



# Localization of Human Glutamate Dehydrogenases Provides Insights into Their Metabolic Role and Their Involvement in Disease Processes

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## Abstract

Glutamate dehydrogenase (GDH) catalyzes the reversible deamination of L-glutamate to  $\alpha$ -ketoglutarate and ammonia. In mammals, GDH contributes to important processes such as amino acid and carbohydrate metabolism, energy production, ammonia management, neurotransmitter recycling and insulin secretion. In humans, two isoforms of GDH are found, namely hGDH1 and hGDH2, with the former being ubiquitously expressed and the latter found mainly in brain, testis and kidney. These two iso-enzymes display highly divergent allosteric properties, especially concerning their basal activity, ADP activation and GTP inhibition. On the other hand, both enzymes are thought to predominantly localize in the mitochondrial matrix, even though alternative localizations have been proposed. To further study the subcellular localization of the two human iso-enzymes, we created HEK293 cell lines stably over-expressing hGDH1 and hGDH2. In these cell lines, immunofluorescence and enzymatic analyses verified the overexpression of both hGDH1 and hGDH2 iso-enzymes, whereas subcellular fractionation followed by immunoblotting showed their predominantly mitochondrial localization. Given that previous studies have only indirectly compared the subcellular localization of the two iso-enzymes, we co-expressed them tagged with different fluorescent dyes (green and red fluorescent protein for hGDH1 and hGDH2, respectively) and found them to co-localize. Despite the wealth of information related to the functional properties of hGDH1 and hGDH2 and the availability of the hGDH1 structure, there is still an ongoing debate concerning their metabolic role and their involvement in disease processes. Data on the localization of hGDHs, as the ones presented here, could contribute to better understanding of the function of these important human enzymes.

**Keywords** Human glutamate dehydrogenases (hGDH1 and hGDH2) · Localization · Human disease · Metabolism

## Introductory Remarks

Glutamate dehydrogenase (GDH) catalyzes the reversible deamination of L-glutamate to  $\alpha$ -ketoglutarate and ammonia using NAD<sup>+</sup> and NADP<sup>+</sup> as cofactors [1]. As shown by its ubiquitous existence in almost all living species, GDH is a key enzyme for metabolism and therefore considered to be essential for cell survival [2]. In mammals, inside mitochondria, its main metabolic function is to fuel aerobic metabolism through the Krebs cycle for energy

production, interconnecting in parallel amino acid and carbohydrate metabolism [3, 4]. In addition, it contributes to other important tissue and non-tissue specific processes, including autophagy, neurotransmitter recycling, ammonia management and insulin secretion [5–11]. To perform these processes, humans possess two GDH isoforms, hGDH1 and hGDH2, respectively, with the latter emerging from the former through retroposition in the common ancestor of modern primates more than 23 million years ago [12–16]. Being endowed by favorable allosteric regulatory mutations that diversified its function from the parental hGDH1 gene, hGDH2 persisted during evolution and adapted to the changing needs of the primate nervous and other systems [13, 17, 18]. Among the properties that distinguish hGDH2 from its parental hGDH1 enzyme are low basal activity that is nonetheless highly amenable to ADP and L-leucine activation, increased GTP resistance and decreased heat stability [19,

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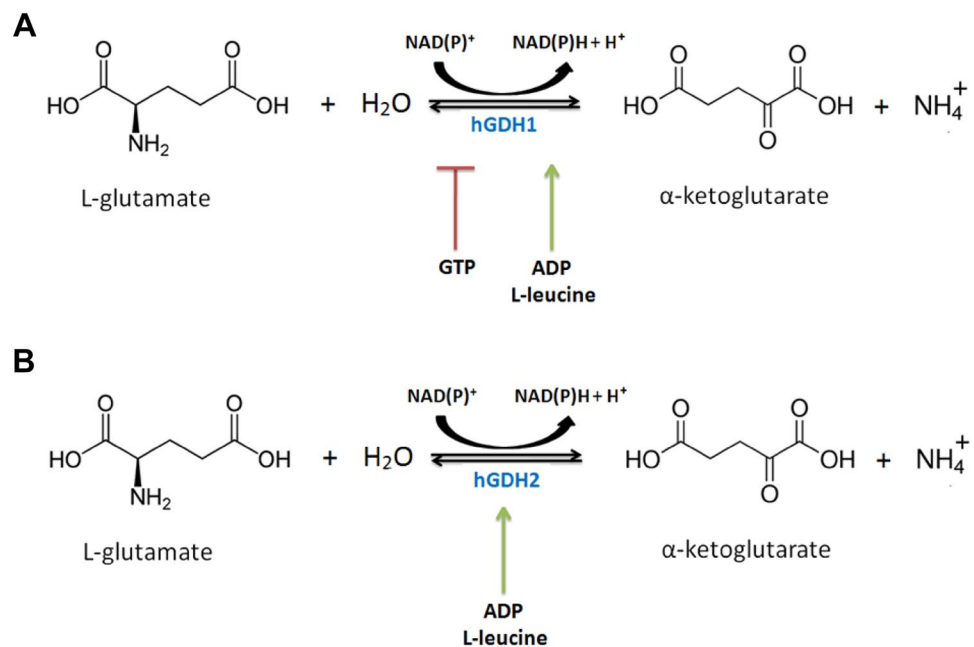
20]. In this work, we will expand further on the localization of the two human iso-enzymes aiming to probe their role in cellular metabolism and human disease processes.

## The hGDH Reaction and its Regulation

Despite decades of extensive research, the direction of the highly-regulated GDH reaction in mammals (Fig. 1), including humans, is a matter of active debate [21–23]. Even though this reaction is reversible, and its rate influenced by multiple metabolic mediators, there seems to be a preference in humans for the GDH reaction to proceed in the direction of  $\alpha$ -ketoglutarate production [24, 25]. The main argument for this is that the high  $K_m$  of the enzyme for ammonia (in the mmolar range) corresponds to ammonia levels that would otherwise be toxic to mammalian cells [10, 26, 27]. As a counterargument, even though under physiological conditions ammonia levels in human tissues are low, they can reach mmolar levels during hypereammonemia states [28]. In addition, inside mitochondria, the ammonia levels can locally increase to levels that would allow the GDH reaction to operate in the reductive amination reaction [22, 29]. Finally, as ammonia accumulates in the tumor micro-environment, due to poor blood supply and high production rates by glutamine catabolism, it is recycled back via GDH to support amino acid synthesis for the rapidly proliferating breast cancer cells, accelerating tumor growth [30].

Irrespective of the preferred direction of the reaction, it seems that the enzyme displays high *in vivo* activity with the flux through its pathway being constantly regulated [31]. This regulation is of special importance for the hGDH2 enzyme, which, in contrast to hGDH1 that displays a significant (35–40%) basal activity in the absence of allosteric effectors, has a negligible (3–5%) activity in the absence of ADP and/or L-leucine [32, 33]. This low basal activity of hGDH2 is though amenable to full activation by ADP and/or L-leucine, permitting the enzyme to act as a molecular switch in metabolic processes accompanied by significant fluctuations of these two metabolic intermediates [20]. This remarkable property was conferred to the hGDH2 enzyme by the Arg443Ser evolutionary amino-acid substitution [17]. Concerning the inhibitory regulation of these human iso-enzymes, hGDH1 is very sensitive to inhibition by GTP, and this inhibition displays a significantly co-operative behavior (Fig. 1) [34]. This property is thought to be of importance for not overloading the Krebs cycle when the cell has been saturated in energy, as GTP produced from the function of the Krebs cycle just downstream of the point of entrance of  $\alpha$ -ketoglutarate produced by the hGDH1 reaction diffuses back to inhibit hGDH1 (negative feedback regulation) [5]. In contrast, hGDH2 shows a marked resistance to GTP inhibition, owing to the Gly456Ala substitution (Fig. 1) [18]. We should note at this point that even though ADP, L-leucine and GTP are the main allosteric regulators of the two human GDH iso-enzymes, there are numerous metabolic mediators and exogenous compounds that exert an effect on hGDH1 and hGDH2, most of the time with differing potencies [20].

