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Biochemistry and Cell Biology
Biochimie et biologie cellulaire

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| Journal: | *Biochemistry and Cell Biology* |
| Manuscript ID | bcb-2016-0009.R1 |
| Manuscript Type: | Article |
| Date Submitted by the Author: | 10-Feb-2016 |
| Complete List of Authors: | Gao, Xuejuan; Jinan University, Key Laboratory of Functional Protein Research of Guangdong Higher Education Institutes, Institute of Life and Health Engineering<br>Xu, Fengmei; Jinan University, Key Laboratory of Functional Protein Research of Guangdong Higher Education Institutes, Institute of Life and Health Engineering<br>Zhang, Huan-Tian; Jinan University, Institute of Orthopedic Diseases and Department of Orthopedics, the First Affiliated Hospital<br>Chen, Miaojuan; Guangzhou Women and Children's Medical Center, Department of Interventional Radiology and Vascular Anomalies<br>Huang, Wensi; Jinan University<br>Zhang, Qihao; Jinan University, Institute of Biomedicine, and National Engineering Research Center of Genetic Medicine; Guangdong Provincial Key Laboratory of Bioengineering Medicine<br>Zeng, Qingzhong; Jinan University, Key Laboratory of Functional Protein Research of Guangdong Higher Education Institutes, Institute of Life and Health Engineering<br>Liu, Langxia; Jinan University, Key Laboratory of Functional Protein Research of Guangdong Higher Education Institutes, Institute of Life and Health Engineering |
| Keyword: | TRIM21, TRAIL, H1299 cells, GSK3β, PKCα |
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# PKCα/GSK3β/NF-κB signaling pathway and the possible involvement of TRIM21 in TRAIL-induced apoptosis

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**Conflict of interest:** The authors declare that they have no conflict of interest.

**Abstract**

Tumor necrosis factor related apoptosis-inducing ligand (TRAIL) is a highly promising therapeutic agent for cancer treatment due to its ability to selectively target tumor cells for cell death, while has little effect on most of normal cells. However, recent research has found that many cancers including non-small cell lung cancer (NSCLC) display resistance to TRAIL. Therefore, it is important to elucidate the molecular mechanism governing the resistance to TRAIL treatment in tumor cells. In this study, we showed the negative regulation of TRAIL-induced apoptosis by GSK3β in H1299 NSCLC cells, and determined the PKCα isozyme as an upstream regulator of GSK3β that phosphorylates and inactivates GSK3β, thereby sensitizing cancer cells to TRAIL-induced apoptosis. Furthermore, we demonstrated that the anti-apoptotic effect of GSK3β is mediated by NF-κB pathway, while the tripartite motif 21 (TRIM21) was able to inhibit the activation of NF-κB by GSK3β, and leads to the promotion of cell apoptosis. Taken together, our study further delineated the underpinning mechanism of resistance to TRAIL-induced apoptosis in H1299 cells and provided new clues for sensitizing NSCLC cells to TRAIL therapy.

**Keywords:** TRIM21; TRAIL; H1299 cells; GSK3β; PKCα.

## Introduction

TRAIL (Tumor necrosis factor related apoptosis-inducing ligand) belongs to the tumor necrosis factor (TNF) family, and is recognized as the most potential therapeutic agent in the TNF superfamily due to its ability to selectively target cancer cells with limited harm toward normal cells (Walczak et al., 1999; Zhuang et al., 2013). It has been reported that soluble TRAIL and agonistic antibodies against TRAIL receptors are currently applied to clinical trials for cancer therapy (Johnstone et al., 2008; Azijli et al., 2013). Unexpectedly, almost 50% of human cancer cell lines, such as lung adenocarcinoma cells and a majority of primary cancer cells, become insensitive to TRAIL treatment, reflecting the poor clinical therapeutic efficacy of the treatment (Todaro et al., 2008). Therefore, the identification of the signaling molecules that are involved in TRAIL-induced cancer cell apoptosis may provide new clues for the development of effective sensitizers for TRAIL therapy (Felber et al., 2007).

Two signaling pathways, namely the mitochondrial intrinsic apoptotic pathway and the death receptor-mediated extrinsic apoptotic signaling pathway, have been reported to mediate apoptosis in the majority of cells. TRAIL induces cancer cell death mainly through activating the death receptor signaling pathway (Wiley et al., 1995; Kischkel et al., 2000). Upon the binding of TRAIL to its receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), TRAIL triggers oligomerization of its receptors and subsequently recruits cytoplasmic proteins FADD (Fas-associated death domain protein) and procaspase 8 (or procaspase 10), thereby forming the death-inducing signaling complex (DISC) (Peter and Krammer, 2003; Johnstone et al., 2008). After the formation of the DISC complex, procaspase 8 can be cleaved and activated by an autocatalytic mechanism, which leads to the subsequent activation of downstream effector caspases (Kaufmann and Hengartner,

2001; Johnstone et al., 2008). In addition to the caspases, several others key factors such as glycogen synthase kinase beta (GSK3β) (Liao et al., 2003; Song et al., 2004; Kotliarova et al., 2008; Beurel et al., 2009), NF-κB (Lin et al., 2000; Zhang and Fang, 2005), PKC (Harper et al., 2003; Azijli et al., 2013; McCray et al., 2014; Hayashi et al., 2015), cellular FLICE-inhibitory protein (c-FLIP) (Leverkus et al., 2000; Chen et al., 2011), and X-linked IAP (XIAP) (Deveraux et al., 1998) have also been demonstrated to be essential for this process.

GSK3β, a member of GSK3 family, was initially identified as an enzyme that is capable of phosphorylating glycogen synthase (Embi et al., 1980). Recent studies have reported that GSK3β is involved in the regulation of various cellular processes, such as survival, apoptosis, cell mobility, and embryonic development (Sanchez et al., 2003; Luo, 2009). The phosphorylation of Ser9 of GSK3β inhibits its enzymatic activity (Cross et al., 1995), while the phosphorylation of Tyr216 of GSK3β enhances its enzymatic activity (Kim et al., 1999). To date, several kinases such as PKC and Akt have been reported to phosphorylate GSK3β at Ser9 (Cross et al., 1995). GSK3β is well-known for its bifunctional effects on apoptotic signaling pathways, facilitating the mitochondrial intrinsic apoptotic pathway while blocking the death receptor-induced apoptosis (Beurel and Jope, 2006 ). Here, we focus on the regulating mechanism of GSK3β involved in the apoptosis induced by TRAIL in human lung adenocarcinoma H1299 cells.

Protein kinase C (PKC), a family of serine/threonine protein kinases, is activated by numerous stimuli and has versatile biological functions including regulating cell proliferation, survival, and apoptosis (Griner and Kazanietz, 2007; Chen et al., 2011; Zhang et al., 2014a). PKC isozymes can be classified into three subtypes (Spitaler and Cantrell, 2004): 1) the conventional cPKCs (α, β, γ), activated by diacylglycerol (DAG), calcium, or phosphatidylserine; 2) the novel

nPKCs (δ, ε, η, θ), activated by DAG and independent on calcium; and 3) the atypical aPKCs (ζ, λ/ι), activated by phosphatidylserine and independent on calcium and DAG. Different PKC isozymes have diverse cellular effects, such as pro-apoptosis or anti-apoptosis, depending on different stimuli and cell types (Gavrielides et al., 2004; Martelli et al., 2004). For example, the activation of the novel PKCδ, PKCε, and PKCη promotes cell survival and inhibits TRAIL-induced apoptosis in melanoma and breast cancer cells (Azijli et al., 2013), whereas PKCα has been suggested to increase the expression of TRAIL-R2, thereby contributing to the sensitization of TRAIL-induced apoptosis in non-small cell lung cancer (NSCLC) cells (Chen et al., 2007; Azijli et al., 2013). Nevertheless, the specific role of PKCα isozyme and its downstream signaling pathway in TRAIL-induced apoptosis in H1299 cells is largely unknown.

NF-κB has been shown to be an important transcription factor in the regulation of the resistance to TRAIL in cancer cells (Hoeflich et al., 2000; Aydin et al., 2010; Wang et al., 2014; Zhang et al., 2014b). Moreover, its transcriptional activity can be regulated by GSK3β (Aydin et al., 2010; Zhang et al., 2014b) on one hand, and on the other hand, by the tripartite motif (TRIM) family members (Tomar and Singh, 2015). TRIM family is defined by the existence of an N-terminal tripartite motif including a RING domain, one or two B-boxes and a Coiled-coil region (Micale et al., 2012). It contains more than 70 members and mediates a diversity of biological processes (Tomar and Singh, 2015). Particularly, TRIM21, one member of TRIM family, has been reported to suppress the transcriptional activity of NF-κB (Niida et al., 2010). However, the role of TRIM21 in the regulation of TRAIL-induced apoptosis, as well as its relationship with GSK3β in such process is unknown.

