MADD/DENN Splice Variant of the IG20 Gene Is a Negative Regulator of Caspase-8 Activation

KNOCKDOWN ENHANCES TRAIL-INDUCED APOPTOSIS OF CANCER CELLS

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The MADD variant of the IG20 gene is necessary and sufficient for cancer cell survival. Abrogation of MADD, but not the other IG20 splice variants, can render cells more susceptible to spontaneous as well as TRAIL (tumor necrosis factor α-related apoptosis-inducing ligand)-induced apoptosis. Both types of apoptosis in cells devoid of MADD can be inhibited by expression of CrmA or dominant-negative FADD, thereby suggesting that endogenous MADD may be targeting caspase-8 activation. Immunoprecipitation studies showed that MADD down-modulation could lead to caspase-8 activation at the death receptors without an apparent increase in the recruitment of death-inducing signaling complex components such as FADD. Further, we found that MADD can directly interact with death receptors, but not with either caspase-8 or FADD, and can inhibit caspase-8 activation. These results clearly demonstrate the importance of MADD in the control of cancer cell survival/death and in conferring significant resistance to TRAIL-induced apoptosis. In addition, our results indicate the therapeutic potential of MADD abrogation in enhancing TRAIL-induced selective apoptosis of cancer cells.

Earlier gain-of-function studies showed that expression of exogenous IG20pa can render cells more susceptible to induced apoptosis, whereas DENN-SV can confer resistance to the above treatments (4, 6–8). Interestingly, loss-of-function studies using oligodeoxynucleotides showed that knockdown of all IG20-SVs can result in spontaneous apoptosis of cancer cells, but not normal cells, in vitro as well as in vivo (5, 9). These studies demonstrate an indispensable role for IG20 in cancer cell survival but fail to reveal the relative importance of different IG20-SVs.

Using shRNAs that specifically target exon 15, which is expressed in all expressed isoforms of IG20 and designated Mid, or exons 13L and 16, which are differentially expressed in IG20-SVs, we were able to selectively knock down either all or select combinations of IG20-SVs in HeLa and PA-1 cells and determine their role in cell survival. Knockdown of MADD but not other splice variants resulted in spontaneous apoptosis of cancer cells; these cells could be rescued only upon re-expression of MADD but not other splice variants. These studies demonstrated that MADD is required and sufficient for the survival of cancer cells (21).

In the present study, we have shown that MADD abrogation can render cells more susceptible to TRAIL-induced apoptosis. Further, we demonstrate that MADD can interact with the death receptors (DRs) but not with either FADD (Fas-associated death domain) or caspase-8, and that the enhanced apoptosis results from activation of caspase-8 at the DRs without an apparent increase in the recruitment of death-inducing signaling complex (DISC) components. These results strongly suggest that under physiological conditions MADD can act as a negative regulator of caspase-8 activity in the DISC.

EXPERIMENTAL PROCEDURES

Cell Culture—293T cells, HeLa and PA-1 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin-G, and 100 µg/ml streptomycin. All cell lines were maintained at 37 °C in a humidified atmosphere with 5.5% CO₂.

Antibodies and Other Reagents—The anti-IG20 peptide polyclonal antibody, raised against three different peptides from the N-terminal, middle, and C-terminal region of IG20, has been described previously (4). Anti-Caspase-8 antibody (C-15) was a gift from Marcus E. Peter (Ben May Institute of Cancer Research, University of Chicago). Anti-FADD antibodies were obtained from BD Pharmingen, and anti-GFP/YFP

The abbreviations used are: SV, splice variant; TRAIL, tumor necrosis factor—related apoptosis-inducing ligand; shRNA, short hairpin RNA; DR, death receptor; DcR, decoy receptor; MAPK, mitogen-activated protein kinase; MADD, MAPK-activating death domain; FADD, Fas-associated death domain; FACS, fluorescence-activated cell sorter; DN, dominant negative; DISC, death-inducing signaling complex; RT, reverse transcription; PBS, phosphate-buffered saline; GFP, green fluorescent protein; YFP, yellow fluorescent protein; PE, phycoerythrin; SCR, scrambled.
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antibody (IL-8 clone) was purchased from Clontech, Palo Alto, CA. Anti-caspase-8 (C-20) for immunoprecipitation, anti-DR5 (IMG 120), anti-DR4 (H-130), anti-caspase-3 (H-277), and anti-DR4 (B-9 monoclonal) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TRAIL-biotinylated antibody and recombinant human TRAIL were obtained from Peprotec Inc. (Rocky Hill, NJ). Anti-actin antibody was obtained from Sigma-Aldrich.