**Fig. 1** Reversible deamination of L-glutamate to  $\alpha$ -ketoglutarate and ammonia by (A) hGDH1 and (B) hGDH2. NAD<sup>+</sup> and NADP<sup>+</sup> act as cofactors of the reaction. In the absence of activators, basal activity of hGDH1 and hGDH2 are 3–8% and 30–35% of their maximal activity, respectively. hGDH1 is allosterically activated by ADP and L-leucine and inhibited by GTP, whereas hGDH2 is insensitive to GTP inhibition but amenable to full activation by ADP and L-leucine

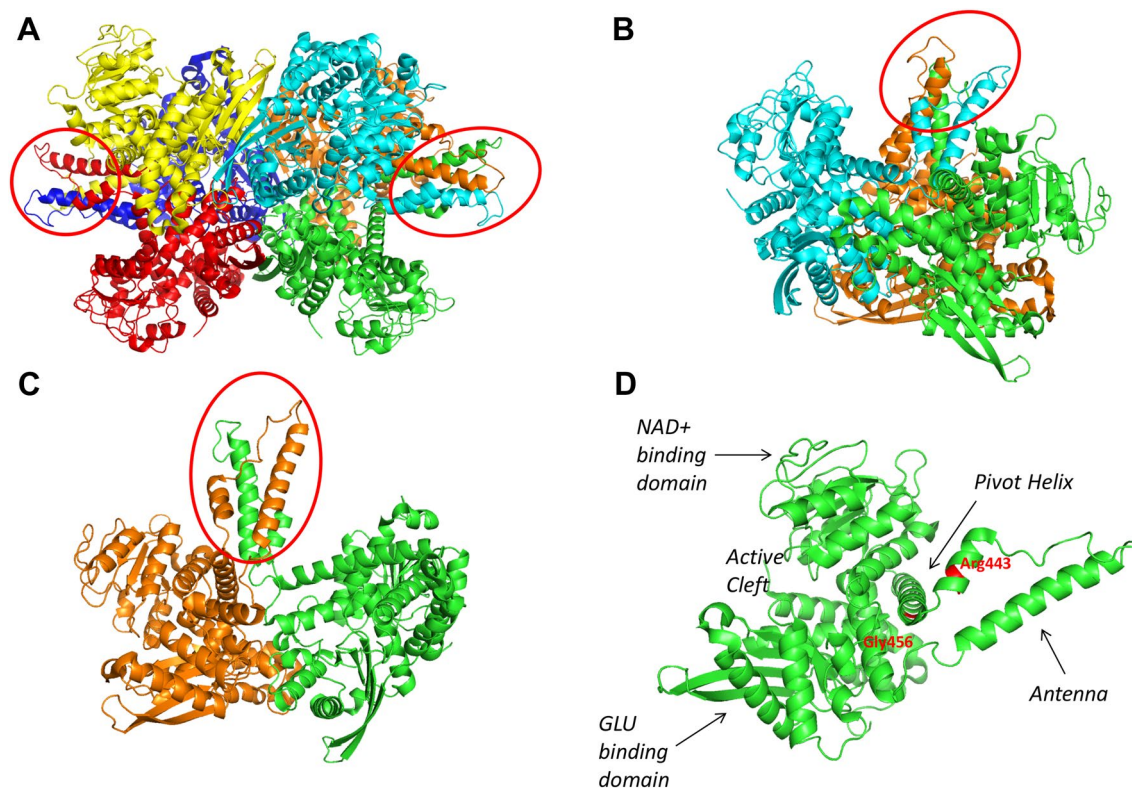


## Structure–Function Relationships of hGDHs

Despite their significantly diverging functional properties, hGDH1 and hGDH2 show very high amino-acid sequence homology, differing in their mature form in only 15 of their 505 amino acids (97% sequence homology), with these amino acid substitutions accumulating during evolution of the primate lineage [5, 14]. Considering their functional divergence, it is expected that these few differences in the primary structure of the two iso-enzymes translate to significant alterations in their tertiary conformation. Indeed, crystallographic studies in the bovine GDH, as well as in hGDH1 [35–38], have indicated that the Arg443Ser and Gly456Ala evolutionary amino acid substitutions (crucial for the molecular evolution of hGDH2) form part of critical regulatory regions of the mammalian enzyme, termed antenna and pivot helix, respectively (Fig. 2) [13, 39]. The antenna protrudes from the main core of the mammalian enzyme, formed by the NAD<sup>+</sup> binding domain and the glutamate binding domain surrounding the active cleft, whereas

the pivot helix forms part of the NAD<sup>+</sup> binding domain (Fig. 2). We have previously shown that of great importance for the allosteric regulation of the enzyme is the interaction of Arg443 in the descending  $\alpha$ -helix of the antenna of one subunit with Ser409 on the ascending  $\alpha$ -helix on a neighboring subunit [40].

In a quaternary level, the mature hGDH1 enzyme forms hexamers of identical subunits, that essentially consist of a dimer of trimers with the subunits of each trimer interacting at their antennas (Fig. 2) [35]. It is also possible that the hexamers form homopolymers, depending on the prevailing conditions [41]. As a crystallographic structure of hGDH2 has not been published yet, we cannot be certain which are the structural consequences of the evolutionary amino acid substitutions that gave hGDH2 its unique functional properties. Also, it is currently unknown if, in the tissues that both hGDH enzymes are expressed, they form hetero-hexamers, which are expected to differ markedly in their functional properties from the homo-hexamers that have been extensively studied thus far.



**Fig. 2** Structure of hGDH1 as (A) hexamer, (B) trimer, (C) dimer and (D) monomer. Each subunit (monomer) of the hGDH1 hexamer (composed essentially from a dimer of trimers) is represented in different color. In sections A, B and C, the red circle indicates the interaction of the antennas of adjacent subunits. In section D (monomer of hGDH1), the antenna, pivot helix, active cleft, and the NAD<sup>+</sup> and glutamate (GLU) binding domains are pointed out. Also in section

D, the Arg443 and Gly456 residues, two of the amino acids differing between hGDH1 and hGDH2 and sites of the two most important evolutionary amino-acid substitutions (Arg443Ser and Gly456Ala, located in the antenna and pivot helix, respectively) are shown in red. The cartoon diagram was created from the coordinates of the hGDH1 hexamer (apo-form, PDB code 1 LIF) using the PyMOL Molecular Graphics System, Version 1.4, Schrodinger, LLC

## Expression of hGDH1 and hGDH2 in Human Tissues

Despite current lack of knowledge on the *in vivo* existence of hGDH1/hGDH2 hetero-hexamers, there has been considerable progress in characterizing the distribution of these iso-enzymes in human tissues [5, 42–45]. As already mentioned, hGDH1 is a housekeeping enzyme, being present in virtually all human tissues, with the highest levels found in the liver. On the other hand, hGDH2 has a significantly different pattern of expression in human tissues, as shown by immunocytochemistry and western blot studies, being expressed in brain (more intensely in astrocytes than in neurons), testis (Sertoli cells), steroid producing tissues and kidney [5, 42, 43].

Concerning the nervous system, where hGDH2, but not hGDH1, has been found in neurons, it is of special interest that both human iso-enzymes are present in astrocytes given the special role these cells have for nervous system function [44–46]. This role includes providing neurons with nutrients or compounds necessary for optimal function and removing excess glutamate from the synaptic cleft to be recycled back to neurons through the consecutive action of astrocytic glutamine synthetase and neuronal phosphate-activated glutaminase [5]. It is possible that the coexistence of both iso-enzymes in astrocytes enables use of hGDH1 for metabolic (housekeeping) purposes, while liberating hGDH2 for use in glutamatergic neurotransmission. The functional properties of hGDH2 (low basal activity amenable to ADP activation and GTP resistance) seem to best serve a role in glutamatergic neurotransmission. Specifically, given that energy-consuming glutamatergic neurotransmission is associated with decrease in ADP levels, this will activate hGDH2, which will continue to function despite Krebs cycle activation replenishing the energy stores of the astrocyte and thus increasing GTP levels [3]. This has recently been shown in transgenic mice overexpressing hGDH2, where under glucose deprived conditions, astrocytes were able to use alternative energy sources [47].

