

Targeting XIAP Bypasses Bcl-2–Mediated Resistance to TRAIL and Cooperates with TRAIL to Suppress Pancreatic Cancer Growth *In vitro* and *In vivo*

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Abstract

Resistance to apoptosis is a hallmark of pancreatic cancer, a leading cause of cancer deaths. Therefore, novel strategies are required to target apoptosis resistance. Here, we report that the combination of X-linked inhibitor of apoptosis (XIAP) inhibition and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is an effective approach to trigger apoptosis despite Bcl-2 overexpression and to suppress pancreatic cancer growth *in vitro* and *in vivo*. Knockdown of XIAP by RNA interference cooperates with TRAIL to induce caspase activation, loss of mitochondrial membrane potential, cytochrome *c* release, and apoptosis in pancreatic carcinoma cells. Loss of mitochondrial membrane potential and cytochrome *c* release are extensively inhibited by a broad range of caspase-3 selective caspase inhibitor and by RNAi-mediated silencing of caspase-3, indicating that XIAP inhibition enhances TRAIL-induced mitochondrial damage in a caspase-3–dependent manner. XIAP inhibition combined with TRAIL even breaks Bcl-2–imposed resistance by converting type II cells that depend on the mitochondrial contribution to the death receptor pathway to type I cells in which TRAIL-induced activation of caspase-3 and caspase-9 and apoptosis proceeds irrespective of high Bcl-2 levels. Most importantly, XIAP inhibition potentiates TRAIL-induced antitumor activity in two preclinical models of pancreatic cancer *in vivo*. In the chicken chorioallantoic membrane model, XIAP inhibition significantly enhances TRAIL-mediated apoptosis and suppression of tumor growth. In a tumor regression model in xenograft-bearing mice, XIAP inhibition acts in concert with TRAIL to cause even regression of established pancreatic carcinoma. Thus, this combination of XIAP inhibition plus TRAIL is a promising strategy to overcome apoptosis resistance of pancreatic cancer that warrants further investigation. [Cancer Res 2008;68(19):7956–65]

Introduction

Despite intensive protocols, treatment resistance of pancreatic cancer considerably contributes to the poor prognosis of this disease (1, 2). Evasion of apoptosis (programmed cell death) can lead to treatment failure in pancreatic cancer because intact apoptosis pathways are crucial for therapy-induced cytotoxicity (3, 4). Thus, new strategies that target apoptosis resistance are required to improve the dismal prognosis of pancreatic cancer patients.

Two principal apoptosis pathways eventually lead to activation of caspases, i.e., the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (4, 5). Ligation of death receptors, e.g., agonistic tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors by TRAIL, results in caspase-8 activation that cleaves effector caspase-3 either directly or indirectly via the mitochondrial route (6). In the mitochondrial pathway, effector caspases are activated upon the release of apoptogenic factors such as cytochrome *c* or second mitochondria-derived activator of caspase (Smac)/direct inhibitor of apoptosis protein (IAP) binding protein with low PI (DIABLO) from mitochondria into the cytosol (7). Cytochrome *c* triggers caspase-3 activation via formation of the cytochrome *c*/caspase-9/Apaf-1 apoptosome complex, whereas Smac/DIABLO promotes apoptosis by neutralizing IAPs (7). The mitochondrial contribution to death receptor signaling is of special relevance in type II cells, for example pancreatic carcinoma, which rely on the apoptotic function of mitochondria for full activation of caspase-3 and apoptosis (8–10). In type I cells, caspase-8 is activated upon death receptor ligation at the death-inducing signaling complex (DISC) in quantities sufficient to directly activate caspase-3 (8). Accordingly, blocking the mitochondrial pathway, e.g., by overexpression of Bcl-2, inhibits death receptor-induced apoptosis in type II but not in type I cells (8).

Among the death receptor ligands, TRAIL is considered as the most promising candidate for medical application because it triggers apoptosis preferentially in cancer cells with no or minimal toxicity to nonmalignant human cells (11). However, many human cancers including pancreatic carcinoma display primary or acquired resistance to TRAIL (9, 12). X-linked inhibitor of apoptosis (XIAP) may contribute to resistance to TRAIL because XIAP blocks apoptosis at a key node of the apoptotic machinery through inhibition of caspase-3 and caspase-9 (13, 14). Searching for strategies to restore sensitivity to TRAIL-induced apoptosis in pancreatic cancer, we investigated XIAP as a molecular target *in vitro* and *in vivo*.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Materials and Methods

Cell culture and reagents. Pancreatic carcinoma cell lines PancTu1 and PaTu11 were established from distinct human poorly differentiated pancreatic adenocarcinoma specimens (15, 16) and cultured in RPMI1640 (Life Technologies, Inc.) as described (12). TRAIL was purchased from R&D Systems, Inc., and all chemicals from Sigma unless indicated otherwise.

Transduction and transfection. To knockdown XIAP, shRNA targeting XIAP sequence 241 (gtggtagtctctgtttcagc; ref. 17) corresponding to mRNA sequence were cloned into pRETRO-SUPER as previously described (12). A sequence with no corresponding part in the human genome (gatcatgtagatagcctca) was used as control. Stable bulk cultures were generated by selection with 2 µg/mL puromycin and clones by limited dilutions. For RNAi-mediated knockdown of caspase-3, cells were transiently transfected twice with 0.06 nmol caspase-3 Stealth or control RNAi (Invitrogen) using TransMessenger (Qiagen). For Bcl-2 overexpression, cells were transduced with pMSCV vector containing mouse Bcl-2 or empty vector using the packaging cell line PT67 (BD Biosciences). Stable cell lines were selected by 10 µg/mL blasticidin (Invitrogen).

Western blot analysis. Western blot analysis was performed as described previously (18) using the following antibodies: mouse anti-caspase-8 (ApoTech Corporation), rabbit anti-caspase-3 (Cell Signaling), rabbit anti-caspase-9, mouse anti-caspase-2, mouse anti-caspase-7,

