

Secretogranin II: a key AP-1-regulated protein that mediates neuronal differentiation and protection from nitric oxide-induced apoptosis of neuroblastoma cells

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Identification of AP-1 target genes in apoptosis and differentiation has proved elusive. Secretogranin II (SgII) is a protein widely distributed in nervous and endocrine tissues, and abundant in neuroendocrine granules. We addressed whether SgII is regulated by AP-1, and if SgII is involved in neuronal differentiation or the cellular response to nitrosative stress. Nitric oxide (NO) upregulated *sgII* mRNA dependent on a cyclic AMP response element (CRE) in the *sgII* promoter, and NO stimulated SgII protein secretion in neuroblastoma cells. Upregulation of *sgII* mRNA, *sgII* CRE-driven gene expression and SgII protein synthesis/export were attenuated in cells transformed with dominant-negative c-Jun (TAM67), which became sensitized to NO-induced apoptosis and failed to undergo nerve growth factor-dependent neuronal differentiation. Stable transformation of TAM67 cells with *sgII* restored neuronal differentiation and resistance to NO. RNAi knockdown of *sgII* in cells expressing functional c-Jun abolished neuronal differentiation and rendered the cells sensitive to NO-induced apoptosis. Therefore, SgII represents a key AP-1-regulated protein that counteracts NO toxicity and mediates neuronal differentiation of neuroblastoma cells.

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The AP-1 family of transcription factors is active as homodimers and heterodimers, consisting of a large number of basic region-leucine zipper proteins that belong to the Jun, Fos and activating transcription factor (ATF) subfamilies.¹ The Jun proteins are versatile and capable of forming Jun–Jun, Jun–Fos and Jun–ATF dimers. Jun–Jun and Jun–Fos dimers bind TRE (12-*O*-tetradecanoylphorbol-13-acetate (TPA) response elements, 5'-TGAG/CTCA-3'), whereas Jun–ATF dimers prefer CREs (cyclic AMP response elements, 5'-TGACGTCA-3').

AP-1 factor components, especially c-Jun, can regulate survival of cells in culture.² However, the physiological significance and the molecular targets of AP-1 in cell death and survival are incompletely understood. AP-1 factors can mediate oncogenic transformation,³ suggesting an involvement in either stimulation of cell proliferation or suppression of cell death. Some AP-1 family members are also implicated in triggering cell death.² Although there are many growth-regulating proteins involved in apoptosis, the AP-1 family are unusual because they respond to growth factors as well as genotoxic stresses.^{2,4} The diverse effects of AP-1 on cell death and survival probably arise from the combinatorial diversity of AP-1 dimers as well as activation of different groups of pro- or antiapoptotic target genes.^{1,2,5}

Secretogranin II (SgII) is a putative AP-1-regulated gene.^{6–10} SgII is a member of the acidic, secretory granin family that is widely distributed in nervous and endocrine

tissues, comprising chromogranin A (CgA), CgB, CgC (SgII) and four other members including 7B2 (SgV).^{11,12} After biosynthesis in the ER, SgII is transported to regulated secretory vesicles via the Golgi apparatus and the *trans* Golgi network. Within these vesicles, SgII is proteolytically processed to varying degrees, leading to the formation of various small peptides, some of which have defined biological functions.^{11,12} For example, SgII is the precursor of a 33 amino-acid peptide secretoneurin (SN), which modulates neurotransmission and inflammatory responses and may be involved in neuronal differentiation.^{12,13} Notably, the highest degree of processing of SgII (>90%) occurs in the brain, producing functional SN at detectable concentrations comparable to established neuropeptides. SgII protein and *sgII* mRNA levels vary considerably in different tissues, implying the existence of cell-specific mechanisms acting through *cis*-acting DNA elements in the *sgII* gene.⁷ It has been reported that a conserved CRE motif located in the promoter region of *sgII* is important for both basal and second messenger-regulated transcription of the *sgII* gene in different species,^{7,8,14} suggesting *sgII* might be regulated by AP-1.

We reported basal activity of c-Jun/AP-1 is required for expression of neural cell adhesion molecule 140 (NCAM140), which is essential for nerve growth factor (NGF)-dependent neuronal differentiation and protection from nitric oxide (NO)-induced apoptosis in neuroblastoma cells.¹⁵ Here, we set out to identify neuroprotective gene(s) whose expression is NO

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Abbreviations: CgA, chromogranin A; CgB, chromogranin B; CRE, cyclic AMP response element; DETA-NO, diethylenetriamine nitric oxide adduct; FSK, forskolin; LDH, lactate dehydrogenase; NCAM140, neural cell adhesion molecule 140; NGF, nerve growth factor; NO, nitric oxide; SgII, secretogranin II; SgV, secretogranin V; SN, secretoneurin; TRE, TPA (12-*O*-tetradecanoylphorbol-13-acetate) response element

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inducible as well as AP-1 dependent. We found that *sgII* is one such gene – both basal and NO-inducible *sgII* expression is dependent on AP-1, and SgII opposes NO-induced apoptosis. Moreover, like NCAM140, SgII is also required for NGF-dependent neuronal differentiation of neuroblastoma cells.

Results

Basal and NO-inducible expression of *sgII* are mediated by AP-1 through a CRE motif. We reported dominant-negative c-Jun (TAM67) potently inhibits AP-1 activity and sensitizes SH-Sy5y cells to NO-induced apoptosis, which is due in part to the loss of basal expression of c-Jun/AP-1-regulated NCAM140.¹⁵ This sensitization is related to loss of both CRE and TRE activities (reflecting the loss of combinatorial AP-1 dimer activities), because untreated SH-Sy5y cells express much higher levels of both activities compared to TAM67 cells (Figure 1a). Substantial CRE element-mediated transcription is activated in SH-Sy5y cells upon stimulation of the cells with the pure NO donor diethylenetriamine NO adduct (DETA-NO), raising the possibility of a NO-inducible, AP-1-dependent transcriptional response (Figure 1b).

A preliminary microarray analysis was carried out to identify NO- and AP-1-regulated genes by comparing the temporal NO-regulated gene expression profiles in SH-Sy5y cells and TAM67 stable cells. Two independent experiments indicated that *sgII* mRNA was markedly lower in the TAM67 cells compared to the SH-Sy5y cells at 6 and 10 h after NO donor treatment (data not shown), suggesting that one or more AP-1 factors may be involved in the NO-induced transcriptional

induction of the *sgII* gene. RT-PCR confirmed and extended the microarray results, showing that basal and NO-inducible *sgII* mRNA expression was markedly reduced in TAM67 cells compared to SH-Sy5y cells treated for various times with DETA-NO (Figure 1c). As a control for the time-course experiments, *sgII* was not induced in SH-Sy5y cells by buffer alone. DETA-NO is one of the purest NO donors. We assayed nitrites/nitrates as a measure of NO production and found that the maximum steady-state levels of nitrites/nitrates of up to 5–6 mM were achieved as early as 0.5 h after DETA-NO treatment (data not shown). To demonstrate that NO itself contributes to *sgII* upregulation, we compared *sgII* transcription in response to fresh DETA-NO (half-life >7.7 h) and DETA-NO inactivated by pre-incubation at 37°C for 96 h, which releases the majority of NO from the diethylenetriamine moiety. Only fresh DETA-NO reduced cell viability and was able to induce *sgII* transcription (Supplementary Figure 1a and b). Taken together, these data strongly suggest that it is NO released from the donor into the culture medium that enhances *sgII* transcription.