In the present study, we confirmed the protective role of GSK3β in TRAIL-induced

apoptosis in H1299 NSCLC cells, and demonstrated PKCα is the specific isozyme capable of phosphorylating and inactivating GSK3β, which enhances the apoptosis induced by TRAIL. Then, we showed that TRIM21 acts downstream of GSK3β in the signaling cascade to negatively regulate NF-κB activation during the cell apoptosis.

## Materials and methods

### Cell culture

Lung adenocarcinoma H1299 cells (Institute of life science Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Grand island, NY) supplemented with 10% fetal bovine serum (PAN-Biotech, Germany), in a humidified atmosphere containing 5% $CO_2$ at 37°C.

### MTT assays

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Beyotime Biotechnology, China) assays were used to evaluated the effects of TRAIL treatment on cell viability. H1299 cells ($4\times10^3$ cells/well) were seeded into 96-well plate (TCP011096, JET, Guangzhou, China) and incubated overnight. Then, the cells were treated with TRAIL (20 ng/ml) for different durations. Lastly, the cells were cultured with MTT solution (final concentration is 20 μg/ml) for 4 h at 37°C incubator and the generated formazan crystals were dissolved in 150 μl DMSO/well. A spectrophotometric microplate reader (ELX800, BioTek Instrument, USA) was used to measure the absorbance of formazan crystals at 570 nm, and cellular viability corresponding to absorbance value was analyzed.



### *In vitro* phosphorylation assay

GST-GSK3β fusion protein was expressed and purified as described previously (Gao et al., 2013; Gao et al., 2014). Active PKCα was purchased from Abcam (USA). PP1 phosphatase was purchased from New England BioLabs (USA). For the phosphorylation experiments, purified

GST-GSK3β and Kinase Assay Buffer (25 mM MOPS, pH 7.2, 12.5 mM β-glycerol-phosphate, 25 mM $MgCl_2$, 5 mM EGTA, 2 mM EDTA; adding 0.25 mM DTT prior to use) were first brought to room temperature for 30 min. Next, 200 ng of purified GST-GSK3β and Kinase Assay Buffer were mixed together for 10 min. Then, 5 μl active PKCα (0.1 μg/μl) and 10 μl ATP (1 mg/ml, Biosharp, China) with or without PP1 phosphatase (2.5 U/μl, 0.4 μl) accompanied by 1×PP1 reaction buffer (50 mM HEPES, 100 mM NaCl, 0.1 mM EGTA, 2 mM DTT, 0.025% Tween 20, Ph 7.5) were added to the mixture for 2 hat 30°C. Then, the Ser9 phosphorylation state of GSK3β was assessed using standard Western Blot assay. The bolts were probed with phospho-GSK3β (Ser9) (Cell Signaling Technology) antibody. CBB (Coomassie brilliant blue) staining of SDS-PAGE gels was used to analyze the level of GST-GSK3β fusion protein.



**Small interfering RNAs (siRNAs) assays**

SiRNAs target against GSK3β or PKCα were synthetized in GenePharma (Shanghai, China). The sense strand sequence of siRNA target GSK3β is: 5'-AAGUAAUCCACCUCUGGCUACTT-3'. The sense strand sequences of siRNA target PKCα is: 5'-AAGAGGTGCCATGAATTTGTT-3' (Cameron et al., 2008). The sequence of 5'-UUCUCCGAACGUGUCACGUTT-3' was used as negative control (NC). These siRNAs (100nM) were transfected into H1299 cells using Lipofectamin 2000 according to the manufacturer's instruction (Invitrogen). The interfered cells were harvested 48 h post-transfection.

**Plasmid transfection and western blot analysis**

Plasmids of Flag-GSK3β1, mCherry-GSK3β1 and mCherry-GSK3β1 S9A mutant were constructed previously (Gao et al., 2014). The cDNA encoding PKCα and TRIM21 (human) were

kindly provided by Han's Lab at Xiamen University, and was subcloned into Flag-vector and HA-vector to generate the corresponding plasmids, Flag-PKCα and HA-TRIM21. H1299 cells at a confluence of 80-90% were transfected with Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). H1299 cells were transfected with Flag-GSK3β1, mCherry-GSK3β1, mCherry-GSK3β1 S9A, Flag-PKCα, HA-TRIM21, siRNA target against GSK3β or PKCα, or combined with treatments of TRAIL (20 ng/ml, Sino Biological Inc. Beijing, China), LiCl (20 mM, Sigma), Gö6983 (1 μM, Beyotime, China), BAY 11-7082 (Beyotime, China), where it is indicated. Total cell lysate was prepared with buffer B, which containing 20 mM pH7.5 Tris, 150 mM NaCl, 1 % Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, $Na_3VO_4$, leupeptin, and 1 % protease inhibitor cocktail (Roche). 10-50 μg of protein was separated by 10 % or 12 % SDS-PAGE.

Blots were probed with antibodies against Flag (A2220, Sigma), HA (H9658, Sigma), GSK3β, phospho-GSK3β (Ser9), Cleaved Caspase 3 (Cell Signaling Technology), GAPDH (ZSGB-BIO, China), mCherry (Abbkine, USA), and p-NF-kB p65 (Ser536) (Santa Cruz) following the standard protocol (Gao et al., 2013; Gao et al., 2014; Gao et al., 2015). The quantitative analysis of immunoblot results was carried out using Image J software.

**Hoechst 33342 staining assay**

H1299 cells seeded in the 12-well plates at $1\times10^5$ cells/well were transfected with Flag-GSK3β, siRNA of GSK3β or their controls, and combined with TRAIL (20 ng/ml, 8 hours) treatment. Then, the cells were incubated with 0.1 mg/ml Hoechst 33342 for 10 min at 37°C in incubator. The stained cells were observed using a fluorescence microscope (Olympus, Japan) with 20× objective.

A total of about 200 cells from 4 random fields were counted. The percent of apoptosis was presented as ration of apoptotic cells to total cells. Apoptotic cells were the cells with chromatin condensation and fragmented fluorescent nuclei.

**Luciferase Assay**

To detect the transcriptional activity of NF-κB, NF-κB luciferase reporter plasmid p-NF-κB-TA-Luc (kindly provided by Prof. Xu Jiake) was used as described previously (Gao et al., 2014). Briefly, after transfection of p-NF-κB-TA-Luc plasmid for 12 h in a 6-well plate, other indicated plasmids or siRNA were co-transfected into H1299 cells for another 4 h and the cells were re-suspended in culture medium and reseeded into a 24-well plate at a density of $2\times10^5$ cells/well. Cells were cultured during 30 hours, then treated with TRAIL (20 ng/ml) or LiCl (20 mM) for 8 more hours. Finally, cells were harvested and prepared for luciferase assays as described previously (Gao et al., 2014).



**Statistics analysis**

Statistical analysis using Student's *t*-test was performed and the statistical significance was defined as $p<0.05$.

## Results

### GSK3β inhibits TRAIL-induced apoptosis in H1299 cells

In order to investigate the functional relationship among GSK3β, NF-κB and TRIM21 in TRAIL-induced apoptosis in H1299 cells, we have firstly set up a cell model by confirming the cytotoxic effect of TRAIL for H1299 cells, and the role of GSK3β in TRAIL-induced apoptosis. Consistent with previously report, our result showed that TRAIL indeed time-dependently inhibited the cell viability of H1299 cells (Fig. 1A). This result was further confirmed by Western blotting showing the increased level of the cleaved caspase 3, a marker for cell apoptosis (Fig. 1B). We next verified the effect of Flag-GSK3β overexpression or GSK3β knockdown on TRAIL-induced apoptosis by assessing the level of cleaved caspase 3. The results showed that overexpression of Flag-GSK3β largely prevented the cleavage of caspase 3, while GSK3β knockdown increased the level of the cleaved caspase 3 (Fig. 1C and D). Furthermore, overexpression of Flag-GSK3β remarkably inhibited the apoptotic ratio assessed by Hoechst 33342 staining (Fig. 1E, F), and GSK3β knockdown increased the apoptotic ratio (Fig. 1G, H). Taken together, these results suggested that GSK3β negatively regulated TRAIL-induced H1299 cell apoptosis.



