β-arrestin2 regulates TRAIL-induced HepG2 cell apoptosis via the Src-extracellular signal-regulated signaling pathway

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Abstract. β-arrestins, including β-arrestin1 and β-arrestin2, two ubiquitously expressed members of the arrestin family in various types of tissue, are adaptor proteins that modulate the desensitization and trafficking of seven membrane-spanning receptors. Recently, β-arrestins have been shown to bind to numerous signaling molecules, including c-Src and mitogen-activated protein kinase family members. In addition, accumulating evidence has suggested that β-arrestins are involved in the anti-apoptosis signaling pathway by associating with kinases, such as Akt and ERK, and altering their activities. However, the role of β-arrestins in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis remains unclear. In the present study, β-arrestin2, but not β-arrestin1, was observed to modulate TRAIL-triggered HepG2 cell apoptosis by regulating activation of the Src-extracellular signal-regulated kinase (ERK) signaling pathway. Using overexpression and RNA interference experiments, β-arrestin2 was demonstrated to prevent TRAIL-induced HepG2 cell apoptosis. Additionally, β-arrestin2 exerted an additive effect on TRAIL-induced activation of Src and ERK. Furthermore, downregulating β-arrestin2 expression attenuated the TRAIL-induced activation of Src and ERK survival signaling and enhanced TRAIL-induced apoptosis. PP2, a pharmacological inhibitor of Src, reduced activation of the Src-ERK signaling pathway and enhanced TRAIL-induced HepG2 cell apoptosis. Co-immunoprecipitation experiments demonstrated a physical association between β-arrestin2 and Src, and TRAIL stimulation resulted in enhanced quantities of the β-arrestin2/Src complex. A notable interaction was identified between β-arrestin2 and death receptors (DR)4 and 5, but only in the presence of TRAIL stimulation. To the best of our knowledge, these findings are the first to demonstrate that β-arrestin2 mediates TRAIL-induced apoptosis by combing with DRs and Src, and regulates the activation of Src-ERK signaling in HepG2 cells. It is hypothesized that the formation of a signaling complex comprising DR, β-arrestin2 and Src is required for the action of TRAIL on HepG2 cell apoptosis, which provides a novel insight into analyzing the effects of β-arrestin2 on protecting cells from TRAIL-induced apoptosis.

Introduction

β-arrestins, including β-arrestin1 and β-arrestin2, two ubiquitously expressed members of the arrestin family in various types of tissues, localize in the cytoplasm and plasma membrane, and modulate the desensitization and trafficking of seven transmembrane receptors (1,2). Recent evidence revealed that β-arrestins also served as multi-functional adaptors that contribute to regulating multiple signaling molecules. For example, β-arrestins associate with c-Src and mitogen-activated protein kinase (MAPK) family members, including extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (3,4). The binding of β-arrestins with these signaling molecules modulates phosphorylation, ubiquitination and/or subcellular distribution of their binding partners (5,6). The biological and clinical behaviors of numerous types of tumor are largely determined by multiple molecular signaling pathways. In addition, it was recently established that β-arrestins were involved in signaling events responsible for tumor viability and metastasis (7). It has also been reported that β-arrestins are involved in the anti-apoptosis pathway by associating with kinases such as Akt and ERK and altering their activities (4,8,9). However, the role of β-arrestins in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis remains unclear. TRAIL induces apoptosis in a variety of cancer cell lines by interacting with their death receptors (DRs) while causing minimal or no toxicity to normal cells, establishing it as an attractive agent for cancer therapy (10). However, numerous
types of cancer cell have been shown to be resistant to TRAIL-induced apoptosis, including hepatic carcinoma and human breast cancer cells (11,12). TRAIL signaling involves the activation of effector caspases and initiation of apoptosis, and thus the activation of pro-survival signaling pathways involving nuclear factor-xB, Akt and MAPKs may have contributed to the development of TRAIL resistance in tumor cells (13,14).

In the present study, the role and molecular mechanisms underlying the modulation of TRAIL-mediated apoptosis by β-arrestin2 in HepG2 cells was investigated.

