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PAS kinase as a nutrient sensor in neuroblastoma and hypothalamic cells required for the normal expression and activity of other cellular nutrient and energy sensors

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Abstract

PAS kinase (PASK) is a nutrient sensor that is highly conserved throughout evolution. PASK-deficient mice reveal a metabolic phenotype similar to that described in S6 kinase-1 S6K1-deficient mice that are protected against obesity. Hypothalamic metabolic sensors, such as AMP-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR), play an important role in feeding behavior, the homeostasis of body weight and energy balance. These sensors respond to changes in nutrient levels in the hypothalamic areas involved in feeding behavior and in neuroblastoma N2A cells, and we have recently reported that those effects are modulated by the anorexigenic peptide glucagon-like peptide-1 (GLP-1). Here we identified PASK in both N2A cells and rat VMH and LH areas and found that its expression is regulated by glucose and GLP-1. High levels of glucose decreased *Pask* gene expression. Furthermore, PASK-silenced N2A cells record an impaired response by the AMPK and mTOR/S6K1 pathways to changes in glucose levels. Likewise, GLP-1 effect on the activity of AMPK, S6K1 and other intermediaries of both pathways and the regulatory role at the level of gene expression were also blocked in PASK-silenced cells. The absence of response to low glucose concentrations in PASK-silenced cells correlates with increased ATP content, low expression of mRNA coding for AMPK upstream kinase LKB1 and enhanced activation of S6K1. Our findings indicate that, at least in N2A cells, PASK is a key kinase in GLP-1 actions and exerts a coordinated response with the other metabolic sensors, suggesting that PASK might play an important role in feeding behavior.

Introduction

The PAS kinase (PASK), also termed PASKIN, has a serine/threonine kinase catalytic domain and a PAS domain designed to detect environmental parameters, such as light, oxygen and redox state. In mammals, it has been proposed that PASK is activated by a small metabolite and may regulate glycogen synthesis and protein translation. PASK is also a nutrient sensor conserved from yeast to humans [1-3] with its role being compared to other metabolic sensors: AMP-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR). AMPK activity is regulated by fluctuations in cellular energy produced by nutritional state or by metabolic stress that reduces ATP production. The activation of AMPK occurs with a high AMP/ATP ratio in low energy states. Once activated, the AMPK will contribute to restore the energy balance [4-6]. By contrast, mTOR and its downstream target, the S6 Kinase 1 (S6K1), respond to nutrients and hormonal signals [7-9]. mTOR complex 1 (mTORC1) is rapamycin and nutrient sensitive. Thus, mTOR is stimulated by an abundance of nutrients and hormones promoting anabolic processes and cell growth [10,11]. Both AMPK and mTOR respond to changes in glucose and other nutrients in a coordinated way and in opposite directions. Therefore, low energy conditions activate AMPK, which mediates the phosphorylation of the tuberous sclerosis complex 2 (TSC2) and the concomitant inhibition of mTOR [12]. Furthermore, AMPK phosphorylates raptor (rapamycin-sensitive adaptor protein of mTOR) in mTORC1, down-regulating this complex [13].

It has been reported that PASK expression is regulated by glucose, being increased at high glucose concentration, which also activates PASK post-translationally in Min-6 cells [14]. In contrast, PASK regulation by glucose was not observed in pancreatic beta-cells from PASKIN knockout and wild-type mice, and glucose-stimulated insulin release was also similar in both types of mice [15].

An initial characterization of PASK-deficient mice showed no abnormalities in development, growth or reproductive functions [16]. Subsequent studies showed that PASK-deficient mice are resistant to diet-induced obesity and that protection seems to be caused by a high metabolic rate in skeletal muscle; these effects were not related to changes in AMPK and S6K1 activation levels [17]. Additionally, the PASK knockdown effect was also observed in cultured myoblasts, which also registered an increase in the oxidation of glucose and palmitate accompanied by elevated cellular ATP levels [17].

In mammals, PASK is thought to be involved in the regulation of glucose and energy metabolism homeostasis, as reviewed by [3,18,19]. The effects of PASK activation have also been reported to be tissue specific [17]. Likewise, studies in recent years have established a direct relationship between the activity of the metabolic sensors in the hypothalamus and the regulation of food intake, body weight and energy homeostasis. Moreover, the function of hypothalamic metabolic sensors is modulated by anorexigenic and orexigenic peptides. We have recently reported that the metabolic sensors in hypothalamic areas and in the neuroblastoma N2A cell line responded to glucose and the anorexigenic peptide glucagon-like peptide 1 (GLP-1), which is also used as an antidiabetogenic agent [20,21]. In addition, the AMPK and mTOR/S6K

pathways respond to changes in glucose concentrations, and GLP-1 treatment reversed the glucose effects [22]. PASK is also expressed in hypothalamic areas and in neuroblastoma N2A cells; hence we studied the glucose and GLP-1 effect on PASK expression and whether there is a coordinated response by metabolic sensors at different glucose concentrations. Accordingly, we investigated the effect of PASK-silencing on the expression of several genes, as well as the metabolic effects on the expression and activity of AMPK and mTOR/S6K1 pathways in neuroblastoma cells.

Materials and methods

Experimental animals

All the procedures involving animals were approved by the appropriate Institutional Review Committee and met the guidelines for the care of animals specified by the European Community. Same-aged male Wistar rats weighing 200-250 g were fed *ad libitum* with a standard pellet diet and housed at a constant temperature (21 ºC) on a 12-h light-dark cycle, with lights on at 08:00.

Cell cultures

Mouse neuroblastoma N2A cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) containing 4.5 g/L glucose and supplemented with 10 % FBS, 2 mM-glutamine and penicillin (100 U/ml), streptomycin (100 mg/ml). The cells were maintained in a humidified atmosphere containing 5 % CO_2 at 37 °C. Only the cells propagating for less than eight passages were used in the experiments.

In situ hybridization histochemistry

The histological examination was conducted using 20 µm of frozen rat brain slices. In situ hybridization was performed as described by [23] using digoxigenin-labeled cRNA probes. Single-stranded Pask RNA sense or antisense probes were prepared by the transcription of a fragment of 1180 pb previously amplified by PCR using the universal forward primers pUC/M13 (5´-GTTTTCCCAGTCACGAC-3´) and reverse primers (5´-CAGGAAACAGCTATGAC3´). T7 and SP6 RNA polymerases (Roche) (Promega, Southampton, UK) were used for preparing antisense and sense probes, respectively. Tissues were incubated with RNA probes at 1 µg/ml concentration in a hybridization mix (50 % formamide, 1.3x SSC [pH 4.5], 5 mM EDTA, 50 µg/ml yeast RNA, 0.2 % Tween 20, 0.5 % CHAPS, and 100 µg/ml heparin). To elicit color, the slices were incubated with a 1/2000 dilution of anti-digoxigenin-alkaline phosphatase antibody (Roche) and developed with 0.26 mg/ml nitroblue tetrazolium salt and 0.175 mg/ml 5-bromo-4-chloro-3-indolyl phosphate prepared in NTMT (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM MgCl2, and 0.1 % Tween 20). Images were taken with a Leica DM RB microscope.

Immunocytochemistry

Immunocytochemistry was developed as described [24]. Briefly, cells were grown attached to glass coverslips of 12 mm in diameter. The cells were then fixed for 15 minutes at 4 °C in 4 % paraformaldehyde and washed three times with PBS at room temperature. The cells were permeabilized for 20 min with PBS 0.4 % (v⁄v) Triton X-100, and non-specific antibody binding sites were blocked by incubation with a blocking solution (PBS, 10 % goat serum, 0.1 % Triton X-100) for 1 h. The cells were incubated with anti-PASK antibody diluted 1:100 in the blocking solution and then washed twice for 10 min with PBS at room temperature. For fluorescence detection, the cells were incubated with Texas Red conjugated donkey anti-rabbit antibody (GeneTex, Inc., San Antonio, CA, USA) diluted 1:200 in the blocking solution. In all cases, the specificity of immunodetection was confirmed by not adding a primary antibody. The nucleus DNA was stained by adding 1 µg/ml of 4,6-diamidino-2-phenylindole (DAPI) in PBS. Finally, the cells were mounted with the Fluoromount-G slide mounting medium (EMS, Hatfield, PA, USA). The images were taken with a TCS SP2 confocal laser microscopy system (Leica Microsystems, Wetzlar, Germany) equipped with an inverted DMIRE2 Leica microscope. Confocal fluorescence images were analyzed using LCS Lite software from Leica.

Procedure for hypothalamic-slice explants cultures

Hypothalamic-slices were obtained as described previously [25]. Briefly, male Wistar rats were decapitated and the brains were quickly removed and immersed in a cold (4 ºC) MEM medium containing 25 mM HEPES, 20 % heat-inactivated horse serum, 4 mM glutamine, 6.5 mg/ml glucose, and 100 U/ml penicillin-streptomycin. The hypothalami were removed from the brain and sections of 300 µm thickness were cut on a Mcllwain tissue chopper (Mickle Laboratory Engineering, Surrey, UK). The slices were cultured at 37 ºC in an atmosphere containing 5 % CO_2 for 5 h in MEM supplemented with 25 mM HEPES and Hank´s salt-enriched with the above components in order to stabilize the cultures. The slices were then incubated in a medium containing 2 % FBS and 5.5 mM glucose for 16 h. Following this, the hypothalamic slices were fasted at 0.5 mM glucose for 2 h. The medium was removed and the slices were incubated for 3 h with 1, 2.5 or 10 mM glucose, in some cases adding 10 nM GLP-1. At the end of the incubations, special care was taken to identify and isolate the VMH and LH areas by micropunching according to the stereotaxic coordinates (Paxinos and Watson 2004).

RNA interference

The short interference RNA (siRNA) targeting *Pask* gene (PASK Silencer® Select Pre-design siRNA) and the negative control siRNA (Silencer® Select Negative Control siRNA) were obtained from Ambion (Life

Technologies). The sequences of *Pask* siRNA were: Sense CGUGUAAUCUGCAUCAGGAtt and Antisense UCCUGAUGCAGAUUACACGtg. N2A cells were grown up to a 50 % confluence in six well plates. siRNA was transfected by using Lipofectamine™ with 0.15 µg of *Pask* or 10 nM control siRNAs, according to the manufacturer's protocol. After 24 h, the cells were starved of serum (0.1 %) and glucose (0.5 mM) for 2 h. They were then incubated in a medium containing either 0.5, 2.5 or 10 mM of glucose for 3 h. In some cases, 10 nM GLP-1 was added during the incubation. Cell lysates were collected and processed for western blot analysis or RNA extraction, cDNA synthesis and quantitative real-time PCR.

Real-time polymerase chain reaction (TaqMan® Assay)

The total RNA from N2A cells or rat hypothalamic-slices was isolated by the acid guanidinium isothiocyanate method (Chomczynski and Sacchi 1987). RNA integrity was tested with the Bioanalyzer 2100 (Agilent), and cDNA synthesis was developed with the "high-capacity cDNA archive kit" (Applied Biosystems), using 1 µg of RNA as template, following the manufacturer's instructions. The mRNA levels of *Pask, Ampkα2, Glp-1r, β-actin, 18s, Sf-1 and Prepro-orexin* were measured by real-time quantitative RT-PCR using TaqMan® probes (Applied Biosystems). The primers and probes (Online Resource1) were designed with Primer Express 2.0 software from Applied Biosystems.

Multiple gene expression analyses in a TaqMan® Gene Expression Assay

RNA integrity was tested with the Bioanalyzer 2100 (Agilent), and cDNA synthesis was developed with the "high-capacity cDNA archive kit" (Applied Biosystems) using 1 µg of RNA as template in a final volume of 20 µl, following the manufacturer's instructions.

Multiple gene expression analyses were conducted by quantity real-time PCR, using the TaqMan® Fast Universal PCR Master Mix (Roche Diagnostic) on a TaqMan® Array Fast 96-well Plate containing 18 customized duplicated genes (Online Resource 2). Two microlitres of a 1/10 dilution of the cDNA was used as a template for the PCR developed in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The conditions were 50 ºC for 2 min, 95 ºC for 10 min, followed by 40 cycles at 95 ºC for 15 s and 60 ºC for 1 min. Two housekeeping genes were used for the normalization procedure, namely, *18s* and *β-actin*. The data were analyzed with RQ Manager Software (Applied Biosystems).

The average *18s* or *β-actin* CT values on each 96-well plate were then subtracted from the CT value for each reaction well on that plate.

Protein expression and activity of PASK, AMPK and mTOR/S6K1 pathways

For the analysis of protein expression and activity by western blot, the cells were cultured for 2 h in the presence of 0.5 mM of glucose. They were then incubated in a medium containing 0.5, 2.5 or 10 mM of glucose for 2, 4, 5, 8, 16 h. The cells were occasionally incubated in the presence or absence of different protein kinase or phosphatase inhibitors at the concentrations described in Table 1 for 30 min. To analyze the effect of AMPK activator 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR) cells were treated with 2 mM AICAR for 1 h. In the case of the activity study, 10 nM GLP-1 was added during the last 10 min of the incubation, while in the protein expression determination the GLP-1 was added from the beginning of the incubation with glucose. The cells were immediately lysed in a RIPA buffer and exposed to microwave irradiation for 5 s. Total and activated forms were detected by western blot using the antibodies described in Table 2. The blots were reprobed for β-ACTIN. Finally, the blots were scanned and quantified using Quantity One software (Bio-Rad, GS800 Densitometer).

ATP concentration

Total cellular ATP concentration was measured using the luciferase bioassay. The bioluminescence assay is based on the reaction of ATP with recombinant firefly luciferase (Sigma-Aldrich, Saint Louis, Missouri, USA) and its substrate luciferin (Sigma). Cells were grown on a 24-well plate. Both the cells previously transfected with negative control siRNA and those with *Pask* siRNA were incubated for 2 h in 0.5 mM glucose and then for a further 2 hours in a medium containing either 0.5 mM or 10 mM of glucose. A 1/1000 dilution of grown cells was harvested by trypsinization and centrifugation in PBS. Pellets were lysed in 20 µl of 1.35 M perchloric acid and then neutralized with 15 µl of 2.8 M $KHCO_3$.0.1M Tris. 10 µl of the supernatant was used for the luciferase reaction, by adding 10 µl of luciferase, and 100 µl luciferin. Luminescence was determined in a luminometer (BG-P luminometer; GEM Biomedical, Hamden, CT).

Statistical analyses

All values are presented as means ± SEM. Groups were compared using t-test analysis. $P<0.05$ was considered statistically significant.

Results

Expression of *Pask* mRNA in the hypothalamus

By means of in situ hybridization with the specific antisense *Pask* probe, labeled cells were found throughout the hypothalamus, whereas no labeling was detected with the sense probe (Fig. 1a). Cells positive for *Pask* mRNA were found to be distributed throughout the hypothalamus. A higher

magnification of the labeled cells in the LH is shown in Fig. 1a. The specificity of the probe and the in situ hybridization background were tested with the sense probe where no labeled cells were found.

Cellular location of PASK

PASK immunocytochemistry was developed in neuroblastoma N2A cells. All the N2A cells contained PASK protein and it was present mainly in the cell cytoplasm (Fig. 1b).

PASK responds to glucose in neuroblastoma cells

PASK is expressed in N2A cells. The *Pask* mRNA expression was measured with real-time RT-PCR after treatment at low and high glucose concentrations (0.5 or 10 mM). Elevated levels of glucose decreased the amount of mRNA coding PASK in neuroblastoma cells (Fig. 1c).

Glucose and GLP-1 regulate PASK expression in hypothalamic-slice explants

We then investigated *Pask* mRNA expression in the hypothalamic areas involved in the control of feeding: ventromedial hypothalamus (VMH) and in the lateral hypothalamic area (LH). In order to ensure the purity of these hypothalamic nuclei obtained by micropunching from hypothalamic-slice explants, *Steroidogenic factor 1* (*Sf-1*) and *Prepro-orexin* genes were used as markers of these specific nuclei respectively. The mRNA encoding SF-1 was mostly present in VMH while, on the contrary *Prepro-orexin* gene expression was highest in LH (Fig 2a)

The mRNA levels of *Pask* were measured with real-time RT-PCR in VMH and LH areas from rat hypothalamic-slice explants after treatment with different glucose concentrations (1, 2.5 or 10 mM) (Fig. 2b,c). Higher glucose concentrations (10 mM) decreased by ≈20 and 66 %, the expression of the mRNA coding PASK in the VMH and LH areas, respectively. The presence of GLP-1 decreased *Pask* mRNA and reversed in part the high glucose concentrations effect in the LH (Fig. 2b,c).

PASK-silencing in neuroblastoma N2A cells

We therefore analyzed the effect of PASK knockdown in neuroblastoma N2A cells. We have previously reported that both AMPK and S6K1 activities respond to glucose concentrations, and GLP-1 also significantly decreased AMPK and S6K1 activation in these cells [22]. PASK-silenced cells recorded a 77% decrease in PASK mRNA compared to siRNA negative control (Fig. 1d).

We then compared the expression of several genes using real-time RT-PCR in PASK knockdown N2A and control cells at low or high glucose concentrations in the presence or absence of GLP-1. Thus, we analyzed the expression of mRNAs coding to PASK and other metabolic sensors as: AMPKα2, mTOR/S6K1;

several genes of proteins that could regulate the metabolic sensors activity: LKB1, TSC2, AKT, PKA; as well as some direct substrates of metabolic sensors: S6, eIF-4B, eEF-2K, eEF1-α1 and nNOS; genes coding to protein decisive for carbohydrate metabolism: GAPDH and PDK1; additionally, genes coding to HIPK2, which was reported to mediate apoptosis in neurons, GLP-1 receptor (GLP-1R) and a housekeeping gene: *β-actin* used for the normalization procedure. The *Pask* mRNA level was higher at low glucose concentration and lower at high glucose, and the treatment with GLP-1 reversed these effects. However, the expression of PASK did not respond to glucose or GLP-1 after PASK-silencing (Fig. 3a). PASK knockdown did not change significantly the expression of mRNAs coding to mTOR and S6K1 (Fig. 3b). Unfortunately, it was impossible to detect the mRNA level of *Ampkα2* in these cells with the probes used. A similar pattern of expression was observed in PASK-silenced and control cells for the genes coding to AKT, PKARIIα and TSC2 (Fig. 3c). However, the expression of *Stk1* mRNAs that codify to LKB1, one of the upstream kinases of AMPK, was enhanced at low glucose concentration, and the presence of GLP-1 reversed the glucose effect, with PASK knockdown impairing both effects (Fig. 3c). The expression of *nNos1* gene responded to glucose levels, being the lower expression observed at high glucose concentration (Fig. 3d). Additionally, we found that *Eif4B* gene that codify to translation initiation factor 4B were downregulated in PASK Knockdown cells (Fig. 3d). The PASK knockdown blocked the glucose response of *nNos1* genes (Fig. 3d). The expression of *Gapdh* gene was increased at low glucose concentration and the presence of GLP-1 reversed the low glucose effect, and PASK knockdown impaired the glucose effect (Fig. 3e).

PASK-silencing in neuroblastoma N2A cells increases levels of ATP at low glucose concentrations

The reduced *Pask* mRNA levels in silenced N2A cells (Fig. 1d) produced eventual reduction in PASK protein of ~68% (Fig. 4a). Treatment of 16 hours with high glucose concentration (10 mM) or 10 nM GLP-1, reduced PASK protein levels in N2A cells relative to the cultured in 0.5 mM glucose (Fig 4b). Both, glucose and GLP-1 effects were abolished in PASK-silenced cells (Fig 4b).

