Resistance to TRAIL Is Mediated by DARPP-32 in Gastric Cancer
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Abstract
Purpose: Dopamine and cAMP-regulated phosphoprotein, Mr 32,000 (DARPP-32), is overexpressed during the gastric carcinogenesis cascade. Here, we investigated the role of DARPP-32 in promoting resistance to treatment with TRAIL.

Experimental Design: In vitro cell models including stable expression and knockdown of DARPP-32 were used. The role of DARPP-32 in regulating TRAIL-dependent apoptosis was evaluated by clonogenic survival assay, Annexin V staining, immunofluorescence, quantitative reverse transcriptase PCR, Western blot, and luciferase reporter assays.

Results: Stable expression of DARPP-32 in MKN-28 cells enhanced cell survival and suppressed TRAIL-induced cytochrome c release and activation of caspase-8, -9, and -3. Conversely, short hairpin RNA-meditated knockdown of endogenous DARPP-32 sensitized the resistant MKN-45 cells to TRAIL-induced apoptosis and enhanced TRAIL-mediated activation of caspase-8, -9, and -3. DARPP-32 induced BCL-xL expression through activation of Src/STAT3 signaling, and treatment with the Src-specific inhibitor PP1 abrogated DARPP-32–dependent BCL-xL upregulation and cell survival in MKN-28 cells. The TRAIL treatment induced caspase-dependent cleavage of NF-kBp65 protein; this cleavage was prevented by DARPP-32, thus maintaining NF-xB activity and the expression of its target, FLIP(S) protein. This suggests that upregulation of BCL-xL could play a possible role in blocking the mitochondria intrinsic apoptosis pathway, whereas the DARPP-32 effect on the NF-kB/FLIP(S) axis could serve as an additional negative feedback loop that blocks TRAIL-induced activation of caspase-8.

Conclusion: Our findings uncover a novel mechanism of TRAIL resistance mediated by DARPP-32, whereby it inhibits the intrinsic apoptosis pathway through upregulation of BCL-xL, and the extrinsic apoptosis pathway through the NF-xB/FLIP(S) axis. Clin Cancer Res; 18(14); 3889–900. ©2012 AACR.

Introduction
Gastric cancer is one of the most frequent malignancies worldwide (1, 2). Although various chemotherapeutic drugs have been used, drug resistance has significantly hampered the effectiveness of chemotherapy leading to poor survival rates in patients with gastric cancer (3, 4).

Death receptor–mediated cell death is one of the major apoptosis pathways. Death receptors including DR4 and DR5, relay a death signal upon binding with TRAIL (5, 6), which shares protein homology with Fas ligand and TNF-α.

Upon treatment with TRAIL, the initiator pro-caspase-8 is recruited to death receptors through the adaptor protein FADD, forming the death-inducing signaling complex (DISC). This results in autocatalytic activation of caspase-8 that directly activates caspase-3 and apoptosis in type I cells. However, in type II cells, caspase-8 through cleavage of Bid activates the intrinsic mitochondrial apoptosis pathway, which is mediated by cytochrome c release and activation of caspase-9 and -3 (8–10). TRAIL selectively induces apoptosis in a variety of tumor cells, whereas several normal cells remain unresponsive to its effects (11, 12). This unique capacity has placed TRAIL in the forefront as a promising anticancer agent. Unfortunately, resistance to TRAIL is not uncommon as TRAIL resistance can be developed by several mechanisms. For instance, overexpression of BCL-xL in type II cells has been shown to protect against TRAIL-mediated apoptosis through inhibition of the intrinsic pathway (13, 14). Several reports have indicated that transcription factors such as NF-xB and STAT3 could upregulate BCL-xL in cancer cells (reviewed by ref. 15). FLIP, which resembles caspase-8 but lacks the enzymatic activity, competes with caspase-8 to bind to FADD, thereby inhibiting TRAIL-induced death receptor signaling (16). Increased expression of FLIP has

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been shown to promote resistance of cancer cells to death receptor-mediated apoptosis (17). In addition, expression of FLIP(S) completely inhibited TRAIL-induced apoptosis through blocking activation of caspase-8 in gastric cancer cells, and AKT activity promoted cancer cell survival through upregulation of FLIP(S) (18). The FLIP gene is regulated by several antiapoptotic pathways including the AKT, MAPK, and NF-κB pathways (19, 20).

We have previously reported that dopamine and cAMP-regulated phosphoprotein, Mr 32,000 (DARPP-32) is amplified and overexpressed in about two-thirds of upper gastrointestinal adenocarcinomas (21). We showed that DARPP-32 expression was associated with the multistep gastric carcinogenesis cascade involving the transition to intestinal metaplasia and the progression to neoplasia (22). Little is known about the mechanisms of TRAIL resistance in gastric cancer. In this study, we uncovered a novel mechanism by which DARPP-32 blocks TRAIL-induced apoptosis in gastric cancer cells.

