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To cite this article: Tania Velletri, Francesco Romeo, Paola Tucci, Angelo Peschiaroli, Margherita Annicchiarico-Petruzzelli, Maria Niklison-Chirou, Ivano Amelio, Richard Knight, Tak Mak, Gerry Melino & Massimiliano Agostini (2013) GLS2 is transcriptionally regulated by p73 and contributes to neuronal differentiation, *Cell Cycle*, 12:22, 3564-3573, DOI: [10.4161/cc.26771](https://doi.org/10.4161/cc.26771)


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# GLS2 is transcriptionally regulated by p73 and contributes to neuronal differentiation

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**Keywords:** p73, GLS2, neuronal differentiation, apoptosis, p53 family, metabolism, neurotransmitter

**Abbreviations:** TAp73, transcriptionally active p73;  $\Delta$ Np73, amino truncated p73; Gln, glutamine; Glu, glutamate; RA, retinoic acid; NB, neuroblastoma; P, postnatal day; ChIP, chromatin immunoprecipitation; GFP, green fluorescent protein; GABA, gamma-aminobutyric acid; VGAT, vesicular GABA transporter; VGLUT, vesicular glutamate transporter; DON, 6-diazo-5-oxo-L-norleucine; DMEM, Dulbecco minimal essential medium; FBS, foetal bovine serum; Ct, cycle

The amino acid Glutamine is converted into Glutamate by a deamidation reaction catalyzed by the enzyme Glutaminase (GLS). Two isoforms of this enzyme have been described, and the GLS2 isoform is regulated by the tumor suppressor gene p53. Here, we show that the p53 family member TAp73 also drives the expression of GLS2. Specifically, we demonstrate that TAp73 regulates GLS2 during retinoic acid-induced terminal neuronal differentiation of neuroblastoma cells, and overexpression or inhibition of GLS2 modulates neuronal differentiation and intracellular levels of ATP. Moreover, inhibition of GLS activity, by removing Glutamine from the growth medium, impairs in vitro differentiation of cortical neurons. Finally, expression of GLS2 increases during mouse cerebellar development. Although, p73 is dispensable for the in vivo expression of GLS2, TAp73 loss affects GABA and Glutamate levels in cortical neurons. Together, these findings suggest a role for GLS2 acting, at least in part, downstream of p73 in neuronal differentiation and highlight a possible role of p73 in regulating neurotransmitter synthesis.

## Introduction

Glutamine (Gln) is an amino acid that plays key roles in many metabolic pathways.<sup>1,2</sup> Mammalian glutaminase (GLS) is an enzyme responsible for the hydrolytic deamidation of Gln into Glutamate (Glu) and ammonium ions. Two paralogous genes on separate chromosomes encode distinct GLS isozymes: the kidney-type isozyme, GLS1, and the liver-type isozyme, GLS2.<sup>3,4</sup> Although early studies showed that GLS1 is ubiquitously expressed,<sup>2</sup> and that GLS2 is mainly expressed in the liver,<sup>4</sup> more recent observations indicate that the expression pattern is more complex. Indeed, GLS2 has also been detected in brain,<sup>5</sup> pancreas,<sup>6</sup> cancer cells,<sup>7</sup> and cells of the immune system.<sup>8</sup> Both isoforms have been identified in human and mouse brain. At the subcellular level, GLS2 localizes to the inner mitochondrial membrane, while in neurons it has been observed in the nucleus.<sup>5</sup> The abundance of a particular glutaminase mRNA species may significantly change depending upon the tissue type and the developmental or

metabolic state of the tissue; therefore, each transcript may represent a specific target for different stimuli. However, although GLS distribution has been extensively investigated, the regulation of GLS1/GLS2 expression is poorly understood. Recently, 2 independent groups have shown that GLS2 is a direct target of the tumor suppressor gene p53,<sup>9,10</sup> and that p53 drives the expression of GLS2 under both non-stressed and stressed conditions. Increased levels of GLS2 promote Gln metabolism, and the Glu produced by Gln hydrolysis is a precursor in the biosynthesis of Glutathione (GSH) and therefore promotes antioxidant defense through regulating the GSH/GSSG ratio. Moreover, Glu can be further transformed into  $\alpha$ -ketoglutarate, which feeds the tricarboxylic acid cycle, resulting in an increase of mitochondrial respiration and ATP production. Therefore, overall, GLS2 plays a key role in energy metabolism and antioxidant defense.

p73 is a pleiotropic protein that belongs to the p53 family.<sup>11–15</sup> It is involved in several biological processes, such as cell death,<sup>16–19</sup> differentiation,<sup>20–22</sup> neuronal stem cell maintenance,<sup>23–26</sup>

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Submitted: 04/17/2013; Revised: 10/09/2013; Accepted: 10/10/2013  
<http://dx.doi.org/10.4161/cc.26771>

aging,<sup>27-29</sup> and metabolism<sup>27</sup> through the regulation of gene<sup>30-32</sup> and microRNA expression. Mice deficient in p73 display neuronal pathologies, including hydrocephalus and hippocampal dysgenesis, with defects in the CA1-CA3 pyramidal cell layers and the dentate gyrus.<sup>33,34</sup> Moreover, they also have a reduction in cortical thickness as a consequence of loss of mature cortical neurons.<sup>35</sup> Recently, it has been demonstrated that TAp73 expression increases in parallel with neuronal differentiation, and its ectopic expression induces neurite outgrowth and expression of neuronal markers in neuroblastoma cell lines, suggesting that it has a pro-differentiation role.<sup>20</sup> In particular, TAp73 acts, at least in part, through microRNA-34a to regulate neuronal differentiation. Here, we demonstrate that, like p53, p73 is able to drive the expression of GLS2. We show that GLS2 expression itself induces neuronal differentiation in a neuroblastoma (NB) cell line, and that modulation of GLS2 influences retinoic acid (RA)-induced NB differentiation and spontaneous differentiation of cortical neurons *in vitro*. These data suggest that GLS2 is one mechanism mediating the neuronal effects of p73.

