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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 neuronally differentiated PC12 cells.

**Results:** To investigate gene expression during ceramide-dependent apoptosis, we carried out a global study of gene expression in neuronally differentiated PC12 cells treated with C<sub>2</sub>-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 C<sub>2</sub>-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.

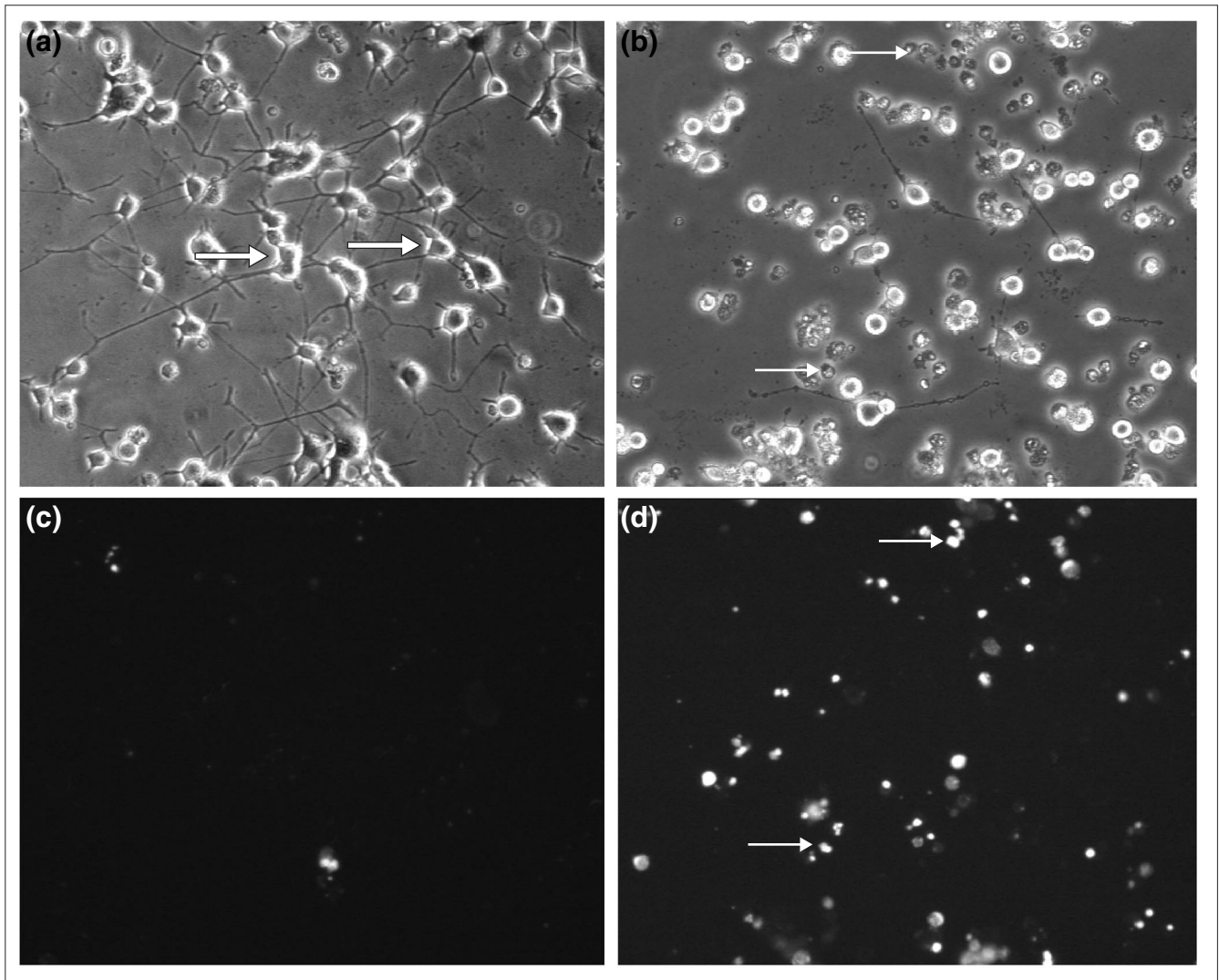
**Conclusions:** Our cell-culture model has enabled us to establish a profile of gene expression during the effector phase of ceramide-mediated cell death. Of the 239 genes that met the criteria for differential hybridization, 10 correspond to genes previously involved in C<sub>2</sub>-ceramide or TNF- $\alpha$  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- $\alpha$ ), interleukin-1 beta (IL-1 $\beta$ ), 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 neuronally differentiated



**Figure 1**

Morphological characteristics of nerve growth factor (NGF)-differentiated PC12 cells during  $C_2$ -ceramide-induced apoptosis. **(a)** Control cultures of PC12 cells after 6 days in the presence of NGF viewed by phase-contrast microscopy; **(b)** NGF-differentiated PC12 cells after 24 h treatment with 25  $\mu$ M  $C_2$ -ceramide. Open arrows, viable cells; white arrows, dead cells. **(c,d)** Condensed and fragmented nuclei of dead cells in (c) control and (d) NGF-differentiated PC12 cells visualized by intercalation of propidium iodide into DNA were viewed under epifluorescence illumination.

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  $C_2$ -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 NF $\kappa$ B [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 neuronally differentiated rat pheocytoma PC12 cells treated with C<sub>2</sub>-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 C<sub>2</sub>-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

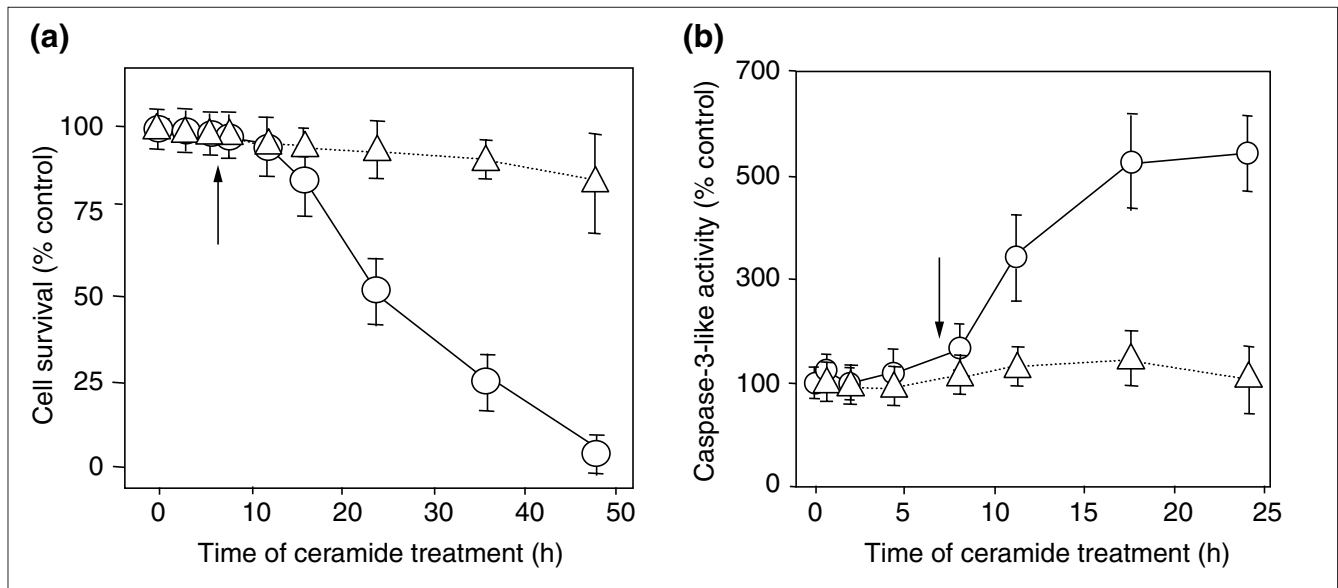
### Cell death induced in neuronally differentiated PC12 cells by C<sub>2</sub>-ceramide

The morphological characteristics of differentiated PC12 cells after 24 hours in the presence of 25 μM C<sub>2</sub>-ceramide were compatible with cell death by apoptosis. Compared with control cultures, as viewed by phase-contrast microscopy (Figure 1a), C<sub>2</sub>-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

C<sub>2</sub>-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 neuronally differentiated PC12 cells or primary cultures of mesencephalic neurons were treated with cell-permeant C<sub>2</sub>-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 C<sub>2</sub> analog of ceramide, C<sub>2</sub>-dihydroceramide (Figure 2).

