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1 **Impact of β -galactosidase mutations on the expression of the**
2 **canine lysosomal multienzyme complex**

3

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11 **Keywords:** differential expression; β -galactosidase variants; protective protein/cathepsin A
12 and neuraminidase 1; lysosomal multienzyme complex; G_{M1}-gangliosidosis

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24Summary

25 Beta-galactosidase (GLB1) forms a functional lysosomal multienzyme complex
26with lysosomal protective protein (PPCA) and neuraminidase 1 (NEU1) which is important
27for its intracellular processing and activity. Mutations in the β -galactosidase gene cause the
28lysosomal storage disease G_{M1}-gangliosidosis. In order to identify additional molecular
29changes associated with the presence of β -galactosidase mutations, the expression of
30canine lysosomal multienzyme complex components in $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$
31fibroblasts was investigated by quantitative RT-PCR, Western Blot and enzymatic assays.

32 Quantitative RT-PCR revealed differential regulation of total β -galactosidase, β -
33galactosidase variants and protective protein for beta-galactosidase gene (*PPGB*) in
34 $GLB1^{+/-}$ and $GLB1^{-/-}$ compared to $GLB1^{+/+}$ fibroblasts. Furthermore, it was shown that
35*PPGB* levels gradually increased with the number of mutant β -galactosidase alleles while
36no change in the *NEU1* expression was observed.

37 This is the first study that simultaneously examine the effect of $GLB1^{+/+}$, $GLB1^{+/-}$
38and $GLB1^{-/-}$ genotypes on the expression of lysosomal multienzyme complex components.
39The findings reveal a possible adaptive process in *GLB1* homozygous mutant and
40heterozygous individuals that could facilitate the design of efficient therapeutic strategies.

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481. Introduction

49 From DNA polymorphisms to protein degradation, eukaryotic gene expression
50 requires chains of complex regulated processes [1]. Due to either structural defects or
51 abnormal processing, impairments in these processes may lead to severe disorders. For
52 several Mendelian diseases, the molecular cause has been successfully identified [2, 3, 4];
53 however, in the majority of the “simple” genetic disorders, a wide phenotypic variability in
54 terms of clinical phenotype and disease severity has been observed [5, 6, 7]. Therefore,
55 clinical manifestations of monogenic disorders seem to be analogous to complex traits [7].
56 One reason for this complexity is the regulation of gene expression at different levels [8,
57 59]. For different genes, certain steps in the regulation of their expression were described
58 after identification of a specific clinico-pathological phenotype. Thus, β -galactosidase
59 (*GLB1*; EC 3.2.1.23) deficiencies were associated with the lysosomal storage disease G_{M1} -
60 gangliosidosis (OMIM # 230500; OMIA # 00402) which displays three major types (type
61 I, II and III) in humans with respect to the age of onset and clinico-pathological
62 manifestations [10, 11, 12]. Other types of β -galactosidase gene (*GLB1*) mutations were
63 also identified in Morquio type B disease, a disease characterized by ganglioside
64 accumulation in several organs without central nervous system involvement [13].
65 Moreover, due to normal alternative splicing, the *GLB1* encodes, additionally to β -
66 galactosidase, the elastin-binding protein (EBP) [14]. As a consequence, structural defects
67 of *GLB1* were associated with impaired elastogenesis [15]. Similar variability of
68 G_{M1} -gangliosidosis has also been described in dogs where the disease resembles either type
69 I (English Springer Spaniel) or type II (Alaskan huskies, Shiba dogs, Portuguese Water
70 Dog, mixed Beagle) human G_{M1} -gangliosidosis [16, 17, 18]. Furthermore, structural
71 defects affecting the co- and post-transcriptional processing of *GLB1* like pre-mRNA
72 splicing or generation of premature termination codons (PTC) were identified in humans

73and dogs [19, 20, 21]. Other studies showed that abnormal transcripts can evade the
74cellular surveillance mechanism when the PTC is located on the last exon [22, 23, 24].

75 After translation, GLB1 forms a functional lysosomal multienzyme complex
76(LMC) with neuraminidase (NEU1) and lysosomal protective protein (PPCA), which is
77important for its intracellular processing and activity [25, 26, 27]. Defects of either the
78hydrolytic or the protective components of LMC lead to abnormal phenotypes with varying
79degrees of clinical severity [28]. Moreover, the senescence-associated GLB1 was identified
80as being the normal lysosomal GLB1 whose activity is increased in physiological
81conditions related to senescence [29, 30].

82 A 19 bp duplication located in exon 15 of the canine *GLB1* gene was identified in
83Alaskan huskies with G_{M1} -gangliosidosis [17, 31, 32]. This genetic modification either
84disrupts a potential exonic splicing enhancer sequence (ESE) leading to the re-framing of
85the mRNA by skipping the mutant exon 15 or generates a PTC on the last exon. Therefore,
86two *GLB1* mRNA populations were identified in diseased homozygous mutant dogs: one
87mRNA isoform lacking the exon 15 (*GLB1* Δ Ex15) and the other one carrying the
88abnormal exon 15 with the 19 bp duplication (*GLB1*dupl) [17, 31, 32].

89 For further therapeutic approaches it is important to understand in which ‘molecular
90environment’ the therapy will be applied. To define this ‘molecular environment’ in a
91canine model of late infantile G_{M1} -gangliosidosis, the regulation of the *GLB1* mRNA
92expression of normal and abnormal variants and the GLB1 activity in fibroblasts with
93different *GLB1* genotypes were investigated.

94 Furthermore, possible mechanisms how the GLB1 activity is maintained at
95sufficient levels in heterozygous individuals were analyzed by investigating the
96relationship between *GLB1* expression and the other LMC components, *PPGB* and *NEU1*.

972. Materials and methods

982.1. Cell culture procedures

99 Primary canine skin fibroblasts from 26 healthy dogs (10 homozygous *GLB1*^{+/+} and
10014 heterozygous *GLB1*^{+/-}) and two diseased homozygous (*GLB1*^{-/-}) were used [31, 33, 34].
101All experiments were performed with fibroblasts at passage 3.