## Results

### TAp73 regulates the expression of GLS2

Since both TAp73 and GLS2 are expressed in the brain, and because of the extensive functional similarities between the p53 family of proteins, we asked whether TAp73, like p53, is able to regulate GLS2 expression. We first employed a SAOS2-TAp73 $\alpha$  inducible cell line treated with Doxycyclin and extracted total RNA after 24 h induction. As expected, the expression of TAp73 $\alpha$  was induced as well as that of p21, a well-known TAp73 target (Fig. 1A). In this cellular context, we also observed a significant upregulation of GLS2 mRNA (Fig. 1B). To confirm that TAp73 is able to regulate the expression of GLS2, we transiently expressed different isoforms of TAp73 in H1299 cells (Fig. 1C). As shown in Figure 1D, GLS2 mRNA was significantly induced (by 6–8-fold) after ectopic expression of TAp73 $\alpha$  and  $\beta$ . No induction of GLS2 expression was observed in cells overexpressing  $\Delta$ Np73 $\alpha$ . Next, we asked if GLS2 is directly regulated by TAp73. We first analyzed the human GLS2 promoter and identified a p53 consensus DNA-binding element located at –787 with respect to the transcription start site. In order to verify if TAp73 $\alpha$  was able to bind this putative responsive element, we performed a chromatin immunoprecipitation assay (ChIP). As shown in Figure 1E, TAp73 binds the GLS2 promoter containing the p53-responsive element. To confirm that this binding was functionally significant, we transfected TAp73 with a GLS2-luciferase reporter construct. Expression of TAp73 $\alpha$  or  $\beta$  resulted in an approximately 6-fold enhancement of luciferase activity (Fig. 1F). To further confirm the ability of TAp73 to modulate the expression of GLS2, we used an *in vitro* model of neuronal terminal differentiation, in which TAp73 has already been shown to be a positive regulator.<sup>20,22</sup> First, we characterized our cellular system for the intracellular localization of GLS2 and expression of the GLS isoforms. In SH-SY5Y cells, GLS2 localizes in the mitochondria (Pearson's correlation coefficient 0.69) as shown in Figure 1G and Figure S1A. No expression of GLS2

was detected at the nuclear level. Moreover, we have found that GLS1 is also expressed in SH-SY5Y cells (Fig. S1B). Then, we treated SH-SY5Y cells with retinoic acid (RA) in order to induce terminal differentiation and isolated total RNA. As shown in Figure 1H, GLS2 RNA expression was increased after treatment with RA. Moreover, this increase in GLS2 expression induced by RA was abolished when TAp73 expression was inhibited by RNA interference (Fig. 1H).

Together these results demonstrate that TAp73 directly regulates the expression of GLS2 and suggest a potential role for GLS2 in the regulation of RA-induced terminal differentiation of neuroblastoma cells.

### GLS2 expression contributes to the terminal neuronal differentiation of neuroblastoma cell lines

Since we have shown that RA induces the expression of GLS2 via a TAp73-dependent mechanism, we asked whether GLS2 could itself play a role in the terminal neuronal differentiation of NB cells. To assess this, we overexpressed GLS2 together with GFP in SH-SY5Y cells (Fig. 2A) and, after 24 h, evaluated neurite outgrowth. Figure 2B shows that overexpression of GLS2 (pGLS2) significantly increases neurite outgrowth though not to the same extent as RA. Transfection of GFP (C+GFP) or GFP+empty vector (pCTR+GFP) had no effect. Moreover, as shown in Figure 2C, cells overexpressing GLS2 show a high frequency of neurites twice, and to a lesser extent, 3 times the length of the soma. GFP+empty vector transfected cells remained in an undifferentiated state (und).

### Role of endogenous GLS2 in neuronal differentiation

To assess whether endogenous GLS2 also contributes to neuronal differentiation, we knocked down GLS2 expression in SH-SY5Y cells treated with RA (Fig. 3A; Fig. S1C). Figure 3B shows that the inhibition of GLS2 expression resulted in a modest but significant ( $P = 0.02$ ) reduction of the neurite extension induced by RA at 24 h. This small effect may be due to the compensatory effect of the concomitant expression of the GLS1 isoform in SH-SY5Y cells as mentioned above. To better understand, in a more physiological context, the role of GLS isoforms in neuronal differentiation, we functionally inhibited GLS activity by Gln withdrawal during *in vitro* differentiation of cortical neurons. Primary cortical neurons were plated, and after 24 h the complete medium was replaced with medium without Gln. After 6 days cortical neurons with or without Gln were processed for immunocytochemistry. As shown in Figure 3C and D, deprivation of Gln results in a reduction in the number of neurites, as shown by  $\beta$ -III-Tubulin staining. This reduction was confirmed by staining the cortical neurons for the specific synaptic proteins VGAT, VGLUT, and Synapsin 1/2. No significant differences in apoptosis were detected between cortical neurons in normal medium and in medium without Gln (Fig. S2).<sup>36</sup>

Taken together, these observations indicate that GLS2 participates in the regulation of neuronal differentiation in both RA-treated NB cells and in cortical neurons.

### Metabolic function of GLS2 in NB cells

It is well known that GLS2 plays a key role in energy metabolism and antioxidant defense. Indeed, it regulates ATP levels and the GSH/GSSG ratio in cells.<sup>9,10,37</sup> Hence, we asked whether