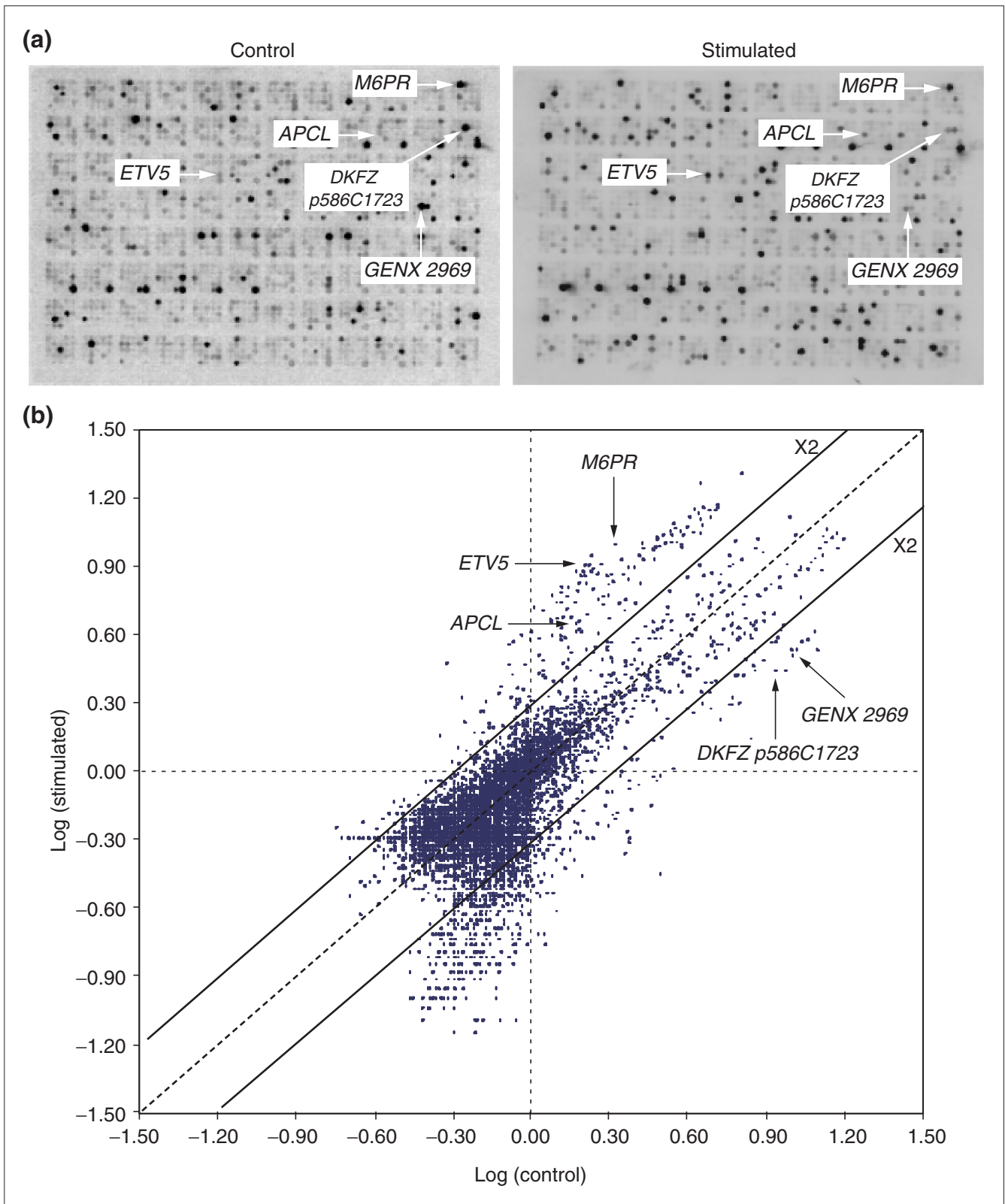
### Validation of hybridization signals

Hybridization of 9,120 cDNA clones with complex cDNA targets from poly(A)<sup>+</sup> RNA extracted from C<sub>2</sub>-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



**Figure 2**

Characterization of C<sub>2</sub>-ceramide-induced apoptosis. **(a)** Time course of cell death induced by 25 μM C<sub>2</sub>-ceramide (circles) or by 25 μM C<sub>2</sub>-dihydroceramide (triangles). Cells were counted 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 C<sub>2</sub>-ceramide treatment with respect to the corresponding control. **(b)** Time course of caspase-3-like activity after 25 μM C<sub>2</sub>-ceramide (circles) or 25 μM C<sub>2</sub>-dihydroceramide (triangles) treatment. Data are mean ± SEM (bars) values of at least three experiments, performed in triplicate. The black arrows indicate the time of C<sub>2</sub>-ceramide treatment of the cell cultures used in the expression study.



**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 C<sub>2</sub>-ceramide treatment (control) or treated with C<sub>2</sub>-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 C<sub>2</sub>-ceramide-treated cDNA target (1,297) were excluded from further analysis. The remaining 6,494 clones were analyzed for differential hybridization.

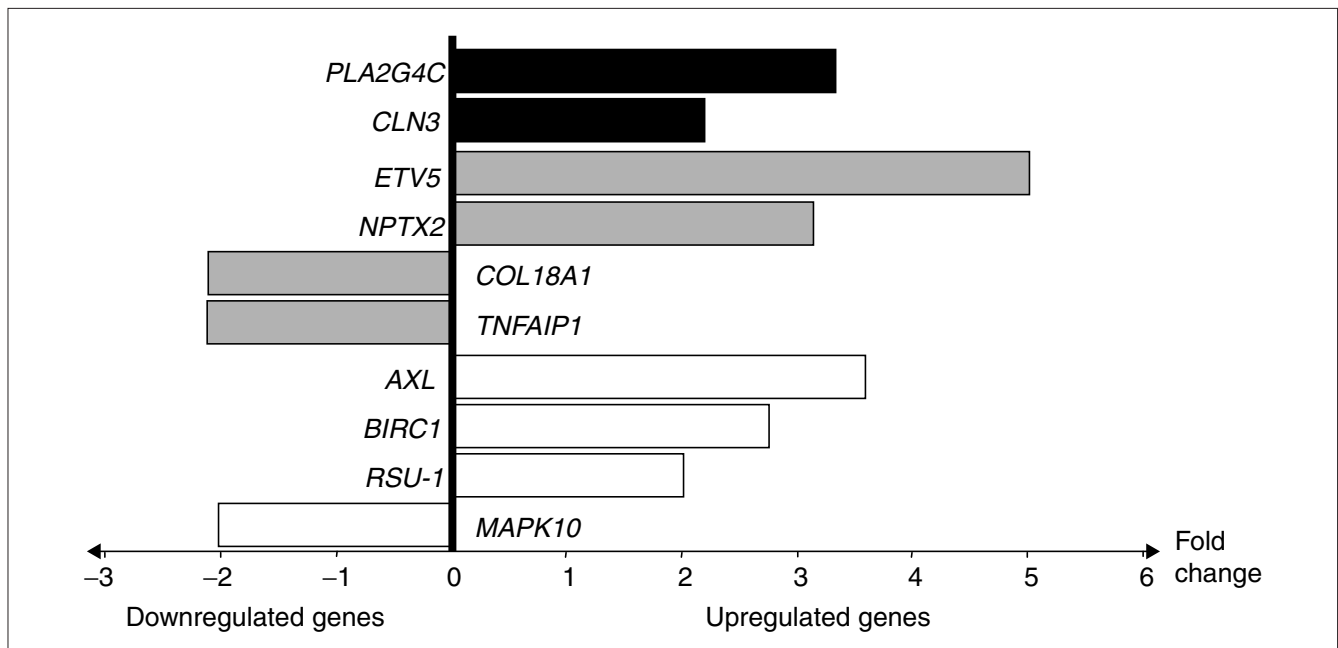
**Differential gene expression in neuronally differentiated PC12 cells treated with C<sub>2</sub>-ceramide compared to controls**

