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GAS1 induces cell death through an intrinsic apoptotic pathway

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Abstract Growth Arrest Specific 1 (GAS1) is a protein expressed when cells are arrested and during development. When ectopically expressed, *GAS1* induces cell arrest and apoptosis of different cell lines, and we have previously demonstrated that the apoptotic process set off by GAS1 is caused by its capacity inhibiting the GDNF-mediated intracellular survival signaling. In the present work, we have dissected the molecular pathway leading to cell death. We employed the SH-SY5Y human neuroblastoma cell line that expresses *GAS1* when deprived of serum. We observed, as we have previously described, that the presence of GAS1 reduces RET phosphorylation and inhibits the activation of AKT. We have now determined that the presence of GAS1 also triggers the dephosphorylation of BAD, which, in turn, provokes the release of Cytochrome-*c* from the mitochondria to the cytosol activating caspase-9, prompting the activity of caspase-3 and resulting in apoptosis of the cells. The apoptotic process is intrinsic, because there is no activation of caspase-8, thus this is consistent with apoptosis induced by the lack of trophic support. Interestingly, in cells where *GAS1* has been silenced there is a significant delay in the onset of apoptosis.

Keywords GAS1 · Apoptosis · Caspases · Cancer · GDNF

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Introduction

Growth Arrest Specific 1 (*GAS1*) was isolated from serum starved NIH-3T3 fibroblast, and thus associated with cell arrest [1]. *GAS1* is an intronless gene, suggesting it originated from a retrotransposon, that codes for a protein linked to the outer cell membrane by a glycosyl phosphatydylinositol (GPI) anchor and possesses a high structural homology with the GDNF family of receptors [2–4]. It was also found that when ectopically expressed *GAS1* could arrest different tumor cells and induced cell death [5–7]. Moreover, we demonstrated that the over-expression of *GAS1* caused cell arrest and apoptosis of glioma cells [8–12], and that GAS1 was acting by inhibiting the GDNF-induced intracellular signaling pathway [4, 13, 14]. Because of its capacity to arrest cell growth and induce apoptosis, GAS1 becomes an attractive option as a potential adjuvant in the treatment of cancer.

Interestingly, GAS1 has also been related with SHH functioning. This interaction was originally interpreted as antagonistic [15], but further work has shown it to be a cooperative interaction, with the participation of the Cdo and Boc proteins [16–18]. *GAS1* is expressed during development [19], and alterations of the GAS1–SHH functional interaction have been associated with developmental abnormalities, such as craniofacial malformations

in rodents [20], and holoprosencephaly in humans [21–23]. The dual effects of GAS1 inhibiting growth and inducing apoptosis in tumor cells, and on the other hand, increasing the effect of SHH during development, appear to be contradictory, also considering that in tumor cells GAS1 induces cell arrest and apoptosis in a manner independent of the presence of SHH [11]. Taken all this information together we propose that GAS1 is a pleiotropic protein that exerts different effects depending on the cellular context where it is expressed; this suggests that GAS1 plays a critical role in the integration of different intercellular signaling systems [14].

Considering the potential importance of GAS1 in the treatment of cancer, and to better understand its function, we decided to carefully analyze the molecular pathway by which GAS1 leads to cell death. For the present experiments, we took advantage of the capacity of SH-SY5Y cells of expressing GAS1 when deprived of serum [13], and then proceeded to silence its expression to determine the consequences of the lack of GAS1 on cell survival. We observed, as we have previously described, that the presence of GAS1 reduces RET tyrosine 1062 phosphorylation and inhibits the activation of AKT [11, 13]. We have now determined that the presence of GAS1 also triggers the dephosphorylation of BAD, which, in turn, provokes the release of Cytochrome-c (Cyt-c) from the mitochondria to the cytosol activating caspase-9, resulting in the consequent activation of caspase-3 and in apoptosis of the cells. The apoptotic process is intrinsic, because there is no activation of caspase-8, thus this is consistent with apoptosis induced by the lack of trophic support.

Materials and methods

Cell culture and treatment

The SH-SY5Y neuroblastoma cell line (ATCC) (passages 5–15) was cultured in DMEM high glucose (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% antibiotic/antimycotic (Gibco) and maintained at 37°C, in a 95% air/5% CO_2 atmosphere, with 100% relative humidity (proliferating conditions). To induce the expression of GAS1, complete medium was withdrawn and substituted with medium without serum for different periods, up to 96 h. Cultures of cells were observed using an Observer D1 (Carl Zeiss) microscope and images acquired with the Axion Vision software Version 4.8.1.0 (Carl Zeiss Imaging Solutions).

GAS1 silencing

To silence GAS1 expression in a stable manner, we employed a complementary mRNA antisense approach.