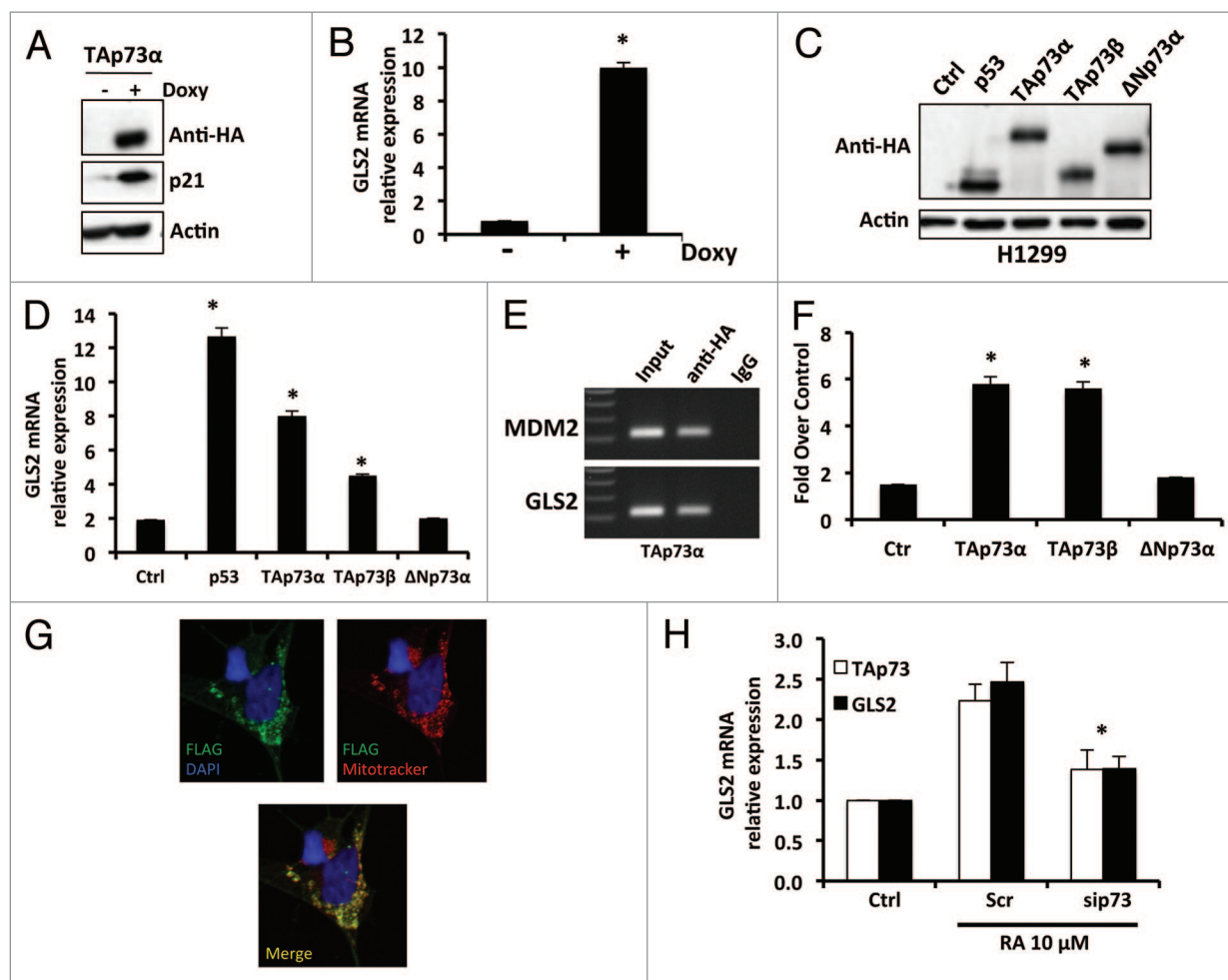
GLS2 could also exert the same functions in SH-SY5Y cells. First, we overexpressed GLS2 (Fig. 4A) and measured the intracellular levels of ATP. As shown in Figure 4C (left hand bars), the ectopic expression of GLS2 led to a significant increase (about +40%) of ATP intracellular levels. In contrast, when we knocked down GLS2 expression by siRNA (Fig. 4B), we observed a significant reduction (about -20%) of ATP levels. The same results were obtained when we inhibited the activity of GLS2 with the glutamine antagonist DON (Fig. 4C). However, the data in Figure 4D show that neither overexpression nor knockdown of GLS2 significantly effects the GSH/GSSG ratio.

#### Expression of GLS2 during neuronal development

To further investigate whether GLS2 is involved in neuronal differentiation in vivo, we monitored its expression during

cerebellar development. We collected mouse cerebellum at different stages (from postnatal day [P] 0 to P8), extracted total RNA, and used qPCR to assess the level of GLS2 expression. Figure 5A shows that the expression of GLS2 increases from P0 to P1 and then remains constant at least until P8.

Our in vitro data prompted us to explore whether TAp73 also regulated the expression of GLS2 in this in vivo context. To do this, we first isolated RNA from hippocampus obtained from wild-type (WT) and  $p73^{-/-}$  mice at different ages and monitored the expression of GLS2. As shown in Figure 5B, no differences in the expression of GLS2 mRNA were found between WT and  $p73^{-/-}$  mice. The same results were obtained when we compared the expression of GLS2 during the in vitro differentiation of hippocampal neurons. Indeed, as shown in Figure 5C, GLS2



**Figure 1.** TAp73 drives the expression of GLS2. (A) SAOS-2-TAp73α inducible cell lines were treated with Doxycyclin (Doxy) for 24 h in order to overexpress the human TAp73α protein, and endogenous levels of GLS2 were assessed by real-time PCR (B). Induction of TAp73α led to a significant ( $P < 0.05$ ) increase of GLS2 expression, as evaluated by real-time PCR. (C) H1299 cells were transfected with the indicated plasmids and expression of GLS2 was evaluated by real-time PCR as in (D). (E) TAp73 binds to the promoter of GLS2 as shown by ChIP. (F) TAp73 activates the GLS2 promoter as evaluated by luciferase activity. Co-transfection of a *Renilla* luciferase control plasmid was used to normalize the transfection efficiency. (G) Exogenous GLS2 localizes in the mitochondria. SH-SY5Y were transfected with FLAG-GLS2 expressing vector and after 24 h stained with MitoTracker<sup>®</sup> Red CMXRos and antibody against FLAG epitope as described in "Materials and Methods". A representative micrograph is shown. Magnification 40x. (H) TAp73 regulates GLS2 expression during neuronal terminal differentiation of neuroblastoma cells. SH-SY5Y cells were treated with 10  $\mu$ M retinoic acid in order to induce differentiation. Retinoic acid (RA) treatment induces the expression of GLS2. Inhibition of TAp73 expression induced by retinoic acid prevents the upregulation of GLS2. Real-time PCR data are normalized to the housekeeping gene GAPDH and relative to control (Ctrl) Data represent mean  $\pm$  s.d. of 3 different experiments. \* $P < 0.05$

expression appears to increase during the differentiation of  $p73^{-/-}$  hippocampal neurons, at least at some time points.