Changes in gene expression were analyzed during the effector phase of neuronal death, 7 hours after the beginning of C<sub>2</sub>-ceramide treatment. This time point was chosen because on the one hand it is preceded by the activation of the transcription factor NFκB and c-Jun observed 4 to 6 hours after C<sub>2</sub>-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 C<sub>2</sub>-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 C<sub>2</sub>-ceramide cDNA target to that obtained with the control target. Clones were considered to be differentially hybridized in C<sub>2</sub>-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)<sup>+</sup> RNA extracted from C<sub>2</sub>-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 C<sub>2</sub>-ceramide (data not shown). Among the 239 clones that met the criteria for differential hybridization, 132 were up-hybridized in C<sub>2</sub>-ceramide-treated cells and 107 were down-hybridized. The distribution of the hybridization-intensity values between the control and the C<sub>2</sub>-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 C<sub>2</sub>-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 laboratory (the Genexpress Index [23]) and in public databases. Of the 239 clones, 179 clones corresponded to already identified 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



**Figure 4** Differentially expressed genes that encode proteins with functions involved in ceramide-dependent apoptosis. Black boxes, Genes involved in the ceramide signaling pathway; gray boxes, genes transcriptionally stimulated by TNF-α; white boxes, genes involved in the TNF-α signaling pathway.

**Table 1****Genes differentially expressed in ceramide-dependent apoptosis and involved in the ceramide and TNF- $\alpha$  pathways**

Clone ID	GENX	GenBank accession number	Unigene	C. int.	C. SD	S. int.	S. SD	Ratio	Similarity	Gene symbol
Genes involved in the C <sub>2</sub> -ceramide signaling pathway										
yf59e08	5705	R13531	Hs.18858	3.56	0.54	12.01	0.86	3.37	Phospholipase A2, group IVC (cytosolic, calcium-independent)	<i>PLA2G4C</i>
yf71a08	115123	R12998; R40387	Hs.194660	1.14	0.23	2.53	0.15	2.21	Ceroid-lipofuscinosis, neuronal 3, juvenile (Batten, Spielmeyer-Vogt disease)	<i>CLN3</i>
Genes transcriptionally stimulated by TNF- $\alpha$										
yg86b08	4272	R53048; R53135	Hs.43697	1.72	0.18	8.77	0.58	5.09	Ets variant gene 5 (ets-related molecule)	<i>ETV5</i>
yc86d06	5838	F12910; T75064	Hs.3281	3.61	0.48	11.54	0.61	3.19	Neuronal pentraxin II	<i>NPTX2</i>
yc88f11	197	F10424; F12821	Hs.78409	1.18	0.21	0.55	0.11	0.47	Collagen, type XVIII, alpha 1	<i>COL18A1</i>
yf78g01	200888	R14176; R40470	Hs.76090	1.19	0.28	0.54	0.06	0.46	TNF- $\alpha$ -induced protein 1 (endothelial)	<i>TNFAIP1</i>
Genes involved in the TNF- $\alpha$ signaling pathway										
yf76d09	1017	R13424; R40936	Hs.83341	1.49	0.23	5.45	0.54	3.65	AXL receptor tyrosine kinase	<i>AXL</i>
yg49f10	116415	R20716	Hs.79019	4.54	0.13	12.69	1.38	2.80	Baculoviral IAP repeat-containing 1	<i>BIRC1</i>
c-26g10	1350	F07467	Hs.75551	1.37	0.27	2.81	0.56	2.05	Ras suppressor protein 1	<i>RSU1</i>
c-08d10	4997	F05370; Z38358	Hs.151051	4.94	1.06	2.41	0.51	0.49	Mitogen-activated protein kinase 10	<i>MAPK10</i>

Clone ID, clone name according to the public databases. GENX, cluster name including the corresponding cDNA sequence in the Genexpress Index 2 ([23] and R. Mariage-Samson *et al.*, unpublished data). UniGene, cluster name in the UniGene database [85]; C. int., mean of the normalized and validated intensity values obtained after filter hybridization with complex cDNA target derived from control mRNA. C. SD, standard deviation derived from the C. int. S. int., mean of the normalized and validated intensity values obtained after filter hybridization with complex cDNA target derived from ceramide-stimulated cultured cell mRNA. S. SD, standard deviation derived from the S. int. Ratio, ratio of S. int. to C. int. Similarity, gene similarity.

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.

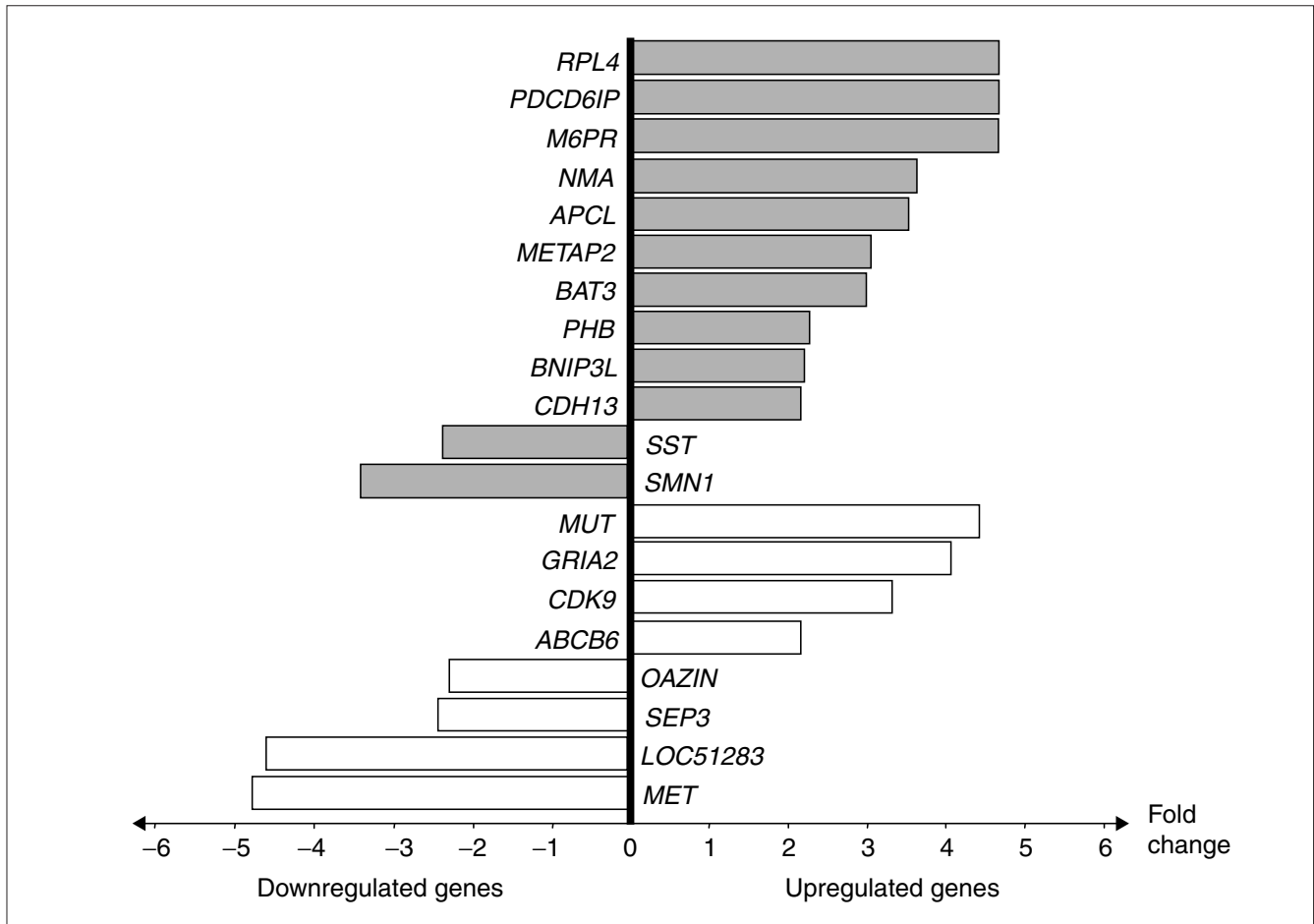
Ten differentially expressed genes encode proteins with a role in ceramide or TNF- $\alpha$  signaling pathways (Figure 4, Table 1; see [24] for links to database entries for each gene). Two of these genes, *PLA2G4C* [25] and *CLN3* [26,27] seem to have a role in ceramide-mediated cell death or survival. Two upregulated genes (*ETV5* [28], *NPTX2* [29,30]) and two downregulated genes (*COL18A1* [31,32], *TNFAIP1* [33]) encode proteins that are modulated by TNF- $\alpha$ . Four genes, three upregulated (*AXL* [34], *BIRC1* [35], *RSU1* [36]) and one downregulated (*MAPK10* [37]) encode proteins with a role in the TNF- $\alpha$  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<sub>2</sub>-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 *ETV5*, *M6PR* and *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.



