



GLS hyperactivity causes glutamate excess, infantile cataract and profound developmental delay

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42

43

44 **Abstract**

45 Loss-of-function mutations in glutaminase (GLS), the enzyme converting glutamine into
46 glutamate, and the counteracting enzyme glutamine synthetase (GS) cause disturbed glutamate
47 homeostasis and severe neonatal encephalopathy. We report a *de novo* Ser482Cys gain-of-
48 function variant in *GLS* encoding glutaminase associated with profound developmental delay
49 and infantile cataract. Functional analysis demonstrated that this variant causes hyperactivity
50 and compensatory downregulation of GLS expression combined with upregulation of the
51 counteracting enzyme GS, supporting pathogenicity. Ser482Cys-GLS likely improves the
52 electrostatic environment of the GLS catalytic site, thereby intrinsically inducing hyperactivity.
53 Alignment of +/-12.000 GLS protein sequences from >1000 genera, revealed extreme
54 conservation of Ser482, to the same degree as catalytic residues. Together with the
55 hyperactivity, this indicates that Ser482 is evolutionarily preserved to achieve optimal -but
56 submaximal- GLS activity. In line with GLS hyperactivity, increased glutamate and decreased
57 glutamine concentrations were measured in urine and fibroblasts. In the brain (both grey and
58 white matter), glutamate was also extremely high and glutamine almost undetectable, using
59 ultra-high field magnetic resonance spectroscopic imaging. Considering the neurotoxicity of
60 glutamate when present in excess, the strikingly high glutamate concentrations measured in the
61 brain provide an explanation for the developmental delay. Cataract, a known consequence of
62 oxidative stress, was evoked in zebrafish expressing the hypermorphic Ser482Cys-GLS and
63 could be alleviated by inhibition of GLS. The capacity to detoxify reactive oxygen species was
64 reduced upon Ser482Cys-GLS expression, providing an explanation for cataract formation. In
65 conclusion, we describe an inborn error of glutamate metabolism caused by a GLS hyperactivity
66 variant, illustrating the importance of balanced GLS activity.

67

68 **Introduction**

69 The amino acid glutamate is best known for its role as excitatory neurotransmitter, but also
70 serves as a substrate for other key metabolites, including the anti-oxidant glutathione(1-3).
71 Glutamate homeostasis is mainly warranted by two enzymes: glutamine synthetase (GS; EC
72 6.3.1.2) and glutaminase (GLS; EC 3.5.1.2). GS converts glutamate into glutamine and is
73 ubiquitously expressed(4). GLS catalyzes the deamination of glutamine into glutamate and
74 ammonia and exists in two isoforms: GLS -present in two splice variants KGA and GAC-
75 mainly expressed in kidney and brain; and GLS2, ubiquitously expressed with the highest
76 expression in the liver(5, 6).

77 Inborn errors of metabolism are usually due to severe loss of function of the involved enzymes,
78 hence recessive inheritance. In line, a disturbed equilibrium of glutamate and glutamine was
79 described in patients with GS deficiency, clinically resulting in glutamine deficiency, neonatal
80 epilepsy and early death(7). Recently, GLS loss of function has been described to cause lethal
81 epileptic encephalopathy and glutamine excess in two families(8). The description of patients
82 with spastic ataxia and optic atrophy harbouring bi-allelic hypomorphic variants in GLS,
83 suggests a phenotypic spectrum –presumably depending on the degree of residual activity- that
84 is yet to be uncovered(9). Theoretically, glutamate homeostasis can also be disturbed by
85 hyperactivity of either enzyme. This option is commonly disregarded as there are only few
86 examples of genetic variants that induce enzyme hyperactivity, GDH and IDH2 gain of
87 functions(10, 11). These examples indicate that a heterozygous variant is sufficient to induce
88 overall enzyme hyperactivity.

89 In this study, we characterize a *de novo* hypermorphic heterozygous *GLS* variant found in a
90 patient with infantile onset cataract, skin abnormalities, profound developmental delay and
91 intracerebral glutamate excess. This new inborn error of metabolism illustrates the importance
92 of regulated GLS activity for lens transparency and brain function.

93 **Results**

94 *Clinical description*

95 In a female patient, bilateral cataract was diagnosed at the age of 3 months after the parents
96 noticed decreased eye contact and loss of the red light reflex of the pupils on photos (Figure.
97 1A, S1). The proband is the first child of healthy non-consanguineous parents of Dutch descent
98 (Figure. S1A) as indicated by family history and SNP-array. Gestation and delivery were
99 uneventful. After lens extraction and replacement, eye contact unexpectedly remained absent.
100 By the age of 8 months, delayed development was noted, along with a relative decrease of the
101 head circumference from 0 SD to -2 SD. She developed recurrent dermatological abnormalities
102 on her extremities, cheeks and ears without pruritus, characterized as erythematic subcutaneous
103 nodules of approximately 1 cm (Figure. 1B). Histopathological analysis of these lesions showed
104 deep perivascular and periglandular lymphohistiocytic infiltrates and pronounced
105 leukocytoclasia at the surface of the dermis and focal vacuolar alterations, hyperkeratosis and
106 parakeratosis of the epidermis. A dermatological diagnosis remained inconclusive. Over time,
107 the girl lost the ability to make meaningful sounds and the ability to sit. She developed profound
108 axial hypotonia leading to kyphoscoliosis. Upon arousal she exhibited uncontrolled motoric
109 agitation and self-injurious behavior. Development remained slow paced. At the most recent
110 follow up at the age of 11 years, she was able to use gestures for communication, to understand
111 verbal single component instructions and to steer her own wheelchair.