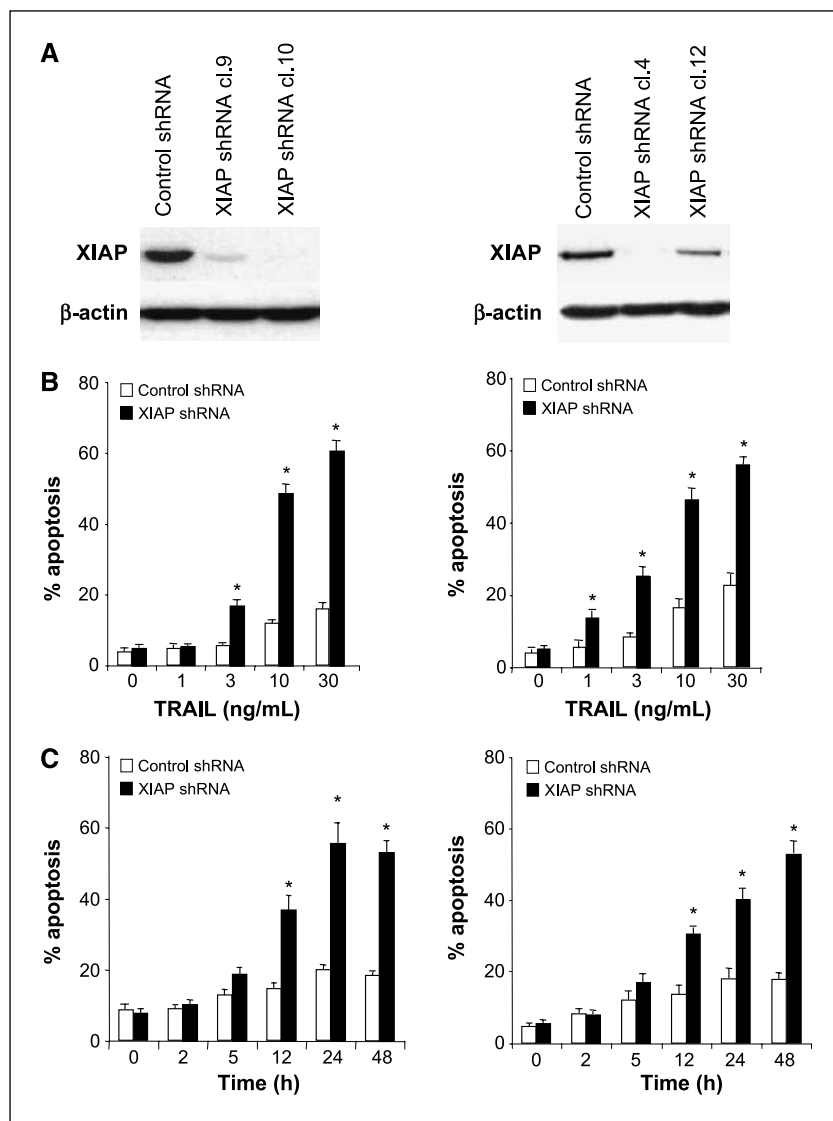
mouse anti-XIAP from BD Biosciences, mouse anti-mouse Bcl-2 (Zymed), or mouse anti-β-actin (Sigma) followed by goat-anti-mouse IgG or goat-anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Enhanced chemiluminescence was used for detection (Amersham Bioscience).

Bax immunoprecipitation. Cells were lysed for 25 min in CHAPS lysis buffer [10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1% CHAPS]. Five hundred micrograms of protein were incubated with 4 µg mouse anti-Bax antibody (6A7; Sigma) overnight at 4°C on rotator followed by addition of 10 µL Dynabeads Pan Mouse IgG (Dako) and incubated for 2 h at 4°C on rotator. Beads were washed thrice with CHAPS lysis buffer. Immune complexes were analyzed by Western blotting using rabbit anti-BaxNT antibody (Upstate Biotechnology).

Determination of apoptosis and cell viability. Apoptosis was determined by Annexin-V staining or by fluorescence-activated cell sorting (FACS) analysis of DNA fragmentation of propidium iodide-stained nuclei as described (12). Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (Roche Diagnostics).

Determination of mitochondrial membrane potential and cytochrome c release. CMXRos (1 µmol/L; Molecular Probes) was used to measure the mitochondrial transmembrane potential. Cells were incubated for 30 min at 37°C in the presence of the fluorochrome and immediately

Figure 1. XIAP knockdown sensitizes pancreatic carcinoma cells for TRAIL-induced apoptosis. *A*, PancTu1 (*left*) and PaTu11 (*right*) pancreatic carcinoma cells were infected with vector containing control shRNA or XIAP shRNA and different clones (*cl.*) were generated. Protein expression of XIAP and β-actin was assessed by Western blotting. A representative experiment of three independent experiments is shown. *B* and *C*, PancTu1 (*left*) and PaTu11 (*right*) cells infected with control shRNA or XIAP shRNA (XIAP shRNA clone 10 for PancTu1 cells, XIAP shRNA clone 4 for PaTu11 cells) were treated with indicated concentrations of TRAIL for 48 h (*B*) or with 10 ng/mL TRAIL for indicated times (*C*). Apoptosis was determined by Annexin-V staining. *Columns*, of three independent experiments; *bars*, SE; *, *P* < 0.01 comparing XIAP shRNA to control shRNA.