Materials and methods

Antibodies and reagents. Monoclonal rabbit antibodies against poly ADP ribose polymerase (PARP; 9532s; 1:1,000), pro-caspase-3 (9665s; 1:1,000), cleaved caspase-3 (9664s; 1:500), Src (2109s; 1:1,000), phosphorylated (p)-Src (Tyr416; 6943s; 1:1,000), ERK (9102s; 1:1,000), p-ERK (Thr202/Tyr204; 4376s; 1:1,000), DR5 (8074s; 1:1,000), β-arrestin2 (3857s; 1:1,000) and β-actin (4970s; 1:1,000) were obtained from Cell Signalling Technology, Inc. (Danvers, MA, USA). In addition, mouse monoclonal antibody against DR4 (sc-8411; 1:500) and monoclonal rabbit antibody against β-arrestin1 (sc-53780; 1:500) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Polyclonal rabbit anti-GAPDH antibody (AP0063; 1:1,000) was purchased from BioWorld Technology, Inc. (St. Louis Park, MN, USA). Anti-green fluorescent protein (GFP) antibody (18114460001; 1:1,000) was obtained from Roche Diagnostics (Indianapolis, IN, USA). Recombinant human TRAIL was produced by PeproTech, Inc. (Rocky Hill, NJ, USA), and PP2, PP3 and U0126 were purchased from BioLabs, Inc. (St. Louis, MO, USA).

DNA constructs. pcDNA3.0-GFP-arrestin1/2 and pBS-U6-β-arrestin1/2 were provided by Dr. Gang Pei (Chinese Academy of Sciences, Shanghai, China). All expression vectors were sequenced and purified using the EndoFree Plasmid Preparation kit (Qiagen China Co., Ltd., Shanghai, China).

Cell culture and transfection. Hepatic carcinoma (HepG2) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Invitrogen Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; HyClone) at 37°C and 5% CO2 in a fully humidified incubator. Cell viability was determined using a Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Briefly, HepG2 cells were seeded at a density of 1x104 into 96-well plates 24 h before treatment. Cells were pretreated with PP2 (5 µM), PP3 (5 µM) or 0.1% dimethyl sulfoxide (DMSO) for 2 h, then exposed to 200 ng/ml TRAIL for 24 h, followed by incubation with 10 µl CCK-8 working solution at 37°C for 2 h. The absorbance of each well at a wavelength of 450 nm was measured using a Synergy2 multi-mode microplate reader (Bio-Tek, Inc.). Three experiments were performed for each of the different treatments.

Co-immunoprecipitation and immunoblotting analysis. Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and lysed on ice in a lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing 20 mM Tris (pH 7.5), 2 mM EDTA, 135 mM NaCl, 2 mM dithiothreitol, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM Na2VO4, 1% Triton X-100, 10 mM NaF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM phenylmethanesulfonyl fluoride supplemented with 0.01% complete protease inhibitor cocktail (Roche) for 30 min. Lysates were centrifuged at 12,500 x g for 15 min at 4°C. Equal quantities of proteins were immunoprecipitated overnight with β-arrestin2 monoclonal antibody at 4°C. The precleared Protein A/G PLUS-Agarose beads (20 µl; Santa Cruz Biotechnology, Inc.) were co-incubated with immunocomplexes for an additional 2 h, then washed four times with the cold lysis buffer. The immunoprecipitates were electrophoresed on 12% SDS-PAGE (135 V; 2 h) and transferred onto nitrocellulose membranes (GE Healthcare Life Sciences). Immunoblotting was subsequently performed. The LI-COR Odyssey Infrared Imaging system and IRDye® 800 fluorophore-conjugated antibody (LI-COR Biosciences, Lincoln, NE, USA) were used to visualize the antibody-antigen complexes. Band intensity quantification was directly performed on the blot using LI-COR Odyssey Analysis software 1.2. Aliquots of whole cell lysates were subjected to immunoblotting analysis to confirm appropriate protein expression.