NO stimulated CRE activity in SH-Sy5y cells (Figure 1b), and a CRE element in the human *sgII* promoter region appears to be important for tissue-specific and inducible expression of the gene.^{7,14} We therefore asked whether the CRE located within the *sgII* promoter is sufficient to mediate NO-induced transcriptional upregulation of *sgII*. A short DNA region of 909 bp comprising the *sgII* promoter and its CRE element (Figure 2a) was placed upstream of a luciferase reporter. NO activated substantial luciferase activity when this construct was transfected in SH-Sy5y cells, as did cAMP and forskolin (FSK), which are known activators of CRE-dependent transcription (Figure 2b). However, neither NO nor cAMP

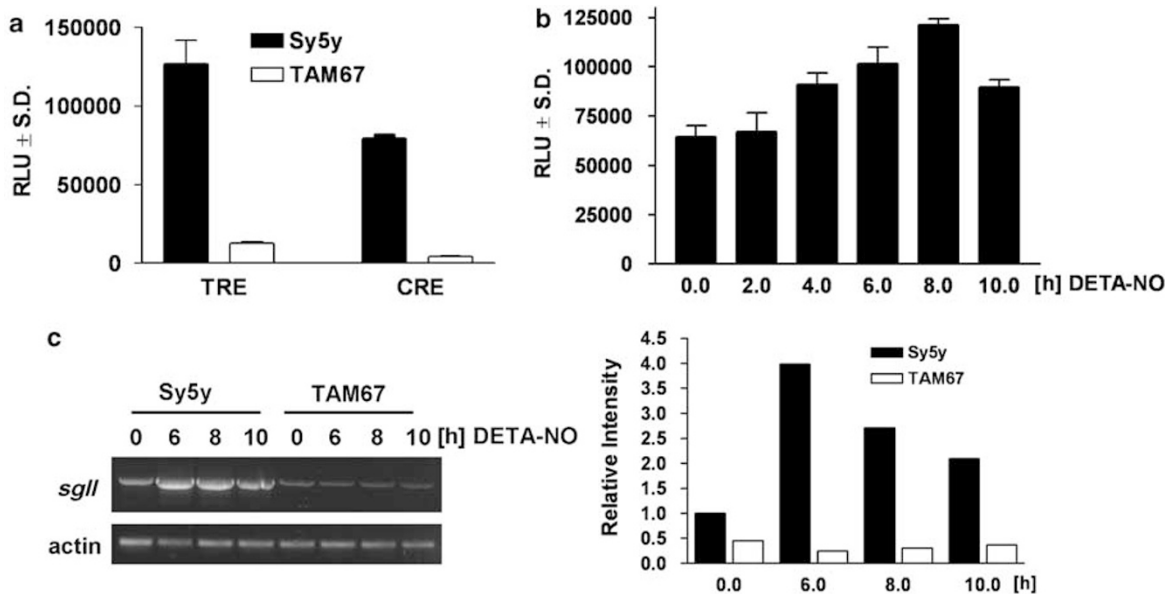


Figure 1 Different reporter gene and *sgII* expression patterns in SH-Sy5y cells and TAM67 cells. (a) SH-Sy5y (filled bars, Sy5y) and TAM67 stable cells (open bars) were transfected with CRE or TRE luciferase reporter plasmids. At 40 h after transfection, cells were harvested and luciferase activity was measured. (b) SH-Sy5y cells were transfected with a CRE luciferase reporter plasmid. At 40 h after transfection, cells were treated with 1.5 mM DETA-NO for the indicated times, harvested and luciferase activity was measured. (a, b) Values are the mean ± S.D. determined from three experiments performed in triplicate. RLU: relative luciferase unit. (c) Left panel: SH-Sy5y cells and TAM67 cells were treated with 1.5 mM DETA-NO for the indicated times, total RNA was extracted and subjected to RT-PCR to detect *sgII* transcripts. Right panel: densitometry analysis of left panel. Relative density was calculated by the ratio of the densities of *sgII* and actin, and the relative density of *sgII* in untreated Sy5y cells was defined as 1

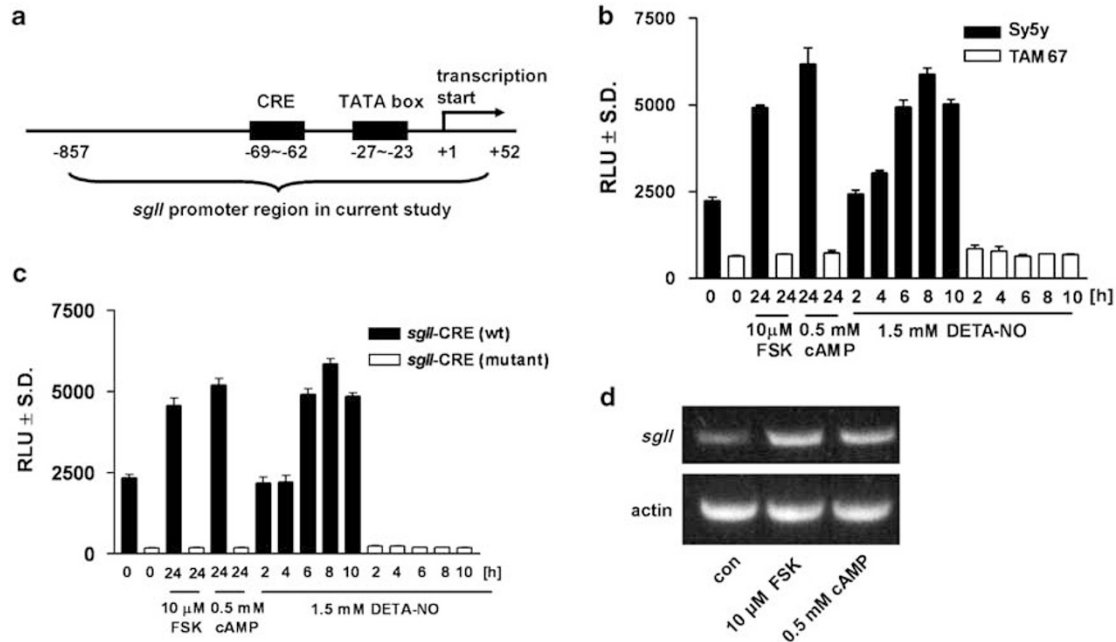


Figure 2 *sgll* expression is mediated by AP-1 and requires a CRE motif in the *sgll* promoter. (a) Schematic representation of the *sgll* promoter region⁷ referred to in (b, c). (b) SH-Sy5y cells (filled bars) or TAM67 cells (open bars) were transfected with a luciferase reporter plasmid bearing the human *sgll* promoter sequence. At 40 h after transfection, cells were treated with 10 μM forskolin (FSK) or 0.5 mM cAMP, or 1.5 mM DETA-NO for the indicated times and harvested. Luciferase activity was measured. RLU: relative luciferase unit. (c) SH-Sy5y cells were transfected with a luciferase reporter plasmid bearing either the wild-type *sgll* CRE (filled bars) or a mutated CRE (open bars). At 40 h after transfection, cells were treated with 10 μM forskolin (FSK), 0.5 mM cAMP or 1.5 mM DETA-NO for the indicated times and harvested. Luciferase activity was measured. (b, c) Values are the mean ± S.D. determined from three experiments performed in triplicate. (d) Upregulation of *sgll* by forskolin or cAMP. SH-Sy5y cells were treated with the indicated concentrations of forskolin or cAMP for 24 h, and total RNA was extracted and subjected to RT-PCR to detect the transcripts of *sgll* by agarose gel electrophoresis