### PKCα enhanced TRAIL-induced H1299 cell apoptosis via phosphorylating GSK3β

PKC is known to be able to phosphorylate GSK3β at Ser9 residue (Cross et al., 1995). Meanwhile, the Ser9 phosphorylation of GSK3β is involved in the process of TRAIL stimulation (Cross et al., 1995; Azijli et al., 2013). So we asked if the PKC-GSK3β signaling pathway could be effective in the process of TRAIL-induced apoptosis in H1299 cells. To this end, we used Gö6983 (having no off target effect on GSK3β (Anastassiadis et al., 2011; Wu-Zhang and Newton,

2013), a pan-inhibitor of PKC, in various combinations with TRAIL and LiCl to treat the cells. LiCl is a widely used GSK3β inhibitor which increases its Ser 9 phosphorylation level by interfering its PP1 (protein phosphatase-1)-mediated dephosphorylation (Zhang et al., 2003). The combination of LiCl with the PKC inhibitor Gö6983 would allow us to access the importance of GSK3β as the effector of PKC in TRAIL-induced apoptosis. As shown in Fig. 2A, upon the treatment of TRAIL, the cleavage of caspase 3 was increased, and LiCl further increased the cleavage of caspase 3, accompanied with the increased Ser9 phosphorylation of GSK3β. Interestingly, Gö6983 significantly reduced GSK3β Ser9 phosphorylation, but had little effect on TRAIL-induced caspase 3 cleavages regardless of LiCl treatment, although a slight increase of cleaved caspase 3 level could be observed with Gö6983 plus LiCl compared with Gö6983 only. This might be explained by the fact that Gö6983, as a pan-inhibitor of PKC isozymes, could have simultaneously blocked the pro-survival and pro-apoptosis PKC subtypes, resulting in the mutual offset between the opposing effects. In order to verify this hypothesis, we sought to determine the specific role of the PKCα isozyme in the Ser9 phosphorylation of GSK3β during the TRAIL-induced apoptosis. As shown in Fig. 2B, we found that Flag-PKCα overexpression dramatically elevated the Ser9 phosphorylation of GSK3β in a dose-dependent manner, which is, more interestingly, echoed by the similar dose-dependent increase of caspase 3 cleavage. In contrast, knockdown of PKCα decreased Ser9 phosphorylation of GSK3β, with the compromise of the apoptotic efficiency upon TRAIL treatment (Fig. 2C). These results suggested that PKCα exerts a pro-apoptotic role in TRAIL-induced apoptosis through Ser9 phosphorylation of GSK3β in H1299 cells. Subsequently, we confirmed the Ser9 phosphorylation of GSK3β by PKCα kinase *in vitro* using purified PKCα kinase, PP1 phosphatase, and GST-GSK3β. As shown in Fig. 2D, the

incubation of purified GST-GSK3β fusion protein with the active PKCα significantly increased its Ser9 phosphorylation level. This effect could be counterweighed by PP1 phosphatase (Fig. 2D).

**GSK3β is an effector of PKCα in TRAIL-induced H1299 cell apoptosis**

Given that GSK3β could be phosphorylated by PKCα in TRAIL-induced H1299 cell apoptosis, we further determine the importance of GSK3β as the downstream effector of PKCα. To this end, we combined the overexpression or knockdown of PKCα with that of GSK3β in the cells treated or not with TRAIL, and evaluated the degree of apoptosis by Western blotting using anti-cleaved caspase 3 antibody. After knockdown of PKCα, cleaved caspase 3 significantly reduced, and simultaneous knockdown of GSK3β blocked this reduction (Fig. 3A). More convincingly, while PKCα overexpression was capable of reducing the anti-apoptotic effect of GSK3β overexpression (Fig. 3B), it seems to be devoid of such capacity on GSK3β-S9A (taken into account the varying expression levels of mCherry-GSK (WT and S9A mutant) and PKCα), the phospho-defective mutant of GSK3β (Fig. 3C). This suggests that PKCα promotes TRAIL-induced apoptosis perhaps through Ser9 phosphorylation of GSK3β.

**Upregulation of NF-κB pathway by GSK3β in TRAIL-induced H1299 cell apoptosis**

Previous studies have demonstrated that NF-κB is a critical transcription factor that mediated TRAIL-induced apoptosis in various cells (Hoeflich et al., 2000; Aydin et al., 2010; Zhang et al., 2014b). As shown in Fig. 4A, we also found that NF-κB inhibitor BAY 11-7082 remarkably sensitized TRAIL-induced apoptosis, as judged by increase of the cleaved caspase 3 level, which peaked when BAY 11-7082 was used at 10 μM. The inhibitory effect of BAY 11-7082 (10 μM, 8

hours) on the activity of NF-κB was confirmed in Fig. S1 and there is no off target effect of BAY 11-7082 on GSK3β (Anastassiadis et al., 2011). We then attempted to confirm the regulation of NF-κB pathway by GSK3β in our cellular model. As shown in Fig. 4B and 4C, without TRAIL treatment, neither the inhibition of GSK3β by LiCl nor its knockdown had obvious effect on the NF-κB transcriptional activity. Induction of H1299 cells by TRAIL exerted an inhibitory effect on NF-κB pathway, which was further strengthened by LiCl treatment or knockdown of GSK3β (Fig. 4B, C). This result confirmed the inhibition of NF-κB pathway by inhibiting or knocking down of GSK3β in the process of TRAIL-induced apoptosis. In support with this, treatment of BAY 11-7082 compromised the reduction of cleaved caspase 3 induced by Flag-GSK3β under TRAIL treatment (Fig. 4D). These results collectively suggest that GSK3β positively regulates NF-κB pathway in TRAIL-induced H1299 cell apoptosis.



**TRIM21 counteracts the promotion of NF-κB pathway by GSK3β under TRAIL treatment**

It has been reported that TRIM21 suppressed the transcriptional activity of NF-κB (Niida et al., 2010), but no study concerning its possible role in TRAIL-induced apoptosis has yet been reported. In order to address this issue, we have firstly determined the effect of TRAIL on the expression of TRIM21. As shown in Fig. 5A, no obvious variation of TRIM21 expression could be detected upon TRAIL treatment. Next, we assessed the effect of TRIM21 overexpression and knockdown on TRAIL induced-apoptosis. As shown in Fig. 5B, overexpression of TRIM21 augmented the level of cleaved caspase-3, and consistently, its knockdown decreased the level of the latter, confirming the pro-apoptosis role of this protein (Fig. 5C). Interestingly, TRIM21 overexpression significantly inhibited NF-κB activation regardless of TRAIL treatment (Fig. 5D).

Moreover, the stimulation of NF-κB activity by GSK3β overexpression under TRAIL treatment was remarkably inhibited by the co-overexpression of TRIM21, further supported by the Western blotting analysis of the cleaved caspase 3 level which was reduced by GSK3β overexpression but re-ascend when TRIM21 was also co-overexpressed (Fig. 5E). Taken together, these results suggest that TRIM21 is a regulator of TRAIL-induced cell apoptosis whose function is possibly linked to the anti-apoptotic GSK3β.

## Discussion

TRAIL is a promising candidate for cancer treatment since it selectively promotes cell death in tumor cells while sparing most normal cells (Walczak et al., 1999; Zhuang et al., 2013). However, many tumor cells including NSCLC cells possessed both intrinsic and acquired resistance to TRAIL, drastically jeopardizing the outcome of TRAIL therapy. Therefore, it is important to investigate in depth the functional mechanisms of TRAIL and identify the effective drug targets, permitting to improve the clinical efficacy by sensitizing NSCLC cells to TRAIL therapy. In this study, we investigated the involvement of GSK3β in TRAIL-induced apoptosis in H1299 NSCLC cells, and demonstrated that PKCα isoform acted at the upstream of GSK3β, phosphorylating and inactivating GSK3β, and sensitizing the cancer cells to TRAIL-induced apoptosis. Furthermore, we also demonstrated the role of NF-κB in mediating the anti-apoptotic role of GSK3β. Finally, tripartite motif 21 (TRIM21) was found to negatively regulate the GSK3β-mediated activation of NF-κB pathway, which suppressed cell apoptosis.

Various isozymes of PKC have been reported to be involved in the regulation of TRAIL-induced tumor cell apoptosis. In melanoma cells and breast cancer cells, the activation of the novel PKC (δ, ε) led to TRAIL resistance (Gillespie et al., 2005; Shankar et al., 2008; Azijli et al., 2013). Novel PKCδ and η isozymes have also been identified as key targets for antagonizing TRAIL's cytotoxic activity (Sonnemann et al., 2004; Hayashi et al., 2015). In addition, inhibition of novel PKC using NPC 15437 significantly augmented sensitivity of A549 cells to TRAIL, however, inhibition of the conventional PKC using Gö6976 did not increase its sensitivity to TRAIL(Felber et al., 2007). The anti-apoptotic mechanism of PKC involves activation of pro-survival Akt and reduction of p53 (Shankar et al., 2008), inhibition of recruitment of FADD

and caspase-8, and the latter leads to the disruption of DISC formation (Harper et al., 2003). On the contrary, the activation of conventional PKC (α, β, γ) by PMA (PKC activator) or bryostatin-1 sensitizes TRAIL-induced apoptosis through up-regulating the expression of Bad and TRAIL receptors (Farrow et al., 2002). In NSCLC cells, the authors suggest that PKCα promotes TRAIL-R2 expression and contributed to the sensitization of TRAIL-induced cytotoxicity by quercetin (Chen et al., 2007; Azijli et al., 2013). In agreement with these reports, our study has demonstrated the role of PKCα as an apoptosis promoter on TRAIL-induced apoptosis in NSCLC H1299 cells (Fig. 2), by phosphorylating and inactivating GSK3β. Our results further clarified the role of PKCα and the related mechanism in TRAIL's cytotoxicity.