RNA interference. HepG2 cells were transfected with short hairpin (sh)RNA constructs against β-arrestin1 or β-arrestin2 (pBS-U6-β-arrestin1 or pBS-U6-β-arrestin2), or a negative control vector using FuGene® HP Transfection Reagent according to the manufacturer's instructions. Interference efficiency was confirmed by immunoblot analysis following a 72-h transfection using β-arrestin1 or β-arrestin2 antibodies.

Flow cytometry. Cell apoptosis was determined using the Annexin V/propidium iodide (PI) double staining assay (Kaiji Materials Co., Ltd., Nanjing, China). Briefly, following transfection with shRNA or a negative control vector, the cells were washed with PBS, harvested by trypsinization, precipitated by centrifugation (2,000 x g for 5 min), rinsed with PBS again, resuspended with 500 µl binding buffer, and stained with Annexin V and PI. Apoptotic cells were detected directly using the Guava Easy Cyte™ system, and the data were analyzed using Guava TUNEL Software (Guava Technologies, Inc., Hayward, CA, USA).

Immunofluorescence microscopy and 4',6-diamidino-2-phenylindole (DAPI) staining. HepG2 cells were precultured with PP2 (5 µM), PP3 (5 µM) or 0.1% DMSO for 2 h, then stimulated with TRAIL; the cells were subsequently fixed with 4% paraformaldehyde (JRDun Biotechnology, Co., Ltd., Shanghai, China) and permeabilized with 0.2% Triton X-100. Following incubation with 1 µg/ml DAPI for 5 min at room
temperature, the cells were washed with PBS again. The immunofluorescence images were captured using a fluorescence microscope (Leica TCS SP8; Leica Microsystems GmbH, Wetzlar, Germany). The number of cells exhibiting nuclear condensation and fragmentation were counted in randomly selected fields, and the ratio of these cells to the total number of cells was calculated.

Statistical analysis. Data are presented as the mean ± standard deviation. One-way analysis of variance was used to determine significant differences between two groups. P<0.05 was considered to indicate a statistically significant difference. Statistical calculations were performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA).

Results

β-arrestin2 inhibited TRAIL-induced apoptosis in HepG2 cells. A previous study demonstrated that β-arrestins prevented cell apoptosis via the ERK, p38 and Akt signaling pathways (4), which are involved in cancer progression (7,15). Furthermore, TRAIL has previously demonstrated marked anticancer effects in numerous tumor types, but not in normal cells (10). Conversely, previous studies reported that certain human cancers were resistant to TRAIL (11,12,16,17), although the underlying molecular mechanisms were unclear. The present study hypothesized that β-arrestins are involved in TRAIL-resistance by combining with pro-survival signaling molecules and regulating their activity.

In the present study, the role of β-arrestins in modulating TRAIL-induced HepG2 cell apoptosis was evaluated. HepG2 cells were transiently transfected with pBS-U6-β-arrestin1 and 2 or control plasmid, pBS-U6. After a 72-h transfection, HepG2 cells were treated with 200 ng/ml TRAIL for the indicated times. (A and B) Expression levels of PARP, pro-caspase-3 and cleaved caspase-3 were detected by western blotting. (C) Apoptotic ratios were detected by flow cytometry. The lower left quartile represents live cells, the lower right quartile represents early apoptotic cells, the upper left quartile represents dead cells and the upper right quartile represents late apoptotic cells. Experiments were performed in triplicate and data are presented as means ± standard deviation. *P<0.05 vs. control. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PARP, poly ADP ribose polymerase; RNAi, RNA interference; Arrb, β-arrestin.