Lentivirus Production—Subconfluent 293T cells grown in 100-mm plates were co-transfected with 10.8 µg of lentivirus vector, 0.6 µg of pcRev, 0.6 µg of pcTat, and 0.3 µg of pHIT/G using calcium phosphate. Culture medium was replaced after 16 h, and the supernatant was harvested at 40 h and filtered using a 0.45-µm filter. HeLa and PA-1 cells were transduced with the respective lentiviruses as described previously (21, 22).

RT-PCR—Total RNA was extracted from 1 × 10⁶ transduced cells using TRIzol (Invitrogen); 1 µg of RNA was used for RT-PCR using the SuperScript One-Step RT-PCR system (Invitrogen). RT-PCR was carried out using F2-B2 and glyceraldehyde-3-phosphate dehydrogenase primers as described previously (4). The PCR products were then separated on a 2% agarose gel.

Hoechst Staining—5 × 10⁵ transduced cells were washed in cold PBS. Five µg/ml Hoechst 33342 and 1 µg/ml propidium iodide (Sigma) were used to stain the cells for 5 min. Cells with condensed chromatin were analyzed using a BD-LSR (BD Biosciences). Highly propidium iodide-positive cells representing necrotic or late apoptotic cells were excluded from the analysis. Only GFP-positive (shRNA-expressing) cells were included in the analysis.

FACS Analysis of Cell Surface Expression of Receptors—Forty-eight hours post-transduction, HeLa cells were collected in enzyme-free cell dissociation buffer (Invitrogen), washed once with PBS containing 0.5% bovine serum albumin, and let stand in the same buffer for 10 min at 4 °C. PE-conjugated anti-DR4 (DJR1 clone), anti-DR5 (DJR2-4 clone), anti-DcR1 (DJR3 clone), anti-DcR2 (DJR4-1 clone), anti-TRAIL (clone RIK 2), and anti-FasL (NOK1) antibodies, purchased from eBiosciences (San Diego, CA), and anti-Fas (BD Pharmingen) were used to stain samples for 30 min at 4 °C. A mouse IgG antibody was used as an isotype control. Cells were washed with PBS, and GFP-positive cells were analyzed by using a FACSCalibur (BD Biosciences).

Suppression of Apoptosis Using Dominant-negative (DN)-FADD and CrmA—HeLa and PA-1 cells were transfected with a DN-FADD, CrmA, or control PCDNA3.1 vector using SuperFect reagent (Qiagen). Permanently transfected cells were selected in 800 µg/ml G418. Post-selection, stably transfected cells were grown in medium containing 400 µg/ml G418. The stable cells were transduced with the respective lentiviruses, and at 72 h post-transduction cells were assayed for spontaneous apoptosis. Cells were treated with 100 ng of TRAIL at 36 h post-transduction and assayed for apoptosis by staining for active caspase-3.

Active Caspase-3 Detection by FACS—Active caspase-3 levels were detected by analyzing PE-positive population using the active caspase-3-PE staining kit (BD Pharmingen). Only GFP-positive cells were included in the analysis using FACSCalibur.

Tetramethylrhodamine Methyl Ester Staining—5 × 10⁵ transduced cells were stained with 100 nM tetramethylrhodamine methyl ester (Invitrogen-Molecular Probes) for 15 min at 37 °C and apoptosis in GFP-positive cells was analyzed by FACS.

Caspase Detection—To detect the inactive as well as active forms of caspase-8, 1 × 10⁷ HeLa and/or PA-1 cells were lysed with radioimmune precipitation assay buffer (Tris-HCl 50 mM, NaCl 150 mM, SDS 0.1%, sodium deoxycholate 0.1%, EDTA 5 mM, dithiothreitol 1 mM, Nonidet P-40 1%, pH 8.0) and normalized for protein concentration. Immunoblots were probed for caspase-8 and pro-caspase-3 using C-15 and H-277 antibodies, respectively.

