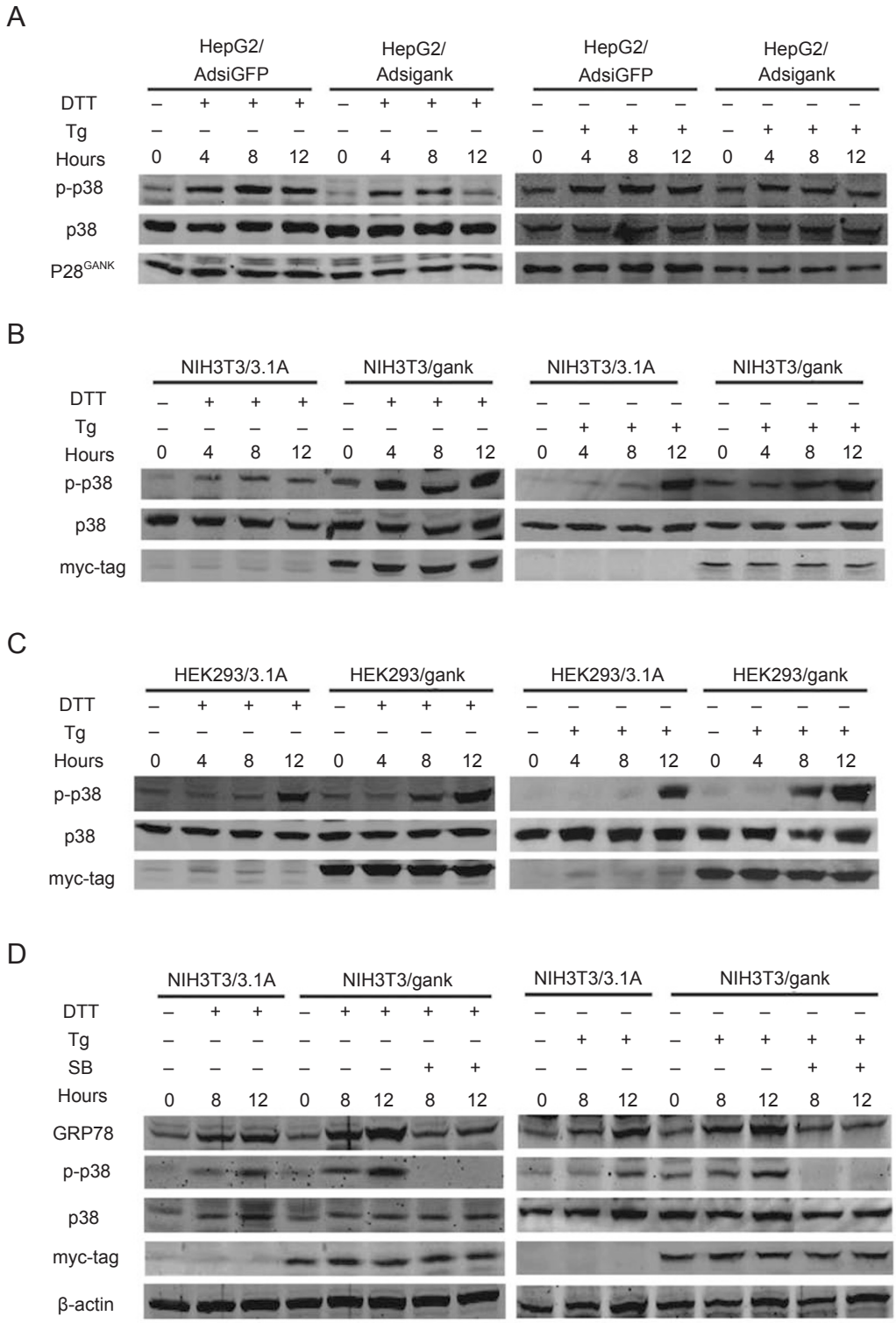


**Figure 6** p38 MAPK is involved in p28$^{GANK}$-induced GRP78 upregulation. **(A)** p28$^{GANK}$ knockdown decreases p38 MAPK activation. After transfected with p28$^{GANK}$ siRNA for 24 h, HepG2 cells were treated with dithiothreitol (2.5 mM) or thapsigargin (1 μM), then subjected to western blot analysis. **(B-C)** p28$^{GANK}$ increases p38 MAPK activation. p28$^{GANK}$-stably overexpressed NIH3T3 **(B)** and p28$^{GANK}$-transiently transfected HEK293 cells **(C)** were treated with dithiothreitol (2.5 mM) or thapsigargin (1 μM), then subjected to western blot analysis. **(D)** SB202190 inhibits p28$^{GANK}$-induced GRP78 upregulation. NIH3T3 cells were treated with dithiothreitol (2.5 mM) or thapsigargin (1 μM) with or without SB202190 (10 μM) pre-incubation for 1 h, then subjected to western blot analysis.