or FSK activated luciferase activity when the same construct was transfected in TAM67 cells, and the basal luciferase activity was much lower in unstimulated TAM67 cells than in SH-Sy5y cells (Figure 2b), suggesting one or more AP-1 factors are important for the expression of *sgll* in SH-Sy5y cells. To obtain direct evidence that the CRE is important for *sgll* transcription, we disabled the CRE core box of the *sgll* promoter by mutation and repeated the reporter analysis. As shown in Figure 2c, the basal, NO-inducible and cAMP- or FSK-inducible luciferase expression were all virtually abolished, indicating the critical importance of the CRE element in mediating *sgll* expression. In addition, cAMP or FSK alone induced *sgll* mRNA expression, again consistent with the importance of a CRE in *sgll* upregulation (Figure 2d). The protein synthesis inhibitor cycloheximide failed to block *sgll* mRNA upregulation by NO (Supplementary Figure 1c), indicating that *de novo* protein synthesis is not necessary for *sgll* induction. This is more consistent with NO-stimulated AP-1 factor(s) acting directly on the *sgll* promoter rather than via intermediate NO-mediated transcription/translation prior to AP-1 induction.

It is well known that NO can mediate downstream oxidative stress in addition to nitrosative stress, and oxidative stress alone can activate AP-1-dependent transcription. Two different experiments were performed to exclude this possibility. In the first, high concentrations of H₂O₂ that induced significant apoptosis failed to upregulate *sgll* (Supplementary Figure 2a); while in the second experiment, the general antioxidant *N*-acetyl cysteine failed to block the NO-induced upregulation

of *sgll* mRNA at concentrations that were sufficient to block NO-induced cell death (Supplementary Figure 2b). These results altogether argue against the possibility that *sgll* induction via AP-1 factor(s) is primarily due to oxidative stress.

Since the genes encoding other chromogranin family proteins (CgA, CgB and SgV) share some common *cis*-regulatory elements in their promoters,¹² we also examined the expression patterns of their mRNAs. Although, *cgA*, *cgB* and *sgV* each exhibited a basal level of expression in SH-Sy5y cells, none showed NO-induced upregulation like *sgll* (Figure 3a and b); but strikingly, the expression of *sgV* was totally dependent on AP-1 (Figure 3b). Altogether, these data show that *sgll* and *sgV* are regulated in large part by AP-1, and suggest that the single CRE located upstream of the *sgll* gene is involved in AP-1-dependent basal expression and NO-stimulated induction of *sgll*.

Consistent with the RT-PCR analyses, Sgll protein was also considerably reduced (by five- to sixfold) in the AP-1-inhibited TAM67 cells (Figure 4a). Unlike the NO-inducible upregulation of *sgll* transcripts, the intracellular Sgll protein remained at a constant level over time following NO treatment of SH-Sy5y cells (Figure 4b, top panel). The apparent failure to detect a corresponding upregulation of Sgll protein in SH-Sy5y cells may be due to a time-dependent, NO-inducible release of Sgll into the culture medium (Figure 4b, middle panel), which represented up to 20% of total detectable Sgll (Figure 4b, bottom panel). As expected, NO-induced Sgll secretion was only detected in wild type SH-Sy5y cells but not in AP-1-inhibited TAM67 stable cells (Figure 4c). Since Sgll is

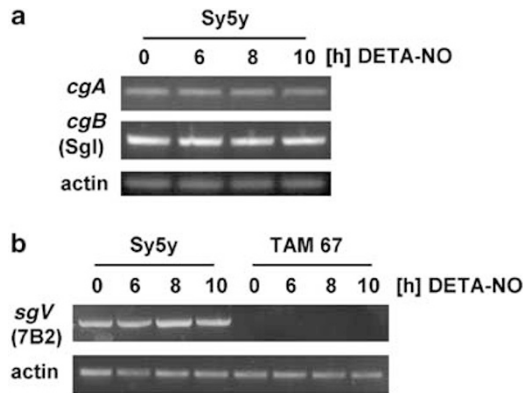


Figure 3 Expression patterns of various chromogranin genes. (a) NO-inducible mRNA expression patterns of members of the chromogranin family in SH-Sy5y cells. SH-Sy5y cells were treated with 1.5 mM DETA-NO for the indicated times, total RNA was extracted and subjected to RT-PCR to detect the transcripts of *cgA* and *cgB* by agarose gel electrophoresis. (b) Basal and NO-inducible mRNA expression patterns of *sgV* in SH-Sy5y cells and TAM67 cells. SH-Sy5y cells and TAM67 cells were treated with 1.5 mM DETA-NO for the indicated times, total RNA was extracted and subjected to RT-PCR to detect *sgV* transcripts by agarose gel electrophoresis

known to undergo some proteolytic processing in secretory granules,^{11,12} generating small peptides too small to be resolved in SDS-PAGE, we cannot exclude the possibility that the failure to detect intracellular SgII protein upregulation is also partly due to the processing of SgII.

SgII protects SH-Sy5y cells from NO-induced apoptosis. We previously showed that c-Jun/AP-1 regulates NCAM140, and loss of NCAM140 in TAM67 cells partially sensitizes SH-Sy5y cells to apoptosis induced by NO and other oxidative stresses.¹⁵ To address whether SgII can also influence NO sensitivity, we stably re-introduced *sgII* cDNA (as a *sgII-myc* fusion) into TAM67 dominant-negative c-Jun cells, and obtained clones with different degrees of SgII-Myc overexpression (Figure 5a) compared with the endogenous SgII expression levels (Figure 5b). Two independent SgII overexpressing clones both exhibited enhanced resistance to NO killing compared to TAM67 cells (Figure 5c and d). As vehicle controls, all the cell lines were incubated for the same times in the absence of DETA-NO, demonstrating no loss of viability (data not shown).

As mentioned above, *sgV* was expressed in SH-Sy5y but not in TAM67 cells (Figure 3b). However, in sharp contrast to *sgII*, stable expression of *sgV* in TAM67 cells failed to enhance resistance to NO donor-induced apoptosis (data not shown). In sum, this is evidence for a selective protective effect of SgII against NO toxicity compared with SgV, another member of the granin family.

SgII is required for NGF-induced neuronal differentiation of SH-Sy5y cells. We previously reported that c-Jun/AP-1 is required for NGF-induced neuronal differentiation, and TAM67 cells failed to undergo neuronal differentiation owing to lack of expression of NCAM140.¹⁵ We asked whether the TAM67 cells stably re-expressing *sgII* would regain competence for NGF-induced neuronal differentiation. As previously reported in PC12 cells,¹⁶ NGF also strongly