Characterization of DISC Immunoprecipitated Using anti-DR4/DR5 Antibody—HeLa cells (2 × 10⁷) and PA-1 cells (5 × 10⁷) transduced for 48 h were collected and washed in cold PBS. Washed cells were lysed in 1 ml of lysis buffer (30 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, protease inhibitors mixture (Roche Applied Science), 1% Triton X-100, and 10% glycerol) on ice for 30 min and clarified by centrifugation at 12,000 rpm for 30 min at 4 °C. Supernatants were normalized for protein concentration and then immunoprecipitated using 2 µg of H-130 DR4/DR5 antibody on a rotoshaker at 4 °C for 4 h followed by the addition of 25 µl of a 50% slurry of protein A/G beads (Amersham Biosciences) for 2 h. Beads were then washed three times with lysis buffer and boiled in SDS lysis buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and bromphenol blue, pH 6.8) for 5 min. Eluates were then subjected to SDS-PAGE using a 12% gel for subsequent immunoblot analysis.

Characterization of DISC Immunoprecipitated Using Anti-TRAIL Antibody—HeLa (2 × 10⁷) cells transduced for 36 h were treated with 250 ng of TRAIL (Peprotech) for 30 min either at 4 °C (untreated) or at 37 °C (treated), collected, washed in cold PBS, and lysed in 1 ml of lysis buffer for 30 min on ice. The lysates were clarified and then normalized for protein concentration. The DISC was then immunoprecipitated overnight using 2 µg/ml of anti-TRAIL-biotinylated antibody. The biotinylated antibody was immunoprecipitated with 35 µl of a 50% slurry of streptavidin-agarose beads. Beads were washed three times with lysis buffer, boiled in SDS sample buffer, and separated on a 12% SDS-polyacylamide gel for immunoblot analysis (23).

Immunoprecipitation of the MADD Complex—HeLa cells were transfected with MADD-YFP. Thirty-six hours post-transduction HeLa cells (2 × 10⁷) were collected and left untreated or treated with 250 ng/ml TRAIL for 30 min at 37 °C. At the end of the treatment, cells were washed in cold PBS, pelleted, and then lysed in 1 ml of DISC lysis buffer for 30 min on ice and clarified by centrifugation. The supernatants were normalized for protein concentration, precleared, and incubated with 10 µl of polyclonal anti-IG20 antibodies on a rotoshaker overnight. The complexes were immunoprecipitated with a 50% slurry of protein A/G beads. The beads were washed three times and boiled in SDS lysis buffer for 5 min. The eluates were then subjected to SDS-PAGE using a 12% gel for subsequent immunoblot analysis.
Immunoblotting—The membranes were blocked in 5% non-fat dry milk in PBS-Tween (PBS with 0.05% Tween 20) for 1 h. Primary antibodies were used at a concentration of 1 μg/ml, and the secondary antibodies were used at a 1:10000 concentration. The blots were developed by enhanced chemiluminescence according to the manufacturer’s protocol (Pierce).

RESULTS
MADD Abrogation Results in Enhanced TRAIL-induced Apoptosis—Down-modulation of IG20 transcripts upon treatment of cells with lentiviruses expressing different shRNAs was monitored by RT-PCR. Our results showed that the IG20 transcripts were significantly down-modulated by 24 h (Fig. 1, A and C). These cells were tested for spontaneous apoptosis at 72 h post-transduction by Hoechst staining (Fig. 1, B and D) or mitochondrial depolarization (not shown). As evident from these data and consistent with our earlier observation, abrogation of MADD, but not other isoforms, resulted in spontaneous apoptosis.

We have observed consistently that although the IG20 transcripts are significantly down-modulated by 24 h, the cells do not undergo spontaneous apoptosis until 72 h post-shRNA induction. This is most likely due to the time required for complete degradation of the remaining endogenous proteins.

**FIGURE 1.** MADD down-modulation in HeLa and PA-1 cells results in spontaneous apoptosis. A and C, RT-PCR profile of IG20-SVs at 24, 48, and 72 h post-shRNA transduction in HeLa (A) and PA-1 cells (B), respectively. B and D, spontaneous apoptosis as measured by Hoechst staining in transduced HeLa (B) and PA-1 cells (D), respectively. Data represent mean ± S.D. of triplicate samples.