A 382 bp fragment comprising sequences in both the 5′UTR and coding regions (178–559 bp with respect to GAS1 mRNA) was amplified by PCR (forward primer 5′-CGGGGTACCAGAGCGGGACCAGAT-3′; reverse primer 5′-CGGGGTACCTGCCAGCAGATGAGG-3′) from the BAC clone RP11-208G24. The PCR fragment was cloned in both sense and antisense orientations in the pcDNA 3.0 plasmid, and sequenced. This fragment was then directionally subcloned into the pAdTrack-CMV expression plasmid which uses the CMV promoter and possesses a green fluorescent protein (GFP) reporter gene. The transfection of the different plasmids; backbone, GAS1 antisense and GAS1 sense, pAdTrack-CMV, pAdANT-Gas1, and pAdSENGas1, respectively, was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were selected with 600 μg/ml of G-418 (Gibco), maintained with 200 μg/ml of G-418, and finally selected by flow cytometry, using a Mo-Flo High-Performance Cell Sorter (Beckman Coulter).

RT-PCR assays

Total RNA was isolated using the Trizol reagent (Invitrogen). Briefly, 5 μg of total RNA were treated with DNAseI (Biolabs), reverse-transcribed with M-MLV reverse transcriptase (Invitrogen), and 2 μl of cDNA amplified by PCR. The amplification of GAS1 mRNA and β-ACTIN mRNA, as a control, were performed as previously described [9, 10]. We determined the expression of the (mRNAs) of members of the SHH pathway, SHH, PTCH1 and GLI1, as had been previously described [11, 24].

Cell viability assays

To determine cell viability, Trypan blue exclusion, and MTT assays were employed. For the Trypan blue assay, 1×10^5 cells were seeded in 24-well culture plates, and serum was withdrawn from the medium as previously described. The number of viable cells was determined at the indicated times. For the MTT assay, the same number of cells was employed, and the capability of cells to reduce MTT (Sigma) (5 mg/ml) to formazan was determined using a colorimetric assay with a Bio Rad 540 plate reader.

Release of cytochrome-c from mitochondria

The mitochondrial and cytosolic fractions were isolated using the Cyt-c Release Apoptosis Assay Kit (Calbiochem), and 10 μg of protein from each fraction were employed to determine the levels of Cyt-c by western blot analysis using a monoclonal antibody against Cyt-c (Calbiochem, catalog number JA5204, 1:200).

Western blot assays

Total protein was isolated in the presence of a protease inhibitor cocktail. Fifty micrograms of total protein were separated on 12 or 6% SDS-PAGE gels and transferred onto nitrocellulose. Membranes were blocked for 1 h at room temperature in 5% non-fat milk/TBST (0.1% Tween-20, TBS) and then incubated with primary polyclonal antibodies against: human GAS1 (ProSci, 1:500) [10, 13], GDNF (Santa Cruz Biotechnology, catalog number sc-328, 1:100), p-RET (Y1062, Santa Cruz Biotechnology, catalog number sc-20252-R, 1:700), t-RET (Santa Cruz Biotechnology, catalog number sc-13104, 1:700), t-AKT (Santa Cruz Biotechnology, catalog number sc-8312, 1:500), p-AKT (Santa Cruz Biotechnology, catalog number sc-7985-R 1:5,000), t-BAD (Cell Signaling, catalog number 9292, 1:500), p-BAD (Cell Signaling, catalog number 5286S, 1:250), Caspase-9 (Santa Cruz Biotechnology, catalog number sc-8355, 1:1,000), Caspase-3 (Cell Signaling, catalog number 9662, 1:500), Caspase-8 (Santa Cruz Biotechnology, catalog number sc-6134, 1:1,000), overnight at 4°C. Blots were washed and incubated with a goat anti-rabbit antibody (Jackson ImmunoResearch, catalog number 111-035-003, 1:5,000) or a rabbit anti-goat antibody (Jackson ImmunoResearch, catalog number 305-035-003, 1:5,000). As a positive control some blots were stripped and re-probed with a monoclonal anti-β-ACTIN antibody [25] and then with a goat anti-mouse (Invitrogen, catalog number 62-6520, 1:5,000). Secondary antibodies were coupled with peroxidase and proteins revealed with enhanced chemiluminescence (PerkinElmer). Images from films were digitally acquired with a BioDoc-It Imaging System (UVP), and densitometric analyses performed with the LabWorks software (UVP).

Apoptosis assays

Apoptosis was determined using two different assays. Annexin-V (Pacific Blue conjugate)/Propidium iodide (Invitrogen, catalog number A35122) assays were performed with flow cytometry, according to the manufacturer's instructions, using a CyAN ADP instrument (DAKO Cytomation) and data were analyzed with the Summit 4.3 software. DNA damage was estimated using a TUNEL immunocytochemical assay according to the manufacturer's instructions (Roche), and cells counted using an Olympus BX-51 microscope.

Statistical analysis

Linear regression analysis was performed and slopes compared with a t test using the SPSS 17 software; a $P < 0.05$ was considered significant.