1022.2. RNA extraction, cDNA synthesis and primer design

103 Total RNA was isolated from primary skin fibroblasts using the RNeasy Mini Kit
104(Qiagen, Hilden, Germany) followed by a DNase treatment (Qiagen, Hilden, Germany)
105according to the manufacturer's instructions. The OmniScript™ kit (Qiagen, Hilden,
106Germany), oligo dT₁₂ (Roche, Mannheim, Germany) and 500 ng RNA were used for cDNA
107synthesis. The primers used for quantification of *GLB1*, *PPGB*, *NEUI* and glyceraldehyde-
1083-phosphate-dehydrogenase (*GAPDH*) mRNA expression were designed using Primer 3 v
1090.4.0 software and the published sequences [(GenBank™ accession no. DQ196436, NW
110876277, XM 538838 and AB038240) [35]. The characteristics of these primers are
111presented in table 1.

112To distinguish between the different populations of *GLB1* mRNAs (total *GLB1*,
113*GLB1*ΔEx15 and *GLB1*dupl) specific primer pairs were designed as shown in figure 1.

1142.3. Quantitative RT-PCR (qRT-PCR)

115 Quantitative RT-PCRs were performed with a Mx3005P™ Instrument (Stratagene,
116La Jolla, CA, USA) in a total volume of 25 µl. For each reaction, 1 µl cDNA was placed in
117a 24 µl reaction mixture containing: 0.25 µl *Taq* DNA Polymerase (5 U/µl) (Invitrogen,
118Karlsruhe, Germany), 2.5 µl supplied 10X buffer, 1.25 µl MgCl₂ (50 mM), 0.5 µl dNTP
119(10 mM) (Invitrogen, Karlsruhe, Germany) and 0.5 µl of each primer (10 µM) diluted in
120distilled water. SYBR® Green I was used as DNA-binding dye while ROX was chosen as
121reference dye. The cycling temperature profile was identical for all transcripts and
122consisted of: 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 30

123seconds, annealing at 68°C for 1 minute and extension at 72°C for 30 seconds. Melting
124curves were generated at the end of each amplification reaction.

125To generate gene specific standard curves, the PCR products were run on a 2% agarose
126gel, excised and eluted using the QIAquick Gel Extraction™ kit (Qiagen, Hilden,
127Germany). The DNA concentration was measured at 260 nm using the GeneQuant Pro™
128RNA/DNA calculator (GE Healthcare, Freiburg, Germany). The identity of each amplicon
129was confirmed by sequencing (Agowa GmbH, Berlin, Germany). All reactions were
130performed in quadruplicate.

1312.4. *Quantification of mRNA expression levels*

132 Each assay included two standard curves (one for the gene of interest and one for
133GAPDH) using serial 10-fold dilutions (ranging from E+08 to E+02 copies). The
134correlation coefficient (R^2) of the standard curves was above 0.985. The slopes of the
135standard curves were used to determine the reaction efficiency (e) using the equation: (e) =
136 $10^{(-1/\text{slope})} - 1$ [32]. Thus, obtained amplification efficiencies were above 95%. Between
137quadruplicates, the median coefficient of variation (CV) was calculated as $CV = SD/\text{mean}$
138and was under 5%. Due to differences in the amplicon sizes, the CT-values were corrected
139for PCR efficiency (e) using the following formula: $CT_{e=100\%} = CT_e [\log(1+e)/\log(2)]$. The
140relative number of molecules for each transcript was determined by interpolating the
141 $CT_{e=100\%}$ values of the gene of interest (GOI) to each standard curve and the obtained values
142were normalized with respect to *GAPDH* for each individual cDNA. The relative
143expression level was calculated by the $2^{-\Delta\Delta Ct}$ method using the expression from *GLBI*^{+/+}
144fibroblasts as calibrator [36, 37, 38]. Statistical differences between groups were assessed
145by t-test and differences with $p < 0.05$ were considered significant.

1462.5. *β -galactosidase enzymatic assays*

147 GLB1 activities were measured as previously described [18, 33, 34] and expressed
148 in mU/mg protein.

149 2.6. Western Blotting and densitometric analysis

150 Proteins were extracted and subjected to SDS-PAGE analysis as described [39]. For
151 detection of PPCA (precursor- 54kD and mature form- 32 and 20kD) and actin, the
152 polyclonal anti-PPCA (Acris GmbH, Hiddenhausen, Germany) and anti-actin antibody
153 (Santa Cruz Biotechnology, Heidelberg, Germany) were used as primary antibodies. As
154 secondary antibodies the anti-rabbit IgG, HRP-linked antibody (Santa Cruz Biotechnology,
155 Hiddenhausen, Germany) and the polyclonal rabbit anti-mouse IgG HRP-linked antibody
156 (Dako GmbH, Hamburg, Germany) were utilized. All antibodies were diluted 1:1000. The
157 antigen-antibody complexes were visualized with the ECL chemiluminescence system
158 (Amersham) and exposed to a Kodak X-OMAT film. Each set of samples (homozygous
159 $GLB1^{+/+}$, heterozygous $GLB1^{+/-}$ and homozygous $GLB1^{-/-}$) was measured in three
160 independent experiments. The densitometric analysis of the resulted protein bands was
161 performed using the Scion Image Beta Software (Scion Corp., Frederick, MD, USA). The
162 integrated optic densities were normalized to actin and the statistical differences between
163 groups were assessed by t-test and differences with $p < 0.05$ were considered significant.

164 3. Results

165 3.1. GLB1 activities in $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts

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167 $GLB1^{+/+}$ and $GLB1^{+/-}$ fibroblasts showed high levels of GLB1 activities whereas
168 $GLB1^{-/-}$ cells had a significant deficiency in the enzymatic activity of GLB1 (Fig. 2).
169 Surprisingly, no significant difference was observed between the GLB1 activities of
170 $GLB1^{+/+}$ and $GLB1^{+/-}$ fibroblasts.