Materials and Methods

Cell lines and reagents

The human gastric cancer cell lines, MKN-28 and MKN-45, were maintained in culture using Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 10% FBS (Invitrogen Life Technologies) and 1% penicillin/streptomycin (GIBCO). Recombinant human TRAIL/Apo2 ligand was purchased from BioVision Research Products. 4-Amino-5-(4-methylphenyl)-7-(1-butyl)pyrazolo[3,4-d]-pyrimidine (PP1), a specific Src inhibitor, was purchased from Enzo Life Sciences. Rabbit anti-DARPP-32 antibody was obtained from Santa Cruz Biotechnology. Mouse monoclonal anti-cytoskeleton antibody was purchased from BD PharMingen. A secondary anti-mouse antibody conjugated to tetramethylrhodamine isothiocyanate (TRITC) and anti-rabbit antibody conjugated to fluorescein isothiocyanate (FITC) were obtained from Invitrogen Life Technologies. Horseradish peroxidase–conjugated rabbit secondary antibody; NF-xBp65, FLIP, BCL-xL, caspase-3, cleaved caspase-3, caspase-9, caspase-8, p-Src (Y416), Src, and β-actin antibodies were purchased from Cell Signaling Technology. STAT3 and p-STAT3 (Y705) antibodies were obtained from GenScript.

DARPP-32 expression and short hairpin RNA vectors

The mammalian expression plasmid for DARPP-32 was produced by PCR amplification of the full-length coding sequence of DARPP-32 and cloned in-frame into pcDNA3 (Invitrogen). MKN-28 cells stably expressing DARPP-32 or pcDNA3 empty vector were generated after standard protocols as previously described (21). After selection with 500 μg/mL neomycin (G418; Invitrogen), DARPP-32 protein expression was evaluated by Western blot analysis and clones were selected. Lentivirus particles expressing DARPP-32 short hairpin RNA (shRNA) or control-shRNA were produced by GeneCopeia and then used to transduce cells.

Clonogenic survival assay

Cells were first washed with PBS, trypsinized, and harvested in single-cell suspension. Cells (1,000 cells per well) were seeded onto 6-well plates. The next day, cells were treated for 24 hours with TRAIL or vehicle. After incubation for 2 weeks, colonies were fixed with 4% paraformaldehyde and stained with 0.05% crystal violet. Colonies with ≥50 cells were counted. Alternatively, the long-term cell viability was determined using the Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega) following the supplier’s instructions.

Apoptosis assay

MKN-28 cells stably expressing DARPP-32 or pcDNA3 empty vector, and MKN-45 cells transduced with lentivirus particles expressing DARPP-32-shRNA or control-shRNA were seeded onto 60-mm culture plates. The next day, cells were treated with TRAIL (200 ng/mL) or vehicle overnight. Cells were then collected and stained with Annexin V-FITC and propidium iodide (PI; R&D Systems), or Annexin V-PE (BioVision). The samples were washed with PBS and resuspended in binding buffer for subsequent fluorescence-activated cell sorting (FACS) analysis. Apoptotic cell death was evaluated by counting the number of cells that stained positive for Annexin V-FITC and positive for PI (MKN-28 cells), or positive for Annexin V-PE (MKN-45 cells).

Immunofluorescence assay

To evaluate cytochrome c release, MKN-28 cells stably expressing DARPP-32 or pcDNA3 empty vector were treated with 200 ng/mL TRAIL or vehicle for 24 hours. Immunofluorescence was conducted with anti-cytoskeleton antibody; NF-xBp65, FLIP, BCL-xL, caspase-3, cleaved caspase-3, caspase-9, caspase-8, p-Src (Y416), Src, and β-actin antibodies were purchased from Cell Signaling Technology. STAT3 and p-STAT3 (Y705) antibodies were obtained from GenScript.
In healthy cells, cytochrome c was depicted by punctuated red fluorescence. In apoptotic cells, cytochrome c release was indicated by diffused red staining. 4',6-Diamidino-2-phenylindole (DAPI) was used as a nuclear counterstain (blue fluorescence). The ratio of cells that presented diffused cytochrome c staining to the total number of cells was averaged from 6 random microscopic fields for more than 100 cells in each category.

To assess NF-kBp65 nuclear localization, MKN-28 cells stably expressing DARPP-32 or pcDNA3 empty vector, were treated with 200 ng/mL TRAIL or vehicle for 24 hours. Cells were subjected to immunofluorescence assay with anti-NF-kBp65 antibody (1:200) and a secondary antibody conjugated to FITC (1:800). DAPI was used as a nuclear counterstain. The cells were visualized by fluorescence microscopy. The ratio of cells that presented nuclear NF-kBp65 staining (indicating activation of NF-kB) to the total number of cells was averaged from 5 random microscopic fields for more than 100 cells in each category.

Quantitative real-time reverse transcriptase PCR
Quantitative real-time reverse transcriptase PCR (qRT-PCR) was carried out in triplicate using an iCycler (Bio-Rad) with a threshold cycle number determined by use of iCycler software version 3.0. Total RNA was isolated from cells using an RNasey Mini Kit (Qiagen). Single-stranded cDNA was synthesized from a total RNA amount of 1 μg by an iScript cDNA synthesis kit (Bio-Rad). mRNA-specific primers for FLIP(L), FLIP(S), BCL-xL, and HPRT1 were designed, and the results were normalized to HPRT1 as a stable reference gene for quantitative real-time RT-PCR. All primer sequences are available upon request. The mRNA fold expression levels were calculated according to the formula 2^((ΔCT)/2), as described previously (24).