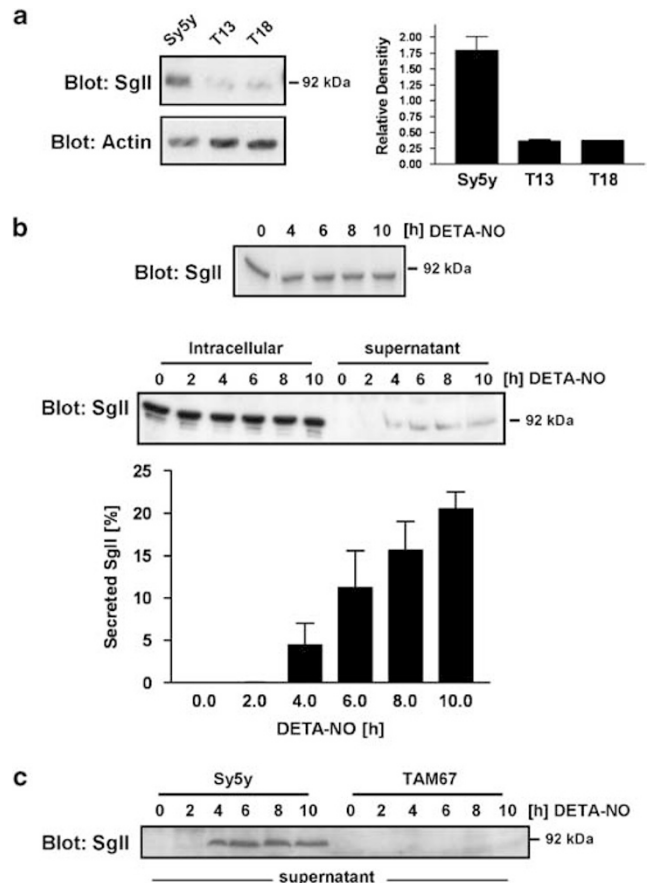


Figure 4 Basal SgII synthesis is mediated by AP-1 and SgII is secreted. (a) Left panel, total proteins were extracted from SH-Sy5y cells and two independent clones of TAM67 stable cells (T13 and T18) were separated in 0.1% SDS–8% polyacrylamide gels followed by western blot analysis to detect SgII. Right panel, densitometry analysis of the western blot shown in (a). Relative density was calculated by the ratios of the densities of SgII and actin. (b) Upper panel, SH-Sy5y cells were treated with 1.5 mM DETA-NO for the indicated times, and total proteins were extracted. Middle panel, SH-Sy5y cells were treated with 1.5 mM DETA-NO for the indicated times. Total intracellular proteins as well as secreted proteins from the cell culture medium were extracted or collected, respectively. In both panels, the proteins were separated in 0.1% SDS–8% polyacrylamide gels followed by western blot analysis to detect SgII. Lower panel, densitometry analysis of the western blot shown in the middle panel. The percentage of secreted SgII is defined as the density of secreted SgII/the density of intracellular SgII plus secreted SgII. (c) SH-Sy5y cells and TAM67 stable cells were treated with 2 mM DETA-NO for the indicated times and secreted proteins from the cell culture medium were collected. The proteins were separated in 0.1% SDS–8% polyacrylamide gels followed by western blot analysis to detect SgII. All western blot experiments in (a–c) were repeated twice, and densitometry results were mean values from two experiments

activated the protein kinase ERK in SH-Sy5y cells (Supplementary Figure 3a), thereby transducing signals for neuronal differentiation. More importantly, NGF-induced ERK activation was not compromised in TAM67 stable cells (Supplementary Figure 3b), suggesting the involvement of AP-1 targets downstream of ERK in neuronal differentiation in these cells. Following NGF treatment for 8 days, two independent clones of TAM67/*sgII* cells (T-S cells) as well as SH-Sy5y cells, but not TAM67 vector control cells, showed the classic neuronal differentiation morphology, including neurite outgrowth, a network of interconnected neurons and shrinkage of the cell bodies (Figure 6a). It is

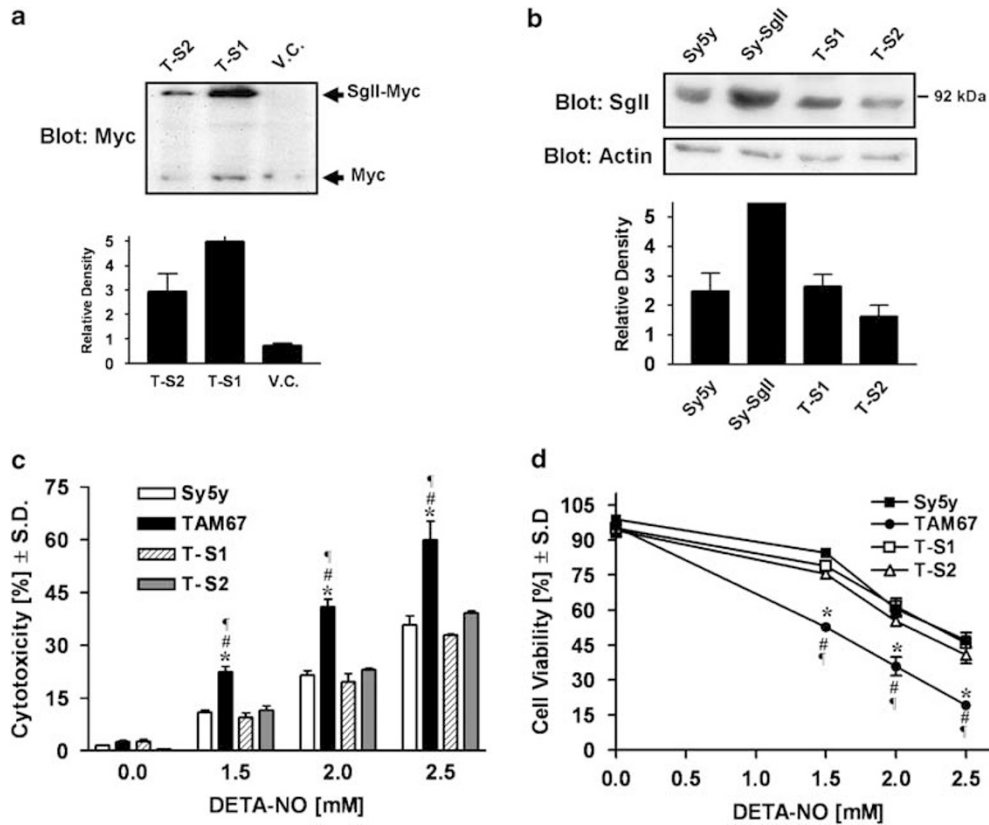


Figure 5 Resistance to NO restored in TAM67 cells overexpressing *sgII*. (a) Upper panel, representative western blot showing overexpression of *sgII* in TAM67 stable cells. Upper bands are SgII-c-Myc fusion proteins, and the lower bands represent endogenous c-Myc. Lower panel, corresponding densitometry analysis. Relative density was calculated by the ratios of the densities of SgII and Myc. V.C.: vector control. (b) Western blot with SgII antibody detects endogenous SgII in different cell lines (upper panel) and the corresponding densitometry analysis (lower panel). Relative density was calculated by the ratios of the densities of SgII and actin. (a, b) Experiments were repeated twice. Sy-SgII is SgII-overexpressing SH-Sy5y cells. Densitometry analysis results in (a, b) were mean values of two experiments. (c) SH-Sy5y cells, TAM67 vector control cells and different *sgII* overexpressing TAM67 cell clones (T-S1 and T-S2) were treated with different concentrations of DETA-NO for 24 h, cells were harvested and the percentage of cell death was measured by LDH release assay. (d) SH-Sy5y, T-S1 or T-S2, and TAM67 vector control cells were treated with different concentrations of DETA-NO for 24 h, cells were harvested and percentage of viable cells was measured using the Trypan blue exclusion assay. (c, d) Values are the mean \pm S.D. determined from three experiments performed in triplicate. Statistical analysis revealed significant differences between TAM67 and SH-Sy5y/T-S1/T-S2 cells as indicated (* $P < 0.05$, TAM67 against SH-Sy5y cells; # $P < 0.05$, TAM67 against T-S1 cells; # $P < 0.05$, TAM67 against T-S2 cells)

established that Bcl-2 is strongly upregulated during neuronal differentiation of some neurons and neuroblastoma cells;^{17,18} and in certain cell types Bcl-2 regulates neurite outgrowth and differentiation.^{19,20} NGF induced a steady upregulation of Bcl-2 in both *sgII*-overexpressing clones similar to that observed in SH-Sy5y cells, but not in TAM67 stable cells that fail to differentiate (Figure 6b). Thus, SgII appears essential for NGF-induced neuronal differentiation by both morphological and biochemical criteria. In contrast, differentiation was not restored in TAM67 cells stably transformed with *sgV*, indicating that SgV may not be required for neuronal differentiation (data not shown).