171 3.2. mRNA levels of total GLB1 in $GLB1^{+/+}$, $GLB1^{+/-}$ and $GLB1^{-/-}$ fibroblasts

172 To further investigate how GLB1 activity is maintained at similar levels in *GLB1*^{+/+}
173 and *GLB1*^{+/-} fibroblasts, *GLB1* mRNA expression was analyzed by qRT-PCRs using a
174 universal GLB1 primer pair (GLB1fw and GLB1rev). These primers were designed to
175 detect the total *GLB1* mRNA expression represented by one (GLB1wild-type) mRNA
176 population in *GLB1*^{+/+} fibroblasts, three (GLB1wild-type, GLB1ΔEx15 and GLB1dupl) in
177 *GLB1*^{+/-} and two (GLB1ΔEx15 and GLB1dupl) mRNA populations in *GLB1*^{-/-} fibroblasts
178 (Fig. 1). Relative quantification revealed the *GLB1* mRNA expression pattern in *GLB1*^{+/+},
179 *GLB1*^{+/-}, *GLB1*^{-/-} genotypes (Fig. 3) with a significant increase in total *GLB1* mRNA
180 expression in heterozygous and homozygous recessive fibroblasts. Thus, in *GLB1*^{+/-} and
181 *GLB1*^{-/-} fibroblasts total *GLB1* mRNA expression showed a 2.68 and 1.85 fold up-
182 regulation respectively compared to *GLB1*^{+/+} fibroblasts.

183 3.3. mRNA levels of *GLB1* variants in *GLB1*^{+/+}, *GLB1*^{+/-} and *GLB1*^{-/-} fibroblasts

184 To determine which *GLB1* population contributed to the high levels of *GLB1*
185 mRNA in *GLB1*^{+/-} fibroblasts and to analyze possible alterations in the alternative splicing
186 process between *GLB1*^{+/-} and *GLB1*^{-/-} fibroblasts, the expression of *GLB1* variants
187 (GLB1ΔEx15 and GLB1dupl) was further investigated. While, as expected, the *GLB1*^{+/+}
188 cells showed no GLB1ΔEx15 mRNA expression, the *GLB1*^{-/-} fibroblasts expressed
189 significantly higher levels of GLB1ΔEx15 mRNA compared to *GLB1*^{+/-} cells (Fig. 4). For
190 the GLB1dupl mRNA population, the expression increased slightly in *GLB1*^{-/-} fibroblasts
191 compared to *GLB1*^{+/-} fibroblasts but was not statistically significant (Fig. 5). Analysis of
192 the absolute GLB1ΔEx15 and GLB1dupl mRNA levels revealed that the GLB1dupl mRNA
193 exceeded the expression of GLB1ΔEx15 mRNA by 10-fold (E+04/E+03). Due to great
194 similarities between GLB1wild-type and GLB1dupl sequences, a specific quantification of
195 GLB1wild-type transcript was not possible. Moreover, eventual interference between
196 *GLB1* and *EBP* expression was excluded while a canine *EBP* expression was not detected

197 using primers for the canine *GLBI* similar with those used to amplify the alternatively
198 spliced variant of the human *GLBI* named the human EBP (data not shown).

199 3.4. mRNA levels of *NEUI* in *GLBI*^{+/+}, *GLBI*^{+/-} and *GLBI*^{-/-} fibroblasts

200 To further investigate whether this increase in total *GLBI* mRNA expression in
201 *GLBI*^{+/-} fibroblasts was associated with modifications in the expression of *NEUI*, additional
202 qRT-PCRs were performed. The *NEUI* mRNA levels in *GLBI*^{+/-} and *GLBI*^{-/-} fibroblasts
203 were not significantly altered compared to *GLBI*^{+/+} fibroblasts (Fig. 6).

204 3.5. mRNA and protein levels of *PPGB* in *GLBI*^{+/+}, *GLBI*^{+/-} and *GLBI*^{-/-} fibroblasts

205 The investigation of the *PPGB* mRNA expression revealed a gradually increased
206 expression in *GLBI*^{+/+}, *GLBI*^{+/-} and *GLBI*^{-/-} fibroblasts with the highest expression in
207 *GLBI*^{-/-} (4.57-fold), intermediate expression in *GLBI*^{+/-} (2.06) and the lowest expression in
208 *GLBI*^{+/+} fibroblasts (Fig. 7). At the protein level, PPCA precursor (54kD) showed a similar
209 pattern of expression as *PPGB* mRNA (Fig. 8). Densitometric analysis of the Western blot
210 bands revealed a statistically significant increase in PPCA precursor expression in *GLBI*^{-/-}
211 (1.14 ± 0.21) compared to *GLBI*^{+/+} fibroblasts (0.11 ± 0.01). In contrast to PPCA
212 precursor, the mature forms of PPCA (32 and 20kD bands) were not present in all samples
213 and their appearance did not correlate with the *GLBI* genotypes.

214 4. Discussion

215 Over the years, the genetic defects causing several Mendelian disorders have been
216 elucidated and it was shown that different mutations can affect the expression of a single
217 gene at multiple levels. Moreover, single genes are involved in various biological
218 processes. Thus, the phenotypical manifestations of a so-called “simple” genetic defect
219 display variability similar to complex traits. Therefore, even for monogenic diseases, the
220 identification of the molecular cause is only the first step in designing further therapeutic
221 approaches [40].

222 In the present study, the expression of *GLB1*, *PPGB* and *NEU1* in *GLB1*^{+/+}, *GLB1*^{+/-}
223and *GLB1*^{-/-} genotypes was comparatively analyzed, to investigate additional molecular
224changes associated with the presence of *GLB1* mutations. The identification and
225characterization of such modifications are essential for the development of efficient
226type-specific therapeutic strategies for the treatment of G_{M1}-gangliosidosis.

227 The majority of lysosomal genes vary substantially in their expression [41].
228Thereby, modifications of the *GLB1* mRNA expression under particular conditions cannot
229be excluded. Differential expression of *GLB1* was investigated by comparing healthy
230individuals and patients suffering from G_{M1}-gangliosidosis. However, as for other
231monogenic diseases, investigating heterozygous *GLB1*^{+/-} individuals is important not only
232for the detection of mutant allele carriers but also for developing strategies to antagonize
233the effect of the mutant allele.