The pro-survival role of GSK3β under the treatment of TRAIL has been reported in numerous tumor cells (Liao et al., 2003; Song et al., 2004; Kotliarova et al., 2008; Beurel et al., 2009). Consistent with these studies, in our results, overexpression of GSK3β blocked caspase 3 cleavage while GSK3β knockdown augmented caspase 3 cleavage (Fig. 1). In addition, our results demonstrated that NF-κB is the downstream effector which is responsible for the anti-apoptotic role of GSK3β under TRAIL treatment in H1299 cells (Fig. 4), reconfirming the previous studies (Hoeflich et al., 2000; Schwabe and Brenner, 2002). The family of NF-κB transcription factors comprises of five members including RelA (p65), RelB, c-Rel, p50 and p52, which form homo- and heterodimer. p50/p65 is well-studied NF-κB dimer, which is in complex with IκBα in resting cells. Upon the activating-signal, TAK1/TAB1 kinase complex activates the IKK, which further phosphorylates IκB. Phosphorylation of IκBα by IKK leads to its subsequent ubiquitination and proteasomal degradation in the cytoplasm (Tomar and Singh, 2015). GSK3β has been shown to positively regulate the transcription activity of NF-κB (Hoeflich et al., 2000; Takada et al., 2004;

Ougolkov et al., 2007). The reported molecular cross-talk between GSK3β and NF-κB pathway included the direct phosphorylation of p65 (Schwabe and Brenner, 2002) and NF-kappa B1/p105 (Demarchi et al., 2003) by GSK3β, the stability regulation of NF-kappa B1/p105 by GSK3β (Demarchi et al., 2003), activation of IKK and promotion of IκB phosphorylation, ubiquitination, and degradation by GSK3β (Takada et al., 2004), up-regulation of NF-κB target gene Bcl-xL by nuclear GSK3β and contribution to TNFα and TRAIL resistance in pancreatic cancer cells (Zhang et al., 2014b).

Recent review has reported that TRIMs act as E3 ligases and are extensively involved in the regulation of NF-κB pathway at different levels (Tomar and Singh, 2015). TRIM21 is shown to induce the mono-ubiquitination and degradation of IKKβ, leading to the suppression of NF-κB activity (Wada et al., 2009; Niida et al., 2010). In agreement with this conclusion, our result showed that overexpression of TRIM21 dramatically reduced NF-κB transcriptional activity under TRAIL treatment (Fig. 5D). Furthermore, TRIM21 negatively regulated the augmented activation of NF-κB induced by GSK3β overexpression (Fig. 5E). Thus, TRIM21 counteracted the protective effect of GSK3β against TRAIL's cytotoxicity and significantly promoted TRAIL-induced apoptosis. Taking into account the previous studies showing that these two proteins are both capable to act on IKK in NF-κB pathway, as cited above, our results may suggest that the functions of GSK3β and TRIM21 in the regulation of NF-κB pathway might be linked by a common molecular mechanism which needs further investigation in the future.

In conclusion, our study delineates the PKCα/GSK3β/NF-κB axis in H1299 cells under the treatment of TRAIL in which PKCα functions as an apoptosis promoter through phosphorylating and inactivating the pro-survival GSK3β. Furthermore, we demonstrate the opposite roles of

GSK3β and TRIM21 proteins in TRAIL-induced apoptosis in H1299 cells. Taken together, our results further clarified the molecular mechanism of TRAIL's cytotoxicity and provided the effective clues for sensitizing NSCLC cells to TRAIL therapy.

## Acknowledgments

This work was partially supported by the National Natural Science Foundation of China (Grant No. 31000628); National Program on Key Basic Research Project (973 Program) (Grant No.2011CB910700); High-Level Talents Project of the Universities of Guangdong (No. [2011]431); Fundamental Research Funds for the Central Universities (Grant No. 21615407; 21609317); Science and Technology Program of Huadu District of Guangzhou, Guangdong Province (15-HDWS-016).

We thank Dr. Serge Benichou at Institute Cochin in France, Prof. Xu Jiake in University of Western Australia, and Han's Lab of Xiamen University for providing us with reagents.

**References**

Anastassiadis, T., Deacon, S.W., Devarajan, K., Ma, H., and Peterson, J.R. 2011. Comprehensive assay of kinase catalytic activity reveals features of kinase inhibitor selectivity. Nat. Biotechnol. **29**:1039-1045. doi:1010.1038/nbt.2017.PMID:22037377.

Aydin, C., Sanlioglu, A.D., Bisgin, A., Yoldas, B., Dertsiz, L., Karacay, B., et al. 2010. NF-κB targeting by way of IKK inhibition sensitizes lung cancer cells to adenovirus delivery of TRAIL. BMC Cancer. **10**:584. doi:510.1186/1471-2407-1110-1584.PMID:20977779.

Azijli, K., Weyhenmeyer, B., Peters, G.J., de Jong, S., and Kruyt, F.A. 2013. Non-canonical kinase signaling by the death ligand TRAIL in cancer cells: discord in the death receptor family. Cell Death Differ. **20**:858-868. doi:810.1038/cdd.2013.1028.PMID:23579241.

Beurel, E., Blivet-Van Eggelpoël, M.J., Kornprobst, M., Moritz, S., Delelo, R., Paye, F., et al. 2009. Glycogen synthase kinase-3 inhibitors augment TRAIL-induced apoptotic death in human hepatoma cells. Biochem. Pharmacol. **77**:54-65. doi:10.1016/j.bcp.2008.1009.1026.PMID:18938143.

Beurel, E., and Jope, R.S. 2006 The paradoxical pro- and anti-apoptotic actions of GSK3 in the intrinsic and extrinsic apoptosis signaling pathways. Prog. Neurobiol. **79**:173-189. doi: 110.1016/j.pneurobio.2006.1007.1006.PMID:16935409.

Cameron, A.J., Procyk, K.J., Leitges, M., and Parker, P.J. 2008. PKC alpha protein but not kinase activity is critical for glioma cell proliferation and survival. Int. J. Cancer. **123**:769-779. doi: 710.1002/ijc.23560.PMID:18508315.

Chen, S., Cao, W., Yue, P., Hao, C., Khuri, F.R., and Sun, S.Y. 2011. Celecoxib promotes c-FLIP degradation through Akt-independent inhibition of GSK3. Cancer Res. **71**:6270-6281.

doi:6210.1158/0008-5472.CAN-6211-0838.PMID:21868755.

Chen, W., Wang, X., Zhuang, J., Zhang, L., and Lin, Y. 2007. Induction of death receptor 5 and suppression of survivin contribute to sensitization of TRAIL-induced cytotoxicity by quercetin in non-small cell lung cancer cells. Carcinogenesis. **28**:2114-2121. doi:2110.1093/carcin/bgm2133.PMID:17548900.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., and Hemmings, B.A. 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature. **378**:785-789. doi:710.1038/378785a378780. PMID:8524413.

Demarchi, F., Bertoli, C., Sandy, P., and Schneider, C. 2003. Glycogen synthase kinase-3 beta regulates NF-kappa B1/p105 stability. J. Biol. Chem. **278**:39583-39590. doi:39510.31074/jbc.M305676200.PMID:312871932.

Deveraux, Q.L., Roy, N., Stennicke, H.R., Van Arsdale, T., Zhou, Q., Srinivasula, S.M., et al. 1998. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J. **17**:2215-2223. doi: 2210.1093/emboj/2217.2218.2215.PMID:9545235.

Embi, N., Rylatt, D.B., and Cohen, P. 1980. Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. Eur. J. Biochem. **107**:519-527. doi:510.1111/j.1432-1033.1980.tb06059.x. PMID:6249596.

Farrow, B., Thomas, R.P., Wang, X.F., and Evers, B.M. 2002. Activation of conventional PKC isoforms increases expression of the pro-apoptotic protein Bad and TRAIL receptors. Int. J. Gastrointest. Cancer. **32**:63-72. doi:10.1385/IJGC:1332:1382-1383:1363.PMID:12794242.

Felber, M., Sonnemann, J., and Beck, J.F. 2007. Inhibition of novel protein kinase C-epsilon augments

TRAIL-induced cell death in A549 lung cancer cells. Pathol. Oncol. Res. **13**:295-301. doi:210.1007/BF02940308.PMID:18158564.

Gao, X., Dan, S., Xie, Y., Qin, H., Tang, D., Liu, X., et al. 2015. 14-3-3ζ reduces DNA damage by interacting with and stabilizing proliferating cell nuclear antigen. J. Cell. Biochem. **116**:158-169. doi:110.1002/jcb.24955.PMID:25169136.

Gao, X., He, Y., Gao, L.M., Feng, J., Xie, Y., Liu, X., et al. 2014. Ser9-phosphorylated GSK3β induced by 14-3-3ζ actively antagonizes cell apoptosis in a NF-κB dependent manner. Biochem. Cell Biol. **92**:349-356. doi:310.1139/bcb-2014-0065.PMID:25138042.

