Research

Identification of genes involved in ceramide-dependent neuronal apoptosis using cDNA arrays
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Abstract

Background: Ceramide is important in many cell responses, such as proliferation, differentiation, growth arrest and apoptosis. Elevated ceramide levels have been shown to induce apoptosis in primary neuronal cultures and neurally differentiated PC12 cells.

Results: To investigate gene expression during ceramide-dependent apoptosis, we carried out a global study of gene expression in neurally differentiated PC12 cells treated with C2-ceramide using an array of 9,120 cDNA clones. Although the criteria adopted for differential hybridization were stringent, modulation of expression of 239 genes was identified during the effector phase of C2-ceramide-induced cell death. We have made an attempt at classifying these genes on the basis of their putative functions, first with respect to known effects of ceramide or ceramide-mediated transduction systems, and then with respect to regulation of cell growth and apoptosis.

Conclusions: Our cell-culture model has enabled us to establish a profile of gene expression during the effector phase of ceramide-mediated cell death. Of the 239 genes that met the criteria for differential hybridization, 10 correspond to genes previously involved in C2-ceramide or TNF-α signaling pathways and 20 in neuronal disorders, oncogenesis or more broadly in the regulation of proliferation. The remaining 209 genes, with or without known functions, constitute a pool of genes potentially implicated in the regulation of neuronal cell death.

Background

Ceramide is an intracellular lipid second messenger generated in response to a large number of extracellular signals [1,2]. These include tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), ionizing and ultraviolet radiation, anti-cancer drugs, growth-factor withdrawal, infection by human immunodeficiency virus (HIV) or bacteria. It is reported to participate in cell differentiation [3], senescence [4], growth arrest or programmed cell death [1,2], depending on the cell type.

The role of ceramide in programmed cell death or apoptosis has been described in lymphocytes [5], macrophages [6], neurons in primary culture [7-8] and neurally differentiated
PC12 cells [9-11]. A number of downstream targets of ceramide have been identified. The best documented are the ceramide-activated protein phosphatases (CAPP) and the ceramide-activated protein kinase (CAPK). The former, represented by the PP1 and PP2A families, mediate the effect of ceramide on the transcription factors c-Myc [12] and c-Jun [13]. CAPK is involved in the mitogen-activated protein (MAP) kinase (MAPK) cascades that include the extracellular-signal regulated kinases (ERK), the c-Jun N-terminal kinases or stress-activated kinases (JNK/SNK/SAPK) and the p38 family [14].

Recently, it has been shown that C₂-ceramide rapidly decreases phosphorylation of ERKs, but increases p38 and JNK phosphorylation, activating the transcription factors c-Fos, c-Jun and p53, during the effector phase of apoptosis in primary cortical neurons [15]. It also regulates the protein kinase B (Akt/PKB)-dependent survival pathways, inactivating Akt by dephosphorylation and activating the Bcl-2-related protein BAD by phosphorylation [16-18]. Ceramide-induced apoptosis in neurons or in neuronally differentiated PC12 cells has been associated with mitochondrially produced reactive oxygen species (ROS) as well as activation and nuclear translocation of the transcription factor NFκB [10,11,19]. All these molecular events are observed during the effector phase of ceramide-induced apoptosis which also includes gene expression and new protein synthesis required for ceramide-mediated cell death, as it has been shown that neuronal cell death can be inhibited by cycloheximide [7].
The genes that are transcriptionally regulated during ceramide-mediated cell death are still poorly documented. To study gene expression during neuronal cell death, we carried out a differential screen of an array of 9,120 cDNA clones from a human infant brain library (library 1NIB [20]) with complex cDNA targets derived from neurally differentiated rat pheochromocytoma PC12 cells treated with C2-ceramide compared to control PC12 cells. This model is particularly suitable for establishing a gene-expression profile during ceramide-mediated neuronal death because first, the neuronal cell population is synchronized and homogeneous, unlike brain tissue or primary neuronal cultures, and second, because the use of exogenous C2-ceramide eliminates the risk of interference by transcripts activated by signal transducers upstream of ceramide in the cell-death pathway or in pathways activated in parallel.

Results

Cell death induced in neurally differentiated PC12 cells by C2-ceramide

The morphological characteristics of differentiated PC12 cells after 24 hours in the presence of 25 μM C2-ceramide were compatible with cell death by apoptosis. Compared with control cultures, as viewed by phase-contrast microscopy (Figure 1a), C2-ceramide-treated cells lost their neurites and became rounded and shrunken after 24 hours of treatment (Figure 1b). The cells that remained viable in the C2-ceramide-treated cultures were refringent (Figure 1b), like those in the control cultures (Figure 1a), and excluded the vital marker propidium iodide (Figure 1c), whereas the dead cells took up propidium iodide that intercalated into their DNA (Figure 1d), revealing condensed and fragmented nuclei. As previously described, when neurally differentiated PC12 cells or primary cultures of mesencephalic neurons were treated with cell-permeant C2-ceramide (10-50 μM), they died in a dose-dependent manner [7,10]. At 25 μM no significant cell death was observed until 12 hours after the initiation of treatment (Figure 2a). After 24 hours, 50% of the cells had died. By 48 hours, no viable cells remained. Furthermore, we observed activation of caspase-3/CPP32, a member of the cysteine-activated aspartate family of cell-death proteases [21], that started 8 hours after the beginning of ceramide treatment and was five times the control value by 18 hours (Figure 2b). No significant cell death and caspase-3/CPP32 activity were observed using the inactive C2 analog of ceramide, C2-dihydroceramide (Figure 2).

Validation of hybridization signals

Hybridization of 9,120 cDNA clones with complex cDNA targets from poly(A)⁺ RNA extracted from C2-ceramide-treated or control cells produced signals of varying intensities (Figure 3a). In order to eliminate clones for which no reproducible hybridization signals were obtained, the signal-intensity values were validated as described in Materials and Methods.
Figure 3
Hybridization signal analysis. (a) Macroarray of 9,120 cDNA clones hybridized with complex cDNA targets derived from mRNA of neuronally differentiated PC12 cells without C2-ceramide treatment (control) or treated with C2-ceramide (stimulated). (b) Distribution of the hybridization signal intensities between control and stimulated cells. Some genes identified in the present study are indicated.
methods. Thus, 7% of the clones hybridized with the control 
cDNA target (634) and 14% of clones hybridized with the 
C2-ceramide-treated cDNA target (1,297) were excluded 
from further analysis. The remaining 6,494 clones were ana-
lyzed for differential hybridization.

Differential gene expression in neuronally 
differentiated PC12 cells treated with C2-ceramide 
compared to controls

Changes in gene expression were analyzed during the effec-
tor phase of neuronal death, 7 hours after the beginning of 
C2-ceramide treatment. This time point was chosen because 
on the one hand it is preceded by the activation of the tran-
scription factor NFκB and c-Jun observed 4 to 6 hours after 
C2-ceramide treatment in PC12 cells [10,22], and on the 
other, the apoptotic process is still not induced by caspase-3 
activation, which occurs 8 hours after the beginning of 
C2-ceramide treatment.

Hybridization between the rat PC12 cell-derived targets and 
the human cDNA macroarray was carried out as described 
in Materials and methods. Modulation of gene expression 
was quantitated by calculating the ratio of the intensity of 
the normalized hybridization signal obtained with the 
C2-ceramide cDNA target to that obtained with the control 
target. Clones were considered to be differentially 
hybridized in C2-ceramide-treated cells compared to control 
cells if the ratio between the corresponding hybridization 
intensity values was ≥ 2 (up-hybridized clones) or ≤ 0.5 
(down-hybridized clones) which are the limits of confidence 
for the method. To decrease the risk of false-positive 
results, clones with hybridization signals that were less than 
twofold above background were also excluded, resulting in 
the elimination of 538 clones. In addition, the remaining 
clones were hybridized with complex cDNA targets from 
poly(A)+ RNA extracted from C2-dihydroceramide-treated 
cells used as negative control and compared to untreated 
cells. No modulation of expression was observed (except for 
one clone excluded from the analysis) in the presence of this 
inactive analog of C2-ceramide (data not shown). Among 
the 239 clones that met the criteria for differential hybridiza-
tion, 132 were up-hybridized in C2-ceramide-treated cells 
and 107 were down-hybridized. The distribution of the 
hybridization-intensity values between the control and the 
C2-ceramide complex cDNA targets is presented in 
Figure 3b. Approximately 55% (72/132) of the up-hybridized 
clones were hybridized 3-6-fold more in C2-ceramide-
treated cells than in the control and 40% (41/107) of the 
down-hybridized clones were hybridized 3-9-fold less.

