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The NRIF3 Family of Transcriptional Coregulators Induces Rapid and Profound Apoptosis in Breast Cancer Cells

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Many anticancer drugs kill cancer cells by inducing apoptosis. Despite the progress in understanding apoptosis, how to harness the cellular death machinery to selectively deliver tumor-specific cytotoxicity (while minimizing damage to other cells) remains an important challenge. We report here that expression of the NRIF3 family of transcriptional coregulators in a variety of breast cancer cell lines induces rapid and profound apoptosis (nearly 100% cell death within 24 h). A novel death domain (DD1) was mapped to a short 30-amino-acid region common to all members of the NRIF3 family. Mechanistic studies showed that DD1-induced apoptosis occurs through a novel caspase 2-mediated pathway that involves mitochondrial membrane permeabilization but does not require other caspases. Interestingly, the cytotoxicity of NRIF3 and DD1 appears to be cell type specific, as they selectively kill breast cancer or related cells but not other examined cells of different origins. Our study demonstrates the feasibility of selectively inducing cytotoxicity in a specific cancer and suggests that breast cancer cells contain a novel "death switch" that can be specifically triggered by NRIF3 or DD1. Strategies utilizing NRIF3 and/or DD1 and/or targeting this death switch may lead to the development of novel and more selective therapeutics against breast cancer.

Apoptosis or programmed cell death is a fundamental cellular process where the affected cell dies by actively executing a coordinately regulated death program (11, 18). For multicellular organisms (e.g., mammals) apoptosis plays important roles in normal development, tissue homeostasis, and diverse pathological processes. Caspases and mitochondria are two key cellular components involved in the execution and regulation of apoptosis (18, 50). Caspases are a group of cysteine proteases that are ordinarily inactive in cells as proenzymes but are activated upon appropriate apoptotic stimuli. Generally, the initiator caspases (e.g., caspases 2, 8, 9, and 10) are activated when complexed with adaptor molecules, resulting in either autoprocessing due to induced proximity or holoenzyme formation (9, 18, 26, 40). The downstream effector caspases (e.g., caspases 3, 6, and 7) are activated through proteolytic cleavage by an initiator caspase(s). Effector caspases then cleave various cellular components, leading to the morphological and biochemical phenotypes characteristic of apoptosis (11, 18).

Mitochondria also play an important role in apoptosis, as various apoptotic stimuli converge on mitochondria and lead to mitochondrial membrane permeabilization (MMP) (25, 38, 50). Upon MMP, mitochondria release a number of factors that are involved in apoptosis initiation and/or execution, such as cytochrome *c*, Smac/Diablo, and apoptosis-inducing factor (AIF) (8, 18, 38, 50). The released cytochrome *c* interacts with the adaptor protein Apaf-1 and pro-caspase 9 to form an activated complex referred to as an apoptosome, which then cleaves and activates downstream effector caspases (e.g., caspase 3) (18, 52). In contrast, AIF released from mitochondria triggers apoptosis (e.g., by inducing chromatin condensation and large-scale DNA fragmentation) independently of effector caspases (8, 31, 46, 51). This caspase-independent apoptogenic function of AIF is evolutionarily conserved and plays an important role both in normal development and in cell death processes whereby caspases are minimally activated or inhibited (e.g., by chemical inhibitors) (8, 38).

There are two major apoptotic pathways in mammalian cells (11, 18). The extrinsic pathway is initiated by the binding of transmembrane death receptors (e.g., Fas, TNF-R1, and TRAIL receptors) with cognate extracellular ligands. Liganded receptors recruit adaptor proteins (e.g., FADD) which interact with and trigger the activation of caspase 8. Activated caspase 8 then cleaves and activates downstream effector caspases such as caspase 3. In contrast, the intrinsic pathway is characterized by disruption of mitochondrial membrane integrity when cells are exposed to various stresses (e.g., DNA-damaging agents). MMP triggers apoptosis via both caspase-dependent (e.g., the cytochrome *c*-caspase 9 pathway) and caspase-independent (e.g., the AIF pathway) mechanisms. Cross talk exists between the extrinsic and intrinsic pathways, as activated caspase 8 can cleave Bid to produce truncated Bid (tBid), which then binds to mitochondria and promotes MMP (30, 32). The subsequent release of cytochrome *c* from mitochondria further facilitates the apoptotic process.

MMP is regulated by the Bcl-2 family of proteins, which act upstream of mitochondria and contain both antiapoptotic (e.g., Bcl-2 and Bcl-xL) and proapoptotic (e.g., Bak, Bax, Bid, and Bad) members (3, 7). The relative balance between the pro- and antiapoptotic members of the Bcl-2 family is critical in controlling MMP. Interestingly, a number of recent studies have shown that caspase 2 acts upstream of mitochondria and is required for MMP during stress-induced apoptosis in certain cell types (17, 27, 42). While these studies implicate caspase 2

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as an initiator caspase in a certain intrinsic pathway(s) of apoptosis, the mechanistic interplay between caspase 2 and members of the Bcl-2 family in controlling MMP is not yet clear (24).

Many anticancer drugs act by inducing apoptosis (20). The rapid progress in understanding mechanisms underlying apoptosis may present opportunities to harness the cellular death machinery for the benefit of treating human diseases such as cancer (20, 35). Ideally, therapeutic strategies targeting an apoptotic pathway(s) should selectively kill cancer but not other cells. At present, however, this remains a very challenging objective.

Breast cancer is the second leading cause of cancer-related deaths in women (23). Currently, effective drug treatment for breast cancer is somewhat limited. Since many early-stage breast tumors express the estrogen receptor (ER) and depend on estrogen for their optimal growth, antiestrogens (ER antagonists) have been widely used in the treatment of ER^+ tumors (5, 41). Antiestrogens, however, are not effective in ER^- tumors. Also, tumors that are initially ER^+ may lose ER expression and become independent of estrogen for their growth and refractory to antiestrogen therapy.

We have been interested in studying the role of nuclear receptors in breast cancer cell proliferation (2). We previously cloned a novel coregulator (designated NRIF3) which specifically interacts with and enhances the activity of ligand-bound thyroid hormone receptors (TRs) and retinoid X receptors (RXRs) (28, 29). Since retinoids are known to inhibit the growth of certain breast cancer cells (2, 15), we examined the effect of NRIF3 on retinoid-mediated growth inhibition in a number of breast cancer cell lines. Surprisingly, we found that NRIF3 expression inhibits cell growth independently of retinoid treatment. Further studies indicate that this apparent growth inhibition results from the induction of rapid and profound apoptosis in these cells by NRIF3 (virtually 100% cell death within 24 h). The apoptogenic function of NRIF3 is independent of its interaction with nuclear receptors and is mapped to a novel death domain (DD1) that is relatively small in size (~30 amino acids). Mechanistic studies suggest that DD1-induced apoptosis occurs through a novel caspase 2-mediated pathway that involves MMP and AIF translocation but does not appear to require other caspases. Interestingly, the cytotoxicity of NRIF3 and DD1 appears to be cell type specific, as their expression leads to efficient apoptosis in all the breast cancer cell lines surveyed (ER^+ T-47D, MCF-7, and MDA-MB-231/ER^+ cells and ER^- MDA-MB-231 and MDA-MB-435 cells) but not in five other cell types of different origins (HeLa, GH4C1, 293, UOK145, and Cos-1). Our study demonstrates that cytotoxicity can be selectively induced in a specific cancer and reveals the presence of a novel "death switch" in breast cancer cells that can be specifically triggered by NRIF3 or DD1. These results may have important implications in development of novel therapeutics against breast cancer.