112 *Identification of the Ser482Cys-GLS de novo variant*

113 Extensive diagnostic workup unexpectedly revealed extremely low glutamine levels and high
114 glutamate levels in both cortex and white matter as detected consistently with quantitative brain
115 proton MRS and MRSI at 1.5Tesla (Figure. 1C, S1B) and recently also shown at 7Tesla (Figure
116 1D, S1C). Interestingly, CSF and plasma levels were unaffected (Table S1). Brain MRI at age
117 16 months showed delayed myelination (Figure 1E). Analyses of stored urine samples similarly

118 showed low concentrations of glutamine and high concentrations of glutamate (Figure. 1F,
119 Table S1). The diagnosis remained enigmatic until trio-based whole exome sequencing (WES)
120 revealed a heterozygous *de novo* *GLS* missense variant (NC_000002.11:g.191795182C>G).
121 Analysis of WES data using recessive filters yielded no rare homozygous damaging variants.
122 The analysis for compound heterozygosity (including correctness of segregation in parents)
123 yielded two genes hit by rare and possibly damaging variants, but based on gene function,
124 absent links with human disease and the high prevalence within the healthy population, these
125 variants were considered as unlikely to contribute to the phenotypes of the patient
126 (supplementary results). The conservative mutation in *GLS* from serine to cysteine at position
127 482 NP_055720.3:p.(Ser482Cys) was confirmed by Sanger sequencing (Figure. 1G,
128 supplementary results). Glutaminase mediates the conversion of glutamine into glutamate,
129 therefore this genetic change could only be explained if the encoded protein would be
130 hyperactive.

131

132 *Ser482Cys-GLS leads to GLS hyperactivity*

133 The effect of the Ser482Cys-*GLS* variant on the activity of the GLS enzyme was assessed in
134 fibroblasts from the patient by quantification of intracellular glutamine and glutamate. The *GLS*
135 variant indeed resulted in an increased intracellular glutamate:glutamine ratio (Figure. 2A, 2B,
136 S2A). To validate enhanced catalytic activity of the GLS variant, a HEK293 cell model with
137 inducible expression of Ser482Cys-*GLS* (KGA, the long splice variant) was generated.
138 Induction of Ser482Cys-*GLS* again strongly increased the glutamate:glutamine ratio while
139 induction of wildtype *GLS* had no effect (Figure. 2C, 2D, S2B). Inhibition of GLS with CB-
140 839 resulted in normalization of glutamate and glutamine concentrations in both fibroblasts and
141 HEK293 cells, providing additional evidence that the Ser482Cys-*GLS* variant leads to GLS
142 hyperactivity.

143 *GLS hyperactivity leads to metabolic compensatory mechanisms*

144 Protein expression of both GLS splice variants KGA and GAC in patient fibroblasts was
145 decreased -rather than increased- compared to controls, ruling out that increased GLS activity
146 was due to increased protein availability. Conversely, the observed down-regulation of GLS
147 protein expression suggests it served as a compensatory mechanism aiming at normalizing
148 glutamine and glutamate concentrations (Figure. 2B). In support, introduction of Ser482Cys-
149 *GLS* in HEK293 cells also evoked decreased GAC expression levels (Figure. 2D, S2C).
150 Furthermore, CB-839-induced GLS inhibition restored GLS expression. GLS expression could
151 also be restored by normalization of glutamate levels through depletion of extracellular
152 glutamine, pointing to glutamate as a regulator of GLS expression (Figure. 2D). Finally, the
153 observation that protein levels of the reciprocal enzyme GS increased upon expression of
154 Ser482Cys-GLS -an effect that could also be reversed through CB-839-mediated GLS
155 inhibition (Figure. 2B, 2D)- underlines that cellular efforts were aimed at normalizing
156 glutamine and glutamate concentrations.

157 *Ser482 functions as a highly conserved intrinsic restrictor of glutaminase activity*

158 Ser482 is located near the catalytic site of GLS, but does not have an identified role in the
159 catalytic process itself (12). Alignment of +/- 12.000 GLS protein sequences from > 1000
160 genera, revealed that Ser482 is a residue with an extremely high degree of evolutionary
161 conservation (conservation score >0.98) along with residues directly involved in the catalytic
162 process (Figure. 2E). The Ser482Cys substitution is absent in healthy populations in the
163 databases GoNL(13), gnomAD(14), ClinVar(15) and ExAC(16) and is expected to be tolerated
164 without overall disturbances of the protein fold. Interestingly, substitution by cysteine -
165 containing a sterically more demanding and less polar thiol group than serine- changes the
166 electrostatic environment of Tyr466, one of the catalytic residues that protonates glutamine and
167 thereby accelerates deamination into glutamate. This change is likely to enhance the propensity

168 for proton donation and thereby to increase the speed of the reaction (Figure. 2F, S3,
169 Supplemental discussion).