234 In the present study, the GLB1 activity of *GLB1*^{+/+}, *GLB1*^{+/-} and *GLB1*^{-/-} fibroblasts
235was analyzed and, as expected, it was observed that *GLB1*^{-/-} fibroblasts have a severe
236deficiency in GLB1 activity compared to *GLB1*^{+/+} and *GLB1*^{+/-} fibroblasts. Interestingly, no
237significant differences between fibroblasts carrying one or two wild-type alleles were
238identified, suggesting a differential regulation of GLB1 activity in these genotypes. To
239further elucidate this observation, the total *GLB1* mRNA expression was investigated. In
240*GLB1*^{+/-} compared to *GLB1*^{+/+} fibroblasts a 2.68 fold increase of total *GLB1* mRNA
241expression was shown. Thus, *GLB1* alleles elevated their expression as a result of a
242mutation in the *GLB1* gene. In addition, *GLB1* mRNA expression was 1.85 fold increased
243in *GLB1*^{-/-}. This observation is in contrast to previous reports which showed no difference
244between the *GLB1* expression in controls and G_{M1}-gangliosidosis patients [4, 42].
245However, the higher levels of total GLB1 mRNA in *GLB1*^{+/-} compared to *GLB1*^{+/+} could
246also result from an increased expression of each GLB1 transcript. The great similarities

247between *GLB1*wild-type and the *GLB1*dupl sequences (wild-type: 5'-
248ggatcccagacttgccccaggacacc-3'; *GLB1*dupl: 5'-ggatcccagacttgccccagga
249tcccagacttgccccaggacacc-3') it) did not allow a direct differentiation between these two
250transcripts. In order to overcome this difficulties the mean β -galactosidase activity was
251compared between *GLB1*^{+/+} and *GLB1*^{+/-} individuals, however no significant differences
252were found ($p < 0,05$). Furthermore, *GLB1*^{+/+} individuals carry two wild-type alleles which
253are responsible for the measured β -galactosidase activity (mean value of 365.1 ± 122.33
254mU/mg), while *GLB1*^{+/-} individuals posses only one normal allele which results in a similar
255mean value of β -galactosidase activity (340 ± 127.87 mU/mg). In addition, only *GLB1*wild-
256type mRNA gives rise to an active protein [18]. Thus, it was concluded that the wild-type
257allele will have higher levels of transcription in *GLB1*^{+/-} individuals in order to maintain
258the β -galactosidase activity at levels similar to those detected in *GLB1*^{+/+} individuals.
259Moreover, human G_{M1} -gangliosidosis patients carrying splicing defects displayed a
260decrease in total *GLB1* mRNA expression [4]. As previously mentioned, the 19 bp
261duplication in canine G_{M1} -gangliosidosis generates a PTC which should trigger nonsense
262mediated decay (NMD) of the abnormal mRNA. However, due to its special position (less
263than 50-55 nucleotides upstream the 3'-most exon-exon junction), this PTC confers
264'resistance' against NMD [22, 24, 43]. As sequel, the mRNA abundance in such NMD-
265resistant mRNA carriers differs from those lacking these RNAs. This could explain why
266the *GLB1* mRNA levels were higher in *GLB1*^{-/-} fibroblasts compared to *GLB1*^{+/+} Alaskan
267huskies fibroblasts. To prove the presence of NMD-resistant mRNA and to further
268investigate the distributions of both *GLB1* variants in *GLB1*^{+/-} and *GLB1*^{-/-} fibroblasts, qRT-
269PCR using variant-specific primers was performed. Thus, NMD-resistant mRNA
270(*GLB1*dupl) was detected in *GLB1*^{+/-} and *GLB1*^{-/-} fibroblasts. In addition, after absolute
271RT-PCR quantification, it was observed that only a small fraction of this mRNA lacks the
272mutant exon 15 (*GLB1* Δ Ex15). The amino acid sequence encoded by exon 15 contains a

273 cleavage site important for the correct lysosomal processing of GLB1.

274 The GLB1 enzymatic activity depends on its correct assembly with PPCA and NEU1 to
275 form the lysosomal multienzyme complex [26, 44, 45]. Albeit the elevated levels of *GLB1*
276 expression in *GLB1*^{+/-} fibroblasts a lack of an appropriate amount of the other LMC
277 components could potentially lead to deficiencies in GLB1 activity. Therefore we further
278 investigated the expression of the *PPGB* and *NEU1* genes in *GLB1*^{+/+}, *GLB1*^{+/-} and *GLB1*^{-/-}
279 fibroblasts. Thus, after analysis of *NEU1* mRNA levels in these fibroblasts no significant
280 difference was observed between *GLB1*^{+/+}, *GLB1*^{+/-} and *GLB1*^{-/-} cells. Furthermore, our
281 previous analyses of the NEU1 enzymatic activity of canine fibroblasts from healthy,
282 carriers and homozygous Alaskan huskies showed no differences among the three
283 genotypes [33]. These results are in concordance with the observation that some human
284 *GLB1* mutant alleles are not associated with modifications in *NEU1* expression [10]. A
285 lack of variation in the *NEU1* expression was also described when sialidosis patients
286 (*NEU1*^{-/-}) were compared to control healthy individuals (*NEU1*^{+/+}) [46].

287 Housekeeping genes characteristics were initially reported for the *PPGB* promoter;
288 however, the distribution pattern of *PPGB* requires a specific regulation that cannot be
289 anticipated by the housekeeping gene properties of its promoter [39, 47]. In the present
290 study, a differential expression of *PPGB* mRNA was observed in *GLB1*^{+/+}, *GLB1*^{+/-} and
291 *GLB1*^{-/-} fibroblasts. This correlates with the increase of total *GLB1* mRNA level in *GLB1*^{+/-}
292 and *GLB1*^{-/-} fibroblasts compared to *GLB1*^{+/+} cells.

293 Previous studies described two distinct and separate functions of the PPCA: the
294 carboxypeptidase function as mature form and the protective role as precursor [48]. In our
295 experiments, the expression of the PPCA precursor correlates with *GLB1* genotypes (low
296 in *GLB1*^{+/+}, medium in *GLB1*^{+/-} and high in *GLB1*^{-/-}) while the mature forms showed not
297 only a GLB1 mutation type-dependent expression [49] but also individual variations.

298 These observations suggest a role of PPCA precursor in GLB1 protection in *GLB1*^{+/-} and
299 *GLB1*^{-/-} fibroblasts. Therefore, the GLB1 activity in *GLB1*^{+/-} fibroblasts seems to be
300 maintained at high levels by a simultaneous increase of GLB1 and PPCA precursor
301 expression. Interestingly, the observation that PPCA precursor expression in *GLB1*^{-/-}
302 fibroblasts was significantly higher compared to *GLB1*^{+/-} fibroblasts, indicates a
303 mechanism whereby the PPCA precursor level is increased in order to antagonize the lack
304 of GLB1 activity. Further studies are necessary to decipher this mechanism and its clinical
305 relevance.