Cellular ATP content was measured at low and high glucose concentration. The ATP levels were increased at high glucose concentration in neuroblastoma cells. Regarding PASK-silenced cells, at low glucose we observed a level of ATP similar to that observed at high glucose levels in the control cells (Fig. 4c). We then checked the expression of peroxisome proliferator-activated receptor (PPAR) γ co-activator 1α (PGC-1α), which can coordinate both mitochondrial biogenesis and oxidative phosphorylation due to its role as a co-activator of PPAR family of nuclear receptors. PGC-1α expression was not altered in PASK knock-down cells and no effect of glucose or GLP-1 was observed (Fig. 4d).

Impaired effects of Glucose and GLP-1 on AMPK activity and ACC phosphorylation in PASK knockdown neuroblastoma N2A cells

The activation of AMPK in N2A cells, was checked using an anti-phospho-AMPKα (Thr172) after a doses-response treatment of glucose (0.5, 2.5 or 10 mM) (online resource 3). Glucose levels that produced the maximum and minimum activation level (0.5 and 10 mM glucose) were used in subsequent studies. The data show that AMPK activities increased at low glucose concentration, while the presence of GLP-1 significantly decreased the AMPK activity previously stimulated by low glucose concentrations (Fig. 5a, d). The glucose effect on AMPK activity was not significant when *Pask* mRNA expression was inhibited by siRNA targeting *Pask* gene and the GLP-1 effect was lost, while no differences on AMPKα2 protein expression were observed at low glucose in PASK knockdown cells compared to the control cells (Fig 5b, d). However, the level of total AMPKα2 decreased after 4-5 h treatment in the presence of high glucose levels in control neuroblastoma cells, and the glucose effect was lost in PASK-silenced neuroblastoma cells (Fig. 5b, d).

The activation of AMPK is associated with the enhanced phosphorylation of its downstream target acetyl-CoA carboxylase (ACC). The level of ACC phosphorylation followed a similar pattern to AMPK at low glucose levels (Fig. 5c, d). An elevated phospho-ACC level was found at low glucose concentration. GLP-1 treatment decreased phospho-ACC level, and the amount of phospho-ACC significantly decreased at high glucose concentration. PASK-silencing decreased the phospho-ACC level at low glucose concentration; the GLP-1 effect was impaired. However, the glucose effect on phospho-ACC level was still detected in PASK knockdown neuroblastoma cells (Fig. 5c, d).

LKB1 and CaMKKβ expression in PASK knockdown neuroblastoma N2A cells

The expression of the AMPK upstream kinases, liver kinase B1 (LKB1) and the Ca^{2+}-calmodulin-dependent protein kinases (CaMKKβ) were also analyzed. LKB1 expression was lower at high glucose both in PASK-silenced and control neuroblastoma cells (Fig.6a, c). Furthermore, PASK-silenced cells showed a significant reduction in the expression of LKB1 compared to control cells. However, CaMKKβ protein expression was similar under all the conditions studied (Fig. 6b, c). PASK-silenced cells recorded a slight decrease in the amount of CaMKKβ protein (Fig. 6b, c).

Impaired effects of Glucose and GLP-1 on S6K1 pathway activity in PASK knockdown neuroblastoma N2A cells

The activation of S6K1 was detected using anti-phospho-S6K1 (Thr389) after treatment with different glucose concentrations (0.5, 2.5 or 10 mM) shown in online resource 3. Glucose levels that produced the

maximum and minimum activation level were used in subsequent studies. The effect of growth factors on the mTOR/S6K1 pathway is exerted through phosphatidylinositol-3,4,5-triphosphate kinase (PI3K) and further activation of AKT. The activation of Ras/MAPK cascade by mitogens can also activate mTOR. Signaling through this pathway is also regulated by the phosphatase PTEN, which controls the phosphorylation state of phosphatidylinositol-3,4,5-triphosphate (PIP3), PI3K and AKT.

We have checked the effect of certain specific inhibitors of protein kinases or phosphatases that might be involved in the regulation of S6K1 activation in the N2A cell line (Fig. 7). The stimulatory effect of high glucose on S6K1 phosphorylation was impaired in the presence of the specific inhibitors of PI3K and mTOR (Fig. 7a). The inhibitory effect of GLP-1 on S6K1 activity was not detected in the presence of a specific PKA inhibitor (Fig. 7b).

The glucose and GLP-1 effect on the mTOR/S6K1 pathway was then analyzed. S6K1 activation was observed at high glucose and the presence of GLP-1 also significantly decreased S6K1 activation (Fig. 8a, d). PASK knockdown cells showed a complete loss of the capability of the S6K1 to sense nutrient levels and respond to GLP-1 (Fig. 8a, d). The activation state of AKT was detected with anti-phospho AKT (Ser 473). The level of phospho-AKT increased at low glucose concentration, while the presence of GLP-1 decreased AKT activation at low glucose (Fig. 8b, d). PASK-silencing decreased the stimulatory effect of low glucose, although AKT activation was still modulated by glucose and GLP-1 in PASK knockdown cells (Fig 8b, d).

The expression of phosphatase PTEN was similar in all the conditions analyzed (Fig 8c, d).

Finally, we have checked the effect of AMPK activator AICAR in PASK knockdown cells. The presence of AICAR activated AMPK in both Pask-deficient and control cells. These effects on AMPK activation, correlated with decreased level of phospho-S6K1 (data not shown).

DISCUSSION

PASK plays different physiological functions in different tissues. Thus, PASK has been considered a nutrient sensor whose expression is upregulated by a high glucose concentration in Min-6 cells [14]. On the other hand, no evidence of PASK regulation by glucose has been observed in pancreatic beta-cells or testicular cell lines from PASKIN knockout and wild-type mice [15]. Further investigation has indicated that PASK-deficient mice have a metabolic phenotype [17] similar to that described in S6K1-deficient mice; in both cases, those mice were protected against obesity [26,27].

We have recently reported that both AMPK and S6K1 activities respond to changes in glucose concentrations in neuroblastoma cells. Low glucose concentrations produced a rapid activation of AMPK. However, S6K1 was activated at high glucose concentrations in N2A cells, and the presence of anorexigenic peptide GLP-1 significantly decreased both AMPK and S6K1 activation. The observed response to glucose and GLP-1 is similar to that found in the rat hypothalamic areas involved in feeding behavior [22]. We contend here that PASK protein is also a nutrient sensor in neuroblastoma cells. Our data indicated that the *Pask* gene is upregulated by low glucose in neuroblastoma cells, and this effect was reversed in the presence of GLP-1.

The observed changes in mRNA coding to PASK were consistent with the changes in PASK protein. Thus, cells exposed to high glucose or to GLP-1 for more than 8 h significantly decreased the amount of PASK protein.

Hypothalamic metabolic sensors play an important role in feeding behavior, bodyweight homeostasis and energy balance. Nutrients, orexigenic and anorexigenic peptides, and the signals that report on the status of energy stores may coordinate the activity of the metabolic sensors regulating food intake.

Here, the response of *Pask* to nutrients and gut peptides was observed not only in neuroblastoma cells but also in VMH and LH on rat hypothalamic slices. Both areas are involved in feeding behavior and energy homeostasis: VMH and LH are considered the satiety and hunger centers, respectively. Rat hypothalamic slices have previously been reported to preserve some tissue architecture and functional connections in order to maintain a selective c-Fos expression in response to different glucose concentrations [25] similar to that found in vivo [28]. We have also reported that glucose and GLP-1 regulate *Ampkα2* gene expression and both AMPK and S6K1 activity in these areas [22]. A similar approach has also been used to analyze neuropeptide expression [29]. In this regard, we show here that higher glucose concentration and GLP-1 treatment downregulate *Pask* mRNA expression in VMH and LH. However, The GLP1 effect at a high glucose concentration differed in both areas: GLP-1 treatment reversed the effect of high glucose specifically in LH, but not in VMH. A distinctive pattern of gene regulation in response to glucose and neuropeptides has also been previously reported in VMH and LH, including the expression of the GLP-1 receptor (GLP-1R) in these areas [22,30]. The differences in the regulation of *Glp-1r* gene expression by glucose and neuropeptides in these areas might explain our results [30]. Our data show that the *Pask* gene is regulated by glucose and GLP-1, an anorexigenic peptide whose level increases after a meal, informing

the central nervous system of nutrient status. This suggests that PASK may be involved in the control of food intake.

Another interesting finding was that the knockdown of more than 75 % *Pask* mRNA is sufficient to impair the response to glucose of the AMPK and mTOR/S6K1 pathways in neuroblastoma cells. However, previous data indicated that the activation of AMPK or mTOR signaling pathways was not dependent on the PASK function [17]. There are two possible explanations: first, the differences may be due to the experimental method used, since we analyzed the response of the metabolic sensors at low and high glucose levels when activation/inhibition was maximal; second, this response may be cell or tissue specific. Nonetheless, the effect mediated by PASK in these pathways was mainly at posttranscriptional level. Thus, PASK knockdown in N2A cells did not significantly modify the expression of gene coding to mTOR and S6K1. Nevertheless, a tendency to decrease the levels of gene coding to mTOR, AKT1, S6K1, eEF-2K, PDK1, GADPH, nNOS and eIF-4B was observed in PASK-silenced cells. Therefore, the assumption that the total knockout of PASK expression could change the expression of these genes cannot be ruled out. However, the expression of mRNA coding to LKB1 decreased in PASK-silenced cells. Unfortunately, we could not measure the expression of the mRNA coding to AMPKα2 in N2A cells using two different probes. This suggests the presence of point mutations, although we have not checked the sequence in these cells. However, AMPKα2 protein was detected by western blot, and a similar amount of AMPKα2 was found at low glucose levels in PASK knockdown and control cells. Nevertheless, previous studies have reported that the level of AMPKα2 decreased after the prolonged presence of elevated glucose [31,32,22]. Here, we confirm this fact in control neuroblastoma cells and also show that the glucose effect was impaired after PASK-silencing.

Previous data have reported the role of ATP levels in stimulating AMPK in neurons and the regulation of neuropeptides that modulate feeding behaviour [29]. Therefore, the activation of AMPK at low energy states correlates with increases in the AMP/ATP ratio. Previous studies have affirmed that PASK increased oxidative metabolism and ATP generation in PASK knockdown L6 myoblast cell lines [17]. However, no differences were found in cellular ATP production in fibroblasts from PASKIN knockout mice [19]. Our data indicate that elevated levels of glucose and ATP in N2A cells corresponded to the lowest level of AMPK activation, while lower levels of ATP are associated with greater AMPK activation. By contrast, the presence of low glucose levels in PASK-deficient cells correlated with the enhanced concentration of ATP and the absence of AMPK activation.

AMPK activation is also regulated by several upstream kinases. In mammals, the two main kinases are LKB1 and CaMKKβ [33,5]. Thus, the low expression of the mRNA coding to LKB1 at a low glucose level in PASK knockdown cells also explains the lack of AMPK activation. Similar data were found for LKB1-deficient muscle [34] or liver [35]. Our data show that the gene coding to LKB1 is regulated by glucose and GLP-1 in neuroblastoma cells. Low glucose increased the mRNA coding to LKB1. Nevertheless, that effect was lost in PASK-silenced cells. In contrast, previous results suggested a constitutive activation of

LKB1 in cell lines and skeletal and cardiac muscle, as reviewed by [36]. Subsequent studies will be needed to clarify whether this finding is characteristic for this cell line.

We have previously reported that AMPK stimulation at low glucose levels was not inhibited by STO-699, a CaMKK inhibitor in N2A cells [22]. Here, we show a slight decrease in the CaMKKβ protein expression in PASK knockdown regarding the control cells. Taking into account the effects on ATP concentrations, LKB1 and CaMKKβ, our data would explain the lack of activation in PASK-deficient cells.

AMPK plays a key role in glucose and lipid metabolism. AMPK thus phosphorylates and inhibits ACC, whose activation prevented the entry into and oxidation of fatty acids in mitochondria. AMPK activation in the hypothalamus promoted by starvation induces ACC inactivation and a subsequent decrease in malonyl-CoA, and also stimulates fatty acid oxidation. Furthermore, it has been reported that ghrelin stimulates AMPK and other signaling pathways promoting the inhibition of lipogenesis and increasing fatty acid oxidation leading to changes in mitochondrial respiration, enhancing the production of reactive oxygen species (ROS) and activating NPY/AgRP neurons [37,38].

Our data confirm that at a high glucose concentration, and besides AMPK inhibition, the amount of phospho-ACC decreased sharply. The presence of GLP-1 did not change this result, according to previous data showing that the action of GLP-1 depends on the maximal activation of AMPK [22]. The level of AMPK activation in PASK-deficient cells at a low glucose concentration was similar to that found in control cells at high glucose according to the levels of ATP. In these PASK deficient cells, an increase in glucose concentration did not significantly lower the level of phospho-AMPK, and phospho-ACC levels were almost undetectable. The differences in the levels of phospho-ACC and phospho-AMPK at high and low glucose concentration may be explained by differences in the localization of these proteins. Thus, the ACC is located exclusively in the cytoplasm, but its phosphorylation depends on the presence of AMPK. However, AMPKα2 may move between the nucleus and the cytoplasm in agreement with previous reports indicating that low energy and increased oxidative stress concentrated AMPK in the nucleus [39]. It cannot be dismissed that the location of AMPKα2 could be affected in PASK-deficient cells.

The lack of AMPK activation at low glucose concentrations was accompanied by an enhanced activation of S6K1. Our data indicated that the specific inhibitors of PI3K and mTOR in these cells impaired the effect of high glucose levels on S6K1 activation. We have therefore checked the activation level of AKT, as well as PTEN protein, at low and high glucose levels. Our data showed that N2A cells exposed to low glucose levels recorded the following: AMPK was activated, AKT was highly activated and S6K1 was inhibited. GLP-1 treatment decreased both AMPK and AKT activity at a low glucose concentration. In contrast, PASK knockdown impaired AMPK activation at a low glucose concentration, and this effect could also explain the full activation of S6K1, while AKT was still activated in the absence of GLP-1 treatment, although the activation level was significantly lower than in the control cells. It has been reported that glucose starvation enhances AKT and GSK3β activity mediated through AMPK activation in both cardiac and skeletal myocytes and in primary skeletal myofilaments [40]. AMPK also phosphorylates the TSC2 inhibiting mTOR/S6K1 pathway. Under a glucose deficit, the feedback inhibition of this pathway mediated

by S6K1 was not active, and the mTORC2 was highly activated by glucose starvation. This pathway will provide survival signals for these cells [40]. Additionally, S6K1 should exert a feedback inhibitory effect on this pathway by phosphorylating the insulin receptor substrate (IRS) [41].

The contra-regulatory effect of AMPK on mTOR/S6K1 pathway was confirmed in *Pask*-silencend cells. As well as in control cells, AICAR treatment decreased S6K1 activity. AKT activation also regulates the phosphatase activity of PTEN, which controls the phosphorylation state of intermediaries of this pathway. AKT activation stimulates PTEN activity producing a negative feedback effect [42,43]. The level of PTEN expression was not modified in PASK-silenced neuroblastoma cells. Our results showed that AKT activation was lower after PASK-silencing. Nevertheless, the activation was still significantly higher than at high glucose levels, suggesting that survival pathways may still be functional, and other signaling proteins besides AMPK may regulate this process in neuroblastoma cells.

In conclusion, our data indicated that PASK is a nutrient and hormonal sensor in neuroblastoma cells and in VMH and LH from rat hypothalamic slices. Low glucose levels upregulated PASK in neuroblastoma cells. In these cells, at least, there is a coordinated response with the other metabolic sensors. Thus, the knockdown of PASK impaired not only the response of the AMPK and mTOR/S6K1 pathways at low and high glucose levels, but also some of the GLP-1 effects observed at the level of both gene expression and AMPK and S6K1 activity (Fig. 9). PASK-deficient N2A cells failed to detect the availability of nutrients. The metabolic sensors in the brain have been directly related to feeding behavior, bodyweight and energy homeostasis. Our data suggest that PASK might also play an important role in feeding behavior. PASK-deficient mice had a phenotype resistant to diet-induced obesity. It would be interesting to analyze the state of the AMPK and mTOR/S6K1 pathways in these mice

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LEGENDS TO FIGURES

Fig. 1 **PASK is expressed in the rat hypothalamus and neuroblastoma cells. *Pask* mRNA expression is regulated by glucose and siRNA targeting *Pask* gene. a)** In situ hybridization to detect the expression of *Pask* mRNA in the hypothalamus. The photomicrographs are of 20 µm sections of rat brain showing the 3rd ventricle (3V) and the hemi-hypothalamus. The sections were processed for in situ hybridization with a digoxigenin labeled Pask RNA antisense probe (upper panels) or sense probe (bottom panels). The right panels show a higher magnification of the squared area corresponding to the LH. The photomicrographs correspond to two independent experiments. **b)** Immunocytochemistry of PASK in N2A cells. N2A fixed cells were incubated in the presence of either an anti-PASK antibody or a preimmune serum (immunostaining negative control). PASK location was visualized in red (Texas Red coupled to a secondary antibody). Nuclei were stained with DAPI (blue fluorescence). **c)** *Pask* mRNA levels in N2A cells treated for 3 h in a medium that contained 0.5 or 10 mM glucose, quantified by real time RT-PCR and normalized to *β-actin* mRNA levels. The bars represent the mean ± SEM of 5 experiments performed in duplicate. The values are relative to the data obtained at 0.5 mM glucose that was considered as 1. **P<0.01 10 mM glucose vs. 0.5 mM glucose treatment**. d)** Inhibition of *Pask* mRNA in PASK-silenced neuroblastoma N2A cells. Bars represent the mean ± SEM (n = 5) of *Pask* mRNA levels normalized to *β-actin* in cells transfected with siRNA targeting *Pask* gene (PASK siRNA) or negative control siRNA (negative control siRNA). The values are relative to the data obtained in cells transfected with negative control siRNA that was considered as 1. ***P<0.001 PASK siRNA vs. negative control siRNA.

Fig. 2 **Glucose and GLP-1 modulate *Pask* gene expression in the VMH and LH areas.** Organotypic hypothalamic slices of 300 µm were glucose-starved for 2 h and then cultured for 3 h in a medium containing 1, 2.5 or 10 mmol/l glucose in the presence or absence of 10 nM GLP-1. The VMH and LH areas were dissected from slices by micropunching. **a)** RT-PCR analysis of *Sf-1*, *Prepro-orexin* and *β-actin* mRNA expression in the VMH and LH. **b, c)** *Pask* mRNA was quantified by real-time RT-PCR analysis. Bars represent *Pask* mRNA levels normalized by RNA *18s* and referred to the value obtained under the 2.5 mM-glucose condition in the absence of GLP-1, considered as 1. Data are expressed as means ± SEM; n = 4-5 independent experiments performed in duplicate.*P<0.05, ***P<0.001 10 mM glucose vs. 2.5 mM glucose; †P<0.05, ††P<0.01, ††† P<0.001 absence of GLP-1 vs. the presence of 10 nM GLP-1.