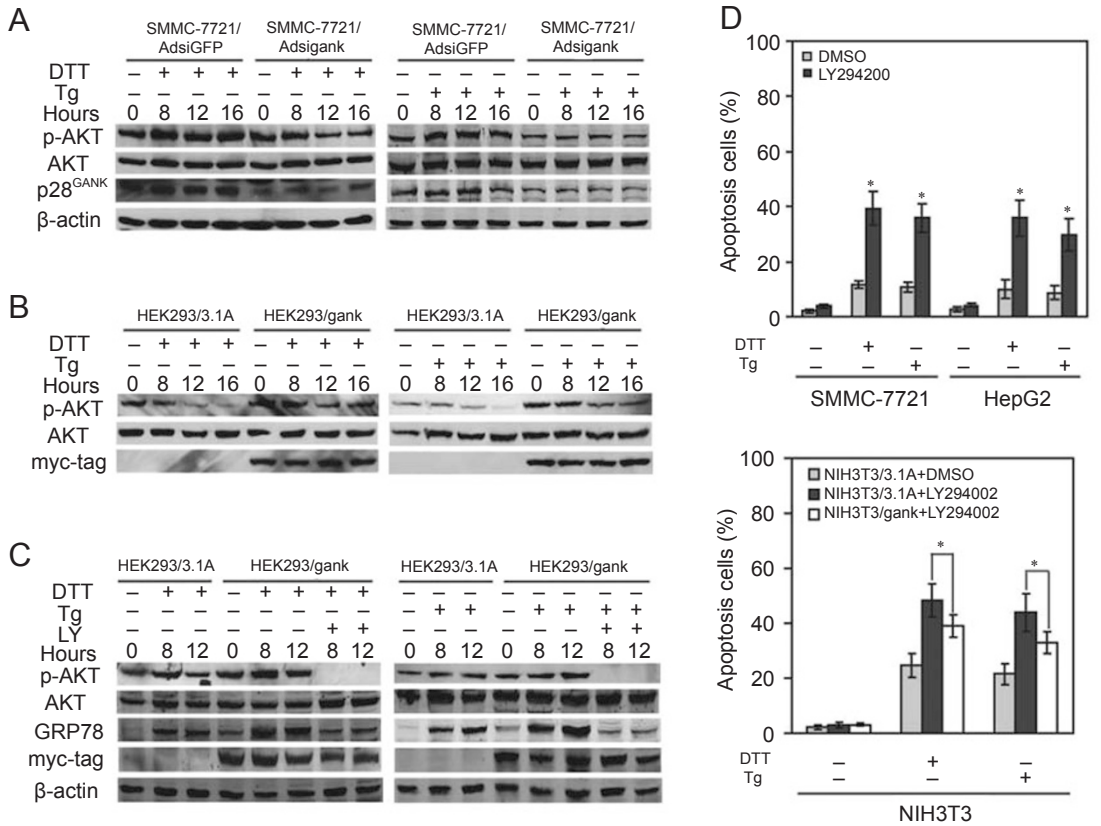


**Figure 7** PI3K/Akt is involved in p28$^{GANK}$-induced GRP78 upregulation and the anti-apoptosis effect of p28$^{GANK}$. **(A)** p28$^{GANK}$ knockdown decreases Akt activation. After being transfected with p28$^{GANK}$ siRNA for 24 h, SMMC-7721 cells were treated with dithiothreitol (2.5 mM) or thapsigargin (1 μM), then subjected to western blot analysis. **(B)** p28$^{GANK}$ overexpression enhances Akt activation. After transient transfection with p28$^{GANK}$ construct for 24 h, HEK293 cells were treated with dithiothreitol (2.5 mM) or thapsigargin (1 μM), then subjected to western blot analysis. **(C)** LY294002 inhibits p28$^{GANK}$-mediated GRP78 upregulation. After transient transfection with p28$^{GANK}$ construct for 24 h, HEK293 cells were treated with dithiothreitol (2.5 mM) or thapsigargin (1 μM) with or without LY294002 (30 μM) pre-incubation for 1 h, then subjected to western blot analysis. **(D)** PI3K/Akt is involved in the anti-apoptosis effect of p28$^{GANK}$. SMMC-7721, HepG2, and NIH3T3 cells were treated with dithiothreitol (2.5 mM) or thapsigargin (1 μM) for 48 h with or without LY294002 (30 μM) pre-incubation for 1 h. Apoptosis was measured using flow cytometry. Columns, mean of three individual experiments; bars, SE. *$P < 0.05$.

We first evaluated whether p28$^{GANK}$ regulates p38 MAPK phosphorylation under ER stress. When p28$^{GANK}$ was silenced by its siRNA, p38 MAPK phosphorylation was downregulated in both DTT- and TG-treated HepG2 cells (Figure 6A). Furthermore, overexpression of p28$^{GANK}$ increased p38 MAPK phosphorylation in DTT- or TG-treated NIH3T3 and HEK293 cells (Figure 6B and 6C).