MATERIALS AND METHODS

Plasmids. Plasmids expressing green fluorescent protein (GFP)-NRIF3, GFP-EnS, GFP-EnL, the GFP control, wild-type NRIF3, Gal4-DD1, and the Gal4 control have been described previously (28, 29). The GFPNLS vector expresses nucleus-localized GFP, where the nuclear localization signal (NLS) is derived from the simian virus 40 T antigen (1). Constructs expressing GFP or GFPNLS fusions of various regions of NRIF3 were generated by PCR-based cloning. Briefly, DNA fragments encoding a desired NRIF3 region were produced by PCR amplification, digested with XhoI and Acc65I, and cloned into either the original GFP vector (for regions corresponding to residues 1 to 86 and 20 to 86) or a GFPNLS vector (for wild-type DD1 and the S28A mutant DD1) digested with the same pair of enzymes. All GFP fusion constructs were confirmed by sequence analysis. The AIF-GFP plasmid was kindly provided by Guido Kroemer (31). Vectors expressing Bcl-2 and Bcl-xL were gifts from Honglin Li.

Cell culture and transfection conditions. All breast cancer cell lines were maintained in Dulbecco modified Eagle medium (DMEM; GIBCO-BRL, Life Technologies) supplemented with 10% fetal bovine serum (FBS). Other cells were cultured in DMEM supplemented with either 10% FBS or 10% HyClone defined-supplemented bovine calf serum. For most of the transient transfections, T-47D cells were plated at a density of 3×10^4 cells/well on coverslips in 24-well tissue culture plates. About 20 h later, the cells were transfected with the indicated plasmid(s) by the use of Genefect transfection reagent (Molecula) according to the manufacturer's protocol. Generally, the amount of plasmids used in transfections was as follows: GFP or GFP fusion, 300 to 500 ng; Bcl-2 or Bcl-xL, 1 to 1.5 μg; AIF-GFP, 500 ng; Gal4-DD1 or Gal4 control, 0.5 to 1 μg. After transfection, cells were incubated in DMEM–10% FBS medium for 5 to 24 h before being harvested and processed for appropriate analyses. Transient transfections of other breast cancer cell lines were carried out similarly, except that the transfection reagent used was Lipofectamine 2000 (Life Technologies). All other cells were transfected using Geneporter 2 (Gene Therapy Systems). When indicated, the following compounds were included in the medium: all-*trans* retinoic acid (tRA; 100 nM), etoposide (100 μM), zVAD-fmk (100 μM), zVDVAD-fmk (20 μM), tumor necrosis factor alpha (TNF-α; 10 ng/ml), and cycloheximide (10 μg/ml).

Flow cytometry and cell sorting analysis. Cells transfected with an appropriate GFP construct were processed for flow cytometry analysis as previously described (2). Flow cytometric analysis for GFP and propidium iodide was performed using four-color FACscan sorting (Becton-Dickinson Immunocytometry Systems, San Jose, Calif.). GFP-positive or -negative cells were analyzed for changes in cell cycle distribution. For reinoculation studies, cells were plated at a density of 3×10^6 cells/plate in a 100-cm^2 tissue culture plate and transfected with 50 μg of indicated GFP or GFP fusion constructs with the use of Genefect. About 24 h after transfection, cells were harvested and sorted by flow cytometry. GFP-positive or -negative cells were collected and replated at 3×10^4 cells/well in 24-well plates. Cells were monitored over a period of 48 to 72 h for attachment, growth, and morphological changes. In some cases, the collected cells were replated onto coverslips and subsequently processed for annexin V staining.

siRNA studies. A small interference RNA (siRNA) duplex that efficiently silences human caspase 2 expression has been previously described (27) and was purchased from Dharmacon. The siRNA was dissolved at 20 pmol/μl in H_2O. T-47D cells were plated at the density of 1.5×10^5 cells/well in six-well plates the day before being transfected with siRNA. Transfection was carried out using Oligofectamine (Invitrogen), with 12 μl of dissolved siRNA and 12 μl of Oligofectamine reagent. Cells were fed with additional DMEM–10% FBS on the second day and harvested about 42 h after siRNA transfection. Mock-transfected cells were treated similarly but did not receive caspase 2 siRNA. The harvested cells were then replated on coverslips in 24-well plates as described above and incubated for a few hours to let attachment occur. Cells were then transfected with the indicated GFP or GFP fusion vectors as described earlier or treated with etoposide (100 μM). Cells were harvested about 20 to 24 h later and processed for appropriate assays. To document caspase 2 knockdown, total lysates from siRNA-transfected or control cells were quantified for protein concentrations. Equal amounts of proteins were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Western analysis using a monoclonal antibody to caspase 2 (11B4; Alexis).

Apoptosis assays. Cells plated on coverslips in 24-well tissue culture plates were transfected and/or treated with appropriate compounds as indicated. Generally, cells were harvested within 20 to 24 h and processed for terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) and/or annexin V assays. For the annexin V assay, cells were washed three times with phosphate-buffered saline and assayed using the ApoAlert nitric oxide-annexin V dual sensor kit (BD Biosciences) according to the manufacturer's protocol. Cells were then mounted on slides with Dako fluorescent mounting medium (DAKO Corporation) and examined by fluorescence microscopy. For TUNEL assays, cells were washed three times with phosphate-buffered saline, fixed in 3.7% formaldehyde, and assayed using the in situ cell death detection kit (TMR red) from Roche Diagnostics GmbH (Mannheim, Germany) according to the manufacturer's protocol. In some cases, cells were also stained with Hoechst dye to visualize nuclei. Cells were then mounted on slides and examined by fluorescence

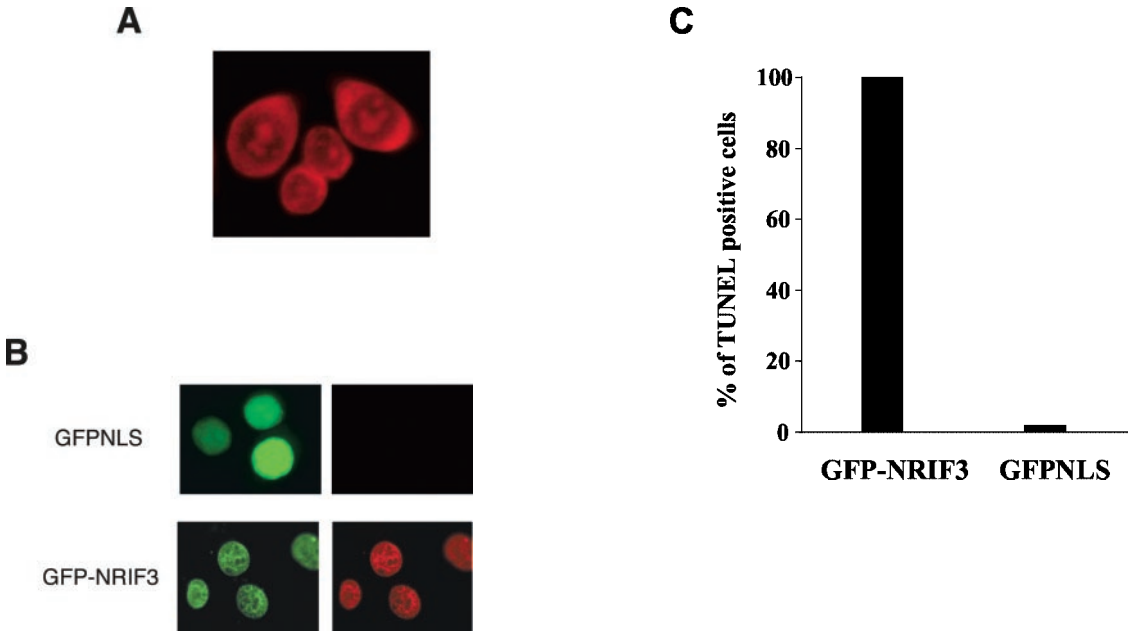


FIG. 1. NRIF3 induces apoptosis in T-47D cells. (A) GFP-NRIF3 was expressed in T-47D cells by transient transfection. Twenty-four hours later the green fluorescent cells were collected after sorting by flow cytometry and reinoculated onto coverslips. Cells were then analyzed for apoptosis by annexin V staining (red). Control cells expressing GFP alone were negative for annexin V staining (data not shown). (B) Representative fluorescence micrographs of T-47D cells transfected with either GFPNLS or GFP-NRIF3. Cells were examined for apoptosis by TUNEL assay (red). (C) Quantitative presentation of the experiments in panel B. The percentages of green fluorescent cells that were TUNEL positive were scored for T-47D cells transfected with either GFP-NRIF3 or GFPNLS.

microscopy. For quantitative analysis, fields consisting of at least several hundred cells were scanned, and a number of representative fields were photographed using GFP and rhodamine filters. Green and red cells from the same field were then counted. Generally, about 100 total green cells were counted for each data point. The percentages of green cells counted that were also red were then calculated.