Fig. 3 **Effects of PASK knockdown on the expression of several genes in neuroblastoma cells**. PASK knockdown N2A and control cells were serum-starved for 2 h and then incubated in a medium containing either 0.5 mM or 10 mM of glucose for 3 h in the presence or absence of 10 nM GLP-1. Quantity real-time PCR was used to analyze the expression of several genes as shown in Online Resource 1. The mRNA

levels of the different genes were normalized by mRNA of *β-actin* used as housekeeping gene. The value obtained at 0.5 mM glucose without GLP-1 was taken as 1. Results are means ± SEM; n = 4-5. *P<0.05, **P<0.01, ***P<0.001 10 mM glucose vs. 0.5 mM glucose; ††P<0.01, †††P<0.001 absence of GLP-1 vs. the presence of 10 nM GLP-1; #P<0.05, ##P<0.01 PASK siRNA vs. negative control siRNA.

Fig. 4 **Effects of glucose and GLP-1 on the expression of PASK, PGC-1α and on the ATP content in *Pask* gene knockdown neuroblastoma cells.** PASK knockdown N2A (PASK siRNA) and control cells (negative control siRNA) were serum-starved for 2 h and then incubated in a medium containing either 0.5 mM or 10 mM of glucose in the presence or absence of 10 nM GLP-1 for 5, 16 h. Neuroblastoma cells were immediately lysed in RIPA buffer and exposed to microwave irradiation for 5 s, after which they were processed for western blot analysis. The blots were reprobed for β-ACTIN to normalize the results **(a, b, d). a)** Inhibition of PASK protein expression in PASK knockdown cells. Immunoblot analysis of PASK. Bar graphs represent the densitometric values, normalized by β-ACTIN. The results are means ± SEM; n = 3-4. **b, d)** Glucose and GLP-1 effect in PASK and PGC-1α expression. Immunoblot analysis of PASK (16 h) **(b)** or PGC-1α (5 h) **(d).** The value of negative control siRNA at 0.5 mM glucose without GLP-1 was taken as 1. ***P<0.001 10 mM glucose vs. 0.5 mM glucose; ††P<0.01 absence of GLP-1 vs. the presence of 10 nM GLP-1; ###P<0.001 PASK siRNA vs. negative control siRNA. The bottom panels correspond to representative western blots of the data indicated in the graphs **(a, b, d). c**) Cellular ATP content normalized by mg of total protein in cell extracts treated for 2 hours with different glucose concentrations. Bars represent the means ± SEM of 5 experiments developed in duplicate. The results are expressed as a percentage of negative control siRNA at 0.5 mM glucose that was considered 100 % *P<0.05 10 mM glucose vs. 0.5 mM glucose; #P<0.05 PASK siRNA vs. negative control siRNA.

Fig. 5 **Impaired effects of glucose and GLP-1 on the expression and activation of AMPK and ACC in *Pask* gene knockdown neuroblastoma cells.** PASK knockdown N2A (PASK siRNA) and control cells (negative control siRNA) were serum-starved for 2 h and then incubated in a medium containing either 0.5 mM or 10 mM of glucose for 2 h with the presence of 10 nM during the last 10 min, in the case of activity determination (**a, c** and **d**). On the other hand, total protein valuation was performed in cells incubated with different glucose concentrations and in the presence or absence of 10 nM GLP-1 for 5 h (**b**). Neuroblastoma cells were immediately lysed in a RIPA buffer and exposed to microwave irradiation for 5 s, after which they were processed for western blot analysis of phospho-AMPK (Thr-172) (P-AMPK) (**a, d**) and total AMPK (AMPK) (**b, d**) and of phospho-ACC (Ser-79) (P-ACC) and total ACC (ACC) (**c, d**). The blots were reprobed for β-ACTIN to normalize the results. Bar graphs represent means ± SEM; n = 3-4 of the densitometric values, normalized by β-ACTIN and by non-phosphorylated forms. Panel **d**) shows representative western blots of the graphs **a**, **b** and **c**, respectively. The value of negative control siRNA at 0.5 mM glucose without GLP-1 was taken as 1. **P<0.01, ***P<0.001 10 mM glucose vs. 0.5 mM

glucose; ††P<0.01,††† P<0.01 absence of GLP-1 vs. the presence of 10 nM GLP-1; #P<0.05, ##P<0.01, ###P<0.001 PASK siRNA vs. negative control siRNA.

Fig. 6 **Impaired effects of glucose and GLP-1 on the expression of LKB1 and CaMKKβ in *Pask* gene knockdown neuroblastoma cells**. PASK knockdown N2A (PASK siRNA) and control cells (negative control siRNA) were serum-starved for 2 h and then incubated in a medium containing either 0.5 mM or 10 mM of glucose for 5 h. In some cases, 10 nM GLP-1 was added during the incubation. Neuroblastoma cells were immediately lysed in a RIPA buffer and exposed to microwave irradiation for 5 s, after which they were processed for western blot analysis of LKB1 (LKB1) (**a, c**) and CaMKKβ (CaMKKβ) (**b, c**). The blots were reprobed for β-ACTIN. Bar graphs represent the densitometric values, normalized by β-ACTIN. The results are means ± SEM; n = 3-4. Panel **c**) shows representative western blots of graphs (**a**) and (**b**), respectively. The value of negative control siRNA at 0.5 mM glucose without GLP-1 was taken as 1. **P<0.01 10 mM glucose vs. 0.5 mM glucose; #P<0.05; ##P<0.01 PASK siRNA vs. negative control siRNA

Fig. 7 **Impact of several protein kinases and phosphatase inhibitors on the GLP-1-induced inhibition of S6K1 activity at high glucose concentration**. N2A cells were incubated in the presence of 10 mM glucose for 4 h, treated or not with several inhibitors for 30 minutes (Table 1), in the absence (**a**) or presence (**b**) of 10 nM GLP-1 over the last 10 min. Cell lysates were exposed to microwave irradiation for 5 s and then processed for western blot analysis of phospho-S6K1 (Thr389) (P-S6K1) and total S6K1 (S6K1) (**a, b**). Densitometric values were normalized by β-ACTIN and by non-phosphorylated forms. **a)** The value of 10 mM glucose without inhibitors was taken as 1 (C). The results are means ± SEM; n = 3-4. **P<0.01, 10 mM glucose compared with 0.5 mM glucose. **b)** The value obtained in the cells treated with 10 mM glucose and 10 nM GLP-1 without inhibitors (C) was taken as 1. The results are means ± SEM; n = 3-4. *P<0.05, **P<0.01, ***P<0.001 vs. without inhibitors.

Fig. 8 **Impaired effects of glucose and GLP-1 on the activity of S6K1 and AKT, and on the expression of PTEN, in *Pask* gene knockdown neuroblastoma cells**. PASK knockdown N2A (PASK siRNA) and control cells (negative control siRNA) were serum-starved for 2 h and then incubated in a medium containing either 0.5 mM or 10 mM of glucose for 2 h with 10 nM during the last 10 min, in the case of activity determination (**a, b** and **d**). On the other hand, total protein valuation was performed in cells incubated with different glucose concentrations and in the presence or absence of 10 nM GLP-1 for 5 h (**c** and **d**). Neuroblastoma cells were immediately lysed in a RIPA buffer and exposed to microwave irradiation for 5 s, after which they were processed for western blot analysis of phospho-S6K1 (Thr389) (P-S6K1) and total S6K1 (S6K1) (**a, d**); of phospho AKT (Ser 473) (P-AKT) and total AKT (AKT) (**b, d**) and

of total PTEN (PTEN). The blots were reprobed for β-ACTIN. Panel d) shows the representative western blots of graphs **a**), **b**) and **d**), respectively. Bar graphs represent the densitometric values, normalized by β-ACTIN and by non-phosphorylated forms. The results are means ± SEM; n = 3-4. The value of negative control siRNA at 0.5 mM glucose without GLP-1 was taken as 1. ***P<0.001 10 mM glucose vs. 0.5 mM glucose; †P<0.05, ††P<0.01, †††P<0.001 absence of GLP-1 vs. the presence of 10 nM GLP-1; ##P<0.01 and ###P<0.001 PASK siRNA vs. negative control siRNA.

Fig. 9 **Representative diagram of glucose and GLP-1 effect on the activity of AMPK and S6K-1.**

The activation of AMPK depends on the glucose levels. **a)** Fall in glucose (dark rows and shapes) leads to an increased transcription of *Lkb1* and AMP/ATP ratio. The elevation of AMP/ATP ratio activates AMPK by phosphorylation. AMPK inactivation is mediated by the action of phosphatases such as PP2B. AMPK activation promotes inactivation of ACC and mTORC1, preventing the activation of S6K1. GLP-1 stimulates PKA protein and exerts some effects in a PASK dependent way: (1) Stimulation of PP2B promoting the inactivation of AMPK and (2) inactivation of S6K1. **b)** Fall glucose in PASK-deficient cells, leads to a decreased expression of *Lkb1* and AMP/ATP ratio and, consequently, inactivation of AMPK and activation of ACC, mTORC2, and S6K1.

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Table 1. Inhibitors of protein kinases and phosphatases

Inhibitors		Manufacturer	Concentration
PKA inhibitor	KT5720	Santa Cruz Biotechnology, California, USA	200 nM
PKC inhibitor	Ro-318220	Bionova Científica, Madrid, Spain	10 mM
PI3K inhibitor	LY294002	Bionova Científica, Madrid, Spain	10 mM
MEK inhibitor	PD 98059	Calbiochem, Darmstadt, Alemania	2 mM
mTOR inhibitor	Rapamycin	Santa Cruz Biotechnology, California, USA	100 nM
Phosphatases inhibitors: PP2B and PP1	Okadaic Acid Sodium Salt	Santa Cruz Biotechnology, California, USA	10 mM 20 nM
CaMK inhibitor	KN62	Santa Cruz Biotechnology, California, USA	3 mM
CaMKK inhibitor	STO-609	Sigma Aldrich, Madrid, Spain	5 mM

Table 2
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Table 2. Antibodies and conditions used for western blot assays

Antibody	Host	Manufacturer	Dilution used
Anti-ACC	Rabbit	Millipore Iberica, Madrid, Spain	1:1000
Anti-phospho-ACC(Ser 79)	Rabbit	Millipore Iberica, Madrid, Spain	1:500
Anti-AKT	Rabbit	Cell Signaling, Danvers, MA, USA	1:1000
Anti-phospho-AKT(Ser 473)	Mouse	Millipore Iberica, Madrid, Spain	1:1000
Anti-AMPKα	Rabbit	Cell Signaling, Danvers, MA, USA	1:1000
Anti-phospho-AMPKα(Thr172)	Rabbit	Cell Signaling, Danvers, MA, USA	1:1000
Anti-β-ACTIN	Mouse	Sigma-Aldrich, Saint Louis, Missouri, USA	1:5000
Anti CaMKKβ	Mouse	Santa Cruz Biotechnology, California, USA	1:1000
Anti LKB-1	Mouse	Abcam, Cambridge,UK	1:100
Anti-PGC-1α	Mouse	Millipore Iberica, Madrid, Spain	1:1000
Anti-S6K1	Rabbit	Cell Signaling, Danvers, MA, USA	1:1000
Anti-phospho-S6K1(Thr389)	Rabbit	Cell Signaling, Danvers, MA, USA	1:1000
Anti-PASK	Rabbit	Cell Signaling, Danvers, MA, USA	1:1000
Anti-PTEN	Rabbit	Millipore Iberica, Madrid, Spain	1:1000
Anti-Rabbit-HRP	Goat	Millipore Iberica, Madrid, Spain	1:5000
Anti-Mouse-HRP	Goat	Bethyl Laboratories, Montgomery, USA	1:5000

Figure 1
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Fig. 1

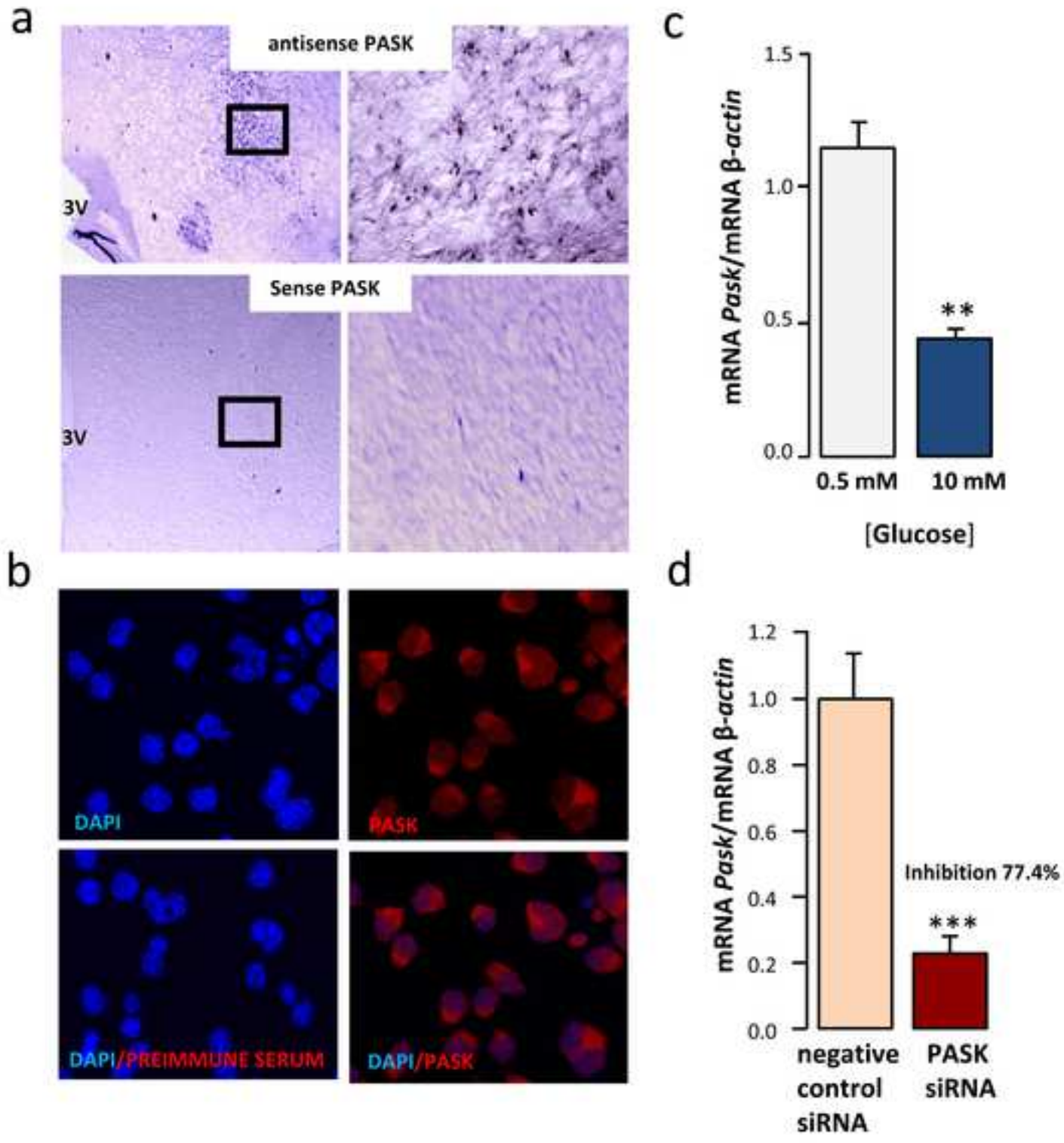

Figure 2
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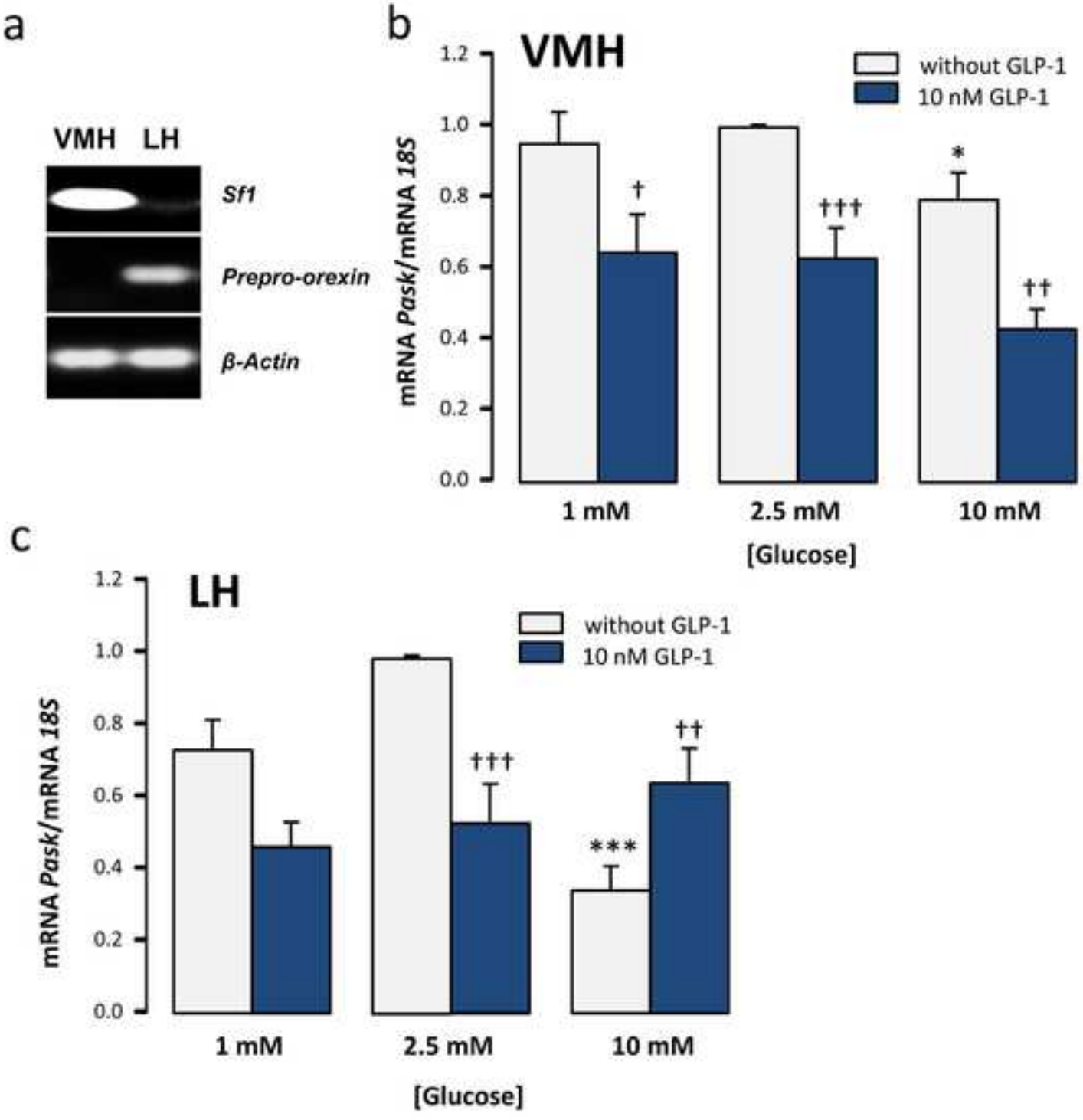