**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.

**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 C<sub>2</sub>-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



**Table 2****Differentially expressed genes that encode proteins involved in the regulation of apoptosis and/or cell growth**

Clone ID	GENX	GenBank accession number	Unigene	C. int.	C. SD	S. int.	S. SD	Ratio	Similarity	Gene symbol
Proteins stimulating apoptosis and/or growth arrest										
yg01b10	2112	R18353; R42557	Hs.286	1.68	0.17	7.95	0.55	4.74	Ribosomal protein L4	<i>RPL4</i>
yl73h11	567	H06473	Hs.9663	1.60	0.15	7.42	0.54	4.64	Programmed cell death 6-interacting protein	<i>PDCD6IP</i>
yc92h11	673	F13260; T77039	Hs.75709	2.12	0.33	9.83	0.95	4.64	Mannose-6-phosphate receptor (cation dependent)	<i>M6PR</i>
yg94h08	6030	R56149	Hs.78776	1.96	0.34	7.09	1.25	3.61	Putative transmembrane protein	<i>NMA</i>
yf90d04	25970	R15366	Hs.20912	1.34	0.22	4.71	0.58	3.51	Adenomatous polyposis coli like	<i>APCL</i>
yg76b02	3804	R51346; R51453	Hs.78935	0.95	0.18	2.89	0.40	3.03	Methionine aminopeptidase; eIF-2-associated p67	<i>METAP2</i>
yd01h06	9451	R39334; T78769	Hs.274348	1.98	0.16	5.89	0.66	2.97	HLA-B associated transcript-3	<i>BAT3</i>
c-22F12	2915	F08770	Hs.75323	1.44	0.23	3.26	0.29	2.26	Prohibitin	<i>PHB</i>
yf69g07	115124	R14126	Hs.132955	1.82	0.30	3.99	0.96	2.19	BCL2/adenovirus E1B 19kD-interacting protein 3-like	<i>BNIP3L</i>
yd02b11	115910	T79985	Hs.63984	0.88	0.19	1.88	0.30	2.14	Cadherin 13, H-cadherin (heart)	<i>CDH13</i>
c-3ke04	781	F10823; F13223	Hs.12409	1.17	0.10	ND	ND	0.43	Somatostatin	<i>SST</i>
yg64g08	115205	R35542; R51110	Hs.288986	3.01	0.41	0.90	0.15	0.30	Survival of motor neuron 1, telomeric	<i>SMN1</i>
Proteins downregulating apoptosis and/or stimulating growth										
yg44d03	408	R25503	Hs.155212	1.74	0.40	7.67	0.40	4.40	Methylmalonyl coenzyme A mutase	<i>MUT</i>
yg68d10	2957	R36284; R49571	Hs.89582	1.80	0.25	7.26	0.57	4.04	Glutamate receptor, ionotropic, AMPA 2	<i>GRIA2</i>
yl81d04	9379	H05457; H07007	Hs.150423	2.64	0.36	8.72	1.06	3.31	Cyclin-dependent kinase 9 (CDC2-related kinase)	<i>CDK9</i>
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	<i>ABCB6</i>
yg51a11	17820	R21694; R46587	Hs.223014	1.09	0.24	ND	ND	0.46	Antizyme inhibitor	<i>OAZIN</i>
yh10g09	4858	R61276; R61277	Hs.8073	1.59	0.23	0.66	0.16	0.41	Septin 3	<i>SEP3</i>
yf53a12	3165	R12025; R37093	Hs.356245	1.14	0.21	0.29	0.02	0.25	Apoptosis regulator	<i>LOC51283</i>
yg67b12	115951	R35827; R49537	Hs.285754	2.38	0.48	0.50	0.12	0.21	Met proto-oncogene	<i>MET</i>

Abbreviations and column headings are as in Table 1.

[41]. 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  $C_2$ -ceramide-induced apoptosis but modulates endogenous ceramide synthesis and suppresses apoptosis by preventing generation of ceramide [42]. Thus,  $C_2$ -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  $TNF-\alpha$  were also modulated by  $C_2$ -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  $TNF-\alpha$  or  $IL-1\beta$  stimulation [28]. *PEA3* (a mouse protein

**Table 3**

**Known genes differentially expressed in ceramide-dependent apoptosis with no identified direct interaction with the ceramide-dependent apoptosis process**

