

## **An Apoptosis-inducing Isoform of Neu Differentiation Factor (NDF) Identified Using a Novel Screen for Dominant, Apoptosis-inducing Genes**

By Stefan Grimm and Philip Leder

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*From the Department of Genetics, Harvard Medical School and Howard Hughes Medical Institute, Boston, Massachusetts 02115*

### **Summary**

Apoptosis is a genetically programmed series of events that results in cell death. As a consequence, it is difficult to identify dominant genes that play a role in this process using genetic selections in conventional cell culture systems. Accordingly, we have established an efficient expression screen to isolate dominant, apoptosis-inducing genes. The assay is based on the apoptotic morphology induced in the human kidney cell line 293 after transient transfection of small plasmid pools from normalized cDNA expression libraries. Using this assay, we isolated a novel isoform of the proto-oncogene Neu differentiation factor (NDF), a ligand for erbB receptor tyrosine kinases. Several lines of experimental evidence indicate that this gene kills in a cell-autonomous fashion and independently of known erbB receptors. This apoptotic property of an NDF isoform is readily contrasted with NDF's transforming potential and might balance the tendency to tumorigenesis in cells that overexpress NDF.

Considerable progress has been made in unraveling the genetic elements of programmed cell death in lower organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* (1, 2). Higher mammalian systems might possess even more elaborate genetic pathways leading to the induction of apoptosis. However, since mammals are less amenable to genetic manipulation, our knowledge of their relevant genes might still be largely incomplete. Two genetic approaches in mammalian cells have been described which facilitate the isolation of genes that inhibit apoptosis (3, 4). Due to the transient apoptotic phenotype and the subsequent disappearance of the dead cells, the isolation of dominant genes that lead to the induction of apoptosis did not seem practical. Nonetheless, here we report a simple genetic screen for dominant, apoptosis-inducing genes that is based on easily scored morphological changes in apoptotic cells. Using this screen, we have isolated a novel isoform of the precursor protein for the protooncogene NDF (Neu differentiation factor) which induces apoptosis on overexpression.

### **Materials and Methods**

*Isolation of Apoptosis-inducing Genes.* Kidney mRNA from 4–6-wk-old FVB mice was normalized as described (5). The procedure uses the rapid association of abundant mRNA species with their cDNAs covalently attached to latex beads which are subsequently removed by centrifugation. After two rounds of hybridization, 200 ng (of initially 2  $\mu$ g) mRNA were retrieved and used to generate a cDNA library with a cDNA synthesis kit (GIBCO

BRL, Gaithersburg, MD). After ligation of a BstXI adaptor (Invitrogen, San Diego, CA) and a NotI digest, the cDNAs were inserted into a modified pcDNA3 vector (Invitrogen) in which the neomycin resistance gene had been deleted. The DNA was electroporated into *Escherichia coli* SURE cells (Stratagene Corp., La Jolla, CA) which were immediately frozen. Plating of aliquots revealed that the library contained  $\sim 1.1 \times 10^5$  independent clones. Aliquots corresponding to 20 clones were thawed and grown up. Miniprep DNA was isolated as described (6) and transfected into 293 cells in 24-well plates using the calcium phosphate co-precipitation method. After 18 h, the cells were inspected for apoptosis induction with phase contrast microscopy. The bacteria pool whose plasmid DNA caused morphological signs of apoptosis in 293 cells was spread on plates. Plasmid DNAs from individual bacteria colonies were again transfected into 293 cells to isolate the active clone.

*Cell Transfections.* Plasmid DNAs were isolated with Qiagen columns (Qiagen, Chatsworth, CA) and transfected into 293 or baby hamster kidney (BHK) cells with the calcium phosphate co-precipitation method as described (7).

*Expression Constructs.* The deletion mutants of  $\beta 2b$  NDF were created with PCR using suitable primers encompassing the  $\beta 2b$  NRG Kozak sequence, 200 ng template, and 20 cycles with Pwo (Boehringer Mannheim, Indianapolis, IN) which has proofreading activity. All constructs were transcribed and translated in vitro (TNT kit; Promega Corp., Madison, WI), and yielded products of the expected sizes (not shown). In each case, two NDF expression constructs were generated by two independent PCR reactions.

*Quantitative Apoptosis Assay.* BHK cells were transfected with the respective plasmids and a  $\beta$ -galactosidase ( $\beta$ -gal) expression vector. When PCR-generated NDF deletion constructs were

used, two independently generated constructs were transfected in two transfections each. 24 h later the cells were stained with X-gal and the extent of apoptosis was measured by counting morphologically apoptotic blue cells and determining their percentage of the total number of blue, transfected cells (8).