Figure 3

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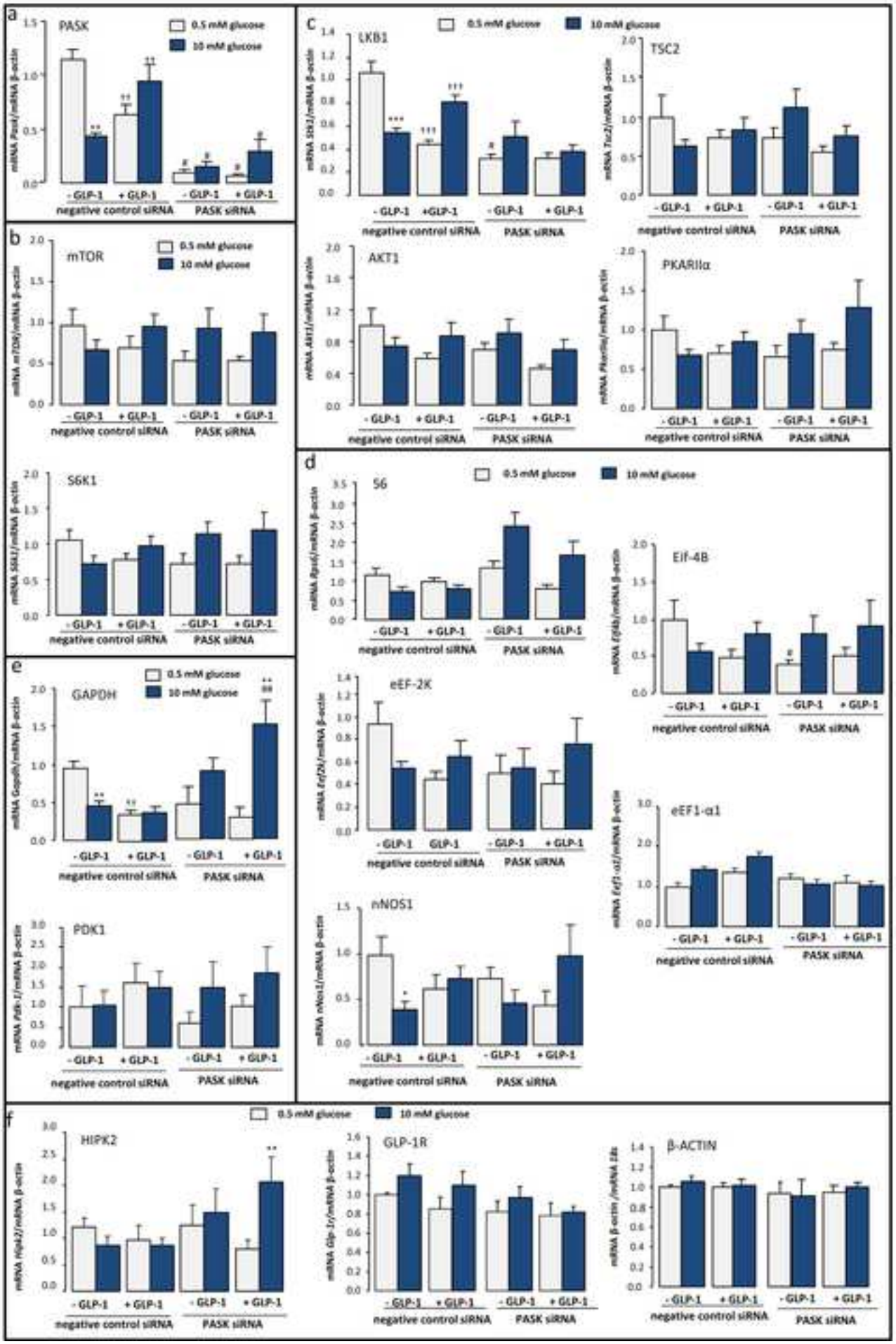

Figure 4

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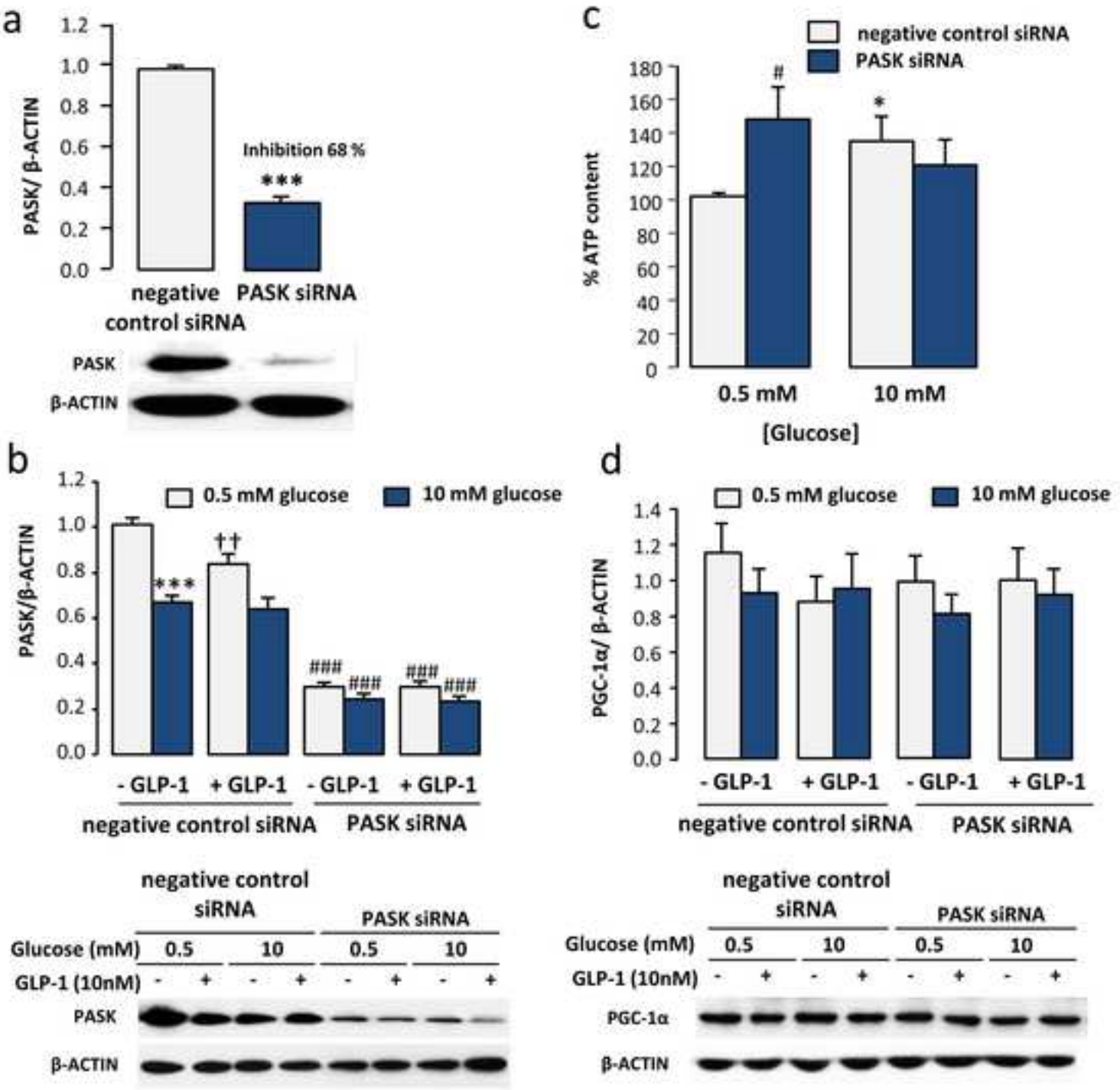

Figure 5

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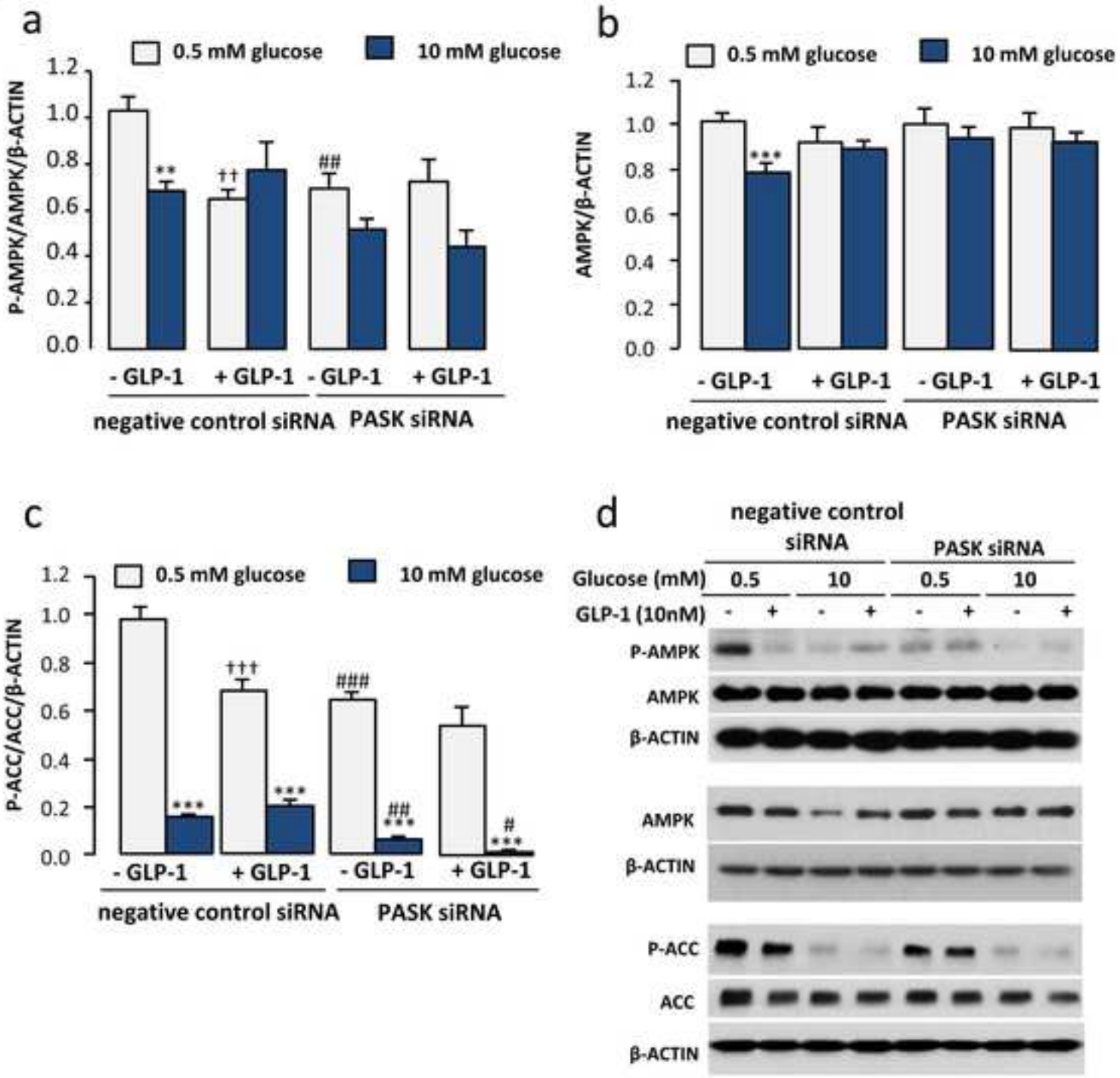

Figure 6

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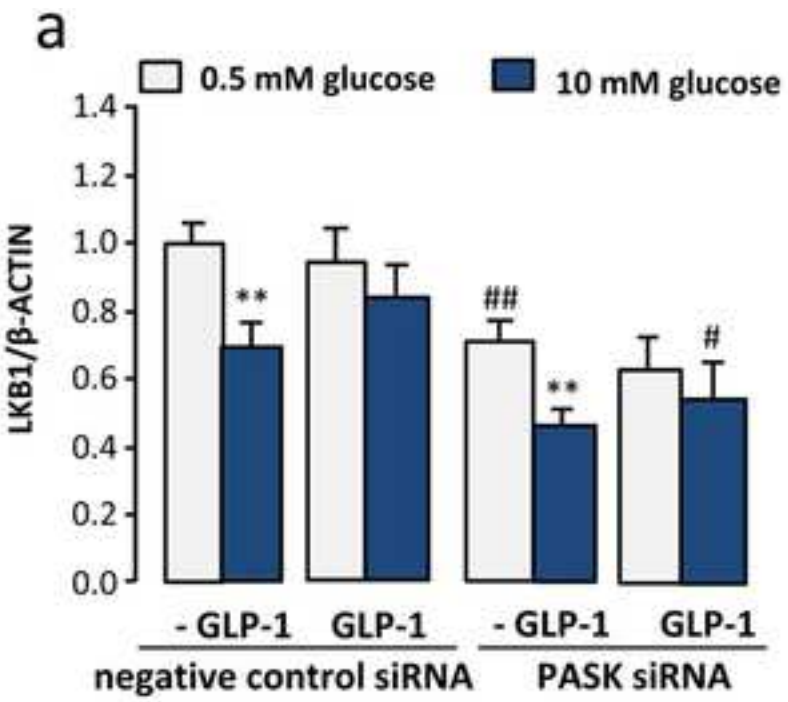

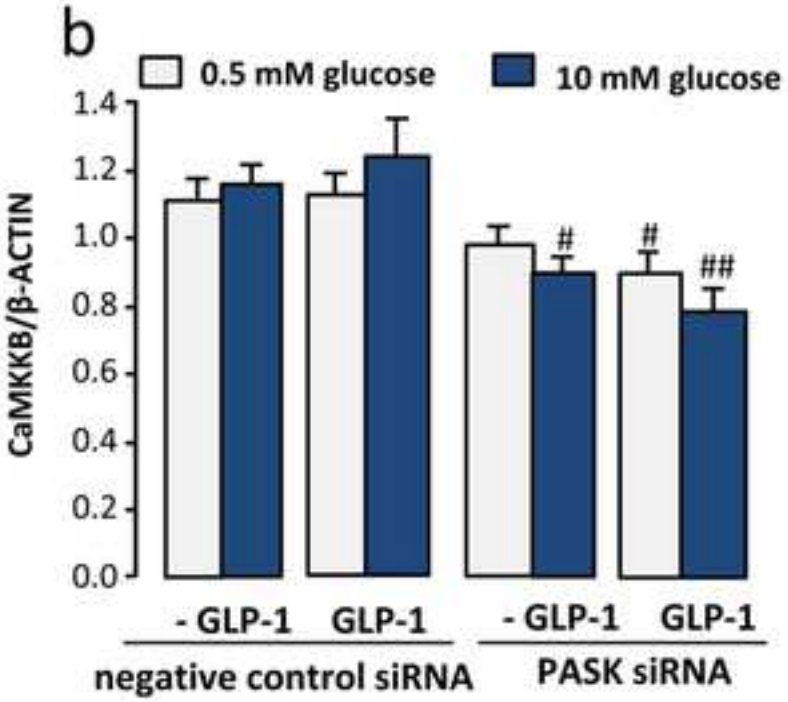

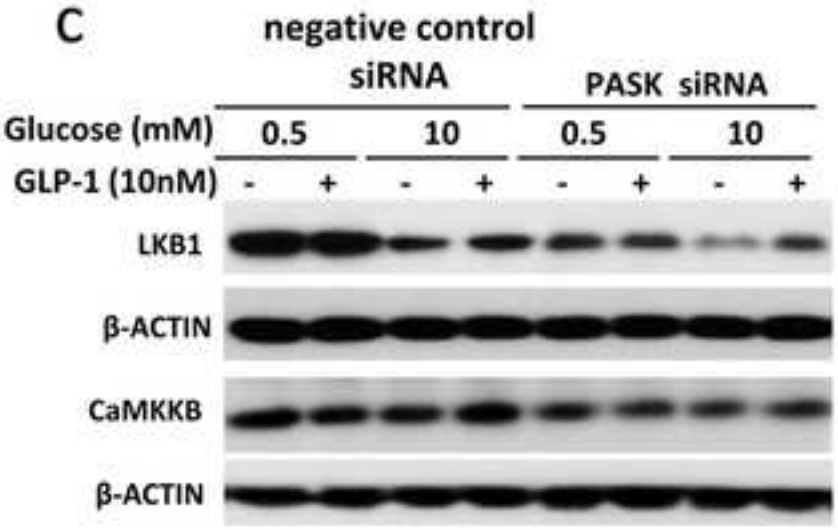

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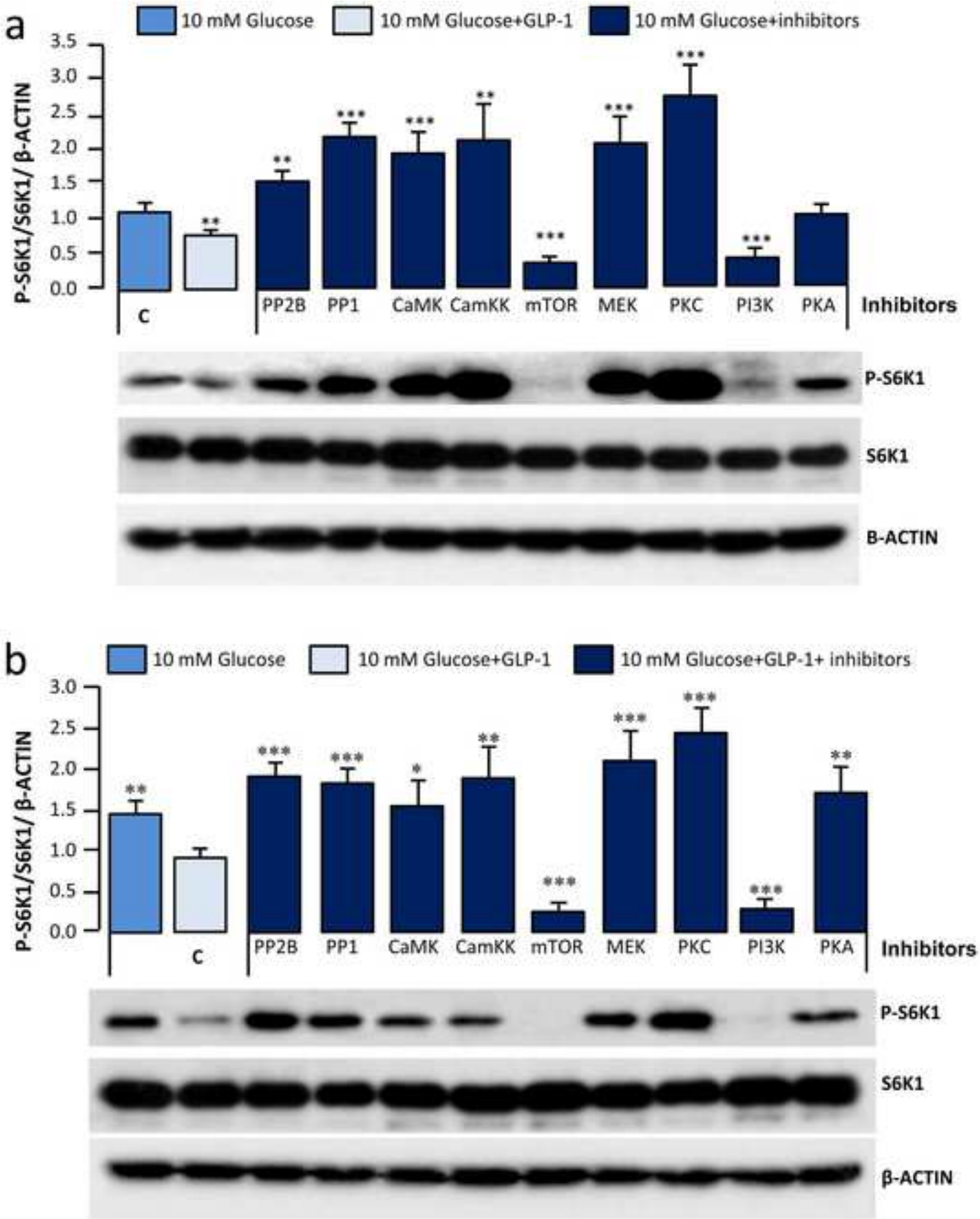