Partial 5´ and 3´ sequences of the 239 clones were compared 
with all the sequences in the database developed in our labo-
atory (the Genexpress Index [23]) and in public databases. 
Of the 239 clones, 179 clones corresponded to already identi-
ﬁed human genes, 113 of which have deﬁned functions. The 
remaining 60 clones corresponded to genes with limited 
characterization. Under the hypothesis that differential 
hybridization of the clones reﬂects linear modulation of
expression of the corresponding genes, we assume that we have detected differential gene expression using cDNA array technology that can be interpreted according to the information available.

Ten differentially expressed genes encode proteins with a role in ceramide or TNF-α pathways (Figure 4, Table 1; see [24] for links to database entries for each gene). Two of these genes, \( \text{PLA2G4C} \) and \( \text{CLN3} \), seem to have a role in ceramide-mediated cell death or survival. Two upregulated genes (\( \text{ETV5} \), \( \text{NPTX2} \)) and two downregulated genes (\( \text{COL18A1} \), \( \text{TNFAIP1} \)) encode proteins that are modulated by TNF-α. Four genes, three upregulated (\( \text{AXL} \), \( \text{BIRC1} \), \( \text{RSU1} \)) and one downregulated (\( \text{MAPK10} \)) encode proteins with a role in the TNF-α signaling pathway.

Twenty clones correspond to genes encoding proteins that have been involved in the regulation of apoptosis and/or cell growth (Figure 5, Table 2, see [24]). Fourteen are up-hybridized and six are down-hybridized by C\(_2\)-ceramide. Ten of the upregulated and two of the downregulated genes encode proteins stimulating apoptosis and/or growth arrest. The other genes (four upregulated and four downregulated) encode proteins downregulating apoptosis and/or stimulating growth.

The remaining 83 clones corresponding to 82 genes with known or putative functions have no obvious relation to the apoptosis process (Table 3, see [24]). Of the total number of differentially hybridized clones, 66 correspond to mRNA sequences (Table 4, see [24]) and 60 to poorly characterized genes (Table 5, see [24]) that encode proteins without known function.

To confirm the results obtained by macroarray analysis, differentially expressed transcripts representing upregulated or downregulated genes were analyzed for differential expression by reverse transcription PCR (RT-PCR) or northern blots. As shown in Figure 6, the upregulation of \( \text{ETV5} \), \( \text{M6PR} \) and \( \text{APCL} \) was confirmed by RT-PCR, and the downregulation of two genes with unknown function (mRNA DKFZp586C1723 and GENX 2969) was confirmed by northern blotting.
Discussion

Extracellular signaling molecules such as cytokines, growth-factor deprivation and DNA damage caused by chemotherapeutic agents or irradiation activate ceramide-mediated signal transduction pathways leading to cell death. These pathways have been investigated in the immune system, where they are known to have an important role, and in neurons, as they are suspected to play a part in neurodegenerative disorders [1]. A number of steps in the signaling cascades have been elucidated. However, although the translation inhibitor cycloheximide inhibits the ceramide-mediated death of mesencephalic neurons [7], the expression patterns of genes modulated during ceramide-mediated cell death remain unknown. In a global approach to this question, we have used cDNA macroarray technology to determine the profile of gene expression in a neuronal model of cell death, neuronally differentiated and C2-ceramide-treated PC12 cells, in which ceramide-dependent changes in gene expression could be isolated from the effects of other transcription modulators.

Identification of genes closely implicated in the ceramide and/or TNF-α signaling pathway

We were able to detect differential expression of 10 genes known to be involved in the ceramide or TNF-α signaling pathways (see Figure 4, Table 1) thus validating our study. A summary illustration of the putative role of these genes is presented in Figure 7. Briefly, two genes, encoding phospholipase A2 group IVC (PLA2G4C) and ceroid-lipofuscinosis, neuronal 3, juvenile (CLN3) are already known to be involved in ceramide-mediated signal transduction. The first, PLA2G4C, belongs to the cytosolic phospholipase A2 gene family that encodes two different proteins: calcium-independent and calcium-dependent cytosolic phospholipases [38]. TNF-α regulates the expression of PLA2G4A mRNA in HeLa cells [39] and in human bronchial epithelial cells [40], which is indirect evidence of modulation by ceramide, but the role of ceramide was not demonstrated directly in these studies. However, ceramide was shown directly to upregulate the expression of the gene encoding cytosolic phospholipase A2 in the fibroblast cell line L929.

Figure 5
Differentially expressed genes that encode proteins involved in the regulation of apoptosis and/or cell growth. Gray boxes, genes stimulating apoptosis and/or growth arrest; white boxes, genes downregulating apoptosis and/or stimulating growth.
Conversely, the activation of this gene was reported to be necessary for ceramide accumulation and cell death in the same cells [25]. We show for the first time that this gene is involved in neuronal apoptosis.

The second gene, CLN3, is expressed in a variety of human tissues including the brain, where the product is necessary for neuronal survival [26,27]. Interestingly, CLN3 does not inhibit C₄-ceramide-induced apoptosis but modulates endogenous ceramide synthesis and suppresses apoptosis by preventing generation of ceramide [42]. Thus, C₄-ceramide can activate a negative feedback mechanism regulating endogenous ceramide generation as well as activate the downstream targets of the endogenous lipid.

Four other genes or families of genes known to be transcriptionally regulated by TNF-α were also modulated by C₄-ceramide in our model (Table 1). Of these, ETS variant 5 (ETV5) belongs to the family of ETS transcription factor genes. Increased expression of both ETS1 mRNA and the protein has been observed in human fibroblasts after TNF-α or IL-1β stimulation [28]. PEA3 (a mouse protein

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### Table 2

Differentially expressed genes that encode proteins involved in the regulation of apoptosis and/or cell growth

| Clone ID | GenBank accession number | Unigene | C. int. | C. SD | S. int. | S. SD | Ratio | Similarity | Gene symbol |
|----------|--------------------------|---------|---------|-------|---------|-------|-------|------------|-------------|
| yg01b10  | 2112 R18353; R42557      | Hs.286  | 1.68    | 0.17  | 7.95    | 0.55  | 4.74  | Ribosomal protein L4 | RPL4        |
| yl73h11  | 567 H06473                | Hs.9663 | 1.60    | 0.15  | 7.42    | 0.54  | 4.64  | Programmed cell death 6-interacting protein | PDCD6IP     |
| yc29h11  | 673 F13260; T77039        | Hs.75709| 2.12    | 0.33  | 9.83    | 0.95  | 4.64  | Mannose-6-phosphate receptor (cation dependent) | M6PR        |
| yg94h08  | 6030 R56149               | Hs.78776| 1.96    | 0.34  | 7.09    | 1.25  | 3.61  | Putative transmembrane protein | NMA         |
| yf90d04  | 25970 R15366              | Hs.20912| 1.34    | 0.22  | 4.71    | 0.58  | 3.51  | Adenomatous polyposis coli like | APC         |
| yg76b02  | 3804 R51346; R51453       | Hs.78935| 0.95    | 0.18  | 2.89    | 0.40  | 3.03  | Methionine aminopeptidase; elf-2-associated p67 | METAP2      |
| yd01h06  | 9451 R39334; T78769       | Hs.27434| 1.98    | 0.16  | 5.89    | 0.66  | 2.97  | HLA-B associated transcript-3 | BAT3        |
| c-22F12  | 2915 F08770               | Hs.75323| 1.44    | 0.23  | 3.26    | 0.29  | 2.26  | Prohibitin | PHB         |
| yf69g07  | 115124 R14126             | Hs.132955| 1.82 | 0.30  | 3.99    | 0.96  | 2.19  | BCL2/adenovirus E1B 19kD-interacting protein 3-like | BNIP3L      |
| yd02b11  | 115910 T79985             | Hs.63984| 0.88    | 0.19  | 1.88    | 0.30  | 2.14  | Cadherin 13, H-cadherin (heart) | CDH13       |
| c-3ke04  | 781 F10823; F13223        | Hs.12409| 1.17    | 0.10  | ND      | ND    | 0.43  | Somatostatin | SST         |
| yg64g08  | 115205 R35542; R51110     | Hs.288986| 3.01    | 0.41  | 0.90    | 0.15  | 0.30  | Survival of motor neuron 1 1, telomeric | SMN1        |