Results

Effects of GAS1 on cell viability

To determine the capacity of our experimental approach of a complementary antisense mRNA to effectively silence the expression of *GAS1* we employed SH-SY5Y cells, derived from a human neuroblastoma. We observed that there is no expression of *GAS1* mRNA in either SH-SY5Y cells or cells transfected with the antisense *GAS1* in normal serum (proliferating conditions), however, after serum withdrawal there is a clear expression of *GAS1* in SH-SY5Y cells that persists for up to 96 h after serum withdrawal, whereas no *GAS1* mRNA is detected in cells expressing antisense *GAS1* (Fig. 1a). We also determined the levels of GAS1 protein, as assayed by western blot analysis, and observed that the expression of *GAS1* mRNA antisense eliminates the expression of GAS1 induced by serum starvation to background noise levels (Fig. 1b, d). Moreover, neither the transfection of the pAd-SEN-Gas1vector nor of the backbone plasmid had any effect on the expression of *GAS1* after serum withdrawal (Supplementary Fig. 1). We also studied the effect of the *GAS1* silencing procedure on cells in proliferating conditions, normal serum, and observed what appears to be normal morphology of the cells expressing the antisense mRNA (Fig. 1c). Photographs of SH-SY5Y cells transfected with pAd-Track-CMV, pAd-ANTGas1 and pAd-SENGas1 are shown in Supplementary Fig. 2, and we can observe similar morphologies in normal serum, and that cells are GFP positive. From these experiments, we conclude that the method employed to silence *GAS1* expression is very effective, and it causes no evident damage to transfected cells.

Serum withdrawal causes cell death, and we wanted to determine the effect of *GAS1* silencing on cell viability. For this series of experiments, we assayed cell viability employing two different methods, using the MTT assay, which reflects the functional capacity of mitochondria, and Trypan blue assays which report the integrity of the membranes. We compared the viability of SH-SY5Y cells, cells in which *GAS1* had been silenced, cells transfected with the pAdTrack-CMV plasmid and cells expressing the sense sequence of *GAS1*, at different times after serum withdrawal. Figure 2a shows a high level of viable *GAS1*-silenced cells, as assayed by MTT, whereas SH-SY5Y cells and the different controls show an important loss of cell viability which is significantly different from the antisense cells. A similar trend is observed when using the Trypan blue assay, because we can observe a significantly higher number of viable antisense-expressing cells, when compared with all other controls, which behave in the same manner (Fig. 2b). Cells of all groups, in conditions of
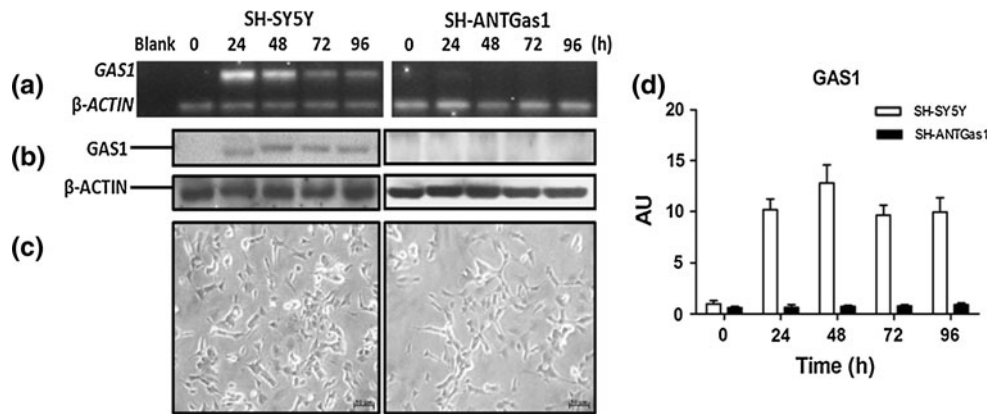
Fig. 1 Silencing of *GAS1* in SH-SY5Y cells. **a** Shows the expression of *GAS1* as determined by RT-PCR in control, non-transfected SH-SY5Y cells, and in cells transfected with pAd-ANTGas1 (SH-ANTGas1 cells), in conditions of proliferation (0 h), and at different times after serum withdrawal. **b** Shows a representative experiment of the presence of GAS1 protein as ascertained by western blot analysis in control and transfected SH-SY5Y cells, in conditions of proliferation and at different times after serum withdrawal.

c Shows photographs of cultures of control and transfected cells in conditions of proliferation. **d** Shows the densitometric analysis of the expression of GAS1 in control SH-SY5Y and cells transfected with pAd-ANTGas1 in conditions of proliferation and at different times after serum withdrawal from three independent experiments. Results are expressed as arbitrary units, normalized with the respective *β*-ACTIN control (mean + SEM). Bar is 50 μm

proliferation and at different times after serum withdrawal are shown in Supplementary Fig. 3; where we can observe the preserved survival of SH-ANTGas1 cells compared to all others after serum withdrawal, approximately 90 and 20%, respectively. These data show that the silencing of *GAS1* preserves the number of viable cells, and that this result is specifically caused by the lack of GAS1, because the decrease in the number of viable cells is the same in control SH-SY5Y cells and in cells transfected with the backbone plasmid, or with the *GAS1* sense sequence.