Figure 8

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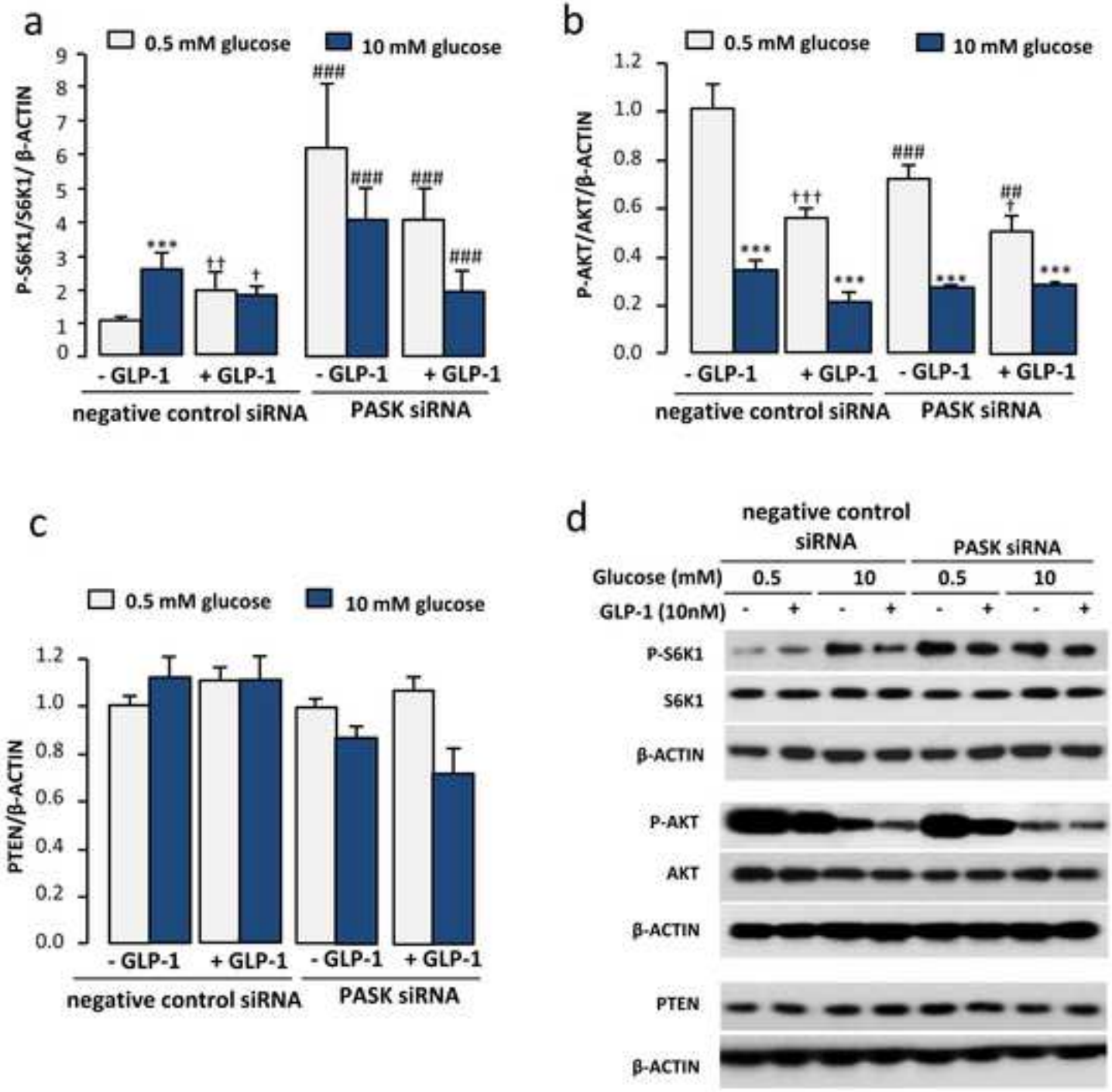

Figure 9
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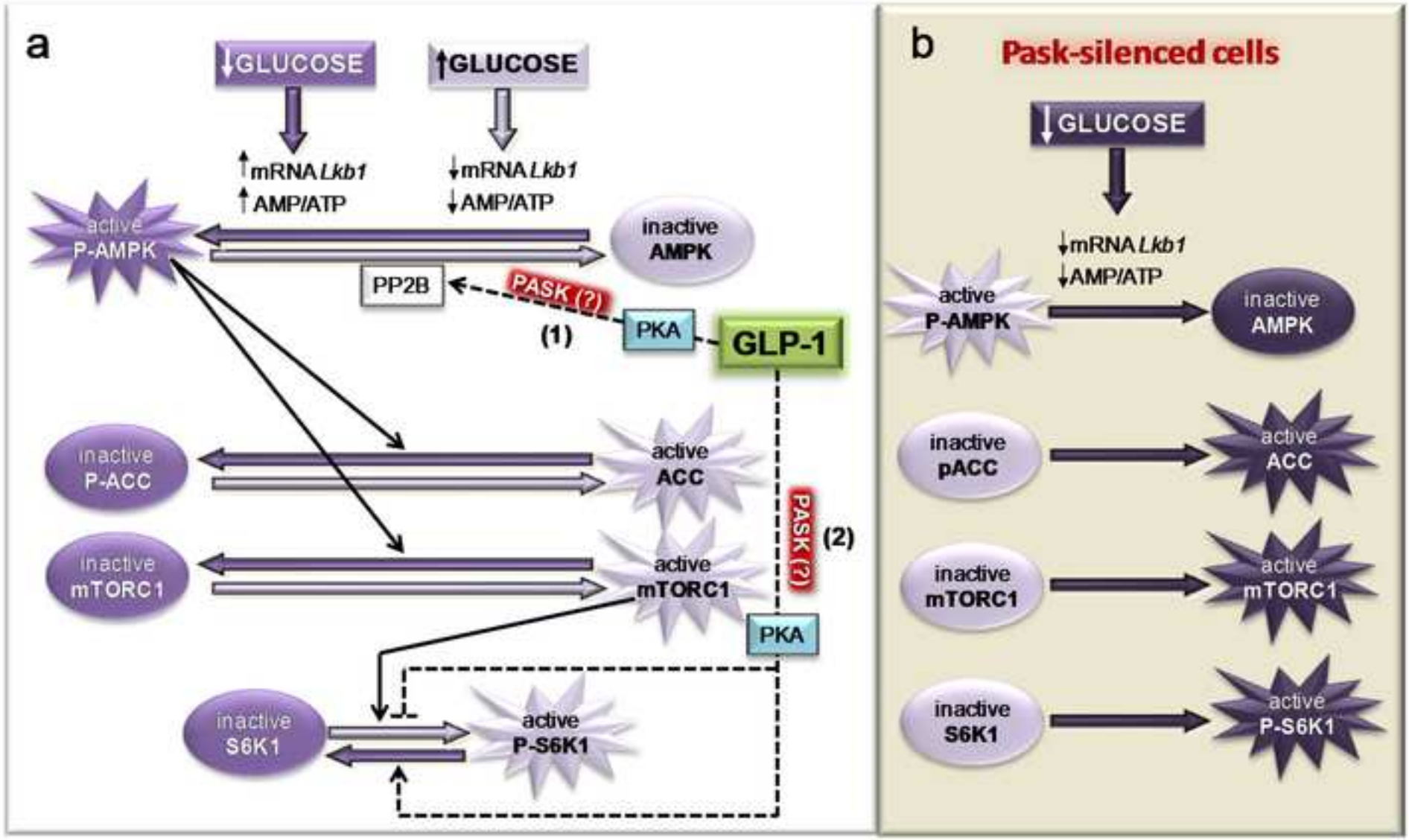

Supplementary Material 1

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Supplementary Material 2

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Supplementary Material 3

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ANSWER TO THE REVIEWERS

Reviewer #2: Molecular Neurobiology PAS Kinase as a nutrient sensor in neuroblastoma and hypothalamic cells required for the normal expression and activity of other cellular nutrient and energy sensors. Hurtado-Carneiro et al.

This manuscript describes the effect of PASK expression on transcription and signaling on other nutrient sensing proteins and pathways. This manuscript offers novelty in the sense that it is one of the first studies of PASK function in neuronal cell lines or tissues. However, it suffers from several key limitations which are needed to be addressed.

General comment: The combination of nutrient (glucose) and hormone (GLP-1) treatment is resulting in confusing transcription and signaling outcome. For example, while PASK expression is lowered by high glucose, the combination of high glucose and GLP-1 actually stimulates increase in PASK expression. It appears that in high glucose condition, GLP-1 may actually rescue PASK expression. For clarity, it is recommended that author draw a model that integrates their findings from two different signaling molecules in regulating PASK expression and its transcription and signaling outcome.

Taking into account your suggestion, we have incorporated a scheme named figure 9

1. The studies involving siRNA against PASK described in this manuscript appears to have used only one targeting sequence. It is generally accepted to use multiple siRNA targeted at different sequences to validate biological phenomenon. We recommend authors to test different PASK targeting siRNA to match extent of knockdown with expression profiles. I also think authors should provide the siRNA targeting sequence in their material and methods. This is a key deficiency in the manuscript and must be addressed.

The details of the sequence of the siRNA have been included in “material and methods” of the revised manuscript. The target sequence is located in exon 10 of the kinase domain. The inhibition achieved in mRNA and protein content of PASK (77% and 68% respectively) is according to the optimal efficiency indicated by the manufacturer (Ambion). Different siRNAs concentrations and times were checked in order to validate the siRNA. The optimal conditions (0.15 µg of siRNA transfected during 6h and 24 hours of siRNA inhibition) are used in this work.

Others authors have used a single siRNA target in this kind of studies (da Silva et al, 2004. Proc Natl Acad Sci USA 101:8319-8324 and da Silva et al, 2001 Diabetologia 54: 819–827)

The reduction of 68% of PASK protein that we observed in these cells caused significant differences in the levels of LKB1, ATP and in the level of activation of AMPK and S6K1. Our data can not rule out that the complete loss of PASK could increase some of these effects. Anyway it does not alter the conclusions of this work. In this sense, we are therefore studying *Pask*-deficient mice, in order to answer those questions.

2. Figure 3: i. While authors suggest that the levels of mTOR, GAPDH, TSC2, PDK-1 are not changed upon PASK knock down, the data definitely suggest that the levels of these genes are lowered in PASK knock down samples in both low glucose alone as well as low glucose + GLP-1. While the decrease in the levels may not be as significant as it is with LKB1, the trend definitely indicates reduction in the mRNA levels of mTOR, GAPDH, TSC2 and PDK-1. It is for these reasons important to use multiple siRNA to clean up data and obtain more robust expression pattern.

Thank you for your advice. At this point, it is out of our reach to use multiple siRNAs for all experimental procedures in this work. However, we have increased the number of experiments and introduced new data, in order to minimize the error. We have reduced the errors in the data of Fig 3. However, no significant differences were found in the mRNA levels of mTOR, TSC2, PDK1 and S6K1 compared to the control and the PASK siRNA. Figure 3 has been modified in the revised manuscript.

ii. Authors claim that nNOS1 is significantly changed by PASK knockdown. However data does not support that. Please correct the statement.

Thank you for your observation. nNOS1 expression was decreased by high glucose and not affected by PASK knockdown. The error was corrected in the revised manuscript.

3. Figure 4. i. Panel b. PASK protein levels do not show any decrease upon shift to high glucose media in negative control samples. Even in PASK siRNA treated cells, one would expect further reduction in PASK protein levels upon glucose stimulation. In absence of any significant effect on protein levels by high glucose stimulation, what is the biological significance of lower PASK mRNA levels? This is a key drawback of this manuscript and significant inconsistency. An explanation or optimization of the condition to examine protein levels of PASK must be made.

The effect of glucose on PASK protein levels was initially analyzed after 4-5 h treatment in the presence of different glucose concentrations. We agree with you that this time of treatment may not be the optimum to observe changes in protein levels. In the revised manuscript we have also analyzed the glucose effect in PASK protein regulation, after 8 h and 16 h of treatments. Exposure to high glucose or also to GLP-1 for more than 8 h significantly decreased the amount of PASK protein. This confirmed the glucose and GLP-1 effects observed in the mRNA coding to PASK. The results obtained after 16 h of treatment are shown in the revised Fig 4

ii. Panel C. Is the increase in ATP content in PASK silenced cells dependent upon AMPK activity?

The activation of AMPK at low energy states correlates with increasing AMP/ATP ratio. Our data indicate that, in N2A cells, elevated levels of glucose and ATP,

corresponded to the lowest level of AMPK activation. On the contrary, lower levels of ATP are associated with greater activation of AMPK. However, the presence of low glucose levels in PASK-deficient cells correlated with an increase in the concentration of ATP and the absence of activation of AMPK.
A comment about this has been introduced in the discussion of revised manuscript

4. Figure 5.

i. AMPK phosphorylation was decreased upon GLP-1 treatment in low glucose, but increased in high glucose when GLP-1 was added. These patterns were suppressed in PASK silenced cells. However, AMPK phosphorylation does not match its activity in that phosphorylation of AMPK substrate ACC is largely unaffected in either negative control samples or PASK silenced samples. For examples, AMPK in GLP-1 treated cells under low glucose condition is inhibited (dephosphorylated), yet ACC phosphorylation at AMPK site is normal. Similarly, AMPK phosphorylation is lower in PASK silenced cells however ACC phosphorylation appears normal. Authors should test some other AMPK substrates to examine if AMPK activity is better reflected in those substrates.

We agree with you. The levels of phospho-AMPK and phospho-ACC have been revised, introducing more new data. The treatment with GLP-1 at high glucose did not modify the level of AMPK activation in accordance with previous results (Hurtado et al, 2012). The level of phosphorylation of AMPK and ACC matched in all the conditions studied at low glucose concentration. The differences in the effects of high glucose concentration on the levels of phospho-ACC and phospho-AMPK may be due to differences in the localization of these proteins. The ACC is located exclusively in the cytoplasm but AMPKα2 may move between the nucleus and the cytoplasm. Previous reports indicated that low energy and increased oxidative stress concentrated AMPKα2 in the nucleus (Kodiha, 2007: Am J Physiol Cell Physiol 293, 5: C1427-36). We can not exclude differences on AMPKα2 localization in control and PASK-silenced cells.
A commentary has been introduced in the discussion of the revised manuscript and the figure 5a has been modified incorporating the additional data.

5. Figure 6. i. Panel C. A better LKB1 blot needs to be shown since apparent visual band pattern does not match LKB1 quantification presented.

A more representative blot is shown, in accordance with the bar graphs results that correspond to means of 4 densitometric values.

6. Figure 8. i. Does AMPK activator suppress increased in p-S6K1 seen in PASK siRNA cells?

In order to reply this question, we have checked the effect of AMPK activator AICAR in PASK knockdown cells. The presence of AICAR activated AMPK in both *Pask*-deficient and control cells and these effects correlated with decreased level of phospho-S6K1.

The absence of AMPK activation effect on S6K1 in PASK-silenced cells was confirmed by the AICAR treatment that decreased S6K1 activity.

Other minor comments:

1. In figure 2b, authors have normalized mRNA expression of PASK in low glucose concentration to 1. It is better to show effect of glucose and GLP-1 based on raw numbers to be able to compare side-by-side the effect of glucose and GLP-1 in VMH and LH.

According to your suggestion, the data in figure 2 was corrected in the revised manuscript.

2. Why LH and VMH tissues show difference in PASK expression pattern in high glucose concentrations with GLP-1. Are there differences in GLP-1 receptor distribution between two neuronal regions? If so, a reference and comment about it should be provided.

We have previously reported differences in the pattern of regulation by glucose and neuropeptides of *Glp-1r* gene expression in VMH and LH which may explain the differences observed (Sanz et al, 2008). Reference to these results has been included in the discussion of the revised manuscript.

3. PASK targeting siRNA sequence, sources of antibodies used should be provided in material and methods.

Sorry for the omission. As indicated above, the sequence of PASK targeting siRNA used has been included as well as a Table with the antibodies details in "material and methods" section of the revised manuscript

Reviewer #3: This study addresses an interesting topic namely energy sensors in hypothalamic neurones. Specifically the main conclusion of the paper is that PASK-silenced N2A cells record an impaired response by the AMPK and mTOR/S6K1 pathways to changes in glucose levels.This is a quite relevant one since these two sensors are felt as the most relevant ones in terms of energy and metabolic homesotasis at the hypothalamic level.

Major concerns:

-General comment. The discussion is quite repetitive in terms of reproducing some entire paragraphs from the introduction and the results. It should be more focused in terms of the interpretation of the data and their limitations.

Discussion section has been modified according to the reviewer's advice.

-The authors should look for statistical advise. In experiments where differences doses are used (e.g. glucose) a dose-effect should be documented.

In the revised manuscript we have included a dose-response glucose effect for AMPK and S6K activation that we have developed, but we did not include it in the original manuscript. These results have been included as supplementary data in online resource 3. Some comments about these controls have also been included in the revised manuscript

Specific comments:

-Data showing that the microlaser dissection of VMH and LH was devoid of "contamination" with other cell types should be provided. This can be accomplished by showing SF-1 measurements (restricted expression in VMH) or preprorexin (LH).

Following your suggestion, the expression of SF-1 and prepro-orexin mRNAs was analyzed in VMH and LH. The data showed the specific expression of SF-1 in VMH and the prepro-orexin in LH, ensuring the purity of the nuclei obtained by this technique. These results are shown in Figure 2 in the revised manuscript.

-Data from Fig 2 appears to indicate that PAS mRNA levels are modestly regulated by glucose in the VMH and that GLP-1 exert an inhibitory effect that is glucose independent. However their comment that GLP-1 increased PAS mRNA levels can be misleading. At 10 mM glucose exert a marked inhibitory effect (66%). My feeling is that GLP-1 is able to rescue to some extent the decreased in PASK mRNA levels exerted by high glucose levels but that if the data was normalized to the levels observed in 2.5 mM glucose without GLP-1 the levels will still be lower.

Thank you, we have taken into account your indication and we agree with you in your appreciation about data in Fig 2. Both, the data and comments about the figure 2 were corrected in the revised manuscript and have improved the content and understanding of the results.

-The quality of the data presented in Fig 3 raise some doubts. In general terms beacuse of the large error bars it appear quite difficult to reach any firm conclusion.For example there appears to be quite marked changes in mTOR in relation to 10 mM glucose and GLP-1 comparing the control and the PASK siRNA. Similar comments apply to TSC2 or S6K1. An increase in the number of experiments/replicates is warranted.