To determine whether the p38 MAPK pathway is involved in p28$^{GANK}$-induced GRP78 upregulation, stable p28$^{GANK}$-expressing NIH3T3 cells and the control cells were treated with DTT or TG in the presence or absence of p38 MAPK inhibitor SB202190 (10 μM). The results showed that SB202190 inhibited p28$^{GANK}$-induced GRP78 upregulation in DTT- or TG-treated NIH3T3 cells (Figure 6D), suggesting that the p38 MAPK pathway is required for p28$^{GANK}$-induced GRP78 upregulation.

*Elevated Akt activation is involved in p28$^{GANK}$-induced GRP78 upregulation*

In addition to p38 MAPK, p28$^{GANK}$ also increased Akt phosphorylation under ER stress. As shown in Figure 7A, p28$^{GANK}$ knockdown decreased Akt phosphorylation in DTT- or TG-treated SMMC-7721 cells. Furthermore, p28$^{GANK}$ overexpression obviously increased Akt phosphorylation in DTT- or TG-treated HEK293 cells (Figure 7B). To test whether the PI3K/Akt pathway contributes to p28$^{GANK}$-induced GRP78 upregulation, p28$^{GANK}$-transiently transfected HEK293 cells and control cells were treated with DTT or TG in the presence or absence of

PI3K-specific inhibitor LY294002 (30 μM). As shown in Figure 7C, LY294002 prevented p28GANK-induced GRP78 upregulation, indicating that PI3K/Akt is involved in the p28GANK-induced GRP78 upregulation.