Western blot analysis
Cell lysates were prepared in 0.5% Triton X-100, 150 mmol/L NaCl, 5 mmol/L Tris supplemented with 1 × Halt protease inhibitor cocktail and 1 × Halt phosphatase inhibitor cocktail (Pierce), centrifuged at 3,500 rpm for 10 minutes at 4°C. Protein concentration was measured using a Bio-Rad protein assay (Bio-Rad Laboratories). Proteins (10–20 μg) from each sample were separated on 10% SDS-PAGE and transferred to Immobilon PVDF membrane (Millipore). Membranes were probed with specific antibodies, and proteins were visualized by using horseradish peroxidase (HRP)-conjugated secondary antibodies and Immobilon Western Chemiluminescent HRP Substrate detection reagent (Millipore). Gel loading was normalized for equal β-actin.

Luciferase reporter assay
To assess the activity of the NF-κB signaling pathway, we used the NF-κB-Luc reporter vector that contains multiple copies of the consensus sequence (Clontech). After endogenous NF-κB proteins bind to the κ enhancer element, transcription is induced and the reporter gene is activated. MKN-28 cells stably expressing DARPP-32 or pcDNA3 empty vector, and MKN-45 cells transfected with lentivirus particles expressing DARPP-32-shRNA or control-shRNA were seeded in 96-well plates (10^3 cells per well). Cells were transiently cotransfected with 60 ng of the NF-κB-Luc and 6 ng of an ubiquitin promoter, Renilla luciferase control plasmid, using Fugene according to the manufacturer’s instructions. The next day, cells were treated with 200 ng/mL TRAIL for 24 hours. Luciferase activity was measured using the dual-luciferase reporter assay kit (Promega) according to the manufacturer’s instructions. Firefly luciferase activities were normalized to Renilla luciferase levels. Results are the average of 3 independent experiments and expressed as mean ± SD.

Statistical analysis
The results were expressed as the mean ± SEM of 3 independent experiments. Statistical significance of the studies was evaluated by the parametric unpaired Student t test. Differences with P ≤ 0.05 are considered significant.

Results
DARPP-32 enhances cell survival of gastric cancer cells
To determine the sensitivity of MKN-28 cells to TRAIL, we used the clonogenic survival assay for long-term assessment of cell viability. The results showed a TRAIL dose-dependent decrease in cell survival (Fig. 1A). MKN-28 cells, which are negative for DARPP-32 expression, were stably transfected with DARPP-32 expression plasmid or pcDNA3 empty vector (Fig. 1B). On the basis of the survival data (Fig. 1A), we selected the TRAIL concentration 200 ng/mL as an approximate IC50 in MKN-28 cells to assess the role of DARPP-32 in regulating long-term cell survival by clonogenic survival assay (2-week-long assay). As expected, following TRAIL treatment, cell survival of control cells decreased by approximately 60% relative to vehicle (P < 0.001; Fig. 1C). In contrast, in response to TRAIL, cell survival of 2 clones of MKN-28/DARPP-32 cells decreased by only 10% to 15% relative to vehicle (Fig. 1C). Interestingly, the size of colonies was relatively larger in pcDNA3 control cells than DARPP-32–expressing cells. This suggests that DARPP-32 may regulate cell aggregation or migration. These results clearly showed that stable DARPP-32 expression promoted cell survival in response to TRAIL in gastric cancer cells. To confirm this finding, we used the resistant MKN-45 cells to knockdown endogenous DARPP-32 by shRNA and subject the cells to clonogenic survival assay after treatment with vehicle or 200 ng/mL TRAIL (Fig. 1D). The data clearly indicated that knocking down of endogenous DARPP-32 alone was sufficient to decrease cell survival by 20% (P < 0.001), implying the important role of DARPP-32 in cell survival (Fig. 1D). In addition, knockdown of DARPP-32 in combination with TRAIL induced approximately 70% decrease in cell survival relative to treatment with TRAIL alone (P < 0.001; Fig. 1D). The results showed that the knocking down of endogenous DARPP-32 sensitized MKN-45 cells to TRAIL.
**DARPP-32 counteracts TRAIL-induced apoptosis by blocking cytochrome c release and activation of caspases in gastric cancer cells**

In addition to long-term clonogenic cell survival assays (Fig. 1C and D), we next used the Annexin V apoptosis assay to evaluate the role of DARPP-32 in regulating TRAIL-induced apoptosis following short-term treatments (24-hour-long assay). The Annexin V staining and FACS analysis results showed that treatment with TRAIL resulted in twice as high apoptotic cells in the MKN-28 control cells as compared with MKN-28/DARPP-32 cells (Fig. 1E, top). This indicated that ectopic expression of DARPP-32 significantly blocked TRAIL-induced apoptosis. Concordant with these findings, the knocking down of endogenous DARPP-32 in MKN-45 cells and treatment with TRAIL induced twice the number of apoptotic cells as compared with control cells (Fig. 1E, bottom). These results showed that knocking down endogenous DARPP-32 sensitized resistant cells to TRAIL. In addition, knocking down endogenous DARPP-32 alone in MKN-45 cells induced approximately 25% increase in apoptotic cells relative to control cells (Fig. 1E, bottom).