According with your suggestion, we have increased the number of experiments and introduced new data, in order to minimize the error. Including the new data, the errors were reduced in some cases. However, no significant differences were found in the mRNA levels of mTOR, TSC2, and S6K1 between the control and the PASK siRNA. Figure 3 has been modified in the revised manuscript.

-Data presented in Gig 4 is interesting in the sense that validates at protein level the effect of PASK silencing (4a). However the data presented in Fig 4b appears to be in conflict with the one shown in Fig 3a where a clear decrease in Pask mRNA was evident after exposure to 10 mM glucose. This may be related to the time-lag effects between changes in mRNA and protein levels but it should be documented.

The effect of glucose on PASK protein levels was initially analyzed after 4-5 h treatment in the presence of different glucose concentrations and/or GLP-1. We agree with you that this time of treatment may not be the optimum to observe changes in protein levels. In the revised manuscript we have also analyzed the glucose and/or GLP-1 effect in regulation of PASK protein levels, after 8 h and 16 h treatments. Exposure to high glucose or GLP-1 for more than 8 h, significantly decreased the amount of PASK protein. This confirmed the glucose and GLP-1 effects on the mRNA coding to PASK. The data obtained after exposure to glucose and GLP-1 for 16 h treatment has been included in Fig 4.

-The authors stated that the levels of pAMPK followed a similar pattern to pACC. However this does not appear to be the case regarding the experiment with the negative control and treated cells with 10 mM glucose plus GLP-1. Please explain.

The levels of phospho-AMPK and phospho-ACC have been revised, introducing more new data. The treatment with GLP-1 at high glucose did not modify the level of AMPK activation in accordance with previous results (Hurtado-Carneiro et al 2012). The level of phosphorylation of AMPK and ACC matched in all the conditions studied at low glucose concentration. The differences in the levels of phospho-ACC and phospho-AMPK at high glucose concentration may be due to differences in the localization of these proteins. The ACC is located exclusively in the cytoplasm but AMPK may move between the nucleus and the cytoplasm. Previous reports indicated that low energy and increased oxidative stress concentrated AMPK in the nucleus (Kodiha, 2007: Am J Physiol Cell Physiol 293, 5: C1427-36). We can not exclude differences on AMPKα2 localization on control and PASK-silenced cells.

-Regarding the main conclusion of the paper: "In conclusion, our data indicated that PASK is a nutrient and hormonal sensor in neuroblastoma cells and in the VMH and LH from rat hypothalamic slices. In neuroblastoma cells, at least, there is a coordinated response with the other metabolic sensors. Thus, the knockdown of PASK impaired not only the response of the AMPK and mTOR/S6K1 pathways at low and high glucose levels, but also some of the GLP-1 effects observed at both the gene expression level and the activity of AMPK and S6K1. PASK-deficient N2A cells failed to detect the availability of nutrients. Our data are consistent with findings that low glucose levels increase the expression of PASK, suggesting that lower levels of PASK in these cells may be used to report the presence of high levels of glucose". I have some difficulties with this conclusion in the light of their data in Fig 4b showing that PASK protein levels are not influenced by glucose. This aspect should be addressed in depth in the discussion.

In the revised manuscript we have also checked the glucose effect after 8 h and 16 h treatments. Exposure to high glucose for longer than 8 h significantly decreased the amount of PASK protein. This confirmed the data on mRNA expression. We have modified the conclusions according to this and other results obtained according to the comments from the reviewers.

PAS kinase as a nutrient sensor in neuroblastoma and hypothalamic cells required for the normal expression and activity of other cellular nutrient and energy sensors

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Keywords: AMPK · Antidiabetogenic agents · Feeding behavior · GLP-1 · Hypothalamus · mTOR/S6K · Obesity

Abstract

PAS kinase (PASK) is a nutrient sensor that is highly conserved throughout evolution. PASK-deficient mice reveal a metabolic phenotype similar to that described in S6 kinase-1 S6K1-deficient mice that are protected against obesity. Hypothalamic metabolic sensors, such as AMP-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR), play an important role in feeding behavior, the homeostasis of body weight and energy balance. These sensors respond to changes in nutrient levels in the hypothalamic areas involved in feeding behavior and in neuroblastoma N2A cells, and we have recently reported that those effects are modulated by the anorexigenic peptide glucagon-like peptide-1 (GLP-1). Here we identified PASK in both N2A cells and rat VMH and LH areas and found that its expression is regulated by glucose and GLP-1. High levels of glucose decreased *Pask* gene expression. Furthermore, PASK-silenced N2A cells record an impaired response by the AMPK and mTOR/S6K1 pathways to changes in glucose levels. Likewise, GLP-1 effect on the activity of AMPK, S6K1 and other intermediaries of both pathways and the regulatory role at the level of gene expression were also blocked in PASK-silenced cells. The absence of response to low glucose concentrations in PASK-silenced cells correlates with increased ATP content, low expression of mRNA coding for AMPK upstream kinase LKB1 and enhanced activation of S6K1. Our findings indicate that, at least in N2A cells, PASK is a key kinase in GLP-1 actions and exerts a coordinated response with the other metabolic sensors, suggesting that PASK might play an important role in feeding behavior.

Introduction

The PAS kinase (PASK), also termed PASKIN, has a serine/threonine kinase catalytic domain and a PAS domain designed to detect environmental parameters, such as light, oxygen and redox state. In mammals, it has been proposed that PASK is activated by a small metabolite and may regulate glycogen synthesis and protein translation. PASK is also a nutrient sensor conserved from yeast to humans (Grose et al. 2007; Hao and Rutter 2008; Smith and Rutter 2007) with its role being compared to other metabolic sensors: AMP-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR). AMPK activity is regulated by fluctuations in cellular energy produced by nutritional state or by metabolic stress that reduces ATP production. The activation of AMPK occurs with a high AMP/ATP ratio in low energy states. Once activated, the AMPK will contribute to restore the energy balance (Hardie et al. 1998; Hardie et al. 2012; Rutter et al. 2003). By contrast, mTOR and its downstream target, the S6 Kinase 1 (S6K1), respond to nutrients and hormonal signals (Alessi et al. 2009; Foster and Fingar 2010; Zoncu et al. 2011). mTOR complex 1 (mTORC1) is rapamycin and nutrient sensitive. Thus, mTOR is stimulated by an abundance of nutrients and hormones promoting anabolic processes and cell growth (Gingras et al. 2001; Proud 2002). Both AMPK and mTOR respond to changes in glucose and other nutrients in a coordinated way and in opposite directions. Therefore, low energy conditions activate AMPK, which mediates the phosphorylation of the tuberous sclerosis complex 2 (TSC2) and the concomitant inhibition of mTOR (Inoki et al. 2003). Furthermore, AMPK phosphorylates raptor (rapamycin-sensitive adaptor protein of mTOR) in mTORC1, down-regulating this complex (Gwinn et al. 2008).

It has been reported that PASK expression is regulated by glucose, being increased at high glucose concentration, which also activates PASK post-translationally in Min-6 cells (da Silva Xavier et al. 2004). In contrast, PASK regulation by glucose was not observed in pancreatic beta-cells from PASKIN knockout and wild-type mice, and glucose-stimulated insulin release was also similar in both types of mice (Borter et al. 2007).

An initial characterization of PASK-deficient mice showed no abnormalities in development, growth or reproductive functions (Katschinski et al. 2003). Subsequent studies showed that PASK-deficient mice are resistant to diet-induced obesity and that protection seems to be caused by a high metabolic rate in skeletal muscle; these effects were not related to changes in AMPK and S6K1 activation levels (Hao et al. 2007). Additionally, the PASK knockdown effect was also observed in cultured myoblasts, which also registered an increase in the oxidation of glucose and palmitate accompanied by elevated cellular ATP levels (Hao et al. 2007).

In mammals, PASK is thought to be involved in the regulation of glucose and energy metabolism homeostasis, as reviewed by (Hao and Rutter 2008; MacDonald and Rorsman 2011; Schlafli et al. 2009). The effects of PASK activation have also been reported to be tissue specific (Hao et al. 2007). Likewise, studies in recent years have established a direct relationship between the activity of the metabolic sensors in

the hypothalamus and the regulation of food intake, body weight and energy homeostasis. Moreover, the function of hypothalamic metabolic sensors is modulated by anorexigenic and orexigenic peptides. We have recently reported that the metabolic sensors in hypothalamic areas and in the neuroblastoma N2A cell line responded to glucose and the anorexigenic peptide glucagon-like peptide 1 (GLP-1), which is also used as an antidiabetogenic agent (Blonde et al. 2006; Niswender 2010). In addition, the AMPK and mTOR/S6K pathways respond to changes in glucose concentrations, and GLP-1 treatment reversed the glucose effects (Hurtado-Carneiro et al. 2012). PASK is also expressed in hypothalamic areas and in neuroblastoma N2A cells; hence we studied the glucose and GLP-1 effect on PASK expression and whether there is a coordinated response by metabolic sensors at different glucose concentrations. Accordingly, we investigated the effect of PASK-silencing on the expression of several genes, as well as the metabolic effects on the expression and activity of AMPK and mTOR/S6K1 pathways in neuroblastoma cells.

Materials and methods

Experimental animals

All the procedures involving animals were approved by the appropriate Institutional Review Committee and met the guidelines for the care of animals specified by the European Community. Same-aged male Wistar rats weighing 200-250 g were fed *ad libitum* with a standard pellet diet and housed at a constant temperature (21 ºC) on a 12-h light-dark cycle, with lights on at 08:00.

Cell cultures

Mouse neuroblastoma N2A cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) containing 4.5 g/L glucose and supplemented with 10 % FBS, 2 mM-glutamine and penicillin (100 U/ml), streptomycin (100 mg/ml). The cells were maintained in a humidified atmosphere containing 5 % CO_2 at 37 °C. Only the cells propagating for less than eight passages were used in the experiments.

In situ hybridization histochemistry

The histological examination was conducted using 20 μm of frozen rat brain slices. In situ hybridization was performed as described by (Leon et al. 1998) using digoxigenin-labeled cRNA probes. Single-stranded Pask RNA sense or antisense probes were prepared by the transcription of a fragment of 1180 pb previously amplified by PCR using the universal forward primers pUC/M13 (5´-GTTTTCCCAGTCACGAC-3´) and reverse primers (5´-CAGGAAACAGCTATGAC3´). T7 and SP6 RNA polymerases (Roche) (Promega, Southampton, UK) were used for preparing antisense and sense probes, respectively. Tissues were incubated with RNA probes at 1 μg/ml concentration in a hybridization

mix (50 % formamide, 1.3x SSC [pH 4.5], 5 mM EDTA, 50 µg/ml yeast RNA, 0.2 % Tween 20, 0.5 % CHAPS, and 100 µg/ml heparin). To elicit color, the slices were incubated with a 1/2000 dilution of anti-digoxigenin-alkaline phosphatase antibody (Roche) and developed with 0.26 mg/ml nitroblue tetrazolium salt and 0.175 mg/ml 5-bromo-4-chloro-3-indolyl phosphate prepared in NTMT (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM MgCl2, and 0.1 % Tween 20). Images were taken with a Leica DM RB microscope.

Immunocytochemistry

Immunocytochemistry was developed as described (Sanz et al. 2010). Briefly, cells were grown attached to glass coverslips of 12 mm in diameter. The cells were then fixed for 15 minutes at 4 °C in 4 % paraformaldehyde and washed three times with PBS at room temperature. The cells were permeabilized for 20 min with PBS 0.4 % (v/v) Triton X-100, and non-specific antibody binding sites were blocked by incubation with a blocking solution (PBS, 10 % goat serum, 0.1 % Triton X-100) for 1 h. The cells were incubated with anti-PASK antibody diluted 1:100 in the blocking solution and then washed twice for 10 min with PBS at room temperature. For fluorescence detection, the cells were incubated with Texas Red conjugated donkey anti-rabbit antibody (GeneTex, Inc., San Antonio, CA, USA) diluted 1:200 in the blocking solution. In all cases, the specificity of immunodetection was confirmed by not adding a primary antibody. The nucleus DNA was stained by adding 1 µg/ml of 4,6-diamidino-2-phenylindole (DAPI) in PBS. Finally, the cells were mounted with the Fluoromount-G slide mounting medium (EMS, Hatfield, PA, USA). The images were taken with a TCS SP2 confocal laser microscopy system (Leica Microsystems, Wetzlar, Germany) equipped with an inverted DMIRE2 Leica microscope. Confocal fluorescence images were analyzed using LCS Lite software from Leica.

Procedure for hypothalamic-slice explants cultures

Hypothalamic-slices were obtained as described previously (Sanz et al. 2007). Briefly, male Wistar rats were decapitated and the brains were quickly removed and immersed in a cold (4 ºC) MEM medium containing 25 mM HEPES, 20 % heat-inactivated horse serum, 4 mM glutamine, 6.5 mg/ml glucose, and 100 U/ml penicillin-streptomycin. The hypothalami were removed from the brain and sections of 300 µm thickness were cut on a Mcllwain tissue chopper (Mickle Laboratory Engineering, Surrey, UK). The slices were cultured at 37 ºC in an atmosphere containing 5 % CO_2 for 5 h in MEM supplemented with 25 mM HEPES and Hank´s salt-enriched with the above components in order to stabilize the cultures. The slices were then incubated in a medium containing 2 % FBS and 5.5 mM glucose for 16 h. Following this, the hypothalamic slices were fasted at 0.5 mM glucose for 2 h. The medium was removed and the slices were incubated for 3 h with 1, 2.5 or 10 mM glucose, in some cases adding 10 nM GLP-1. At the end of the

incubations, special care was taken to identify and isolate the VMH and LH areas by micropunching according to the stereotaxic coordinates (Paxinos and Watson 2004).

RNA interference

The short interference RNA (siRNA) targeting *Pask* gene (PASK Silencer® Select Pre-design siRNA) and the negative control siRNA (Silencer® Select Negative Control siRNA) were obtained from Ambion (Life Technologies). The sequences of *Pask* siRNA were: Sense CGUGUAAUCUGCAUCAGGAtt and Antisense UCCUGAUGCAGAUUACACGtg. N2A cells were grown up to a 50 % confluence in six well plates. siRNA was transfected by using Lipofectamine™ with 0.15 µg of PASK or 10 nM control siRNAs, according to the manufacturer's protocol. After 24 h, the cells were starved of serum (0.1 %) and glucose (0.5 mM) for 2 h. They were then incubated in a medium containing either 0.5, 2.5 ~~mM~~ or 10 mM of glucose for 3 h. In some cases, 10 nM GLP-1 was added during the incubation. Cell lysates were collected and processed for western blot analysis or RNA extraction, cDNA synthesis and quantitative real-time PCR.

Real-time polymerase chain reaction (TaqMan® Assay)

The total RNA from N2A cells or rat hypothalamic-slices was isolated by the acid guanidinium isothiocyanate method (Chomczynski and Sacchi 1987). RNA integrity was tested with the Bioanalyzer 2100 (Agilent), and cDNA synthesis was developed with the "high-capacity cDNA archive kit" (Applied Biosystems), using 1 µg of RNA as template, following the manufacturer's instructions. The mRNA levels of *Pask*, *Ampkα2*, *Glp-1r*, *β-actin*, *18s*, *Sf-1* and *Prepro-orexin* ~~*18s RNA*~~ were measured by real-time quantitative RT-PCR using TaqMan® probes (Applied Biosystems). The primers and probes (Online Resource1) were designed with Primer Express 2.0 software from Applied Biosystems.

Multiple gene expression analyses in a TaqMan® Gene Expression Assay

RNA integrity was tested with the Bioanalyzer 2100 (Agilent), and cDNA synthesis was developed with the "high-capacity cDNA archive kit" (Applied Biosystems) using 1 µg of RNA as template in a final volume of 20 µl, following the manufacturer's instructions.

Multiple gene expression analyses were conducted by quantity real-time PCR, using the TaqMan® Fast Universal PCR Master Mix (Roche Diagnostic) on a TaqMan® Array Fast 96-well Plate containing 18 customized duplicated genes (Online Resource 2). Two microlitres of a 1/10 dilution of the cDNA was used as a template for the PCR developed in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The conditions were 50 ºC for 2 min, 95 ºC for 10 min, followed by 40 cycles at 95 ºC for 15 s and 60 ºC for 1 min. Two housekeeping genes were used for the normalization procedure, namely, *18s* and *β-actin*. The data were analyzed with RQ Manager Software (Applied Biosystems).

The average *18s* or *β-actin* CT values on each 96-well plate were then subtracted from the CT value for each reaction well on that plate.

Protein expression and activity of PASK, AMPK and mTOR/S6K1 pathways

For the analysis of protein expression and activity by western blot, the cells were cultured for 2 h in the presence of 0.5 mM of glucose. They were then incubated in a medium containing ~~either~~ 0.5, 2.5 ~~mM~~ or 10 mM of glucose for 2, 4, ~~-~~5, 8, 16 h. The cells were occasionally incubated in the presence or absence of different protein kinase or phosphatase inhibitors at the concentrations described in Table 1 for 30 min. To analyze the effect of AMPK activator 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR) cells were treated with 2 mM AICAR for 1 h. In the case of the activity study, 10 nM GLP-1 was added during the last 10 min of the incubation, while in the protein expression determination the GLP-1 was added from the beginning of the incubation with glucose. The cells were immediately lysed in a RIPA buffer and exposed to microwave irradiation for 5 s. Total and activated forms were detected by western blot using the antibodies described in Table 2.~~Online Resource 3.~~ The blots were reprobed for β-ACTIN. Finally, the blots were scanned and quantified using Quantity One software (Bio-Rad, GS800 Densitometer).

ATP concentration

Total cellular ATP concentration was measured using the luciferase bioassay. The bioluminescence assay is based on the reaction of ATP with recombinant firefly luciferase (Sigma-Aldrich, Saint Louis, Missouri, USA) and its substrate luciferin (Sigma). Cells were grown on a 24-well plate. Both the cells previously transfected with negative control siRNA and those with *Pask* siRNA were incubated for 2 h in 0.5 mM glucose and then for a further 2 hours in a medium containing either 0.5 mM or 10 mM of glucose. A 1/1000 dilution of grown cells was harvested by trypsinization and centrifugation in PBS. Pellets were lysed in 20 µl of 1.35 M perchloric acid and then neutralized with 15 µl of 2.8 M $KHCO_3$-0.1M Tris. 10 µl of the supernatant was used for the luciferase reaction, by adding 10 µl of luciferase, and 100 µl luciferin. Luminescence was determined in a luminometer (BG-P luminometer; GEM Biomedical, Hamden, CT).

Statistical analyses

All values are presented as means ± SEM. Groups were compared using t-test analysis. P<0.05 was considered statistically significant.

Results

Expression of *Pask* mRNA in the hypothalamus

By means of in situ hybridization with the specific antisense *Pask* probe, labeled cells were found throughout the hypothalamus, whereas no labeling was detected with the sense probe (Fig. 1a). Cells positive for *Pask* mRNA were found to be distributed throughout the hypothalamus. A higher magnification of the labeled cells in the LH is shown in Fig. 1a. The specificity of the probe and the in situ hybridization background were tested with the sense probe where no labeled cells were found.

Cellular location of PASK

PASK immunocytochemistry was developed in neuroblastoma N2A cells. All the N2A cells contained PASK protein and it was present mainly in the cell cytoplasm (Fig. 1b~~1a~~).