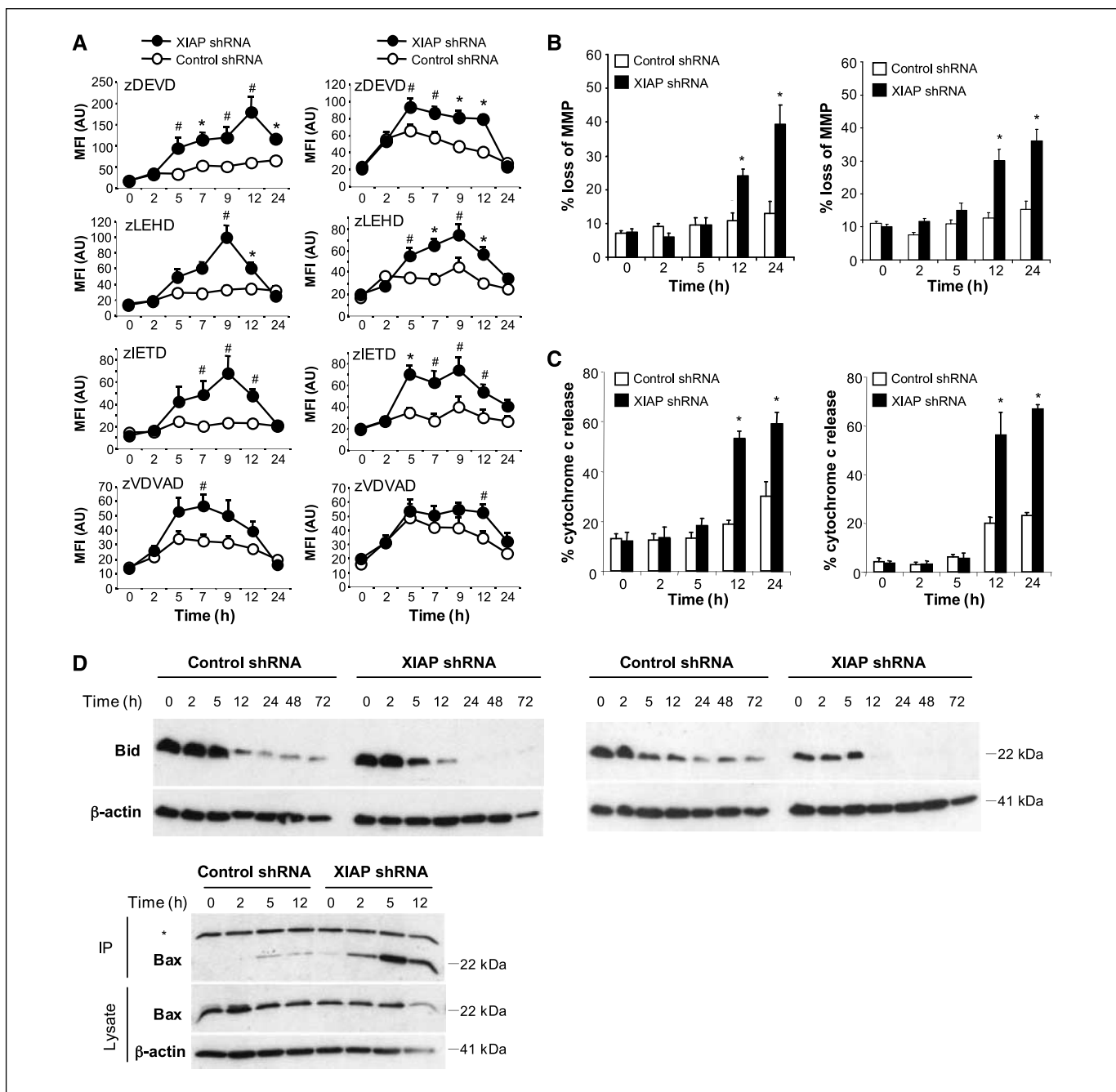


Figure 2. XIAP knockdown enhances TRAIL-induced activation of caspases and mitochondrial perturbations. PancTu1 (left) and PaTull (right) cells infected with control shRNA or XIAP shRNA were treated with 10 ng/mL TRAIL for indicated times. **A**, caspase activity was determined by FACS analysis using rhodamine-conjugated caspase substrates (zDEVD for caspase-3/7, zLEHD for caspase-9, zIETD for caspase-8, and zVDVAD for caspase-2); mean fluorescence intensity (MFI) is shown in arbitrary units (AU). **B** and **C**, mitochondrial transmembrane potential (**B**) and cytochrome *c* release (**C**) were assessed by FACS analysis. **D**, Bid cleavage was analyzed by Western blotting (top) in PancTu1 (left) and PaTull cells (right). Bax conformational change was determined in PancTu1 cells by immunoprecipitation (IP) using an active conformation specific Bax antibody (bottom). Expression levels of Bax in the corresponding lysates served as controls. *, light chains detected in immunoprecipitation samples. **A** to **C**, mean + SE of three independent experiments performed in triplicate are shown; #, $P < 0.05$; *, $P < 0.01$ comparing XIAP shRNA to control shRNA. **D**, a representative experiment of three independent experiments is shown.

analyzed by flow cytometry. Cytochrome *c* release was determined in permeabilized cells using mouse anti-cytochrome *c* monoclonal antibody (BD Biosciences) as described (19).

Determination of caspase activity. Caspase activity was determined in living nonfixed, nonlysed cells as described (20) using caspase substrates conjugated to rhodamine R110: zDEVD-R110 for caspase-3/7, zVDVAD-R110 for caspase-2, zIETD-R110 for caspase-8, and zLEHD-R110 for caspase-9, all

from Molecular Probes. Caspase inhibitors (zVAD.fmk, zDEVD.fmk) were purchased from Bachem.

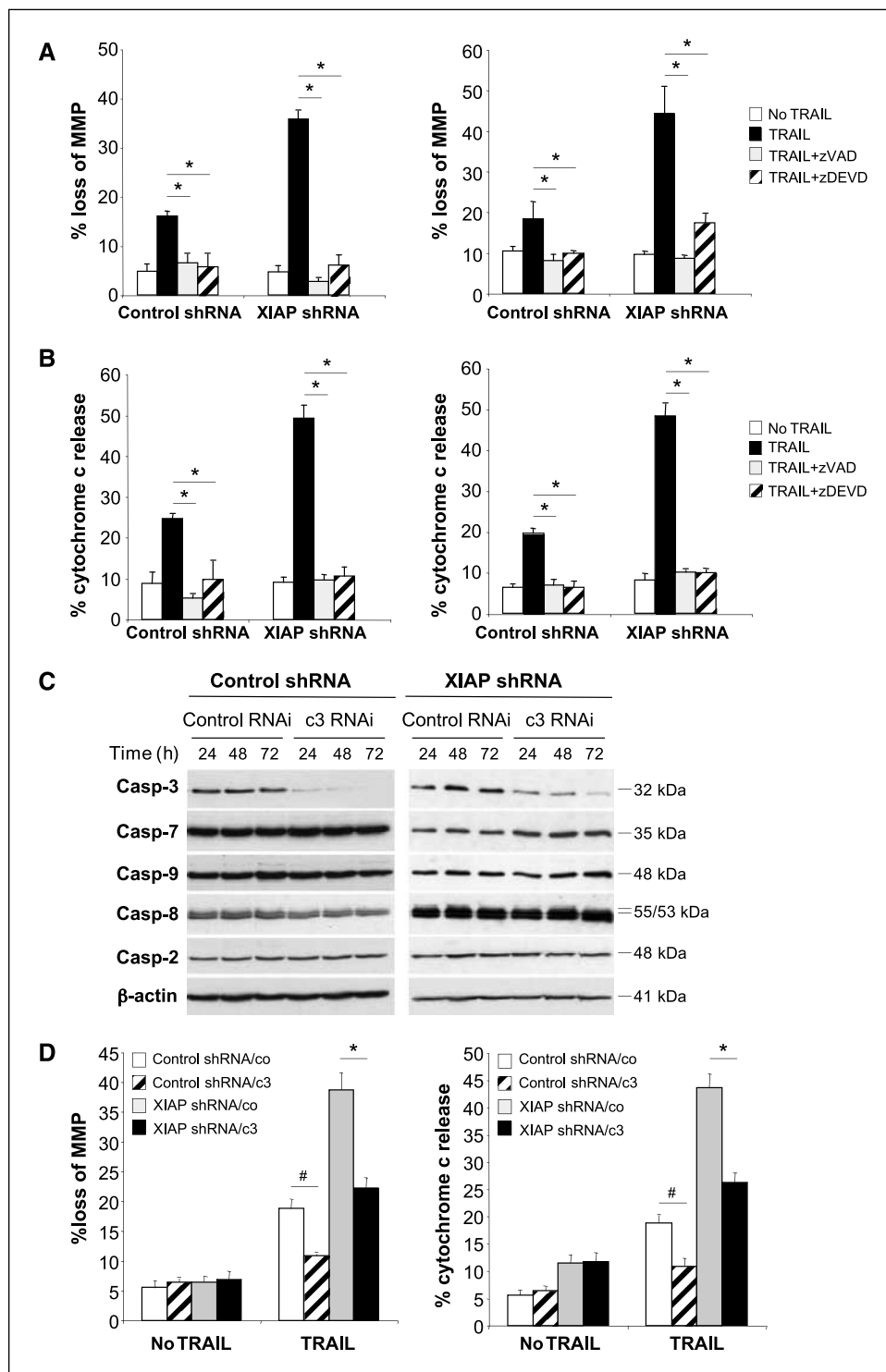
Chorioallantoic membrane assay. Chorioallantoic membrane (CAM) assay was done as described previously (21). Briefly, 1×10^6 tumor cells were resuspended in 25 μ L serum-free medium and 25 μ L Matrigel Matrix (BD Biosciences) and implanted on fertilized chicken eggs on day 8 of incubation. Tumors were topically treated with 0.7 ng TRAIL in 15 μ L PBS