**FIGURE 2.** Down-modulation of MADD enhances susceptibility to TRAIL. A and C, kinetics of response. Thirty-six hours post-transduction, HeLa cells were treated with 50 ng of TRAIL for different durations. B and D, dose response. HeLa cells were treated for 5 h with the indicated concentrations of TRAIL (A and B). Active caspase-3 was detected using a PE-conjugated antibody specific for active caspase-3 by FACS. Data presented are representative of three different experiments; p < 0.005. C and D, apoptosis was also measured by tetramethylrhodamine methyl ester (TMRM) exclusion using FACS. Data represent mean ± S.D. of triplicate samples.
Therefore, at 36 h post-shRNA transduction, when there was no indication of spontaneous apoptosis, HeLa cells were treated with various concentrations of TRAIL for different durations and assayed for apoptosis. Cells devoid of MADD showed enhanced TRAIL-induced apoptosis as indicated by significant increases in caspase-3 activation (Fig. 2, A and B) and mitochondrial depolarization (Fig. 2, C and D). Similar results were obtained in PA-1 ovarian carcinoma cells (not shown).

Down-modulation of MADD Results in Activation of Caspase-8 at the DRs, Which Can Be Inhibited by DN-FADD and CrmA—To gain insight into the mechanism underlying MADD-mediated cell survival, we examined whether caspases were activated upon abrogation of MADD expression. Only MADD-depleted HeLa and PA-1 cells (Mid and 13L cells) showed processing of pro-caspase-8 (p55/53), as indicated by the higher amounts of the intermediate active caspase-8 proteins (p43/41) (Fig. 3). Similarly, HeLa cells treated with 100 ng of TRAIL for the indicated durations (Fig. 4) showed sustained caspase-8 activation and subsequent caspase-3 activation, as evident from the increased levels of p43/p41 and p18 subunits of caspase-8 and a concomitant decrease in pro-caspase-3 levels only in MADD-depleted cells.

Next, we made use of two caspase-8 inhibitors that are known to inhibit TRAIL-mediated apoptosis. HeLa and PA-1 cell lines stably expressing DN-FADD or CrmA were infected with lentiviruses that could express scrambled (SCR) or Mid- or 13L-specific shRNAs, and at 36 h post-transduction they were treated with TRAIL (50 ng/ml) for 5 h. Knockdown of MADD (13L-transduced) or all IG20 isoforms (Mid-transduced) caused a significant increase in both spontaneous (Fig. 5) and TRAIL-induced (Fig. 6) apoptosis, which could be almost completely inhibited in matched cells expressing either CrmA or DN-FADD.

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FIGURE 3. Down-modulation of MADD results in caspase-8 activation. HeLa cells (A) and PA-1 cells (B) were collected 48 h post-transduction and probed for caspase-8 and FADD, which are components of the DISC. As observed, the cleaved intermediate fragments of caspase-8 (early indication of caspase-8 activation) can be detected only upon MADD abrogation, although the levels of pro-caspase-8, FADD, and actin were comparable in all cells. These results were reproduced three times.

FIGURE 4. Enhanced TRAIL-mediated apoptosis results from increased caspase-8 activation. TRAIL treatment results in recruitment of pro-caspase-8 to the receptors followed by its cleavage resulting in its activation. The cleaved intermediate fragments (p43/p41) and the catalytically active subunits of caspase-8 (p18) were detected upon TRAIL treatment. Caspase-8 is the initiator caspase that can cause activation of the effector caspase-3. A decrease in the amount of pro-caspase-3 indicates its cleavage to form active caspase-3. Actin served as a loading control.

FIGURE 5. CrmA and DN-FADD can inhibit the onset of spontaneous apoptosis. HeLa (A) and PA-1 cells (B) stably expressing control vector, CrmA, or DN-FADD were transduced with SCR, Mid, and 13L shRNAs. Spontaneous apoptosis was assessed by Hoechst staining 72 h post-transduction. Data shown are from three different experiments carried out in triplicates. Error bars indicate mean ± S.D. The difference in apoptosis of cells treated either with Mid-C and 13L-C relative to the corresponding control or with DN-FADD and CrmA was highly significant; p < 0.005.