Clone ID	GENX	GenBank accession number	Unigene	C. int.	C. SD	S. int.	S. SD	Ratio	Similarity	Gene symbol
Signal transduction										
y185b10	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	Myotubularin-related protein 2	MTMR2
yg11b08	107475	R17181; R41731	Hs.5462	0.72	0.12	1.54	0.33	2.14	Solute carrier family 4, sodium bicarbonate cotransporter, member 4	SLC4A4
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	PPAP2B
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	RGS14
yg16c08	5294	R17962; R43452	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	TBPL1
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	114423	F07382	Hs.75678	1.38	0.23	0.66	0.16	0.47	FBJ murine osteosarcoma viral oncogene homolog B	FOSB
yg90e12	10904	R56427; R56428	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)	HNRPC
yf64g02	993	R37803	Hs.6151	4.87	0.77	1.87	0.46	0.38	Pumilio homolog 2 ( <i>Drosophila</i> )	PUM2
yg53f10	1678	R62465; R25720	Hs.520	1.41	0.20	ND	ND	0.35	Nuclear receptor subfamily 2, group C, member 2	NR2C2
yg47e10	1548	R21283; R45373	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	C. int.	C. SD	S. int.	S. SD	Ratio	Similarity	Gene symbol
Cellular traffic or structure proteins										
yg19f05	200119	R20424; R43544	Hs.169793	1.51	0.36	7.37	1.32	4.86	Ribosomal protein L32	<i>RPL32</i>
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	<i>VAPB</i>
yc87f04	5084	R38549; T75126	Hs.22826	1.83	0.11	5.59	0.64	3.06	Tropomodulin 3 (ubiquitous)	<i>TMOD3</i>
yf98g01	8512	R18713	Hs.75196	2.96	0.63	9.29	0.80	3.14	Ankyrin repeat-containing protein	<i>G9A</i>
yh17e09	1304	R59488; R59489	Hs.30991	0.78	0.19	2.32	0.11	2.97	Ankyrin repeat domain 6	<i>ANKRD6</i>
yf76d11	424	R13426; R40938	Hs.119324	0.84	0.08	2.07	0.35	2.48	Kinesin-like 4	<i>KNSL4</i>
c-27f03	1382	F07488	Hs.89497	2.32	0.31	5.60	0.64	2.42	Lamin B1	<i>LMNB1</i>
yc96a12	11155	F13331; T77651	Hs.159613	4.50	0.32	10.84	2.13	2.41	Thyroid hormone receptor binding protein	<i>AIB3</i>
yf57c11	1225	R12822; r20734	Hs.1501	0.94	0.22	2.25	0.22	2.39	Syndecan 2	<i>SDC2</i>
yl71a06	10804	H05894	Hs.6682	1.33	0.11	2.94	0.20	2.21	Solute carrier family 7, cationic amino acid transporter, $\gamma^+$ system, member 11	<i>SLC7A11</i>
yc99f07	11082	T78361	Hs.103042	2.21	0.07	0.98	0.19	0.44	Microtubule-associated protein 1B	<i>MAP1B</i>
yf72e08	2558	R13080; R40510	Hs.7979	2.05	0.34	0.80	0.15	0.39	Likely ortholog of mouse synaptic vesicle glycoprotein 2a	<i>SV2</i>
yc87h12	2952	F10545; F12946	Hs.21611	5.68	0.59	1.93	0.17	0.34	Kinesin family member 3C	<i>KIF3C</i>
yg54d05	604	R25813; R46810	Hs.117977	1.62	0.33	0.50	0.11	0.31	Kinesin 2 (60-70 kD)	<i>KNS2</i>
yf91b02	1980	R16352; R42300	Hs.103042	3.50	0.41	1.01	0.24	0.29	Microtubule-associated protein 1B	<i>MAP1B</i>
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	<i>SLC6A8</i> , <i>DXS1357E</i>
Immunity/inflammatory response										
yg75d06	25621	R54423	Hs.179661	1.88	0.18	8.01	1.02	4.26	FK506-binding protein 1A (12 kD)	<i>FKBP1A</i>
yg65b03	2453	R35324	Hs.9688	0.86	0.13	3.67	0.60	4.26	Leukocyte membrane antigen	<i>IRCI</i>
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	<i>RCD-8</i>
c-2bh04	190137	F03851; F07604	Hs.284394	1.13	0.07	0.56	0.07	0.50	Complement component 3	<i>C3</i>
yf59h02	5580	R13549; R20669	Hs.82689	1.05	0.21	0.47	0.10	0.44	Tumor rejection antigen (gp96) I	<i>TRA1</i>
yc86g03	8628	F10456; F12856	Hs.302749	1.51	0.35	0.58	0.09	0.39	FK506-binding protein 9 (63 kD)	<i>FKBP9</i>
Protein processing										
yf68a10	1071	R40190;	Hs.75890	0.55	0.13	2.09	0.23	3.80	Site-1 protease (subtilisin-like, sterol-regulated, cleaves sterol regulatory element binding proteins)	<i>SIP</i>
c-2na07	2001	F04230; F07978	Hs.102	1.01	0.16	0.46	0.10	0.45	Aminomethyltransferase (glycine cleavage system protein T)	<i>AMT</i>
yc85d05	6301	F10498; F12892	Hs.170197	1.45	0.22	0.59	0.10	0.41	Glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)	<i>GOT2</i>
c-2ge12	2793	Z40826; Z46090	Hs.183212	1.14	0.19	0.45	0.09	0.39	Isoprenylcysteine carboxyl methyltransferase	<i>ICMT</i>
yg52f04	202164	R21082; R46258	Hs.235887	1.55	0.31	0.54	0.08	0.34	HMT1 (hnRNP methyltransferase, <i>Saccharomyces cerevisiae</i> )-like 1	<i>HRMT1L1</i>

**Table 3** (continued)

Clone ID	GENX	GenBank accession number	Unigene	C. int.	C. SD	S. int.	S. SD	Ratio	Similarity	Gene symbol
<b>Proteases</b>										
yf64f07	2813	R13707; R37801	Hs.171501	1.27	0.24	ND	ND	0.39	Ubiquitin specific protease 11	<i>USP11</i>
<b>Metabolism</b>										
yc97f08	1805	R39698; T78043	Hs.2838	2.73	0.24	9.35	0.86	3.42	Malic enzyme 3, NADP(+)-dependent, mitochondrial	<i>ME3</i>
yg97d06	3929	R59198; R59256	Hs.78989	0.68	0.07	1.99	0.18	2.94	Alcohol dehydrogenase 5 (class III), chi polypeptide	<i>ADH5</i>
c-2ca07	1549	F03858; F07608	Hs.180616	1.04	0.12	0.50	ND	0.48	CD36 antigen (collagen type I receptor, thrombospondin receptor)-like 1	<i>CD36L1</i>
yc95g06	3164	R39463; T77281	Hs.155247	1.01	0.20	0.31	0.05	0.30	Aldolase C, fructose-bisphosphate	<i>ALDOC</i>
<b>Miscellaneous</b>										
yg24g06	3097	R19249; R44514	Hs.22654	0.48	0.08	2.93	0.67	6.12	Sodium channel, voltage-gated, type I, alpha polypeptide	<i>SCN1A</i>
yc89d05	10816	F10796; F13191	Hs.12365	1.26	0.20	5.46	0.36	4.34	Synaptotagmin XIII	<i>SYT13</i>
c-28e05	4334	F07514	Hs.6126	2.04	0.50	7.19	1.11	3.52	Mannosidase, beta A, lysosomal-like	<i>MANBAL</i>
yg35g09	4463	R20330	Hs.88764	3.30	0.19	9.88	2.40	2.99	Male-specific lethal-3 ( <i>Drosophila</i> )-like 1	<i>MSL3L1</i>
yg36c01	292	R24560; R44360	Hs.6430	1.50	0.22	4.40	0.78	2.93	Protein with polyglutamine repeat; calcium (Ca <sup>2+</sup> ) homeostasis endoplasmic reticulum protein	<i>ERPROT213-21</i>
yc98a06	1475	R37847; T78111	Hs.301789	1.55	0.27	4.52	0.35	2.92	Capping protein (actin filament) muscle Z-line, alpha 1	<i>CAPZA</i>
yf54h02	924	R11969	Hs.4865	1.41	0.17	3.05	0.18	2.17	Voltage-gated sodium channel beta-3 subunit ( <i>scn3b</i> gene)	<i>HSA243396</i>
yf91a04	434	R16348; R42296	Hs.12152	1.00	0.24	2.03	0.16	2.04	APMCF1 protein	<i>APMCF1</i>
c-1ia09	4199	Z39718; Z43661	Hs.8834	0.64	0.15	1.30	0.19	2.02	Ring finger protein 3	<i>RNF3</i>
yg83b04	4211	R53332; R53937	Hs.7022	1.19	0.28	0.59	0.09	0.50	Dedicator of cytokinesis 3	<i>DOCK3</i>
yf57d02	4610	R12627; R20528	Hs.334688	1.51	0.25	0.75	0.13	0.50	Phytanoyl-CoA hydroxylase interacting protein	<i>PHYHIP</i>
yg36h06	3087	R24595; R44400	Hs.7122	1.08	0.13	ND	ND	0.46	Scrapie responsive protein 1	<i>SCRGI</i>
yf99b05	2822	R18211; R42149	Hs.79284	1.07	0.21	0.48	0.07	0.45	Mesoderm specific transcript (mouse) homolog	<i>MEST</i>
c-24h06	92359	Z40467; Z44591	Hs.171545	1.02	0.20	0.45	0.11	0.44	HIV-1 Rev binding protein	<i>HRB</i>
yd05d01	2346	R38832; T80384	Hs.13493	1.06	0.24	0.44	0.06	0.42	Like mouse brain protein E46	<i>E46L</i>
yf74e11	2106	R13277; R40723	Hs.334851	1.95	0.41	0.80	0.17	0.41	LIM and SH3 protein 1	<i>LASP1</i>
yd01e11	3181	T78746	Hs.168640	1.17	0.27	0.47	0.10	0.40	Homolog of mouse Ank	<i>ANK</i>
yf48e09	414	R12292; R12804	Hs.21050	1.22	0.26	0.47	0.07	0.38	g20 protein	<i>LOC51161</i>
yg16d07	1087	R43459; R17969	Hs.87125	9.91	2.06	3.35	0.51	0.34	EH-domain containing 3	<i>EHD3</i>
yf57d07	12763	R12632; R20533	Hs.109706	1.78	0.23	0.58	0.09	0.33	Hematological and neurological expressed 1	<i>HNI</i>
c-2la12	200991	F04056; F07796	Hs.74376	1.00	0.07	0.33	0.01	0.33	Olfactomedin related ER localized protein	<i>NOE1</i>