daily for 3 d, sampled with the surrounding CAM 4 d after seeding, fixed in 4% paraformaldehyde, paraffin embedded, cut in 5- μ m sections, and analyzed by immunohistochemistry.

Xenograft mouse model. PancTu1 cells (1×10^6) transduced with control or XIAP shRNA were s.c. inoculated at left and right dorsal sides, respectively, of 6-wk-old female NMRI/nu-nu mice (Janvier Laboratories). Treatment started 1 wk after tumor cell inoculation by daily i.p. injection of isoleucine zipper-TRAIL (22) for 10 consecutive d, TRAIL maintenance buffer served as control. Tumor size was assessed twice weekly by caliper; the tumor volume

was calculated according to the formula $0.5 \times L \times W \times T$ (L , length; W , width; T , thickness). Relative tumor growth ratio (treated versus control, T/C) was calculated by tumor volume in treated group divided by tumor volume in control group. At the end of experiments, tumors were recovered for histologic and pathologic analysis. Additional 3 mice were sacrificed 24 h after the second injection of TRAIL on day 10 of the experiment, and tumor tissue was analyzed by immunohistochemistry. Animal experiments were performed in accordance with relevant institutional and national regulations; research protocols were approved by relevant authorities.

Figure 3. XIAP knockdown promotes TRAIL-induced mitochondrial perturbations in a caspase-dependent manner. *A* and *B*, PancTu1 (*left*) and PaTu11 (*right*) cells infected with control shRNA or XIAP shRNA were treated with 10 ng/mL TRAIL for 24 h in the presence or absence of 25 μ mol/L zVAD.fmk or 25 μ mol/L zDEVD.fmk. Mitochondrial transmembrane potential (*A*) and cytochrome *c* release (*B*) were assessed by FACS analysis. *C* and *D*, PancTu1 cells infected with control or XIAP shRNA were transiently transfected with caspase-3 (*c3*) or control siRNA (*co*). *C*, expression of caspase-3, caspase-7, caspase-9, caspase-8, caspase-2, and β -actin was determined by Western blotting. *D*, mitochondrial transmembrane potential (*left*) and cytochrome *c* release (*right*) after treatment with 25 ng/mL TRAIL for 12 h were assessed by FACS analysis. Columns, mean of three independent experiments performed in triplicate; bars, SE (*A*, *B*, and *D*); #, $P < 0.05$; *, $P < 0.01$ comparing XIAP shRNA to control shRNA. *C*, a representative experiment of two independent experiments is shown.



Immunohistochemistry. Immunohistochemical staining was performed with 1:1 hematoxyline and 0.5% eosin, anti-Ki-67 (Dako) or anticleaved caspase-3 antibody (Biozol) as previously described (23). Apoptosis was assessed by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL; Roche Diagnostics) according to the manufacturer's instructions. Negative controls were performed by omitting the first step with primary antibody and yielded negative results. The percentage of positive cells was independently determined by two examiners.

Statistical analysis. Statistical significance was assessed by Student's *t* test or Mann-Whitney *U* test, where appropriate, using Winstat (R. Fitch Software) or SPSS (SPSS GmbH Software) software.

Results

XIAP knockdown sensitizes pancreatic carcinoma cells for TRAIL-induced apoptosis. To antagonize XIAP in pancreatic cancer, we generated clones of PancTu1 and PaTuII pancreatic carcinoma cells in which XIAP was substantially down-regulated by retroviral short-hairpin RNA (shRNA) vectors (Fig. 1A). Knockdown of XIAP significantly enhanced TRAIL-induced apoptosis in a concentration- and time-dependent manner (Fig. 1B and C; XIAP shRNA clone 10 for PancTu1 cells, XIAP shRNA clone 4 for PaTuII cells). Similar results were obtained in distinct clones with XIAP knockdown (Supplementary Fig. S1A) and also in another pancreatic carcinoma cell line, i.e., Colo357 (12). For subsequent experiments, XIAP shRNA clone 10 were used for PancTu1 cells and XIAP shRNA clone 4 for PaTuII cells. Apoptotic cell death was confirmed by analysis of DNA fragmentation and by typical morphologic features, e.g., nuclear condensation and fragmentation (data not shown). Furthermore, XIAP knockdown cooperated with TRAIL to suppress clonogenic survival (Supplementary Fig. S1B). By comparison, XIAP knockdown did not significantly alter chemotherapeutic drug-induced cytotoxicity in PancTu1 and PaTuII cells (Supplementary Fig. S1C). We also extended our study to another IAP protein, i.e., survivin (24). Knockdown of survivin significantly increased apoptosis without the addition of TRAIL and also acted in concert with TRAIL to induce apoptosis (Supplementary Fig. S2). Together, these findings show that inhibition of XIAP or survivin sensitizes pancreatic carcinoma cells to TRAIL-induced apoptosis.

XIAP knockdown enhances TRAIL-induced caspase activation and mitochondrial perturbations. Next, we explored molecular mechanisms responsible for the cooperative action of XIAP inhibition and TRAIL in pancreatic carcinoma cells. Analysis of caspase activity by enzymatic caspase assay revealed that XIAP knockdown significantly increased TRAIL-induced activation of caspase-3, caspase-9, caspase-8, and caspase-2 (Fig. 2A). Activation of caspases was also confirmed by detection of active caspase cleavage fragments by Western blotting (data not shown). Addition of the broad range caspase inhibitor zVAD.fmk extensively inhibited TRAIL-induced apoptosis in cells with XIAP knockdown (Supplementary Fig. S3), demonstrating that apoptosis occurs in a caspase-dependent manner.