RESULTS

NRIF3 induces rapid and profound cell death in T-47D cells. We previously identified NRIF3 as a coactivator for certain members of the nuclear hormone receptor superfamily, including TR and RXR (28, 29). A unique feature of RXR is that it serves as the common heterodimeric partner for many other members of the nuclear receptor superfamily (33). Thus, a heterodimer composed of the retinoic acid receptor (RAR) and RXR is the functional unit that transduces retinoid signaling in vivo (21, 22). Retinoid signaling plays important roles in both development and homeostasis (21, 22). In addition, retinoids are known to inhibit the proliferation of certain breast cancer cells (2, 15), although the underlying molecular mechanism(s) has not been fully defined.

Our identification of NRIF3 as a coactivator for RXR prompted us to test whether NRIF3 would enhance the antiproliferative effect of retinoids in responsive breast cancer cells. We and others have previously shown that retinoid treatment inhibits the proliferation of T-47D and MCF-7 breast cancer cell lines (2, 39, 43). This results in an increase in distribution of cells in the G_0/G_1 phase of the cell cycle and a concomitant decrease in the number of cells in S phase (2). To test the effect of NRIF3, we transfected T-47D cells with a vector expressing GFP or GFP-NRIF3 and incubated the cells with or without tRA. Green fluorescent and nongreen cells were then sorted by flow cytometry and analyzed for cell cycle distribution. Intriguingly, we found that expression of GFP-NRIF3 (but not the GFP control) was sufficient to inhibit the proliferation of T-47D cells, whether or not the cells were incubated with tRA (data not shown). This result suggested that NRIF3 mediates an antiproliferative effect on T-47D cells independently of retinoid treatment.

To examine this further, we collected sorted green fluorescent cells expressing either GFP-NRIF3 or GFP and replated them in culture dishes to monitor cell growth. While GFP-expressing cells attached and grew normally, cells expressing GFP-NRIF3 attached to the dish inefficiently and failed to divide. Microscopy examination revealed that the GFP-NRIF3-expressing cells displayed morphological changes suggestive of apoptosis (rounded-up cell shape and cytoplasmic shrinkage). Thus, we examined these cells for apoptosis using an annexin V assay (19, 49), and found that virtually all cells expressing GFP-NRIF3 were positive for annexin V staining while the control cells expressing GFP were negative (Fig. 1A and data not shown).

To further confirm that NRIF3 induces apoptosis, T-47D cells expressing GFP-NRIF3 or GFP fused to an NLS (GFPNLS) were examined by a TUNEL assay (16). GFPNLS was included as a control since NRIF3 is a nuclear protein (28). Initial experiments suggested that the number of green fluorescent cells is maximal 20 to 24 h after transfection. Therefore, in most of our studies, a TUNEL assay was carried out about 20 h after transfection. We found that GFP-NRIF3-expressing cells were TUNEL positive while GFPNLS-express-

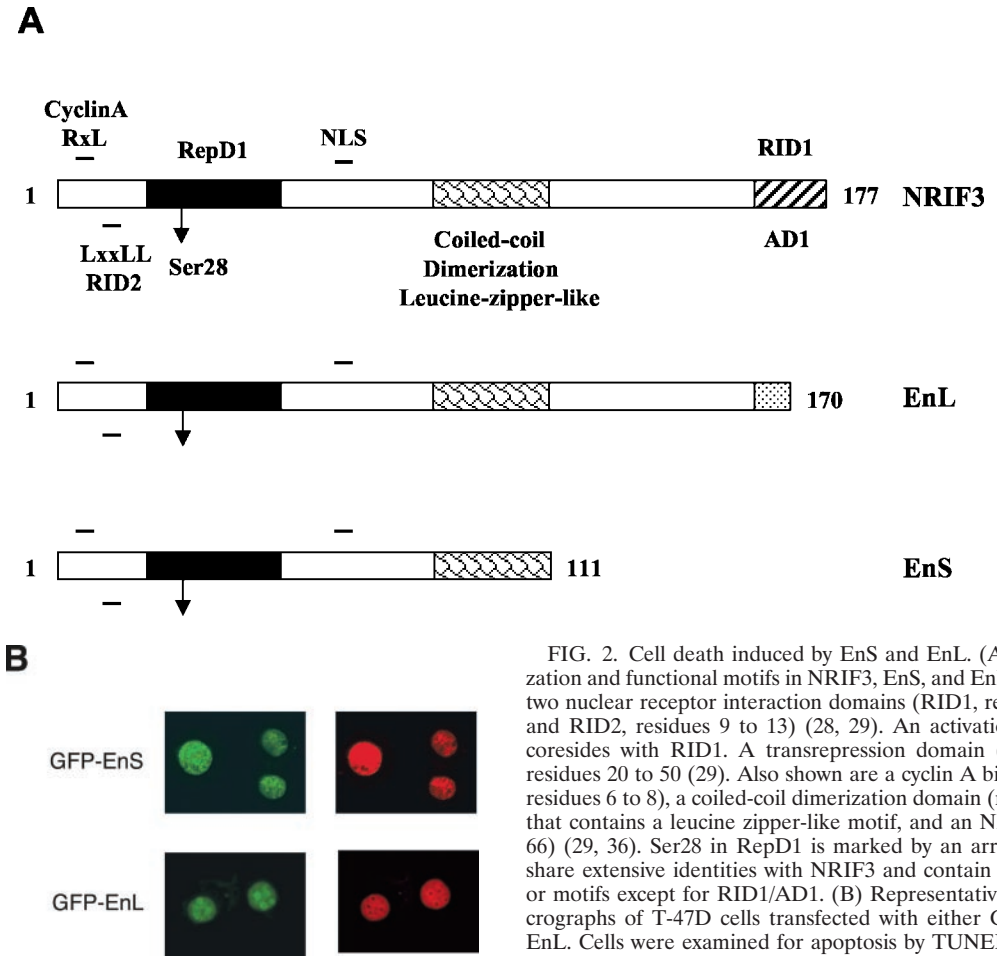


FIG. 2. Cell death induced by EnS and EnL. (A) Domain organization and functional motifs in NRIF3, EnS, and EnL. NRIF3 contains two nuclear receptor interaction domains (RID1, residues 162 to 177, and RID2, residues 9 to 13) (28, 29). An activation domain (AD1) coresides with RID1. A transrepression domain (RepD1) maps to residues 20 to 50 (29). Also shown are a cyclin A binding motif (RxL; residues 6 to 8), a coiled-coil dimerization domain (residues 86 to 112) that contains a leucine zipper-like motif, and an NLS (residues 63 to 66) (29, 36). Ser28 in RepD1 is marked by an arrow. EnS and EnL share extensive identities with NRIF3 and contain the same domains or motifs except for RID1/AD1. (B) Representative fluorescence micrographs of T-47D cells transfected with either GFP-EnS or GFP-EnL. Cells were examined for apoptosis by TUNEL assay (red).

ing cells were TUNEL negative (Fig. 1B). Quantitative analyses revealed that nearly 100% of GFP-NRIF3-expressing cells were TUNEL positive within 24 h after transfection, compared with little or no TUNEL reaction for GFPNLS-expressing cells (Fig. 1C). To rule out the possibility that cell death mediated by GFP-NRIF3 results from the fusion of GFP and NRIF3 instead of NRIF3 itself, we also transfected T-47D cells with a vector expressing full-length wild-type NRIF3 (not as a GFP fusion) and found that the transfected cells also underwent apoptosis (data not shown). Taken together, our results indicate that expression of NRIF3 induces rapid and profound death in T-47D cells via apoptosis or an apoptosis-like process.