**Table 3** (continued)

Clone ID	GENX	GenBank accession number	Unigene	C. int.	C. SD	S. int.	S. SD	Ratio	Similarity	Gene symbol
Miscellaneous (continued)										
yf61b05	1882	R13783; R37641	Hs.297743	2.04	0.16	0.63	0.12	0.31	Carbonic anhydrase X	CA10
yf61a10	3960	R39112; R13989	Hs.2288	12.49	1.82	3.38	0.84	0.27	Visinin-like 1	VSNL1
yg53b12	8924	R25707; R62451	Hs.169047	2.34	0.46	0.54	0.12	0.23	Chondroitin sulfate proteoglycan 3 (neurocan)	CSPG3

Abbreviations and column headings are as in Table 1.

corresponding to ETV5) inhibits tumorigenesis *in vivo* [43]. Moreover, ETV5 and ETS1 can cooperate with c-Jun/c-Fos [44,45], potential regulators of apoptosis in many cell types and specially in the mammalian nervous system [46]. The second gene regulated by TNF- $\alpha$  is *NPTX2*, encoding neuronal pentraxin II. Pentraxins are a family of proteins that include C-reactive protein and serum amyloid P. They have been found in the brain plaques characteristic of Alzheimer's disease and are toxic to neuronal cell cultures [47,48]. Furthermore, the expression of *NPTX3* is increased in response to TNF- $\alpha$  or IL-1 $\beta$  stimulation via activation of NF $\kappa$ B [29,30]. The regulation of the pentraxin gene family by C<sub>2</sub>-ceramide treatment is consistent with our previous studies showing NF $\kappa$ B activation by C<sub>2</sub>-ceramide in PC12 cells and in primary cultures of neurons [10,19]. The last two genes known to be regulated by TNF- $\alpha$  and identified in our model are *COL18A1*, encoding type XVIII collagen alpha 1, and *TNFAIP1*, encoding TNF- $\alpha$ -induced protein 1. These proteins, downregulated by C<sub>2</sub>-ceramide, are modulated by TNF- $\alpha$  in various cell types [31-33].

We also identified four genes encoding proteins known to participate in TNF- $\alpha$ -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- $\alpha$ -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- $\alpha$  [35]. The third identified gene, *RSU1*, encodes Ras suppressor protein 1, which is involved in TNF- $\alpha$  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 C<sub>2</sub>-ceramide in our model.

The identification of these eight genes, which are involved in the TNF- $\alpha$  signaling pathway, in C<sub>2</sub>-ceramide treated PC12 cells, suggests that their modulation of expression by TNF- $\alpha$  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 C<sub>2</sub>-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 C<sub>2</sub>-ceramide in our model, encodes a protein that interacts with ALG2, a Ca<sup>2+</sup>-binding protein that is required for apoptosis induced by diverse stimuli, including ceramide

Table 4

## Messenger RNA or protein sequences 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									
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	<i>Homo sapiens</i> cDNA: FLJ23285 fis, clone HEP09071
yc94b11	223	F13362; T77404	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.26409	0.83	0.18	3.55	0.67	4.27	cDNA DKFZp547K204 (from clone DKFZp547K204)
yf79e07	2479	R14269; R40562	Hs.19150	0.93	0.15	3.91	0.25	4.22	cDNA DKFZp564A2164 (from clone DKFZp564A2164)
yf49e01	6657	R11708	Hs.21710	1.64	0.20	6.61	1.00	4.04	Hypothetical protein DKFZp761G0313
yg07h12	200578	R22668	Hs.7734	1.00	0.09	3.71	0.56	3.70	<i>H. sapiens</i> cDNA: FLJ21380 fis, clone COL03329
yg32e10	5469	R23681	Hs.106825	1.38	0.15	5.09	0.55	3.68	Hypothetical protein FLJ20300
yf90d07	6203	R15369; R42110	Hs.323396	1.28	0.20	4.66	0.41	3.63	Hypothetical protein RPI-317E23 (LOC56181)
yg91g03	1624	R56083; R56195	Hs.272814	0.83	0.15	2.89	0.30	3.48	Chromosome 20 open reading frame 67
yg11e11	2086	R17284	Hs.106210	4.25	0.56	14.04	2.67	3.30	Hypothetical protein FLJ10813
ym11b06	6715	H11788	Hs.125034	1.98	0.16	6.52	0.85	3.29	<i>H. sapiens</i> cDNA FLJ10733 fis, clone NT2RP3001392
yf80c08	772	R14304; R40254	Hs.59236	1.21	0.25	3.94	0.63	3.24	Hypothetical protein DKFZp434L0718
yg18e11	4391	R20224	Hs.41185	3.49	0.86	11.27	1.23	3.23	cDNA DKFZp564O1262 (from clone DKFZp564O1262)
yc90h10	1343	F13218; T75433	Hs.141003	1.32	0.24	4.19	1.05	3.18	<i>H. sapiens</i> cDNA: FLJ21691 fis, clone COL09555
yg42a11	200671	R24764; R45496	Hs.288368	0.45	0.10	1.41	0.11	3.14	<i>H. sapiens</i> cDNA: FLJ21314 fis, clone COL02248
yc85f03	255	F12760; T74722	Hs.318401	3.03	0.42	9.46	0.80	3.12	HSPC039 protein (LOC51124)
yc86g12	32	F12859; T75226	Hs.180948	4.61	0.28	14.27	1.89	3.10	KIAA0729 protein
c-21b03	1917	Z45263	Hs.155182	6.48	1.55	19.98	3.34	3.08	KIAA1036 protein
yf94d09	2836	R16328; R41404	Hs.6343; HS.306400	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	<i>H. sapiens</i> 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	<i>H. sapiens</i> cDNA: FLJ20859 fis, clone ADKA01617
yg73c09	51540	R51740	Hs.288959	1.31	0.18	3.70	0.65	2.83	<i>H. sapiens</i> cDNA: FLJ20920 fis, clone ADSE00877
yf50h09	9583	R11919;	Hs.11637	3.87	0.33	10.71	1.31	2.77	<i>H. sapiens</i> mRNA; cDNA DKFZp547J125 (from clone DKFZp547J125)
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	<i>H. sapiens</i> cDNA FLJ12150 fis, clone MAMMA1000422
c-24b10	1689	Z44563	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	<i>H. sapiens</i> 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; R48960	Hs.326416	0.90	0.16	1.98	0.35	2.20	cDNA DKFZp564H1916 (from clone DKFZp564H1916)
yg15g12	5559	R18075; R42970	Hs.22370	0.66	0.14	1.45	0.21	2.19	cDNA DKFZp564O0122 (from clone DKFZp564O0122)
yg97d02	1018	R59194; R59252	Hs.5324	0.64	0.07	1.32	0.16	2.06	Hypothetical protein (CL25022)
yc95f04	3851	F13386; R39459	Hs.7888	0.58	0.07	1.16	0.25	2.02	<i>H. sapiens</i> 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	<i>H. sapiens</i> cDNA: FLJ21721 fis, clone COLF0381
yg33e09	5446	R20455; R44158	Hs.333389	1.39	0.19	0.67	0.17	0.48	Hypothetical protein MGCI3090
c-2aa11	1485	Z40609; Z44824	Hs.13485	1.44	0.23	0.70	0.16	0.48	KIAA1918 protein
yf65e06	5690	R13865; R37007	Hs.301685	1.03	0.21	ND	ND	0.48	KIAA0620 protein
yl76d07	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	<i>H. sapiens</i> cDNA: FLJ22696 fis, clone HSII1696
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	10.44	1.29	3.36	0.75	0.32	<i>H. sapiens</i> cDNA FLJ31803 fis, clone NT2RI2009101
yf61c10	1067	R13997; R39120	Hs.5008; Hs.21515	1.11	0.09	0.35	0.08	0.32	CG-87 protein
yd06g01	2455	R38891; T81283	Hs.165570	1.41	0.07	0.45	0.10	0.32	<i>H. sapiens</i> 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)