Proteins regulating apoptosis and/or stimulating growth

| Clone ID | GenBank accession number | Unigene | C. int. | C. SD | S. int. | S. SD | Ratio | Similarity | Gene symbol |
|----------|--------------------------|---------|---------|-------|---------|-------|-------|------------|-------------|
| yg44d03  | 408 R25503                | Hs.155212| 1.74   | 0.40  | 7.67    | 0.40  | 4.40  | Methylmalonyl coenzyme A mutase | MUT        |
| yg68d10  | 2957 R36284; R49571       | Hs.89582| 1.80    | 0.25  | 7.26    | 0.57  | 4.04  | Glutamate receptor, ionotropic, AMPA 2 | GRIA2      |
| yl81d04  | 9379 H05457; H07007       | Hs.150423| 2.64   | 0.36  | 8.72    | 1.06  | 3.31  | Cyclin-dependent kinase 9 (CDC2-related kinase) | CDK9        |
| yd02a11  | 78693 T79973              | Hs.107911| 2.52   | 0.42  | 5.38    | 0.44  | 2.14  | ATP-binding cassette, sub-family B (MDR/TAP), member 6 | ABCB6      |
| yg51a11  | 17820 R21694; R46587      | Hs.223014| 1.09   | 0.24  | ND      | ND    | 0.46  | Antizyme inhibitor | OAZIN       |
| yh10g09  | 4858 R61276; R61277       | Hs.8073  | 1.59    | 0.23  | 0.66    | 0.16  | 0.41  | Septin 3 | SEP3        |
| yf53a12  | 3165 R12025; R37093       | Hs.356245| 1.14   | 0.21  | 0.29    | 0.02  | 0.25  | Apoptosis regulator | LOCS1283   |
| yg67b12  | 115951 R35827; R49537     | Hs.285754| 2.38   | 0.48  | 0.50    | 0.12  | 0.21  | Met proto-oncogene | MET         |

Abbreviations and column headings are as in Table 1.
| Clone ID  | GENX | GenBank accession number | Unigene | C. int. | C. SD | S. int. | S. SD | Ratio | Similarity                                      | Gene symbol |
|----------|------|--------------------------|---------|---------|-------|---------|-------|-------|------------------------------------------------|-------------|
| Signal transduction                                                                                                                                       |
| yf85b10 | 1842 | H05211                   | Hs.22003| 1.62    | 0.40  | 7.99    | 0.37  | 4.94  | Solute carrier family 6 (neurotransmitter transporter, GABA), member 1 | SLC6A1      |
| yf77g11 | 3900 | R14207; R37490           | Hs.75819| 1.12    | 0.12  | 5.02    | 0.43  | 4.46  | Glycoprotein M6A                                   | GPM6A       |
| yg63f10 | 1552 | R26636; R49665           | Hs.24212| 1.01    | 0.15  | 4.02    | 0.61  | 3.98  | Latrophilin                                        | KIAA0786    |
| c-2ee07 | 116218| Z45003                   | Hs.107979| 1.75    | 0.35  | 6.17    | 0.85  | 3.52  | Small membrane protein 1                            | SMP1        |
| yf60h11 | 12653| R13771                   | Hs.61628| 1.43    | 0.18  | 4.68    | 0.61  | 3.28  | Calcium binding atopy-related autoantigen 1          | CBARA1      |
| yf88a09 | 9668 | R15201                   | Hs.181326| 4.01    | 0.50  | 11.65   | 2.01  | 2.90  | Myotubulin-related protein 2                        | MTMR2       |
| yg1l08 | 107475| R17181; R41731           | Hs.5462 | 0.72    | 0.12  | 1.54    | 0.33  | 2.14  | Solute carrier family 4, sodium bicarbonate cotransporter, member 4 | SLCA4        |
| c-2mh12 | 1997 | Z41050; Z45338           | Hs.108787| 1.08    | 0.21  | 0.52    | 0.04  | 0.47  | Phosphatidylinositol glycan, class N                 | PIGN        |
| yc87e10 | 115203| F10343; F12737           | Hs.173717| 1.24    | 0.17  | 0.50    | 0.06  | 0.40  | Phosphatidic acid phosphatase type 2B                | PPA2B       |
| yf48c10 | 9043 | R12286; R12797           | Hs.10842| 1.10    | 0.24  | 0.43    | 0.03  | 0.39  | RAN, member RAS oncogene family                      | RAN         |
| yd09f12 | 2991 | R39085                   | Hs.306359| 2.39    | 0.46  | 0.90    | 0.22  | 0.38  | Hect domain and RCC1 (CHC1)-like domain (RLD) 1      | HERC1       |
| c-3ie05 | 5307 | F10685; F13091           | Hs.9347 | 1.48    | 0.24  | 0.53    | 0.02  | 0.36  | Regulator of G-protein signaling 14                  | RGSI4       |
| yg16c08 | 5294 | R17962; R41352           | Hs.1440 | 1.05    | 0.23  | 0.29    | 0.04  | 0.27  | Gamma-aminobutyric acid (GABA) A receptor, beta 3    | GABRB3      |
| yf50c04 | 1366 | R11777; R37698           | Hs.5985 | 1.13    | 0.12  | 0.17    | 0.01  | 0.15  | Non-kinase Cdc42 effector protein SPEC2              | LOC56990    |
| Transcription/translation                                                                                                                                  |
| yf71g02 | 5232 | R40420                   | Hs.16313| 0.90    | 0.13  | 2.30    | 0.15  | 2.55  | Kruppel-like zinc-finger protein GLIS2               | GLIS2       |
| c-26a02 | 451  | F07446                   | Hs.13993| 1.64    | 0.38  | 3.39    | 0.73  | 2.07  | TBP-like 1                                          | TBP1        |
| c-05c07 | 4917 | Z38284; Z41997           | Hs.26973| 1.21    | 0.20  | 2.45    | 0.52  | 2.02  | Bromodomain adjacent to zinc-finger domain, 2B       | BAZZ2B      |
| c-24a11 | 11423| F07382                   | Hs.75678| 1.38    | 0.23  | 0.66    | 0.16  | 0.47  | FBJ murine osteosarcoma viral oncogene homolog B     | FOSB        |
| yg90e12 | 10904| R56427; R56248           | Hs.239 | 1.28    | 0.23  | 0.59    | 0.03  | 0.46  | Forkhead box M1                                     | FOXM1       |
| yf61e03 | 4401 | R13803; R37662           | Hs.182447| 7.20    | 1.01  | 2.75    | 0.60  | 0.38  | Heterogeneous nuclear ribonucleoprotein C (C1/C2)    | HNRPCC      |
| yf64g02 | 993  | R37803                   | Hs.6151 | 4.87    | 0.77  | 1.87    | 0.46  | 0.38  | Pumilio homolog 2 (Drosophila)                       | PUM2        |
| yg53f10 | 1678 | R62465; R25370           | Hs.520 | 1.41    | 0.20  | ND      | ND    | 0.35  | Nuclear receptor subfamily 2, group C, member 2      | NR2C2       |
| yg47e10 | 1548 | R21283; R45337           | Hs.14520| 1.55    | 0.26  | 0.53    | 0.13  | 0.34  | Eukaryotic translation initiation factor 2C, 1       | EIF2C1      |
| yg36d06 | 1872 | R24568; R44373           | Hs.76177| 10.91   | 1.23  | 3.67    | 0.10  | 0.34  | Transcription factor CP2                             | TFCP2       |
| yg60b12 | 303  | R35123; R49511           | Hs.2186 | 3.12    | 0.63  | 0.94    | 0.07  | 0.30  | Eukaryotic translation elongation factor 1 gamma     | EEF1G       |
| yg27a08 | 4127 | R43968                   | Hs.278589| 9.43    | 1.24  | 2.76    | 0.40  | 0.29  | General transcription factor II, i, pseudogene 1     | GTF2IP1     |
### Table 3 (continued)