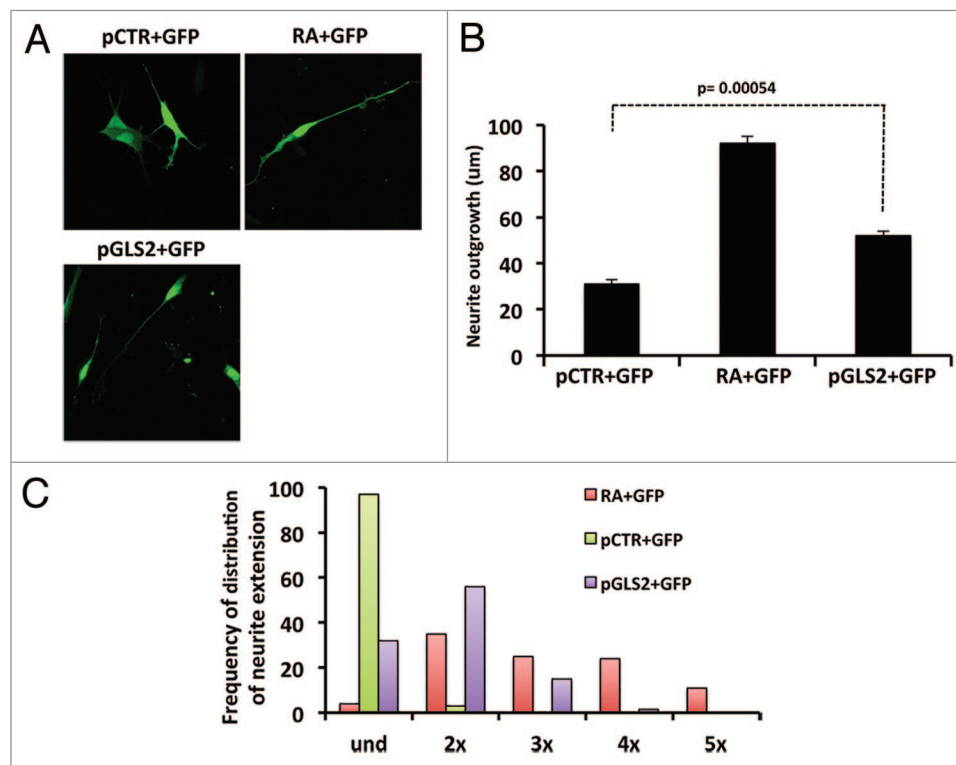
Although our results suggest that  $p73$  is not required for the *in vivo* expression of GLS2, we sought to investigate whether the absence of  $p73$  could affect glutamate metabolism (Fig. 5D). Cortical neurons derived from WT,  $TAp73^{-/-}$ , and  $\Delta Np73^{-/-}$  mice were plated, harvested after 7 days, and subjected to mass spectrometry analysis. Interestingly, as shown in Figure 5E, cortical neurons from the  $TAp73^{-/-}$  appeared to have reduced levels of GABA, which may be driven by slightly lower levels of glutamate. However, aspartate, which is also transported by glutamine transporters, and levels of N-acetylaspartylglutamate (NAAG), are unchanged in the  $TAp73^{-/-}$  neurons. Thus, reduction of GABA in these neurons is more likely to be a result of altered glutamic acid decarboxylase (GAD) activity. Conversely, NAAG and its precursor N-acetylaspartate (NAA), as well as the inhibitory neurotransmitter glycine (Fig. S3A), were reduced in  $\Delta Np73^{-/-}$  neurons at DIV 7. Decreased levels of these molecules in  $p73^{-/-}$  neurons could result in altered neuronal function or may reflect a delay in neuronal differentiation during *in vitro* culture.

Glutamate is one of the precursor amino acids in the biosynthesis of GSH. We observed that cortical neurons derived from  $TAp73^{-/-}$  mice show a reduction in GSH levels and in the GSH/GSSG ratio (Fig. S3B). However, these differences fail to reach statistical significance.

## Discussion

The  $p53$  family is involved in many aspects of development<sup>38-41</sup> and, in particular,  $p73$  regulates neuronal differentiation. First,  $p73$  deletion results in neuronal defects, including hydrocephalus and hippocampal dysgenesis, with abnormalities in the CA1-CA3 pyramidal cell layers and in the dentate gyrus.<sup>33,34</sup> Second,  $TAp73$  regulates neuronal differentiation of cortical neurons at least in part throughout the transcription of the microRNA,  $miR-34a$ .<sup>20,42</sup> Third,  $TAp73$  is implicated in the terminal differentiation of NB cells induced by retinoic acid (RA) and which is associated with an increased expression of  $TAp73$ . In addition, ectopic expression of  $TAp73$  itself induces terminal neuronal differentiation.<sup>22</sup> Recently, we have also demonstrated that  $TAp73$  regulates RA-induced differentiation of NB cells through  $miR-34a$ , since RA-induced differentiation of NB cells is inhibited both by knockdown of  $TAp73$  and by  $antagomiR-34a$ .<sup>20</sup> In this report we show that  $TAp73$  is able to directly regulate the expression of GLS2. Our results suggest that GLS2 is under the control of  $TAp73$  during neuronal differentiation of NB cells induced by retinoic acid, and that GLS2 itself can modulate neuronal differentiation.