Knockdown of *sgII* by siRNA prevents neuronal differentiation and sensitizes neuroblastoma cells to NO-induced apoptosis. Six different oligonucleotides were chosen to knock down *sgII*, and independent stable SH-Sy5y cell lines were generated. Independent clones derived from two of the oligonucleotides (Figure 7a) demonstrated a

70–80% reduction in SgII protein (Figure 7b). Neither of the *sgII* knockdown stable cell lines underwent NGF-mediated neuronal differentiation on the basis of both morphological criteria (Figure 8a) and the failure of Bcl-2 upregulation 8 days after NGF addition (Figure 8b). In contrast, two control stable cells which expressed different scrambled sequences of oligonucleotides 551 or 619 (551-SC and 619-SC; Figure 7a) underwent NGF-mediated neuronal differentiation normally (Figure 8a and b). Interestingly, the NGF-treated, undifferentiated *sgII* knockdown cells underwent massive cell death (Figure 8a), as did NGF-treated SgII-deficient TAM67 cells (Figure 6a), suggesting that the absence of differentiation when SgII is down-regulated could be due either to failure of neuritogenesis or loss of apoptosis suppression (or both). Moreover, reduction in SgII protein in two *sgII* knockdown clones sensitized SH-Sy5y cells to apoptosis induced by various concentrations of NO donor by two different methods (measuring cell death or survival) (Figure 8c and d).

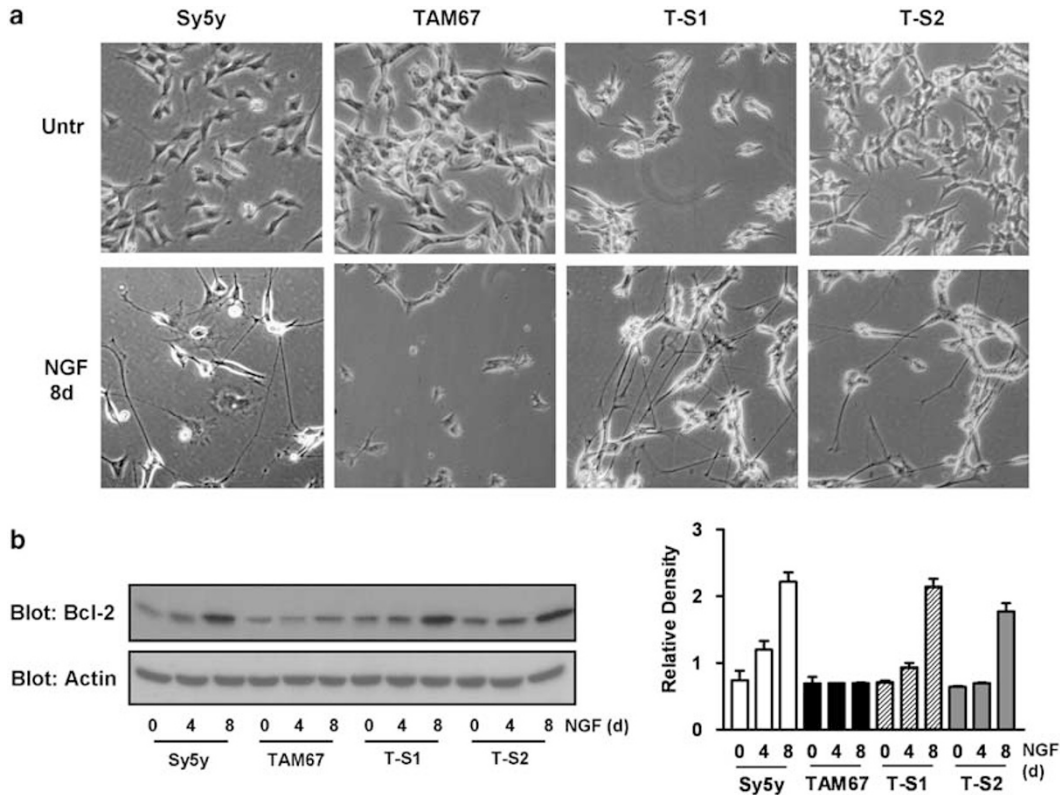


Figure 6 *sgII* overexpression restores NGF-induced neuronal differentiation in TAM67 cells. SH-Sy5y cells, TAM67 vector control cells and two independent clones of T-S stable cells (TAM67 cells overexpressing *sgII*) were induced into neuronal differentiation by NGF and aliphidocolin for up to 8 days. (a) Morphological changes described in the text are shown before (untr) and after differentiation (NGF 8d). (b) Left panels, cells were harvested after the indicated number of days, and total proteins in the lysates were separated in 0.1% SDS–15% polyacrylamide gels. Bcl-2 protein was revealed by western blotting. Actin was the loading control. Right panel, corresponding densitometry analysis. Relative density was calculated by the ratio of the densities of Bcl-2 and actin. Densitometry analysis results were mean values of two experiments

Discussion

Our observation of an early (30 min) and substantial production (low millimolar) of nitrites/nitrates was surprising, because DETA-NO releases two molecules of NO with a half-life reported to be in the range of 7.7–36 h.^{21–23} Moreover, according to first-order kinetic laws, 2 mM DETA-NO theoretically generates only 6.6 nM NO/min.²¹ High concentrations of short half-life NO donors (unlike DETA-NO) release a quick burst of NO, some of which is rapidly auto-oxidized to nitrates before it can reach the cells; but this cannot be the explanation for the 5–6 mM concentrations of nitrites/nitrates we measured following 1.5 mM DETA-NO treatment of SH-Sy5y cells. Therefore, the only conceivable explanation for the high and early saturation of nitrites/nitrates we observed is that auto-oxidation of NO within the DETA-NO adduct occurs rapidly in the medium independently of NO generation. In support, there are reports that steady-state nitrite/nitrate concentrations of up to 200 μ M are rapidly reached in 15–30 min following DETA-NO treatment.^{22,24,25} It is likely that in these reports DETA-NO is also directly and rapidly auto-oxidized to nitrates in large excess of spontaneously released NO.

It is unlikely that nitrites/nitrates are by themselves toxic. First, millimolar concentrations of added sodium nitrite are not toxic to SH-Sy5y cells (unpublished observations). Second, all the experiments we performed with DETA-NO shown in Figures 1, 2, 4 and 5 were replicated using a fast release NO

donor sodium nitroprusside (SNP; half-life ~20 min). We found that 1.5 mM SNP generates ~60–200-fold lower levels of nitrites/nitrates than DETA-NO. Yet the DETA-NO- and SNP-treated cells die by apoptosis with similar kinetics, and DETA-NO is no more toxic than SNP (data not shown). Therefore, it is highly unlikely that the millimolar nitrites/nitrates generated from DETA-NO are by themselves toxic or account for the protective function of SgII we have uncovered. This also provides a general note of caution that measurement of nitrites/nitrates produced from DETA-NO gives a vast overestimate of the NO actually released.