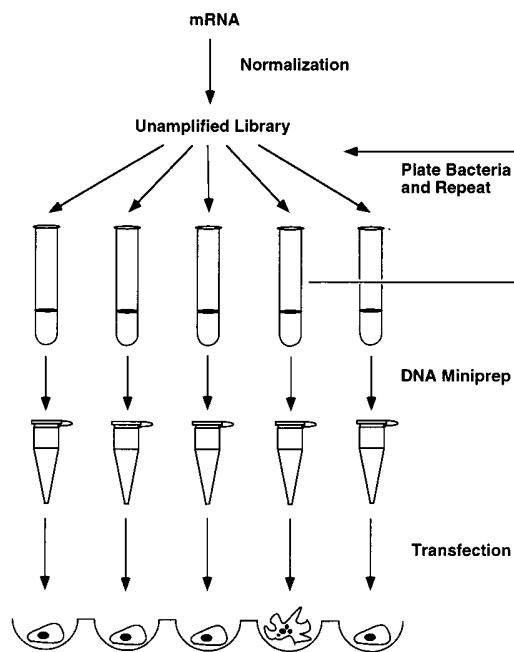
**DNA Isolation.** Low molecular weight DNA was isolated by lysing the cells with 0.2% Triton X-100 on ice for 10 min. After centrifugation, low molecular weight DNA was recovered in the supernatant and phenol-extracted. The samples were loaded on a 2% agarose gel after RNA digestion.

**Reverse Transcription PCR.** Total RNA was isolated (RNastat; Tel-Test Inc., Friendswood, TX) from the indicated tissues from two 6-wk-old FVB mice. For the reverse transcriptase (RT) PCR, 2  $\mu$ g of total RNA were reverse transcribed with oligo dT primers in a volume of 30  $\mu$ l. 5  $\mu$ l were used in a PCR reaction with primers for  $\beta$ -actin (Clontech, Palo Alto, CA), an oligo (5'-AGCTTCTACAAAGCGGAG-3') that spans the junction between the  $\beta$  and the 2 exon of  $\beta$ 2b NDF (hybridizing to 8 bp of the 2 exon and 10 bp of the b exon) and a primer (5'-ATATCTAGATAAAGGCCAAGGGGTC-3') that was complementary to the  $\beta$  exon of  $\beta$ 2b NDF. DNA templates for  $\beta$ -actin (~17 pg) and  $\beta$ 2b NDF (~200 pg) were used as positive controls, reverse transcribed tRNA was used as a negative control. An initial denaturation time of 1 min and 25 cycles of 30 s at 94°C, 1 min at 52°C, and 1 min at 68°C were used in the PCR. The reaction products were separated on 1% agarose gels and blots probed with mouse  $\beta$ -actin or  $\beta$ 2b NDF, respectively.

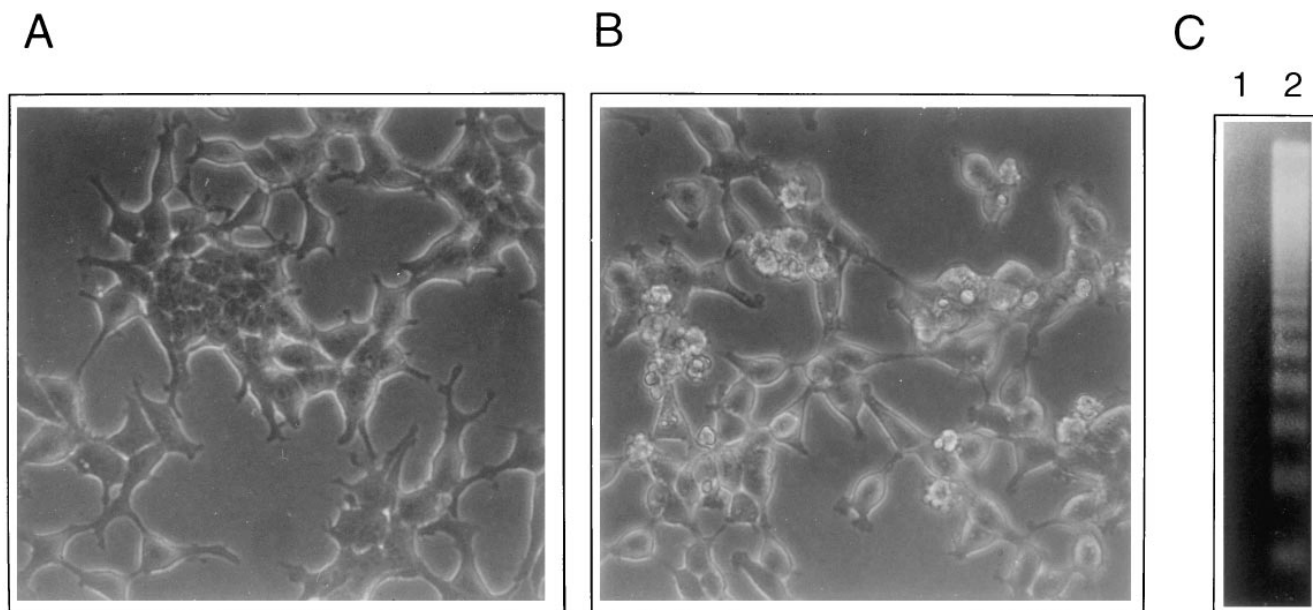
Primers for erbB receptors, amplification conditions, and hybridization with internal oligonucleotide probes were as described (9).