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462**Fig. 1.** Design of the primer pairs used to detect different populations of GLB1 mRNA. Ex14
463= canine *GLB1* exon 14; Ex15 = canine *GLB1* exon 15; Ex16 = canine *GLB1* exon 16; fw =
464sense; rev = antisense primers; GLB1 = wild type canine *GLB1*; GLB1 Δ Ex15 = mutant
465canine *GLB1* lacking exon 15; GLB1dupl = mutant canine *GLB1* with the abnormal exon 15
466carrying the 19bp duplication. Different populations of GLB1 mRNA: total GLB1,
467GLB1 Δ Ex15 and GLB1dupl were specifically detected by using GLB1fw-GLB1rev, Ex14fw-
468Ex14/16rev and Duplfw-Ex15/16rev primers.

469**Fig. 2.** GLB1 activity in *GLB1*^{+/+}, *GLB1*^{+/-}, *GLB1*^{-/-} fibroblasts. GLB1^{+/+}, GLB1^{+/-}, GLB1^{-/-}
470correspond to GLB1 activities in homozygous dominant (*GLB1*^{+/+}), heterozygous (*GLB1*^{+/-})
471and homozygous recessive (*GLB1*^{-/-}) fibroblasts. GLB1 activities are given as mU/mg protein.
472The GLB1 activity in *GLB1*^{-/-} fibroblasts represented 2.73% (10 \pm 1.43 mU/mg) of those of
473*GLB1*^{+/+} and *GLB1*^{+/-} [365.1 \pm 122.33 and 340 \pm 127.87 mU/mg respectively; * - statistically
474significant differences ($p < 0,05$)]

475**Fig. 3.** Expression of total *GLB1* mRNA in *GLB1*^{+/+}, *GLB1*^{+/-}, *GLB1*^{-/-} fibroblasts. GLB1^{+/+},
476GLB1^{+/-}, GLB1^{-/-} correspond to *GLB1* mRNA expression levels in homozygous dominant
477(*GLB1*^{+/+}), heterozygous (*GLB1*^{+/-}) and homozygous recessive (*GLB1*^{-/-}) fibroblasts. Columns
478show relative expression levels of GLB1 normalized to canine GAPDH analyzed by
479quantitative RT-PCR. Statistically significant differences (* - $p < 0.05$) were observed between
480*GLB1* mRNA levels of *GLB1*^{+/+} compared to *GLB1*^{+/-} and *GLB1*^{-/-} fibroblasts.

481**Fig. 4.** Expression of GLB1 Δ Ex15 mRNA (lacking exon 15) in *GLB1*^{+/-} and *GLB1*^{-/-}
482fibroblasts. GLB1^{+/-} and GLB1^{-/-} correspond to GLB1 Δ Ex15 mRNA expression values in
483heterozygous (*GLB1*^{+/-}) and homozygous recessive (*GLB1*^{-/-}) fibroblasts. Columns show
484relative expression levels of GLB1 Δ Ex15 normalized to canine GAPDH analyzed by
485quantitative RT-PCR. Statistically significant differences (* - $p < 0.05$) of GLB1 Δ Ex15 mRNA
486were observed between *GLB1*^{+/-} and *GLB1*^{-/-} fibroblasts.

487**Fig. 5.** Expression of GLB1dupl mRNA (with the exon 15 carrying the 19bp duplication) in
488 *GLB1*^{+/-}, *GLB1*^{-/-} fibroblasts. *GLB1*^{+/-} and *GLB1*^{-/-} correspond to GLB1dupl mRNA
489 expression levels in heterozygous (*GLB1*^{+/-}) and homozygous recessive (*GLB1*^{-/-}) fibroblasts.
490 Columns show relative expression levels of GLB1dupl normalized to canine GAPDH
491 analyzed by quantitative RT-PCR. No significant differences ($p < 0.05$) in the expression levels
492 of GLB1dupl mRNA in *GLB1*^{+/-} and *GLB1*^{-/-} cells were observed.

493**Fig. 6.** Expression of *NEUI* mRNA in *GLB1*^{+/+}, *GLB1*^{+/-} and *GLB1*^{-/-} fibroblasts. *GLB1*^{+/+},
494 *GLB1*^{+/-}, *GLB1*^{-/-} correspond to *NEUI* mRNA expression levels in homozygous dominant
495 (*GLB1*^{+/+}), heterozygous (*GLB1*^{+/-}) and homozygous recessive (*GLB1*^{-/-}) fibroblasts. Columns
496 show relative expression levels of *NEUI* normalized to canine GAPDH analyzed by
497 quantitative RT-PCR. No significant differences ($p < 0.05$) in the expression levels of *NEUI*
498 mRNA in *GLB1*^{+/+}, *GLB1*^{+/-} and *GLB1*^{-/-} cells were observed.