## Subcellular Localization of hGDHs

As the tissue distribution of the hGDH1 and hGDH2 iso-enzymes provides clues to their biological role, the same holds true for their subcellular localization. Extensive studies on the mammalian GDHs, have shown that the enzyme is predominantly localized in the mitochondrial matrix [48–50], even though membrane-bound [51–53] and nuclear forms have been described [54–56].

Despite the numerous studies on the mammalian non-human GDH, hGDH1 and hGDH2 subcellular localization has been less well studied. Concerning the human brain, it has been recently shown using immunohistochemistry that there is hGDH1 and hGDH2 immunostaining in the nuclear membrane of glial and neuronal cells [46, 57]. Using the GFP system, we have in the past few years expressed either hGDH1 or hGDH2 tagged with this fluorescent marker and compared its subcellular distribution with that of appropriate cellular markers. In this way, we have been able to show that both hGDH1 and hGDH2 localize in the mitochondria and the endoplasmic reticulum [32, 58]. Using the same technique, Rosso et al. [59] have concluded that while hGDH2 is specifically targeted to mitochondria, hGDH1 localizes both in mitochondria and the cytoplasm. This enhanced mitochondrial targeting of hGDH2 compared to hGDH1 was attributed to the Glu7Lys evolutionary amino acid substitution in the long cleavable presequence [59]. The importance of this Glu7Lys substitution, even though this time as part of a triple substitution, as well as the slightly more efficient targeting of hGDH2 into mitochondria, were verified in our recent study using isolated yeast mitochondria and human cell lines [60]. In this work, we also showed the importance of the two  $\alpha$ -helices of the 53-amino acid long presequence for the highly efficient import into mitochondria [60].

The studies mentioned above rely on the indirect comparison of tagged hGDH1 and hGDH2 distribution through the use of another subcellular marker and as such they do not perform a direct comparison of the two iso-enzymes. To further characterize the localization of the two human iso-enzymes, we studied the endogenous hGDH localization in HEK293 (Human Embryonic Kidney) cells using anti-hGDH1 and anti-hGDH2 specific antibodies, and then overexpressed hGDH1 and hGDH2 in the same cell line (as well as in HeLa cells; human cervical adenocarcinoma), to verify our results with the endogenous proteins. In addition, to perform a direct comparison of the localization of the two human iso-enzymes, we have concurrently tagged hGDH1 and hGDH2 with different fluorescent dyes (GFP and RFP, respectively) and co-expressed them in mammalian cell lines (HEK293 and HeLa cells). Results showed that both human iso-enzymes colocalize in cellular structures corresponding to mitochondria.



## Methods

### Creation of HEK293 Cell Lines Stably Overexpressing hGDH1 and hGDH2

HEK293 cells stably over-expressing hGDH1 or hGDH2 were created according to the vector (pcDNA<sup>TM</sup>3.1) manufacturer's protocol (Invitrogen, CA, USA). The construct used for this purpose was pcDNA<sup>TM</sup>3.1 (Invitrogen, CA, USA) containing either *GLUD1* or *GLUD2* cDNA. The cell cultures were maintained in 100 mm dishes treated with Dulbecco's Modified Eagles Medium enriched with 10% fetal bovine serum at 37 °C in 5% CO<sub>2</sub>. We transfected HEK293 cells using Lipofectamine 2000 and 24 h post transfection, a medium containing selective antibiotic (Geneticin, Life Technologies) in final concentration of 600 µg/ml was introduced to the cell culture. The cell culture was incubated for another 2–3 weeks until single colonies were visible, while fresh medium with geneticin was introduced every 48 h. Single colonies were transferred in 24 well plates for further expansion and positive colonies were selected based on Western blot, immunocytochemistry and enzymatic activity analyses. Two colonies (col2 and col6, respectively) were selected from both the hGDH1 and hGDH2 overexpressing cells for use in the experiments described below. Each of these experiments was performed at least in triplicate (≥ three biological replicates used).

### Western Blot Analyses

HEK293 cells were harvested from cultures and lysed using ice-cold lysis buffer (10 mM Tris-HCl, 0.5M NaCl, 0.5 mM EDTA, pH 7.4, 1% triton, protease inhibitor mixture). A Bradford kit (Bio-Rad Laboratories, Hercules, CA) was used for total protein quantification. Whole cell lysates were loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on nitrocellulose membrane at 350 mA for 1 h. The nitrocellulose membranes were incubated overnight with anti-GDH1 (1:5000) (Aviva Systems Biology, CA, USA) and anti-hGDH2 (1:5000) [5] primary specific polyclonal antibodies. Next, the samples were incubated with horseradish peroxidase-coupled secondary antibodies (goat anti-rabbit, 1:1000) and visualization of the proteins was done using ECL kit (Thermo Fisher scientific, Life Technologies).

### Subcellular Fractionation Analyses

For the cell compartmentation assay, cells were harvested from 100 mm dishes and centrifuged at 850 g for 2 min. After the cell pellet was obtained, Mitochondria Isolation

Kit for cultured cells (Thermo Fisher Scientific, Life Technologies) was used according to the reagent-based method and following the manufacturer's instructions. In brief, cells were resuspended in Reagent A buffer, supplemented with protease inhibitor, and incubated for 2 min on ice. Reagent B was then added, and samples were vortexed and incubated on ice for 5 min, followed by centrifugation at 700×g for 10 min at 4 °C with Reagent C. The resulting supernatant was collected and subjected to centrifugation at 12,000×g for 15 min at 4 °C, to pellet mitochondria [61]. Total protein quantification of the samples was performed also using the Bradford kit (Bio-Rad Laboratories, Hercules, CA) and samples were loaded on 12% SDS-PAGE gel for electrophoresis. An antibody against MnSOD (also known as SOD2), which is an integral mitochondrial matrix protein [62, 63], was used as a mitochondrial marker evaluating the proper mitochondrial fractions preparation.

### Enzymatic Activity Measurements

Whole cell lysates from HEK293 cells, obtained as described above, were used for enzymatic activity measurements. Total protein quantification of the samples was performed using Bradford kit (Bio-Rad Laboratories, Hercules, CA). Samples was stored at –80 °C and handling for the experiments was always performed on ice. Enzymatic activity measurements were performed spectrophotometrically at 340 nm in the reductive amination direction measuring the concentration reduction of NADPH because of the reaction. The reaction mixture was 1 ml of final volume containing 50 mM triethanolamine-HCl buffer pH 8, 100 mM ammonium acetate, 100 µM NADPH and 2.6 mM EDTA. A final concentration of 10 mM α-ketoglutarate and 1 mM ADP was also added simulating maximal activating conditions for both enzymes. Specific activity was calculated in µmol/mg total protein of the samples/min.

### Immunocytochemistry

Immunocytochemistry was performed in HEK293 cells cultured in 24-well plates with bottom coverslips. When cells reached ~70% confluency, they were fixed with 5% paraformaldehyde (PFA) at room temperature (RT) for 15 min. After fixation, cells were washed twice with phosphate-buffered saline (PBS) (pH 7.5). Blocking was performed using 0.5% triton and 2% normal goat serum (NGS) in PBS for 1 h at RT. Cells were incubated over night at 4 °C with primary antibodies against hGDH1 or hGDH2 diluted 1:5000 in blocking solution. Cells were washed in PBS and incubated with fluorescence-conjugated secondary antibodies (goat anti-rabbit, 1:200) and TO-PRO 3 for nuclear staining (1:1000, Thermo Fisher Scientific, Life Technologies) in PBS. Coverslips were mounted in Dako

(Agilent Technologies) and samples were visualized using laser scanning SP2 confocal fluorescence microscopy (Leica TCS-NT Laser Scanning). Image acquiring was performed using lenses  $\times 10$ ,  $\times 40$  or  $\times 63$  with oil objective. The results were processed in Adobe Photoshop CS6.