MADD Abrogation Does Not Affect the Expression of Death (DR) or Decoy Receptors (DcR) or Their Cognate Ligands—One underlying possibility for enhanced caspase-8 activation is that loss of endogenous MADD could positively regulate the expression of various death receptors and their ligands or down-regulate decoy death receptors. Because increases in the levels of expression of DRs and their ligands or decreases in DcRs can result in oligomerization of death receptors followed by apoptotic cell death, we tested for the levels of their expression in HeLa (Fig. 7) and PA-1 cells (not shown). Our results showed no
significant difference in the levels of expression in various shRNA-transduced cells relative to control cells and indicated that spontaneous apoptosis resulting from MADD down-modulation was not because of perturbations in the levels of DR4, DR5, Fas, FasL, TRAIL, DcR1, and DcR2 expression on the cell surface.

Endogenous MADD Negatively Regulates Caspase-8 Activity at the Death Receptors—As caspase-8 plays an essential role in receptor-mediated apoptosis characterized by DISC formation, we examined the status of DISC in cells undergoing apoptosis due to loss of endogenous MADD expression. We immunoprecipitated DR4 and DR5 from HeLa and PA-1 cells, respectively, and probed for known DISC components (Fig. 8). FADD and pro-caspase-8 were found to be associated constitutively with the DRs in cells with and without MADD abrogation. However, the intermediate cleaved fragments of caspase-8 (p43/p41) were detected only in MADD-depleted cells, which suggested that activation of caspase-8 at the DRs was associated with spontaneous apoptosis resulting from MADD abrogation.

To more specifically analyze the DISC associated with TRAIL-bound death receptors, TRAIL-induced DISC was immunoprecipitated from HeLa cells using a TRAIL-specific antibody and subjected to SDS-PAGE followed by Western blotting. Staining for various TRAIL-DISC components revealed co-precipitation of DR4, FADD, and caspase-8 (Fig. 9). Relative to control cells (SCR), TRAIL immunoprecipitates from MADD-depleted cells (13L and Mid) showed increased levels of intermediate fragments of caspase-8 (p43/41). This observation correlated with enhanced caspase-8 activity that we had observed earlier in these cells upon TRAIL treatment (Fig. 4).

MADD Binds to DR4 but Not to Caspase-8 or FADD—To examine whether MADD confers resistance to apoptosis by interacting with caspase-8, we expressed MADD-YFP in HeLa cells and immunoprecipitated it using an IG20-specific antibody from lysates of cells that were either left untreated or treated with TRAIL (250 ng) for 30 min. These proteins were separated by SDS-PAGE and subjected to Western blot analysis to probe for DISC components. Interestingly, endogenous DR4
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FIGURE 9. Down-modulation of MADD results in increased caspase-8 activation at the TRAIL DISC. The TRAIL DISC was immunoprecipitated using a biotinylated-antibody specific for TRAIL from HeLa cells that were either left untreated or treated with 250 ng of TRAIL for 30 min. Complexes were separated on 12% SDS-PAGE and subjected to immunoblotting with the indicated antibodies. Increased recruitment of FADD and pro-caspase-8 was observed after TRAIL treatment, but increased caspase-8 activation (p43/41) was more apparent upon MADD down-modulation.

FIGURE 10. MADD exerts its anti-apoptotic effect by binding to DR4 but not other components of the DISC. To determine whether MADD prevents caspase-8 activation by directly binding to caspase-8, HeLa cells were transfected with MADD-YFP. Thirty-six hours post-transduction, cells were either left untreated or treated with 250 ng of TRAIL for 30 min. Samples were lysed, and the lysates were normalized for protein concentration and preclarified. Equal amounts of proteins were incubated with either anti-IG20 or anti-caspase-8 antibody. Complexes were subjected to immunoblotting (WB, Western blot) as described under “Experimental Procedures.” Results show MADD binding to DR4 but not to caspase-8 or FADD. IP, immunoprecipitation.

but not caspase-8 co-precipitated with MADD in both untreated and TRAIL-treated cells. On the other hand, immunoprecipitation using a caspase-8-specific antibody followed by Western blotting showed co-precipitation of FADD with caspase-8 upon TRAIL treatment; whereas MADD and DR4 could not be co-precipitated with caspase-8 under either condition (Fig. 10). The above results clearly demonstrated that MADD may exert its anti-apoptotic effect by directly binding to DR4 but not to caspase-8 or FADD.