Since previous findings indicated that the endogenous Akt activity plays a critical role in cell survival by suppressing ER stress-induced cell death [24], we investigated the role of PI3K/Akt in protecting HCC cells against ER stress-induced apoptosis. SMMC-7721 and HepG2 cells were treated with LY294002 1 h before the addition of DTT or TG for another 24 h. The results showed that LY294002 significantly sensitized SMMC-7721 and HepG2 cells to DTT- and TG-induced apoptosis (Figure 7D). These observations suggest that increased Akt activation contributes to the anti-apoptosis effect of p28GANK. Furthermore, when incubated with LY294002 before the addition of DTT or TG, control cells showed more sensitivity to apoptosis than the stable p28GANK-expressing NIH3T3 cells (Figure 7D), indicating that LY294002 reduced, only in part, the cytoprotective ability of p28GANK under ER stress.

*p28GANK promotes translational recovery*

During ER stress, the phosphorylation of eIF2α on Ser51 elicits a global translational repression to protect cells from protein misfolding overload, and allow time to reestablish ER homeostasis. However, translational recovery is needed for stressed cells to survive when protein misfolding overload is attenuated. To determine whether p28GANK can affect ER stress-induced translational repression, we analyzed the effect of p28GANK on eIF2α phosphorylation in NIH3T3 and L02 cells. The results showed that phosphorylation of eIF2α was decreased by p28GANK in the course of the ER stress response in both cell lines (Figure 8), indicating that p28GANK promotes translational recovery.

## Discussion

In this study, we demonstrated that p28GANK is a potent protective molecule against ER stress-induced apoptosis. We have previously shown the pro-carcinogenesis activity of p28GANK in HCC cells *in vitro* and *in vivo* [18], but the role of p28GANK in regulating ER stress-induced cell death has not been characterized. The UPR is an important cytoprotective response for cells to cope with ER stress, but prolonged ER stress can activate mitochondria-dependent or -independent apoptosis [25]. Although