### Construct Creation, Transfection and Visualization of Co-expressed Human Iso-enzymes

The constructs used to study the co-localization of the two iso-enzymes, hGDH1 and hGDH2, were the hGDH1/green fluorescent protein (GFP) and the hGDH2/red fluorescence protein (RFP), respectively. To obtain the hGDH1/GFP construct, the cDNA sequence of the *GLUD1* gene was firstly amplified via PCR and fused to the GFP sequence, as previously described [58]. The hGDH2/RFP construct was designed to chimerically express hGDH2 conjugated with RFP at its C-terminus. In more detail, the plasmid vector used was pmCherry-N1 (Clontech Laboratories, Inc., CA, USA) and the entire *GLUD2* sequence was fused into the vector. The forward primer was designed to create an *EcoRI* restriction site (5'-CCGGAATTCATGTACCGCTACCTG GCC-3'). The reverse primer (5'-ATTTCGCGGATCCCC TTTGTGAAGGTCACACCA-3') creates a *BamHI* restriction site and converts the stop codon TAG to AAG. The amplified product was then digested using *EcoRI* and *BamHI* restriction enzymes and introduced in to the pmCherry vector. Ligation was performed overnight at 16 °C using the T4 DNA ligase kit (New England Biolabs), according to the manufacturer's instructions. Bi-directional Sanger sequencing was performed for verification of the proper orientation and sequence of the constructs. Moreover, for fluorescent labeling of mitochondria, the mammalian expression vector *pDsRed2-Mito* was used (BD Biosciences Clontech, Palo Alto, CA, USA).

The two constructs, hGDH1/GFP and hGDH2/RFP, respectively, were co-transfected, in final concentration of 3  $\mu\text{g}/\text{well}$ , in HEK293 and HeLa cell lines using the transfection reagent Lipofectamine 2000 (Invitrogen, CA, USA). Also, 2  $\mu\text{g}$  of plasmid *pDsRed2-Mito* were co-transfected with 2  $\mu\text{g}$  of hGDH1/GFP in HEK293 cells, to label the mitochondrial compartment with red fluorescence. The cells were maintained in six well plates with bottom coverslip and treated with Dulbecco's Modified Eagles Medium enriched with 10% fetal bovine serum at 37 °C in 5%  $\text{CO}_2$  conditions. Transfected cells were incubated for 48 h at 37 °C, and when the expression of the enzymes was maximized, cells were fixed with 5% PFA in slides and visualized using laser scanning SP2 confocal fluorescence microscopy (Leica TCS-NT laser scanning). The lenses used for image acquiring were  $\times 10$ ,  $\times 40$  or  $\times 63$  with oil objective. The results were exported as TIFF files and processed in Adobe Photoshop CS6.

### Statistical Analyses

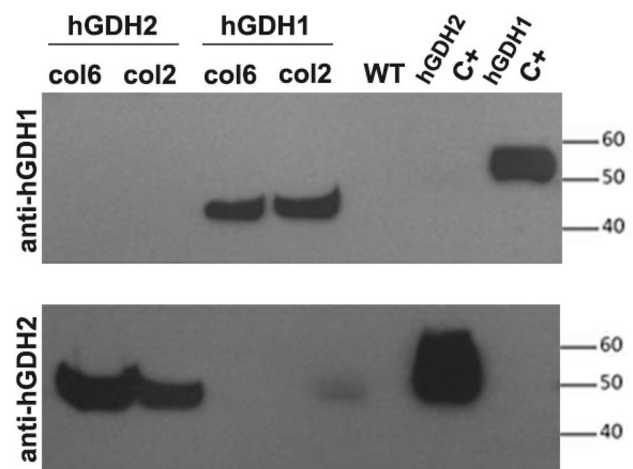
For the statistical analyses, one-way ANOVA was performed using Graphpad prism 6.

### Results

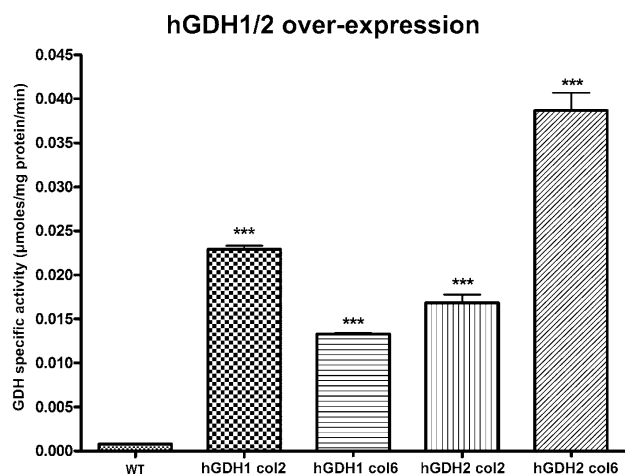
#### Overexpression of Both hGDH1 and hGDH2 Several Times Over the Endogenous Proteins

To verify the stable transformation of the HEK293 cells and study the expression levels of hGDH1 and hGDH2 in these cell lines, we initially performed western blot analyses in whole cell lysates from two different colonies for each iso-enzyme (Fig. 3). For hGDH2, comparing the signal intensity between stable cell lines overexpressing hGDH2 with the signal of the native hGDH2 enzyme showed that the exogenous protein was highly over-expressed (Fig. 3). For hGDH1, under the conditions the experiment was performed, no signal for the endogenous protein was detected.

To functionally quantify the overexpression of hGDHs in stably transfected HEK 293 cells, we measured the enzymatic specific activity in whole cell lysates. As shown in Fig. 4, the hGDH specific activity in the stably over-expressing cell lines was several times over the endogenous hGDH activity. The highest activity was found in the hGDH2 over-expressing col6 cell line (Fig. 4), an activity accounting for



**Fig. 3** Overexpression of the hGDH1 and hGDH2 in stably transfected HEK293 cell lines. Shown are western blot analyses of whole cell lysates from wild-type (WT) and cell lines stably overexpressing hGDH1 and hGDH2 (two different colonies for each enzyme). In the upper panel the anti-hGDH1 antibody was used, while in the lower panel the membrane was blotted for hGDH2 with the specific anti-hGDH2 antibody. Equal amounts of total protein were loaded in each of the first five lanes. In the last two lanes, purified hGDH1 and hGDH2 were used as a control



**Fig. 4** GDH specific activity ( $\mu\text{mol}/\text{mg}$  protein/min) in whole HEK293 cell lysates. The samples used were wild-type (WT) HEK293 cells, stable cell lines overexpressing hGDH1 (hGDH1 col2 and col6, respectively) and stable cell lines overexpressing hGDH2 (hGDH2 col2 and col6, respectively). The GDH specific activity was significantly enhanced in stable cell lines compared to the WT cells (one-way ANOVA,  $p < 0.0001$ ). The highest expression levels were found in the hGDH2 col6 cell line ( $0.03867 \pm 0.003$   $\mu\text{mol}/\text{mg}$  protein/min), an activity 48 times more than the endogenous GDH activity found in the WT HEK293 cells ( $0.0008047 \pm 0.0001$   $\mu\text{mol}/\text{mg}$  protein/min). The activities recorded for the rest of the stable cell lines were  $0.02290 \pm 0.0007$ ,  $0.01329 \pm 0.0002$ , and  $0.01683 \pm 0.0015$   $\mu\text{mol}/\text{mg}$  protein/min for the hGDH1 col2, hGDH1 col6 and hGDH2 col2 cell lines, respectively (28.5, 16.5 and 21 times the endogenous GDH activity).  $***p < 0.0001$  compared to the WT HEK293 cell GDH specific activity

an over-expression level of 48 times over the endogenous GDH activity.

### Mitochondrial Localization of Both Overexpressed hGDH1 and hGDH2

Next, we studied the HEK293 stable cell lines by immunocytochemistry using specific anti-hGDH1 and anti-hGDH2 antibodies. In both cases, we were able to verify the overexpression of the exogenous iso-enzyme over the endogenous, as well as its presence in coarse cytoplasmic structures reminiscent of mitochondria (Figs. 5 and 6). Interestingly, in both cases, a similar immunofluorescence pattern, even though of considerably less intensity, was found in the wild-type HEK293 cells, indicative of a comparable distribution of the endogenous hGDH1/2 iso-enzyme (Figs. 5 and 6).