Other members of the NRIF3 family induce apoptosis in T-47D cells. The human NRIF3 gene encodes several different proteins as a result of alternative splicing, including NRIF3, EnS (endonexin short form), and EnL (endonexin long form) (28, 29, 44). Both EnS and EnL share extensive identity with NRIF3 at the amino acid level (28, 29) (Fig. 2A). EnS is identical to the first 111 amino acids of NRIF3 and thus can be viewed as a naturally occurring truncation of NRIF3. Like NRIF3, EnS and EnL are primarily nucleus localized and together with NRIF3 constitute a new family of transcriptional coregulators (29). Given the similarity among the NRIF3 family members, we asked whether EnS and EnL also induce apoptosis in T-47D cells. To this end, T-47D cells were transfected with vectors expressing GFP-EnS or GFP-EnL and, 20 h later, were analyzed for expression of the GFP fusion proteins and for apoptosis. We found that expression of GFP-EnS or GFP-EnL resulted in profound apoptosis (nearly 100% of green cells were TUNEL positive), indicating that the N-terminal portion of NRIF3 (residues 1 to 111) is sufficient to induce death in T-47D cells (Fig. 2B and data not shown).

NRIF3 contains a novel death domain (DD1). The region comprising the first 111 amino acids of NRIF3 (equivalent to EnS), which is sufficient to induce apoptosis, contains a number of structural and functional features identified in previous studies (29, 36). These include a coiled-coil domain (residues 84 to 112) that mediates protein-protein interactions, a putative NLS (residues 63 to 66), a transrepression domain (residues 20 to 50, RepD1), an LXXLL motif (residues 9 to 13) that plays a role in interaction with certain nuclear receptors, and an RxL motif (residues 6 to 8) that binds cyclin A and mediates interaction with cyclin A-Cdk2 (Fig. 2A). To test whether any of these known domains or motifs are required for NRIF3-mediated apoptosis and to further map the functional death domain in NRIF3, we generated a series of GFP vectors expressing various regions of the N-terminal 111 amino acids of NRIF3. These GFP fusion constructs were then individually expressed in T-47D cells to examine induction of apoptosis (Fig. 3A).

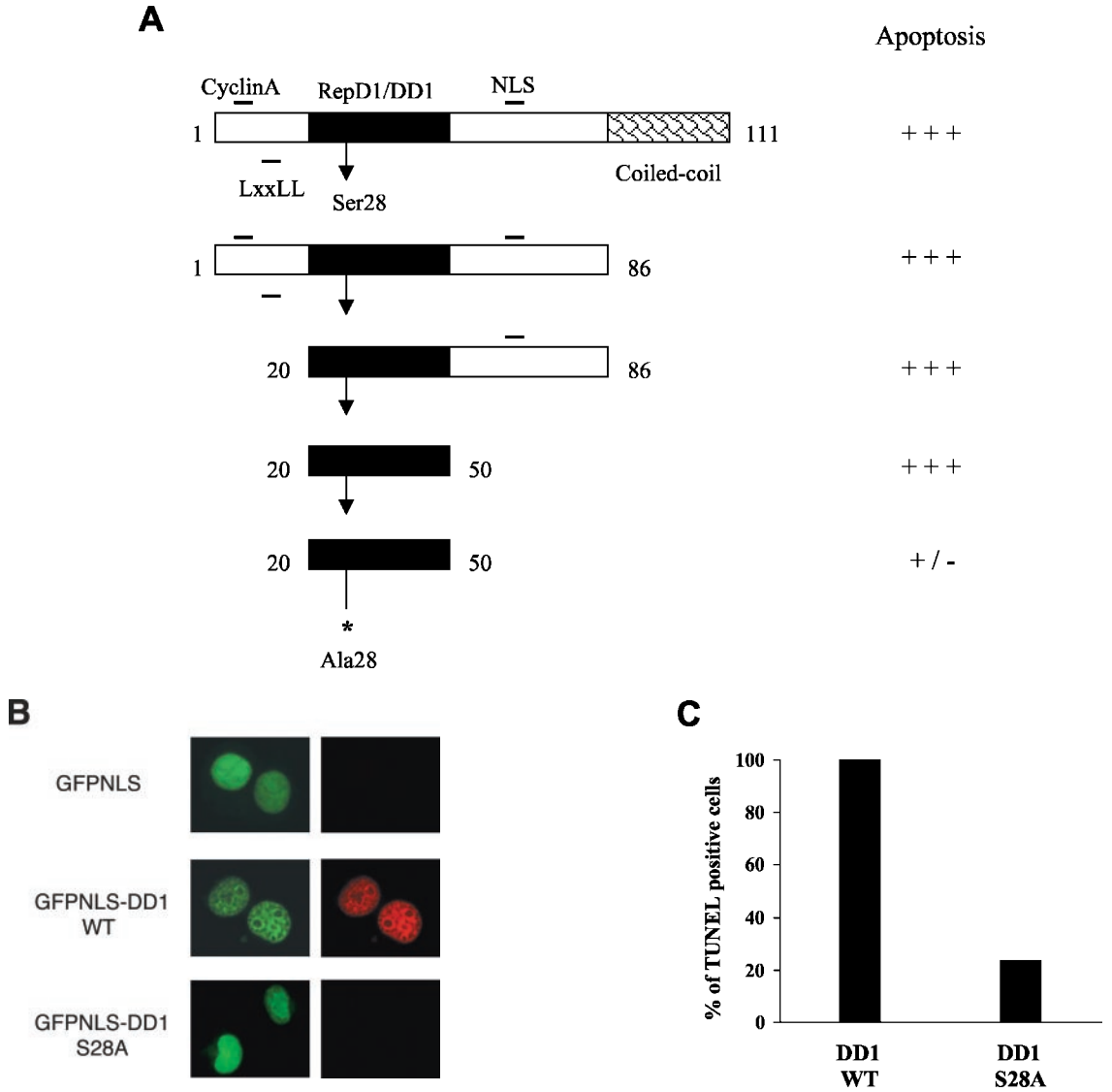


FIG. 3. NRIF3 contains a novel death domain (DD1). (A) T-47D cells were transfected with each of the indicated constructs expressing various regions of NRIF3 fused to GFP or GFPNLS. Ser28 in wild-type DD1 is marked by an arrow, while Ala28 in the mutant DD1 is marked by an asterisk. These regions were expressed either as a GFP fusion or, for those lacking an intrinsic NLS, as a GFPNLS fusion. Green fluorescent cells were scored for apoptosis by TUNEL assay, annexin V staining, or both. "+++" indicates profound cell death, where nearly 100% of green cells displayed positive staining for TUNEL and/or annexin V, while "−" indicates no apoptosis (less than 2%). (B) Representative fluorescence micrographs of T-47D cells transfected with GFPNLS, wild-type (WT) GFPNLS-DD1 (residues 20 to 50 of NRIF3), or the GFPNLS-DD1 mutant (S28A). Cells were examined for apoptosis by TUNEL assay (red). (C) The Ser28-to-Ala mutation severely compromises the apoptogenic function of DD1. The percentages of green fluorescent cells that were TUNEL positive were scored for T-47D cells transfected with either wild-type (WT) GFPNLS-DD1 or the mutant GFPNLS-DD1S28A.