| Clone ID | GENX | GenBank accession number | Unigene symbol | C. int. | C. SD | S. int. | S. SD | Ratio | Similarity | Gene symbol |
|----------|------|--------------------------|----------------|--------|------|--------|------|------|------------|-------------|
| **Cellular traffic or structure proteins** | | | | | | | | | | |
| yg1905 | 200119 | R20424; R43544 | Hs.169793 | 1.51 | 0.36 | 7.37 | 1.32 | 4.86 | Ribosomal protein L32 | RPL32 |
| yc86h03 | 2760 | F12918; T75229 | Hs.182625 | 2.38 | 0.26 | 7.78 | 1.17 | 3.27 | Vamp (vesicle-associated membrane protein)-associated protein B and C | VAPB |
| yc87f04 | 5084 | R38549; T75126 | Hs.22826 | 1.83 | 0.11 | 5.59 | 0.64 | 3.06 | Tropomodulin 3 (ubiquitous) | TMOD3 |
| yf98g01 | 8512 | R18713 | Hs.75196 | 2.96 | 0.63 | 9.29 | 0.80 | 3.14 | Ankyrin repeat-containing protein | G9A |
| yh17e09 | 1304 | R59488; R59489 | Hs.30991 | 0.78 | 0.19 | 2.32 | 0.11 | 2.97 | Ankyrin repeat domain 6 | ANKR6D |
| yf76d11 | 424 | R13426; R40938 | Hs.119324 | 0.84 | 0.08 | 2.07 | 0.35 | 2.48 | Kinesin-like 4 | KNSL4 |
| c-27f03 | 1382 | F07488 | Hs.89497 | 2.32 | 0.31 | 5.60 | 0.64 | 2.42 | Lamin B1 | LMNB1 |
| yc96a12 | 11155 | F13331; T77651 | Hs.159613 | 4.50 | 0.32 | 10.84 | 2.13 | 2.41 | Thyroid hormone receptor binding protein | ALB3 |
| yf57c11 | 1225 | R12822; t20734 | Hs.1501 | 0.94 | 0.22 | 2.25 | 0.22 | 2.39 | Syndecan 2 | SDC2 |
| yf71a06 | 10804 | H05894 | Hs.6682 | 1.33 | 0.11 | 2.94 | 0.20 | 2.21 | Solute carrier family 7, cationic amino acid transporter, y+ system, member 11 | SLC7A11 |
| yc99f07 | 11082 | T78361 | Hs.103042 | 2.21 | 0.07 | 0.98 | 0.19 | 0.44 | Microtubule-associated protein 1B | MAP1B |
| yf72e08 | 2558 | R13080; R40510 | Hs.7979 | 2.05 | 0.34 | 0.80 | 0.15 | 0.39 | Likely ortholog of mouse synaptic vesicle glycoprotein 2a | SV2 |
| yc87h12 | 2952 | F10545; F12946 | Hs.21611 | 5.68 | 0.59 | 1.93 | 0.17 | 0.34 | Kinesin family member 3C | KIF3C |
| yg54d05 | 604 | R25813; R46810 | Hs.117977 | 1.62 | 0.33 | 0.50 | 0.11 | 0.31 | Kinesin 2 (60-70 kD) | KNS2 |
| yf91b02 | 1980 | R16352; R42300 | Hs.103042 | 3.50 | 0.41 | 1.01 | 0.24 | 0.29 | Microtubule-associated protein 1B | MAP1B |
| yf72a03 | 115963 | R13048; R40479 | Hs.187958 | 1.46 | 0.34 | 0.40 | 0.06 | 0.28 | Solute carrier family 6, member 8, accessory proteins BAP31/BAP29 | SLC6A8, DXS1357E |
| **Immunity/inflammatory response** | | | | | | | | | | |
| yg75d06 | 25621 | R54423 | Hs.179661 | 1.88 | 0.18 | 8.01 | 1.02 | 4.26 | FKS56-binding protein 1A (12 kD) | FKBPA1 |
| yg65b03 | 2453 | R35324 | Hs.9688 | 0.86 | 0.13 | 3.67 | 0.60 | 4.26 | Leukocyte membrane antigen | IRC1 |
| yg57f05 | 190007 | R34428 | Hs.181244 | 3.83 | 0.24 | 9.76 | 1.22 | 2.55 | MHC class I gene family | |
| yf51e08 | 2563 | R12005; R39844 | Hs.75682 | 0.89 | 0.04 | 2.05 | 0.21 | 2.31 | Autoantigen | |
| c-2bh04 | 190137 | F03851; F07604 | Hs.284394 | 1.13 | 0.07 | 0.56 | 0.07 | 0.50 | Complement component 3 | C3 |
| yf59h02 | 5580 | R13549; R20669 | Hs.82689 | 1.05 | 0.21 | 0.47 | 0.10 | 0.44 | Tumor rejection antigen (gp96) | TRA1 |
| yc86g03 | 8628 | F10456; F12856 | Hs.302749 | 1.51 | 0.35 | 0.58 | 0.09 | 0.39 | FKS56-binding protein 9 (63 kD) | FKBPA9 |
| **Protein processing** | | | | | | | | | | |
| yf68a10 | 1071 | R40190; R46258 | Hs.75890 | 0.55 | 0.13 | 2.09 | 0.23 | 3.80 | Site-I protease (subtilisin-like, sterol-regulated, cleaves sterol regulatory element binding proteins) | S1P |
| c-2na07 | 2001 | F04230; F07978 | Hs.102 | 1.01 | 0.16 | 0.46 | 0.10 | 0.45 | Aminomethyltransferase (glycine cleavage system protein T) | AMT |
| yc85d05 | 6301 | F10498; F12892 | Hs.170197 | 1.45 | 0.22 | 0.59 | 0.10 | 0.41 | Glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2) | GOT2 |
| c-2ge12 | 2793 | Z40826; Z46090 | Hs.183212 | 1.14 | 0.19 | 0.45 | 0.09 | 0.39 | Isoprenylcysteine carboxyl methyltransferase | ICMT |
| yg5204 | 202164 | R21082; R46258 | Hs.235887 | 1.55 | 0.31 | 0.54 | 0.08 | 0.34 | HMT1 (hnRNP methyltransferase, Saccharomyces cerevisiae)-like 1 | HRMT1LI |
| Clone ID | GENX | GenBank accession number | Unigene | C. int. | C. SD | S. int. | S. SD | Ratio | Similarity | Gene symbol |
|----------|------|--------------------------|---------|--------|-------|--------|-------|-------|------------|-------------|
| yf6407   | 2813 | R13707; R37801           | Hs.171501 | 1.27   | 0.24  | ND     | ND    | 0.39  | Ubiquitin specific protease 11 | USP11       |
| yc9708   | 1805 | R39698; T78043           | Hs.2838  | 2.73   | 0.24  | 9.35   | 0.86  | 3.42  | Malic enzyme 3, NADP(+)-dependent, mitochondrial | ME3         |
| yg9706   | 3929 | R59198; R59256           | Hs.78989  | 0.68   | 0.07  | 1.99   | 0.18  | 2.94  | Alcohol dehydrogenase 5 (class III), chi polypeptide | ADH5        |
| c-2ca07  | 1549 | F01858; F07608           | Hs.180616 | 1.04   | 0.12  | 0.50   | ND    | 0.48  | CD36 antigen (collagen type I receptor, thrombospondin receptor)-like 1 | CD36L1      |
| yc9506   | 3164 | R39463; T77281           | Hs.155247 | 1.01   | 0.20  | 0.31   | 0.05  | 0.30  | Aldolase C, fructose-bisphosphate | ALDOC       |
| yc9806   | 1475 | R37847; T78111           | Hs.301789 | 1.55   | 0.27  | 4.52   | 0.35  | 2.92  | Sodium channel, voltage-gated, type I, alpha polypeptide | SCNIA       |
| yc5402   | 924  | R11969                   | Hs.4865  | 1.41   | 0.17  | 3.05   | 0.18  | 2.17  | Synaptotagmin XIII | SYT13       |
| c-11a09  | 4199 | Z39718; Z43661           | Hs.8834  | 0.64   | 0.15  | 1.30   | 0.19  | 2.02  | Ring finger protein 3 | RNF3        |
| yg33b04  | 4211 | R53332; R53937           | Hs.7022  | 1.19   | 0.28  | 0.59   | 0.09  | 0.50  | Capping protein (actin filament) muscle Z-line, alpha 1 | CAPZA       |
| yf5702   | 4610 | R12627; R20528           | Hs.334688 | 1.51   | 0.25  | 0.75   | 0.13  | 0.50  | Mannosidase, beta A, lysosomal-like | MANB1       |
| yg36h06  | 3087 | R24595; R44400           | Hs.7122  | 1.08   | 0.13  | ND     | ND    | 0.46  | Scare study protein 1 | SCG51       |
| yf9905   | 2822 | R18211; R24149           | Hs.79284  | 1.07   | 0.21  | 0.48   | 0.07  | 0.45  | Protein with polyglutamine repeat; calcium (Ca2+)-homeostasis endoplasmic reticulum protein | EHD3        |
| c-19a06  | 9239 | Z40467; Z44591           | Hs.171545 | 1.02   | 0.20  | 0.45   | 0.11  | 0.44  | Mesoderm specific transcript (mouse) homolog | MEST        |
| yd05d01  | 2346 | R38832; T80384           | Hs.13493  | 1.06   | 0.24  | 0.44   | 0.06  | 0.42  | Like mouse brain protein E46 | E46L        |
| yf74e11  | 2106 | R13277; R40723           | Hs.334851 | 1.95   | 0.41  | 0.80   | 0.17  | 0.41  | Lim and SH3 protein 1 | LASP1       |
| yd01e11  | 3181 | T78746                   | Hs.168640 | 1.17   | 0.27  | 0.47   | 0.10  | 0.40  | Homolog of mouse Ank | ANK         |
| yf48e09  | 414  | R12292; R12804           | Hs.21050  | 1.22   | 0.26  | 0.47   | 0.07  | 0.38  | HIV-1 Rev binding protein | HIRB        |
| yg16d07  | 1087 | R43459; R17969           | Hs.87125  | 9.91   | 2.06  | 3.35   | 0.51  | 0.34  | Hematological and neurological expressed | HN1         |
| yf57d07  | 12763| R12632; R20533           | Hs.109706 | 1.78   | 0.23  | 0.58   | 0.09  | 0.33  | Hematological and neurological expressed | HN1         |
| c-2la01  | 200991| F04056; F07796          | Hs.74376  | 1.00   | 0.07  | 0.33   | 0.01  | 0.33  | Olfactomedin related ER localized protein | NOEL1       |

**Table 3 (continued)**
C2-ceramide treatment is consistent with our previous [29,30]. The regulation of the pentraxin gene family by TNF-α/H9251 suppressor protein 1, which is involved in TNF-α signaling, is reported to protect against TNF-α-stimulated signaling via activation of NFκB pathways. Thus AXL, encoding type XVIII collagen alpha 1, and identified in our model are COL18A1, encoding type XVIII collagen alpha 1, and TNFAIP1, encoding TNFα-induced protein 1. These proteins, downregulated by C2-ceramide, are modulated by TNF-α in various cell types [31-33].