To confirm the mitochondrial localization of the enzyme, we performed subcellular fractionation experiments followed by western blot analyses. In all four stable cell line clones studied (two for each iso-enzyme), we detected the presence of hGDH1 and hGDH2 in the mitochondrial fraction (Fig. 7). For the cytosolic fraction, there was absence of hGDH1, and only small amounts of hGDH2 (Fig. 7). This

could represent alternative localization of the overexpressed hGDH2 iso-enzyme or be due to small amount of cross-contamination, as shown by experiments using MnSOD as a mitochondrial marker (Fig. 8).

### Co-expressed hGDH1/GFP and hGDH2/RFP Co-localize in Human Cell Lines

When we co-expressed *hGDH1/GFP* and *hGDH2/RFP* constructs in HEK293 cells, we observed that both displayed a similar punctate distribution, reminiscent of mitochondria (Fig. 9). The immunofluorescence of both chimeric proteins was overlapping (Fig. 9). The same results were obtained when we co-transfected *hGDH1/GFP* and *hGDH2/RFP* in HeLa cells (Fig. 10). The co-transfection experiment using *hGDH1/GFP* and *pDsRed2-Mito* in HEK293 cells, produced a yellow fluorescent signal on dual-color imaging, corroborating the *hGDH1/GFP* mitochondrial localization (Fig. 11).

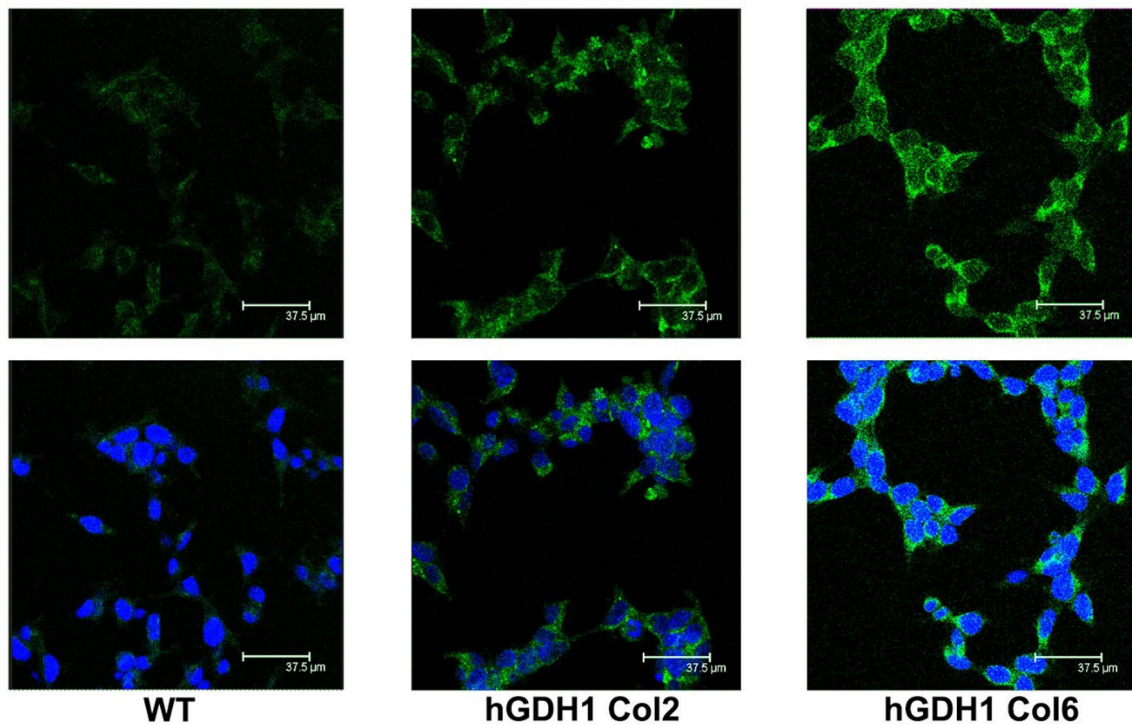
### Discussion

The results of the studies presented above point towards a similar behavior of both iso-enzymes when over-expressed in human cell lines (either separately, or, for the first time, concurrently) as far as their localization is concerned, consistent with predominantly mitochondrial localization. This is of importance, given that these iso-enzymes co-exist in several tissues and cell types, where they are thought to play different roles due to their different regulatory properties [3, 5, 43]. Thus, it is probable that during primate evolution the new hGDH2 iso-enzyme adopted a novel role mainly due to its unique enzymatic properties rather than by being differentially targeted inside cells. This is consistent with the experiments we have recently performed in the yeast *S. cerevisiae* as well as in human cell lines to elucidate the properties of the targeting pre-sequence [60].

One could argue that the current results could be due to the over-expression of the two hGDH iso-enzymes. However, we got similar results in western blot studies using two colonies of hGDH1 overexpressing cells (col2 and col6, respectively) and two colonies of hGDH2 overexpressing cells (col2 and col6, respectively), with each of the four colonies showing different levels of overexpression. Also, in cells with transient hGDH1/GFP and hGDH2/RFP overexpression, we observed similar results in immunofluorescence experiments across several levels of overexpression. Finally, we noted similar immunofluorescence patterns using the anti-hGDH1 and anti-hGDH2 antibodies in wild-type HEK293 cells and hGDH1 and hGDH2 overexpressing cells, even though, as expected, the signal was less intense in the WT cells (Figs. 5, 6). These results are compatible to similar experiments performed in different cell systems using



## anti-hGDH1 / TO-PRO3



**Fig. 5** Exogenously introduced hGDH1 localizes in coarse cellular structures reminiscent of mitochondria. Immunocytochemistry was performed using the anti-hGDH1 specific antibody in WT HEK293 and two colonies of HEK293 cell lines stably overexpressing hGDH1

(hGDH1 col2 and col6, respectively). TO-PRO3 was used for nuclei staining and is depicted in blue. The same laser intensity was applied in all cases during confocal microscopy

different methods [32, 58–60]. Also, there have been several reports in the literature of models of hGDH overexpression that were used to study the metabolic role of mammalian GDH without being compromised by the overexpression of the exogenous enzyme. For example, Carobbio et al. overexpressed GDH using a retrovirus expression system in rat and mouse pancreatic islets, as well as in a beta-cell line, for studying the glucose-induced insulin secretion profile [64]. Also, Tanizawa et al. [65, 66] used both the pcDNA3.1 vector used here and a retroviral vector to overexpress hGDH1 in COS-7 and MIN6 cells, respectively, again to study insulin secretion.

There are, also, several papers focusing on over-expression of mammalian GDH in neural tissue. These studies used transgenic mice modified to overexpress mouse mGDH1 or human hGDH2 [67, 68]. One of the pioneers in this field of research is the group of Eli Michaelis, to whom this issue is dedicated [67, 69–73]. Based on an over-expression model of mouse *Glud1* enzyme, they were able to show age-associated changes due to excess glutamate release in the hippocampus and other areas, a phenotype similar to progressive neurodegeneration (see below) [67, 73]. Establishing models of stable overexpression of hGDH1 and hGDH2,

as done here, are needed to study these important enzymes, given their significance for human disorders, as described below.