GLS2 has recently attracted attention because it is a target of the  $p53$  tumor suppressor gene.<sup>9,10</sup> In particular, GLS2 regulates energy metabolism and antioxidant defense in cancer cells. Indeed our results indicate and confirm that GLS2 is a key player



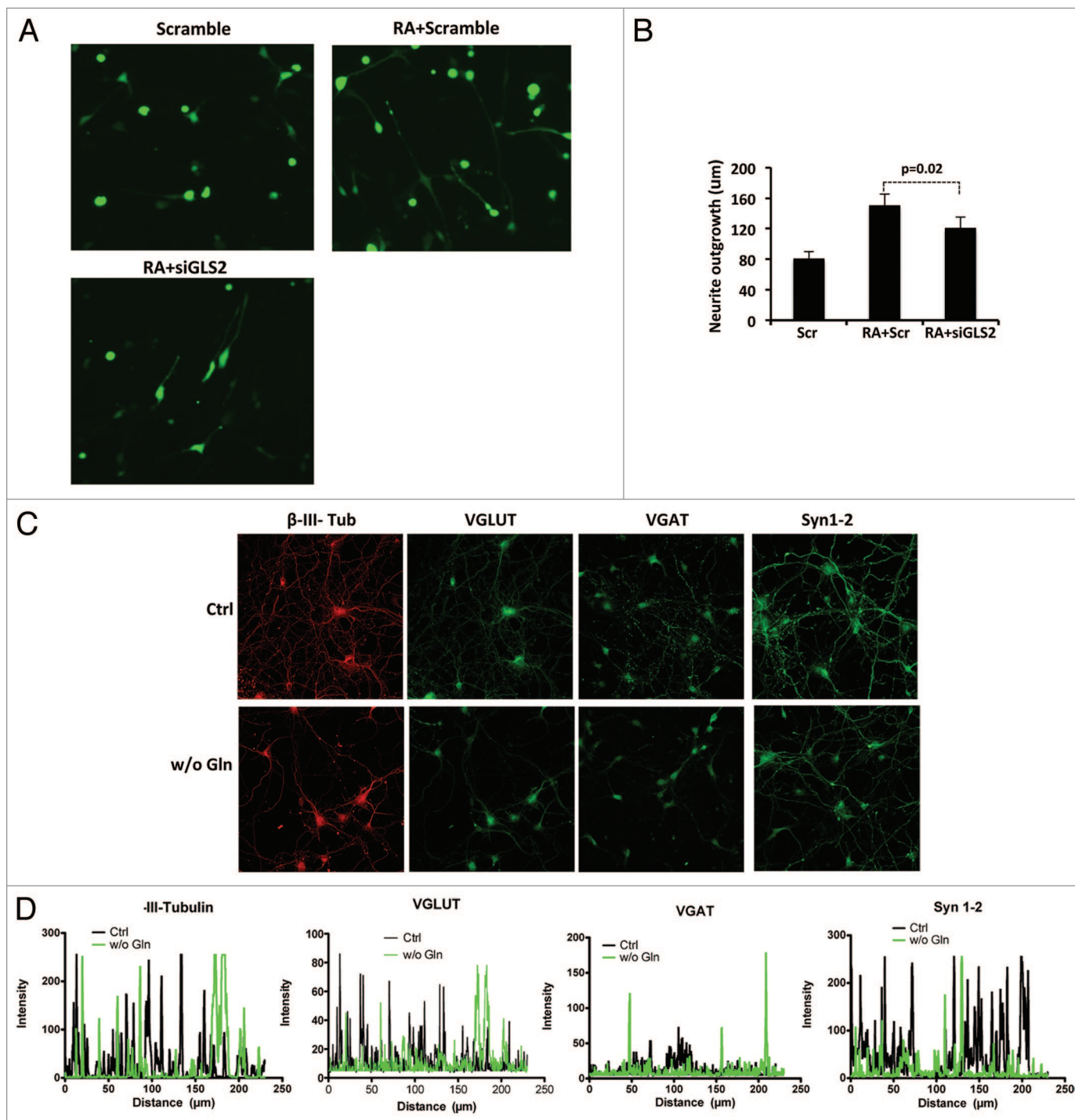
**Figure 2.** Ectopic expression of GLS2 increases neurite outgrowth. (A) Representative images of neurite outgrowth in the SHSY-5Y cell line. Cells were transfected with the indicated plasmids and either left untreated or treated with RA. A representative micrograph is shown. Magnification 40× (B) After 48 h cells were fixed and analyzed for neurite extension as described in “Materials and Methods”. (C) SH-SY5Y cells overexpressing GLS2 (violet columns) have neurites 2–3 times longer than the cell body. Data represent mean ± s.d. of 3 different experiments.



in the regulation of, at least, intracellular ATP levels. However, a role in cell differentiation has also been attributed to GLS2.<sup>7</sup> Our findings reinforce this possible role of GLS2 in cell differentiation, particularly neuronal differentiation. Thus, GLS2 gain- and

loss-of-function experiments result in an increase and decrease of neuronal differentiation, respectively.

Therefore, the role of TAp73 in neuronal differentiation, as revealed by the TAp73-knockout mouse phenotype, is complex



**Figure 3.** Inhibition of endogenous levels of GLS2 reduce neurite outgrowth induced by RA. **(A)** Representative images of neurite outgrowth in the SHSY-5Y cell line. Cells were transfected with scramble + GFP or siGLS2 + GFP and untreated or treated with retinoic acid (RA). **(B)** After 48 h cells were fixed and analyzed for neurite extension as described in Methods. Data represent mean  $\pm$  s.d. of 3 different experiments. Glutamine withdrawal impairs in vitro terminal differentiation of cortical neurons. **(C)** Cortical neurons were cultured either in the presence or absence of glutamine and fixed and stained for the indicated proteins after 7 days. Magnification 40 $\times$ . **(D)** Quantification of fluorescence intensity of the neuronal marker  $\beta$ -III-Tubulin and of the indicated synaptic proteins was performed using Zeiss LSM 510 software analysis.

and involves several downstream pathways. The demonstration in this report that GLS2, in addition to miR-34a, is a TAp73 target involved in the differentiation of NB cells, and, in particular, that direct manipulation of GLS2 expression itself modulates NB

differentiation, and that Gln deprivation influences the differentiation of cortical neurons in vitro, suggests that the neuronal effects of TAp73 are partly due to its effects on metabolism, since p53 family members, including TAp73, have been reported to have metabolic effects<sup>43-45</sup> in addition to regulating Gln metabolism. Despite the fact that TAp73 is not essential for the in vivo regulation of GLS2 expression, our results suggest that TAp73 loss affects glutamate metabolism. Indeed, cortical neurons derived from TAp73<sup>-/-</sup> mice show a reduction in the levels of the neurotransmitters glutamate and GABA. Because neuronal differentiation is a complex biological process that is regulated by intrinsic pathways<sup>46-51</sup> and extrinsic signals,<sup>52,53</sup> it will be of interest to see whether these other metabolic effects of TAp73 also affect this biological process.