This evidently underscored the prosurvival properties of DARPP-32 in gastric cancer. To investigate whether DARPP-32 regulated the mitochondrial intrinsic apoptosis pathway, we assessed cytochrome c release in response to TRAIL in MKN-28 cells by immunofluorescence assay. In healthy cells, cytochrome c was punctuated. In contrast, in apoptotic cells, cytochrome c release was indicated by diffused staining, which depicted the onset of mitochondria-dependent apoptosis. The results showed that treatment with 200 ng/mL TRAIL led to diffused cytochrome c staining of approximately a 6-fold increase in pcDNA3 control cells ($P < 0.0001$) as opposed to less than a 2-fold increase in 2 clones of MKN-28/DARPP-32 cells relative to vehicle (Fig. 2A). The results clearly indicated that DARPP-32 blocked TRAIL-induced cytochrome c release. We next investigated the role of DARPP-32 in blocking TRAIL-induced activation of caspases associated with the extrinsic and intrinsic apoptosis pathways in MKN-28 and MKN-45 cell models. Western blot analysis showed that treatment with 200 ng/mL TRAIL led to a significant activation of caspase-3, -9, and -6 in MKN-28/pcDNA3 control cells (Fig. 2B).
DARPP-32 blocks cytochrome c release and activation of caspases. A, MKN-28/pcDNA3 and MKN-28/DARPP-32 cells were treated with vehicle or 200 ng/mL TRAIL for 24 hours. Immunofluorescence was conducted with anti-cytochrome c antibody and a secondary antibody conjugated to TRITC. DAPI was used as a nuclear counterstain. White arrows indicate TRAIL-induced chromatin condensation and nuclear fragmentation. In response to TRAIL, approximately 10% of DARPP-32–expressing cells released cytochrome (cyto) c as opposed to 30% of control cells. Results are representative of 3 experiments and shown as the mean ± SEM. Significance of difference was calculated using the Student t-test analysis. B, MKN-28/DARPP-32 and MKN-28/pcDNA3 cells were treated with vehicle or TRAIL, as in A. Western blotting data showed that treatment with TRAIL led to more activation of caspase-8, -9, and -3 in control cells than in DARPP-32–expressing cells. C, MKN-45/ control-shRNA and MKN-45/ DARPP-32-shRNA cells were treated with vehicle or 200 ng/mL TRAIL for 24 hours. Western blotting results showed that knocking down endogenous DARPP-32 in combination with TRAIL led to activation of caspase-8, -9, and -3. Gel loading was normalized for equal β-actin.

contrast, TRAIL had no significant effect on the activation of caspase-8, -9, and -3 in 2 clones of MKN-28/DARPP-32 cells (Fig. 2B). The results indicated that TRAIL induced more significant activation of caspase-8, -9, and -3 in MKN-45/ DARPP-32-shRNA cells than MKN-45/control-shRNA cells (Fig. 2C). Interestingly, knockdown of DARPP-32 alone and without TRAIL treatment led to some activation of caspase-8 and -9 as opposed to control cells (Fig. 2C). Together, these results showed that ectopic expression of DARPP-32 blocked TRAIL-induced activation of caspases in MKN-28 cells. Conversely, knocking down endogenous DARPP-32 enhanced TRAIL-induced activation of caspases in MKN-45 cells.

DARPP-32 promotes the prosurvival BCL-xL expression through activation of Src/STAT3 signaling

As the prosurvival BCL2 family members are mainly mitochondrial membrane proteins, and their expression inhibits cytochrome c release and activation of caspase-9 (reviewed by ref. 25), we evaluated BCL-xL mRNA and protein expression in the 2 cell models described earlier. The qRT-PCR results indicated that the BCL-xL mRNA level was higher in 2 clones of MKN-28/DARPP-32 cells than control cells (P < 0.01; Fig. 3A). In agreement with these findings, the knocking down of endogenous DARPP-32 with shRNA alone or in combination with TRAIL treatment led to a significant reduction (P = 0.01) in BCL-xL mRNA levels in MKN-45 cells (Fig. 3B). We also noticed that the increase in the levels of BCL-xL in DARPP-32 cells was more robust at the protein level. The Western blot analysis results showed higher BCL-xL protein levels in the 2 clones of MKN-28/DARPP-32 cells than control cells (Fig. 3C). Similarly, Western blot analysis data showed that the BCL-xL protein level was significantly lower in MKN-45/DARPP-32-shRNA cells than MKN-45/control-shRNA cells (Fig. 3D). Of note, treatment with TRAIL had no effect on BCL-xL expression levels in MKN-45 cells (Fig. 3D). Therefore, these results established the role of DARPP-32 in regulating BCL-xL expression, thus potentially suppressing the intrinsic apoptosis pathway and promoting cell survival; a finding supported by the effect of DARPP-32 on cytochrome c release as shown in Fig. 2A. In an attempt to identify the mechanism by which DARPP-32 regulates BCL-xL expression, we investigated whether DARPP-32 activated NF-xB signaling in MKN-28 cell model. Immunofluorescence assay data indicated that stable expression of DARPP-32
had no significant effect on NF-κB activation as depicted by nuclear NF-κBp65 staining (Supplementary Fig. S1). On the basis of this observation, we examined the possibility of DARPP-32 regulation of BCL-xL and cell survival through activation of Src/STAT3 signaling in MKN-28 cells. We first confirmed the role of this pathway in DARPP-32-mediated TRAIL resistance by treating cells with PP1, a specific Src inhibitor, alone or in combination with TRAIL. Indeed, clonogenic survival assay data indicated that after TRAIL treatment cell survival of control cells decreased by 70% relative to vehicle \( (P < 0.0001, \text{Fig. 4A and B}) \). In contrast, in response to TRAIL, cell survival of 2 clones of MKN-28/DARPP-32 cells decreased by approximately 20% relative to vehicle \( (P < 0.05, \text{Fig. 4A and B}) \). However, the protective effect of DARPP-32 was completely reversed when the cells were treated with TRAIL in combination with PP1, as cell survival was reduced to the level of TRAIL-treated control cells \( (P < 0.0001, \text{Fig. 4A and B}) \). Of note, combining PP1 with TRAIL had no significant effect on survival as compared with treatment with TRAIL alone in control cells \( (\text{Fig. 4A and B}) \). These data indicated that the Src tyrosine kinase activity contributes to the prosurvival effect of DARPP-32 in MKN-28 cells.