## Results

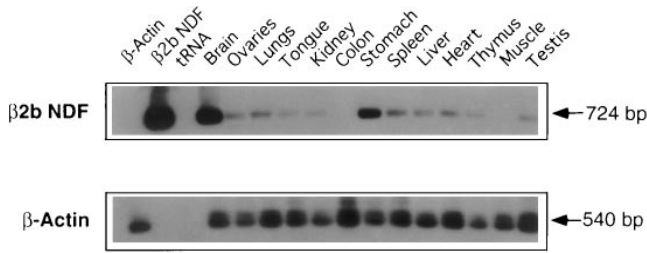
Reasoning that the machinery for apoptosis might already exist in latent form in dynamic organs (10), we used normal mouse kidney as a RNA source for constructing an



**Figure 1.** Outline of the assay for cloning apoptosis-inducing genes. After normalization of the mRNA, a library was constructed in a mammalian expression vector. DNAs from pools of ~20 bacteria clones were transiently transfected into 293 cells. The bacteria of the pool which induced morphological signs of apoptosis in cells (as indicated in the figure by a cell with altered shape) were plated and individual clone DNAs were once more transiently transfected. The positive clone was again identified by inspecting the cells for apoptosis induction.



**Figure 2.** Apoptosis induction by the isolated expression plasmid. 293 cells were transiently transfected with the empty vector (A) or the isolated plasmid clone (B). After 12 h, incubation phase contrast pictures at a 320-fold magnification were taken. (C) Expression of the isolated clone leads to DNA degradation. 293 cells were transfected with the vector (lane 1) or with the expression plasmid DNA (lane 2). After 16 h, low molecular weight DNA was isolated, separated on a 2% agarose gel, and stained with ethidium bromide.

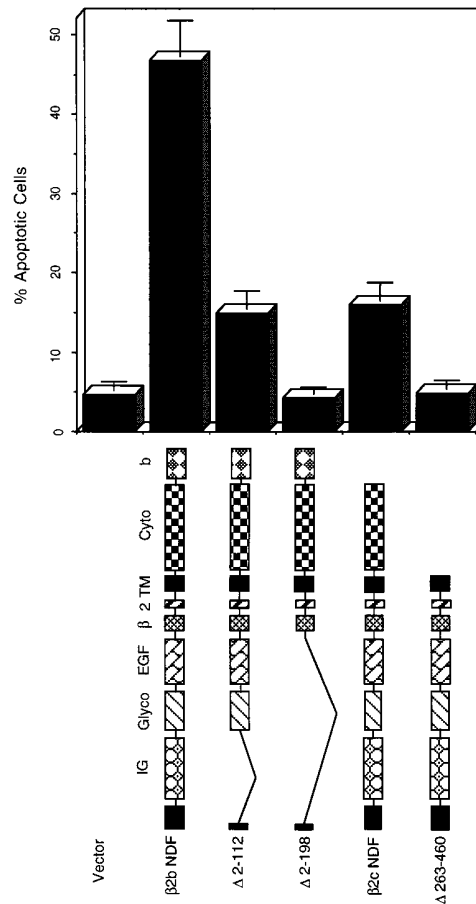


**Figure 3.** Expression profile of  $\beta 2b$  NDF in mice as measured by RT-PCR.  $\beta 2b$  NDF-specific primers were used to amplify a 724-bp long DNA. The efficiency of the RT reaction was controlled by co-amplifying a 540-bp long  $\beta$ -actin fragment. Both products were detected by Southern blotting. The  $\beta 2b$  NDF blot was exposed for 12 h, and the  $\beta$ -actin blot for 2 h.