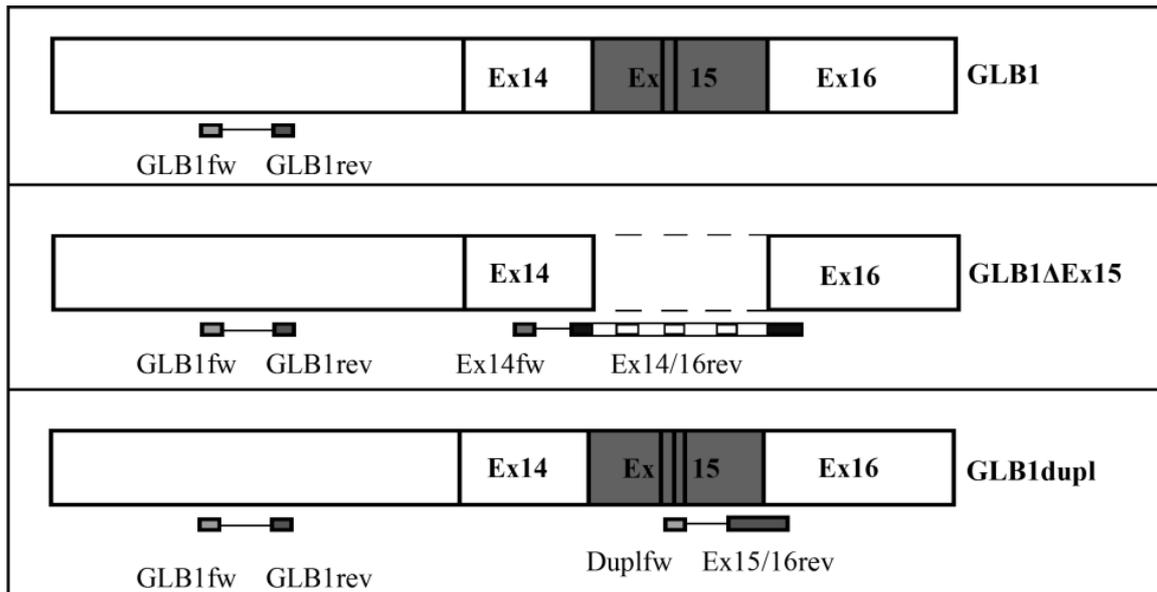
499**Fig. 7.** Expression of *PPGB* mRNA in *GLB1*^{+/+}, *GLB1*^{+/-} and *GLB1*^{-/-} fibroblasts. *GLB1*^{+/+},
500 *GLB1*^{+/-}, *GLB1*^{-/-} correspond to *PPGB* mRNA expression levels in homozygous dominant
501 (*GLB1*^{+/+}), heterozygous (*GLB1*^{+/-}) and homozygous recessive (*GLB1*^{-/-}) fibroblasts. Columns
502 show relative expression levels of *PPGB* mRNA normalized to canine GAPDH analyzed by
503 quantitative RT-PCR. Statistically significant differences (* - $p < 0.05$) were observed between
504 *PPGB* mRNA levels from *GLB1*^{+/+} compared to *GLB1*^{+/-} and *GLB1*^{-/-} fibroblasts.

505**Fig. 8.** Expression of PPCA protein in *GLB1*^{+/+}, *GLB1*^{+/-} and *GLB1*^{-/-} fibroblasts. *GLB1*^{+/+},
506 *GLB1*^{+/-}, *GLB1*^{-/-} correspond to PPCA protein levels in homozygous dominant (*GLB1*^{+/+}),
507 heterozygous (*GLB1*^{+/-}) and homozygous recessive (*GLB1*^{-/-}) fibroblasts normalized to actin
508 protein. Statistically significant differences (* - $p < 0.05$) were observed between the PPCA
509 protein of *GLB1*^{-/-} compared to *GLB1*^{+/-} and *GLB1*^{+/+} fibroblasts.

510**Table 1.** Characteristics of the primer pairs used for quantification of GLB1 variants,
 511PPCA and NEU1. *GLB1*- canine β -galactosidase gene; *GLB1* Δ Ex15 – mutant canine β -
 512galactosidase lacking exon 15; *GLB1*dupl – mutant canine β -galactosidase with the
 513abnormal exon 15 carrying the 19 bp duplication; *PPCA* – protective protein/cathepsin A
 514gene; *NEU1*- neuraminidase 1 gene; *GAPDH* - glyceraldehyde-3-phosphate-
 515dehydrogenase; fw = sense; rev = antisense; Ex14 = canine *GLB1* exon 14; Ex14/16 =
 516boundary between exon 14 and 16 in the mutant canine *GLB1* lacking exon 15; dupl =
 51719bp duplication in exon 15 of canine *GLB1*; * - primers were generated using wild type
 518(DQ 196436) and mutant specific *GLB1* sequences.

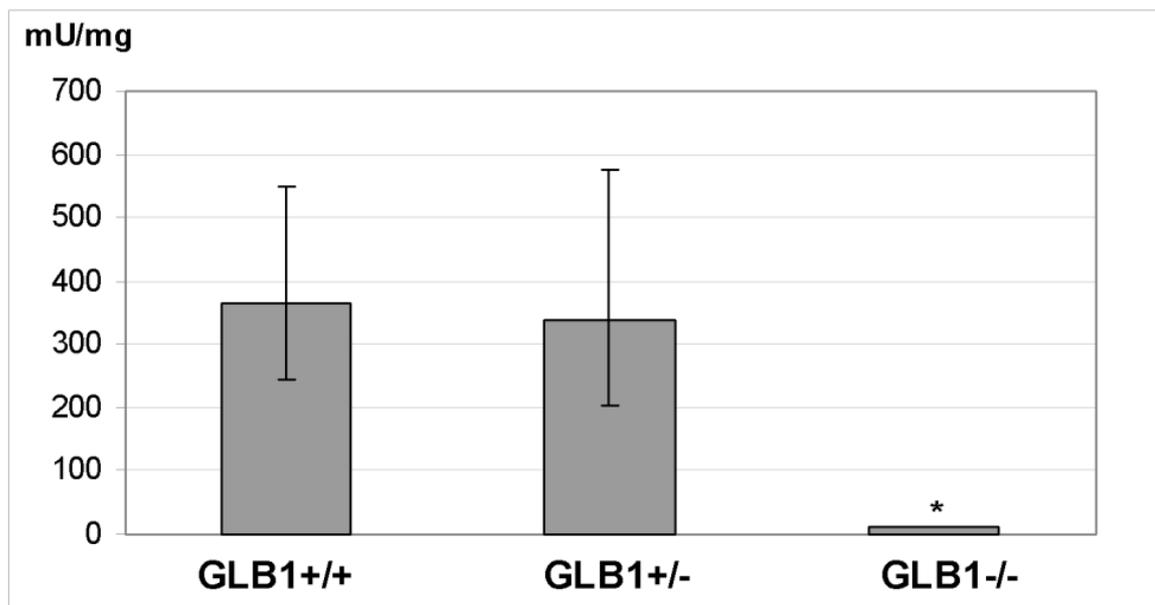
Gene name GeneBank Acc. No.	Primer Name	Sequence	Positions	Size of PCR product (bp)
<i>GLB1</i>	97fw	5'-TTC ACA ATT GAC TAC AGC CAC- 3'	79-100	326
DQ 196436	98rev	5'- TAA TAG CCA AGC AGG TAA TC- 3'	384-405	
<i>GLB1</i> Δ Ex15	Ex14fw	5'-TCT GAA CAT AAC CCG GAA GG- 3'*	1390-1409	107
DQ 196436*	Ex14/16rev	5'-TCC ACA GAC CCT TAA AAT C- 3'*	1475-1488	
<i>GLB1</i> dupl	Dupl fw	5'-TTG CCC CAG GAT CCC AGA C- 3'*	1697-1715	76
DQ 196436*	Ex15/16	5'-TAA TCC ACA CCT GAC CCT TG- 3' *	1754-1773	
<i>PPCA</i>	PPCAfw	5'-CCT ATG AGC AGA ATG ACA ACT CC- 3'	687-709	290
NW 876277	PPCArev	5'-GGC AGG CGA GTG AAG ATG TT- 3'	954-983	
<i>NEU1</i>	NEU1fw	5'-CAT GTT GGT GTG GAG CAA AG- 3'	507-526	195
XM 538838	NEU1rev	5'-CAC TGA GGA GGC AGA AGA CC- 3'	681-700	
<i>GAPDH</i>	GAPDHfw	5'-GCC AAA AGG GTC ATC ATC TC- 3'	340-359	229
AB 038240	GAPDHrev	5'-GGC CAT CCA CAG TCT TCT- 3'	548-565	