Effects of GAS1 silencing the GDNF signaling pathway

Our previous work showed that GAS1 induced apoptosis by blocking the GDNF-mediated signaling pathway. Here, we wanted to describe in greater detail the molecular pathway leading to cell death. We took advantage of the property of SH-SY5H cells to express *GAS1* after serum withdrawal, and to analyze the results comparing them with cells in which *GAS1* had been silenced.

We first demonstrated that both, control and *GAS1*-silenced cells, expressed GDNF (Fig. 3). We then proceeded to determine the activity of the intracellular components of the GDNF pathway on both cell types, at different times after serum withdrawal. We first observed decreased levels of phosphorylation of RET in SH-SY5Y cells after serum withdrawal, whereas RET phosphorylation is preserved in *GAS1*-silenced cells (Fig. 3). An even more intense effect was observed regarding AKT activity; in this case the levels of phosphorylated AKT were undetectable in control cells, after serum withdrawal, but in cells that do not express *GAS1* a robust signal is seen

(Fig. 3). Another intermediate in the GDNF-induced signaling is BAD, here we observe that in response to serum withdrawal, there is a striking decrease of protein phosphorylation, but the level remains high in *GAS1*-silenced cells (Fig. 3). With these experiments, we demonstrate that in the presence of GAS1, there is a blockade of the GDNF-induced signaling pathway, an effect that is overcome when *GAS1* is silenced.

Since there is evidence indicating the effect of GAS1 on the SHH pathway we determined, by RT-PCR, the expression of the mRNAs of *SHH*, its receptor *PTCH1*, and *GLI1* the most important mediator of this pathway, and observed they are present in SH-SY5Y cells and in silenced cells both in the presence and absence of serum (Supplementary Fig. 4).

GAS1 and apoptosis

A critical component of the apoptotic process is the release of Cyt-*c* from the mitochondria to the cytosol, an event demonstrating mitochondrial membrane permeabilization. In this work, we show that in conditions of serum withdrawal, control cells release Cyt-*c* from the mitochondria to the cytosol, an event that does not occur in *GAS1*-silenced cells, where Cyt-*c* remains in the mitochondria, even after serum has been removed from the culture medium (Fig. 4).

The release of Cyt-*c* is necessary for the activation of caspases leading to apoptotic cell death. Figure 4 shows, that in the presence of GAS1, there is activation of both caspase-9 and caspase-3 in serum-starved SH-SY5Y cells, but that this does not occur in the cells that do not express
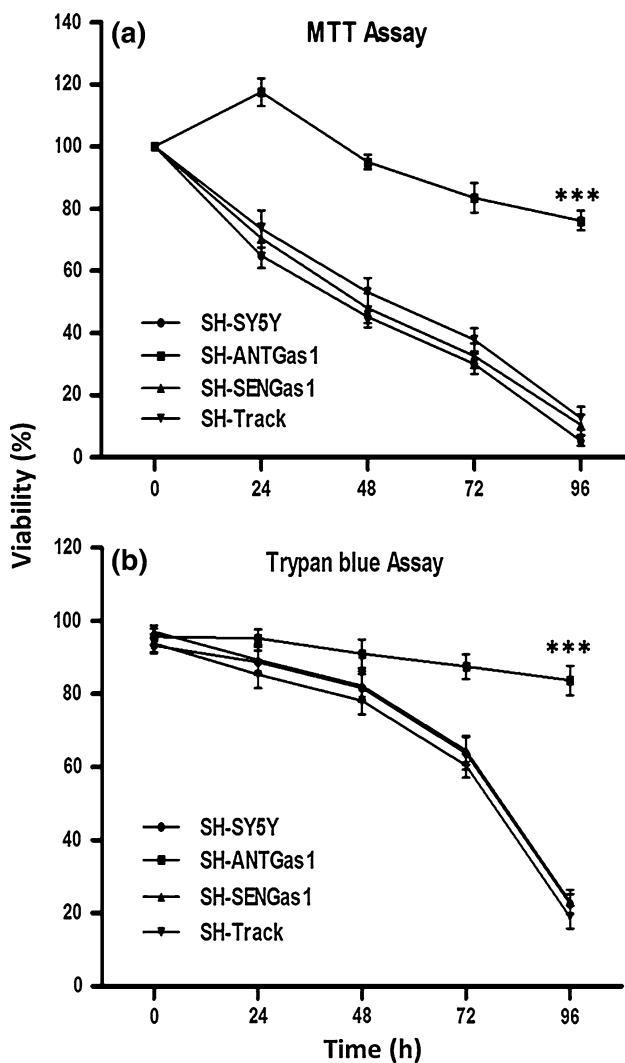
Fig. 2 Effect of GAS1 on cell viability. **a** Shows the viability of cells as determined by the MTT assay, in conditions of proliferation and at different times after serum withdrawal. **b** Shows the percentage of viable cells according to the Trypan blue assay, in conditions of proliferation and at different times after serum withdrawal. Cells evaluated are: SH-SY5Y cells, control non-transfected, and: cells transfected with *GAS1* antisense (SH-ANTGas1); transfected with *GAS1* in the sense orientation (SH-SENGas1) and transfected with the pAdTrack-CMV plasmid (SH-Track). Data represent mean $\pm$ SEM, $N = 3$; ***$P < 0.001$, SH-ANTGas1cells different from all other groups