Figure 1. β-arrestin2 mediated TRAIL-induced apoptosis. HepG2 cells were transiently transfected with pBS-U6-β-arrestin1 and 2 or control plasmid, pBS-U6. After a 72-h transfection, HepG2 cells were treated with 200 ng/ml TRAIL for the indicated times. (A and B) Expression levels of PARP, pro-caspase-3 and cleaved caspase-3 were detected by western blotting. (C) Apoptotic ratios were detected by flow cytometry. The lower left quartile represents live cells, the lower right quartile represents early apoptotic cells, the upper left quartile represents dead cells and the upper right quartile represents late apoptotic cells. Experiments were performed in triplicate and data are presented as means ± standard deviation. *P<0.05 vs. control. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PARP, poly ADP ribose polymerase; RNAi, RNA interference; Arrb, β-arrestin.
detected using the AnnexinV/PI assay. The TRAIL-induced apoptotic ratio in β-arrestin2 RNA interference (RNAi) cells was 62.12% at 24 h, whereas the apoptotic ratio was 40.24% in the control plasmid transfected cells; however, no significant difference was observed between the apoptotic ratio of β-arrestin1 RNAi cells and control plasmid transfected cells (Fig. 1C).

To further clarify the role of β-arrestin2 in TRAIL-induced apoptosis, GFP-tagged β-arrestin2 (GFP-Arrb2) was overexpressed in HepG2 cells. Following a 48-h transfection, the cells were stimulated with TRAIL for 6, 12 or 24 h. As presented in Fig. 2A-C, caspase-3 and PARP cleavage, and the apoptotic ratio induced by TRAIL, were attenuated by overexpression of β-arrestin2 in HepG2 cells. To further demonstrate the role of β-arrestin2 in TRAIL-induced apoptosis, β-arrestin2 shRNA was transiently transfected into HepG2 cells for 72 h, followed by transfection with GFP-Arrb2 or empty vectors for 48 h. As shown in Fig. 2D, the levels of cleaved caspase-3 and PARP in β-arrestin2 RNAi cells were reversed following overexpression with β-arrestin2 plasmid. These results demonstrate that β-arrestin2 exerts an anti-apoptotic role in TRAIL-induced HepG2 cell apoptosis.

β-arrestin2 mediated activation of the Src-ERK signaling pathway in response to TRAIL. It has been reported that TRAIL activates apoptosis signaling pathways, as well as survival signaling pathways, which are involved in the development of TRAIL resistance. The Src-ERK signaling pathway...
was demonstrated to counteract TRAIL toxicity in tumor cells (18). Therefore, the activation of Src-ERK signaling upon TRAIL stimulation was investigated in the present study by western blotting. HepG2 cells were treated with 200 ng/ml TRAIL for 0.25, 0.5, 1, 2 or 4 h and the cell lysates were detected by immunoblotting with the indicated antibodies. The band intensities were quantified using densitometry using LI-COR Odyssey Analysis software 1.2. *P<0.05, **P<0.01 vs. the TRAIL group. HepG2 cells were transfected with (B) pBS-U6-β-arrestin2 or control plasmid for 72 h or (C) pcDNA3.0 and GFP-Arrb2 plasmids for 48 h. (B and C) Cells were stimulated with TRAIL for 1 or 2 h and the levels of p-, total Src and ERK were detected by western blotting. Equal protein loading was confirmed by GAPDH. (D) Cells were pretreated with 0.1% DMSO or 5 µM PP2 or 5 µM PP3 for 2 h, then treated with 200 ng/ml TRAIL for 4 h. Cell viability was detected by CCK-8 and experiments were independently repeated three times. *P<0.05 vs. DMSO. (F) Cells were stained with DAPI and the nuclear morphology was observed by immunofluorescent microscopy (magnification, x200). (G) Cells were pretreated with 0.1% DMSO or 20 µM U0126 for 2 h, then exposed to 200 ng/ml TRAIL for 24 h. Cell viability was detected by CCK-8 and experiments were independently repeated for three times. *P<0.05 vs. DMSO. (H) Cells were stained with DAPI and the nuclear morphology was observed by immunofluorescent microscopy (magnification, x200). TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; p, phosphorylated; RNAi, RNA interference; Arrb, β-arrestin; GFP, green fluorescent protein; ERK, extracellular signal-regulated kinase; DMSO, dimethyl sulfoxide; CCK-8, Cell Counting Kit-8; DAPI, 4,6-diamidino-2-phenylindole.