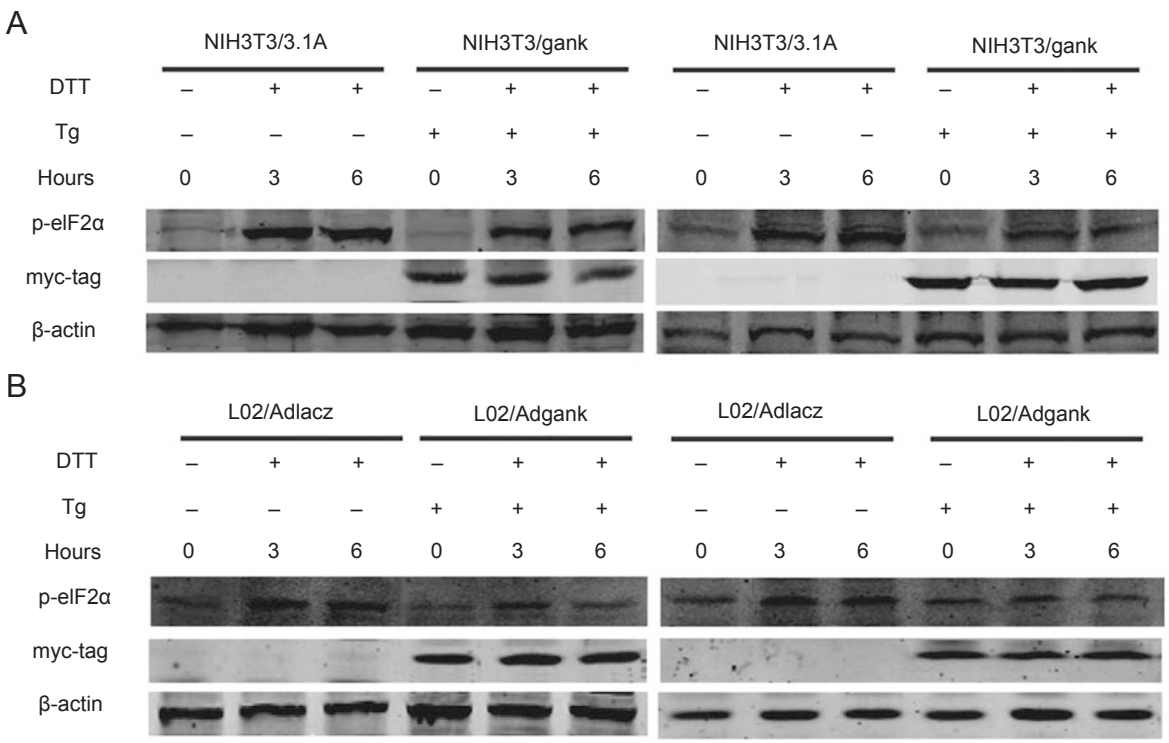


**Figure 8** p28GANK promotes recovery from translational repression. **(A-B)** p28GANK decreases eIF2α phosphorylation in NIH3T3 **(A)** and L02 **(B)** cells. p28GANK-overexpressed NIH3T3 and L02 cells were treated with dithiothreitol (2.5 mM) or thapsigargin (1 μM), then subjected to western blot analysis.

the UPR is clearly activated in solid tumors, it is still unclear how tumor cells adapt to long-term ER stress [20, 26-28]. In addition to the UPR, tumor cells adopt other survival mechanisms to cope with ER stress. We hypothesize that some oncoproteins, such as Ras, c-myc, and $p28^{GANK}$, are involved in these mechanisms. ER stress induces a global downregulation of protein synthesis through the PERK/eIF2α pathway [6], but $p28^{GANK}$ protein level did not change in response to ER stress in HCC cells. More importantly, treatment with the ER stress inducer, DTT, or TG did not induce significant apoptosis in SMMC-7721 and HepG2 cells, whereas the resistance of both cell lines to these agents was reversed by $p28^{GANK}$ suppression. In addition, overexpression of $p28^{GANK}$ significantly reduced ER stress-induced apoptosis in L02 and QSG-7701 cells. We therefore conclude that $p28^{GANK}$ promotes HCC cell survival under ER stress.

How does $p28^{GANK}$ protect HCC cells from ER stress-induced cell death? It has been demonstrated that cell survival under ER stress is regulated by NF-κB, JNK, GADD153, PI3K/Akt, and MEK/ERK pathways [24, 29-31]. In this study, we found that $p28^{GANK}$ did not affect the activities of JNK and ERK under ER stress (Supplementary information, Figure S3). As $p28^{GANK}$ facilitates p53 degradation through binding to MDM2 [17], we also tested the effect of $p28^{GANK}$ on p53 protein level under ER stress, and we found that $p28^{GANK}$ had no appreciable effect on p53 protein level (Supplementary information, Figure S3). These data suggest that p53, ERK, and JNK pathways are not involved in the anti-apoptosis effect of $p28^{GANK}$ under ER stress. Instead, our results suggest that $p28^{GANK}$ inhibits ER stress-induced apoptosis by enhancing the adaptive UPR signaling system and promoting the recovery of ER function.

As a central regulator of ER homeostasis and the UPR, GRP78 plays multiple roles in protein folding, ER calcium homeostasis, and controlling of the activation of transmembrane ER stress sensors. Considering that hepatocytes possess highly developed secretory systems and that hepatocyte proliferation can activate $p28^{GANK}$ expression [32], it seems that $p28^{GANK}$ might play some roles in hepatocyte homeostasis regulation. This hypothesis is supported by our data, which demonstrated that $p28^{GANK}$ increased ER stress-induced GRP78 protein accumulation, thereby enhancing the folding capacity of the ER and promoting recovery from ER stress. It has been reported that the induction of GRP78 is essential for the survival of tumor cells under ER stress, and suppression of GRP78 inhibits tumor growth *in vivo* [33]. Consistent with these data, our results demonstrated that GRP78 inhibition by siRNA resulted in substantially increased the sensitivity of HCC cells to DTT- and TG-induced apoptosis. We therefore propose that $p28^{GANK}$ plays a cytoprotective role, at least in part, through upregulating GRP78 induction.