Gao, X., Wang, J.Y., Gao, L.M., Yin, X.F., and Liu, L. 2013. Identification and analysis of glycogen synthase kinase 3 beta1 interactome. Cell. Biol. Int. **37**:768-779. doi:710.1002/cbin.10095. PMID: 23505128.

Gavrielides, M.V., Frijhoff, A.F., Conti, C.J., and Kazanietz, M.G. 2004. Protein kinase C and prostate carcinogenesis: targeting the cell cycle and apoptotic mechanisms. Curr. Drug Targets. **5**:431-443. doi:410.2174/1389450043345380.PMID:1389450015216909.

Gillespie, S., Zhang, X.D., and Hersey, P. 2005. Variable expression of protein kinase C epsilon in human melanoma cells regulates sensitivity to TRAIL-induced apoptosis. Mol. Cancer Ther. **4**:668-676. doi: 610.1158/1535-7163.MCT-1104-0332.PMID:15827341.

Griner, E.M., and Kazanietz, M.G. 2007. Protein kinase C and other diacylglycerol effectors in cancer. Nat. Rev. Cancer. **7**:281-294. doi:210.1038/nrc2110.PMID:17384583.

Harper, N., Hughes, M.A., Farrow, S.N., Cohen, G.M., and MacFarlane, M. 2003. Protein kinase C modulates tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by targeting the apical events of death receptor signaling. J. Biol. Chem. **278**:44338-44347.

doi:44310.41074/jbc.M307376200.PMID:312920112.

Hayashi, K., Tabata, S., Piras, V., Tomita, M., and Selvarajoo, K. 2015. Systems Biology Strategy Reveals PKCδ is Key for Sensitizing TRAIL-Resistant Human Fibrosarcoma. Front. Immunol. **5**:659. doi:610.3389/fimmu.2014.00659.PMID:25601862.

Hoeflich, K.P., Luo, J., Rubie, E.A., Tsao, M.S., Jin, O., and Woodgett, J.R. 2000. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature. **406**:86-90. doi:10.1038/35017574. PMID:10894547.

Johnstone, R.W., Frew, A.J., and Smyth, M.J. 2008. The TRAIL apoptotic pathway in cancer onset, progression and therapy. Nat. Rev. Cancer. **8**:782-798. doi:710.1038/nrc2465.PMID:18813321.

Kaufmann, S.H., and Hengartner, M.O. 2001. Programmed cell death: alive and well in the new millennium. Trends Cell Biol. **11**:526-534. doi:510.1016/S0962-8924(1001)02173-02170.PMID:11719060.

Kim, L., Liu, J., and Kimmel, A.R. 1999. The novel tyrosine kinase ZAK1 activates GSK3 to direct cell fate specification. Cell. **99**:399-408. doi:310.1016/S0092-8674(1000)81526-81523. PMID:10571182.

Kischkel, F.C., Lawrence, D.A., Chuntharapai, A., Schow, P., Kim, K.J., and Ashkenazi, A. 2000. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. Immunity. **12**:611-620. doi:610.1016/S1074-7613(1000)80212-80215.PMID:10894161.

Kotliarova, S., Pastorino, S., Kovell, L.C., Kotliarov, Y., Song, H., Zhang, W., et al. 2008. Glycogen synthase kinase-3 inhibition induces glioma cell death through c-MYC, nuclear factor-kappaB,

and glucose regulation. Cancer Res. **68**: 6643-6651. doi:6610.1158/0008-5472.CAN-6608-0850.PMID:18701488.

Leverkus, M., Neumann, M., Mengling, T., Rauch, C.T., Bröcker, E.B., Krammer, P.H., et al. 2000. Regulation of tumor necrosis factor-related apoptosis-inducing ligand sensitivity in primary and transformed human keratinocytes. Cancer Res. **60**:553-559.PMID:10676636.

Liao, X., Zhang, L., Thrasher, J.B., Du, J., and Li, B. 2003. Glycogen synthase kinase-3beta suppression eliminates tumor necrosis factor-related apoptosis-inducing ligand resistance in prostate cancer. Mol. Cancer Ther. **2**:1215-1222.PMID:14617795.

Lin, Y., Devin, A., Cook, A., Keane, M.M., Kelliher, M., Lipkowitz, S., et al. 2000. The death domain kinase RIP is essential for TRAIL (Apo2L)-induced activation of IkappaB kinase and c-Jun N-terminal kinase. Mol. Cell. Biol. **20**:6638-6645. doi:6610.1128/MCB.6620.6618.6638-6645.2000.PMID:10958661.

Luo, J. 2009. Glycogen synthase kinase 3beta (GSK3beta) in tumorigenesis and cancer chemotherapy. Cancer Lett. **273**:194-200. doi:110.1016/j.canlet.2008.1005.1045.PMID:18606491.

Martelli, A.M., Mazzotti, G., and Capitani, S. 2004. Nuclear protein kinase C isoforms and apoptosis. Eur. J. Histochem. **48**:89-94.PMID:15145780.

McCray, A.N., Desai, S., and Acevedo-Duncan, M. 2014. The interruption of PKC-ι signaling and TRAIL combination therapy against glioblastoma cells. Neurochem. Res. **39**:1691-1701. doi:1610.1007/s11064-11014-11361-11068.PMID:24965532.

Micale, L., Chaignat, E., Fusco, C., Reymond, A., and Merla, G. 2012. The tripartite motif: structure and function. Adv. Exp. Med. Biol. **770**:11-25.PMID:23630997.

Niida, M., Tanaka, M., and Kamitani, T. 2010. Downregulation of active IKK beta by Ro52-mediated

autophagy. Mol. Immunol. **47**:2378-2387.doi:2310.1016/j.molimm.2010.2305.2004.PMID:20627395.

Ougolkov, V., Bone, N.D., Fernandez-Zapico, M.E., Kay, N.E., and Billadeau, D.D. 2007. Inhibition of glycogen synthase kinase-3 activity leads to epigenetic silencing of nuclear factor Blood. **110**: 735-742. doi: 710.1182/blood-2006-1112-060947.PMID:17463171.

Peter, M.E., and Krammer, P.H. 2003. The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ. **10**:26-35. doi:10.1038/sj.cdd.4401186.PMID:12655293.

Sanchez, J.F., Sniderhan, L.F., Williamson, A.L., Fan, S., Chakraborty-Sett, S., and Maggirwar, S.B. 2003. Glycogen synthase kinase 3beta-mediated apoptosis of primary cortical astrocytes involves inhibition of nuclear factor kappaB signaling. Mol. Cell. Biol. **23**:4649-4662. doi:4610.1128/MCB.4623.4613.4649-4662.2003.PMID:12808104.

Schwabe, R.F., and Brenner, D.A. 2002. Role of glycogen synthase kinase-3 in TNF-α-induced NF-κB activation and apoptosis in hepatocytes. Am. J. Physiol. Gastrointest. Liver Physiol. **283**:G204-G211. doi:210.1152/ajpgi.00016.02002.PMID:12065308.

Shankar, E., Sivaprasad, U., and Basu, A. 2008. Protein kinase C epsilon confers resistance of MCF-7 cells to TRAIL by Akt-dependent activation of Hdm2 and downregulation of p53. Oncogene. **27**:3957-3966. doi:3910.1038/onc.2008.3939.PMID:18317451.

Song, L., Zhou, T., and Jope, R.S. 2004. Lithium facilitates apoptotic signaling induced by activation of the Fas death domain-containing receptor. BMC Neurosci. **5**:20. doi:10.1186/1471-2202-1185-1120.PMID:15157283.

Sonnemann, J., Gekeler, V., Sagrauske, A., Müller, C., Hofmann, H.P., and Beck, J.F. 2004. Down-regulation of protein kinase Ceta potentiates the cytotoxic effects of exogenous tumor

necrosis factor-related apoptosis-inducing ligand in PC-3 prostate cancer cells. Mol. Cancer Ther. **3**:773-781.PMID:15252138.

Spitaler, M., and Cantrell, D.A. 2004. Protein kinase C and beyond. Nat. Immunol. **5**:785-790. doi:710.1038/ni1097.PMID:15282562.

Takada, Y., Fang, X., Jamaluddin, M.S., Boyd, D.D., and Aggarwal, B.B. 2004. Genetic Deletion of Glycogen Synthase Kinase-3β Abrogates Activation of IκBα Kinase, JNK, Akt, and p44/p42 MAPK but Potentiates Apoptosis Induced by Tumor Necrosis Factor. J. Biol. Chem. **279**: 39541-39554. doi:39510.31074/jbc.M403449200.PMID: 415252041.

Todaro, M., Lombardo, Y., Francipane, M.G., Alea, M.P., Cammareri, P., Iovino, F., et al. 2008. Apoptosis resistance in epithelial tumors is mediated by tumor-cell-derived interleukin-4. Cell Death Differ. **15**:762-772. doi:710.1038/sj.cdd.4402305.PMID:18202702.

Tomar, D., and Singh, R. 2015. TRIM family proteins: emerging class of RING E3 ligases as regulator of NF-κB pathway. Biol. Cell. **107**:22-40. doi:10.1111/boc.201400046.PMID:225319221.