Furthermore, we investigated whether XIAP inhibition has an effect on the mitochondrial pathway during TRAIL-induced apoptosis. XIAP knockdown significantly enhanced TRAIL-induced loss of mitochondrial membrane potential and cytochrome *c* release from mitochondria in a time-dependent manner (Fig. 2B and C). We also assessed Bid cleavage and Bax conformational change as possible links between caspase activation and mitochondrial injury. XIAP knockdown enhanced TRAIL-induced Bid cleavage as well as Bax activation as indicated by higher levels of conformationally active Bax in TRAIL-treated cells with XIAP

knockdown compared with control cells, whereas total levels of Bax protein were not up-regulated in cells with XIAP knockdown (Fig. 2D). Together, this shows that XIAP inhibition cooperates with TRAIL to trigger caspase activation and mitochondrial perturbations in pancreatic carcinoma cells.

XIAP knockdown promotes TRAIL-induced mitochondrial perturbations in a caspase-dependent manner. Because we observed an increase in caspase activity before mitochondrial perturbations in TRAIL-treated cells with XIAP knockdown (Fig. 2A–C), we asked whether caspases are involved in causing mitochondrial damage. To address this issue, we examined the effect of the broad range caspase inhibitor zVAD.fmk and the relatively specific caspase-3 inhibitor zDEVD.fmk on mitochondrial apoptogenic functions upon treatment with TRAIL. zVAD.fmk as well as zDEVD.fmk extensively blocked TRAIL-induced loss of mitochondrial membrane potential and cytochrome *c* release (Fig. 3A and B). In a second approach, we specifically knocked down caspase-3 by RNA interference because chemical inhibitors of caspases lack absolute substrate specificity (25). Control experiments showed that caspase-3 siRNA caused down-regulation of caspase-3 with no detectable effects on expression levels of caspase-7, caspase-9, caspase-8, and caspase-2 (Fig. 3C). Interestingly, silencing of caspase-3 significantly reduced TRAIL-induced loss of mitochondrial membrane potential and cytochrome *c* release (Fig. 3D). This indicates that XIAP inhibition promotes TRAIL-induced mitochondrial damage in a caspase-3-dependent manner.

XIAP knockdown overcomes Bcl-2-mediated resistance to TRAIL. Because pancreatic carcinoma cells have been reported to classify as type II cells that express antiapoptotic Bcl-2 family proteins at high levels and depend on the mitochondrial contribution to death receptor-induced apoptosis (9), we next asked whether blockade of the mitochondrial pathway impairs apoptosis induced by the combination of XIAP knockdown and TRAIL. To address this issue, we stably overexpressed Bcl-2 in cells with XIAP knockdown (Fig. 4A). Interestingly, XIAP knockdown enhanced TRAIL-induced cytotoxicity irrespective of Bcl-2 overexpression, whereas Bcl-2 overexpression conferred resistance to TRAIL in control cells with high XIAP expression (Fig. 4B). Also, XIAP knockdown increased TRAIL-induced cleavage of caspase-3 into the active p17/p12 cleavage fragments and cleavage of caspase-9 regardless of high Bcl-2 levels (Fig. 4C).

To elucidate whether XIAP knockdown bypasses the requirement of the mitochondrial pathway for TRAIL-induced apoptosis in Bcl-2-overexpressing cells or whether it alternatively breaks mitochondrial resistance, we examined variables of mitochondrial apoptosis that are controlled by Bcl-2 (26). Bcl-2 overexpression failed to inhibit loss of mitochondrial membrane potential and cytochrome *c* release in TRAIL-treated cells with XIAP knockdown, whereas it significantly reduced these mitochondrial perturbations in control cells with high XIAP expression (Fig. 4D). This set of experiments shows that XIAP knockdown converts type II pancreatic carcinoma cells, in which Bcl-2 confers protection against TRAIL, to type I cells in which TRAIL triggers activation of caspase-3 and caspase-9 and apoptosis regardless of high Bcl-2 levels.

XIAP knockdown enhances TRAIL-induced antitumor activity against pancreatic carcinoma *in vivo*. We then evaluated the antitumor activity of concomitant XIAP inhibition and TRAIL *in vivo* using the CAM model, an established *in vivo* tumor model (21, 27). To this end, PancTu1 cells transduced with XIAP or control shRNA were seeded on the CAM of chicken embryos, allowed to form tumors, and then treated with TRAIL. Importantly, XIAP