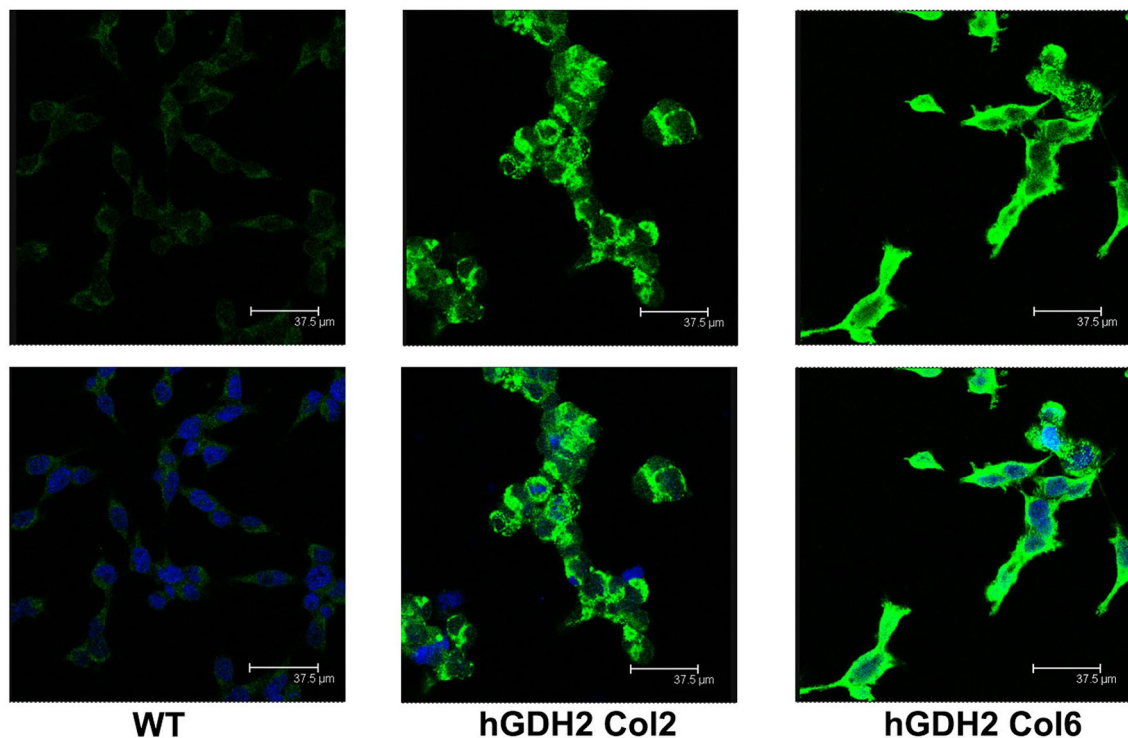
### hGDH and Human Disease

Given the importance of hGDHs for cell metabolism and several tissue-specific functions, such as insulin secretion and neurotransmission, it is not unexpected that several human disorders, especially relating to carbohydrate metabolism and nervous system function, are associated to hGDH1 and hGDH1 deregulation [57, 74, 75]. Inversely, several studies have focused on the pathogenetic role of hGDHs in these human diseases aiming to develop GDH modulating strategies as a therapeutic means.

The first human disease found to be associated with mutations in a hGDH gene was the hyperinsulinism–hyperammonemia (HI/HA) syndrome, a rare autosomal dominant disorder, characterized by hypoglycemic episodes provoked by fasting or high-protein meals [76]. In a breakthrough research effort, HI/HA was found to be caused by activating mutations in the *GLUD1* gene, encoding for hGDH1



## anti-hGDH2 / TO-PRO3



**Fig. 6** Exogenously introduced hGDH2 localizes in coarse cellular structures reminiscent of mitochondria. Immunocytochemistry was performed using the anti-hGDH2 specific antibody in WT HEK293 cells and two colonies of cell lines stably overexpressing hGDH2

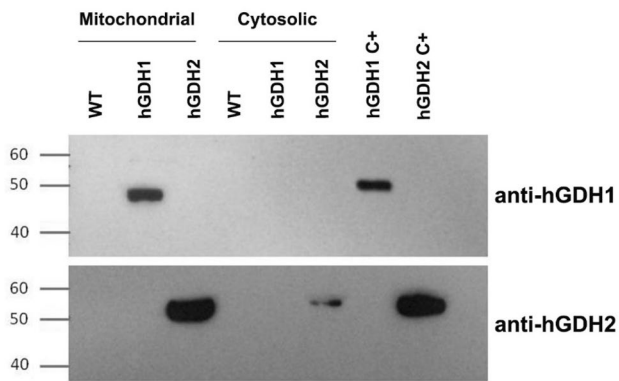
(hGDH2 col2 and col6, respectively). TOPRO3 was used for nuclei staining and is depicted in blue. The same laser intensity was applied in all cases during confocal microscopy

[77]. Since then, many more disease-causing mutations have been identified in exons 6, 7, 11 and 12 in the *GLUD1* gene [78–90]. Most of these missense variants affect the GTP-binding site of the enzyme, abrogating its sensitivity to GTP inhibition, with even the mutations that are remote from the GTP binding site indirectly interfering with the allosteric regulation by GTP. The resulting hyperactive hGDH1 enzyme produces increased ATP levels (increased ATP/ADP ratio) in pancreatic  $\beta$ -cells. Since the ATP/ADP ratio regulates insulin secretion from  $\beta$ -cells, hGDH1 hyperactivity leads to excess insulin release that is inappropriate to the serum glucose levels [91]. It is also possible that these activating mutations in hGDH1 lead to impaired glucagon release from pancreatic  $\alpha$ -cells and possibly increased glucagon-like peptide 1 secretion from L-cells [92, 93]. The evidence above renders hGDHs an attractive target for diabetes treatment [94, 95].

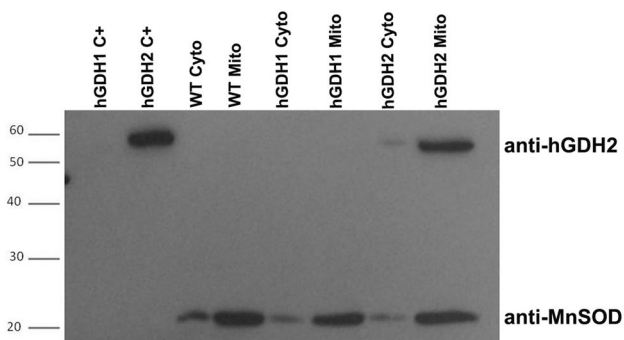
It is interesting that there is another form of congenital hyperinsulinism caused by deregulation of hGDH1 function brought about by deficiency of the short-chain 3-hydroxyacyl-CoA dehydrogenase enzyme (SCHAD, encoded by the *HADH1* gene) [96, 97]. It has been shown that SCHAD strongly interacts with hGDH1 and inhibits its function [98],

possibly in the context of a multiprotein metabolic complex (metabolon) [99]. Interestingly, hGDH1 forms part of other metabolons, as in the case of the BCAT metabolizing enzymes [4, 9].

In cases of the HI/HA syndrome caused by activating mutations of hGDH1, it has been shown that these patients develop neurological manifestations that extend beyond those expected by the hypoglycemic episodes and the coexisting hyperammonemia [100, 101]. Specifically, these patients develop psychomotor retardation, behavioral disorders and a high rate of epilepsy unrelated to hypoglycemia or hyperammonemia [85, 100, 102, 103]. It is possible that the hyperactivity of hGDH1 in brain can lead to depletion of glutamate and consequently depletion of GABA, which is synthesized in neurons by the action of glutamate decarboxylase on glutamate. Reduction of GABA, which is the main inhibitory neurotransmitter in the central nervous system, could lead to epilepsy and dystonia [104–106]. Alternatively, it is possible that the high ammonia levels in the context of the HI/HA syndrome could shift the balance of the hGDH1 reaction towards glutamate formation, creating excess glutamate locally that could create epileptic predisposition and excitotoxicity,



**Fig. 7** Mitochondrial localization of over-expressed hGDH1 and hGDH2. Western blot analyses after subcellular fragmentation (into mitochondrial and cytosolic fractions) of cell lysates of WT and stable cell line colonies (col2 for both hGDH1 and hGDH2 stable cell line, respectively). In the upper panel the anti-GDH1 antibody was used, while the lower membrane was blotted for hGDH2. These results indicate that the overexpressed enzyme is driven into mitochondria, given that under these conditions the endogenous iso-enzyme(s) are not detected. The small cytosolic band in hGDH2 over-expressing cell lines could represent alternative localization of the enzyme or be the result of negligible contamination due to excess of the enzyme (see below). The last two lanes (controls; hGDH1 C+ and hGDH2 C+) were loaded with purified recombinant hGDH1 and hGDH2, respectively



**Fig. 8** Co-localisation of hGDH2 with the mitochondrial marker MnSOD. Western blot analyses after subcellular fragmentation (into mitochondrial-Mito and cytosolic-Cyto fractions) of cell lysates of WT and stable cell line colonies (col2 for both hGDH1 and hGDH2 stable cell line). The anti-hGDH2 antibody was used, while reprob- ing was performed using an anti-MnSOD antibody. Results show that hGDH2 was predominantly localized in the mitochondrial fraction, with only a small amount present in the cytosolic fraction, a pattern comparable to that of MnSOD. The first two lanes (controls; hGDH1 C+ and hGDH2 C+) were loaded with purified recombinant hGDH1 and hGDH2