Wada, K., Niida, M., Tanaka, M., and Kamitani, T. 2009. Ro52-mediated monoubiquitination of IKK{beta} down-regulates NF-{kappa}B signaling. J. Biochem. **146**:821-832. doi:810.1093/jb/mvp1127.PMID:19675099.

Walczak, H., Miller, R.E., Ariail, K., Gliniak, B., Griffith, T.S., Kubin, M., et al. 1999. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat. Med. **5**:157-163. doi:110.1038/5517.PMID:9930862.

Wang, F., Lin, J., and Xu, R. 2014. The molecular mechanisms of TRAIL resistance in cancer cells: help in designing new drugs. Curr. Pharm. Des. **20**:6714-6722. doi:6710.2174/1381612820666140929100735.PMID:1381612820666140925269558.

Wiley, S.R., Schooley, K., Smolak, P.J., Din, W.S., Huang, C.P., Nicholl, J.K., et al. 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity. **3**:673-682. doi:610.1016/1074-7613(1095)90057-90058.PMID:8777713.

Wu-Zhang, A.X., and Newton, A.C. 2013. Protein kinase C pharmacology: refining the toolbox. Biochem J. **452**:195-209. doi:110.1042/BJ20130220.PMID:23662807.

Zhang, F., Phiel, C.J., Spece, L., Gurvich, N., and Klein, P.S. 2003. Inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) in response to lithium. Evidence for autoregulation of GSK-3. J. Biol. Chem. **278**:33067-33077. doi:33010.31074/jbc.M212635200.PMID:212796505.

Zhang, H.T., Zhang, D., Zha, Z.G., and Hu, C.D. 2014a. Transcriptional activation of PRMT5 by NF-Y is required for cell growth and negatively regulated by the PKC/c-Fos signaling in prostate cancer cells. Biochim. Biophys. Acta. **1839**:1330-1340. doi:1310.1016/j.bbagrm.2014.1309.1015.PMID:25281873.

Zhang, J.S., Herreros-Villanueva, M., Koenig, A., Deng, Z., de Narvajas, A.A., Gomez, T.S., et al. 2014b. Differential activity of GSK-3 isoforms regulates NF-κB and TRAIL- or TNFα induced apoptosis in pancreatic cancer cells. Cell Death Dis. **5**:e1142. doi:1110.1038/cddis.2014.1102.PMID:24675460.

Zhang, L., and Fang, B. 2005. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. Cancer Gene Ther. **12**:228-237. doi:210.1038/sj.cgt.7700792.PMID:15550937.

Zhuang, H., Jiang, W., Zhang, X., Qiu, F., Gan, Z., Cheng, W., et al. 2013. Suppression of HSP70 expression sensitizes NSCLC cell lines to TRAIL-induced apoptosis by upregulating DR4 and DR5 and downregulating c-FLIP-L expressions. J. Mol. Med (Berl). **91**:219-235.

doi:210.1007/s00109-00012-00947-00103.PMID:22948392.

**Figure legends**

**Fig. 1** The effect of GSK3β on TRAIL-induced apoptosis in lung adenocarcinoma H1299 cells. (A) MTT assays of cellular viability after TRAIL treatment. H1299 cells were seeded into 96-well plate ($4\times10^3$ cells/well) and treated with TRAIL (20 ng/ml) for different durations as indicated. MTT assays were then carried out to test cellular viability by monitoring the absorbance of formazan crystals at 570 nm. The absorbance of untreated control group was set as 100 %. **: $p<0.01$. (B) The level of cleaved Cas 3 in H1299 cells after TRAIL treatment with different durations. H1299 cells were treated with TRAIL (20 ng/ml) for 4-24 hours and subjected to Western blot analysis using cleaved caspase 3 antibody. The effects of overexpression of Flag-GSK3β (C) or knockdown of GSK3β (D) on TRAIL-induced apoptosis. H1299 cells were transfected with Flag-GSK3β or siGSK3β and treated with TRAIL (20 ng/ml, 8 hours). Then, the cells were analyzed by Western blot with the indicated antibodies. All results are representative of three repeated experiments. Cleaved Cas 3/GAPDH values indicated on B, C, D represent the ratio between the gray values of Cleaved Cas 3 and GAPDH protein bands. Hoechst 33342 staining assay was used to detect the apoptotic ratio in Flag-GSK3β overexpressed cells (E-F) or in GSK3β knockdown cells (G-H) after TRAIL treatment. H1299 cells transfected with Flag-GSK3β or GSK3β siRNA were treated with TRAIL (20 ng/ml, 8 hours) and then stained with Hoechst 33342. The representative images were shown in (E, G), and the corresponding quantitative analysis were shown in (F, H). **: $p<0.01$.

**Fig. 2** The effects of PKCα isozyme on apoptosis induced by TRAIL and phosphorylation of GSK3β. (A) The effects of inhibition of PKC on cleaved caspase 3 and GSK3β Ser9

phosphorylation. H1299 cells were treated with TRAIL (20 ng/ml, 8 hours) combined with or without Gö6983 (1 μM, 8 hours), LiCl (20 mM, 8 hours). Then, the cells were analyzed by Western blot with the indicated antibodies. The effects of overexpression of Flag-PKCα (B) or knockdown of PKCα (C) on cleaved caspase 3 and GSK3β Ser9 phosphorylation. H1299 cells transfected with Flag-PKCα (0-2 μg) in 6-well plate or with siPKCα (100 nM) and treated with TRAIL (20 ng/ml, 8 hours) were analyzed by Western blot with the indicated antibodies. Cleaved Cas 3/GAPDH values indicated on A, B, C represent the ratio between the gray values of Cleaved Cas 3 and GAPDH protein bands. (D) Active PKCα phosphorylated GST-GSK3β *in vitro*. 200 ng purified GST-GSK3β and Kinase Assay Buffer were mixed together for 10 min. Then, 5 μl active PKCα (0.1 μg/μl) and 10 μl ATP (1 mg/ml) with or without PP1 phosphatase (0.4 μl) were added to the mixture and incubated for 2 hours at 30°C. Then, the mixture was performed the standard Western Blot assay with the indicated antibody. CBB: Coomassie Brilliant Blue staining.



**Fig. 3** GSK3β is the major effector of PKCα in TRAIL-induced H1299 cell apoptosis. H1299 cells were transfected with siPKCα + siGSK3β (A), Flag-PKCα + mCherry-GSK3β (B), Flag-PKCα + mCherry-GSK3β S9A mutant (C), and treated with TRAIL (20 ng/ml, 8 h). Then, the cells were analyzed by Western blot with the indicated antibodies. Cleaved Cas 3/GAPDH values indicated on A, B, C represent the ratio between the gray values of Cleaved Cas 3 and GAPDH protein bands.

**Fig. 4** The effect of GSK3β/NF-κB on TRAIL-induced apoptosis. (A) The effect of inhibition of NF-κB on TRAIL-induced apoptosis. H1299 cells were treated with BAY 11-7082 (0-20 μM, 8

hours) combined with TRAIL (20 ng/ml, 8 hours) as indicated. Then, the cells were analyzed by Western blot with the indicated antibodies. (B) The effects of LiCl on NF-κB transcriptional activity. H1299 cells were transfected with p-NF-κB-TA-Luc plasmid and combined with TRAIL (20 ng/ml, 8 hours) or LiCl (20 mM, 8 hours) treatment as indicated. (C) The effects of GSK3β knockdown on NF-κB transcriptional activity. H1299 cells were transfected with p-NF-κB-TA-Luc plasmid combined with siGSK3β, and treated with TRAIL (20 ng/ml, 8 hours) as indicated. (D) The effect of GSK3β/NF-κB on TRAIL-induced apoptosis. H1299 cells transfected with Flag-GSK3β or Flag-vector were co-treated with BAY 11-7082 (10 μM, 8 hours) and TRAIL (20 ng/ml, 8 hours) as indicated. Then, the cells were analyzed by Western blot with the indicated antibodies. Cleaved Cas 3/GAPDH values indicated on A, D represent the ratio between the gray values of Cleaved Cas 3 and GAPDH protein bands.