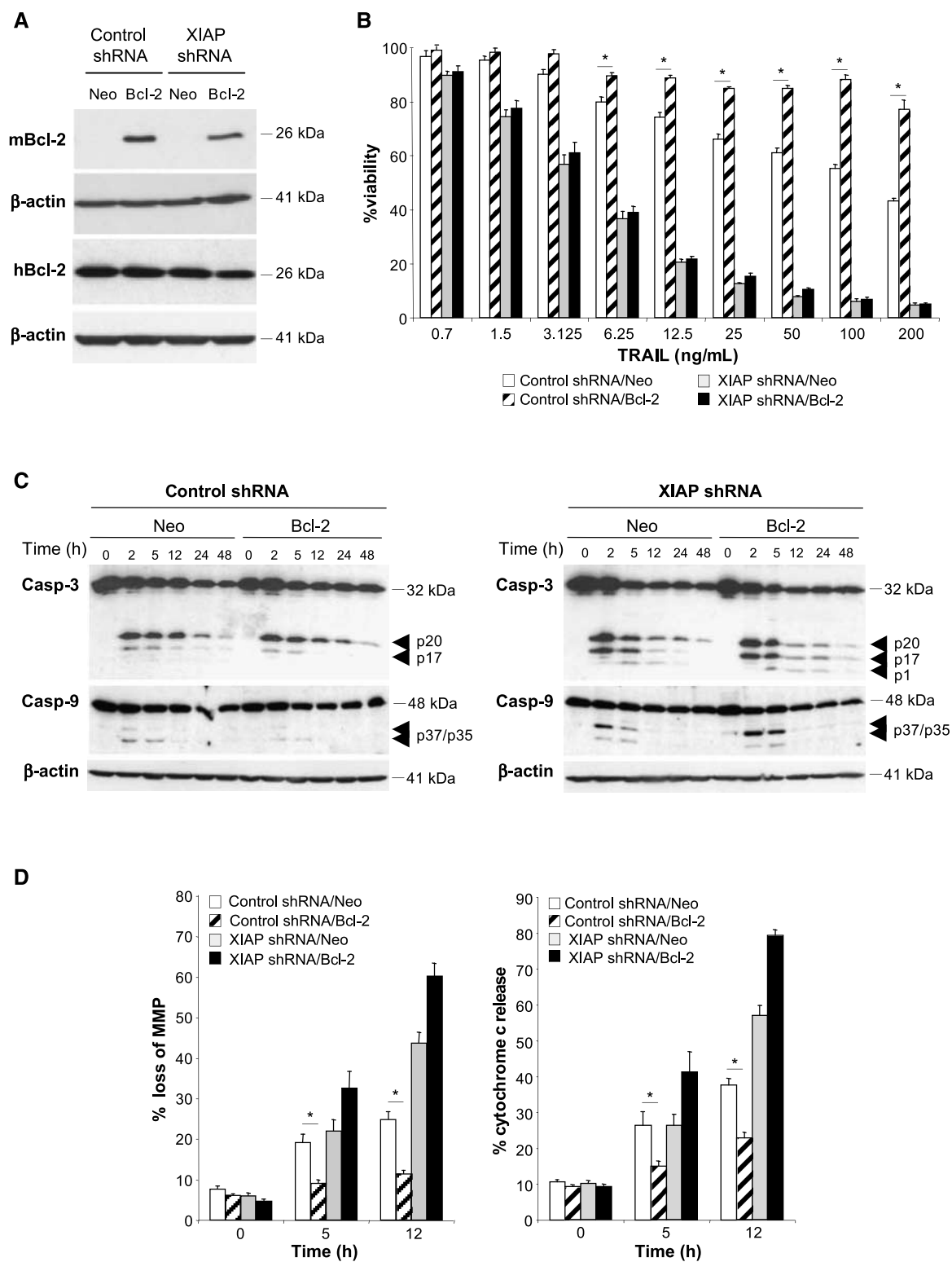


Figure 4. XIAP knockdown overcomes Bcl-2-mediated resistance to TRAIL. PancTu1 cells infected with control or XIAP shRNA were transfected with a vector containing mouse Bcl-2 or empty vector (*Neo*). **A**, expression levels of ectopically expressed mouse Bcl-2 (*mBcl-2*) or endogenous human Bcl-2 (*hBcl-2*) were determined by Western blotting. **B**, cell viability was determined after treatment with indicated concentrations of TRAIL for 72 h by MTT assay and expressed as percentage of untreated control. **C**, activation of caspase-3 and caspase-9 after treatment with 25 ng/mL TRAIL for indicated times was assessed by Western blotting; *arrowheads*, active cleavage fragments. **D**, mitochondrial transmembrane potential (*left*) and cytochrome *c* release (*right*) were determined by FACS analysis. *Columns*, mean of three independent experiments performed in triplicate; *bars*, SE (**B** and **D**); *, $P < 0.01$. **A** and **C**, representative experiments of three independent experiments are shown.

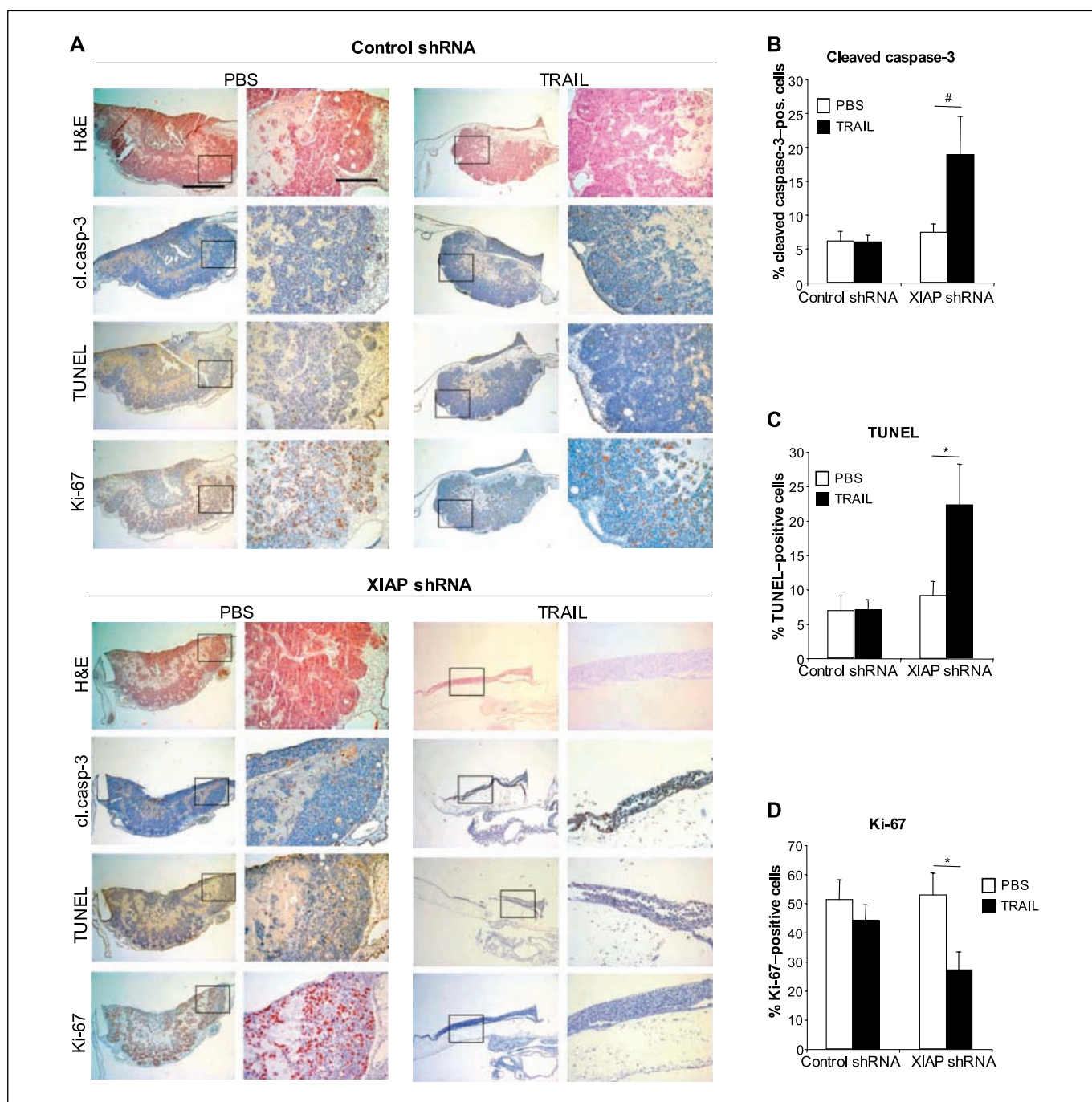


Figure 5. XIAP inhibition enhances TRAIL-induced antitumor activity against pancreatic carcinoma *in vivo*. PancTu1 cells infected with control or XIAP shRNA were seeded on the CAM of chicken embryos, allowed to establish, and treated for 3 d with 0.7 ng TRAIL or PBS. The CAM was excised on day 4, fixed, and stained with H&E, anti-Ki-67 antibody, TUNEL, or an antibody recognizing cleaved caspase-3. Representative pictures (A) and the percentage of cells positive for cleaved caspase-3 (B), TUNEL (C), and Ki-67 (D) are shown; bars, 800 μ m (left) and 200 μ m (right). Columns, mean of 12 samples per group of 2 independent experiments; bars, SE (B–D); #, $P < 0.05$; *, $P < 0.01$. Cleaved caspase pos., cleaved caspase positive.