170 *GLS hyperactivity decreases redox buffer capacity*

171 Oxidative stress is a known consequence of glutamate excess and a common cause of cataract
172 and neuronal injury(17, 18). In HEK293 cells expressing Ser482Cys-*GLS*, clearance of a sub-
173 lethal pulse of hydrogen peroxide was impaired with normal basal reactive oxygen species
174 (ROS) levels. (Figure 3). This indicates that Ser482Cys-*GLS* results in a lower capacity for
175 ROS scavenging.

176 *Ser482Cys-GLS induces lens opacification*

177 To explore the causal relationship between Ser482Cys-*GLS* expression and cataract, we
178 examined the effect of this variant in developing zebrafish embryos. Lens transparency at 5
179 days post fertilization (dpf) in zebrafish embryos injected with Ser482Cys-*GLS* cDNA was
180 compared to that in control embryos injected with wildtype *GLS* or uninjected embryos (Figure.
181 S4A). Of the embryos expressing the Ser482Cys-*GLS* variant, 34/47 (72%) developed
182 structural opacities in the lens, which were not observed in any of the control embryos (Figure.
183 4A-C, S4B-D). *GLS* inhibition with CB-839 from 6 hours post fertilization (hpf) resulted in
184 profoundly decreased formation of structural opacities in the lens of the Ser482Cys-*GLS*
185 zebrafish embryos (Figure. 4D, Figure. S4E).

186 **Discussion**

187 We characterize a *de novo* heterozygous, hyperactive *GLS* variant found in a patient with
188 infantile onset cataract, skin abnormalities, profound developmental delay and intracerebral
189 glutamate excess. The increased conversion of glutamine into glutamate observed upon
190 introduction of this variant provides a compelling explanation for the strikingly elevated
191 glutamate levels in cerebro and -in view of the central role of glutamate in brain functioning-

192 likely explains the developmental delay. Furthermore, zebrafish studies unexpectedly reveal
193 that introducing the hypermorphic GLS variant induces lens opacities. Together with the
194 observations that the lens opacities are amenable to GLS inhibition, this supports a role for
195 glutaminase activity in cataract formation.

196 Inborn errors of metabolism are usually due to bi-allelic or mono-allelic loss-of-function
197 variants with few exceptions. Of these, hyperinsulinism-hyperammonemia syndrome is caused
198 by increased sensitivity of the enzyme GDH to allosteric activation and D-2-hydroxyglutaric
199 aciduria is caused by a neomorphic function of the enzyme IDH2(19, 20). The variant described
200 here truly increases enzymatic activity (Supplemental discussion) likely due to an improved
201 electrostatic environment of the GLS catalytic site. To the best of our knowledge, this nature of
202 hypermorphic gain-of-function in which activity is intrinsically increased by improvement of
203 the catalytic machinery has not been described before. Although rare by nature, it is possible
204 that the current paradigm –heterozygous variants in enzyme encoding genes are usually
205 harmless- hampers identification of comparable disease causing hypermorphic variants in
206 enzyme encoding genes.

207 The cellular efforts, aimed at counteracting the effects of the hyperactive enzyme by decreasing
208 GLS protein availability while increasing the reciprocal enzyme GS, underline that increased
209 GLS activity is detrimental. Our data underline the observation by Krebs in 1935 that glutamate
210 acts as a sensor for GLS regulation and reveal that glutamate not only affects GLS enzyme
211 kinetics but also its expression. The extremely high degree of conservation of the hypermorphic
212 residue across > 1000 genera -comparable only to residues directly involved in the enzymatic
213 conversion of glutamine into glutamate- suggests that the serine residue serves as a built-in
214 restrictor, ensuring submaximal activity rather than maximal enzyme activity of GLS.

215 A point of interest is the observation that the ratio between glutamate and glutamine was
216 increased in brain and urine, while it remained unaltered in CSF and plasma. We postulate that
217 this discrepancy is explained by the degree to which glutamine and glutamate levels are
218 controlled by GLS. Tissues with abundant expression of GLS -neurons and kidney- are mainly
219 under GLS control (6). The relative importance of GLS within the brain is illustrated by a high
220 glutamate/glutamine ratio (2:1) in normal population (21). GLS overactivity may be masked in
221 other tissues in which GLS is only one of several players -including GS- that together regulate
222 glutamine and glutamate levels. The reduced importance of GLS in plasma is reflected by the
223 significantly lower ratio of glutamate/glutamine (1:15) (22). Further supporting this hypothesis,
224 the ratio is even lower (~1:100) in CSF, which is produced by choroid plexus from plasma by
225 glial cells that are known to have high GS expression (4) (22). The striking contrast between
226 CSF and brain could be regarded as a cautionary note: CSF should not be readily regarded as a
227 proxy for the brain.