In this study, we made several novel findings. First, the expression and NO-mediated upregulation of *sgII* require a CRE element located just upstream of the *sgII* promoter.¹⁴ There is one perfect consensus CRE plus two TRE-like elements upstream of the *sgII* promoter,¹⁴ yet mutation of the *sgII*-CRE core box alone abolished the basal as well as inducible *sgII* promoter activity, suggesting a crucial role of this CRE in maintaining *sgII* expression. TAM67 is a potent dominant-negative form of c-Jun and is able to block both CRE- and TRE-mediated transcription when overexpressed in cells due to the versatile dimerization capacity between Jun and other AP-1 subfamily proteins.^{1,3} This explains why basal and inducible *sgII* promoter activities are compromised in TAM67 stable cells compared to Sy5y cells. Overall, our data show that NO-mediated upregulation of *sgII* relies on AP-1

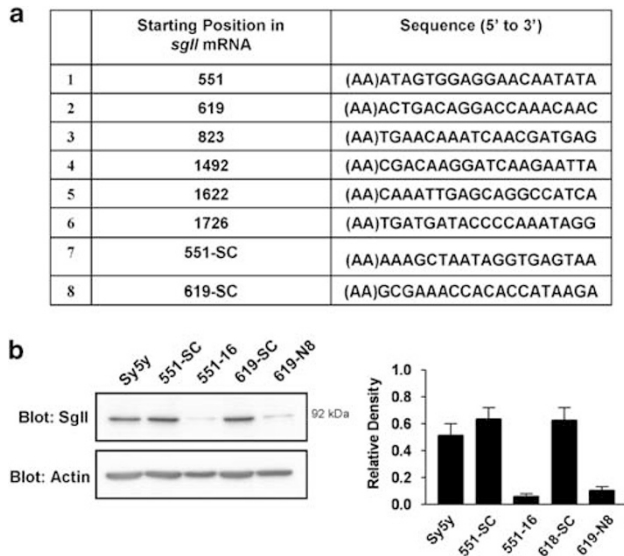


Figure 7 Construction of stable *sgII* knockdown cells mediated by siRNA. (a) Six targeting sequences were used for knock down of *sgII*, and numbers 1 and 2 (551 and 619) were successful. Oligonucleotides 7 and 8 are the scrambled controls. (b) SH-Sy5y cells were stably transfected with p*Silencer-sgII* constructs, hygromycin-resistant clones 551 and 619 or their respective scrambled control (SC) clones were isolated, and total proteins were subjected to electrophoresis in 0.1% SDS–8% polyacrylamide gels followed by western blot analysis detecting SgII. Actin was the loading control. The corresponding densitometry analysis is shown in the right panel. Relative density was calculated by the ratio of the densities of SgII and actin. Densitometry analysis results were mean values of two experiments

factors in an *sgII*-CRE-dependent manner. These findings are in accord with published evidence that other stimuli, such as certain cytokines and hormones can stimulate *sgII* gene transcription via CRE-binding proteins and AP-1 factors, including Fos, c-Jun and CREB/ATF.^{6,9} *cgA*, *cgB* and *sgII* all bear similar conserved CRE elements in their promoter regions,¹² yet only *sgII* is expressed and upregulated by NO in an AP-1-dependent manner. Because these conserved CRE elements dictate cell-type specific and inducible expression of granins, it is possible SH-Sy5y cells lack one or more additional factors needed for the CREs in the *cgA* and *cgB* promoters to respond to NO.

Other novel findings in our study are that SgII (but not the family member SgV) mediates NGF-stimulated neuronal differentiation and protection from NO-induced apoptosis in an AP-1-dependent manner. These functions of SgII were rigorously demonstrated with two opposite but complementary approaches. In one, SgII synthesis was selectively restored in TAM67 cells that express very low levels of *sgII*. In another approach, *sgII* was specifically knocked down with siRNA in cells with normal AP-1 function.

How could SgII fulfill a dual function in neuroprotection and neuronal differentiation? *sgII* transcriptional activation was reported in neuronal cells challenged with different stresses.^{26,27} In one study, *sgII* upregulation was dependent on phosphatidylinositol-3-kinase in response to oxidative stress.²⁷ Since phosphatidylinositol-3-kinase is a well-established survival signal transducer in neurons, it is conceivable that SgII is an important component of the phosphatidylinositol-3-kinase-dependent survival pathway. Alternatively,

secreted SgII might be the biologically active species, since diverse stimuli have previously been shown to cause its secretion from primary cells and cell lines.¹¹ Our detection of both intracellular and NO-induced, secreted SgII in SH-Sy5y cells suggests that SgII might mediate protection from NO either directly or indirectly via secreted SgII in an autocrine/paracrine manner (or both). The presence of SgII in the growth medium probably explains why NO stimulated an increase in *sgII* mRNA but not intracellular SgII protein in SH-Sy5y cells. In contrast to SH-Sy5y cells, the more NO-sensitive TAM67 cells failed to secrete SgII in response to NO (Figure 4c). Moreover, SH-EP neuroblastoma cells, which are markedly more sensitive to NO-mediated killing than SH-Sy5y cells, upregulated *sgII* in response to DETA-NO, but like TAM67 cells failed to secrete SgII (unpublished). These results are all consistent with the idea that secreted SgII might be biologically active in counteracting NO-induced cell death.

It will be important to elucidate whether SgII might act through a receptor, and to learn the downstream intracellular signaling mechanisms. Alternatively, the action of SgII may be receptor independent, because it was previously reported that some secreted granins can be recovered intracellularly via endocytosis and recycled for re-use.²⁸ These considerations further support the notion that secreted SgII might make a significant contribution in protecting neuroblastoma cells from NO toxicity. However, re-introduction of SgII into TAM67 cells only partially rescues TAM67 cells from NO killing; therefore, it is likely that some other protective factors, such as Bcl-2, Nrf2 and NCAM140^{15,29,30} contribute along with SgII in counteracting NO toxicity in neuroblastoma cells. For example, we previously showed that very similar to SgII, c-Jun/AP-1 regulates NCAM140, and re-introduction of NCAM140 in TAM67 cells restores NGF-induced neuronal differentiation and protection from NO killing.¹⁵ This implies that NCAM140 and SgII may either function in a single pathway or engage in cross-talk downstream of AP-1. Our preliminary unpublished data showing that re-introduction of *sgII* into TAM67 cells lacking AP-1 function also permits the re-expression of NCAM140 supports this idea. However, SgII knockdown cells that express functional AP-1 still produce NCAM140 (unpublished). Thus, much more still needs to be done to understand the interrelationships of NCAM140 and SgII downstream of AP-1.

Several reports proposed SgII as a marker of neuronal differentiation, based on observations that SgII is upregulated at the transcriptional and translational levels in several systems.^{31,32} In differentiated neuroblastoma cells, SgII was present in the Golgi and at the periphery of neurites and in growth cones, but was virtually undetectable in undifferentiated cells.³³ However, neither CgA nor CgB was found colocalized with SgII in the same organelles in differentiated cells. SgII always shows higher expression levels in different cells of the neuronal type.^{31,34,35} Thus, NGF might trigger differentiation-inducing signal transduction pathways leading to activation of transcription factors (i.e. AP-1) and their target genes including *sgII*.