knockdown together with TRAIL, but neither approach alone, suppressed the growth of pancreatic carcinoma *in vivo* (Fig. 5A). Immunohistochemical analysis of tumor tissue revealed that XIAP knockdown significantly increased TRAIL-induced caspase-3 activation and apoptosis and also acted in concert with TRAIL to suppress proliferation (Fig. 5B–D). This shows that XIAP inhibition cooperates with TRAIL to induce apoptosis and to inhibit growth of pancreatic cancer *in vivo*.

XIAP knockdown cooperates with TRAIL to cause regression of established pancreatic cancer in a xenograft mouse model. Finally, we assessed the antitumor activity of XIAP inhibition and TRAIL in a tumor regression model of pancreatic cancer in xenograft-bearing mice. In this model, PancTu1 cells transduced with control or XIAP shRNA were s.c. injected into the left and right dorsal sides of nude mice, respectively, and TRAIL treatment was initiated 1 week afterwards when pancreatic carcinoma had

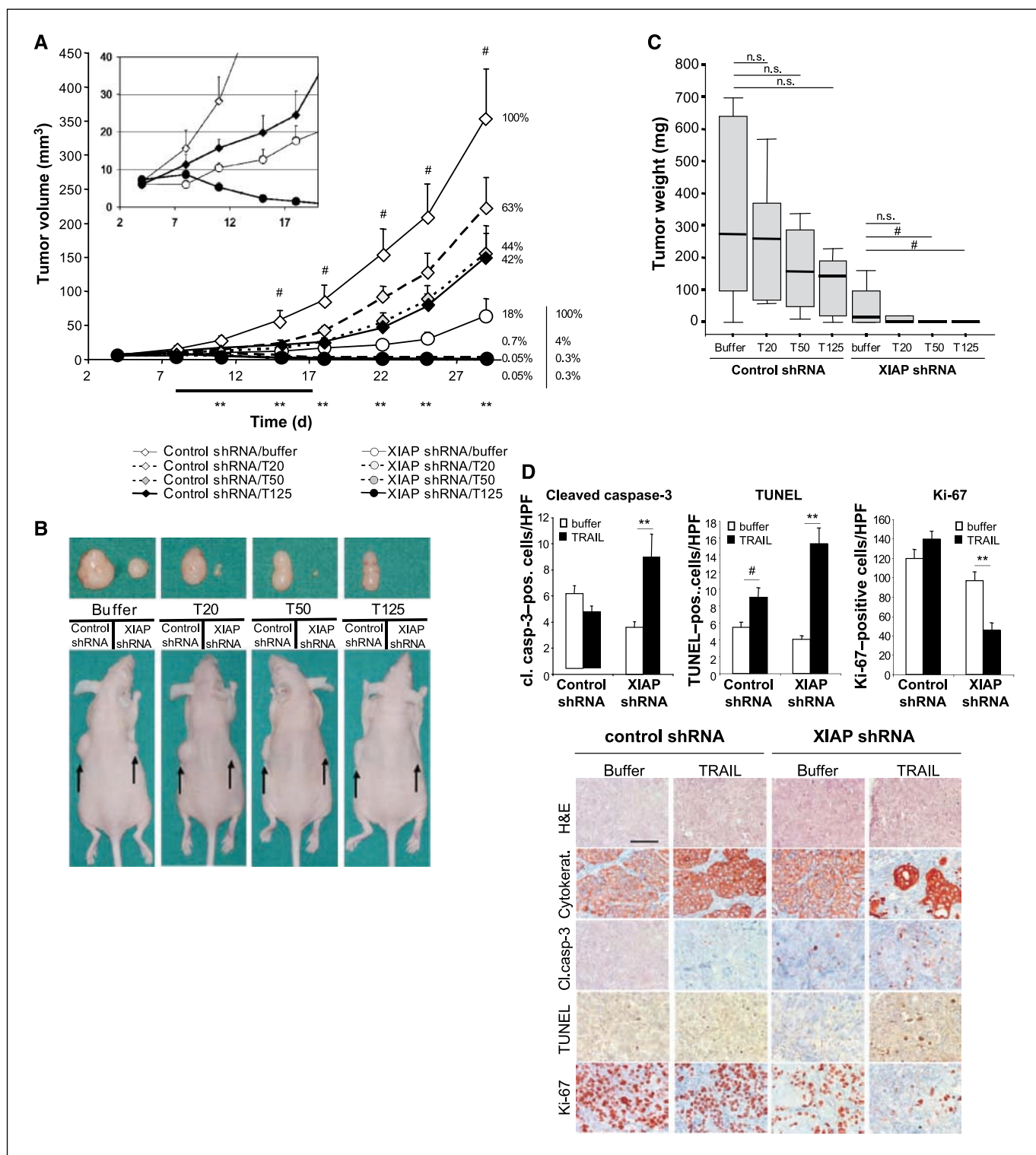


Figure 6. XIAP knockdown cooperates with TRAIL to cause regression of established pancreatic carcinoma in a xenograft mouse model. PancTu1 cells transduced with control or XIAP shRNA were s.c. inoculated at left and right dorsal site, respectively, of mice. Treatment started 1 wk after tumor cell inoculation by daily i.p. injection of 20, 50, or 125 μ g TRAIL (T20, T50, T125) or buffer for 10 d; mice were sacrificed on day 29. Bar below X-axis marks the TRAIL treatment period. **A**, tumor volumes are shown (mean \pm SE); #, $P < 0.05$; **, $P < 0.001$ comparing TRAIL (125 μ g) versus buffer treatment in control tumors (marked above graphs) and in tumors with XIAP knockdown (marked below X-axis). Therapeutic efficacy of TRAIL was estimated by T/C ratio (%) at day 29 relative to buffer-treated control tumors (left column) or buffer-treated tumors with XIAP knockdown (right column). *Insert*, higher magnification tumor growth from day 2 to day 19 of tumors treated with 125 μ g TRAIL or buffer showing that TRAIL causes tumor regression only in tumors with XIAP knockdown. **B**, representative animals and tumors are shown for each group; arrows, tumors. **C**, tumor weight is depicted by box plots; line inside each box, median; boxes, 25th and 75th percentiles; error bars, minimum and maximum; #, $P < 0.05$; n.s., not significant. **D**, immunohistochemical analysis of tumors 24 h after the second dose of 125 μ g TRAIL (day 10) is shown for H&E, cytokeratin, cleaved caspase-3, TUNEL, and Ki-67 stainings. Number of positive cells per high power field (HPF, top) and representative pictures (bottom) are shown; columns, mean; bars, SE; #, $P < 0.05$; **, $P < 0.001$; bar, 100 μ m ($\times 40$).