We also identified four genes encoding proteins known to participate in TNF-α-activated signal transduction pathways. Thus AXL, upregulated by a factor of 3.65 (Table 1), encodes a tyrosine kinase receptor. Signaling through this receptor is reported to protect against TNF-α-induced apoptosis in fibroblasts and its absence increases apoptosis after serum deprivation [34]. Interestingly, ARK, the mouse protein corresponding to AXL, activates the survival pathway mediated by the serine-threonine kinase Akt [49], which is negatively regulated by ceramide [16,17,50], and is also reported to modulate ceramide synthesis [51]. The second gene we identified is BIRC1, encoding baculoviral IAP repeat-containing 1 protein. This protein, putatively involved in spinal muscular atrophy [52], is an inhibitor of cell death induced by various apoptotic stimuli, including TNF-α [35]. The third identified gene, RSU1, encodes Ras suppressor protein 1, which is involved in TNF-α signaling by blocking the Ras-dependent response. Levels of both RSU1 mRNA and protein have been correlated with a decrease in growth rate and tumorigenic potential in U251 glioblastoma cells [53] and it induces growth arrest in PC12 cells [36]. This is consistent with the report that ceramide regulates apoptosis via modulation of the Ras signaling pathway [18]. In addition, RSU1 has been identified as an inhibitor of Jun kinase activation [37]. This point is interesting, as the fourth gene presenting in this group, MAPK10/J.NK3, encoding the JNK family member mitogen-activated protein kinase 10, is downregulated by C2-ceramide in our model.

The identification of these eight genes, which are involved in the TNF-α signaling pathway, in C2-ceramide treated PC12 cells, suggests that their modulation of expression by TNF-α could be the result of a ceramide-dependent mechanism.

Commitment to apoptosis: upregulation of pro-apoptotic genes and downregulation of anti-apoptotic genes by the ceramide pathway

Twenty genes regulated by C2-ceramide correspond to genes known to be involved in regulation of apoptosis and/or cell growth (Figure 5, Table 2). Twelve of these genes are known to be associated with oncogenesis and four with neuronal disorders. Of the upregulated genes, 10 out of 14 are known to be associated with a pro-apoptotic or anti-proliferation process and 3 out of 14 are mainly implicated in protection of the cell against cytotoxicity or damage. Of the downregulated genes, 4 out of 6 are associated with an anti-apoptotic or a proliferation process. This highlights the fact that the cells are engaged in programmed cell death. The putative roles of these genes are illustrated in Figure 7, which focuses on the pro-apoptotic or anti-proliferation process versus anti-apoptotic or proliferation processes.

Briefly, of the known pro-apoptotic or anti-proliferative genes that are upregulated in our model, RPL4 encodes the ribosomal protein L4 that has been shown to be transcriptionally stimulated prior to apoptosis induced by the 5-azacytidine in the PC12 cells [54]. PDCD6IP, upregulated by C2-ceramide in our model, encodes a protein that interacts with ALG2, a Ca++-binding protein that is required for apoptosis induced by diverse stimuli, including ceramide.
| Clone ID  | GENX | GenBank accession number | Unigene C. int. | C. SD | S. int. | S. SD | Ratio | Similarity |
|----------|------|--------------------------|----------------|-------|---------|-------|-------|------------|
| yg51f11  | 229  | R21710;                 | Hs.64691       | 1.32  | 0.16    | 6.34  | 0.43  | 4.80       | KIAA0483 protein |
| yg30b04  | 5093 | R44721;                 | Hs.12896       | 1.31  | 0.12    | 5.99  | 0.29  | 4.57       | KIAA1034 protein |
| yf53g09  | 13   | R12046;                 | Hs.90424       | 1.56  | 0.10    | 7.09  | 0.67  | 4.55       | Homo sapiens cDNA: FLJ23285 fis, clone HEP09071 |
| yc94b11  | 223  | F13362; R77404;         | Hs.101375      | 1.40  | 0.15    | 6.38  | 1.10  | 4.55       | cDNA DKFZp434H205 (from clone DKFZp434H205) |
| yg37d06  | 2008 | R19640;                 | Hs.264636      | 1.70  | 0.39    | 7.39  | 1.60  | 4.34       | KIAA0781 protein |
| yf75c06  | 425  | R13300; R40783;         | Hs.106825      | 1.40  | 0.15    | 5.99  | 1.10  | 4.57       | Homo sapiens cDNA: FLJ21380 fis, clone COL03329 |
| yc94b01  | 6715 | R12046                  | Hs.90424       | 1.56  | 0.10    | 7.09  | 0.67  | 4.55       | cDNA DKFZp434H205 (from clone DKFZp434H205) |
| yg42a11  | 20067| R24764; R45496;         | Hs.21710       | 1.64  | 0.20    | 6.61  | 1.00  | 4.04       | Hypothetical protein DKFZp761G0313 |
| yc85f03  | 255  | F12760; T74722;         | Hs.125034      | 1.98  | 0.16    | 6.52  | 0.85  | 3.29       | Homo sapiens cDNA: FLJ10733 fis, clone NT2RP001392 |
| yc86g12  | 32   | R14304; R40254;         | Hs.59236       | 1.21  | 0.25    | 3.94  | 0.63  | 3.24       | Hypothetical protein DFKZp434L0718 |
| yg18e11  | 4391 | R20224;                 | Hs.41185       | 3.49  | 0.86    | 11.27 | 1.23  | 3.23       | cDNA DKFZp434H205 (from clone DKFZp434H205) |
| yc90h10  | 1343 | F13218; T55433;         | Hs.141003      | 1.32  | 0.24    | 4.19  | 1.05  | 3.18       | Homo sapiens cDNA: FLJ21691 fis, clone COL09555 |
| yg42a11  | 20067| R24764; R45496;         | Hs.288368      | 0.45  | 0.10    | 1.41  | 0.11  | 3.14       | Homo sapiens cDNA: FLJ21314 fis, clone COL02428 |
| yc85f03  | 255  | F12760; T74722;         | Hs.318401      | 3.03  | 0.42    | 9.46  | 0.80  | 3.12       | HSPC039 protein (LOC51124) |
| yc86g12  | 32   | R12859; T75526;         | Hs.180948      | 4.61  | 0.28    | 14.27 | 1.89  | 3.10       | KIAA0729 protein |
| c-2lb03  | 1917 | Z45263                  | Hs.155182      | 4.68  | 1.55    | 19.98 | 3.34  | 3.08       | KIAA1036 protein |
| yf94d09  | 2836 | R16328; R41404;         | Hs.6343;       | 3.16  | 0.48    | 9.58  | 0.97  | 3.03       | KIAA1464 protein |
| yf49g10  | 2696 | R11887;                 | Hs.40094       | 4.42  | 0.68    | 13.38 | 1.70  | 3.03       | Human DNA sequence from clone 167A19 on chromosome 1p32.1-33 |
| yg67h02  | 1136 | R35733; R49366;         | Hs.325825      | 3.76  | 0.34    | 11.27 | 0.80  | 3.00       | Homo sapiens cDNA: FLJ20848 fis, clone ADKA01732 |
| yc89d09  | 2388 | F13194; T75317;         | Hs.22109       | 3.52  | 0.07    | 10.11 | 1.40  | 2.87       | KIAA0945 protein |
| yf72d11  | 4469 | R13137; R40616;         | Hs.6311        | 2.38  | 0.54    | 6.80  | 0.92  | 2.86       | Homo sapiens cDNA: FLJ20859 fis, clone ADKA01617 |
| yg73c09  | 51540| R51740;                 | Hs.288959      | 1.31  | 0.18    | 3.70  | 0.65  | 2.83       | Homo sapiens cDNA: FLJ20920 fis, clone ADSE00877 |
| yf50h09  | 9583 | R11191;                 | Hs.11637       | 3.87  | 0.33    | 10.71 | 1.31  | 2.77       | Homo sapiens mRNA; cDNA DKFZp47J125 (from clone DKFZp47J125) |
| c-2ba02  | 4345 | Z41723; Z44845;         | Hs.15921       | 1.93  | 0.37    | 5.31  | 1.02  | 2.75       | Hypothetical protein FLJ10759 |
| yg36f12  | 11000| R25011; R45019;         | Hs.118983      | 1.13  | 0.27    | 3.00  | 0.46  | 2.65       | Homo sapiens cDNA: FLJ21505 fis, clone MAMMA1000422 |
| c-24b10  | 1689 | Z45463                  | Hs.154919      | 2.67  | 0.43    | 6.60  | 1.44  | 2.47       | KIAA0625 protein |
Table 4 (continued)