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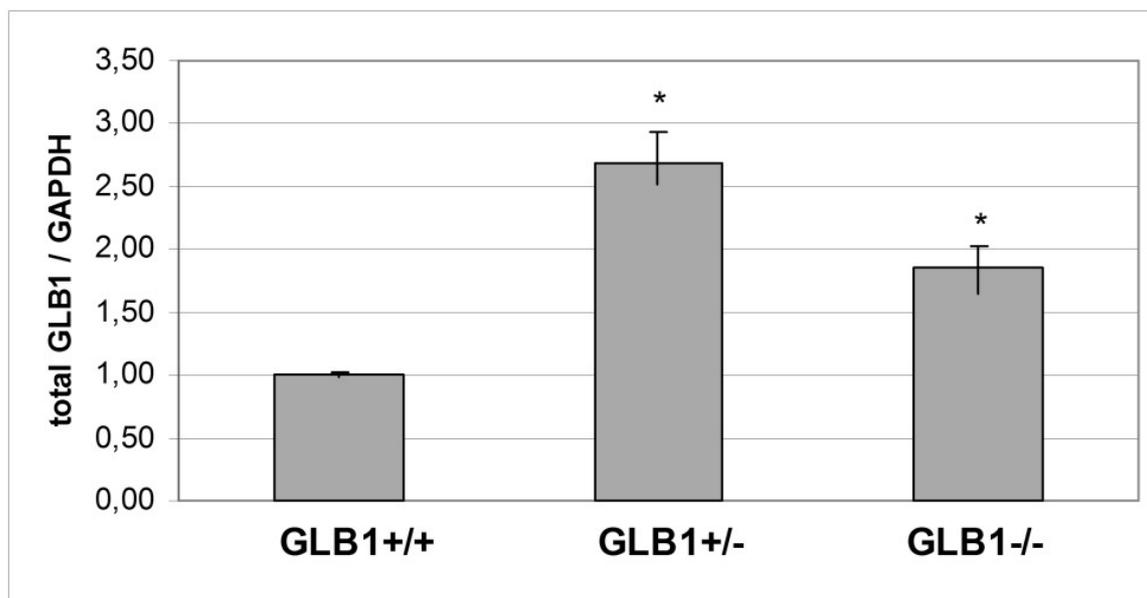
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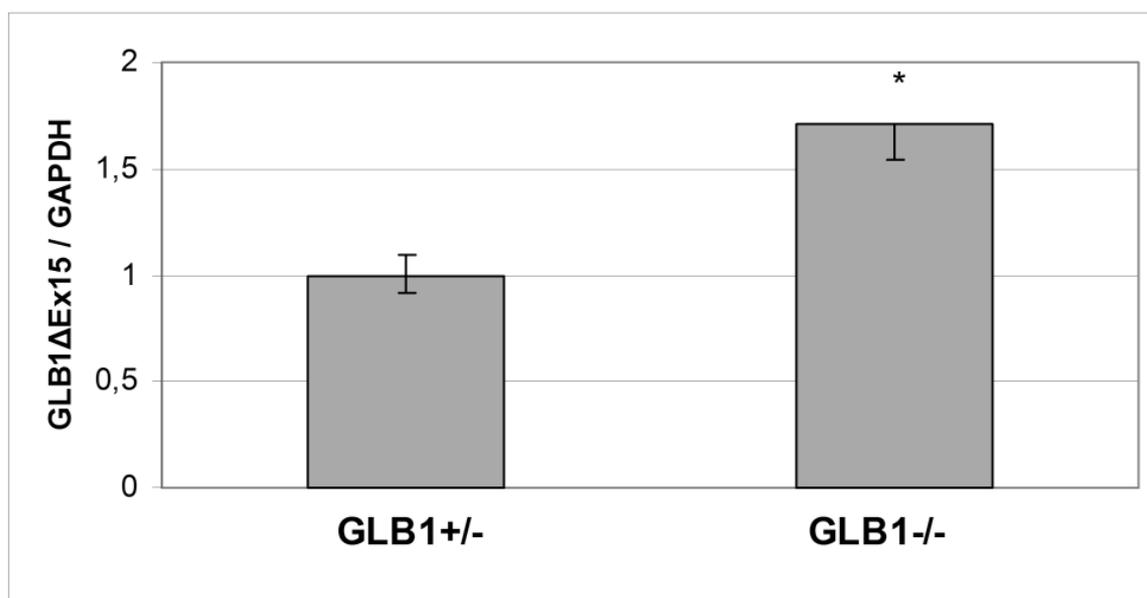