In parallel, we examined BCL-xL mRNA and protein expression after treatment with vehicle, TRAIL, TRAIL in combination with PP1, or PP1 in MKN-28/DARPP-32#1 cells or MKN-28/pCDNA3 control cells. Quantitative real-time PCR and Western blot analysis showed that BCL-xL mRNA \( (P < 0.01, \text{Fig. 4C}) \) and protein \( (\text{Fig. 4D}) \) levels were significantly higher in DARPP-32–expressing cells than control cells. In addition, treatment with TRAIL sustained DARPP-32–induced increase in BCL-xL mRNA and protein levels relative to control cells \( (\text{Fig. 4C and D}) \). In contrast, TRAIL in combination with PP1 abrogated the effect of DARPP-32 and decreased BCL-xL expression to approximately the levels of TRAIL-treated control cells \( (\text{Fig. 4C and D}) \). BCL-xL mRNA and protein expression levels directly correlated with the cell survival data shown in \( \text{Fig. 4A and B} \). In fact, Western blot analysis data indicated that cleavage of caspase-3 was induced by TRAIL in control cells but not in DARPP-32–expressing cells \( (\text{Fig. 4D}) \). Confirming the prosurvival role of BCL-xL in DARPP-32–expressing cells, TRAIL in combination with PP1 induced cleavage of caspase-3 \( (\text{Fig. 4C}) \). Interestingly, although treatment with PP1 alone induced a significant decrease in BCL-xL mRNA and protein levels relative to vehicle in DARPP-32–expressing cells \( (\text{Fig. 4C and D}) \), cell survival was not affected as the cells were not challenged by TRAIL \( (\text{Fig. 4A and B}) \). In line with these results, Western blot analysis showed that treatment with PP1 alone failed to induce cleavage of caspase-3 \( (\text{Fig. 4C}) \).

We next examined the role of DARPP-32 in regulating Src/STAT3 signaling pathway. Western blot analysis data indicated that DARPP-32–induced upregulation of BCL-xL...
expression was associated with a significant increase in p-Src (Y416) and p-STAT3 (Y705) protein levels as compared with control (Fig. 4D). In addition, we showed that treatment with PP1 reversed the effects of DARPP-32 on Src/STAT3 signaling and BCL-xL expression (Fig. 4D). Together, these data showed that the upregulation of BCL-xL via the Src/STAT3 signaling mediates the prosurvival function of DARPP-32 in MKN-28 cells.

DARPP-32 suppresses TRAIL-induced downregulation of NF-κB activity and FLIP(S) expression

We have shown that DARPP-32 upregulated the prosurvival BCL-xL expression and blocked TRAIL-induced activation of caspases through regulation of Src/STAT3 signaling in gastric cancer cells. It is well established that BCL-xL expression inhibits the intrinsic apoptosis pathway through blocking activation of caspase-9 in response to multiple apoptotic stimuli. Interestingly, our cell models indicated that DARPP-32 blocked TRAIL-induced activation of caspase-8 in addition to caspase-9 and -3. Therefore, we next tested our hypothesis that DARPP-32 may regulate the NF-κB after TRAIL treatment, thus explaining the suppression of TRAIL-induced activation of caspase-8 (Fig. 2B). We could not detect any significant effect on nuclear localization of NF-κBp65 in absence of TRAIL treatment (Supplementary Fig. S1).
However, the results were in agreement with our assumption and TRAIL treatment induced a decrease of approximately 50% in nuclear NF-κBp65 in MKN-28/pcDNA3 control cells as compared with MKN-28/DARPP-32 cells (P < 0.001; Fig. 5A). Accordingly, DARPP-32 expression was required to maintain the nuclear NF-κBp65 protein levels in response to TRAIL.