GAS1, where there is no activation of these caspases. On the other hand, caspase-8 is not activated in response to serum withdrawal in neither cell type, control or *GAS1* silenced cells. Finally, to demonstrate that serum withdrawal is inducing apoptotic death, we ascertained this type of cell death by two different methods. The first one was to determine the translocation of phosphatidylserine to the external side of the cell membrane, an early event of apoptosis, and the second was to establish the oligonucleosomal fragmentation of DNA, a late event in apoptosis, as

revealed by a TUNEL assay. The number of viable SH-SY5Y cells is significantly decreased from 48 to 96 h after serum deprivation, when compared with cells not expressing *GAS1*, as determined by the Annexin V/propidium iodide assays; so that 96 h after serum withdrawal only 16% of SH-SY5Y cells remain viable, whereas 54% of SH-ANTGas1 cells are viable (Fig. 5a). The same tendency is observed when determining the amount of TUNEL-positive cells. There is a significantly higher number of TUNEL-positive control cells after removing the serum from the culture medium, compared with *GAS1*-silenced cells. This difference is over tenfold 96 h after serum withdrawal (Fig. 5b). Based on the results of these experiments, we surmise that GAS1 induces cell death by an intrinsic apoptotic process that can be significantly delayed or reduced by silencing *GAS1*, thus showing that GAS1 is a key factor in the death induced by serum withdrawal of SH-SY5Y cells.

Discussion

GAS1 has been associated with cell arrest, and when ectopically expressed it induces arrest and apoptosis of different tumor cell lines and primary cultures of human gliomas [5–12]. Furthermore, the expression and levels of *GAS1* are related with the malignancy of tumors and with their metastatic potential, suggesting that *GAS1* is a relevant tumor biomarker and thus of clinical relevance [26–31].

The effects of GAS1 inducing growth arrest and apoptosis are caused by its capacity inhibiting the GDNF-mediated survival pathway due to its structural similarity with the GDNF-α-receptors [4, 14]. Based on this concept, we showed that GAS1 decreases the phosphorylation of the GDNF co-receptor RET, and the activation of AKT [11, 13]. The fact that SH-SY5Y cells where *GAS1* had been silenced showed a significant larger number of viable cells, compared with all different controls that do not affect *GAS1* expression, proves that it is the absence of GAS1 what is protecting the cells from damage and death. Furthermore, cells transfected with either the backbone plasmid or the *GAS1* sense vector are not different regarding their viability compared with the control non-transfected SH-SY5Y cells. This indicates that the effects on cell survival and mitochondrial failure are caused by the lack of GAS1, and that neither the transfection procedure nor the expression of unrelated RNAs influence cell functioning.