228 Under physiological conditions, glutamate is important for redox homeostasis as it is the
229 precursor of the anti-oxidant glutathione(2). Glutamate excess, however, is associated with
230 oxidative stress, a common cause of cataract and neuronal damage(17, 18). We show that GLS
231 hyperactivity indeed leads to decreased capacity for redox buffering, which can result in
232 oxidative stress. We therefore postulate that glutamate excess contributes to the ophthalmologic
233 phenotype of our patient. In the aqueous humor -nourishing the lens- glutamate concentrations
234 are strictly regulated and even kept low by metabolism and transport(23). Exposure to glutamate
235 causes cataract in chick and rat embryos(17, 24, 25). In line with the phenotype of the affected
236 patient, zebrafish expressing Ser482Cys-GLS develop lens opacities which could be largely
237 prevented by GLS inhibition. Interestingly, neurons and lens cells are both of ectodermal origin,
238 as is the skin, and share similarities in expression and regulation of glutamate receptors,
239 supporting the notion that disturbed glutamate homeostasis not only affects the brain, but also

240 skin and lens(26). Glutamate excitotoxicity has been associated with epilepsy, numerous
241 neurodegenerative diseases, self-injury and agitated behavior(18, 27). The measured glutamate
242 excess in the brain of our patient might therefore provide a plausible explanation for the self-
243 injury behavior and developmental delay of our patient.

244 Interestingly, other defects affecting glutamate homeostasis lead to neurological phenotypes as
245 well. Under physiological circumstances, homeostasis of glutamine and glutamate in the brain
246 is strictly regulated by neuronal GLS and astrocytic GS via the glutamine-glutamate shuttle(4).
247 GLS loss of function variants lead to a phenotypic spectrum. The first description was of a late
248 childhood onset disease, including optic atrophy and spastic ataxia(9). Recently, bi-allelic loss
249 of function variants in *GLS* were described to cause lethal, neonatal onset encephalopathy
250 characterized by respiratory failure, status epilepticus and early death within weeks after
251 birth(8). These patients had simplified gyral patterns and showed destructions of initially
252 normal appearing brain structures. Both the reported hiccups during pregnancy and the
253 simplified gyral patterns on imaging suggest the damage has its onset prenatally. Given the
254 truncating mutations present in the latter phenotype it is tempting to speculate a dose effect
255 relationship explaining the phenotypic spectrum. This inborn error –together with GLS
256 hyperactivity- illustrates the importance of proper GLS activity for both brain physiology and
257 morphology.

258 Deficiency of GS –performing the reversed reaction of GLS- results in decreased glutamine
259 levels but normal glutamate levels and hyperammonemia. This has been reported in three
260 individuals which exhibited neonatal encephalopathy, seizures and respiratory failure and early
261 death(28). The absence of epilepsy in our patient with GLS hyperactivity despite increased
262 glutamate levels on brain MRSI is unexpected as glutamate excitotoxicity is considered a
263 critical factor in the initiation of epileptic seizures(29). Seizures can be provoked by either
264 increased glutamate release into the synaptic cleft or decreased re-uptake or recycling from the

265 synaptic cleft, which implies that glutamate levels in the synaptic cleft of our patient are
266 unaffected despite overall brain glutamate abundance. The phenotypic neurological spectrum
267 of these patients show the importance of strictly regulated glutamate homeostasis for
268 neurological functioning.

269 A limitation of our study is that only a single patient with a hyperactive variant in GLS could
270 be identified. Hyperactive variants are extremely uncommon, especially in a well conserved
271 catalytic area like in GLS. Intolerance to loss-of-function in GLS is likely high, meaning that
272 mutations will likely be lethal. These factors contribute to a limited patient pool. While definite
273 pathogenic conclusions are considered difficult based on evidence from unique subjects, when
274 adequately studied, these cases can be regarded as experiments of nature and provide invaluable
275 insights. Such is the case here, where we provide strong evidence that GLS hyperactivity causes
276 a new metabolic disorder of glutamate metabolism. Our study furthermore provides insight into
277 the regulation of GLS activity and illustrates the importance of appropriate GLS activity for
278 human brain function, skin and lens transparency.

279 **Materials and Methods**

280 *Clinical phenotyping, diagnostics and exome sequencing*

281 Clinical phenotyping was performed and diagnostic tests were requested by metabolic
282 pediatricians, clinical geneticists, an ophthalmologist, neurologists and dermatologist. Amino
283 acids analyses in urine were performed on a Biochrom30 analyzer. In the brain, these were
284 determined with quantitative Magnetic Resonance Spectroscopic (MRS) and Spectroscopic
285 Imaging (MRSI) at 1.5Tesla at age 2 and 3 years and with MRSI at 7Tesla at age 14 years.
286 Genetic analysis was performed by trio-based whole-exome sequencing and Sanger
287 sequencing. See supplementary methods for details.

288 *GLS activity*

289 GLS activity was determined in patient fibroblasts and in human embryonic kidney 293
290 (HEK293) cells stably transfected with either wildtype or Ser482Cys-*GLS* (KGA isoform) or
291 an empty vector, in absence or presence of different concentrations of the allosteric GLS
292 inhibitor CB-839(30). GLS activity was defined as the formation of glutamate from glutamine,
293 quantified by ultra-performance liquid chromatography tandem mass spectrometry(31). Protein
294 expression was assessed by Western Blot.