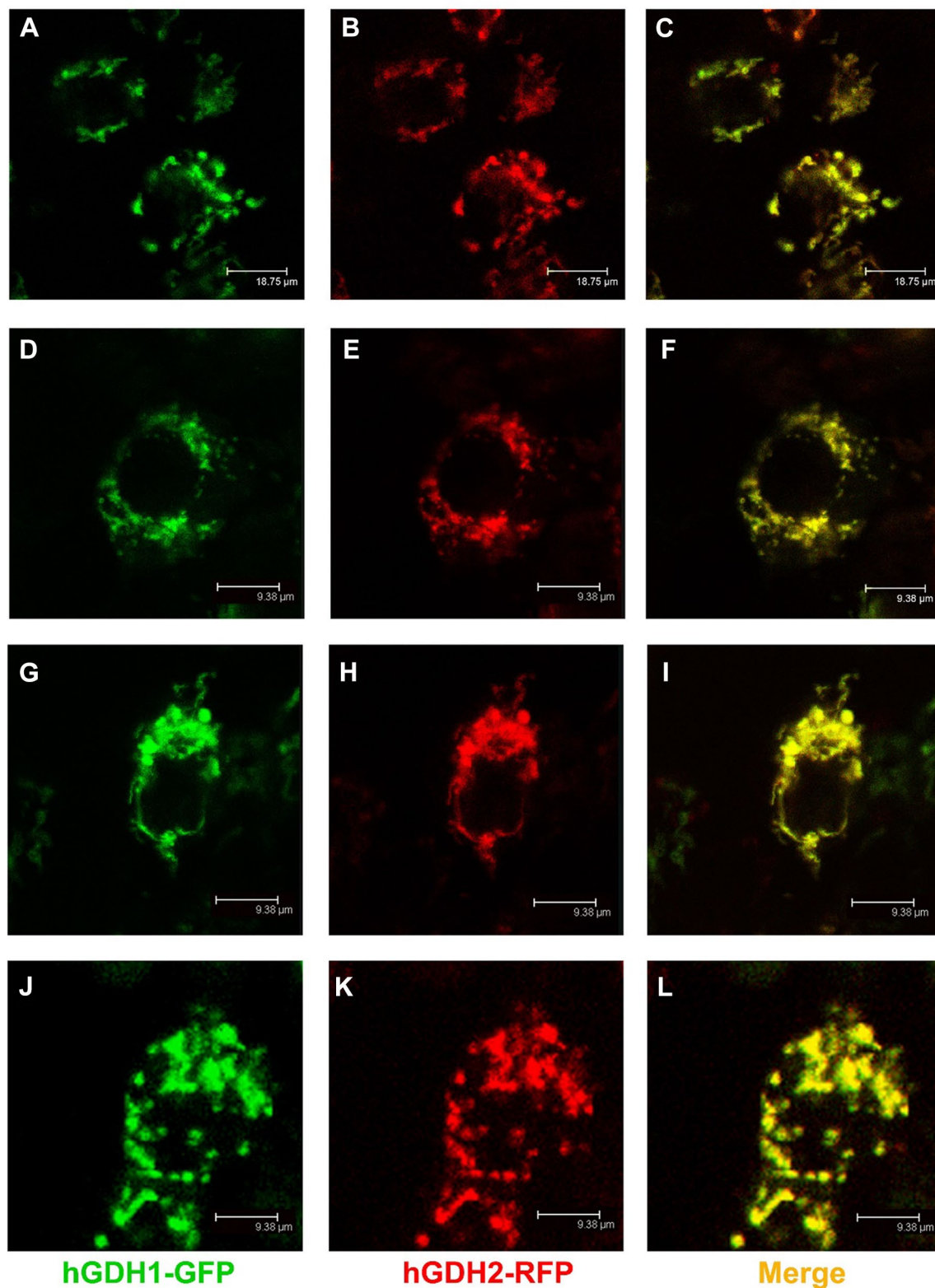
leading to mental retardation. In any case, this syndrome adds evidence for the existing evidence for a role of hGDH1 deregulation in epilepsy [107, 108], paving the way for use of pharmacologic manipulation of the enzyme as an antiepileptic strategy [109].

There are also reports for a possible role of mammalian GDH in attenuating glutamate induced injury following brain ischemia. In the mouse model of Michaelis group with mouse Glud1 overexpression in neurons, the transgenic mice showed increased tolerance to brain ischemia, produced by middle cerebral artery occlusion, an effect attributed to glutamate preconditioning [110]. In this context, it is possible that hGDH modulators could serve as neuroprotective agents in brain ischemia [111]. For example, it has been shown that in a mouse model of middle cerebral artery occlusion, GDH activators provided potent neuroprotection by increasing  $\alpha$ -ketoglutarate levels and, through the TCA cycle, replenishing ATP stores [112]. This could indicate that mouse Glud1 (the orthologous enzyme of hGDH1) mediated glutamate oxidation serves as an energy source in ischemic or other energy or nutrient deprived conditions [113].

Beyond the effects of hGDH1 and mouse Glud1 hyper-activity in the nervous system described above, there is ample evidence that hGDH1/2 deregulation could affect neurodegenerative processes. A recent study from our group [114] showed that a mutation in the *GLUD2* gene (encoding for hGDH2) accelerates Parkinson's disease onset by 6–13 years in men. This *GLUD2* variant (c.T1492G) leads to the Ser445Ala change in hGDH2 and causes a gain of function by increasing the enzyme's basal activity [114]. We can only speculate about the consequences on human brain of the hGDH2Ser445Ala substitution, but it is likely that increased glutamate oxidation could accelerate an ongoing degenerative process [114]. In other words, it could be that glutamate needs to retain appropriate levels in brain tissue, and both glutamate excess and glutamate deficiency could be harmful. On the other hand, it is possible that hGDH2 hyperactivity leads to excess synaptic glutamate, as shown for mouse Glud1 overexpression in the Michaelis transgenic model (see below). Regardless of that and even though the role of hGDH2 in the pathophysiology of Parkinson's disease remains to be determined, these findings build upon existing data of an important contribution of hGDH1/2 deregulation on two other neurodegenerative disorders, namely olivopontocerebellar atrophy and amyotrophic lateral sclerosis [115–120]. This contribution has been known since the 1980s, when the work of Andreas Plaitakis group has shown deregulation of glutamate metabolism and GDH deficiency in patients with various cerebellar, brainstem and anterior horn disorders [115, 117–120].

Increased interest has been shown in recent years in the role of deregulation of glutamate metabolism in Alzheimer's disease, another, far more common, age-related neurodegenerative disorder [121]. In this respect, there are several observations that point towards involvement of hGDH1/2 mediated glutamate metabolism pathways in the neurodegeneration that accompanies Alzheimer's disease or other age-related disease phenotypes. As discussed