expression library. To reduce the redundancy of the mRNA, we normalized its sequence representation (5). From the resulting, unamplified cDNA library, small aliquots were grown up and plasmid miniprep DNAs were transfected into the human kidney cell line 293 (Fig. 1). This cell line has the advantage of being highly transfectable and producing an obvious apoptotic phenotype with membrane blebbing and subsequent shrinkage of the cytoplasm. After screening 480 pools (~10% of the library) by phase contrast microscopy for apoptosis induction, a positive plasmid pool was detected. A single apoptosis-inducing cDNA clone was isolated from this pool (Fig. 2). Transfection of as little as 200 ng of this plasmid brought about apoptosis (not shown). Subsequent experiments demonstrated that this gene could also induce apoptosis in the hamster BHK cell line (see Fig. 4), emphasizing the phylogenetic conservation of this function. Isolation of low molecular weight DNA after transfecting the expression plasmid into 293 cells revealed nucleosomal DNA fragmentation, a biochemical hallmark of apoptosis (11; Fig. 2 C).

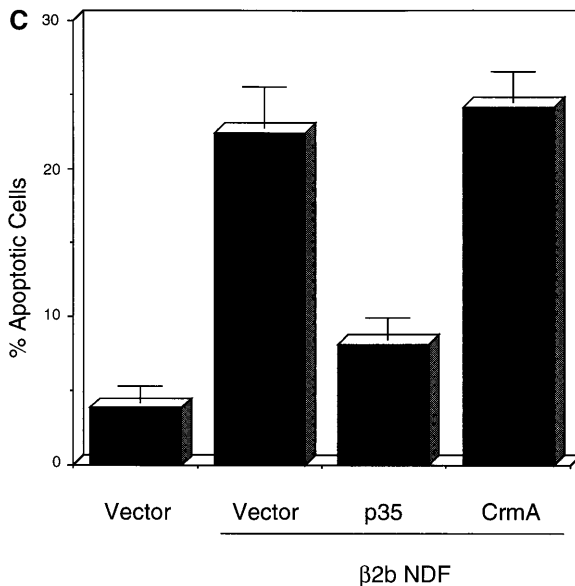
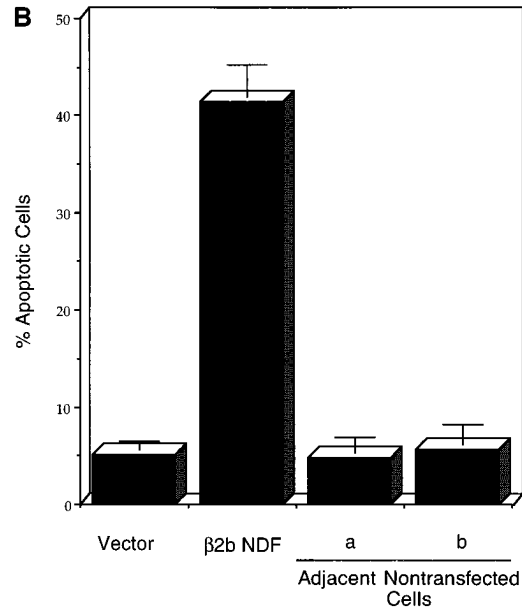
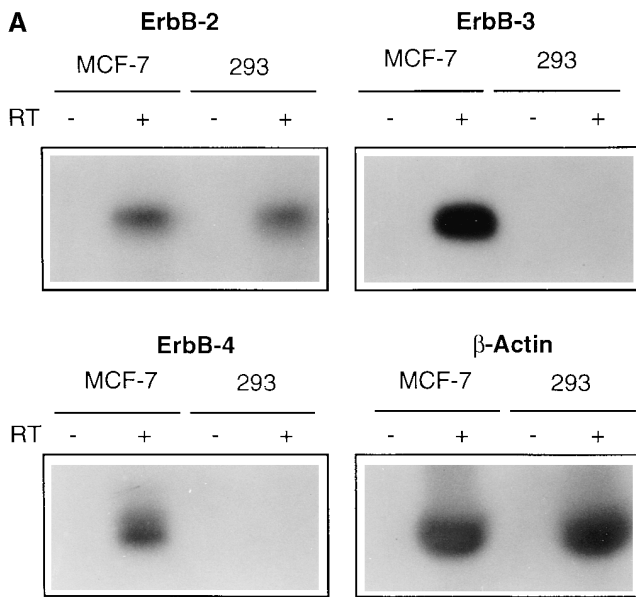
The 2.7-kb insert cDNA was sequenced and was surprisingly identified as a member of the NDF family (12, 13). NDF is a ligand for erbB receptor tyrosine kinases, some of which have been implicated in a variety of human carcinomas (14). NDF gives rise to multiple alternative splice forms which code for transmembrane precursor proteins from which the secreted NDF molecules are proteolytically generated. The particular isoform described here contains the  $\beta$ , 2, and b exons (see Fig. 4). This combination of exons has not been previously described, establishing  $\beta 2b$  NDF as a novel isoform of NDF. No sequence resemblance to genes known to be involved in apoptosis could be detected in the database. Subsequently, RT-PCR was used to determine the expression pattern of  $\beta 2b$  NDF in mice (Fig. 3). The results indicate that it is expressed at similar levels in most tissues examined with higher amounts in brain and in stomach. Colon and muscle did not produce a signal in this experiment; however, extensive PCR amplification revealed low levels of expression in these organs as well (not shown).