Expression of regions comprising amino acids 1 to 86 or 20 to 86 was found to efficiently induce apoptosis in T-47D cells, indicating that the coiled-coil domain (residues 84 to 112), the cyclin A binding motif (residues 6 to 8), and the LxxLL motif (residues 9 to 13) are all dispensable for the apoptogenic effect of NRIF3 (Fig. 3A). The region comprising residues 20 to 86 of NRIF3 contains a previously identified transrepression domain, RepD1 (residues 20 to 50) (29). Thus, we further examined RepD1 and found that its expression efficiently induced death in T-47D cells (Fig. 3A and B). Taken together, these results identify a novel death domain (residues 20 to 50, designated here as DD1) in the NRIF3 family, which, interestingly, coresides with RepD1 (Fig. 3A). We detected no homology for DD1 with other known death domains in the database.

Our previous study of other cell lines identified a putative phosphorylation site (Ser28) in RepD1 (29). Phosphorylation of Ser28 appears to be essential for the transcriptional repression function of RepD1, as a change of Ser28 to Ala (S28A) abolishes repression (29). To test the potential role of Ser28 phosphorylation in apoptosis, we examined the DD1/RepD1 S28A mutant and found that this mutation markedly reduced the ability of DD1 to induce apoptosis in T-47D cells (Fig. 3B

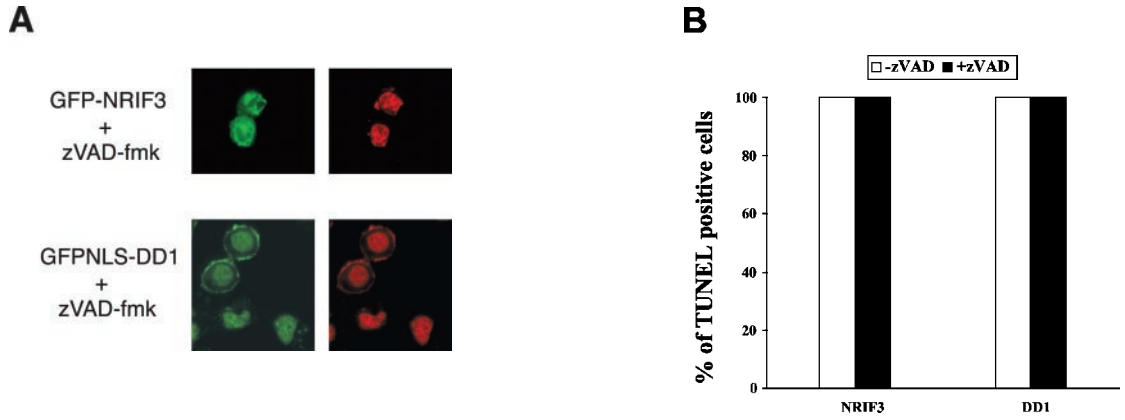


FIG. 4. Cell death mediated by NRIF3 or DD1 is insensitive to zVAD-fmk. (A) T-47D cells were transfected with either GFP-NRIF3 or GFPNLS-DD1 in the absence or presence of the broad-spectrum caspase inhibitor zVAD-fmk. When present, the inhibitor was incubated with the cells before and after transfection. Cells were examined for apoptosis by TUNEL assay (red). Representative fluorescence micrographs are shown for cells treated with zVAD-fmk. (B) Quantitative presentation of the experiments in panel A. The percentages of green fluorescent cells that were TUNEL positive were scored for T-47D cells transfected with either GFP-NRIF3 or GFPNLS-DD1 in the absence or presence of zVAD-fmk.

and C), suggesting that phosphorylation of Ser28 in vivo is important for the apoptogenic function of DD1.

NRIF3- and DD1-mediated apoptosis is insensitive to zVAD-fmk. Since activation of caspases is central to many cell death programs, we tested whether zVAD-fmk, a broad-spectrum irreversible caspase inhibitor (14, 47), has any effect on NRIF3- and DD1-mediated apoptosis in T-47D cells. For these studies, T-47D cells were incubated with 100 μM zVAD-fmk prior to and after transfection with appropriate GFP fusion constructs. We found that zVAD-fmk did not inhibit apoptosis mediated by GFP-NRIF3 or GFPNLS-DD1 (Fig. 4). zVAD-fmk alone did not cause cell death (data not shown). The same dose of zVAD-fmk was found to significantly inhibit apoptosis of HeLa cells treated with TNF-α and cycloheximide (data not shown), where the apoptotic process is dependent on caspase 8 and a downstream effector caspase(s) (10, 18, 34). Taken together, our results indicate that NRIF3- and DD1-mediated apoptosis in T-47D is insensitive to zVAD-fmk.

Role of mitochondria in DD1-mediated cell death. MMP is a critical event in the intrinsic apoptosis pathway as it results in the release of a number of death-promoting molecules such as cytochrome *c* and AIF (38, 50). Members of the Bcl-2 family regulate mitochondrion-mediated cell death by controlling MMP, which is determined by the relative balance of proapoptotic (e.g., Bak, Bax, Bad, and Bid) and antiapoptotic (e.g., Bcl-2 and Bcl-xL) members of the family (3, 7). To assess whether DD1-induced cell death involves a mitochondrion-mediated pathway, we examined whether the apoptogenic effect of DD1 was inhibited by Bcl-2. We found that coexpression of Bcl-2 significantly inhibited DD1-mediated apoptosis in T-47D cells (Fig. 5A and B). Similar inhibition was found with Bcl-xL (data not shown). These results suggest that MMP plays a role in DD1-induced apoptosis.

To further document that DD1 induces MMP, we examined whether AIF is translocated from mitochondria to the nucleus during DD1-mediated cell death, using a vector expressing AIF-GFP (31). Consistent with a previous study (31), expression of AIF-GFP alone resulted in a mitochondrial pattern of distribution (Fig. 5C). Interestingly, coexpression of DD1 resulted in the translocation of AIF-GFP from mitochondria to the nucleus as early as 5 h after transfection (Fig. 5C). Cells containing nucleus-localized AIF-GFP also underwent apoptosis as assessed by TUNEL assay (Fig. 5C). Taken together, our results in Fig. 5 support a model whereby the DD1 of NRIF3 promotes apoptosis in T-47D cells through a mitochondrion-mediated pathway that is regulated by Bcl-2 and involves the translocation of AIF. Since AIF promotes effector caspase-independent apoptosis (8, 38), its translocation during DD1-induced cell death is consistent with our earlier finding that this death program is insensitive to zVAD-fmk.

Requirement for caspase 2 in DD1-mediated apoptosis. A number of recent studies suggest an important role for caspase 2 in the intrinsic (stress-induced) apoptosis pathway in certain cell types where its activity is required for promoting MMP (17, 24, 27, 42). Although caspase 2 was reported to be present in a number of cellular compartments (for a review, see reference 48), recent studies suggest that it is mainly a nuclear protein and that it can trigger MMP and apoptosis from the nucleus without redistribution to the cytoplasm (4, 12, 37, 45). The fact that members of the NRIF3 family are nuclear proteins raises the possibility that the DD1 of NRIF3 might act through activation of nucleus-localized caspase 2. The finding that DD1-induced cell death is not inhibited by zVAD-fmk does not exclude a potential role for caspase 2, as caspase 2 is several orders of magnitude more resistant to zVAD-fmk than are other caspases (14).