PASK responds to glucose in neuroblastoma cells

PASK is expressed in N2A cells. The *Pask* mRNA expression was measured with real-time RT-PCR after treatment at low and high glucose concentrations (0.5 or 10 mM). Elevated levels of glucose decreased the amount of mRNA coding PASK in neuroblastoma cells (Fig. 1c~~1b~~).

Glucose and GLP-1 regulate PASK expression in hypothalamic-slice explants

We then investigated *Pask* mRNA expression in the hypothalamic areas involved in the control of feeding:~~.~~ ~~The mRNA levels of *Pask* were measured with real-time RT-PCR in the~~ ventromedial hypothalamus (VMH) and in the lateral hypothalamic area (LH). In order to ensure the purity of these hypothalamic

nuclei obtained by micropunching from hypothalamic-slice explants, *Steroidogenic factor 1* (*Sf-1*) and *Prepro-orexin* genes were used as markers of these specific nuclei respectively. The mRNA encoding SF-1 was mostly present in VMH while, on the contrary *Prepro-orexin* gene expression was highest in LH (Fig 2a)

The mRNA levels of *Pask* were measured with real-time RT-PCR in VMH and LH areas~~)~~ from rat hypothalamic -slice explants after treatment with different glucose concentrations (1, 2.5 or 10 mM) (Fig. 2b,c~~2a~~). Higher glucose concentrations (10 mM) decreased by ≈20 and 66 %, the expression of the mRNA coding PASK in the VMH and LH areas, respectively.

The presence of GLP-1 decreased *Pask* mRNA and reversed in part~~in~~ the ~~VMH at low and~~ high glucose ~~levels, but only at low glucose~~ concentrations effect in the ~~case of the~~ LH~~. However, the presence of GLP-1 increased the expression of *Pask* at high glucose concentrations in the LH area~~ (Fig. 2b,c).

PASK-silencing in neuroblastoma N2A cells

We therefore analyzed the effect of PASK knockdown in neuroblastoma N2A cells. We have previously reported that both AMPK and S6K1 activities respond to glucose concentrations, and GLP-1 also significantly decreased AMPK and S6K1 activation in these cells (Hurtado-Carneiro et al. 2012). PASK-silenced cells recorded a 77 % decrease in PASK mRNA compared to siRNA negative control (Fig. 1d~~1c~~).

We then compared the expression of several genes using real-time RT-PCR in PASK knockdown N2A and control cells at low or high glucose concentrations in the presence or absence of GLP-1. Thus, we analyzed the expression of mRNAs coding to PASK and other metabolic sensors as: AMPKα2, mTOR/S6K1; several genes of proteins that could regulate the metabolic sensors activity: LKB1, TSC2, AKT, PKA; as well as some direct substrates of metabolic sensors: S6, eIF-4B, eEF-2K, eEF1-α1 and nNOS; genes coding to protein decisive for carbohydrate metabolism: GAPDH and PDK1; additionally, genes coding to HIPK2, which was reported to mediate apoptosis in neurons, GLP-1 receptor (GLP-1R) and a housekeeping gene: *β-actin* used for the normalization procedure. The *Pask* mRNA level was higher at low glucose concentration and lower at high glucose, and the treatment with GLP-1 reversed these effects. However, the expression of PASK did not respond to glucose or GLP-1 after PASK-silencing (Fig. 3a). PASK knockdown did not change significantly the expression of mRNAs coding to mTOR and S6K1 (Fig. 3b). Unfortunately, it was impossible to detect the mRNA level of *Ampkα2* in these cells with the probes used. A similar pattern of expression was observed in PASK-silenced and control cells for the genes coding to AKT, PKARIIα and TSC2 (Fig. 3c). However, the expression of *Stk1* mRNAs that codify to LKB1, one of the upstream kinases of AMPK, was enhanced at low glucose concentration, and the presence of GLP-1 reversed the glucose effect, with PASK knockdown impairing both effects (Fig. 3c). The expression of *nNos1* gene responded to glucose levels, being the lower expression observed at high glucose concentration (Fig. 3d). Additionally, we found that ~~*Eef1a1* and~~ *Eif4B* genegenes that codify to ~~eukaryotic translation~~

~~elongation factor 1 and~~ translation initiation factor 4B were downregulated in PASK Knockdown cells~~upregulated by GLP-1 at high glucose concentrations~~ (Fig. 3d). The PASK knockdown blocked the glucose response of *nNos1* ~~gene as well as the upregulation by GLP-1 of *Eef1a1* and *Eif4B*~~ genes (Fig. 3d). The expression of *Gapdh* gene was increased at low glucose concentration and the presence of GLP-1 reversed the low glucose effect, and PASK knockdown impaired the glucose effect (Fig. 3e).

PASK-silencing in neuroblastoma N2A cells increases levels of ATP at low glucose concentrations

The reduced *Pask* mRNA levels in silenced N2A cells (Fig. 1d) produced eventual reduction in PASK protein of ~68% (Fig. 4a). Treatment of 16 hours with high glucose concentration (10 mM) or 10 nM GLP-1, reduced PASK protein levels in N2A cells relative to the cultured in 0.5 mM glucose (Fig 4b). Both, glucose and GLP-1 effects were abolished in PASK-silenced cells (Fig 4b).~~We confirmed that the PASK protein expression was ~68% lower in PASK-silenced N2A cells (Fig. 4a). However, although the amount of PASK protein decreased at high glucose concentrations, no significant differences were detected (Fig. 4b).~~

Cellular ATP content was measured at low and high glucose concentration. The ATP levels were increased at high glucose concentration in neuroblastoma cells. Regarding PASK-silenced cells, at low glucose we observed a level of ATP similar to that observed at high glucose levels in the control cells (Fig. 4c). We then checked the expression of peroxisome proliferator-activated receptor (PPAR) γ co-activator 1α (PGC-1α), which can coordinate both mitochondrial biogenesis and oxidative phosphorylation due to its role as a co-activator of PPAR family of nuclear receptors. PGC-1α expression was not altered in PASK knock-down cells and no effect of glucose or GLP-1 was observed (Fig. 4d).

Impaired effects of Glucose and GLP-1 on AMPK activity and ACC phosphorylation in PASK knockdown neuroblastoma N2A cells

The activation of AMPK in N2A cells, was checked using an anti-phospho-AMPKα (Thr172) after a doses-response treatment of glucose (0.5, 2.5 or 10 mM) (online resource 3). Glucose levels that produced the maximum and minimum activation level (0.5 and 10 mM glucose) were used in subsequent studies.~~).~~ The data show that AMPK activities increased at low glucose concentration, while the presence of GLP-1 significantly decreased the AMPK activity previously stimulated by low glucose concentrations (Fig. 5a, d). The glucose effect~~and GLP-1 effects~~ on AMPK activity was not significant~~were lost~~ when *Pask* mRNA expression was inhibited by siRNA targeting *Pask* gene and the GLP-1 effect was lost, while no differences on AMPKα2 protein expression were observed at low glucose in PASK knockdown cells compared to the control cells (Fig 5b, d).~~.~~ However, the level of total AMPKα2 decreased after 4-5 h treatment in the presence of high glucose levels in control neuroblastoma cells, and the glucose effect was lost in PASK-silenced neuroblastoma cells (Fig. 5b, d).

The activation of AMPK is associated with the enhanced phosphorylation of its downstream target acetyl-CoA carboxylase (ACC). The level of ACC phosphorylation followed a similar pattern to AMPK at low glucose levels (Fig. 5c, d). An elevated phospho-ACC level was found at low glucose concentration. GLP-1 treatment decreased phospho-ACC level, and the amount of phospho-ACC significantly decreased at high glucose concentration. PASK-silencing decreased the phospho-ACC level at low glucose concentration; the GLP-1 effect was impaired. However, the glucose effect on phospho-ACC level was still detected in PASK knockdown neuroblastoma cells (Fig. 5c,d).

LKB1 and CaMKKβ expression in PASK knockdown neuroblastoma N2A cells

The expression of the AMPK upstream kinases, liver kinase B1 (LKB1) and the Ca^{2+}-calmodulin-dependent protein kinases (CaMKKβ) were also analyzed. LKB1 expression was lower at high glucose both in PASK-silenced and control neuroblastoma cells (Fig.6a, c).~~.~~ Furthermore, PASK-silenced cells showed a significant reduction in the expression of LKB1 compared to control cells. However, CaMKKβ protein expression was similar under all the conditions studied (Fig. 6b,c). PASK-silenced cells recorded a slight decrease in the amount of CaMKKβ protein (Fig. 6b,c).

Impaired effects of Glucose and GLP-1 on S6K1 pathway activity in PASK knockdown neuroblastoma N2A cells

The activation of S6K1 was detected using anti-phospho-S6K1 (Thr389) after treatment with different glucose concentrations (0.5, 2.5 or 10 mM) shown in online resource 3. Glucose levels that produced the maximum and minimum activation level were used in subsequent studies.~~)~~. The effect of growth factors on the mTOR/S6K1 pathway is exerted through phosphatidylinositol-3,4,5-triphosphate kinase (PI3K) and further activation of AKT. The activation of Ras/MAPK cascade by mitogens can also activate mTOR. Signaling through this pathway is also regulated by the phosphatase PTEN, which controls the phosphorylation state of phosphatidylinositol-3,4,5-triphosphate (PIP3), PI3K and AKT.

We have checked the effect of certain specific inhibitors of protein kinases or phosphatases that might be involved in the regulation of S6K1 activation in the N2A cell line (Fig. 7). The stimulatory effect of high glucose on S6K1 phosphorylation was impaired in the presence of the specific inhibitors of PI3K and mTOR (Fig. 7a). The inhibitory effect of GLP-1 on S6K1 activity was not detected in the presence of a specific PKA inhibitor (Fig. 7b).

The glucose and GLP-1 effect on the mTOR/S6K1 pathway was then analyzed. S6K1 activation was observed at high glucose and the presence of GLP-1 also significantly decreased S6K1 activation (Fig. 8a, d). PASK knockdown cells showed a complete loss of the capability of the S6K1 to sense nutrient levels and respond to GLP-1 (Fig. 8a,d). The activation state of AKT was detected with anti-phospho AKT (Ser

473). The level of phospho-AKT increased at low glucose concentration, while the presence of GLP-1 decreased AKT activation at low glucose (Fig. 8b, d). PASK-silencing decreased the stimulatory effect of low glucose, although AKT activation was still modulated by glucose and GLP-1 in PASK knockdown cells (Fig 8b,d).

The expression of phosphatase PTEN was similar in all the conditions analyzed (Fig 8c, d).

Finally, we have checked the effect of AMPK activator AICAR in PASK knockdown cells. The presence of AICAR activated AMPK in both Pask-deficient and control cells. These effects on AMPK activation, correlated with decreased level of phospho-S6K1 (data not shown).

DISCUSSION

PASK plays different physiological functions in different tissues. Thus,

~~**Discussion**~~

PASK has been considered a nutrient sensor whose expression is upregulated by a high glucose concentration in Min-6 cells (da Silva Xavier et al. 2004). On the other hand~~. However~~, no evidence of PASK regulation by glucose has been~~was~~ observed in pancreatic beta-cells or testicular cell lines from PASKIN knockout and wild-type mice (Borter et al. 2007)~~. The first study of PASK-deficient mice indicates that these mice have a normal phenotype as regards embryonic development, growth and reproductive capacity~~ (Katschinski et al. 2003).~~.~~ Further investigation has indicated that PASK-deficient mice have~~had~~ a metabolic phenotype (Hao et al. 2007) similar to that described in S6K1-deficient mice; in both cases, those mice were protected against obesity (Pende et al. 2000; Um et al. 2004).

PASK activation effects are tissue specific. Therefore, PASK activation in β-pancreatic cells regulates insulin release; in liver it enhances the synthesis and storage of triglycerides and in skeletal muscle it inhibits metabolic oxidation (Hao and Rutter 2008). Increased oxidative metabolism and ATP generation were also observed in PASK knockdown L6 myoblast cell lines (Hao et al. 2007). However, no differences were found in cellular ATP production in fibroblasts from PASKIN knockout mice (Schlafli et al. 2009).

Hypothalamic metabolic sensors play an important role in feeding behavior, the homeostasis of body weight and energy balance. Nutrients, orexigenic, anorexigenic peptides and signals that report on the status of energy stores may coordinate the activity of the metabolic sensors regulating food intake. We have recently reported that both AMPK and S6K1 activities respond to changes in glucose concentrations in neuroblastoma cells. Low glucose concentrations produced a rapid activation of AMPK. However, S6K1 was activated at high glucose concentrations in ~~the~~ N2A cells, and the presence of anorexigenic peptide GLP-1 ~~also~~ significantly decreased both AMPK and S6K1 activation. The observed response to glucose and GLP-1 is similar to that found in the rat hypothalamic areas involved in feeding behavior (Hurtado-Carneiro et al. 2012). We contend here that PASK protein is also a nutrient sensor in neuroblastoma cells. ~~*Pask* gene expression is regulated by glucose levels in neuroblastoma cells.~~ Our data indicated that the *Pask* gene is upregulated by low glucose in neuroblastoma cells, and this effect was reversed in the presence of GLP-1.

The observed changes in mRNA coding to PASK were consistent with the changes in PASK protein. Thus, cells exposed to high glucose or to GLP-1 for more than 8 h significantly decreased the amount of PASK protein.

Hypothalamic metabolic sensors play an important role in feeding behavior, bodyweight homeostasis and energy balance. Nutrients, orexigenic and anorexigenic peptides, and the signals that report on the status of energy stores may coordinate the activity of the metabolic sensors regulating food intake.

Here, The response of *Pask* to nutrients and gut peptides was observed not only in neuroblastoma cells but also ~~observed~~ in ~~the~~ VMH and LH on rat hypothalamic slices. Both areas are involved in feeding behavior and energy homeostasis: ~~the~~ VMH and LH are considered the satiety and hunger centers, respectively. Rat hypothalamic slices have previously been reported to preserve some tissue architecture and functional connections in order to maintain a selective c-Fos expression in response to different glucose concentrations (Sanz et al. 2007) similar to that found ~~after~~ in vivo ~~administration~~ (Solomon et al. 2006). We have also reported that glucose and GLP-1 regulate *Ampkα2* gene expression and both AMPK and S6K1 activity in these areas .~~.~~ A similar approach has also been used to analyze neuropeptide expression (Lee et al. 2005). ~~We have previously reported that glucose and GLP-1 regulate *Ampkα2* gene expression and both AMPK and S6K1 activity~~ (Hurtado-Carneiro et al. 2012). In this regard, we show here that higher glucose concentration and GLP-1 treatment downregulate *Pask* mRNA expression in VMH and LH. However, The GLP1 effect at a high glucose concentration differed in both areas: GLP-1 treatment reversed the effect of high glucose specifically in LH, but not in VMH. A distinctive pattern of gene regulation in response to glucose and neuropeptides has also been previously reported in VMH and LH, including the expression of the GLP-1 receptor (GLP-1R) in these areas . The differences in the regulation of *Glp-1r* gene expression by glucose and neuropeptides in these areas might explain our results . Our data show~~. The presence of a high glucose concentration decreased *Pask* mRNA expression. However, GLP-1 treatment at elevated glucose concentrations decreased *Pask* mRNA in the VMH and increased its expression in the LH. Our data suggest~~ that the *Pask* gene is regulated by glucose and GLP-1, an anorexigenic peptide whose level increases after a meal, informing~~and it informs~~ the central nervous system of nutrient status. ~~The effect observed was distinctive in each specific area; similar results have been reported previously with the expression of *Ampkα2* gene~~ (Hurtado-Carneiro et al. 2012)This suggests~~.~~ ~~Our data suggest~~ that PASK may~~might~~ be involved in the control of food intake.

Another interesting finding was~~We also show here~~ that the knockdown of more than 75 % *Pask* mRNA is sufficient to impair~~impaired~~ the response to glucose of the AMPK and mTOR/S6K1 pathways in neuroblastoma cells. However, previous data indicated that the activation of ~~PASK function was not dependent on the~~ AMPK or mTOR signaling pathways (Hao et al. 2007)was not dependent on the PASK function .~~.~~ There are two possible explanations: first, the differences may be due to the experimental method used, since we analyzed the response of the metabolic sensors at low and high glucose levels when ~~the~~ activation/inhibition was maximal; second, this response may be cell or tissue specific. Nonetheless, the effect mediated by PASK in these pathways was mainly at posttranscriptional level. Thus, PASK knockdown in N2A cells did not significantly modify the expression of gene~~genes~~ coding to mTOR and S6K1. Nevertheless, a tendency to decrease the levels of gene coding to mTOR, AKT1, S6K1, eEF-2K, PDK1, GADPH, nNOS and eIF-4B was observed in PASK-silenced cells. Therefore, the assumption that the total knockout of PASK expression could change the expression of these genes cannot be ruled out. However, the expression of mRNA coding to LKB1 decreased in PASK-silenced cells~~, and we have also detected a tendency toward the inhibition of the expression of the *Gapdh* gene, a glycolytic enzyme that~~

responds to oxidative stress and reportedly might link the metabolic status to the process of gene transcription (Ralser et al. 2007; Zheng et al. 2003) and of the *Pdk1* gene, although no significant differences were found. PDK1 phosphorylates and inhibits the multi-enzymatic complex that catalyzes the oxidative decarboxylation of pyruvate, and therefore its entry into the citric acid cycle. In yeast, PASK homologs are involved in coordinating the fate of carbohydrates (Smith and Rutter 2007). .

Unfortunately, we could not measure the expression of the mRNA coding to AMPKα2 in N2A cells using two different probes. This suggests the presence of point mutations, although we have not checked the sequence in these cells. However, the amount of AMPKα2 protein was detected by western blot, and a similar amount of AMPKα2 was found at low glucose levels in PASK knockdown and control cells. Nevertheless, previous studies have reported thatHowever, the level of AMPKα2 decreased after the prolonged presence of elevated glucose . Here, we confirm this fact in control neuroblastoma cells, and also show that the glucose effect was impaired after PASK-silencing. The glucose effect on the AMPKα2 expression has been previously described (Hurtado-Carneiro et al. 2012; McCrimmon et al. 2006; Seo et al. 2008).

Previous data have reported the role of ATP levels in stimulating AMPK in neurons and the regulation of neuropeptides that modulate feeding behaviour . Therefore, The activation of AMPK at low energy states correlates with increases in the AMP/ATP ratio. Previous studies have affirmed that PASK increased oxidative metabolism and ATP generationThe lack of activation of AMPK at low glucose concentrations in PASK knockdown L6 myoblast cell lines . However, no differences were found in cellular ATP production in fibroblasts from PASKIN knockout mice . Our data indicate that cells is further supported by the elevated levels of glucose and ATP in N2A cells corresponded to the lowest level of AMPK activation, while lower levels of ATP are associated with greater AMPK activation. By contrast, the presence ofcontent of ATP observed at low glucose levels in PASK-deficientsilenced cells. Our results agree with the data reported by Hao et al. (Hao et al. 2007) correlated with the enhanced. We have also observed that ATP content increased at high glucose concentration in neuroblastoma cells. Previous data have reported the role of ATP levels to stimulate AMPK in neurons and the regulation of neuropeptides that modulate feeding behavior (Lee et al. 2005)of ATP and the absence of AMPK activation.. However, the expression of PGC-1α was not modified in PASK-knockdown neuroblastoma cells.