One of the possibilities of $p28^{GANK}$-induced GRP78 upregulation is through the augmented UPR. However, our results suggest that other mechanisms might also participate in $p28^{GANK}$-induced GRP78 upregulation. GRP78 expression is primarily regulated by ATF6, XBP1, and activating transcription factor 4 (ATF4) [34-36] at the transcriptional level. ATF6 undergoes posttranslational modifications, such as phosphorylation by p38 MAPK, further enhancing its efficacy as an activator to induce GRP78 expression [22]. Our data showed that $p28^{GANK}$ promoted ER stress-induced p38 MAPK phosphorylation, and blocking the p38 MAPK pathway by SB202190 inhibited $p28^{GANK}$-induced GRP78 upregulation. The mechanism of ER stress-induced p38 MAPK activation is obscure, and a most recent study reported that an IRE1-TRAF2-ASK1 module mediates the p38 MAPK pathway activation [37]. This raises the question whether $p28^{GANK}$-increased p38 MAPK activity is dependent on augmented UPR. As $p28^{GANK}$ also increased $H_2O_2$-induced p38 MAPK phosphorylation in HCC cells (our unpublished results), it seems that $p28^{GANK}$ can increase p38 MAPK activity independent of augmented UPR. This speculation is supported by our demonstration that $p28^{GANK}$ did not affect the phosphorylation of JNK, which is also activated by the IRE1-TRAF2 module under ER stress [38]. These data suggest that $p28^{GANK}$ elevates p38 MAPK activation independently of IRE1-TRAF2-ASK1 under ER stress, which subsequently contributes to GRP78 upregulation.

Although the PI3K/Akt pathway, a critical mediator of cell survival under a variety of apoptotic stimuli [39], has been well documented in preventing ER stress-induced cell death [24], the regulation of Akt activation during ER stress is rarely discussed. In the present study, we found $p28^{GANK}$ obviously increased Akt activation under ER stress, and PI3K inhibitor LY294002 inhibited $p28^{GANK}$-induced GRP78 upregulation, indicating that Akt mediates $p28^{GANK}$-induced GRP78 upregulation. As Akt has no effect on GRP78 mRNA induction (Supplementary information, Figure S4), this suggests that Akt regulates GRP78 induction independently of ATF6. Moreover, PI3K/Akt inhibition significantly sensitized HCC cells to DTT- and TG-induced apoptosis, indicating that $p28^{GANK}$ may promote HCC cell survival through elevated Akt activation. The mechanism of ER stress-induced PI3K/Akt activation remains unclear, and more studies are needed to unravel the link between $p28^{GANK}$ and PI3K/Akt. In addition, NIH3T3 cells stably expressing $p28^{GANK}$ showed less sensitivity to ER stress-induced

apoptosis than the control cells upon treatment with LY294002, indicating that p28GANK also protects cells from ER stress-induced apoptosis by other mechanisms, such as through upregulating GRP78 expression.

It has been reported that p28GANK binds to RelA/p50 directly and suppresses its transcriptional activity [21, 40]. Thus, it is conceivable that p28GANK can regulate ER stress response through the NF-κB pathway. Indeed, p28GANK inhibited ER stress-induced p65 activation. More interestingly, we found that p65 inhibited GRP78 induction and this inhibition can be reversed by p28GANK. These data suggest that p28GANK could promote ER stress-induced GRP78 expression through attenuating p65 activation.