**Table 4** (continued)

Clone ID	GENX	GenBank accession number	Unigene	C. int.	C. SD	S. int.	S. SD	Ratio	Similarity
Downregulated clones (continued)									
yc91e03	11140	F13018; T77597	Hs.337629	5.27	1.08	1.67	0.22	0.32	cDNA DKFZp434D115 (from clone DKFZp434D115)
yf65b02	8918	R13839; R36985	Hs.227913	1.09	0.24	0.32	0.05	0.30	API5-like I
yf60a03	1485	R13618; R38474	Hs.13485	2.13	0.36	0.65	0.09	0.30	KIAA1918 protein
yf56a05	5140	R12419	Hs.7132	1.97	0.33	0.57	0.08	0.29	KIAA0574 protein
yg78h08	884	R51917; R54309	Hs.6449	1.20	0.28	0.34	0.08	0.28	CGI-87 protein (LOC51112)
yf69g12	713	R40161	Hs.288776	1.21	0.23	0.29	0.04	0.24	<i>H. sapiens</i> cDNA: FLJ21304 fis, clone COL02111
yf93b11	2192	R16295; R40219	Hs.108504	1.25	0.16	0.24	0.05	0.20	Hypothetical protein FLJ20113
yf51g08	2572	R12017; R39856	Hs.20977	1.11	0.20	0.19	0.04	0.17	Human DNA sequence from clone RP5-881L22 on chromosome 20
yg26c11	904	R19006; R44076	Hs.226396	3.13	0.50	0.35	0.08	0.11	Hypothetical protein FLJ1126

Abbreviations and column headings are as in Table 1.

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 the tumor-suppressor protein DAN that contains growth or tumor suppressive activity *in vitro* [63]. *PHB* encodes the protein prohibitin, a potential tumor-suppressor protein that binds to the retinoblastoma (Rb) protein and represses E2F transcriptional activity [64,65]. *BNIP3L*, encoding BCL2/adenovirus E1B 19kD-interacting protein 3-like, is a pro-apoptotic gene which has a growth-inhibitory effect on cancer cells [66]. *CDH13*, encoding cadherin 13, is significantly downregulated in human breast carcinoma cell lines and breast cancer, whereas its overexpression decreases tumor-cell growth [67,68].

Of the known anti-apoptotic or proliferative genes that are downregulated in our model, *LOC51582* encodes an antizyme inhibitor, which regulates the antizyme activity proposed to be involved in the polyamine biosynthesis pathway [69,70]. Interestingly, overexpression of antizyme inhibits cell growth [71,72], whereas *LOC51582* is downregulated in our model. This observation is consistent with a role

of antizyme in the apoptotic process and suggests that ceramide can regulate its activity. *LOC51283*, a regulator of the activity of the Bcl-2 family proteins, encodes a novel apoptosis regulator, which has been identified as an inhibitor of Bax-induced cell death [73]. Its downregulation by C<sub>2</sub>-ceramide confirms its involvement in the ceramide-dependent regulation of cell death. The last gene presented here, *MET*, encodes the MET proto-oncogene, known to be a receptor of the hepatocyte growth factor that has been described to protect neuronal cells from apoptosis via the phosphatidylinositol-3 kinase/Akt pathway [74].

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 C<sub>2</sub>-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		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		3.08	0.02	10.53	2.42	3.42	ESTs
yf58e03	1072	R12737; R39789	Hs.119714	3.06	0.60	10.30	0.39	3.36	ESTs
yl69a01	160	H00104	Hs.21417	3.24	0.54	10.72	1.23	3.31	ESTs
yc93d09	438	T77119	Hs.21417	2.08	0.45	6.77	1.60	3.25	ESTs
c-28f03	1425	F07517; Z40576		2.49	0.35	7.77	0.52	3.12	ESTs
yg60e11	2509	R35134		4.12	0.92	12.87	1.02	3.12	ESTs
yl96g09	11047	H09060		2.97	0.51	9.23	1.00	3.11	ESTs
yg02f03	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
yf63f02	201117	R13594	Hs.155639	1.92	0.25	5.75	0.77	3.00	ESTs
yf98b09	16058	R18177; R42241	Hs.106359	1.07	0.24	3.17	0.27	2.95	ESTs
yf80c07	1885	R14303	Hs.32565	0.76	0.04	2.18	0.33	2.85	ESTs
yc92a01	11141	F13028; T76925		4.87	0.12	13.72	2.61	2.82	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
yc88c03	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
yh15d09	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, moderately similar to alternatively spliced product using exon 13A ( <i>H. sapiens</i> )
yg33b02	4208	R20161; R44947	Hs.22905	0.91	0.19	2.05	0.17	2.26	ESTs
yg44c04	3106	R25497; R45563	None	1.11	0.27	2.60	0.50	2.33	ESTs
yg46g12	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; Z44804	Hs.184780	1.06	0.15	0.52	0.11	0.49	ESTs
c-2la08	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 ( <i>H. sapiens</i> )

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
Downregulated clones ( <i>continued</i> )									
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
yg96d11	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
yf66f03	978	R37086	Hs.23210	1.72	0.18	ND	ND	0.29	ESTs
yf67b06	115094	R18860	Hs.203213	1.72	0.28	ND	ND	0.29	ESTs
yl91f12	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
yf84f08	2237	R14545; R41206	Hs.349648	1.06	0.17	0.19	0.03	0.18	ESTs, weakly similar to KIAA1157 protein ( <i>H. sapiens</i> )

Abbreviations and column headings are as in Table 1.

correspond to genes up- or downregulated by C<sub>2</sub>-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). As a result of our study, these genes now have tentative functions. The full list can be consulted with the relevant data on the dedicated website [24].

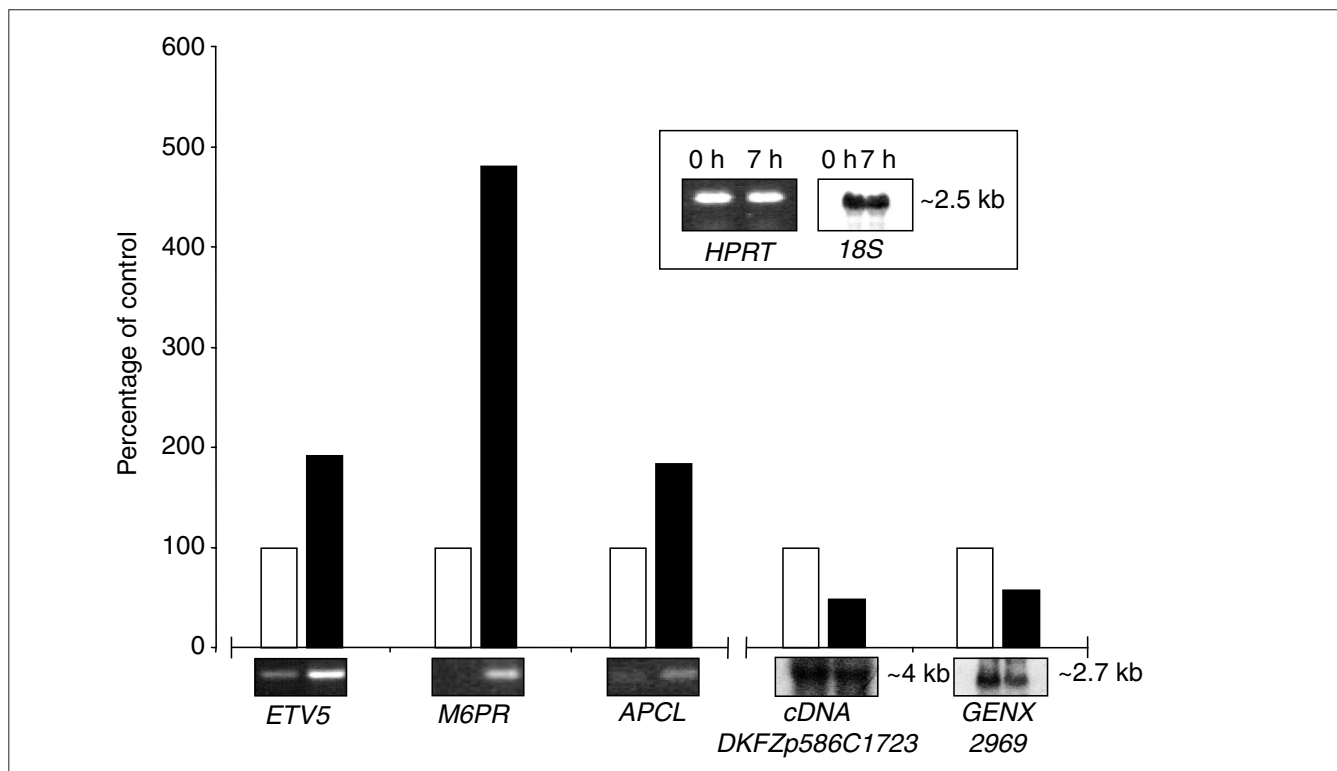
Interestingly, given the large number of genes known to be modulated by NFκB in the immune system [79], it was sur-

prising 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/cm<sup>2</sup> in 75 cm<sup>2</sup> culture flasks coated with polyethylenimine (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,