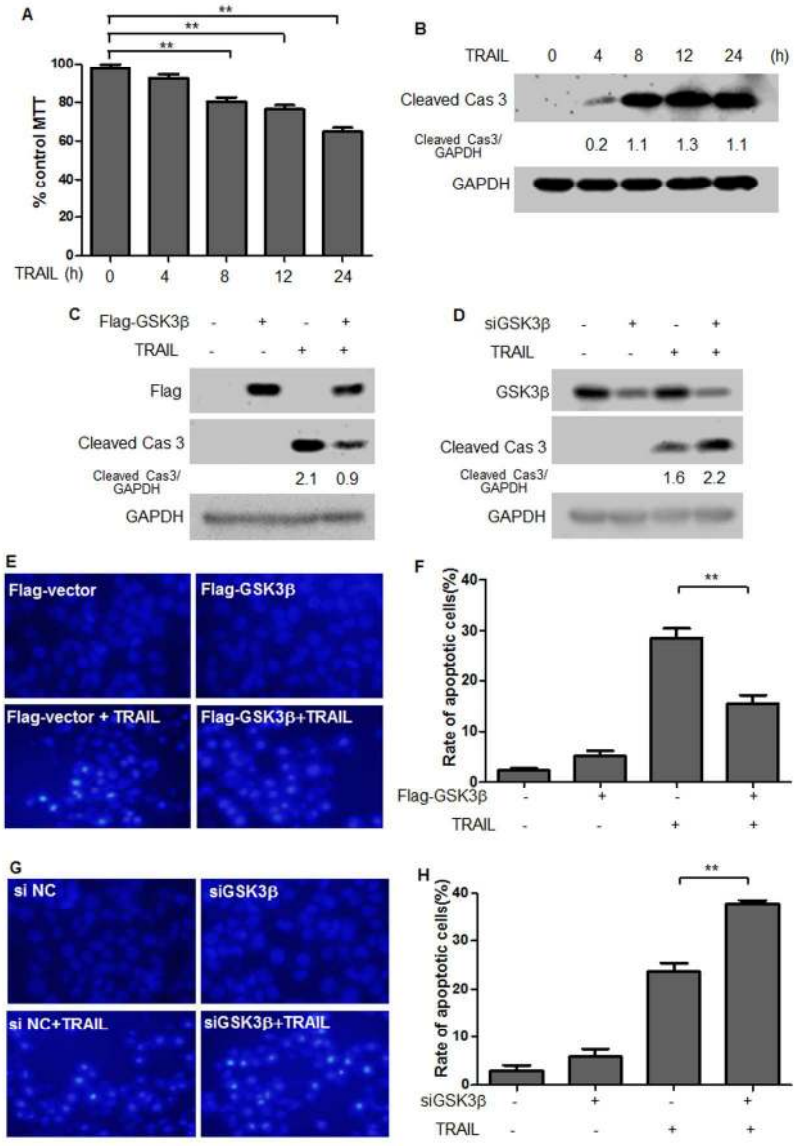


**Fig. 5** TRIM21 counteracts the stimulation of NF-κB pathway by GSK3β. (A) The effect of TRAIL on the expression of TRIM21. H1299 cells were treated with or without TRAIL (20 ng/ml, 8 hours) and then harvested for Western blot assay with the indicated antibodies. The effects of overexpression of TRIM21 (B) or knockdown of TRIM21 (C) on the TRAIL-induced apoptosis. H1299 cells were transfected with HA-TRIM21 or siRNA of TRIM21 and treated with TRAIL (20 ng/ml, 8 hours). Then, the cells were analyzed by Western blot with the indicated antibodies. (D) The effects of TRIM21 on NF-κB transcriptional activity. H1299 cells were cotransfected with p-NF-κB-TA-Luc and HA-TRIM21 plasmids and combined with TRAIL (20 ng/ml, 8 hours) treatment. (E) The effects of TRIM21 on transcriptional activation of NF-κB mediated by GSK3β. H1299 cells were cotransfected with p-NF-κB-TA-Luc, HA-TRIM21, or Flag-GSK3β plasmids

and combined with TRAIL (20 ng/ml, 8 hours) treatment. All the cell samples were harvested and prepared for luciferase assays (upper panel) and Western blot assays (bottom panel) with the indicated antibodies. The data were shown as mean+SEM. *: $p<0.05$, **: $p<0.01$. All results are representative of three repeated experiments. Cleaved Cas 3/GAPDH values indicated on B, C, E represent the ratio between the gray values of Cleaved Cas 3 and GAPDH protein bands.

**Supplemental Information**

**Fig. S1** The inhibitory effect of BAY 11-7082 on the activity of NF-κB. (A) Ser536 phosphorylation of p65 was used to test the effect of BAY 11-7082 on NF-kB activity. H1299 cells were treated with BAY 11-7082 (10 μM, 8 hours) and analyzed by Western blot with p-NF-kB p65 (Ser536) antibody. (B) The effect of BAY 11-7082 on the transcriptional activity of NF-kB. H1299 cells transfected with p-NF-κB-TA-Luc plasmid were treated with BAY 11-7082 (10 μM, 8 hours). Then the cells were harvested and prepared for luciferase assays.

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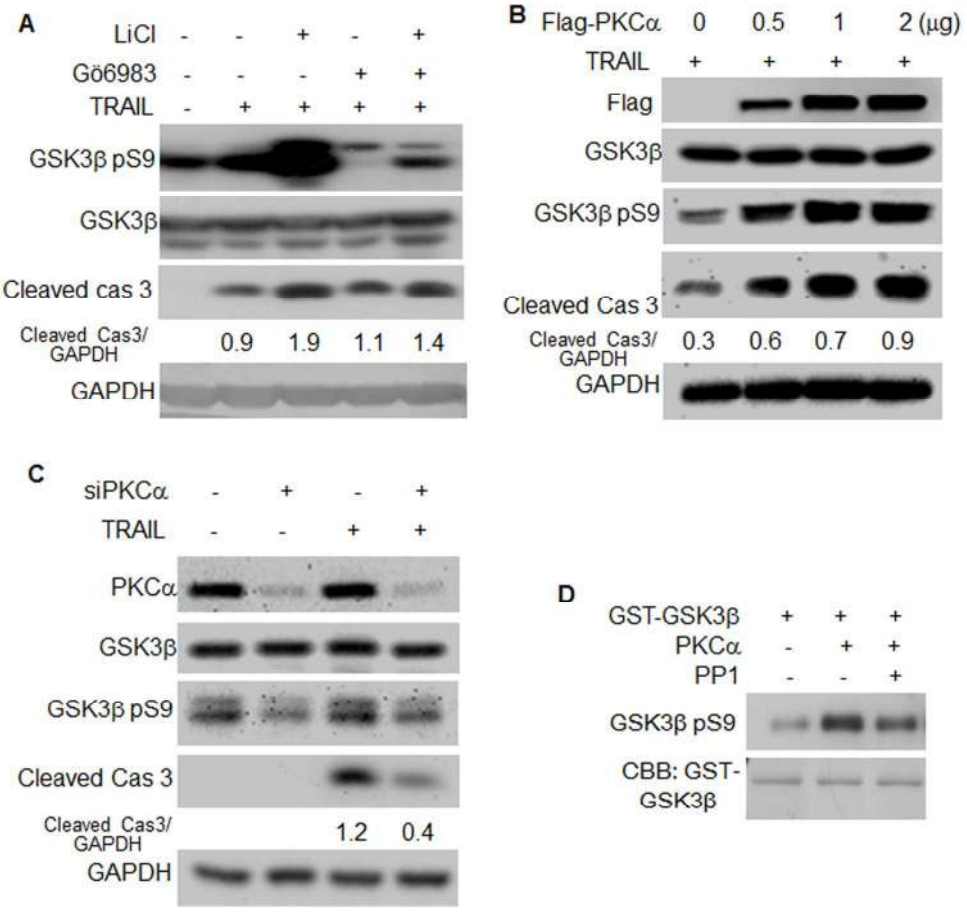


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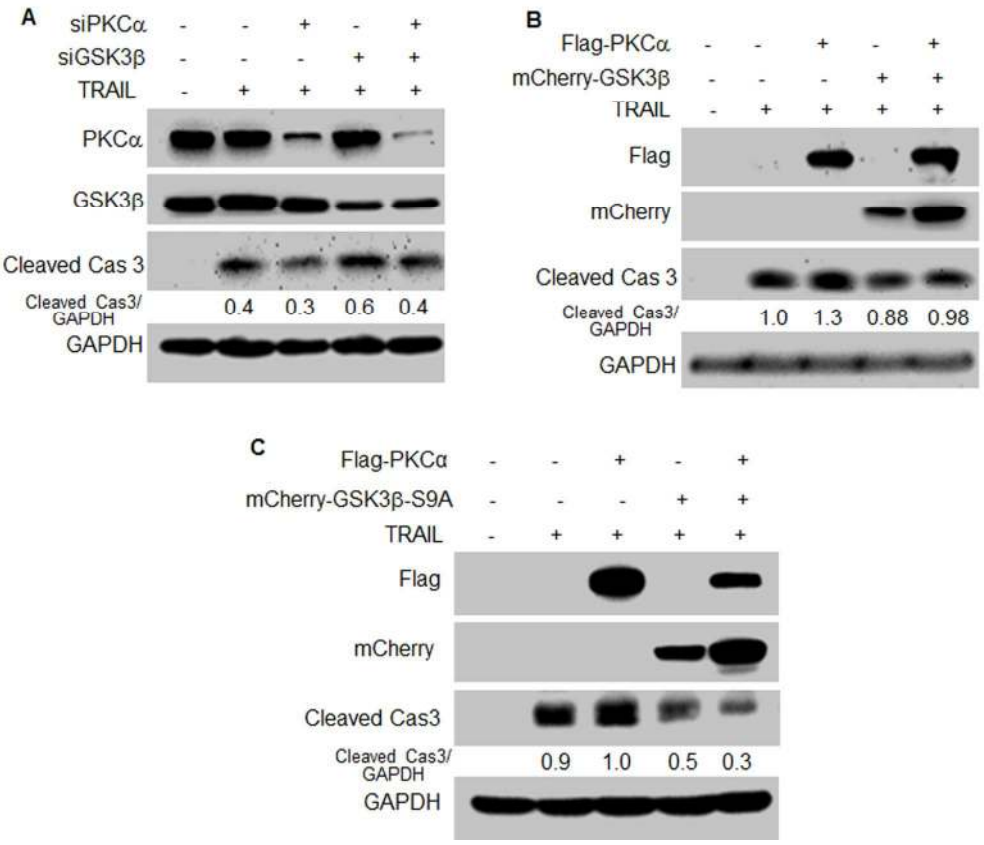