To further confirm the role of DARPP-32 in blocking TRAIL-induced inhibition of NF-κB, we used the luciferase reporter assay using a pNF-κB-Luc plasmid with TRAIL treatment in MKN-28 and MKN-45 cell models. The results indicated that NF-κB luciferase activity was 2.5- to 3-fold lower in MKN-28/pcDNA3 control cells than in MKN-28/DARPP-32 cells (P < 0.01; Fig. 5B). In addition, knocking down endogenous DARPP-32 in MKN-28 cells (treatment with 200 ng/mL TRAIL for 24 h) was required to maintain the nuclear NF-κBp65 expression, as compared with MKN-28/DARPP-32 cells (Fig. 5B). The quantitative results showed that treatment with TRAIL led to a decrease of approximately 50% in nuclear NF-κBp65 in control cells relative to DARPP-32-expressing cells (P < 0.0001). B, the luciferase reporter assay using a pNF-κB-Luc plasmid in MKN-28/pcDNA3, MKN-28/DARPP-32, MKN-45/control-shRNA, and MKN-45/DARPP-32-shRNA cells. After treatment with 200 ng/mL TRAIL for 24 hours, luciferase activity was 2.5- to 3-fold lower in MKN-28 control cells than in DARPP-32-expressing MKN-28 cells (P < 0.01). Knocking down endogenous DARPP-32 in MKN-45 cells and treatment with TRAIL resulted in a 3-fold decrease in luciferase activity relative to control cells (P < 0.0001). C, left, MKN-28/pcDNA3 and MKN-28/DARPP-32 cells were treated with vehicle or 200 ng/mL TRAIL for 24 hours. Western blot analysis of NF-κBp65 expression indicated that, unlike DARPP-32-expressing cells, treatment of control cells with TRAIL induced cleavage of the p65 protein; right, MKN-45/control-shRNA and MKN-45/DARPP-32-shRNA cells were treated with vehicle or TRAIL, as in C. Immunoblotting data showed that knocking down endogenous DARPP-32 significantly enhanced TRAIL-induced cleavage of the p65 protein as opposed to control cells. D, MKN-28 cells were treated with vehicle, 200 ng/mL TRAIL alone or in combination with 10 μmol/L z-VAD-fmk inhibitor for 24 hours. Western blot analysis results indicated that treatment with TRAIL alone induced activation of caspases and cleavage of p65. However, TRAIL in combination with z-VAD-fmk failed to activate caspases and cleave the p65 protein. Downregulation of FLIP(L) was correlated with TRAIL-induced activation of caspases and cleavage of p65. Gel loading was normalized for equal β-actin. Results are representative of 3 experiments and shown as the mean ± SEM. Significance of difference was calculated using the Student t-test analysis.

Figure 5. DARPP-32 abrogation of TRAIL-induced inhibition of NF-κB is associated with blocking caspase-dependent cleavage of NF-κBp65 protein. A, MKN-28/DARPP-32 and MKN-28-pcDNA3 cells were treated with vehicle or 200 ng/mL TRAIL for 24 hours. Cells were subjected to immunofluorescence with anti-NF-κBp65 antibody and a secondary antibody conjugated to FITC. DAPI was used as a nuclear counterstain. Left, representative immunofluorescence images of cells after treatment with TRAIL showing localization of NF-κBp65. Right, the quantitative results showed that treatment with TRAIL led to a decrease of approximately 50% in nuclear NF-κBp65 in control cells relative to DARPP-32-expressing cells (P < 0.0001). B, the luciferase reporter assay using a pNF-κB-Luc plasmid in MKN-28/pcDNA3, MKN-28/DARPP-32, MKN-45/control-shRNA, and MKN-45/DARPP-32-shRNA cells. After treatment with 200 ng/mL TRAIL for 24 hours, luciferase activity was 2.5- to 3-fold lower in MKN-28 control cells than in DARPP-32-expressing MKN-28 cells (P < 0.01). Knocking down endogenous DARPP-32 in MKN-45 cells and treatment with TRAIL resulted in a 3-fold decrease in luciferase activity relative to control cells (P < 0.0001).
down endogenous DARPP-32 in MKN-45 cells and treatment with TRAIL led to a 3-fold decrease in NF-κB luciferase activity relative to control cells (P < 0.0001; Fig. 5B). In an attempt to identify the mechanism by which TRAIL inhibited NF-κB activity and how DARPP-32 abrogated this effect, we evaluated NF-κBp65 protein expression in MKN-28 and MKN-45 cells. Surprisingly, the Western blot analysis results showed that TRAIL-induced cleavage of the p65 protein produced a lower molecular weight, which was prominent in MKN-28/pcDNA3 control cells (Fig. 5C). In contrast, there was no apparent cleavage of the NF-κBp65 protein relative to control cells (Fig. 5C). Knocking down endogenous DARPP-32 with shRNA in MKN-45 cells significantly enhanced TRAIL-induced cleavage of the p65 protein relative to control cells (Fig. 5C). These results suggest a direct correlation between TRAIL-induced cleavage of the p65 protein and NF-κB activity.

We also found that TRAIL-induced cleavage of the p65 protein was dependent on the activation of caspases and was associated with a significant decrease in the protein expression of FLIP(S), a transcriptional target of NF-κB, in MKN-28 cells (Fig. 5D). Of note, TRAIL had no significant effect on the expression of BCL-xL, another transcriptional target of NF-κB, in MKN-28 cells (Fig. 5D). We investigated the role of the extrinsic apoptosis pathway in mediating the effects of TRAIL in MKN-28/DARPP-32 cells (Fig. 5C). In MKN-28/DARPP-32 cells, the z-VAD-fmk inhibitor blocked TRAIL-induced activation of caspases and cleavage of the p65 protein (Fig. 5D). These results showed that TRAIL-induced cleavage of the p65 protein was dependent on activation of caspases.