DISCUSSION

The current study demonstrates that MADD abrogation can lead to spontaneous as well as enhanced TRAIL-induced apoptosis resulting from caspase-8 activation at the DRs and strongly indicates that MADD can act as a negative regulator of caspase-8 activation in cancer cells.

The levels of expression of DRs and DcRs or their ligands were unperturbed (Fig. 7). Expression of CrmA, a known inhibitor of caspase-1 and -8 (24), or DN-FADD, which competes with endogenous FADD (25), conferred resistance to spontaneous apoptosis (Fig. 5). Increased activation of caspase-8 at the DISC was evident from an increase in the p43/p41 fragments (26). Interestingly, caspase-8 activation resulting from MADD abrogation was not accompanied by an increase in the recruitment of FADD or caspase-8 to the DISC (Fig. 8).

Although MADD transcripts were depleted by 24 h post-transduction of shRNAs, it took 72 h for spontaneous apoptosis to begin (Fig. 1). This timing allowed us to determine the susceptibility to TRAIL-induced apoptosis of MADD-depleted cells. These results showed that MADD abrogation can render cells more susceptible to TRAIL-induced apoptosis by causing increased caspase-8 activation at the TRAIL-DISC with consequent caspase-3 activation (27), again, without enhancing the DISC formation (Figs. 2, 4, and 9).

Although recent knockdown studies have revealed an important pro-survival role for MADD (21), earlier overexpression studies failed to indicate a role for MADD in enhanced cell survival (4). This would suggest that endogenous MADD might be sufficient to fully exert its function and that the effects of exogenous MADD, if any, thus may not be apparent. Similarly, although exogenous IG20pa (a pro-apoptotic variant of IG20) can enhance apoptosis, including TRAIL-induced apoptosis (4, 7–8), IG20pa knockdown failed to confer resistance to TRAIL-induced apoptosis. IG20pa is a part of the TRAIL-induced DISC (23), and exogenous IG20pa can enhance DISC formation and thus increase susceptibility to TRAIL-induced apoptosis. This increased DISC formation may result from IG20pa acting as a dominant-negative MADD. In contrast, although overexpression of exogenous DENN-SV enhanced cell proliferation and resistance to apoptosis (4, 7), its expression in the absence of MADD did not prevent enhanced apoptosis (21). We suspect that DENN-SV, because of its ability to enhance NFκB activation, might complement the pro-survival function of MADD and thus, upon overexpression, could enhance cell survival and proliferation. A clearer understanding of the interplay between various IG20-SVs could aid in understanding their differential effects and in the development of novel methods to enhance spontaneous as well as TRAIL-induced apoptosis of cancer cells.

The mode of action of MADD is not yet known. However, we do know that it can bind to DRs, but not to FADD or caspase-8, and prevent activation of caspase-8 without affecting DR-FADD or FADD/caspase-8 interactions (Fig. 10). The proximity-induced dimerization model for caspase-8 activation suggests that increased proximity of pro-caspase-8 molecules at the receptor allows them to dimerize and undergo activation (28–30). Therefore, MADD could sequester death receptors and prevent their oligomerization or sterically hinder caspase-8 homodimerization and/or activation through its interaction with the DRs. It is also possible that MADD association with the DRs can lead to recruitment of other molecules that can either antagonize caspase-8 (e.g. c-FLIP (31)) or are required for the activation of an alternate survival pathway (e.g. MAPKs) that could counteract caspase-8. Nevertheless, our results show that MADD can constitutively bind to DRs, and not to FADD or caspase-8, and prevent caspase-8 activation. The fact that loss of endogenous MADD can render cells more
susceptible to spontaneous as well as TRAIL-induced apoptosis makes this a desirable therapeutic target that can be exploited either alone or in conjunction with TRAIL to develop novel cancer therapies.

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