| Clone ID | GENX | GenBank accession number | Unigene | C. int. | C. SD | S. int. | S. SD | Ratio | Similarity |
|----------|------|--------------------------|---------|---------|-------|---------|-------|-------|------------|
| Upregulated clones (continued) | yf76a11 | 1849 | R13420; R40930 | Hs.7822 | 1.12 | 0.09 | 2.73 | 0.33 | 2.43 | cDNA DKFZp564C1216 (from clone DKFZp564C1216) |
| yc91c07 | 162 | F10758; F13156 | Hs.140833 | 0.61 | 0.14 | 1.46 | 0.32 | 2.41 | H. sapiens mRNA full length insert cDNA clone EUROIMAGE 29222 |
| yc94c08 | 4310 | R38361; T77413 | Hs.119004 | 0.53 | 0.05 | 1.25 | 0.22 | 2.36 | KIAA0665 gene product |
| yg57f04 | 3072 | R34427; Hs.140833 | 0.61 | 0.14 | 1.46 | 0.32 | 2.41 | H. sapiens mRNA full length insert cDNA clone EUROIMAGE 29222 |
| yg15g12 | 5559 | R18075; R44290 | Hs.22370 | 0.66 | 0.14 | 1.45 | 0.21 | 2.19 | cDNA DKFZp564A00122 (from clone DKFZp564A00122) |
| yg97d02 | 1018 | R59194; R59252 | Hs.5324 | 0.64 | 0.07 | 1.32 | 0.16 | 2.06 | Hypothetical protein (CL25022) |
| yc95f04 | 3851 | F13386; Hs.7888 | 0.58 | 0.07 | 1.16 | 0.25 | 2.02 | H. sapiens clone 23736 mRNA sequence |
| Downregulated clones | yg42e05 | 4312 | R45416; R25077 | Hs.169330 | 1.05 | 0.23 | 0.52 | 0.05 | 0.49 | Neuronal protein (NP25) |
| yg89f11 | 2081 | R55970; R55969 | Hs.16443 | 1.16 | 0.27 | 0.56 | 0.06 | 0.49 | H. sapiens cDNA: FLJ21721 fis, clone COLF0381 |
| yg33e09 | 5446 | R20455; R44290 | Hs.333389 | 1.39 | 0.19 | 0.67 | 0.17 | 0.48 | Hypothetical protein MGC13090 |
| c-2aa11 | 1485 | Z40609; Z44824 | Hs.13485 | 1.44 | 0.23 | 0.70 | 0.16 | 0.48 | KIAA00018 protein |
| yf65e06 | 5690 | R13865; R37007 | Hs.301685 | 1.03 | 0.21 | ND | ND | 0.48 | KIAA0620 protein |
| yg76d07 | 37588 | H05960; H06010 | Hs.92418; Hs.63510 | 3.95 | 0.73 | 1.85 | 0.18 | 0.47 | KIAA0141 |
| c-2cg09 | 201091 | F03885; F07635 | Hs.288361 | 1.06 | 0.10 | 0.49 | 0.02 | 0.47 | H. sapiens cDNA: FLJ22696 fis, clone HSI11696 |
| yg64h02 | 2829 | R35543; R51112 | Hs.12239 | 2.94 | 0.61 | 1.35 | 0.30 | 0.46 | CGI-10 protein (LOC51004) |
| yf49c08 | 23982 | R11699; R17677 | Hs.322844 | 1.29 | 0.32 | 0.58 | 0.12 | 0.45 | Hypothetical protein DKFZp564A176 |
| yg33g08 | 636 | R20203; R44989 | Hs.7750 | 8.39 | 1.43 | 3.60 | 0.64 | 0.43 | Novel human gene mapping to chromosome 1 |
| yf53d08 | 532 | R11837; R36955 | Hs.246885 | 1.07 | 0.05 | 0.44 | 0.08 | 0.42 | Hypothetical protein FLJ20783 |
| yg65h10 | 10701 | R35431; R49229 | Hs.222746 | 1.04 | 0.25 | 0.42 | 0.09 | 0.40 | KIAA1610 protein |
| yg69e11 | 1257 | R36317; R49249 | Hs.216958 | 1.16 | 0.28 | 0.44 | 0.10 | 0.38 | KIAA0194 protein |
| yf79f12 | 5599 | R14349; R40677 | Hs.179946 | 2.55 | 0.37 | 0.86 | 0.20 | 0.34 | KIAA1100 protein |
| yf86c11 | 1909 | R15181; R41632 | Hs.286013 | 1.06 | 0.14 | 0.34 | 0.08 | 0.32 | Short coiled-coil protein |
| yf78c09 | 1664 | R14217; R40635 | Hs.351029 | 1.04 | 1.29 | 3.36 | 0.75 | 0.32 | H. sapiens cDNA FLJ31803 fis, clone NT2R12009101 |
| yf61c10 | 1067 | R13997; R39120 | Hs.5008; Hs.21515 | 1.11 | 0.09 | 0.35 | 0.08 | 0.32 | CG-87 protein |
| yd06g01 | 2455 | R38891; R81283 | Hs.165570 | 1.41 | 0.07 | 0.45 | 0.10 | 0.32 | H. sapiens clone 25052 mRNA sequence |
| yf64f10 | 111134 | R36936 | Hs.80285 | 8.72 | 0.83 | 2.76 | 0.35 | 0.32 | mRNA cDNA DKFZp586C1723 (from clone DKFZp586C1723) |
treatment [55-57]. M6PR encodes the cation-dependent mannose-6-phosphate receptor, which has been implicated in retinoid-induced apoptosis [58]. NMA, encoding a putative transmembrane protein, is expressed at low levels in metastatic human melanoma cell lines and xenografts, and is completely absent in highly metastatic human melanoma cell lines [59]. APCL, encoding adenomatous polyposis coli like protein, is a tumor-suppressor gene [60]. METAP2 encodes methionine aminopeptidase eIF-2-associated p67, which interacts with eukaryotic translation initiation factor eIF-2 [61] and could regulate p53 signaling [62]. BAT3, downregulated in some transformed cells, encodes HLA-B associated transcript-3, which interacts with eukaryotic translation initiation factor eIF-2 [61] and could regulate p53 signaling [62]. BAT3, downregulated in some transformed cells, encodes HLA-B associated transcript-3, which interacts with eukaryotic translation initiation factor eIF-2 [61] and could regulate p53 signaling [62]. BAT3, downregulated in some transformed cells, encodes HLA-B associated transcript-3, which interacts with eukaryotic translation initiation factor eIF-2 [61] and could regulate p53 signaling [62]. BAT3, downregulated in some transformed cells, encodes HLA-B associated transcript-3, which interacts with eukaryotic translation initiation factor eIF-2 [61] and could regulate p53 signaling [62].

Four genes out of the other genes presented in Table 2 have already been implicated in neuronal disorders, suggesting that ceramide may be a key second messenger in these pathologies. The upregulation of the glutamate receptor gene (GRIA2) seems to be an indicator of tolerance to ischemia [75]. The absence of somatostatin, encoded by SST (downregulated in our model), is associated with apoptotic neurons in patients with Alzheimer’s disease [76]. SMN, encoding Survival of motor neuron 2, downregulated by C2-ceramide, strongly contributes to the severity of the spinal muscular atrophy [77]. MUT mRNA is upregulated in ischemia, in relation to a decrease in the accumulation of its neurotoxic metabolite [78].