Figure 3. β-arrestin2 mediated activation of the TRAIL-induced Src-ERK signaling pathway. (A) HepG2 cells were treated with TRAIL for 0.25, 0.5, 1, 2 or 4 h and the cell lysates were subjected to immunoblotting with the indicated antibodies. The band intensities were quantified by densitometry using LI-COR Odyssey Analysis software 1.2. *P<0.05, **P<0.01 vs. the TRAIL group. HepG2 cells were transfected with (B) pBS-U6-β-arrestin2 or control plasmid for 72 h or (C) pcDNA3.0 and GFP-Arrb2 plasmids for 48 h. (B and C) Cells were stimulated with TRAIL for 1 or 2 h and the levels of p-, total Src and ERK were detected by western blotting. Equal protein loading was confirmed by GAPDH. (D) Cells were pretreated with 0.1% DMSO or 5 µM PP2 or 5 µM PP3 for 2 h, then treated with 200 ng/ml TRAIL for 4 h. Cell viability was detected by CCK-8 and experiments were independently repeated three times. *P<0.05 vs. DMSO. (F) Cells were stained with DAPI and the nuclear morphology was observed by immunofluorescent microscopy (magnification, x200). (G) Cells were pretreated with 0.1% DMSO or 20 µM U0126 for 2 h, then exposed to 200 ng/ml TRAIL for 24 h. Cell viability was detected by CCK-8 and experiments were independently repeated for three times. *P<0.05 vs. DMSO. (H) Cells were stained with DAPI and the nuclear morphology was observed by immunofluorescent microscopy (magnification, x200). TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; p, phosphorylated; RNAi, RNA interference; Arrb, β-arrestin; GFP, green fluorescent protein; ERK, extracellular signal-regulated kinase; DMSO, dimethyl sulfoxide; CCK-8, Cell Counting Kit-8; DAPI, 4,6-diamidino-2-phenylindole.

 Src-ERK signaling pathway is involved in TRAIL-induced HepG2 apoptosis. It has been recognized that Src may mediate ERK activation and is an upstream kinase in various stimulus-induced signaling cascades (19,20). Therefore, PP2 was used to evaluate the effect of Src on the activation of ERK upon TRAIL stimulation. Blocking Src activity using PP2
Figure 4. β-arrestin2 formed a ternary complex with Src and DRs upon stimulation with TRAIL. HepG2 cells were treated with TRAIL for the indicated times, and the combinations of β-arrestin2 + Src, and β-arrestin2 + DRs were detected by co-immunoprecipitation analysis. The aliquot samples from the same lysates were used as inputs to determine the internal expression of β-arrestin2, DR4/DR5 and Src by immunoblotting. IgG served as a negative control. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DR, death receptor; IgG, immunoglobulin G.

Discussion

Recent studies have demonstrated that β-arrestins contribute to anti-apoptotic effects in apoptosis that is induced by a variety of stimuli (5,8,22). However, the role of β-arrestins in TRAIL-induced apoptosis remains unclear. In the present study, knockdown of β-arrestin2 in HepG2 cells increased cell apoptosis, and reduced activation of the Src-ERK signaling pathway upon TRAIL stimulation. Notably, TRAIL treatment enhanced the quantity of β-arrestin2/Src complexes and the association between β-arrestin2 and DR4/5 was observed only in the presence of TRAIL. These results indicate that β-arrestin2 acts as a negative regulator in TRAIL-induced HepG2 cell apoptosis via formation of ternary complexes and mediating activation of the Src-ERK signaling pathway.