To explore this, we used an siRNA that had been previously shown to efficiently silence human caspase 2 expression in transfected cells (27). T-47D cells were first transfected with this caspase 2 siRNA. Forty-eight hours later the cells were then harvested, replated, and transfected with GFPNLS-DD1 to monitor apoptosis induced by DD1. We found that DD1-mediated apoptosis was dramatically reduced in cells treated with the caspase 2 siRNA (Fig. 6A and C). In contrast, mock-treated cells that did not receive caspase 2 siRNA underwent rapid apoptosis upon expression of DD1 (Fig. 6A and C).

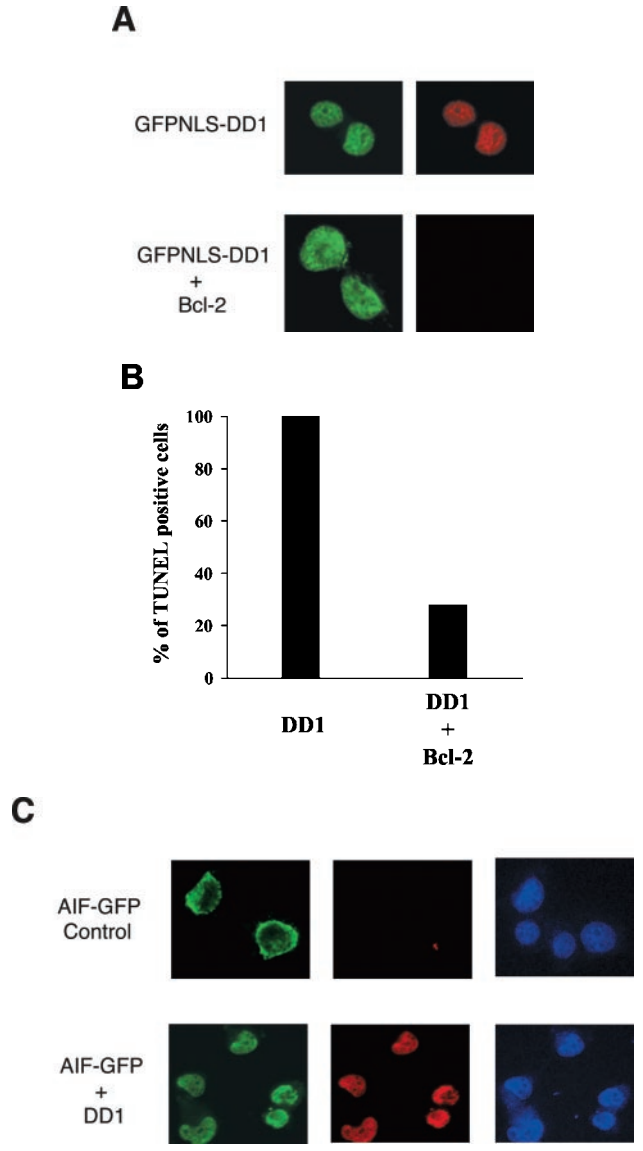


FIG. 5. DD1-mediated cell death involves MMP. (A) Representative fluorescence micrographs of T-47D cells transfected either with GFPNLS-DD1 or with GFPNLS-DD1 and Bcl-2. Cells were examined for apoptosis by TUNEL assay (red). (B) Quantitative presentation of the experiments in panel A. The percentages of green fluorescent cells that were TUNEL positive were scored for T-47D cells transfected either with GFPNLS-DD1 or with GFPNLS-DD1 and Bcl-2. (C) T-47D cells were transfected to express AIF-GFP along with either a control vector or a vector expressing DD1. Approximately 5 h after transfection the cells were fixed and subjected to TUNEL assay. The cells were then examined by fluorescence microscopy for subcellular localization of AIF-GFP (green) and for apoptosis (red). Nuclei were stained with Hoechst dye (blue). Representative fluorescence micrographs of cells transfected with the control vector or DD1 are compared.

Western analysis indicated that the level of caspase 2 protein was reduced by more than fivefold in specific siRNA-treated cells, demonstrating the effectiveness of the siRNA technique (data not shown). Consistent with the siRNA study, we found that zVDVAD-fmk, a chemical inhibitor of caspase 2, also efficiently blocked DD1-induced apoptosis in T-47D cells, suggesting a requirement for caspase 2 activity (data not shown). Interestingly, pretreatment with the same caspase 2 siRNA had no effect on etoposide-induced apoptosis in T-47D cells (Fig. 6B), indicating that caspase 2 is not universally required for initiating apoptosis per se in these cells. The result of our etoposide study is reminiscent of the finding by Lassus et al. showing that MCF-7 cells did not require caspase 2 for DNA damage-induced release of cytochrome *c* (27). Thus, while caspase 2 is not essential for every intrinsic apoptotic program, our study here identifies a specific role for caspase 2 in DD1-mediated apoptosis in T-47D cells.

Cell type specificity in cytotoxicity mediated by NRIF3 and DD1. We previously reported that expression of NRIF3 enhances ligand-dependent transactivation by TR or RXR in HeLa cells (28). Thus, the finding of an apoptogenic function for NRIF3 in T-47D cells was unexpected. One possibility is that the apoptogenic effect of NRIF3 or DD1 is cell type specific. In support of this notion, we found that expression of GFP-NRIF3 or GFPNLS-DD1 did not lead to apoptosis in HeLa cells (Fig. 7A).

The dramatic difference in cellular response to the expression of NRIF3 in T-47D cells (which exhibit 100% apoptosis) and HeLa cells (which show no apoptosis) suggests cell type specificity in the cytotoxic effect mediated by NRIF3 and DD1. To further explore this, we examined 11 cell lines for the induction of apoptosis upon expression of NRIF3 or DD1. Five were breast cancer cell lines, including ER^+ T-47D and MCF-7 cells and ER^- MDA-MB-231 and MDA-MB-435 cells, as well as an ER^+ derivative of MDA-MB-231 (2). We also examined HBL100 cells, a nonmalignant but immortalized breast epithelial cell line (13). Remarkably, we found that expression of NRIF3 or DD1 resulted in efficient apoptosis in all five breast cancer cell lines, as well as in the immortalized HBL100 cells (Fig. 7 and data not shown).

In contrast, expression of NRIF3 or DD1 did not lead to apoptosis in any of the other five tumor cell lines examined (HeLa, 293, Cos-1, UOK145, and GH4C1) which are not derived from breast epithelium (Fig. 7B). Thus, NRIF3 and DD1 appear to selectively induce apoptosis in breast cancer or related cells but not in the other cell types examined. This finding suggests that breast cancer cells contain a novel death switch that is specifically triggered by NRIF3 or DD1 (Fig. 8). We propose that triggering of this switch by NRIF3 (or DD1) results in activation of caspase 2, which in turn leads to further downstream events of apoptosis (Fig. 8).

DISCUSSION

In this study we present a novel finding that expression of members of the NRIF3 family of coregulators leads to rapid and profound apoptosis in a number of different breast cancer cell lines. Deletion analysis showed that the apoptogenic effect of the NRIF3 family is mediated by a novel death domain (DD1) (residues 20 to 50 [Fig. 3]). Interestingly, the cytotoxicity of NRIF3 and DD1 appears to be specific to breast cancer or related cells, as their expression does not lead to apoptosis in other cell types such as HeLa cells (Fig. 7). Consistent with this, our previous study showed that NRIF3 acts as a coactivator for TR and RXR in HeLa cells (28).