In conclusion, our cell culture model has enabled us to establish a profile of gene expression during the effector phase of ceramide-mediated cell death. In spite of the stringency of the criteria adopted for differential hybridization, a large number of cDNA clones, 239 of the 9,120 in our cDNA array derived from a normalized infant brain library,
Table 5

Unknown genes differentially expressed in ceramide-dependent apoptosis

| Clone ID | GENX | GenBank accession number | Unigene | C. int. | C. SD | S. int. | S. SD | Ratio | Similarity |
|----------|------|--------------------------|---------|---------|-------|---------|-------|-------|------------|
| **Upregulated clones** |
| yf66a04 | 17755 | R18781 | 1.04 | 0.24 | 5.19 | 0.59 | 4.98 ESTs |
| yf88d07 | 3024 | R15141; R41563 | Hs.12381 | 1.21 | 0.09 | 5.83 | 0.85 | 4.80 ESTs |
| yc85h07 | 11132 | F12902; T74741 | 1.38 | 0.16 | 5.75 | 0.53 | 4.16 ESTs |
| yg38a10 | 2564 | R19870; R45098 | Hs.182503 | 1.32 | 0.23 | 5.46 | 0.63 | 4.15 ESTs |
| c-25h01 | 1301 | Z44625 | Hs.29672 | 4.51 | 0.68 | 18.31 | 3.99 | 4.06 ESTs |
| yg53c11 | 5862 | R25710; R62454 | Hs.238956 | 1.83 | 0.19 | 7.36 | 0.59 | 4.02 ESTs |
| yg02a02 | 5691 | R18381; R42444 | Hs.240816 | 1.52 | 0.23 | 6.08 | 0.94 | 4.00 ESTs |
| yh09g12 | 4411 | R61781; R61782 | 1.20 | 0.30 | 4.55 | 0.24 | 3.78 ESTs |
| yf80c09 | 943 | R14362 | 2.24 | 0.32 | 8.34 | 0.16 | 3.73 ESTs |
| yd02e05 | 761 | R39357; T80134 | Hs.306425; Hs.327350 | 1.20 | 0.19 | 4.47 | 0.73 | 3.72 ESTs |
| yg17c05 | 5291 | R18746; R43067 | Hs.238956 | 1.09 | 0.11 | 3.79 | 0.39 | 3.48 ESTs |
| c-2ef12 | 1659 | F07687 | 2.97 | 0.12 | 8.10 | 0.31 | 3.36 ESTs |
| yf58c03 | 1072 | R12737; R39789 | Hs.119714 | 3.06 | 0.60 | 10.30 | 0.39 | 3.36 ESTs |
| yf69b01 | 160 | H00104 | 5.20 | 2.54 | 10.72 | 1.23 | 3.11 ESTs |
| yc93d09 | 438 | T77119 | Hs.21417 | 2.08 | 0.45 | 6.77 | 1.60 | 3.25 ESTs |
| c-28b03 | 1425 | F07517; Z40576 | 2.49 | 0.35 | 7.77 | 0.52 | 3.12 ESTs |
| yg60e11 | 2509 | R35134 | 4.12 | 0.92 | 13.72 | 2.61 | 2.82 ESTs |
| yf96g09 | 11047 | H09060 | 2.97 | 0.51 | 9.23 | 1.00 | 3.11 ESTs |
| yg02b03 | 2758 | R18419 | Hs.18585 | 3.51 | 0.62 | 10.83 | 1.03 | 3.09 ESTs |
| yf94d10 | 11844 | R16329; R41405 | Hs.197143 | 2.73 | 0.46 | 8.41 | 1.14 | 3.08 ESTs |
| yf63h02 | 201117 | R13594 | Hs.155639 | 1.92 | 0.25 | 5.75 | 0.77 | 3.00 ESTs |
| yf98c09 | 16058 | R18177; R42241 | Hs.106359 | 1.07 | 0.24 | 3.17 | 0.27 | 2.95 ESTs |
| yf80c07 | 1885 | F07517; Z40576 | 2.49 | 0.35 | 7.77 | 0.52 | 3.12 ESTs |
| yc92a01 | 11141 | F13028; T76925 | 2.08 | 0.45 | 6.77 | 1.60 | 3.25 ESTs |
| yf76a02 | 711 | R13339 | Hs.7913 | 5.21 | 0.78 | 14.52 | 3.06 | 2.79 ESTs |
| yf55h04 | 664 | R12357 | 3.64 | 0.18 | 9.99 | 1.24 | 2.75 ESTs |
| yc85h06 | 11131 | F12901; T74740 | 5.20 | 0.54 | 14.15 | 2.43 | 2.72 ESTs |
| yf88c03 | 10642 | F12878; R38624 | Hs.106313 | 1.50 | 0.30 | 4.04 | 0.70 | 2.70 ESTs |
| yg39a10 | 10317 | R19899; R45120 | Hs.89388 | 4.91 | 0.67 | 12.91 | 2.40 | 2.63 ESTs |
| yc15d09 | 6818 | R61465 | 4.60 | 0.37 | 11.88 | 1.21 | 2.58 ESTs |
| yg02g01 | 1987 | R18425; R42486 | Hs.4983 | 1.11 | 0.27 | 2.75 | 0.51 | 2.47 ESTs |
| yg08h03 | 201114 | R22721; R43427 | Hs.244482 | 0.70 | 0.17 | 1.60 | 0.08 | 2.28 ESTs |
| yg33b02 | 4208 | R20161; R44947 | Hs.22905 | 0.91 | 0.19 | 2.05 | 0.17 | 2.26 ESTs |
| yf44c04 | 3106 | R25497; R45563 | None | 1.11 | 0.27 | 2.60 | 0.50 | 2.33 ESTs |
| yf46g12 | 5388 | R20696; R45358 | Hs.311444; Hs.6591 | 0.90 | 0.17 | 1.95 | 0.48 | 2.16 ESTs |
| yg42a06 | 2573 | R25050; R45389 | Hs.23558 | 0.57 | 0.14 | 1.22 | 0.22 | 2.13 ESTs |
| yf63f11 | 5521 | R36919 | Hs.25205 | 0.99 | 0.14 | 2.11 | 0.19 | 2.13 ESTs |
| **Downregulated clones** |
| c-2eg10 | 1662 | F03955; F07692 | 1.04 | 0.19 | 0.51 | 0.08 | 0.49 ESTs |
| c-29f04 | 201571 | Z40598; Z45048 | Hs.184780 | 1.06 | 0.15 | 0.52 | 0.11 | 0.49 ESTs |
| c-2ia08 | 1913 | Z40977; Z45261 | Hs.125266 | 1.03 | 0.22 | ND | ND | 0.49 ESTs |
| c-2ch10 | 3050 | F03889; F07637 | Hs.27278 | 2.42 | 0.49 | 1.12 | 0.24 | 0.46 ESTs, weakly similar to chain A, cyclophilin A complexed with cyclosporin A (H. sapiens)
correspond to genes up- or downregulated by C₂-ceramide treatment. Already-known genes account for 179 of the transcripts, 113 of which have a putative function.

On the basis of their putative functions, we have made an attempt at classifying these transcripts, first with respect to known effects of ceramide or ceramide-mediated transduction systems, then with respect to regulation of cell growth and apoptosis. The 30 genes in Tables 1 and 2 met these criteria, validating the approach and suggesting that the other modulated genes may also be relevant with regard to the progression of the cell-death mechanisms. These genes were classified as having no obvious relation to cell death or survival (Table 3), no known function (Table 4) or as poorly characterized (Table 5).

Interestingly, given the large number of genes known to be modulated by NFκB in the immune system [79], it was surprising that only pentraxin was detected in our model. This suggests either that NFκB is less important in neurons than in lymphocytes, or that its targets are different. Conversely, the transcriptional regulators responsible for the differential expression of the genes detected in our study remain to be discovered. In any case, our results show that transcriptional regulation plays an important role in ceramide-mediated cell death and that some of the modulated transcripts, in agreement with published studies, are involved in other cell-death mechanisms as well.

### Materials and methods

#### Cell culture

Rat PC12 cells [80], which acquire a neuronal phenotype in the presence of nerve growth factor (NGF), were plated at a density of 2,000-3,000 cells/cm² in 75 cm² culture flasks coated with polyethyleneimine (1 mg/ml) in Leibovitz modified L15 medium (Gibco BRL) supplemented with 2% horse serum and 150 ng/ml NGF (grade II; Alomone Labs, Jerusalem, Israel). Rat PC12 cells were plated at a density of 2,000-3,000 cells/cm², then incubated with 10 ng/ml C₂-ceramide (naturals) for 24 h. The cells were used for experimentation after this incubation period.