TRAIL activates apoptosis signaling pathways and also survival signaling pathways (13), which may contribute to the development of TRAIL resistance (23). The Src-ERK signaling pathway is an important pro-survival signaling pathway and responds to different stimuli (20,24). In addition, recent studies revealed that β-arrestins serve as adaptors for scaffolding intracellular signaling networks to modulate downstream kinase activity (3). Therefore, the present study hypothesized that β-arrestins mediate Src recruitment, which could be involved in the effect of TRAIL on HepG2 cell apoptosis.
A cytoprotective role of β-arrestins was initially verified in HepG2 cells upon TRAIL stimulation. RNAi of β-arrestin2, but not β-arrestin1, potentiated TRAIL-induced apoptosis. Recent studies identified that β-arrestin1 exerted a significant role in the proliferation and anti-apoptotic activity of nicotine-induced human non-small cell lung cancer (25,26); the explanations were associated with differences in cell types and the specificity of the action of different arrestin subtypes. Recently, Kook et al (27) reported that β-arrestin1 was cleaved by caspases during apoptosis, which was not consistent with the present study. Kook et al (27) used mouse embryonic fibroblasts (MEFs) whereas the current study adopted HepG2 cells. β-arrestin has been demonstrated to have diverse roles via distinct mechanisms in various experimental models (22). In the present study, overexpression of β-arrestin2 using GFP-Arb2 plasmids demonstrated that β-arrestin2 exerted anti-apoptotic role in HepG2 cells. Using β-arrestin2 RNAi and β-arrestin2 overexpression, the current results revealed that β-arrestin2 has a significant role in TRAIL-induced HepG2 cell apoptosis. The contribution of β-arrestin2 to TRAIL-induced Src-ERK signaling activation was also demonstrated. Downregulating β-arrestin2 markedly attenuated the phosphorylation of Src and ERK upon TRAIL stimulation, and β-arrestin2 overexpression enhanced Src-ERK signaling pathway activation. To identify the role of Src activation in TRAIL-induced HepG2 cell apoptosis, PP2 was used to block Src phosphorylation, and the results demonstrated that suppression of Src activation following TRAIL treatment reduced the activation of ERK, and enhanced TRAIL-induced HepG2 cell apoptosis. In addition, U0126, an inhibitor of ERK, was used to block ERK phosphorylation, and a CCK-8 assay and DAPI staining revealed that inhibiting ERK activation prevented TRAIL-mediated cell survival. As a multifunctional scaffold, β-arrestins regulate various key signaling molecules through protein-protein interactions (28), thus, the association between β-arrestin2 and Src was examined further. The present study demonstrated that β-arrestin2 physically combined with Src, and the quantity of β-arrestin2 and Src complexes was enhanced upon TRAIL stimulation. It is proposed that β-arrestin2 regulated the activation of Src by increasing the number of β-arrestin2/Src complexes in response to TRAIL stimulation. It was reported that β-arrestins bind to Src family kinases and recruit them to activated GPCRs (29,30), which results in numerous physiological effects, including the generation of signal complexes where β-arrestins scaffold various proteins to potentiate distinct downstream signaling events (31). Using β-arrestin2 RNAi, β-arrestin2 was demonstrated to be important in regulating Src-ERK activation. Further investigation was performed to evaluate whether β-arrestin2 recruited Src to the TRAIL-associated DR. The present study demonstrated that TRAIL stimulation induced the formation of DR/β-arrestin2/Src ternary complexes. However, the formation of ternary complexes could be validated more effectively with protein purification/co-elution experiments, or with FRET-based technology; therefore, further studies are required. The current data indicates that TRAIL may induce activation of the Src-ERK signaling pathway via formation of DR/β-arrestin2/Src complexes. However, this requires further investigation. In addition, further studies are required to establish whether the ternary complex was necessary in β-arrestin2-mediated, TRAIL-induced HepG2 cell apoptosis.

In future studies, whether the formation of ternary complexes is cell-type specific or universal and the mechanism by which β-arrestin2 exerted its action on activation of the Src-ERK signaling pathway requires investigation.

In conclusion, the present study elucidated that β-arrestin2 protects HepG2 cells from TRAIL-induced apoptosis by facilitating the activation of Src-ERK pro-survival signaling. This may have been achieved by the recruitment of Src to DR by β-arrestin2 and the formation of DR/β-arrestin2/Src ternary complexes in response to TRAIL stimulation. These findings provide novel insight into the mechanism by which β-arrestin2 protects cells against TRAIL-induced apoptosis.

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