### Co-transfection in HEK293 cells

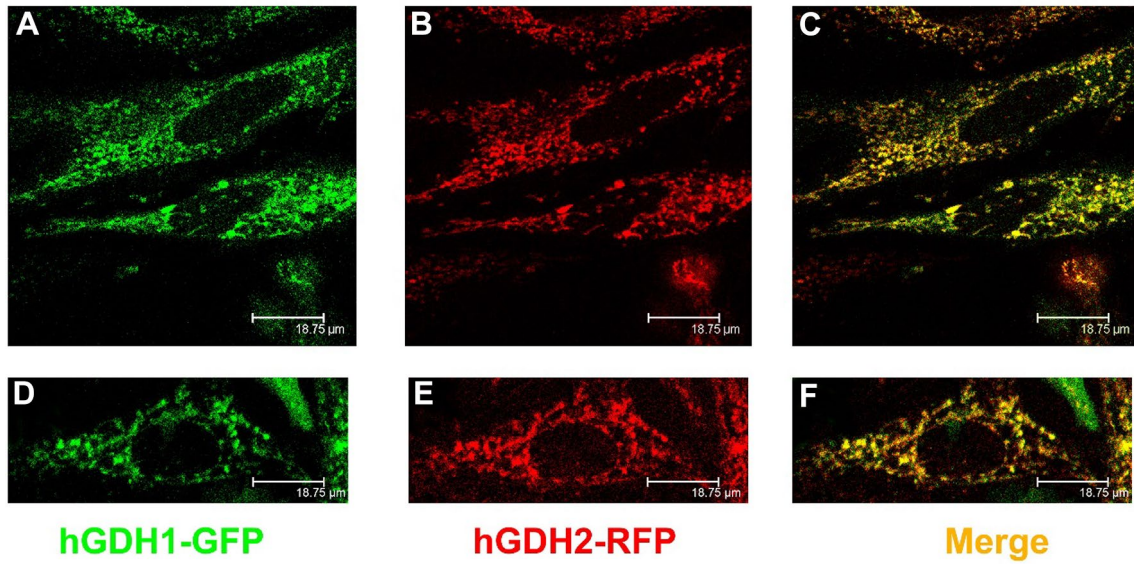


**Fig. 9** Colocalization of hGDH1/GFP and hGDH2/RFP in HEK293 cells. Here we performed confocal imaging of the co-transfection of hGDH1/GFP and hGDH2/RFP constructs in HEK293 cells. The signal originating from hGDH1/GFP is shown in **A**, **D**, **G** and **J**,

whereas in **B**, **E**, **H** and **K** the signal from hGDH2/RFP is detected. Finally, in **C**, **F**, **I** and **L**, the merged signal of the two fluorescent chimeric proteins is shown. Both chimeric iso-enzymes co-localize in structures reminiscent of mitochondria

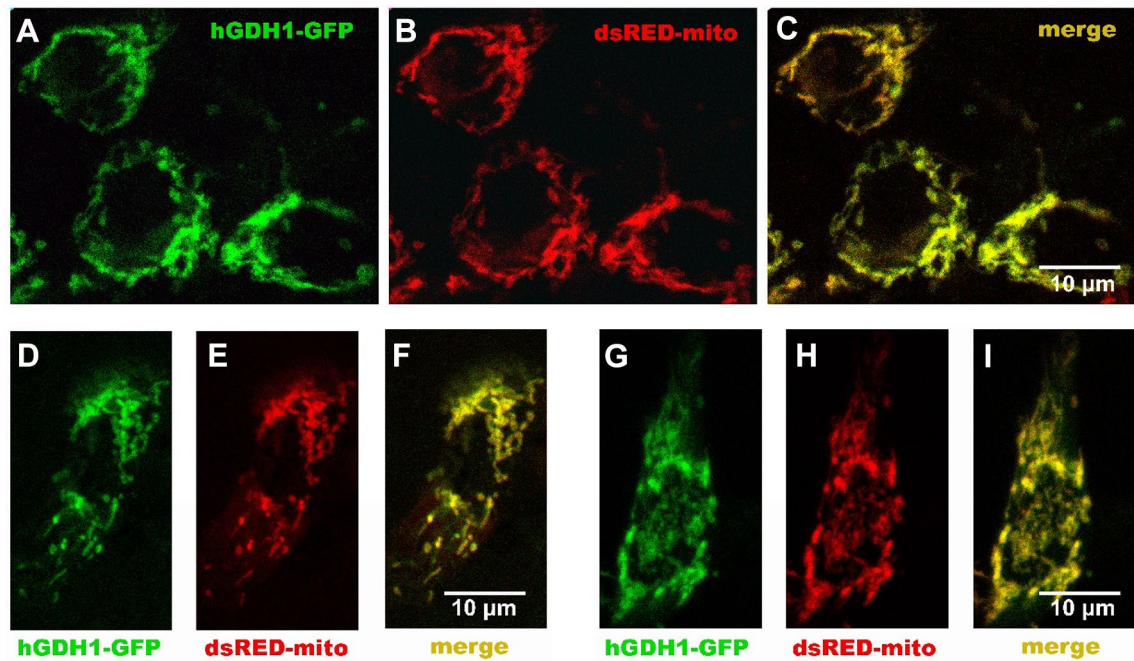


### Co-transfection in HeLa cells



**Fig. 10** Colocalization of hGDH1/GFP and hGDH2/RFP in HeLa cells. Here we performed co-transfection of the hGDH1/GFP and hGDH2/RFP constructs in HeLa cells followed by confocal imaging. **A, D** Signal originating from hGDH1/GFP. **B, E** Signal from

hGDH2/RFP. **C, F** The merged signal of the two fluorescent chimeric proteins. As in similar experiments performed in HEK293 cells, both chimeric iso-enzymes co-localize in structures probably representing of mitochondria



**Fig. 11** Colocalization of hGDH1/GFP and *pDsRed2-Mito* in HEK293 cells. Here we performed co-transfection of the hGDH1/GFP (green) and *pDsRed2-Mito* (red, a mitochondrial marker) constructs in HEK293 cells followed by confocal imaging. **A, D, G** Sig-

nal originating from hGDH1/GFP. **B, E, H** Signal from *pDsRed2-Mito*. **C, F, I** The merged signal of the two fluorescent chimeric proteins. Both chimeric proteins co-localize in mitochondria



above, the group of Elias Michaelis [67] created a transgenic (Tg) mouse model overexpressing mouse *Glud1* that showed constant excess synaptic glutamate release in specific brain regions such as the striatum and the CA1 region of the hippocampus. This was associated with progressive neurodegeneration in many brain areas, but especially in the CA1 hippocampal region where a significant reduction in the number of dendritic spines and axonal nerve terminals was observed. Notably, the degeneration process became more intense and extended to other brain regions as the animals were growing older. Based on these findings, Elias Michaelis and his group quite insightfully suggested that GDH1 overexpression can accelerate age-related neuronal loss and dendritic dysfunction in a manner analogous to that previously observed in Alzheimer's disease [67, 73]. This suggestion, when examined under the light of the above described hGDH1/2 alterations in other degenerative disorders, possibly opens a new path for the elucidation of the pathophysiology of Alzheimer's disease, a disease that poses an overwhelming burden in modern societies with aging populations.

Overall for the role of hGDH1 and hGDH2 in human neurodegenerative disorders, there is an apparent discrepancy among various published results on the effect of hGDH1/2 overactivity and of hGDH1/2 loss of function, both in animal models and in human studies. However, given the heterogeneity of the experimental models used and the complexity of the neurodegenerative processes involved, it could be possible that hyperactivity or loss of function of hGDH1 and/or hGDH2 could be protective in one clinical context and deleterious in another.

Finally, there is an emerging role for hGDH1 and hGDH2 in tumorigenesis [122]. As described above, human breast cancer cells use the GDH reaction to incorporate ammonia into amino acid biosynthesis and promote their growth [30]. This is of special importance under conditions of glutamine shortage. Specifically, under glutamine depletion, these breast cancer cell lines exploit the GDH reaction for biosynthetic purposes, and the main iso-enzyme contributing to this processes is hGDH2 [123]. Concerning the role of the GDH reaction in the metastatic potential of cancer cells, there is evidence that upregulation of hGDH1 activity could mediate lung cancer metastasis through metabolic reprogramming of glutaminolysis [124]. Back to the brain, there is increasing literature on the role of hGDH1 and hGDH2 in human gliomas [125, 126]. Specifically, for hGDH2, it has been recently shown that it promoted glioma growth by compensating for mutant isocitrate dehydrogenase (IDH<sup>R132H</sup>) metabolic derangements and that this effect was mediated through the adaptively evolved allosteric domain [127, 128]. These observations open the way for possibly using hGDH1 and hGDH2 inhibitors [129, 130] in cancer therapeutics.

In summary, hGDH1 and hGDH2 have important roles in metabolism and are also thought to be involved in the pathogenesis of various neurological and non-neurological syndromes, including HI/HA, diabetes, epilepsy, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and various tumors, including gliomas. It is expected that in the near future pharmacological manipulation of the two human GDH iso-enzymes could be of therapeutic potential in at least some of these disease processes.

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## Compliance with Ethical Standards

**Conflict of interest** All authors declared that they have no conflict of interest.

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