Figure 6. DARPP-32 blocks TRAIL-induced downregulation of FLIP expression. A, MKN-28/pcDNA3 and MKN-28/DARPP-32 cells were treated with vehicle or 200 ng/mL TRAIL for 24 hours. Western blot analysis results showed that treatment with TRAIL induced a significant decrease in protein expression of FLIP(S) but not FLIP(L) in control cells. In contrast, TRAIL failed to affect both FLIP(S) and FLIP(L) protein levels in DARPP-32-expressing cells. B, qRT-PCR results indicated that TRAIL significantly decreased mRNA level of FLIP(S) in control cells as opposed to DARPP-32-expressing cells (P < 0.05). Overall, TRAIL had no significant effect on FLIP(L) mRNA expression in all cells. C, MKN-45/control-shRNA and MKN-45/DARPP-32-shRNA cells were treated with vehicle or 200 ng/mL TRAIL for 24 hours. Western blot analysis data indicated that knocking down endogenous DARPP-32 enhanced TRAIL-induced downregulation of FLIP(S) without affecting FLIP(L) protein expression. D, qRT-PCR results showed that knockdown of endogenous DARPP-32 in combination with TRAIL led to a 2-fold decrease in FLIP(S) mRNA level as compared with control cells (P < 0.05). FLIP(L) mRNA levels were not affected in all cells. E, a schematic representation: DARPP-32 inhibits TRAIL-induced apoptosis through regulating both the intrinsic and extrinsic apoptosis pathways in gastric cancer cells.
whether DARPP-32 could block TRAIL-induced downregulation of FLIP expression in MKN-28 and MKN-45 cells. The Western blot analysis results indicated that TRAIL induced a significant decrease in protein expression in FLIP(L) but not FLIP(S) in MKN-28 cells (Fig. 6A). Conversely, TRAIL failed to affect the protein levels of both FLIP(S) and FLIP(L) in MKN-28/DARPP-32 cells (Fig. 6A). The qRT-PCR results revealed that TRAIL significantly downregulated FLIP(S) mRNA expression in MKN-28/pcDNA3 control cells, but had no significant effects on both FLIP(S) and FLIP(L) mRNA levels in MKN-28/DARPP-32 cells (Fig. 6B). We next investigated whether TRAIL-induced downregulation of FLIP(S) expression was time dependent in MKN-28 cells. The qRT-PCR data showed that the effect of TRAIL was evident as early as 2 hours after treatment (Supplementary Fig. S2). In the MKN-45 cell model, we showed that knocking down endogenous DARPP-32 significantly enhanced TRAIL-induced downregulation of protein expression of FLIP(S) without affecting the FLIP(L) protein level (Fig. 6C). Similarly, the qRT-PCR results showed that knocking down endogenous DARPP-32 significantly decreased the mRNA level of FLIP(S) (P < 0.05), but not FLIP(L) in response to TRAIL in MKN-45 cells (Fig. 6D). Together, these results showed that DARPP-32 blocked TRAIL-induced cleavage and inhibition of NF-κB activity, which was associated with caspase-dependent cleavage of the p65 protein. Furthermore, DARPP-32 suppressed TRAIL-induced downregulation of FLIP(S), thereby counteracting TRAIL-mediated activation of caspase-8 in gastric cancer cells.

Discussion
Recombinant TRAIL proteins and TRAIL receptor agonist antibodies have been tested in clinical trials, showing promising antitumor activities with mild side effects (reviewed by ref. 26). Although TRAIL could specifically induce tumor cell death, there are many cancer cells that are TRAIL resistant (27). Resistance to TRAIL is a major clinical challenge that leads to failure of treatment, poor prognosis, and reduced cancer patient survival. Little is known about the mechanisms of TRAIL resistance in gastric cancer. In this study, we show a novel signaling mechanism for TRAIL resistance mediated by DARPP-32, which is overexpressed in more than two-thirds of adenocarcinomas of the stomach and esophagus (21, 22). Given the fact that DARPP-32 is also overexpressed in several other malignancies such as breast and colon cancers, findings from this study provide novel insights into understanding the complexity of TRAIL resistance, which can guide the development of new approaches to circumvent apoptotic blockades or suppress survival signals.