In the present work we sought to carefully examine the molecular pathway by which GAS1 induces apoptosis, and precisely define the type of apoptosis by which tumor cells die. To accomplish these objectives we used SH-SY5Y cells that express *GAS1* when deprived of serum, and
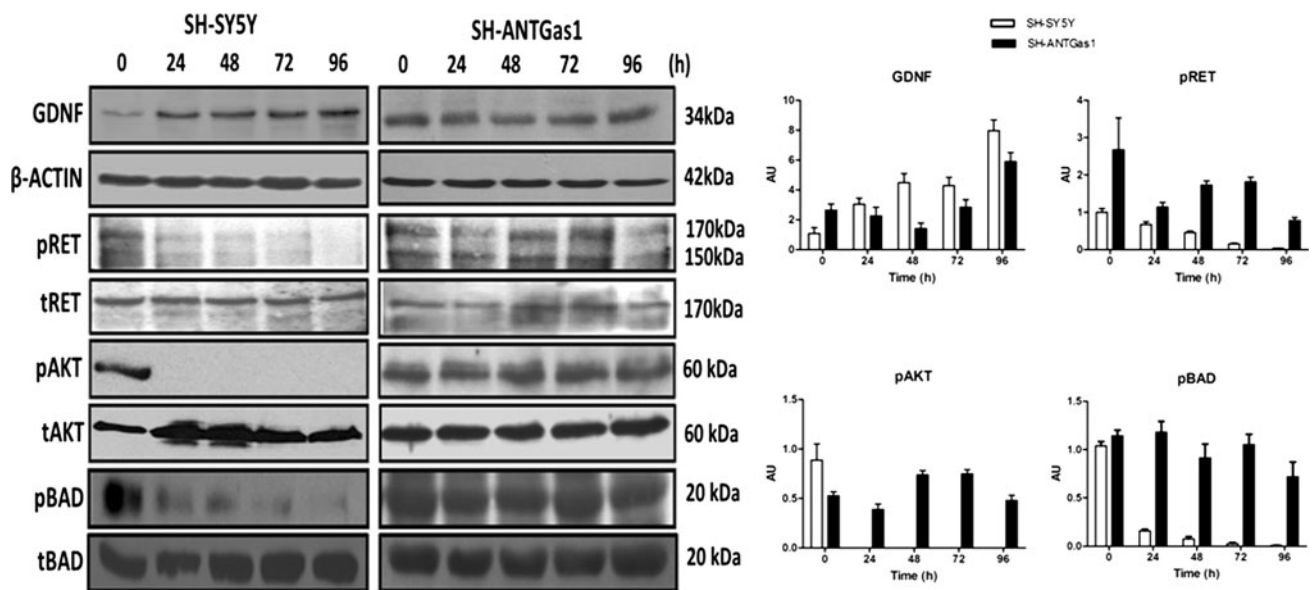
Fig. 3 Signaling pathway leading to apoptosis. The presence and activity of different components of the GDNF signaling pathway as assayed by western blot analysis, in control SH-SY5Y and SH-ANTGas1 transfected cells, in conditions of proliferation (0 h) and at different times after serum withdrawal. *Left panel* shows a representative experiment; from top to bottom: expression of GDNF, and β-ACTIN as its control; RET phosphorylated on Y-1062 (p-RET) and total RET as its control (t-RET); phosphorylated AKT (p-AKT) and total AKT (t-AKT) as its control; phosphorylated BAD (p-BAD) and total BAD (t-BAD) as its control. The *right panels* show the densitometric analysis of the components of the pathway from three independent experiments. Results are expressed as arbitrary units normalized with their respective controls (mean $\pm$ SEM)

Fig. 4 Release of Cyt-*c* and activation of caspases by GAS1. Levels of Cyt-*c* in mitochondria and cytosol and assessment of the activation of caspases by western blot assays in control SH-SY5Y cells and *GAS1*-antisense expressing cells in conditions of proliferation (0 h) and at different times after serum withdrawal. From *top to bottom*: Cyt-*c* in mitochondria (Cyt–Mit) and in cytosol (Cyt–Cyt); assay for caspase-9; assay for caspase-3, and *lower panel* assay for caspase-8
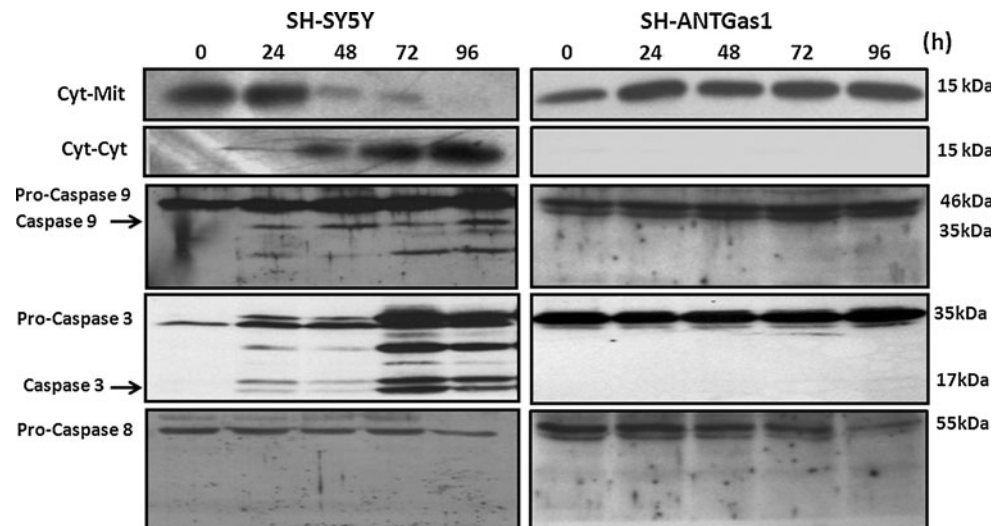
studied the specific effects of GAS1 by silencing it in serum deprived cells. Here, we confirmed previous results showing that GAS1 reduces the phosphorylation of RET and inhibits the activity of AKT. We followed the intracellular signaling cascade downstream and observed that in the presence of GAS1, BAD is dephosphorylated allowing it to translocate to the mitochondria where it binds to BcL-XL and facilitates the release of Cyt-*c* to the cytosol, a critical event in the apoptotic pathway. In the presence of GAS1 there is activation of both caspase-9 and caspase-3