We showed that, unlike SH-Sy5y cells, SgII-deficient TAM67 cells and *sgII* knockdown SH-Sy5y cells failed to differentiate and underwent massive cell death after NGF treatment. These observations permit the speculation that the

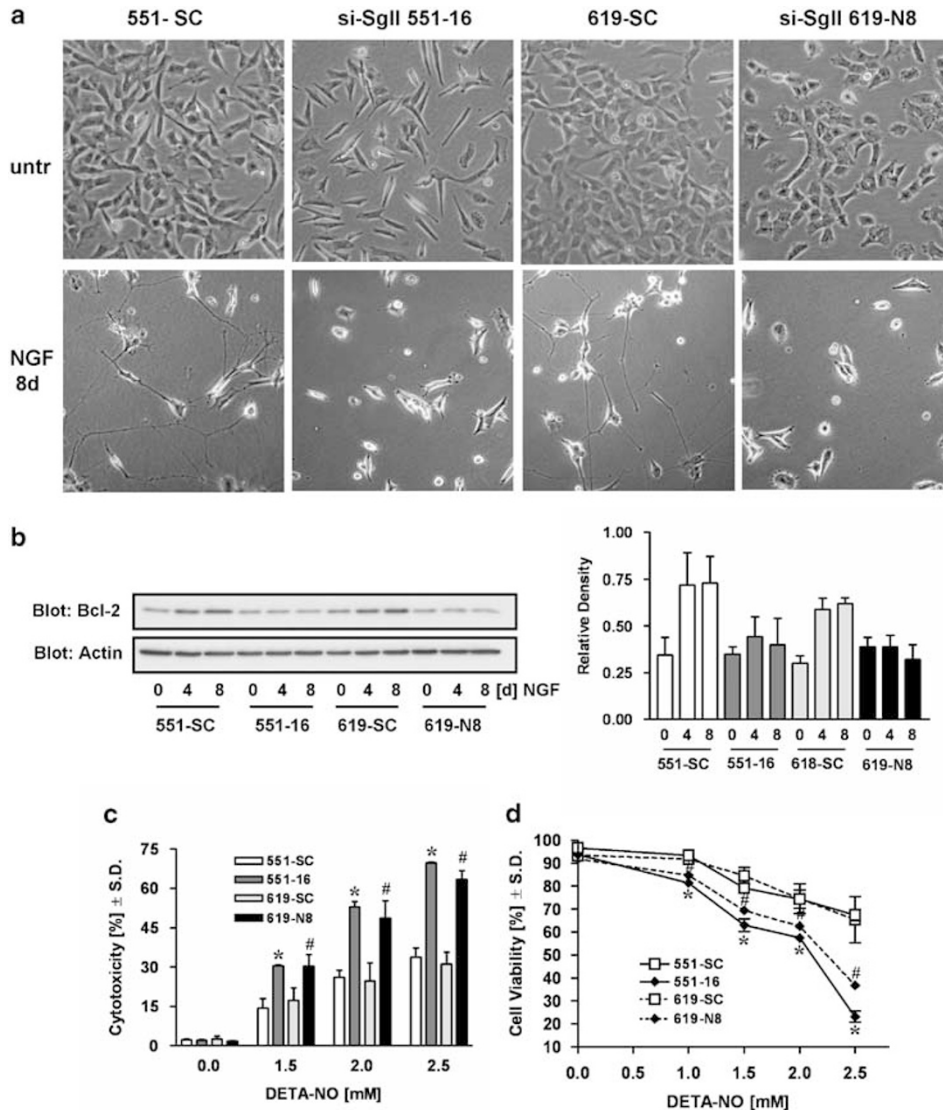


Figure 8 Knock down of *sgII* expression inhibits neuronal differentiation and sensitizes SH-Sy5y cells to NO-induced apoptosis. (a) Scrambled control (551-SC and 619-SC) cells and two independent clones of *sgII*-siRNA (551 and 619) were subjected to NGF-induced neuronal differentiation for 8 days. Morphological changes are shown before (untr) and after differentiation (NGF 8d). (b) Scrambled control (551-SC and 619-SC) and siRNA-expressing clones 551 and 619 cells were treated with NGF for the indicated number of days, harvested and total proteins in the lysates were separated in 0.1% SDS–15% polyacrylamide gels. Bcl-2 protein was revealed by western blotting. Corresponding densitometry analysis is shown in the right panel. Relative density was calculated by the ratio of the densities of Bcl-2 and actin. Densitometry analysis results were mean values of two experiments. (c, d) Scrambled control (551-SC and 619-SC) cells and two different *sgII*-siRNA stable cell clones (551 and 619) were treated with different concentrations of DETA-NO for 24 h, and the percentage of cell death or viability was determined by LDH release assay (c) or the Trypan blue exclusion assay (d). (c, d) Values are the mean \pm S.D. determined from two experiments performed in triplicate. Statistical analysis revealed significant differences between two individual si-SgII stable cells and scrambled control cells as indicated (* $P < 0.05$, 551 against 551-SC cells; # $P < 0.05$, 619 against 619-SC cells)

ability of SgII to counteract NO-induced apoptosis might also be a reflection of an important neuroprotective function for SgII during neuroblastoma cell differentiation. It can now be investigated whether SgII plays analogous roles in NO resistance and differentiation of neural progenitor/stem (and other neural) cells *in vivo*. Our preliminary experiments indicate that NO strongly upregulates *sgII* expression in cultured mouse neural progenitor/stem cells, raising the intriguing possibility of a role for SgII in response to NO not only in human neuronal cell lines but also in primary cells of the CNS.

Materials and Methods

Materials and plasmid construction. FSK and cAMP were from Sigma (St Louis, MO, USA). H_2O_2 was from Merck (Darmstadt, Germany). *N*-Acetyl cysteine was from Calbiochem (Darmstadt, Germany). Polyclonal antibodies against human SgII and actin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-human SgII antibody was raised against a peptide mapping at the carboxy terminus of human SgII. Monoclonal antibodies against Myc and Bcl-2 were from Invitrogen Life Technologies (Carlsbad, CA, USA) and BD Bioscience (Palo Alto, CA, USA), respectively. Polyclonal antibodies against ERK and phospho-ERK were from Cell Signaling Technology Inc. (Beverly, MA, USA). Lipofectin, Opti-Mem and Trizol were from Invitrogen Life Technologies. The p*Silencer*TM 2.1 U6 hygro vector was from Ambion (Austin, TX, USA). The pgl3-basic and pgl3-enhancer

vectors and the dual-luciferase assay kit were from Promega (Madison, WI, USA). TRE and CRE reporter plasmids were kindly provided by S Dhakshinamoorthy of our institute. The genomic DNA extraction kit was from Qiagen (Hilden, Germany). Superscript™ first-strand synthesis system for RT-PCR was from Invitrogen Life Technologies. All other reagents were from Sigma. Primers used for amplification and cloning of the *sgll* promoter were forward 5'-CCGGTACCGTACGAAGCTTCCTTTCGATTGCAAATGAATTC-3'; reverse 5'-CCGCTCGAGGCTCCACAGCATATTCTCCCGTTCTCCGGG-3'. Genomic DNA (200 ng) from SH-Sy5y cells was used for amplification of the *sgll* promoter with expand Hifi DNA polymerase (Roche, Mannheim, Germany). The PCR product (~900 bp, covering nucleotides -857 to +52 of the human *sgll* promoter, harboring a unique CRE located at nucleotides -62 to -69)⁷ was cloned into the pgl3-basic and pgl3-enhancer vectors at *KpnI* and *XhoI* sites. The primers used for mutagenesis of the *sgll*-CRE were forward 5'-GCTGAACCCGGAGTGGTCACTGTGGC-3'; reverse 5'-GCCA CACTGACCACTCCGGGTTCCAGC-3'. The parental and mutated CRE-containing PCR-amplified regions were verified by sequencing. The mutated CRE core box is underlined. Six different oligonucleotides (listed in Figure 7) were chosen for the siRNA knock down of *sgll* using the siRNA target finder software at www.ambion.com. Scrambled control oligonucleotides 551-SC and 619-SC are also listed in Figure 7a. The hairpin oligonucleotides were synthesized with the loop sequence TTCAAGAGA and *Bam*HI and *Hind*III sites (added to the 5' and 3' end of the DNA oligonucleotides, respectively), and cloned into the siRNA vector p*Silencer*™ 2.1 U6.