Since the NDF precursor proteins are composed of multiple subdomains, we wished to delineate those domains that are involved in apoptosis induction. Therefore, we en-



**Figure 4.** Mapping of the apoptosis-inducing domains in  $\beta 2b$  NDF with deletion mutants in BHK cells. The various domains are indicated on top of the diagrammatic representation of wild-type  $\beta 2b$  NDF. IG, immunoglobulin domain; Glyco, glycosylated domain; EGF, epidermal growth factor homology domain;  $\beta$ , sequences of  $\beta$  exon; 2, sequences from exon 2; TM, transmembrane domain; Cyto, cytoplasmic domain; b, sequences from exon b. The names of the deletion mutants indicate the deleted amino acids. Wild type or the deletion mutants of  $\beta 2b$  NDF (4  $\mu$ g) were transfected into BHK cells together with a marker plasmid (1  $\mu$ g) for  $\beta$ -gal staining. 24 h after transfection the cells were stained with X-gal and the extent of apoptosis was measured by counting morphologically apoptotic, blue cells and determining their percentage of the total number of blue, transfected cells. Shown are the means and the standard deviation of a total of at least 1,000 counted cells from four independent experiments.

gineered several deletion mutants of  $\beta 2b$  NDF and scored their ability to induce apoptosis after transient transfection in BHK cells. Fig. 4 shows that deleting the immunoglobulin homology domain (IG) reduced apoptosis induction by 68%. Further deletions into the epidermal growth factor homology domain abrogated the ability of  $\beta 2b$  NDF to cause cell death. However, mutant  $\Delta 2-198$  could weakly induce apoptosis in 293 cells which are more susceptible to cell death induction (not shown). Overexpression of another mutant encompassing only the cytoplasmic domain of  $\beta 2b$  NDF was also inactive in 293 cells (not shown). Interestingly, the intracellular portion, which is not part of the secreted moiety (15), was necessary for apoptosis induc-



**Figure 5.** Characterization of the apoptotic signal generated by β2b NDF. (A) Absence of NDF-binding erbB receptors in 293 cells as detected by RT-PCR. RNAs of the human mammary cell line MCF-7 and 293 cells were amplified, with or without earlier treatment with RT, with specific primers for the indicated genes. The PCR products were separated in agarose gels, blotted onto filters, and hybridized with gene-specific probes. β-actin was amplified to demonstrate the efficiency of the RT reactions. (B) Cells adjacent to NDF-transfected cells do not undergo apoptosis. BHK cells were transfected with the empty vector or β2b NDF (4 μg each) together with the marker plasmid for β-gal (1 μg), and apoptosis was monitored as in Fig. 4. Apoptosis was also measured in cells surrounding NDF-transfected, nonapoptotic (a) and apoptotic (b) cells. (C) β2b NDF-induced apoptosis is inhibitable by the ICE proteases inhibitor p35. BHK cells were transfected with β2b NDF (2 μg) and vector, CrmA, or p35 expression plasmids (2 μg). Apoptosis induction was measured as in Fig. 4.

tion (construct Δ 263–460 in Fig. 4). The importance of the intracellular domain of β2b NDF is also emphasized by a mutant with a deletion of the b exon corresponding to the recently described β2c NDF isoform (16). Its expression led to a 66% reduction in apoptosis. These data suggest that NDF does not act as a secreted molecule in cell death induction (see below).