295 *Conservation analysis*

296 Sequences homologous to human GLS from the non-redundant protein collection at NCBI were
297 aligned in SeaView(32). Obvious partial sequences as well as all pdb sequences were removed
298 which resulted in about 12.000 sequences. The consensus were determined in JalView(33) and
299 fraction of the modal residue in a column were used for generating a color gradients which was
300 mapped onto the GLS structure as a measure of conservation (consensus score).

301 *Reactive oxygen species*

302 ROS-levels were quantified by flow cytometry (BD FACSCalibur™) as previously described
303 in wildtype *GLS* or Ser482Cys-*GLS* transfected HEK293 cells(34).

304 *Animal model*

305 Zebrafish (*Danio rerio*) embryos were microinjected at the 1-cell stage with DNA constructs
306 coding for wildtype or Ser482Cys-*GLS* (KGA isoform). Uninjected zebrafish embryos were
307 used as controls. The embryos were kept under standard laboratory conditions, either in the
308 absence or in the presence of the GLS inhibitor CB-839, prior to assessment of glutamine and
309 glutamate concentrations and lens opacity at 5 days post fertilization (dpf).

310 *Statistics*

311 Statistical analyses were performed by ANOVA, post-hoc Tukey's test using IBM SPSS
312 statistics 21.

313

314 *Study approval*

315 The proband's parents provided written informed consent for all aspects of the study.

316 Zebrafish experiments were carried out in accordance with the guidelines of the Animal
317 Experimentation Committee of the Royal Netherlands Academy of Arts and Sciences
318 (KNAW).

319 For more detailed information, see supplementary materials and methods.

320

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328 The authors declare no competing financial interests.

329

330 **Author contributions**

331 LR, FT, HCMTP, FJTZ, GH, NMVD, JJMJ and PMH contributed to the concept and design of
332 the study. PMH and JJMJ coordinated the study. PJWP, HYK, HCMTP, MSK, GH, NMVD,
333 JJMJ and PMH provided clinical phenotyping, diagnostics, patient care and (genetic)
334 counseling. LR, FT, PJWP, EV, JPW, AAB, SMCS, KJD, MJGB, RJJR, PAWS, DWJK,

335 GCMB, RLT, JPWB, TBD, HR and FJTZ contributed to the acquisition and interpretation of
 336 the data. LR and FT wrote the manuscript and all co-authors critically revised the manuscript
 337 and provided final approval of the manuscript to be published.

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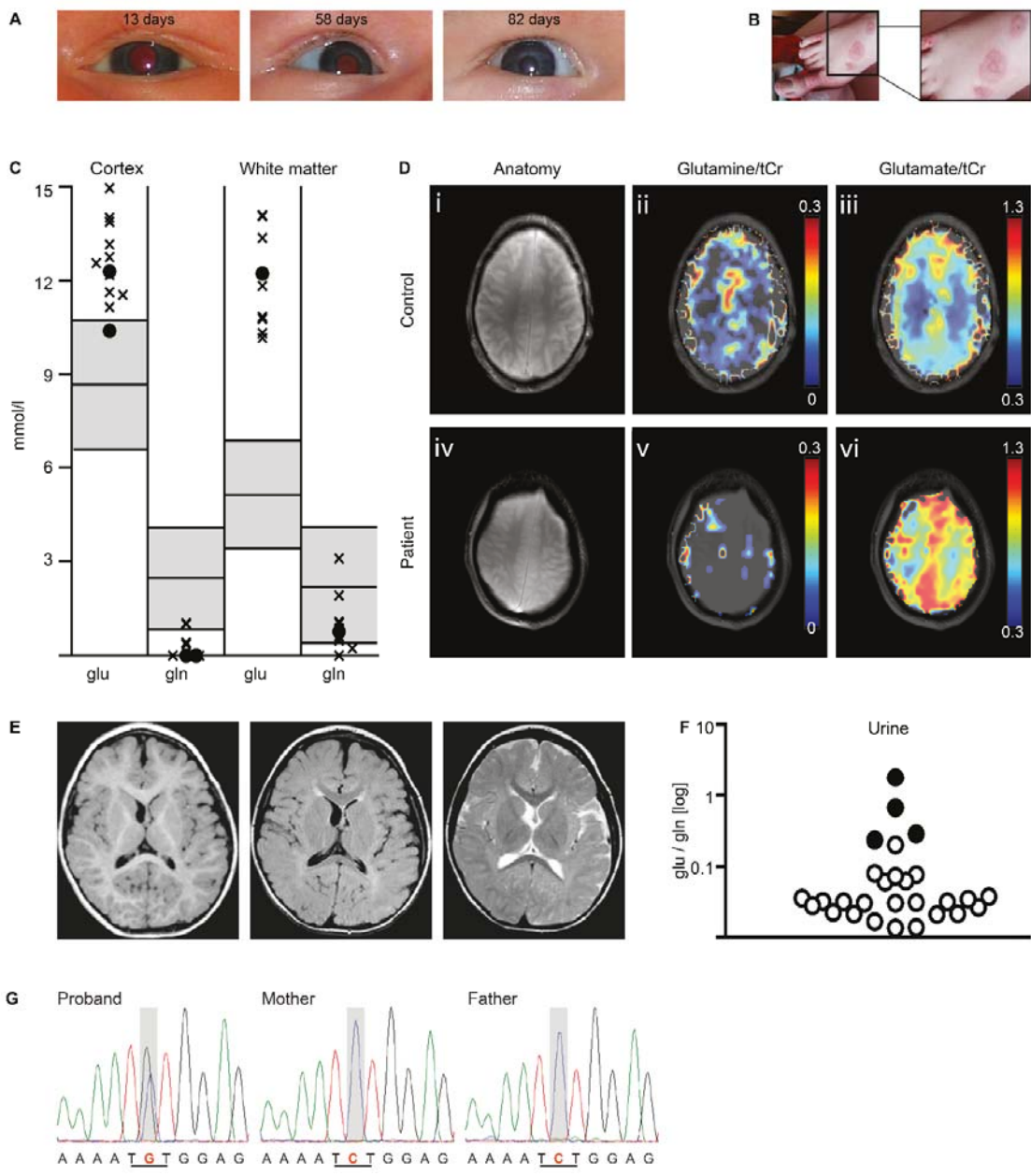
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435 **Figure legends**