## Materials and Methods

### Mice

Mice were bred and subjected to listed procedures under the Project License released from the Home Office. TAp73<sup>-/-</sup> and  $\Delta$ Np73<sup>-/-</sup> mice were generated as previously described.<sup>40,42</sup>

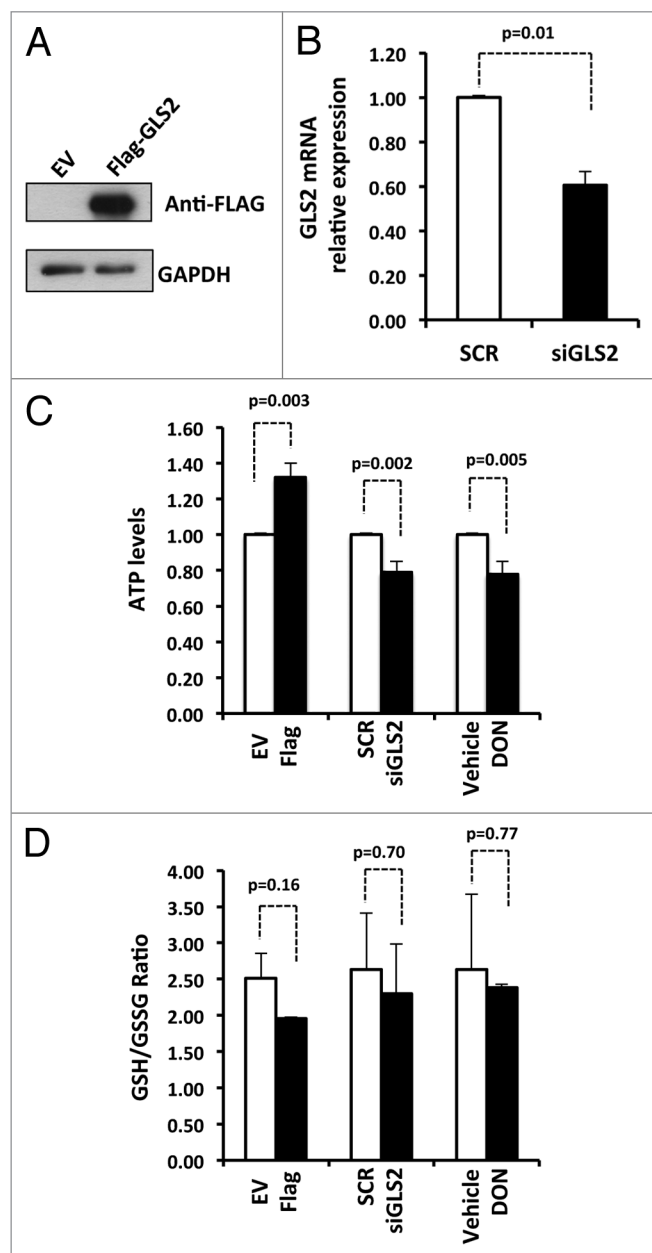
### Cell culture and transfection

TAp73 $\alpha$ -SAOS-2-Tet-On cells were grown in RPMI, 250  $\mu$ M L-glutamine (Gibco), penicillin/streptomycin 1 U/ml (Gibco), and 10% Tet-free FCS (Clontech); HEK 293E cells were grown in DMEM high glucose, 10% FBS, penicillin (100 U), streptomycin (100  $\mu$ g) (Invitrogen); SH-SY5Y cells were maintained in DMEM 10% FCS, penicillin/streptomycin 1 U/ml (Gibco), and H1299 cells were grown in RPMI, 250  $\mu$ M L-glutamine (Gibco), penicillin/streptomycin 1 U/ml (Gibco), 10% FCS (Invitrogen). For differentiation, SH-SY5Y cells were cultured in DMEM supplemented with 1% FCS and 10  $\mu$ M RA.

Cells were transfected by Lipofectamine 2000 according to the manufacturer's protocols (Invitrogen). Human GLS2-expressing vector was kindly provided by Dr Zhaohui Feng (Cancer Institute of New Jersey). Primary cortical neuronal cultures were prepared from E17.5 embryos mouse as previously described.<sup>42</sup>

### RNA extraction and real-time PCR

Total RNA from cells or tissues was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Total RNA (3  $\mu$ g) was reverse transcribed using RevertAid H Minus Reverse Transcriptase and oligo(dT) (Thermo Scientific). qRT-PCR was performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystem) with SYBR green ready mix (Applied Biosystem) and specific primers. The expression of each gene was defined from the threshold cycle (Ct), and relative expression levels were calculated by using the  $2^{-\Delta\Delta C_t}$  method after normalization with reference to expression of the housekeeping gene GAPDH. The following primers were used: mouse GAPDH Fwd 5'-CAATGAATAC GGCTACAGCA AC-3' and mouse GAPDH Rev 5'-AGGGAGATGC TCAGTGTG-3'; mouse GLS2 Fwd 5'-AGCGTATCCC TATCCACAAG TTCA-3' and mouse GLS2 Rev 5'-GCAGTCCAGT GGCCTTCAGA G-3'; human TAp73 Fwd 5'-CTCTGGAGCT CTCTGGAACC A-3' and human TAp73 Rev 5'-CGCCCCACCAC CTCATTATTTC-3'; human GLS2 Fwd 5'-TGCCTATAGT