been established. Strikingly, XIAP knockdown cooperated with TRAIL to cause regression of established pancreatic carcinoma in a dose-dependent manner, an effect that was sustained also after discontinuation of TRAIL treatment (Fig. 6A and B). By comparison, TRAIL treatment alone only slowed down growth of control tumors (Fig. 6A and B). XIAP knockdown substantially enhanced the TRAIL-induced reduction in tumor volume even at the lowest dose of TRAIL, with T/C ratios of 4%, 0.3%, or 0.3% upon treatment with 20, 50, or 125 μg TRAIL in tumors with XIAP knockdown compared with T/C ratios of 63%, 44%, or 42% upon treatment with 20, 50, or 125 μg TRAIL in control tumors (Fig. 6A). Similarly, analysis of tumor weight showed that TRAIL treatment was more effective in pancreatic carcinoma with XIAP knockdown compared with control tumors (Fig. 6C). The suppressive effect of XIAP knockdown alone on tumor growth in xenografted tumors suggests that the XIAP-mediated survival effect may become relevant under cellular stress conditions *in vivo*, i.e., under hypoxia, as XIAP is translationally up-regulated via an internal ribosome entry site during hypoxia (28).

In parallel, we monitored apoptosis in pancreatic carcinoma tissue *in situ* during the course of TRAIL treatment. To this end, three additional mice of each treatment group were sacrificed 24 h after the second injection of TRAIL on day 10 of the experiment and tumor tissue was analyzed by immunohistochemistry. TRAIL-treated pancreatic carcinoma with XIAP knockdown displayed a significant increase in cells with cleaved caspase-3 and TUNEL positivity and a reduction of Ki-67-positive cells (Fig. 6D). These findings reflect at the cellular level in tumor tissue *in vivo* that XIAP inhibition acts in concert with TRAIL to trigger apoptosis and to suppress tumor growth. Notably, no signs of acute or delayed toxicity were observed during treatment or at the end of the experiment as assessed by clinical observation, body weight, blood cell counts, and liver enzymes (Supplementary Fig. S4). Together, this set of experiments in xenograft-bearing mice shows that XIAP inhibition strongly augments the antitumor activity of TRAIL, causing regression of established pancreatic carcinoma *in vivo* without detectable toxicities to normal tissues.

Discussion

Evasion of apoptosis is a key factor preventing treatment responses in pancreatic cancer, a leading cause of cancer deaths (3). This highlights the need for novel strategies to overcome apoptosis resistance to improve the outcome of this lethal disease.

In response to this demand, we develop in the present study the combination of XIAP inhibition plus TRAIL as an effective approach to trigger apoptosis in pancreatic carcinoma, which overcomes Bcl-2-mediated resistance and even causes regression of established tumors *in vivo*. This conclusion is supported by the following pieces of evidence. First, neutralizing XIAP by RNA interference acts in concert with TRAIL to trigger apoptosis in pancreatic carcinoma cells *in vitro*. Second, the combination of XIAP inhibition and TRAIL breaks Bcl-2-imposed resistance by converting type II pancreatic carcinoma cells to type I cells. Third, inhibition of XIAP substantially enhances the antitumor activity of TRAIL against pancreatic cancer in two preclinical models *in vivo*. Importantly, the combination of XIAP inhibition and TRAIL causes regression of established pancreatic carcinoma in xenograft-bearing mice. Together, these results show that XIAP inhibition is a powerful strategy to potentiate the antitumor activity of TRAIL against pancreatic cancer *in vitro* and *in vivo*.

Conceptually, we show for the first time that down-regulation of XIAP breaks Bcl-2-mediated resistance to TRAIL, switching type II pancreatic carcinoma cells that depend on the mitochondrial contribution to the death receptor pathway to type I cells in which TRAIL-induced apoptosis proceeds irrespective of high Bcl-2 levels. Neutralizing XIAP enhances TRAIL-induced apoptosis at the postmitochondrial level by relieving the XIAP-mediated inhibition of caspase-3 and caspase-9. Thus, XIAP inhibition elicits apoptosis also in cells in which the mitochondrial pathway is blocked, e.g., by antiapoptotic Bcl-2 family proteins. It is interesting to note that there is recent evidence that caspase-9 activation can occur in certain cell types in the absence of a functional apoptosome as long as XIAP is kept in check (29). Also, our data indicate that inhibition of XIAP enhances TRAIL-induced mitochondrial breakdown by promoting caspase-3 activation. Caspase-3 may act back on mitochondria by cleaving Bid, antiapoptotic Bcl-2 family proteins, and/or components of the mitochondrial respiratory chain (26, 30–33). By comparison, γ -irradiation and DNA-damaging anticancer drugs were shown to cooperate with TRAIL in Bcl-2 overexpressing cells by bypassing the mitochondrial pathway via enhanced TRAIL DISC formation without causing mitochondrial disassembling (34, 35). Because antiapoptotic Bcl-2 family proteins have been reported to be expressed at high levels in pancreatic cancer and to contribute to apoptosis resistance (2, 9), bypassing the requirement for mitochondrial apoptogenic events in the course of TRAIL-induced apoptosis, e.g., by inhibition of XIAP, may have important implications to restore sensitivity to TRAIL in pancreatic cancer. Furthermore, it will be interesting to explore whether the combination of TRAIL and XIAP inhibition may bypass c-FLIP-imposed apoptosis resistance because a novel caspase-8 activation pathway has very recently been identified in the course of TNF α plus Smac mimetic-triggered apoptosis that is less sensitive to c-FLIP inhibition (36).

Our study also underscores the relevance of TRAIL-based combination protocols for the treatment of pancreatic cancer. The antitumor activity of TRAIL receptor agonists that are currently under evaluation in early clinical trials in various human malignancies (37) may be impaired by resistance of pancreatic carcinoma to TRAIL. This calls for rational combinations to target defective apoptosis programs in pancreatic cancer. Our data provide, for the first time, evidence that the combination of XIAP inhibition plus TRAIL is a potent strategy to trigger regression of established pancreatic carcinoma in xenograft-bearing mice without detectable toxicities to normal tissues. Thus, our findings provide the rationale to further develop the combination of XIAP inhibition and TRAIL to overcome apoptosis resistance of pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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