The findings in this study showed that DARPP-32 promoted cell survival and blocked apoptosis in response to treatment with TRAIL. In an attempt to uncover DARPP-32–mediated signaling mechanism, we found that overexpression of DARPP-32 blocked the TRAIL-induced release of cytochrome c and activation of caspase-8, -9, and -3. Conversely, knockdown of endogenous DARPP-32 enhanced TRAIL-induced activation of caspase-8, -9, and -3. These results suggested that DARPP-32 regulates both the death receptor–mediated extrinsic and the mitochondrial intrinsic apoptosis pathways. Cytochrome c release occurs in response to several apoptotic stimuli and it is a prerequisite for caspase-9 activation (8, 28). This implies that DARPP-32–mediated suppression of TRAIL-induced activation of the intrinsic apoptosis pathway might involve upregulated expression of the prosurvival BCL2 family members. Overexpression of BCL-xl and BCL2 has been shown to protect type II cells against TRAIL-mediated apoptosis (13). The fact that DARPP-32 upregulated BCL-xl expression at the protein and mRNA levels through activation of Src/STAT3 signaling, provides a plausible explanation for suppression of the intrinsic apoptosis. Accordingly, DARPP-33–induced upregulation of BCL-xl may be responsible for blocking TRAIL-mediated cytochrome c release, activation of caspase-9, and subsequently caspase-3. Our finding is in agreement with previous reports that have shown that BCL-xl promotes TRAIL resistance through increased activation of the intrinsic mitochondrial apoptosis pathway in cancer cells (13, 14). Several reports show that transcription factors such as NF-κB and STAT3 could upregulate BCL-xl in cancer cells (reviewed by ref. 15). In our MKN-28 cell model, we showed that DARPP-32–induced BCL-xl expression involved activation of STAT3 transcription factor, and excluded a role for NF-κB as a mediator.

Caspase-8 is normally associated with the extrinsic apoptosis pathway, whereby its activation is regulated by the proapoptotic molecules (TRAIL, FasL, TNF-α) or by a negative feedback loop mediated by NF-κB and FLIP (16, 29). NF-κB activation prevents apoptosis by induction of antiapoptotic proteins such as BCL-xl, cIAP1&2, XIAP, and FLIP (29–33). We hypothesized that TRAIL inhibits NF-κB/FLIP signaling, thereby amplifying apoptosis through increased activation of caspase-8. To uncover the signaling mechanism by which DARPP-32 blocks TRAIL-induced activation of caspase-8, we investigated whether the NF-κB/FLIP negative feedback loop was regulated by DARPP-32 in response to TRAIL.

The results indicated that TRAIL-induced apoptosis was accompanied by the inhibition of NF-κB activity, cleavage of NF-kBp65 protein, and downregulation of FLIP(S) expression. We further confirmed that TRAIL-induced cleavage of the p65 protein and downregulation of FLIP(S) was dependent on active caspases. This is consistent with the notion that activation of caspases can block NF-κB activity and downstream signaling by inducing cleavage of NF-kBp65 protein (34, 35). Caspase-mediated cleavage of NF-kBp65 does not simply lead to its inactivation but converts it into a dominant-negative variant of its noncleaved p65 protein (35). Although DARPP-32 did not significantly activate NF-κB in basal conditions, it suppressed activation of caspases and maintained NF-κB activity and FLIP(S) expression by blocking the cleavage of the NF-kBp65 protein in response to TRAIL treatment. FLIP proteins consist of the short form [FLIP(S)], which contains 2 death effector domains, and the long form [FLIP(L)].
which contains an additional pseudo-caspase domain. Both FLIP(S) and FLIP(L) can interfere with the FADD-caspase-8 interaction, thus blocking the recruitment and activation of caspase-8 during TRAIL-mediated apoptosis (36). Our results, showing TRAIL-dependent downregulation of FLIP(S) but not FLIP(L), were consistent with the study on the role of FLIP(S) in modulating TRAIL-induced apoptosis in cancer (18). Although we cannot rule out other potential mechanisms by which DARPP-32 suppresses TRAIL-induced activation of caspase-8, our findings strongly suggest that DARPP-32 blocked activation of caspase-8 and promoted TRAIL resistance through the inhibition of the mitochondrial apoptosis pathway and maintaining activation of the NF-κB-FLIP feedback loop based on the following lines of evidence: (1) DARPP-32 upregulated the prosurvival BCL-xL expression through activation of Src/STAT3 signaling; (2) DARPP-32 inhibited TRAIL-mediated cytochrome c release; (3) DARPP-32 blocked TRAIL-induced activation of caspase-8, -9, and -3; (4) DARPP-32 suppressed TRAIL-mediated inhibition of NF-κB and caspase-dependent degradation of the p65 protein; and (5) DARPP-32 inhibited TRAIL-induced downregulation of FLIP(S) expression.

In conclusion, our findings indicate that DARPP-32 promotes TRAIL resistance and suggest that the frequent expression of DARPP-32 underscores a clinical challenge in gastric cancers. The capacity of DARPP-32 to block the mitochondrial apoptosis pathway by upregulating BCL-xL expression, and suppress TRAIL-induced activation of caspase-8 through maintaining the activity of the NF-κB-FLIP feedback loop can modulate the response to TRAIL and possibly other chemotherapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed. The contents of this work are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute or Vanderbilt University.

Authors’ Contributions

Conception and design: A. Belkhiri, M. Souotto, W. El-Rifai. Development of methodology: A. Belkhiri, M. Souotto, W. El-Rifai. Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Belkhiri, W. El-Rifai. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Belkhiri, M. Souotto, W. El-Rifai. Writing, review, and/or revision of the manuscript: A. Belkhiri, M. Souotto, W. El-Rifai. Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Zhu, Z. Chen, W. El-Rifai. Study supervision: W. El-Rifai.

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References


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## Resistance to TRAIL Is Mediated by DARPP-32 in Gastric Cancer


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