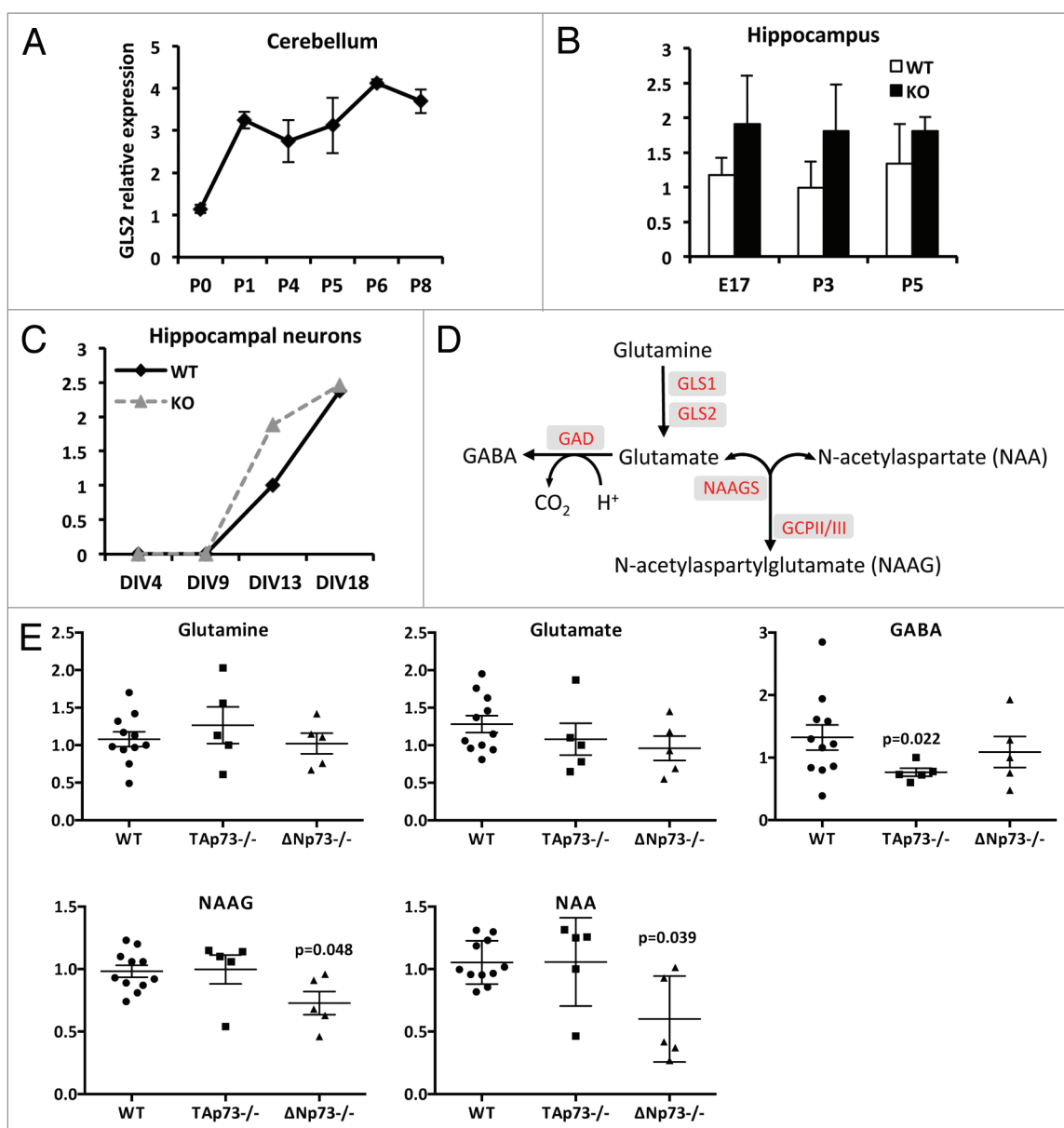
**Figure 4.** GLS2 regulates intracellular levels of ATP. (A) A representative western blot showing expression of GLS2 after transfection of SHSY-5Y cells with empty vector (EV) or FLAG-GLS2. (B) Real-time PCR showing inhibition of GLS2 expression after transfection of SHSY-5Y cells with short interfering RNA against GLS2 (siGLS2). Scramble (SCR). Real-time PCR data are normalized to the housekeeping gene GAPDH relative to scramble (SCR). Data represent mean  $\pm$  s.d. of 3 different experiments. (C) Intracellular levels of ATP in SHSY-5Y transfected or treated as indicated were measured as described in "Materials and Methods". The inhibitor of Glutaminase DON (6-Diazo-5-oxo-L-norleucine) was used at 200  $\mu$ M. (D) The intracellular levels of GSH and GSSG are not affected by GLS2 in SHSY-5Y cells. Ratio of GSH/GSSG was measured as described in "Materials and Methods". Data represent mean  $\pm$  s.d. of 3 different experiments.

GGCGATGTCT CA -3' and human GLS2 Rev 5'-GTTCCATATC CATGGCTGAC AA -3'; human GAPDH Fwd 5'-AGCCACATCG CTCAGACAC-3' and human GAPDH Rev 5'-GCCCCAATACG ACCAAATCC-3'; human GLS1 Fwd 5'-GCTGTGCTCC ATTGAAGTGA CT-3'; and human GLS1 Rev 5'-TTGGGCAGAA ACCACCATTA G-3'.

#### Chromatin immunoprecipitation

TAp73 $\alpha$ -SaOS-2-inducible cells were crosslinked for 10 min in a solution containing 1% formaldehyde and ChIP assays

were performed using a MAGnify ChIP system (Invitrogen), as specified in the manufacturer's instructions. Cell lysates were sonicated in order to obtain chromatin fragments of ~700 bp. The immune complex was immunoprecipitated using an anti-HA specific antibody (16B12, Covance) and nonspecific IgG as a control. Collected DNA fragments were tested by PCR. The following oligos were used for amplifying the p53 putative responsive elements (RE) found in the GLS2 promoter: Fwd 5'-GGCCTCCCAA GTCACCAGTT CA-3' Rev



**Figure 5.** In vivo expression of GLS2. (A) GLS2 expression increases during mouse cerebellar development. The cerebellum was isolated from wild-type mice (n = 4 per each point) and expression of GLS2 was evaluated during cerebellar development by real-time PCR at birth (P0) and subsequent postnatal (P) days. (B) GLS2 expression in the hippocampus dissected from wild-type and p73<sup>-/-</sup> mice at the indicated age (E, embryo; P, postnatal). (C) GLS2 expression during in vitro terminal differentiation of hippocampal neurons derived from wild-type and p73<sup>-/-</sup> mice. Real-time data are normalized to the housekeeping gene GAPDH relative. Data represent mean  $\pm$  s.d. of 3 different experiments. (D) Schematic representation of neurotransmitters biosynthesis. In red the enzymes involved in each reaction. GLS, Glutaminase; GAD, Glutamic acid decarboxylase; NAGGS, N-acetylglutamate synthase; GCP, glutamate carboxypeptidase. (E) Effect of p73 deficiency on intracellular levels of neurotransmitters. Cortical neurons derived from wild-type (WT), TAp73<sup>-/-</sup> and ΔNp73<sup>-/-</sup> were harvested after 7 days of culture and subjected to mass spectrometry analysis as described in "Materials and Methods".