436 **Figure 1. Identification of a *GLS de novo* variant in a patient with bilateral infantile**
437 **cataract. (A)** Photographs of the eyes of the patient at different ages depict a decrease in light
438 reflex, indicating the formation of cataract before the age of three months. **(B)** Dermatological
439 manifestation of erythematic nodules of approximately 1 cm, here on the dorsum of the foot.
440 **(C)** Glutamine and glutamate concentrations assessed by Magnetic Resonance Spectroscopy
441 (1.5 Tesla, both STEAM, TR/TM/TE 6000/30/20 ms, and PRESS, TR/TE 3000/30 ms) in the
442 parietal cortex and white matter of the patient at ages 2 and 3 years. The normal range, +/- 2
443 SD from mean based on control values of children between 2 and 5 years of age(35) is depicted

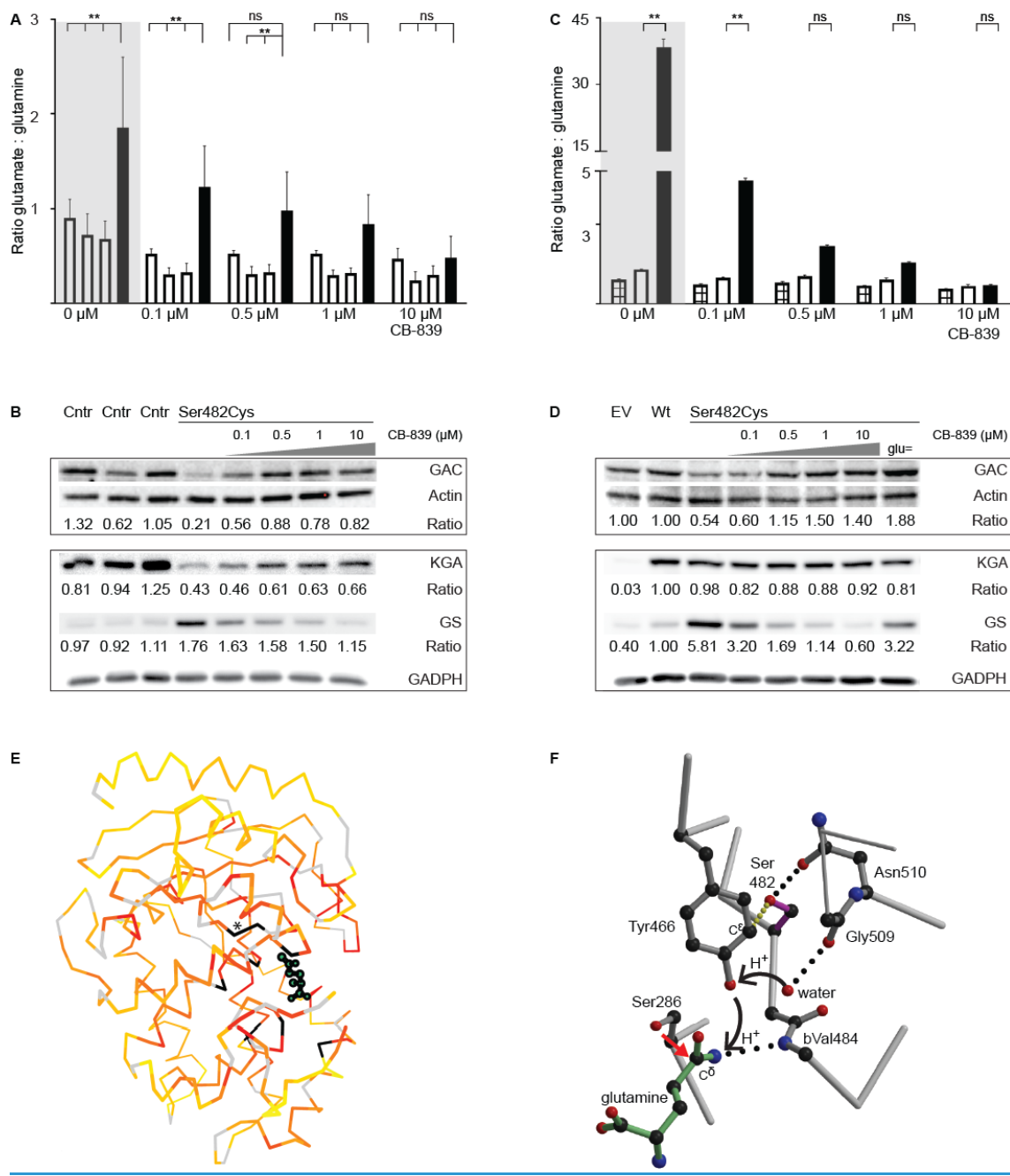
444 in grey. Data represent concentrations in single voxel MRS (o) and in multiple voxels from
445 MRSI (x). **(D)** Maps of glutamine (middle panel) and glutamate (right panel) levels in the brain
446 of the patient at age 14 years (lower row) and a control (top row), generated from 2D MRSI
447 acquisitions (7T, pulse-acquire, matrix 44x44, 0.5x0.5x1.0 cm³, TR/TE 300/2.5 ms) overlaid
448 on anatomical magnetic resonance images (left panel). **(E)** Magnetic resonance imaging of the
449 patient at age 16 months, revealing delayed myelination. The transverse T1-weighted image
450 (left) shows the myelinated cerebral white matter as white. The FLAIR (middle) and T2-
451 weighted (right) images have a lack of contrast between cerebral hemispheric white matter and
452 cortex, indicating that myelination is incomplete. Better myelinated structures, including corpus
453 callosum and internal capsule, have a lower signal. **(F)** Urinary excretion of glutamate and
454 glutamine, presented as ratios on a logarithmic scale in the urine of the patient (black dots)
455 compared to controls (white dots). **(G)** DNA Sanger sequencing trio analysis shows the
456 Ser482Cys-*GLS* *de novo* variant in the patient, which is absent in the unaffected parents. The
457 underlined sequence indicates the nucleic acid change causing the substitution of the amino
458 acid serine for cysteine.