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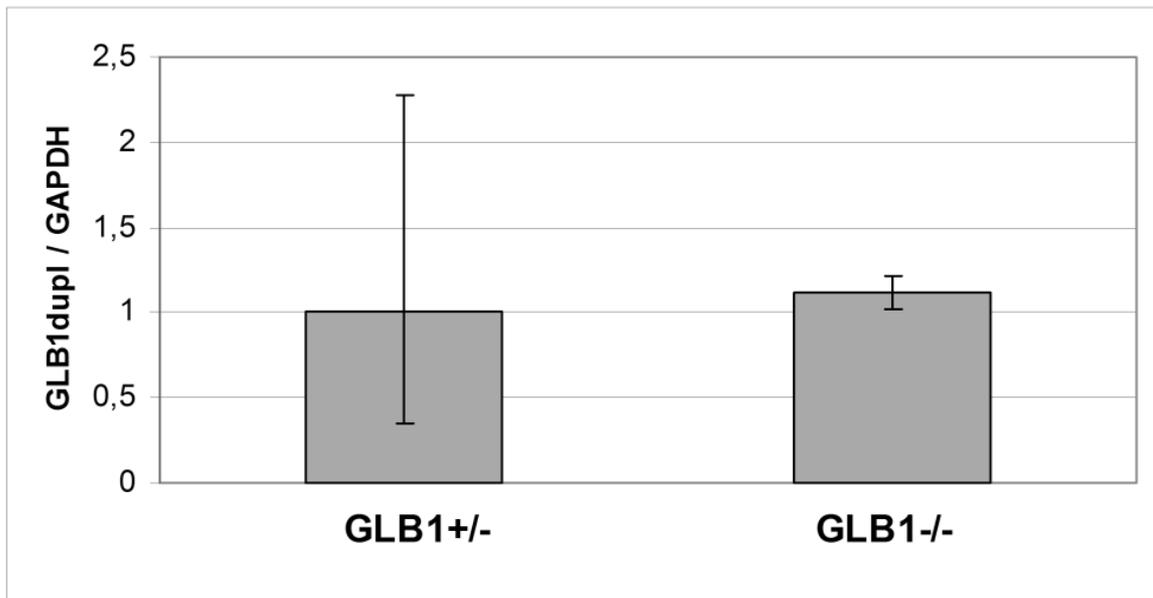
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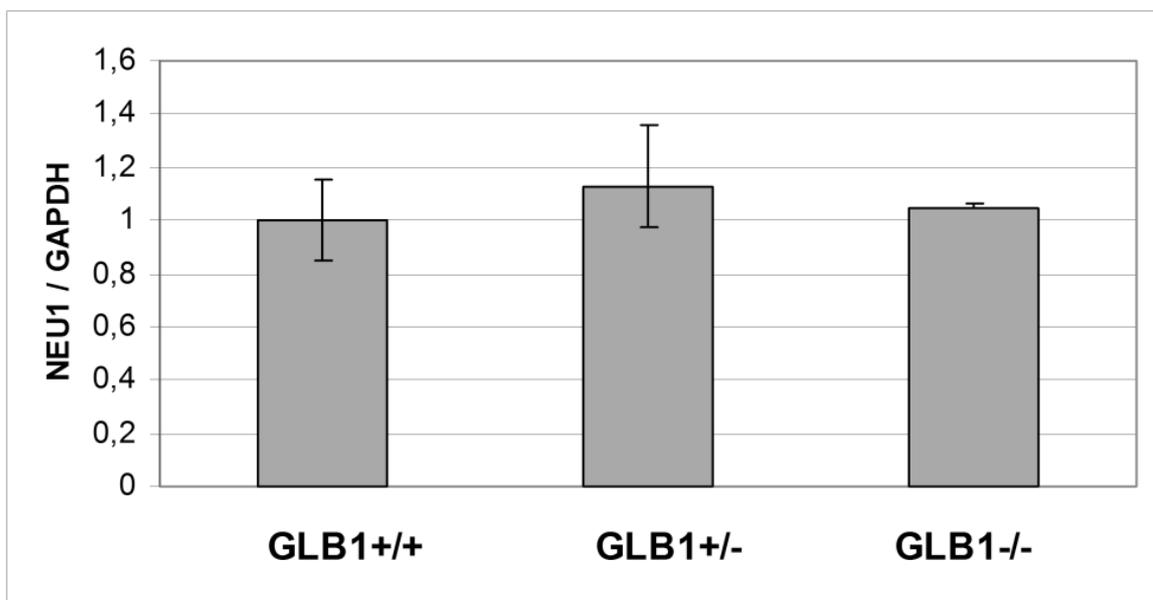


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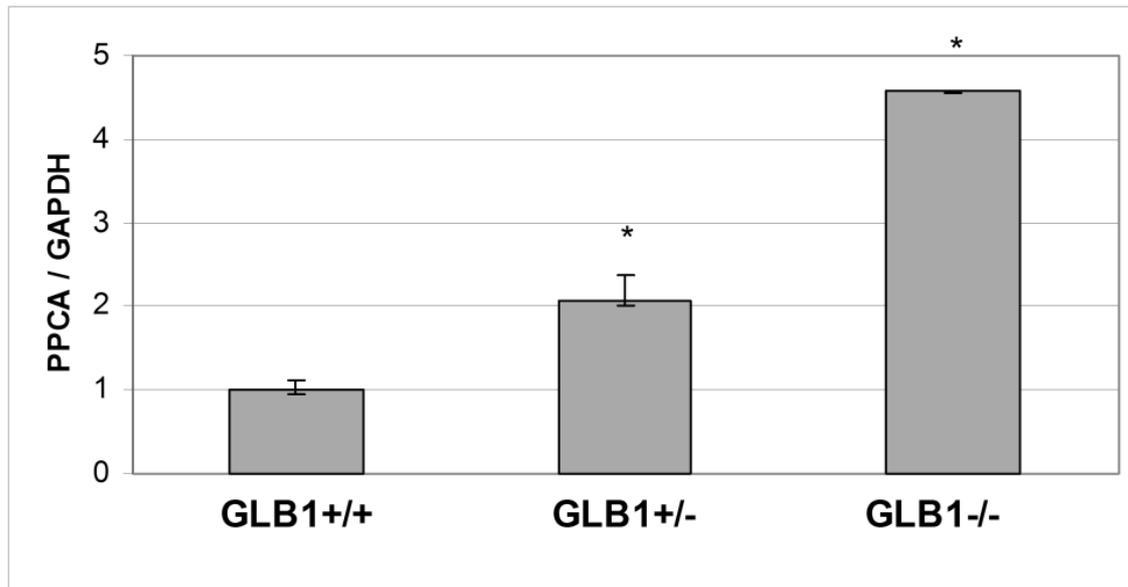
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