which lead to apoptosis, demonstrated in this case both by the translocation of phosphatidyl serine to the outer cell membrane leaflet, and by the oligonucleosomal fragmentation of DNA. We observed that caspase-8 was not involved in this process, thus indicating this is an intrinsic apoptotic pathway. The present data are consistent with the fact that RET acts as a dependence receptor that induces caspase-3-mediated apoptosis in the absence of GDNF [32]. We showed that cells continue to produce GDNF even in the absence of serum (Fig. 3), indicating that in
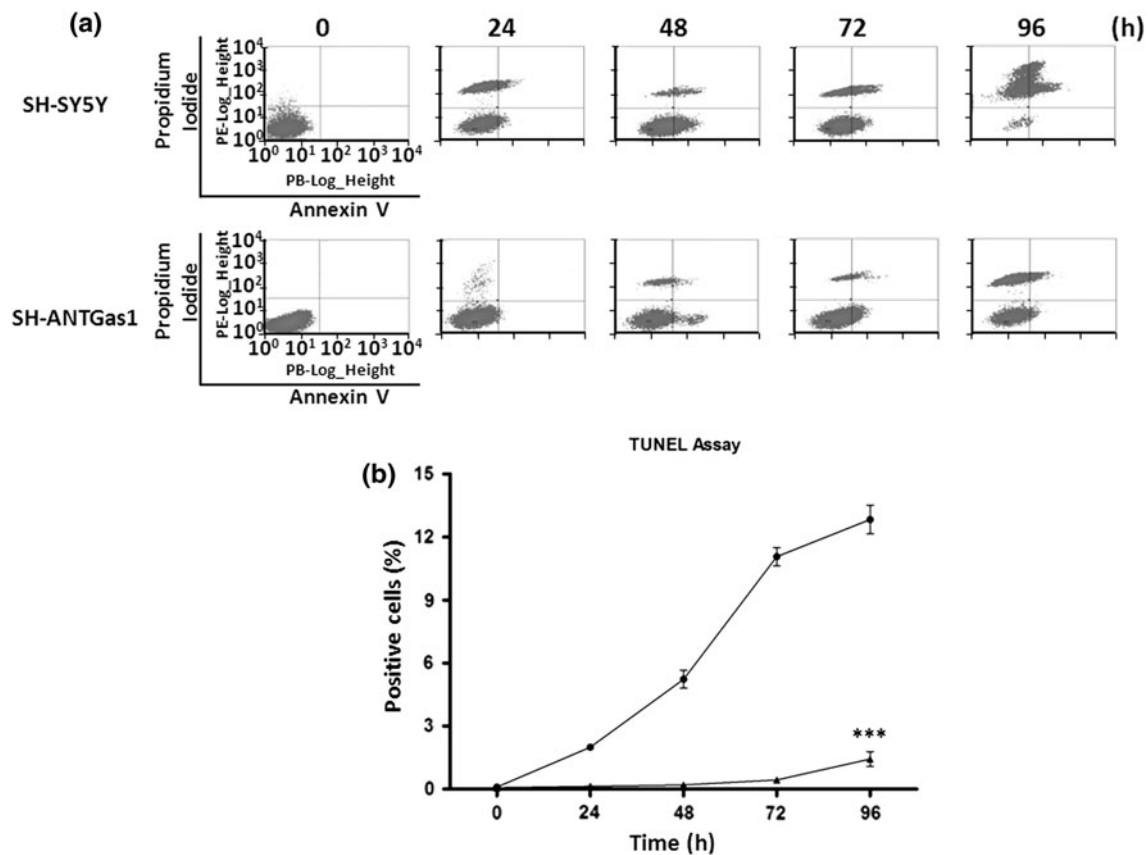
Fig. 5 Determination of apoptosis. **a** Apoptosis determined by Annexin V/propidium iodide by flow cytometry in SH-SY5Y control and SH-ANTGas1 transfected cells, in conditions of proliferation (0 h) and at different times after serum withdrawal. **b** Number of TUNEL positive cells in SH-SY5Y control and SH-ANTGas1 transfected cells, in conditions of proliferation and at different times after serum withdrawal. Data represent means ± SEM, $N = 3$; ***$P < 0.001$, SH-ANTGas1cells different from SH-SY5Y control cells

GAS1-silenced cells the endogenous production of GDNF maintains the survival pathway active. In Fig. 6, we submit a schematic representation of the model we propose to explain the molecular mechanism by which GAS1 induces cell death of tumor cells.