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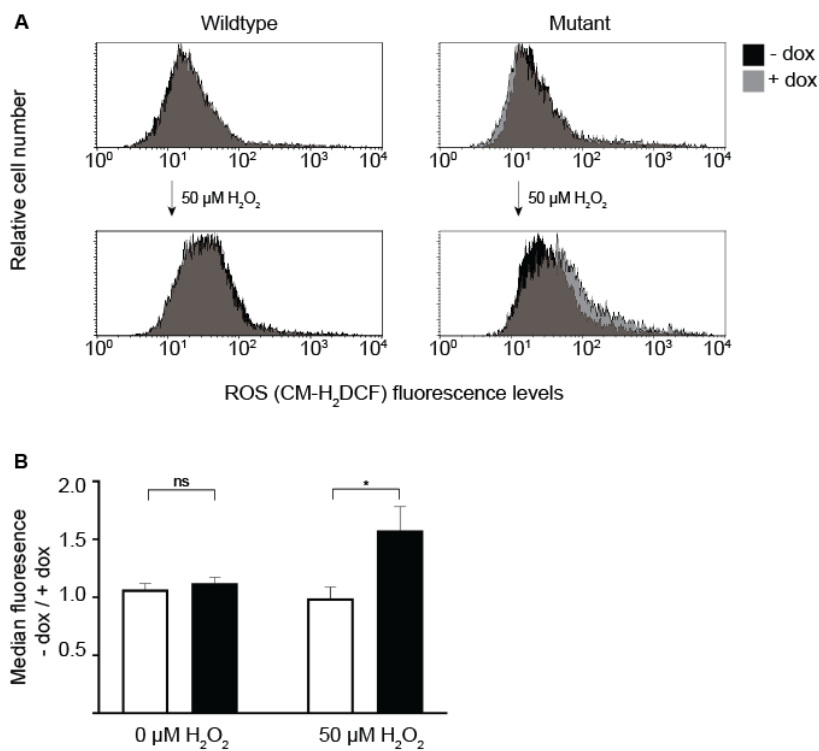
465 **Figure 2. Impact of Ser482Cys-GLS on enzyme activity, expression and structure. (A-C)**
466 Glutamate and glutamine values measured with UPLC-MS/MS, expressed as the ratio of
467 glutamate:glutamine in (A) fibroblasts of 3 controls (white) and the proband expressing
468 Ser482Cys-GLS (black) and (C) HEK293 cells stably transfected with an empty vector
469 (checked), wildtype *GLS* (white) or Ser482Cys-*GLS* (black). Cells containing the variant were
470 untreated (highlighted) or treated with 0.1 μ M, 0.5 μ M, 1 μ M or 10 μ M CB-839. Data represent
471 the mean of biological triplicates with standard deviations. * $p < 0.05$ (ANOVA, Tukey's test)
472 ** $p < 0.01$ (ANOVA, Tukey's test) ns: not significant. (B-D) Western blots of both GLS splice
473 variants -kidney type glutaminase (KGA) and glutaminase C (GAC)- and GS. (B) In fibroblasts
474 of 3 controls and the patient expressing Ser482Cys-GLS, the latter treated with CB-839
475 corresponding to panel a. The mean of the expression levels in control fibroblasts is arbitrarily
476 set at 1. (D) In HEK293 cells stably transfected with an empty vector (EV), wildtype *GLS* (Wt)
477 or Ser482Cys-*GLS* (KGA), the latter treated with CB-839 (corresponding to panel c) or
478 deprived from glutamine to normalize glutamate concentrations (glu=). Expression levels in
479 cells expressing wildtype GLS are arbitrarily set at 1. Results are normalized to actin or
480 GADPH. Analyses performed on the same blot are delineated. (E) Conservation analysis of
481 GLS, in which residues with conservation scores from 0 to 0.98 are represented by a color
482 gradient from yellow to red and the most conserved residues (> 0.98) are represented in black.
483 These residues are clustered around the catalytic site and most of them are directly involved in
484 the catalytic reaction: Ala339 (0.994), Lys481 (0.992), Asn335 (0.991), Lys289 (0.990), Ser286
485 (0.990), Tyr414 (984), Tyr466 (0.986) and Asn388 (0.983). Among these is Ser482 (0.983),
486 indicated by the asterisk symbol. Glutamine is shown in green ball-and-stick representation.
487 Glycine and proline residues -often conserved for pure structural reasons- were omitted and are
488 shown in light grey. (F) Zoom-in on the catalytic site of GLS in complex with glutamine (green)
489 shows that Ser482 (magenta) is located near the catalytic site. The deamination reaction of