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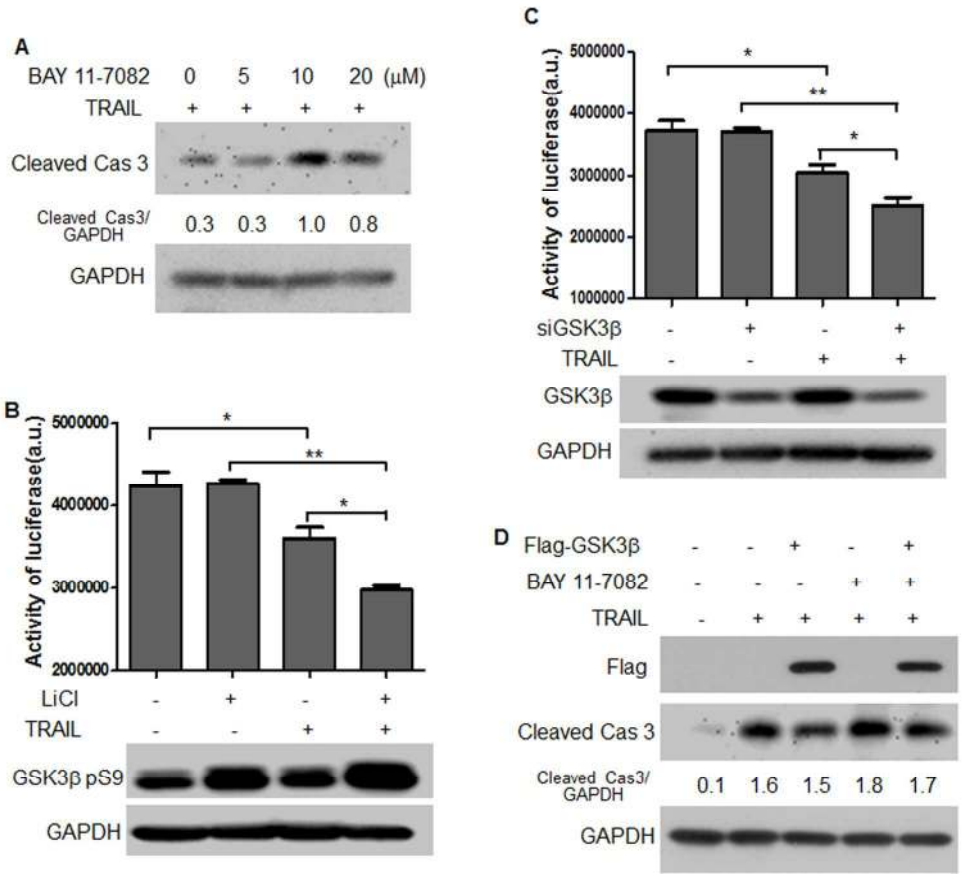


Fig. 4 The effect of GSK3β/NF-κB on TRAIL-induced apoptosis. (A) The effect of inhibition of NF-κB on TRAIL-induced apoptosis. H1299 cells were treated with BAY 11-7082 (0-20 μM, 8 hours) combined with TRAIL (20 ng/ml, 8 hours) as indicated. Then, the cells were analyzed by Western blot with the indicated antibodies. (B) The effects of LiCl on NF-κB transcriptional activity. H1299 cells were transfected with p-NF-κB-TA-Luc plasmid and combined with TRAIL (20 ng/ml, 8 hours) or LiCl (20 mM, 8 hours) treatment as indicated. (C) The effects of GSK3β knockdown on NF-κB transcriptional activity. H1299 cells were transfected with p-NF-κB-TA-Luc plasmid combined with siGSK3β, and treated with TRAIL (20 ng/ml, 8 hours) as indicated. (D) The effect of GSK3β/NF-κB on TRAIL-induced apoptosis. H1299 cells transfected with Flag-GSK3β or Flag-vector were co-treated with BAY 11-7082 (10 μM, 8 hours) and TRAIL (20 ng/ml, 8 hours) as indicated. Then, the cells were analyzed by Western blot with the indicated antibodies. Cleaved Cas 3/GAPDH values indicated on A, D represent the ratio between the gray values of Cleaved Cas 3 and GAPDH protein bands.
167x146mm (300 x 300 DPI)

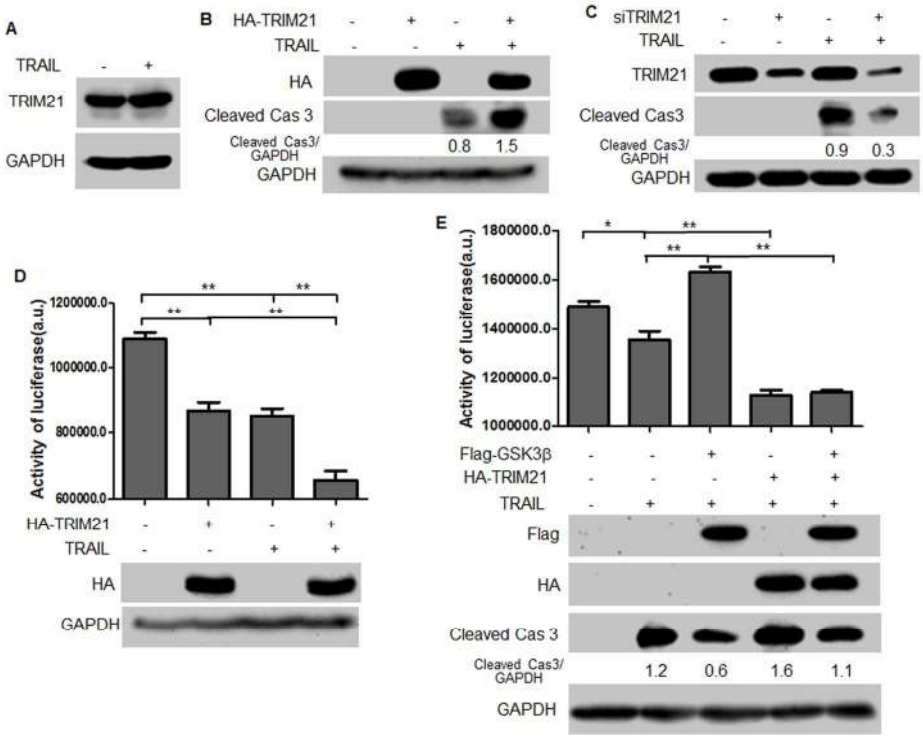


Fig. 5 TRIM21 counteracts the stimulation of NF-κB pathway by GSK3β. (A) The effect of TRAIL on the expression of TRIM21. H1299 cells were treated with or without TRAIL (20 ng/ml, 8 hours) and then harvested for Western blot assay with the indicated antibodies. The effects of overexpression of TRIM21 (B) or knockdown of TRIM21 (C) on the TRAIL-induced apoptosis. H1299 cells were transfected with HA-TRIM21 or siRNA of TRIM21 and treated with TRAIL (20 ng/ml, 8 hours). Then, the cells were analyzed by Western blot with the indicated antibodies. (D) The effects of TRIM21 on NF-κB transcriptional activity. H1299 cells were cotransfected with p-NF-κB-TA-Luc and HA-TRIM21 plasmids and combined with TRAIL (20 ng/ml, 8 hours) treatment. (E) The effects of TRIM21 on transcriptional activation of NF-κB mediated by GSK3β. H1299 cells were cotransfected with p-NF-κB-TA-Luc, HA-TRIM21, or Flag-GSK3β plasmids and combined with TRAIL (20 ng/ml, 8 hours) treatment. All the cell samples were harvested and prepared for luciferase assays (upper panel) and Western blot assays (bottom panel) with the indicated antibodies. The data were shown as mean+SEM. *: $p<0.05$, **: $p<0.01$. All results are representative of three repeated experiments. Cleaved Cas 3/GAPDH values indicated on B, C, E represent the ratio between the gray values of Cleaved Cas 3 and GAPDH protein bands.
136x100mm (300 x 300 DPI)