5'-TGTTTGTCT TGTTCGCGC TTCT-3'. The p53 RE located on hMDM2, used as a positive control, was amplified with one set of primers: Fwd 5'-GGTTGACTCA GCTTTTCCTC TTG-3' and Rev 5'-GGAAAATGCA TGGTTTAAAT AGCC-3' (119 bp).

#### Luciferase assay

HEK 293E cells were plated in 12-well plates ( $1 \times 10^5$  and  $1.5 \times 10^5$  per well, respectively). After 24 h, pGL3control vectors (200 ng) were cotransfected with TAp73 $\alpha$ ,  $\beta$ , and  $\Delta$ Np73 $\alpha$  or empty vector and *Renilla* luciferase pRL-CMV vector (10 ng), using Lipofectamine 2000. Luciferase activities were measured 24 h after transfection using a Dual Luciferase Reporter Assay System (Promega); light emission was measured over 10 s using an OPTOCOMP I luminometer. Efficiency of transfection was normalized using *Renilla* luciferase activity.

#### Western blot

Proteins were extracted with RIPA buffer containing an inhibitor cocktail (Roche) and protein concentration was determined using a Bradford dye-based assay (Biorad). Total protein (30  $\mu$ g) was subjected to SDS-PAGE followed by immunoblotting with appropriate antibodies at the recommended dilutions. The blots were then incubated with peroxidase-linked secondary antibodies followed by enhanced chemiluminescent detection using the Super Signal chemiluminescence kit (Thermo scientific). Antibodies: HA-HRP (1:5000; Sigma), p21 (1:1000, Santa Cruz), FLAG M2 clone (1:5000; Sigma-Aldrich), and GAPDH (1:10000; Sigma-Aldrich).

#### Neurite outgrowth assay

SHSY-5Y cells were transfected as indicated using Lipofectamine 2000 and then treated with RA (10  $\mu$ M) and analyzed 24 h or 48 h later. The projection images were semi-automatically traced with NIH ImageJ using the NeuronJ plugin. The neurite length of each individual GFP positive cell was analyzed.

#### ATP and GSH/GSSG measurements

Intracellular levels of ATP were measured by using the ELITEN ATP Assay System Bioluminescence Detection Kit (Promega). GSH and GSSG levels were measured by using the GSH/GSSG-Glo Assay (Promega). Briefly, cells were transfected either with FLAG-GLS2 or siGLS2 and after 24 h or 48 h, respectively, harvested and processed according to the manufacturer's instructions.

#### Immunofluorescence

SH-SY5Y cells were transfected with human-FLAG-GLS2 and after 24 h were incubated for 30 min at 37 °C with MitoTracker® Red CMXRos (Invitrogen). Then cells were washed twice with warm medium, fixed, and processed for immunofluorescence. SHSY-5Y cells were stained with anti-FLAG (1:1000, Sigma-Aldrich) antibody. Cortical neurons cultured with or without glutamine were fixed with 3% paraformaldehyde in PBS followed by treatment with 0.1% Triton X-100 for 5 min and 10% normal goat serum in PBS for 1 h at RT. Then, cells were stained at 4 °C overnight with the following primary antibodies:

neuronal-specific anti- $\beta$ -III-Tubulin (1:2000; Promega), anti-VGAT (1:500; Synaptic System), anti-VGLUT1 (1:500; Synaptic System), and anti-Synapsin1-2 (1:500; Synaptic System). After 3 washes (10 min in PBS), the cells were incubated with Alexa Fluor conjugated secondary antibody (1:1000; Jackson Immuno Research Laboratories). After 3 washes (10 min, PBS), nuclei were stained with DAPI (1:10 000) for 10 min; this step was followed by a further wash (10 min in PBS). The coverslips were then mounted with Aquapolymount antifading solution (Polysciences) onto glass slides and observed under a confocal microscope (Zeiss LSM 510). Colocalization analysis was performed with Velocity 3D image analysis software version 6.3 (Perkin Elmer).

#### Metabolomic analysis of cortical neurons

DIV7 cortical neurons were harvested and immediately stored at -80°C and samples were processed as previously described.<sup>54,55</sup> Briefly, the sample preparation process was performed using the automated MicroLab STAR® system from the Hamilton Company. Recovery standards were added prior to the first step in the extraction process for QC purposes. Sample preparation was conducted using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into 2 fractions: one for analysis by LC and one for analysis by GC. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. Each sample was then frozen and dried under vacuum. Samples were then prepared for the appropriate instrument either LC/MS or GC/MS. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Identification of known chemical entities was based on comparison to metabolomic library entries of purified standards.

#### Statistical analysis

All results are expressed as means  $\pm$  s.d.  $P < 0.05$  was considered significant. For metabolomics analysis we perform Welch 2-sample  $t$  tests.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Acknowledgments

We thank David J Read for technical support. This work has been supported by the Medical Research Council, UK; grants from "Alleanza contro il Cancro" (ACC12), MIUR/PRIN (20078P7T3K\_001)/FIRB (RBIP06LCA9\_0023, RBIP06LCA9\_0C), AIRC (2011-IG11955), AIRC 5xmille (#9979), Telethon Grant GGPO9133, Min. Salute (Ricerca oncologica 26/07), and IDI-IRCCS (RF06 c.73, RF07 c.57, RF08 c.15, RF07 c.57) to GM.

#### Supplemental Materials

Supplemental materials may be found here:  
[www.landesbioscience.com/journals/cc/article/26771](http://www.landesbioscience.com/journals/cc/article/26771)

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