Although GADD153 expression is primarily regulated at the transcriptional level, our data showed that p28GANK not only promoted GADD153 transcription but also enhanced the stability of GADD153 transcripts. It has been reported that ER stress-induced GADD153 expression can be repressed by p65 [41, 42], which is consistent with our data demonstrating that p65 overexpression decreased GADD153 expression under ER stress. More importantly, p28GANK overexpression dose-dependently attenuated the inhibitory effect of p65 on GADD153 expression (Supplementary information, Figure S5), suggesting that p28GANK promotes ER stress-induced GADD153 expression by suppressing NF-κB activity. In addition to ATF6, p38 MAPK also mediates the phosphorylation of GADD153 and enhances its transcription activity during ER stress [43], suggesting that p28GANK may affect GADD153 posttranslational modifications through the p38 MAPK pathway. GADD153 is considered as a key effector of ER stress-induced apoptosis, but some genes regulated by GADD153 mediate an adaptive response rather than apoptosis induction [44]. Moreover, in certain cellular contexts, GADD153 exhibits anti-apoptotic activity [45]. We therefore suggest that p28GANK-mediated GADD153 upregulation mediates an adaptive response in our studies.

PERK-dependent eIF2α phosphorylation induces a global downregulation of protein synthesis [1], which protects cells from protein misfolding overload and allows time to reestablish ER homeostasis. However, translational recovery is needed for stressed cells to survive when protein misfolding overload is attenuated [46]. Therefore, profound and persistent translational repression is not conducive to coping with ER stress. In this study, we found that p28GANK decreased eIF2α phosphorylation, which could promote the restoration of eIF2α activity.

In brief, we provide evidence that p28GANK protects HCC cells from ER stress-induced apoptosis through enhancement of the ER adaptive capacity. The cytoprotective role of p28GANK under ER stress is mediated, at least in part, by enhanced UPR signaling and GRP78 induction. We also showed that the p38 MAPK, PI3K/Akt, and NF-κB pathways were involved in p28GANK-enhanced GRP78 induction. Further studies on the function of p28GANK under ER stress will contribute to the understanding of molecular mechanisms of hepatocarcinogenesis and the development of new therapeutic strategies against HCC.

## Materials and Methods

*Human tissues*

Human liver biopsies were obtained from Shanghai Eastern Hepatobiliary Surgery Hospital. The use of the samples has been approved by the local ethical committee.

*Antibodies and chemicals*

TG, DTT, and actinomycin D were purchased from Sigma Chemical Company. The p38 MAPK-specific inhibitor SB202190 and PI3K-specific inhibitor LY294002 were purchased from Merck Chemicals. Antibodies against gankyrin (p28GANK), GRP78, GADD153, and phospho-PERK (Thr981) were purchased from Santa Cruz Biotechnology. Antibodies against myc-tag, phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-Akt (Ser473), Akt, phospho-eIF2α (Ser51), Histone H3, and β-actin were purchased from Cell Signaling Technology. Antibody against Flag-tag was purchased from Sigma. Antibody against phospho-Ire1α (Ser724) was purchased from Abcam.

*Cell culture and treatments*

SMMC-7721, HepG2, QSG-7701, L02, NIH3T3, and HEK293 cell lines were maintained at 37 ºC in a humidified incubator containing 5% $CO_2$, in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. DTT and TG were used for inducing the ER stress response at indicated dosage for indicated time periods.

*Transfections*

The whole cDNA sequence of p28GANK (from pCMV-HA-p28GANK) was cloned into the pCDNA-3.1A-myc vector and obtained myc-p28GANK construct. The expression vectors Akt (HA-Akt) were kindly provided by Professor Jin Q Cheng. NIH3T3 cells were transfected by Lipofectamine 2000 (Invitrogen) with the control plasmids (pcDNA3.1A) or myc-p28GANK (GANK) construct. Stable clones were selected in the presence of 400 μg/ml Geneticin (G418). Transient transfection of myc-p28GANK and the control constructs into HEK293 cells were performed using polyethylenimine (Polyplus transfection). The protocol used for the production and application of Adsigank, AdsiGFP, Adgank, or Adlacz has been previously described [21]. The protocol used for GRP78 knockdown is as described previously [47].