Semi-quantitative RT-PCR. SH-Sy5y and TAM67 stable cells were treated with 1.5 mM DETA-NO for various times as indicated, total RNA was prepared, followed by reverse transcription with superscript II polymerase and PCR amplification with Taq polymerase (Qiagen). The PCR temperatures were 5 min denaturation at 95°C, followed by the indicated numbers of cycles at 95°C for 30 s, annealing at 55°C or 60°C for 30 s and extension at 72°C for 45 s or 1.5 min according to the size of the amplified fragments. The numbers of PCR cycles, annealing temperatures and primers used were as follows: *sgll*, 25 cycles, annealing temperature 55°C, forward: 5'-CGACGGGATCCACCATGGCTGAAGC AAAGACCCACTGGCTTGGAG-3'; reverse: 5'-GCAGCACTCGAGCATATTTTCC ATTGCTCTCTTAGCAATATGC-3'. The primers were also used for cloning of *sgll* into the pcDNA4/*myc*-His mammalian expression vector (Invitrogen Life Technologies). *cga*, 25 cycles, annealing temperature 60°C, forward: 5'-GCGCA AGCTTGCCACCATGCGCTCCGCCGCTGTCC-3'; reverse: 5'-GCGCGAATTCGC CCCGCCGTAGTCCTGC-3'. *cgb*, 35 cycles, annealing temperature 55°C, forward: 5'-CGACGAAGCTTGCCACCATGCAGCCAACGCTGCTTCTCAGCC-3'; reverse: 5'-GCAGGGATCCGCCCTTTGGCTGAATTTCTCAGC-3'. *sgV* (7B2), 25 cycles, annealing temperature 55°C, forward: 5'-CGACGAAGCTTGCCACCA TGGTCTCCAGGATGGTCTTACC-3'; reverse: 5'-GCAGGAATCTCTGGATC CTTATCCTCATC-3'. The primers were also used for cloning of *sgll* into the pcDNA4/*myc*-His mammalian expression vector. *actin*, 20 cycles, annealing temperature 55°C, forward: 5'-GATGCATTGTTACGGAAAGT-3'; reverse: 5'-TCATACATCTCAAGTTGGGGG-3'.

Cell culture and transfection. SH-Sy5y cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. The p*Silencer*-Sgll plasmids were transfected into SH-Sy5y cells using lipofectin following the manufacturer's instructions. The stably transfected cells were selected in medium with 200 µg/ml hygromycin (BD Bioscience) and maintained with 50 µg/ml hygromycin. To reconstitute the expression of *sgll* in TAM67 cells, the cells were transfected with pcDNA4-Sgll plasmids using lipofectin, and stable cells were selected and maintained in medium with 100 µg/ml zeocin (Invitrogen Life Technologies).

Reporter assays. Reporter plasmid (1 µg; CRE, TRE or *sgll* promoter reporter with parent or mutant CRE) and 10 ng RPL-TK plasmid were co-transfected in SH-Sy5y cells at 40–50% cell confluency in six-well plates. After 40 h, cells were treated with 1.5 mM DETA-NO for various times. Cells were harvested with 400 µl passive reporter lysis buffer (provided in the dual luciferase assay kit from Promega). Firefly luciferase activity was measured immediately in a GloMax™ 96 Microplate luminometer (Promega) by adding 10 µl of lysates and 100 µl firefly luciferase substrate. *Renilla* luciferase activity, as internal control, was measured by adding 100 µl Stop&Glo solution in the same tube.

Western blot analysis. Except for phospho-ERK analysis, cells were disrupted in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% Triton X-100 and protease inhibitor (Roche Applied Science)) on ice for 30 min and subjected to centrifugation at 14 000 r.p.m. for 30 min at 4°C. Supernatant was collected and protein concentration was measured. Proteins (20–30 µg) were separated on 0.1% SDS–8% (to 15%) polyacrylamide gels and transferred onto PVDF membranes (Millipore). Detection of bands was performed using the ECL™ Western Blot Detection Reagents (Amersham Biosciences). Detection of secreted Sgll protein was performed using StrataClean™ Resin (Stratagene) to concentrate the proteins from the tissue culture medium³⁶ followed by separation on polyacrylamide gels and western blot analysis. For phospho-ERK analysis, cells were harvested at different times following 1 µg/ml NGF treatment and lysed in 200 µl sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue and 50 mM DTT), followed by sonication to shear the genomic DNA and reduce the viscosity. The sonicated lysate was then heated at 99°C for 5 min and subjected to centrifugation at 14 000 r.p.m. for 5 min at 4°C. Supernatants (40 µl) were separated in 0.1% SDS–12% polyacrylamide gels and transferred onto PVDF membranes. Detection of bands was performed using the Phototope™-HRP Western Blot Detection System (Cell Signaling Technology Inc.).

Cell death assays. Two different methods of assaying cell death were used. Lactate dehydrogenase (LDH) release assay: cells (1–2 × 10⁴ per well) were plated onto 96-well plates and treated with different concentrations of DETA-NO for up to 24 h. The cell supernatants were collected, and the cell layer was lysed with an equal volume of lysis buffer (DME plus 0.1% Triton X-100). LDH activity in the supernatant and the lysate was quantitated. Cytotoxicity was calculated as percentage of LDH release by the ratio of supernatant/(lysate + supernatant). Cell viability assay: cells (2.5 × 10⁵ per well) were plated onto six-well plates and subjected to the desired treatments; then both supernatant and cells were harvested in one tube and subjected to centrifugation at 1000 r.p.m. for 10 min at 4°C. Cells were re-suspended in 0.5 ml fresh cell culture medium and the percentage of viable cells was measured based on the Trypan blue exclusion method³⁷ using the cell viability analyzer ViaCell™ XR (Beckman Coulter Inc., Fullerton, CA, USA).

Microarray analysis. SH-Sy5y cells and TAM67 stable cells were treated with 2 mM SNP (a NO donor) for 6 or 10 h, total RNA was extracted using Trizol reagent (Invitrogen Life Technologies) and mRNA was purified using the Oligotex™ mRNA kit (Qiagen). The mRNA concentration was measured and its integrity was confirmed by PCR amplification of randomly selected genes. Microarray analysis was carried out by Incyte Genomics (St Louis, MO, USA).

Induction of neuronal differentiation. Differentiation was induced as described with modifications.³⁸ Cells were plated onto 60 mm tissue culture dishes at a density of 5–6 × 10⁵ cells per dish. After overnight culturing, NGF (NGF-7S; Sigma) was added to the medium at a final concentration of 1 µg/ml. On day 2, the medium was refreshed with 5 µg/ml aliphidicolin (Sigma) in the same medium. On the third day, the medium was refreshed with 1 µg/ml NGF and 5 µg/ml aliphidicolin. The medium was refreshed every 2 days with NGF and aliphidicolin for 8–10 days.

Statistical evaluation. Results are given as mean ± S.D. of mean values. Statistical significance was determined by the two-factor ANOVA test followed by student *t*-test.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)