Apoptosis plays important roles in both normal tissue ho-

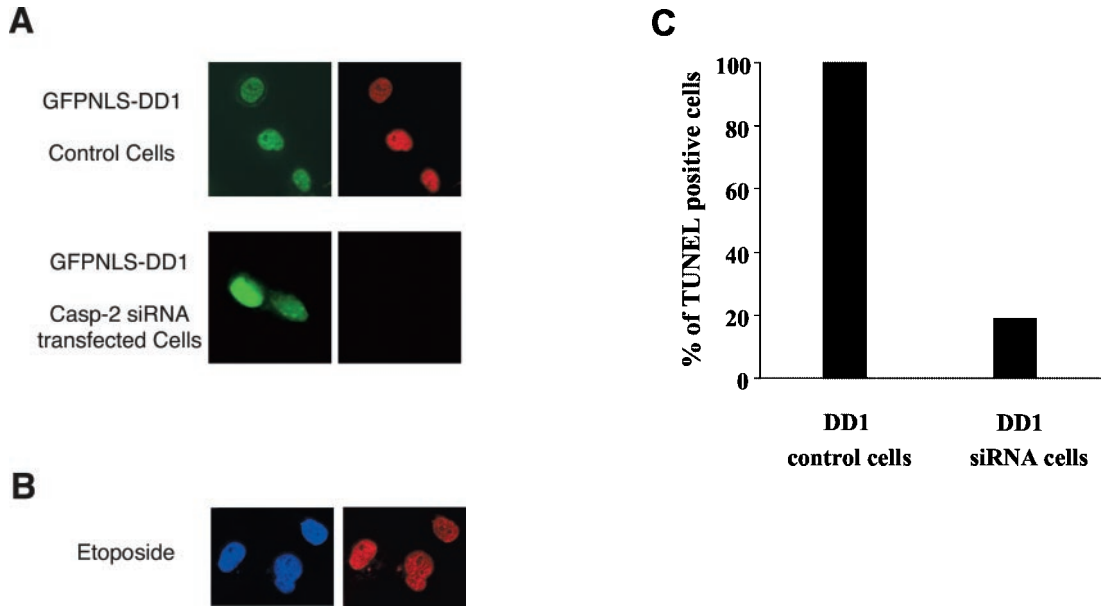


FIG. 6. Requirement for caspase 2 in DD1-mediated apoptosis. (A) T-47D cells pretreated with caspase 2 siRNA or mock-treated control cells were transfected with GFPNLS-DD1. Cells were examined for apoptosis by TUNEL assay (red). Representative fluorescence micrographs of siRNA-treated or mock-treated cells are compared. (B) T-47D cells pretreated with caspase 2 siRNA or mock-treated control cells were incubated with etoposide. Cells were examined for apoptosis by TUNEL assay (red), while the nuclei were visualized by Hoechst staining (blue). Representative fluorescence micrographs are shown for the siRNA-treated cells. Similar results were observed for the mock-treated control cells (data not shown). (C) Quantitative presentation of the experiments in panel A. The percentages of green fluorescent cells (expressing GFPNLS-DD1) that were TUNEL positive were scored for cells pretreated with caspase 2 siRNA or for mock-treated control cells.

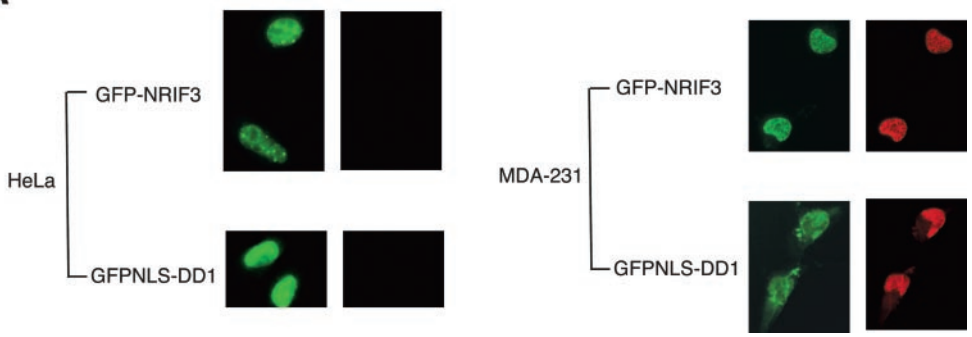
meostasis and pathological processes such as tumorigenesis. Disruption of cellular apoptotic pathways often accompanies tumorigenesis and likely confers a survival advantage on tumor cells (20). Since many anticancer drugs kill by inducing apoptosis, dysregulation of apoptosis could also lead to drug resistance (20). Although the progress in understanding mechanisms underlying apoptosis and its regulation and dysregulation in cancer cells presents opportunities to utilize the cellular death machinery for treating human diseases such as cancer (20, 35), how to deliver tumor-specific cytotoxicity without killing other innocent cells remains an important challenge.

Our study here provides an example in which cytotoxicity can be selectively induced in a specific cancer, as NRIF3 or DD1 appears to kill breast cancer but not other cells (Fig. 7). Since DD1 is devoid of regions involved in interaction with nuclear receptors (28, 29), it is unlikely that apoptosis mediated by NRIF3 or DD1 results from perturbation of nuclear receptor functions. DD1-induced apoptosis is inhibited by coexpression of Bcl-2 or Bcl-xL (Fig. 5A and B and data not shown) and is associated with the translocation of AIF from mitochondria to the nucleus (Fig. 5C), suggesting that cell death is mediated by a mitochondrial pathway (Fig. 8). Since AIF is capable of triggering apoptosis independently of effector caspases (8, 31, 46, 51), its rapid translocation from mitochondria to the nucleus is consistent with the finding that the broad-spectrum caspase inhibitor zVAD-fmk fails to block NRIF3- or DD1-induced cell death (Fig. 4).

The results of our siRNA study indicate that DD1-mediated apoptosis requires caspase 2 (Fig. 6). The same caspase 2 siRNA has no effect on etoposide-induced apoptosis in T-47D cells (Fig. 6B), suggesting that its effect on DD1-mediated cell death is specific. The requirement for caspase 2 is not inconsistent with our zVAD-fmk results, as caspase 2 is much more resistant to zVAD-fmk than are other caspases (14). Interestingly, several recent studies have identified a novel apical initiator role for caspase 2 during stress-induced apoptosis, where it acts upstream of mitochondria and is required for MMP (17, 27, 42). Thus, our results are best interpreted in a model whereby expression of NRIF3 or DD1 in breast cancer cells triggers activation of caspase 2, which then promotes MMP, leading to the release of AIF and subsequent downstream events of apoptosis (Fig. 8). Interestingly, expression of caspase 2-GFP in T-47D cells results in nuclear localization of the GFP signal (H. Li, personal communication). This finding is consistent with our model, as members of the NRIF3 family and DD1 trigger apoptosis in breast cancer cells while being primarily (if not exclusively) localized to the nucleus (Fig. 1B, 2B, and 3B).