*RNA preparation and RT-PCR analysis*

Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The reverse tran-

**Table 1** RT-PCR primers for 18S, XBP1, GRP78, GADD153, and p28$^{GANK}$

| Gene | | Primer sequences (5′⟶3′) |
|---|---|---|
| 18S | human | F: GGG AGG TAG TGA CGA AAA AT |
| | | R: ACC AAC AAA ATA GAA CCG CG |
| | mouse | F: TAA CGA ACG AGA CTC TGG CAT |
| | | R: CGG ACA TCT AAG GGC ATC ACA G |
| XBP1 | human | F: CCT TGT AGT TGA GAA CCA GG |
| | | R: GGG GCT TGG TAT ATA TGT GG |
| | mouse | F: AAA CAG AGT AGC AGC GCA GAC TGC |
| | | R: GGA TCT CTA AAA CTA GAG GCT TGG TG |
| GRP78 | human | F: ATC ACG CCG TCC TAT GTC GC |
| | | R: TCT CCC CCT CCC TCT TAT CC |
| | mouse | F: GAA AGG ATG GTT AAT GAT GCT GAG |
| | | R: GTC TTC AAT GTC CGC ATC CTG |
| GADD153 | human | F: AGT CAT TGC CTT TCT CTT CG |
| | | R: GGT GCA GAT TCA CCA TTC GG |
| | mouse | F: CAT ACA CCA CCA CAC CTG AAA G |
| | | R: CCG TTT CCT AGT TCT TCC TTG C |
| P28$^{GANK}$ | human | F: GCA TGC TCA GCT GGA CAT ACA G |
| | | R: GTA GCC TCA TAA TGG TCC TTA GC |
| | mouse | F: GCT GTC AAT CAA AAC GGC TG |
| | | R: GCC TCT TCA CTT TCT GCT AG |

scription reactions (RT-PCR) were carried out using the M-MLV Reverse Transcriptase (Promega) according to the manufacturer's protocol. The PCRs were optimized for a number of cycles to ensure product intensity within the linear phase of amplification. PCR products were resolved by electrophoresis in a 2% or 4% agarose gel, stained with ethidium bromide. The gel images were digitally captured with a SynGene gel documentation system and analyzed with the Genetools analysis software (Syngene, Frederick, MD, USA). All tests were repeated thrice, and one of the repeats was shown in the results. The primers used in this study are shown in Table 1.

*Nuclear protein extraction and western blot analysis*

Cells were lysed in Triton lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 10 mM NaF, 5 mg/ml aprotinin, 20 mM leupeptin, and 1 mM sodium orthovanadate) and centrifuged at 12 000× *g* for 15 min. Protein concentrations were measured using the BCA assay (Santa Cruz Biotech). Proteins were applied to SDS-PAGE. After electrophoresis, proteins were blotted to polyvinylidene fluoride (PVDF) membranes and then blocked with 5% skim milk powder with 0.1% Tween-20. The blots were then probed at 4 ºC overnight with relevant primary antibodies, washed by TBST (TBS containing 0.1% Tween-20) thrice, and probed with the appropriate horseradish-peroxidase-conjugated secondary antibodies at room temperature for 2 h. Immunoreactive material was detected using the ECL kit according to manufacturer's instruction (Santa Cruz Biotech). The protocol used for nuclear protein extraction has been previously described [21].

*Apoptosis analysis*

Cells were treated with DTT and TG for the indicated time. Apoptosis was detected using Annexin V-FITC Apoptosis Detection Kit (PharMingen) according to the manufacturer's manual. Annexin V staining was analyzed by flow cytometry within 1 h. Cells negative for both PI and Annexin V staining are live cells, Annexin V-positive staining cells are early apoptotic cells, and PI-positive and Annexin V-positive staining cells are primarily cells in late stage of apoptosis. The experiments were repeated thrice.

*Statistical analysis*

Results are expressed as the mean ± standard deviation. Statistical analysis was performed using Student's *t*-test in Microsoft Excel.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)