490 glutamine is initiated by a nucleophilic attack of Ser286 on C^δ of glutamine (red arrow) and is
491 accelerated by Tyr466 via protonation (black arrows indicate proton transfer). The electrostatic
492 environment of Tyr466 is determined by the hydroxyl-group of Ser482 (yellow dotted line).
493 Hydrogen bonds are shown by dotted black lines. Supplemental Figure. 3 provides additional
494 insight into the enzymatic reaction and the possible consequences of the Ser482Cys
495 substitution. Figures are based on pdb entry 3vp0 and were generated with molscript(36) and
496 raster3D(37)



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502 **Figure 3. ROS levels and redox buffering capacity.** HEK293 cells were transfected with
 503 wildtype *GLS* or Ser482Cys-*GLS*, with and without exposure to H₂O₂, measured with flow
 504 cytometry, using CM-H₂DCF-DA. (A) Histograms of non-induced (-dox, black) and induced
 505 (+dox, grey) cells, showing the shift in CM-H₂DCF fluorescence intensity after induction with
 506 doxycycline and exposure to 50 μ M H₂O₂. Data are normalized to total cell counts and are
 507 representative for biological triplicates. (B) Ratio of the median CM-H₂DCF fluorescence
 508 intensities of cells induced with doxycycline over uninduced cells. Data present the mean of
 509 biological triplicates with standard errors of the mean. ns: not significant *p<0.05 (ANOVA,
 510 Tukey's test).



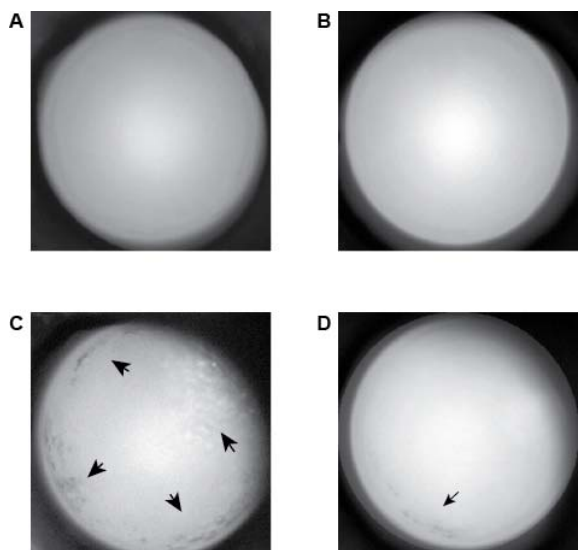
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515 **Figure 4. Lens opacity of zebrafish expressing Ser482Cys-GLS.** Representative images of
516 the lenses of 5 dpf zebrafish embryos of (A) uninjected n=30 or injected with vectors containing
517 (B) wildtype *GLS* cDNA n=28 or (C) Ser482Cys-*GLS* cDNA KGA isoform n=63. (D)
518 Zebrafish embryos expressing Ser482Cys-GLS were treated with 10 μ M CB-839 from 6 hpf
519 n=10. Opacities in the lens are indicated with arrows. See Supplemental Figure. 4 for all images.
520 Images were obtained with a fluorescence microscope. Linear image editing was performed.



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