Although caspase 2 functions upstream of mitochondria in certain intrinsic apoptotic pathways, the molecular mechanism(s) underlying activation of caspase 2 (upon apoptotic stimuli) is not yet understood (24, 48). Activation of other initiator caspases such as caspases 8 and 9 is thought be to be mediated by a dimerization mechanism that does not require proteolytic cleavage (6). It is conceivable that caspase 2 could be activated similarly by complex formation and/or dimerization (9, 40, 48). In this respect, NRIF3 or DD1 may function in breast cancer cells by regulating and/or participating in the process of caspase 2 activation. Interestingly, Western blot analysis showed that caspase 2 is expressed at similar levels in T-47D, MDA-MB-231, HeLa, and 293 cells (unpublished ob-

A



B

Cell lines		Comments	Apoptosis Induction	
			NRIF3	DD1
Breast Cancer Cells	T-47D	Human, ER+, ductal carcinoma	+++	+++
	MCF-7	Human, ER+, adenocarcinoma	+++	+++
	MDA-231	Human, ER-, adenocarcinoma	+++	+++
	MDA-231/ER+	MDA-231 stably expressing ER	+++	+++
	MDA-435	Human, ER-, ductal carcinoma	+++	+++
Non-Breast-Cancer Cells	HeLa	Human cervix adenocarcinoma	-	-
	293	Human kidney transformed	-	-
	UOK-145	Human kidney carcinoma	-	-
	Cos-1	Monkey kidney transformed	-	-
	GH4C1	Rat pituitary tumor	-	-

FIG. 7. Cell type specificity in cytotoxicity mediated by NRIF3 and DD1. (A) Various breast cancer cell lines and other cells were transfected with either GFP-NRIF3 or GFPNLS-DD1. Cells were examined for apoptosis by TUNEL assay (red). Representative fluorescence micrographs of transfected HeLa and MDA-MB-231 cells are compared. (B) Summary of results from all cell lines examined in panel A. "+++" indicates profound apoptosis (>90% of green fluorescent cells were TUNEL positive) while "−" indicates no apoptosis (less than 2% positive).

servations), despite the fact that NRIF3 or DD1 induces cytotoxicity only in T-47D and MDA-MB-231 cells, not in HeLa and 293 cells (Fig. 7). Thus, a simple mechanism such as direct activation of caspase 2 by NRIF3 or DD1 via a bilateral protein-protein interaction seems unlikely.

The detailed mechanism(s) notwithstanding, our results indicate that cytotoxicity of NRIF3 and DD1 is cell type specific. It is possible that NRIF3 or DD1 could act by specifically tipping the balance between pro- and antiapoptotic factors in breast cancer cells, e.g., by blocking the effect of a specific antiapoptotic factor(s) or by enhancing the effect of a specific proapoptotic factor(s). However, a survey of a number of such factors (including Bcl-2, Bcl-xL, Bax, Bak, caspase 3, and p53) in a variety of breast cancer cells did not identify any unique pattern of expression among these cells (53). Thus, we suggest that the apoptogenic function of NRIF3 or DD1 is mediated by a yet-to-be-defined breast cancer cell-specific death switch. Triggering of this switch by NRIF3 or DD1 results in activation of caspase 2, which in turn leads to further downstream events of apoptosis such as MMP (model shown in Fig. 8). Despite an extensive literature on apoptosis in breast cancer cells, very few if any studies have explored the role of caspase 2. Elucidating the molecular nature of the death switch identified by our study should enhance not only the understanding of regulation of apoptosis in breast cancer cells but also provide insight into the mechanism(s) underlying caspase 2 activation in general.

The finding that the S28A mutation significantly reduced the ability of mutant DD1 to induce apoptosis in T-47D cells (Fig. 3B and C) suggests that apoptogenic activity of DD1 (or NRIF3) can be regulated by phosphorylation. However, the S28A mutant can still manifest some apoptogenic activity (about 20% cell death [Fig. 3C]), indicating that phosphorylation of Ser28 is not absolutely required for induction of apoptosis. In contrast, expression of DD1 results in no detectable cell death in nonresponsive cell lines such as HeLa and 293 (Fig. 7). In addition, our previous study indicated that phos-

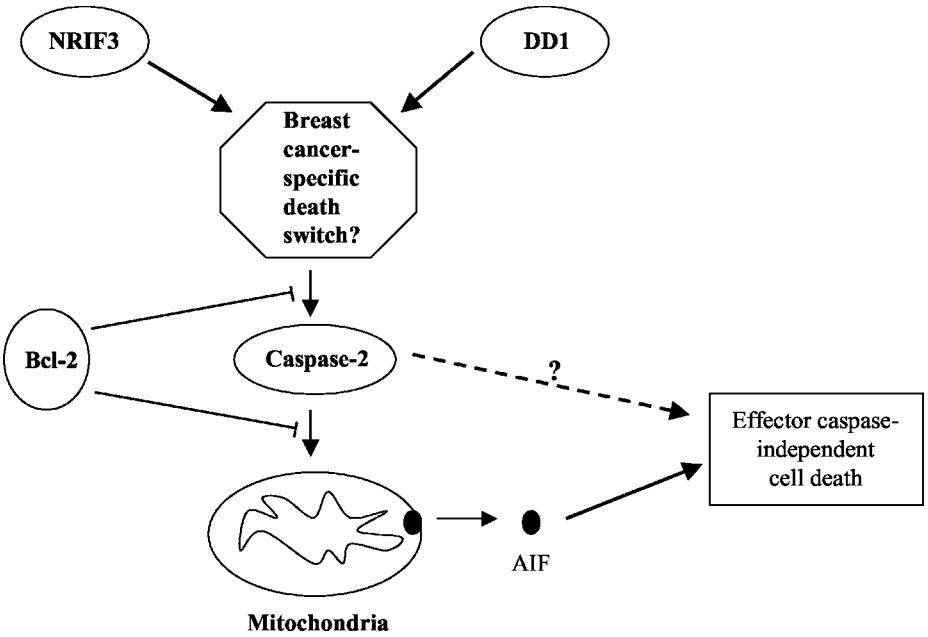


FIG. 8. Model for NRIF3- or DD1-induced apoptosis. We suggest that breast cancer cells contain a specific death switch that can be selectively triggered by NRIF3 or its death domain DD1. Triggering of this switch by NRIF3 or DD1 leads to activation of caspase 2. Activated caspase 2 promotes MMP, which results in the release of AIF. The released AIF then mediates effector caspase-independent cell death (which is insensitive to zVAD-fmk). The antiapoptotic factor Bcl-2 could inhibit this pathway by acting either upstream of caspase 2 (to prevent its activation) or downstream of caspase 2 (to prevent caspase 2-mediated MMP) (24). It is also possible that activated caspase 2 can directly elicit cell death (dashed line) in addition to the depicted mitochondrion-mediated pathway.

phorylation of Ser28 is essential for the transrepression function harbored by the same DD1 region (29). Interestingly, wild-type DD1 mediates significant repression in cell lines such as HeLa and GH4C1 while the repression is abolished by the S28A mutation (29). These results suggest that Ser28 is phosphorylated in HeLa or GH4C1 cells despite the finding that they do not undergo apoptosis in response to DD1 expression. Thus, differential (or lack of) phosphorylation of DD1 in different cell types is unlikely to be the main mechanism accounting for the breast cancer cell-specific death switch proposed in Fig. 8.

Our results here bear a number of potential therapeutic implications. Strategies utilizing the breast cancer-specific cytotoxicity of NRIF3 or DD1 may allow for future development of novel antitumor agents against breast cancer. The relatively compact size of DD1 (only ~30 amino acids) is an attractive feature in this respect. It is also interesting that phosphorylation of Ser28 appears to be important for the apoptogenic function of DD1 (Fig. 3B and C). Identification of the candidate kinase that phosphorylates DD1 and understanding the underlying regulatory mechanism may provide an opportunity to enhance apoptogenic function of NRIF3 or DD1 for therapeutic purposes. Low levels of NRIF3 mRNA can be detected in T-47D and MCF-7 cells by reverse transcription-PCR (unpublished observations). Future study of the mechanism(s) regulating expression of the NRIF3 family in breast cancer cells may lead to novel means to promote apoptosis in these cells by up-regulating their expression. Finally, our study raises an intriguing possibility that a cell-specific mechanism(s) might be employed in activation of caspase 2 and, thus, that similar but not identical death switches may exist in other cancer cells as well. Targeting these switches would represent a novel strategy in developing new and more selective therapeutics against cancer.

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