The supernatant from NDF-transfected 293 cells was able to induce receptor tyrosine phosphorylation in MCF-7 cells indicating that 293 cells can faithfully process β2b NDF proteins (not shown). To further investigate whether a receptor–ligand interaction is involved in this apoptosis induction, we used a sensitive RT-PCR approach to detect the presence of erbB receptors. In accordance with previous reports (9, 17), no expression of NDF-binding erbB genes (erbB-3 and erbB-4) could be detected in 293 cells. How-

ever, erbB-2, which is unable to bind NDF (18), is expressed in 293 cells (Fig. 5 A). Stably transfecting erbB-2 and erbB-3 or transient co-transfection of a dominant-negative erbB-2 mutant (19) had no effect on β2b NDF's apoptosis induction (not shown). Furthermore, cells lying adjacent to NDF-transfected cells showed no signs of apoptosis (Fig. 5 B).

These results, the isolated appearance of apoptotic cells after transient transfection of β2b NDF (see Fig. 2 B), and the observation that the supernatant from transfected 293 cells did not induce cell death in untransfected cells (not shown) indicate that β2b NDF exerts its apoptotic effect cell-autonomously and independently of known erbB receptors.

To integrate the apoptotic signal initiated by β2b NDF into the cell death signal transduction pathway, we tried to block cell death with known inhibitors of apoptosis. Both,

Bcl-2 and Bcl-x, the prototypical apoptosis repressors (20), were capable of reducing cell death when co-transfected with  $\beta$ 2b NDF (not shown). A 2.8-fold repression of apoptosis was observed when baculovirus p35, an ICE protease inhibitor (21), was co-transfected indicating an activation of ICE-cysteine proteases in NDF-mediated apoptosis (Fig. 5 C). Interestingly, other inhibitors of ICE-like proteases, such as the cowpox gene CrmA (22), was without any effect (Fig. 5 C), as were the tetrapeptide ZVAD-FMK (23) and Ich-1s (24) (not shown).

## Discussion

In this study we have described the isolation of the novel  $\beta$ 2b NDF splice variant using a genetic screen for dominant, apoptosis-inducing genes. The isolation of this gene was unexpected. Though NDF was known to cause cellular responses of proliferation or growth arrest (13, 25), its isolation as an inducer of apoptosis was surprising. Since the  $\beta$ 2c isoform of NDF has been shown to function as an oncogene when overexpressed (16), it is worth noting that several other oncogenes, most notably c-myc (26), participate in seemingly contradictory responses characterized by proliferation or cell death. However, in contrast to these oncogenes, NDF's apoptosis induction is separated from the transformation process, since it can induce apoptosis in cells which do not express NDF-binding receptors. The secreted portion of NDF, which is identical between the  $\beta$ 2c and  $\beta$ 2b NDF isoforms, could therefore act as an oncoprotein

by engaging erbB receptor tyrosine kinases in neighboring cells, while the intracellular portion determines its apoptosis induction. Both functions are activated when NDF is overexpressed, but apoptosis induction can kill the NDF-overproducing cell and might thereby protect the organism against tumorigenesis. A functional role for the intracellular domain of NDF has hitherto been unclear, but is suggested by its evolutionary conservation (15). The different apoptotic activities of NDF splice variants in the intracellular domain might allow distinct expression levels in normal development without the induction of apoptosis.

Tumorigenesis by NDF overexpression would therefore require the inactivation of its apoptosis pathway. The genes in this signaling cascade are therefore good candidates for the "second hits" required for tumorigenesis which renders our findings an interesting subject for further studies.

Our assay system uses gene overexpression after transient transfection since most known apoptosis-inducing genes, like the ICE family of cysteine proteases, the "death domain" proteins and p53, achieve their effect at elevated concentrations. This might be the result of overcoming inhibitors and forming protein-protein interactions that stimulate the signal cascades leading to cell death. In any case, the power of our assay is demonstrated here by the identification of an unexpected and dominant property of the NDF precursor. We believe that this screen will uncover additional genes that lie at critical points in the genetically determined pathway that leads to programmed cell death.

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Address correspondence to Philip Leder, Department of Genetics, Harvard Medical School and Howard Hughes Medical Institute, 200 Longwood Ave., Boston, MA 02115.

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