GAS1 is a protein involved with two major signaling pathways, namely GDNF and SHH, and in this paper, we have analyzed a potential interaction between the two intracellular signaling cascades. Abnormal SHH signaling has been associated with the origin and maintenance of neuroblastomas, and several of the components of this pathway have been detected in primary human neuroblastomas and cell lines, including SH-SY5Y [33–36]. In the present work, we detected the expression of the mRNAs for *SHH, PTCH1* and *GLI1*, in both SH-SY5Y cells and cells expressing *GAS1* mRNA antisense, in conditions of proliferation and after serum withdrawal (Supplementary Fig. 4). These data show that the SHH pathway is active, because we can observe the expression of *GLI1*, its major functional mediator, and that neither the expression of

GAS1, nor its silencing affect SHH signaling. From this information, we conclude that GAS1 is capable of inducing apoptosis in tumor cells in a manner independent of the SHH pathway, as we had previously observed for human primary gliomas [11].

In tumor cells, GAS1 acts as an antitumoral agent, and is also an important biomarker of the malignity of tumors. Moreover, when ectopically expressed, GAS1 has strong antitumoral effects, making it a potentially interesting therapeutic agent. In this paper, we present evidence showing that GAS1 induces an intrinsic apoptotic process, due to its capacity inhibiting the GDNF-induced survival pathway. Succinctly, GAS1 causes cell death by withdrawing the GDNF trophic support, an effect consistent with the previously described results of GDNF stimulating C6 cell proliferation [37]. Thus, we provide evidence to better understand the mechanism of action of GAS1 causing tumor cell death, and to assist providing tools to design new therapeutic strategies for treating cancer, as well as identifying potential target molecules involved in cell death.
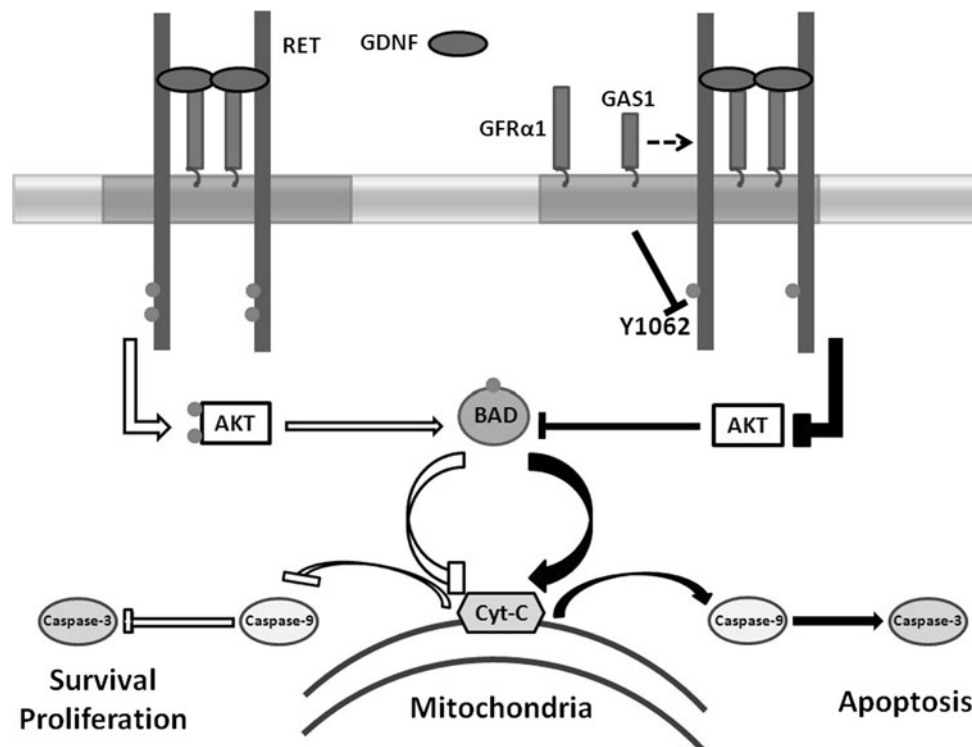
RET GDNF

GFRα1 GAS1

Y1062

BAD AKT

AKT

Cyt-C

Caspase-3 Caspase-9

Caspase-9 Caspase-3

Survival Proliferation

Mitochondria

Apoptosis

Fig. 6 Proposed model of the action of GAS1 inducing apoptosis. The panel represents the GDNF family of ligands (GFLs) signaling pathway; in this representation (*left side*) the binding of GDNF to GFRα1 recruits RET to form a molecular complex allowing the autophosphorylation of RET which in turn interacts with PI3K to activate AKT, this permits AKT to phosphorylate BAD so it remains in the cytosol. On the *right side* we represent the effects of GAS1 on this signaling pathway. The presence of GAS1 impedes the autophosphorylation of RET on tyrosine 1062, decreasing the activity of AKT; this facilitates the dephosphorylation of BAD which translocates to the mitochondria and participates in the release of Cyt-*c* to the cytosol where it forms part of the apoptosome that activates caspase-9 and then caspase-3, leading to apoptosis of the cell. Phosphorylation is represented by *circles* on the illustration

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