**Figure 6**

Confirmation of macroarray results by RT-PCR and northern blotting. The percentage of signal modulation (PCR amplification signal or hybridization signal) in relation to control cells (without  $C_2$ -ceramide treatment) has been calculated in each condition to compare the expression of each gene in neuronally differentiated PC12 cells with (black boxes) or without (white boxes)  $C_2$ -ceramide treatment. The PCR amplification signal and the hybridization signal for the positive controls (*HPRT* and *18S* rRNA genes, respectively) are indicated.

Israel) as previously described [81]. Apoptosis was induced, after 6 days in the presence of NGF, with the cell-permeant  $C_2$  analog of ceramide ( $C_2$ -ceramide), *N*-acetylsphingosine (Biomol Research Laboratories, Plymouth Meeting, PA), at a concentration of 25  $\mu$ M. As negative control, an inactive  $C_2$  analog of ceramide ( $C_2$ -dihydroceramide), *N*-acetylsphinganine (Biomol Research Laboratories), was used in the same condition as  $C_2$ -ceramide.

#### Morphological characterization of apoptosis and cell counts

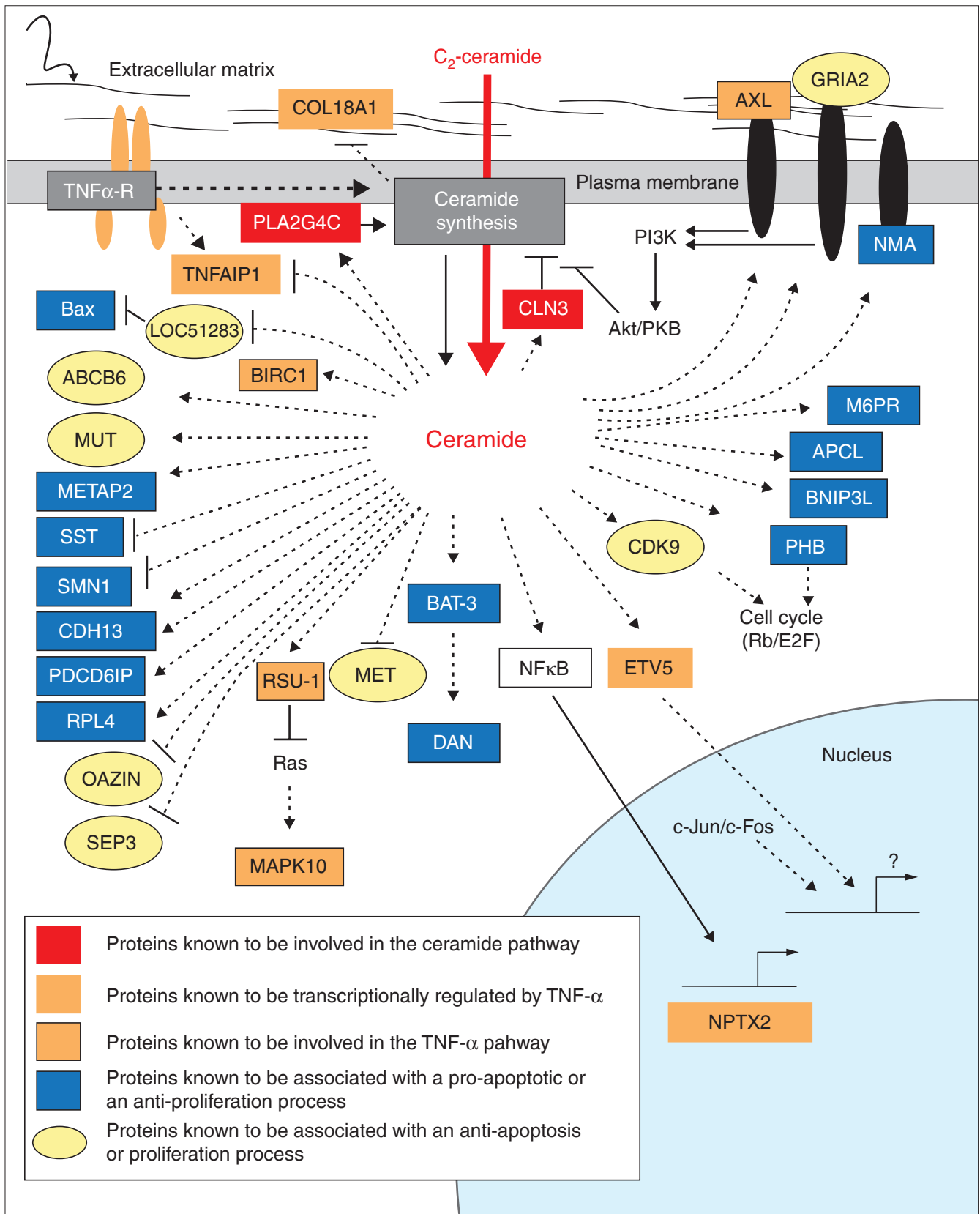
Neurite retraction and cell shrinkage were visualized by phase-contrast microscopy. Condensed and fragmented nuclei were made visible *in situ* as described in [7], by intercalation into nuclear DNA of the fluorescent probe propidium iodide. Propidium iodide, which only enters dead cells that have become 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  $C_2$ -ceramide or  $C_2$ -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/cm<sup>2</sup>) 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)<sup>+</sup> mRNA

Total RNA was extracted from control PC12 cultures and from PC12 cultures treated with C<sub>2</sub>-ceramide or C<sub>2</sub>-dihydroceramide (approximately 10<sup>6</sup> cells) with the RNeasy midi kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. The integrity of the RNA was confirmed by agarose gel electrophoresis. Poly(A)<sup>+</sup> 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)<sup>+</sup> mRNA extracted from control, C<sub>2</sub>-dihydroceramide- or C<sub>2</sub>-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-<sup>33</sup>P]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 phosphorus screens (Molecular Dynamics, Sunnyside, 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 the local background signal was subtracted. The intensity of the hybridization signal for each clone was then divided by the average intensity of all the clones on each filter to obtain normalized values. Hybridization was done in quadruplicate so that, for each clone/target combination, four values were obtained, compared and validated if at least three out of the four values were similar (SD ± 25%). The final value assigned to each clone was the average of the validated values.

### Northern blotting

Total RNA (20 μg) were fractionated under denaturing conditions in a 1.2% agarose gel and transferred onto a Hybond-N+ membrane (Amersham). Specific probes were generated from cDNA clones of interest by PCR using vector-specific primers. The PCR products were purified using the microcon kit (Amicon, Wageningen, The Netherlands) and radio-labeled by random priming (Gibco BRL). Oligonucleotides corresponding to 18S rRNA (control probe) were <sup>32</sup>P-labeled

using [γ-<sup>33</sup>P]ATP and T4 RNA kinase. 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 10<sup>6</sup> 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 phosphorus 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 C<sub>2</sub>-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 phosphoribosyl transferase) as control gene.

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