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**Table 5**

| Clone ID | GENX | GenBank accession number | Unigene | C. int. | C. SD | S. int. | S. SD | Ratio | Similarity |
|----------|------|--------------------------|---------|---------|--------|---------|--------|-------|------------|
| yg83b05 | 20476| R53938; R53333            |         | 1.27    | 0.10   | 0.56    | 0.13   | 0.44  | ESTs      |
| yg36f04 | 5214 | R24580                    | Hs.27104| 2.18    | 0.53   | 0.95    | 0.17   | 0.43  | ESTs      |
| yf60a12 | 3065 | R38592; R13746            |         | 6.52    | 1.15   | 2.61    | 0.42   | 0.40  | ESTs      |
| yc86e07 | 2935 | F10326; F12716            | Hs.227993| 7.76   | 0.94   | 3.02    | 0.59   | 0.39  | ESTs      |
| yc90f10 | 10752| F10679; F13085            | Hs.12395| 1.12    | 0.20   | 0.43    | 0.08   | 0.38  | ESTs      |
| yc97e12 | 4395 | T78036                    | Hs.23213| 1.11    | 0.20   | 0.41    | 0.05   | 0.37  | ESTs      |
| yf50h10 | 477  | R11920; R39108            | Hs.6777 | 2.39    | 0.56   | 0.82    | 0.16   | 0.34  | ESTs      |
| yf74a06 | 16024| R13206; R40294            |         | 1.32    | 0.28   | 0.45    | 0.10   | 0.34  | ESTs      |
| yg96e11 | 3143 | R59141; R59142            |         | 1.30    | 0.19   | 0.43    | 0.08   | 0.33  | ESTs      |
| yf51a04 | 958  | R11976; R39818            | Hs.4241 | 1.25    | 0.23   | 0.40    | 0.09   | 0.32  | ESTs      |
| yg51e05 | 5025 | R46483; R21387            | Hs.23187| 6.26    | 0.92   | 1.98    | 0.19   | 0.32  | ESTs      |
| yg02h09 | 2969 | R17514; R42608            | Hs.139270| 10.09  | 0.93   | 3.19    | 0.46   | 0.32  | ESTs      |
| yf66e03 | 978  | R37086                    | Hs.23210| 1.72    | 0.18   | ND      | ND     | 0.29  | ESTs      |
| yf76b06 | 115094| R18860                   | Hs.203213| 1.72   | 0.28   | ND      | ND     | 0.29  | ESTs      |
| yf91f12 | 4185 | H08130; H08131            | Hs.19515| 2.86    | 0.33   | 0.70    | 0.17   | 0.25  | ESTs      |
| yg14a03 | 2782 | R17432; R42778            | Hs.22217| 1.57    | 0.27   | 0.34    | 0.06   | 0.22  | ESTs      |
| yf52e12 | 4147 | R12228; R39947            | Hs.7237 | 1.57    | 0.30   | 0.34    | 0.06   | 0.22  | ESTs      |
| yf50g11 | 1829 | R11917; R39107            | Hs.352354; Hs.244624| 2.37   | 0.24   | 0.48    | 0.10   | 0.20  | ESTs      |
| yf84b08 | 2237 | R14545; R41206            | Hs.349648| 1.06   | 0.17   | 0.19    | 0.03   | 0.18  | ESTs, weakly similar to KIAA1157 protein (H. sapiens) |

Abbreviations and column headings are as in Table 1.
Israel) as previously described [81]. Apoptosis was induced, after 6 days in the presence of NGF, with the cell-permeant C2 analog of ceramide (C2-ceramide), N-acetylsphingosine (Biomol Research Laboratories, Plymouth Meeting, PA), at a concentration of 25 μM. As negative control, an inactive C2 analog of ceramide (C2-dihydroceramide), N-acetylsphinganine (Biomol Research Laboratories), was used in the same condition as C2-ceramide.

**Morphological characterization of apoptosis and cell counts**

Neurite retraction and cell shrinkage were visualized by phase-contrast microscopy. Condensed and fragmented nuclei were made visible in situ as described in [7], by intercalation into nuclear DNA of the fluorescent probe propidium iodide. Propidium iodide, which only enters dead cells that have become permeable, was visualized by epifluorescence with a rhodamine filter (excitation, 548-580 nm; emission, 580-610 nm). Viability was quantified by counting cells in at least 10 randomly chosen fields with a 20x objective. The percentage of cells excluding the vital dye propidium iodide was calculated at each time point after the beginning of C2-ceramide or C2-dihydroceramide treatment with respect to the corresponding control.

**Measurement of caspase-3-like activity**

Caspase-3-like activity was measured using the CaspACE Assay system (Promega, Madison, WI). Cell extracts containing equivalent amounts of protein were used to measure DEVDase (caspase-3-like) activity: the chromophore p-nitroaniline (pNA), released from the colorimetric substrate (Ac-DEVD-pNA) upon cleavage by DEVDase produces a yellow color that is monitored by a photometer at 405 nm.

**Preparation of the cDNA macroarray**

cDNA clones from a normalized infant brain library (library 1NIB; [20]) were randomly selected to provide a set of 9,120 cDNA clones. The 3’ and/or 5’ ends of these clones had been previously sequenced [25]. The sequences, registered in GenBank [82], were compared to those in public data bases, permitting tentative identification of the corresponding gene transcripts. The cDNA clones were used to prepare PCR products using oligonucleotide primers complementary to sequences in the vector. They were spotted by robot (Flexis; Perkin Elmer, Shelton, CT) at medium density (25 PCR products/cm²) on nylon membranes (Hybond-N+; Amersham Biosciences, Uppsala, Sweden) as previously described [83]. The entire collection of 9,120 cDNA clones was spotted on a set of four filters.
Figure 7
Schematic illustrating the putative roles of the proteins encoded by the genes noted in Figures 4 and 5.
Purification of poly(A)+ mRNA
Total RNA was extracted from control PC12 cultures and from PC12 cultures treated with C2-ceramide or C2-dihydroceramide (approximately 10⁶ cells) with the RNasea midi kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. The integrity of the RNA was confirmed by agarose gel electrophoresis. Poly(A)+ mRNA was extracted from total RNA with oligo(dT)-conjugated magnetic beads (Dynabeads; Dynal, Oslo, Norway), as described in the manufacturer’s protocols.

Complex cDNA target synthesis
Complex cDNA targets were synthesised by reverse transcription of 500 ng poly(A)+ mRNA extracted from control, C2-dihydroceramide- or C2-ceramide-treated PC12 cells. The reaction was performed with the SuperScript™ Preamplification System (Invitrogen) as previously described [84]. The reaction mixture contained random-oligonucleotide primers (500 ng), 50 µCi [α-33P]dATP, 3,000 Ci/mmol (Amersham), 500 µM d(T, C, G)TP (Amersham) and 50 µM dideoxyGTP (Invitrogen).

Filter hybridization
The filters were prehybridized at 68°C for 30 min in ExpressHyb hybridization solution (Clontech, Palo Alto, CA), hybridized for 2 h in the same solution to which the radiolabeled complex cDNA target was added, then washed twice for 30 min at 25°C in standard saline citrate (SSC) 1x/0.1% sodium dodecyl sulfate (SDS) and twice for 30 min at 25°C in SSC 0.1x/0.1% SDS. The washed filters were exposed to phosphor screens (Molecular Dynamics, Sunnyvale, CA) for 16 h.

Hybridization signal quantitation
Image acquisition was carried out with the PhosphorImager (Molecular Dynamics). The hybridization signal corresponding to each cDNA clone was quantitated with a specifically designed software (XdotsReader; Cose, Dugny, France) and compared to the signal obtained with the local background signal was subtracted. The intensity of the hybridization signal for each clone was then divided by the hybridization signal corresponding to 18S rRNA (control probe). For northern blot analysis, the blots were prehybridized 2 h in ULTRAhyb hybridization buffer (Ambion, Austin, TX), hybridized with the labelled probe (1-2 x 10⁶ cpm/ml) for 16 h at 42°C in the same solution, and washed as for the high-density filters. The washed filters were exposed to phosphor screens (Molecular Dynamics) for 48 h. The hybridization signal of the specific probes was analyzed with the ImageQuant software (Molecular Dynamics) and compared to the signal obtained with the control probe.

RT-PCR
Total RNA of PC12 cells cultured with or without C2-ceramide was purified according to the protocol described above. Total RNA (2 µg) were reverse transcribed using the SuperScript™ Preamplification System (Invitrogen) according to the manufacturer’s protocol. An aliquot of the reaction was then used for PCR amplification with the Advantage PCR kit (Clontech) and primers specific to the gene of interest. The amplification products were visualized after electrophoresis in a 1.5% agarose gel with ethidium bromide. The signals were analyzed with ImageQuant software and compared to HPRT (hypoxanthine phosphoribosyltransferase) as control gene.

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