AMPKThe activation process of AMPK is also regulated by several upstream kinases. In mammals, the two main kinases are LKB1 and CaMKKβ (Hardie et al. 2012; Viollet et al. 2009). Thus, the low expression of the mRNA coding to LKB1 at a low glucose level in PASK knockdown cells also explains the lack of AMPK activation. Similar data were found forin LKB1-deficient muscle (Sakamoto et al. 2005) or liver (Shaw et al. 2005). Our data show that the gene coding to LKB1 is regulated by glucose and GLP-1 in neuroblastoma cells. Low glucose increased the mRNA coding to LKB1. Nevertheless, that effect was lost in PASK-silenced cells. In contrast, Previous results suggested a constitutive activation of LKB1 in cell

lines and skeletal and cardiac muscle, as reviewed by (Sanders et al. 2007). Subsequent studies will be needed to clarify whether this finding is characteristic for this cell line.

. We have previously reported that AMPK stimulation at low glucose levels was not inhibited by STO-699, a CaMKK inhibitor in N2A cells (Hurtado-Carneiro et al. 2012). Here, we show a slight decrease in the CaMKKβ protein expression in PASK knockdown regarding the control cells. Taking into account the effects on ATP concentrations, LKB1 and CaMKKβ, our data would explain the lack of activation in PASK-deficient cells.

AMPK plays a key role in glucose and lipid metabolism. AMPK thus phosphorylates and inhibits ACC, whose activation prevented the entry into and oxidation of fatty acids in mitochondria. AMPK activation in the hypothalamus promoted by starvation induces ACC inactivation and a subsequent decrease in malonyl-CoA, and also stimulates fatty acid oxidation. Furthermore, it has been reported that ghrelin stimulates AMPK and other signaling pathways promoting the inhibition of lipogenesis and increasing fatty acid oxidation leading to changes in mitochondrial respiration, enhancing the production of reactive oxygen species (ROS) and activating NPY/AgRP neurons .and oxidation of fatty acids. The level of ACC activity at low glucose levels in PASK-silenced neuroblastoma cells was similar to that found at high glucose levels in the control cells. Therefore, it seems that the increased ATP levels observed in these cells was not due to an increased transport and fatty acid oxidation in mitochondria.

Our data confirm that at a high glucose concentration, and besides AMPK inhibition, the amount of phospho-ACC decreased sharply. The presence of GLP-1 did not change this result, according to previous data showing that the action of GLP-1 depends on the maximal activation of AMPK . The level of AMPK activation in PASK-deficient cells at a low glucose concentration was similar to that found in control cells at high glucose according to the levels of ATP. In these PASK deficient cells, an increase in glucose concentration did not significantly lower the level of phospho-AMPK, and phospho-ACC levels were almost undetectable. The differences in the levels of phospho-ACC and phospho-AMPK at high and low glucose concentration may be explained by differences in the localization of these proteins. Thus, the ACC is located exclusively in the cytoplasm, but its phosphorylation depends on the presence of AMPK. However, AMPKα2 may move between the nucleus and the cytoplasm in agreement with previous reports indicating that low energy and increased oxidative stress concentrated AMPK in the nucleus . It cannot be dismissed that the location of AMPKα2 could be affected in PASK-deficient cells.

The lack of AMPK activation at low glucose concentrations was accompanied by an enhanced activation of S6K1. Our data indicated that the specific inhibitors of PI3K and mTOR in these cells impaired the effect of high glucose levels on S6K1 activation. We have thereforethen checked the activation level of AKT, as well as and PTEN protein, at low and high glucose levels. Our data showed that N2A cells exposed to low glucose levels recorded the following: AMPK was activated, AKT was highly activated and S6K1 was inhibited. GLP-1 treatment decreased both AMPK and AKT activity at a low glucose concentration. In contrast, PASK knockdown impaired AMPK activation at a low glucose concentration, and this effect could also explain the full activation of S6K1, while AKT was still activated in the absence of GLP-1

treatment, although the activation level was significantly lower than in the control cells. It has been reported that glucose starvation enhancesenhanced AKT and GSK3β activity mediated through AMPK activation in both cardiac and skeletal myocytes and in primary skeletal myofilaments (Chopra et al. 2012). AMPK also phosphorylates the TSC2 inhibiting mTOR/S6K1 pathway. Under a glucose deficitthese conditions, the feedback inhibition of this pathway mediated by S6K1 was not active, and the mTORC2 was highly activated by glucose starvation. This pathway will provide survival signals for these cells (Chopra et al. 2012). Additionally. In accordance with these results, our data showed that AMPK was activated, AKT was highly activated and S6K1 was inhibited in neuroblastoma cells exposed to low glucose levels. GLP-1 treatment decreased both AMPK and AKT activity at low glucose concentration. In contrast, PASK knockdown impaired AMPK activation at low glucose concentration, and this effect could also explain the full activation of S6K1, while AKT was still activated in the absence of growth factors, although the activation level was significantly lower than in the control cells. Under these conditions, S6K1 should exert a feedback inhibitory effect on this pathway by phosphorylating the insulin receptor substrate (IRS) (Khamzina et al. 2005).

The contra-regulatory effect of AMPK on mTOR/S6K1 pathway was confirmed in *Pask*-silencend cells. As well as in control cells, AICAR treatment decreased S6K1 activity. AKT activation. The activation of AKT also regulates the phosphatase activity of PTEN, which controls the phosphorylation state of intermediaries of this pathway. AKT activation stimulates PTEN activity producing a negative feedback effect (Al-Khouri et al. 2005; Blanco-Aparicio et al. 2007). The level of PTEN expression was not modified in PASK-silenced neuroblastoma cells. Our results showed that AKT activation was lower after PASK-silencing. Nevertheless, the activation was still significantly higher than at high glucose levels, suggesting that survival pathways may be still be functional, and perhaps other signaling proteins besides AMPK may regulate this process in neuroblastoma cells.

In conclusion, our data indicated that PASK is a nutrient and hormonal sensor in neuroblastoma cells and in the VMH and LH from rat hypothalamic slices. Low glucose levels upregulated PASK In neuroblastoma cells. In these cells, at least, there is a coordinated response with the other metabolic sensors. Thus, the knockdown of PASK impaired not only the response of the AMPK and mTOR/S6K1 pathways at low and high glucose levels, but also some of the GLP-1 effects observed at the level of both the gene expression level and the activity of AMPK and S6K1 activity (Fig. 9).. PASK-deficient N2A cells failed to detect the availability of nutrients. Our data are consistent with findings that low glucose levels increase the expression of PASK, suggesting that lower levels of PASK in these cells may be used to report the presence of high levels of glucose. The metabolic sensors in the brain have been directly related to feeding behavior, bodyweightbody weight and energy homeostasis. Our data suggest that PASK might also play an important role in feeding behavior. PASK-deficient mice had a phenotype resistant to diet-induced obesity. It would be interesting to analyze the state of the AMPK and mTOR/S6K1 pathways in these mice.

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LEGENDS TO FIGURES

Fig. 1 **PASK is expressed in the rat hypothalamus and neuroblastoma cells. *Pask* mRNA expression is regulated by glucose and siRNA targeting *Pask* gene. a)** In situ hybridization to detect the expression of *Pask* mRNA in the hypothalamus. The photomicrographs are of 20 µm sections of rat brain showing the 3rd ventricle (3V) and the hemi-hypothalamus. The sections were processed for in situ hybridization with a digoxigenin labeled Pask RNA antisense probe (upper panels) or sense probe (bottom panels). The right panels show a higher magnification of the squared area corresponding to the LH. The photomicrographs correspond to two independent experiments. **b)** Immunocytochemistry of PASK in N2A cells. N2A fixed cells were incubated in the presence of either an anti-PASK antibody or a preimmune serum (immunostaining negative control). PASK location was visualized in red (Texas Red coupled to a secondary antibody). Nuclei were stained with DAPI (blue fluorescence). **c)** *Pask* mRNA levels in N2A cells treated for 3 h ~~hours~~ in a medium that contained 0.5 or 10 mM glucose, quantified by real time RT-PCR and normalized to *β-actin* mRNA levels. The bars represent the mean ± SEM of 5 experiments performed in duplicate. The values are relative to the data obtained at 0.5 mM glucose that was considered as 1. **p<0.01 10 mM glucose vs. 0.5 mM glucose treatment**. d)** Inhibition of *Pask* mRNA in PASK-silenced neuroblastoma N2A cells. Bars represent the mean ± SEM (n = 5) of *Pask* mRNA levels normalized to *β-actin* in cells transfected with siRNA targeting *Pask* gene (PASK siRNA) or negative control siRNA (negative control siRNA). The values are relative to the data obtained in cells transfected with negative control siRNA that was considered as 1. ***P<0.001 PASK siRNA vs. negative control siRNA.

Fig. 2 **Glucose and GLP-1 modulate *Pask* gene expression in the VMH and LH areas.** Organotypic hypothalamic slices of 300 µm were glucose-starved for 2 h and then cultured for 3 h in a medium containing 1, 2.5 or 10 mmol/l glucose in the presence or absence of 10 nM GLP-1. The VMH and LH areas were dissected from slices by micropunching. **a)** RT-PCR analysis of *Sf-1*, *Prepro-orexin* and *β-actin* mRNA expression in the VMH and LH. **b, c)** *Pask* mRNA was quantified by real-time RT-PCR analysis. ~~a)~~ Bars represent *Pask* mRNA levels normalized by RNA *18s* and referred to the value obtained under the 2.5 mM-glucose condition~~, considered as 1. ***P<0.001 10 mM glucose vs. 2.5 mM glucose. b) Bars represent *Pask* mRNA levels normalized by RNA *18s* and referred to the value obtained~~ in the absence of GLP-1, ~~which was~~ considered as 1. Data are expressed as means ± SEM; n = 4-5 independent experiments performed in duplicate.*P<0.05,~~.~~ ***P<0.001 10 mM glucose vs. 2.5 mM glucose; †P<0.05, ††P<0.01, ††† P<0.001~~,~~ absence of GLP-1 vs. the presence of 10 nM GLP-1.

Fig. 3 **Effects of PASK knockdown on the expression of several genes in neuroblastoma cells**. PASK knockdown N2A and control cells were serum-starved for 2 h and then incubated in a medium containing

either 0.5 mM or 10 mM of glucose for 3 h in the presence or absence of 10 nM GLP-1. Quantity real-time PCR was used to analyze the expression of several genes as shown in Online Resource 1. The mRNA levels of the different genes were normalized by mRNA of *β-actin* used as housekeeping gene. The value obtained at 0.5 mM glucose without GLP-1 was taken as 1. Results are means ± SEM; n = ~~3~~ 4~~-5~~. *P<0.05, **P<0.01, ***P<0.001 10 mM glucose vs. 0.5 mM glucose; ~~†P<0.05,~~ ††P<0.01, †††P<0.001~~,~~ absence of GLP-1 vs. the presence of 10 nM GLP-1~~; and~~ #P<0.05, ##P<0.01 PASK siRNA vs. negative control siRNA.

Fig. 4 **Effects of glucose and GLP-1 on the expression of PASK, PGC-1α and on the ATP content in *Pask* gene knockdown neuroblastoma cells.** PASK knockdown N2A (PASK siRNA) and control cells (negative control siRNA) were serum-starved for 2 h and then incubated in a medium containing either 0.5 mM or 10 mM of glucose in the presence or absence of 10 nM GLP-1 for 5, 16 h. Neuroblastoma cells were immediately lysed in RIPA buffer and exposed to microwave irradiation for 5 s, after which they were processed for western blot analysis. The blots were reprobed for β-ACTIN to normalize the results **(a, b, d). a)** Inhibition of PASK protein expression in PASK knockdown cells. Immunoblot analysis of PASK. Bar graphs represent the densitometric values, normalized by β-ACTIN. The results are means ± SEM; n = 3-4. **b, d)** Glucose and GLP-1 effect in PASK and PGC-1α expression. Immunoblot analysis of PASK **(**16 h) **(b)** or PGC-1α **(**5 h) **(d).** The value of negative control siRNA at 0.5 mM glucose without GLP-1 was taken as 1. ***P<0.001 10 mM glucose vs. 0.5 mM glucose; ††~~,~~ ~~##~~P<0.01 absence of GLP-1 vs. the presence of 10 nM GLP-1;~~and~~ ###P<0.001 PASK siRNA vs. negative control siRNA. The bottom panels correspond to representative western blots of the data indicated in the graphs **(a, b, d). c**) Cellular ATP content normalized by mg of total protein in cell extracts treated for 2 hours with different glucose concentrations. Bars represent the means ± SEM of 5 experiments developed in duplicate. The results are expressed as a percentage of negative control siRNA at 0.5 mM glucose that was considered 100 % *P<0.05 10 mM glucose vs. 0.5 mM glucose; #P<0.05 PASK siRNA vs. negative control siRNA.~~%.~~

Fig. 5 **Impaired effects of glucose and GLP-1 on the expression and activation of AMPK and ACC in *Pask* gene knockdown neuroblastoma cells.** PASK knockdown N2A (PASK siRNA) and control cells (negative control siRNA) were serum-starved for 2 h and then incubated in a medium containing either 0.5 mM or 10 mM of glucose for 2 h with the presence of 10 nM during the last 10 min, in the case of activity determination (**a, c** and **d**). On the other hand, total protein valuation was performed in cells incubated with different glucose concentrations and in the presence or absence of 10 nM GLP-1 for 5 h (**b**). Neuroblastoma cells were immediately lysed in a RIPA buffer and exposed to microwave irradiation for 5 s, after which they were processed for western blot analysis of phospho-AMPK (Thr-172) (P-AMPK) (**a, d**) and total AMPK (AMPK) (**b, d**) and of phospho-ACC (Ser-79) (P-ACC) and total ACC (ACC) (**c, d**). The blots were reprobed for β-ACTIN to normalize the results. Bar graphs represent means ± SEM; n = 3-4 of

the densitometric values, normalized by β-ACTIN and by non-phosphorylated forms. Panel **d**) shows representative western blots of the graphs **a**, **b** and **c**, respectively. The value of negative control siRNA at 0.5 mM glucose without GLP-1 was taken as 1.~~,~~ **P<0.01, ***P<0.001 10 mM glucose vs. 0.5 mM glucose; ††P<0.01,~~†††~~ ~~, ‡‡‡~~P<0.01~~001,~~ absence of GLP-1 vs. the presence of 10 nM GLP-1;~~ and~~ #P<0.05, ##P<0.01, ###P<0.001 PASK siRNA vs. negative control siRNA.

Fig. 6 **Impaired effects of glucose and GLP-1 on the expression of LKB1 and CaMKKβ in *Pask* gene knockdown neuroblastoma cells**. PASK knockdown N2A (PASK siRNA) and control cells (negative control siRNA) were serum-starved for 2 h and then incubated in a medium containing either 0.5 mM or 10 mM of glucose for 5 h. In some cases, 10 nM GLP-1 was added during the incubation. Neuroblastoma cells were immediately lysed in a RIPA buffer and exposed to microwave irradiation for 5 s, after which they were processed for western blot analysis of LKB1 (LKB1) (**a, c**) and CaMKKβ (CaMKKβ) (**b, c**). The blots were reprobed for β-ACTIN. Bar graphs represent the densitometric values, normalized by β-ACTIN. The results are means ± SEM; n = 3-4. Panel **c**) shows representative western blots of graphs (**a**) and (**b**), respectively. The value of negative control siRNA at 0.5 mM glucose without GLP-1 was taken as 1.~~ **~~,~~ ††~~P<0.01 10 mM glucose~~absence of GLP-1~~ vs. 0.5 mM glucose;~~ the presence of 10 nM GLP-1 and~~ #P<0.05;~~ and~~ ##P<0.01 PASK siRNA vs. negative control siRNA

Fig. 7 **Impact of several protein kinases and phosphatase inhibitors on the GLP-1-induced inhibition of S6K1 activity at high glucose concentration**. N2A cells were incubated in the presence of 10 mM glucose for 4 h, treated or not with several inhibitors for 30 minutes (Table 1), in the absence (**a**) or presence (**b**) of 10 nM GLP-1 over the last 10 min. Cell lysates were exposed to microwave irradiation for 5 s and then processed for western blot analysis of phospho-S6K1 (Thr389) (P-S6K1) and total S6K1 (S6K1) (**a, b**). Densitometric values were normalized by β-ACTIN and by non-phosphorylated forms. **a)** The value of 10 mM glucose without inhibitors was taken as 1 (C). The results are means ± SEM; n = 3-4. **P<0.01, 10 mM glucose compared with 0.5 mM glucose. **b)** The value obtained in the cells treated with 10 mM glucose and 10 nM GLP-1 without inhibitors (C) was taken as 1. The results are means ± SEM; n = 3-4. *P<0.05, **P<0.01, ***P<0.001 vs. without inhibitors.

Fig. 8 **Impaired effects of glucose and GLP-1 on the activity of S6K1 and AKT, and on the expression of PTEN, in *Pask* gene knockdown neuroblastoma cells**. PASK knockdown N2A (PASK siRNA) and control cells (negative control siRNA) were serum-starved for 2 h and then incubated in a medium containing either 0.5 mM or 10 mM of glucose for 2 h with 10 nM during the last 10 min, in the case of activity determination (**a, b** and **d**). On the other hand, total protein valuation was performed in cells incubated with different glucose concentrations and in the presence or absence of 10 nM GLP-1 for 5 h (**c**

and **d**). Neuroblastoma cells were immediately lysed in a RIPA buffer and exposed to microwave irradiation for 5 s, after which they were processed for western blot analysis of phospho-S6K1 (Thr389) (P-S6K1) and total S6K1 (S6K1) (**a, d**); of phospho AKT (Ser 473) (P-AKT) and total AKT (AKT) (**b, d**) and of total PTEN (PTEN). The blots were reprobed for β-ACTIN. Panel d) shows the representative western blots of graphs **a**), **b**) and **d**), respectively. Bar graphs represent the densitometric values, normalized by β-ACTIN and by non-phosphorylated forms. The results are means ± SEM; n = 3-4. The value of negative control siRNA at 0.5 mM glucose without GLP-1 was taken as 1.~~,~~ ***P<0.001 10 mM glucose vs. 0.5 mM glucose; †P<0.05, ††P<0.01, †††P<0.001~~,~~ absence of GLP-1 vs. the presence of 10 nM GLP-1~~; and~~ ##P<0.01 and ###P<0.001 PASK siRNA vs. negative control siRNA.

Fig. 9 **Representative diagram of glucose and GLP-1 effect on the activity of AMPK and S6K-1.**

The activation of AMPK depends on the glucose levels. **a)** Fall in glucose (dark rows and shapes) leads to an increased transcription of *Lkb1* and AMP/ATP ratio. The elevation of AMP/ATP ratio activates AMPK by phosphorylation. AMPK inactivation is mediated by the action of phosphatases such as PP2B. AMPK activation promotes inactivation of ACC and mTORC1, preventing the activation of S6K1. GLP-1 stimulates PKA protein and exerts some effects in a PASK dependent way: (1) Stimulation of PP2B promoting the inactivation of AMPK and (2) inactivation of S6K1. **b)** Fall glucose in PASK-deficient cells, leads to a decreased expression of *Lkb1* and AMP/ATP ratio and, consequently, inactivation of AMPK and activation of ACC, mTORC2, and S6K1.

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