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# Evolution of gremlin 2 in cetartiodactyl mammals: gene loss coincides with lack of upper jaw incisors in ruminants

Juan C Opazo <sup>Corresp.</sup> <sup>1</sup>, Kattina Zavala <sup>1</sup>, Paola Krall <sup>2</sup>, Rodrigo Arias <sup>3</sup>

<sup>1</sup> Instituto de Ciencias Ambientales y Evolutivas, Universidad Austral de Chile, Valdivia, Chile

<sup>2</sup> Unidad de Nefrología, Universidad Austral de Chile, Valdivia, Chile

<sup>3</sup> Instituto de Producción Animal, Universidad Austral de Chile, Valdivia, Chile

Corresponding Author: Juan C Opazo

Email address: jopazo@gmail.com

Understanding the processes that give rise to genomic variability in extant species is an active area of research within evolutionary biology. With the availability of whole genome sequences, it is possible to quantify different forms of variability such as variation in gene copy number, which has been described as an important source of genetic variability and in consequence of phenotypic variability. Most of the research on this topic has been focused on understanding the biological significance of gene duplication, and less attention has been given to the evolutionary role of gene loss. Gremlin 2 is a member of the DAN gene family and plays a significant role in tooth development by blocking the ligand-signaling pathway of BMP2 and BMP4. The goal of this study was to investigate the evolutionary history of gremlin 2 in cetartiodactyl mammals, a group that possesses highly divergent teeth morphology. Results from our analyses indicate that gremlin 2 has experienced a mixture of gene loss, gene duplication, and rate acceleration. Although the last common ancestor of cetartiodactyls possessed a single gene copy, pigs and camels are the only cetartiodactyl groups that have retained gremlin 2. According to the phyletic distribution of this gene and synteny analyses, we propose that gremlin 2 was lost in the common ancestor of ruminants and cetaceans between 56.3 and 63.5 million years ago as a product of a chromosomal rearrangement. Our analyses also indicate that the rate of evolution of gremlin 2 has been accelerated in the two groups that have retained this gene. Additionally, the lack of this gene could explain the high diversity of teeth among cetartiodactyl mammals; specifically, the presence of this gene could act as a biological constraint. Thus, our results support the notions that gene loss is a way to increase phenotypic diversity and that gremlin 2 is a dispensable gene, at least in cetartiodactyl mammals.

1 **Research paper**

2

3 **Evolution of gremlin 2 in cetartiodactyl mammals: gene loss**  
4 **coincides with lack of upper jaw incisors in ruminants**

5

6 Juan C. Opazo<sup>1</sup>, Kattina Zavala<sup>1</sup>, Paola Krall<sup>2</sup>, Rodrigo A. Arias<sup>3</sup>

7 <sup>1</sup>Instituto de Ciencias Ambientales y Evolutivas, Facultad de Ciencias, Universidad Austral de

8 Chile, Valdivia, Chile. <sup>2</sup>Unidad de Nefrología, Facultad de Medicina, Universidad Austral de

9 Chile, Valdivia, Chile. <sup>3</sup>Instituto de Producción Animal, Facultad de Ciencias Agrarias,

10 Universidad Austral de Chile, Valdivia, Chile

11

12

13 **Corresponding author:**

14 Juan C. Opazo

15 Email: [jopazo@gmail.com](mailto:jopazo@gmail.com)

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19

**20 Abstract**

21 Understanding the processes that give rise to genomic variability in extant species is an active  
22 area of research within evolutionary biology. With the availability of whole genome sequences,  
23 it is possible to quantify different forms of variability such as variation in gene copy number,  
24 which has been described as an important source of genetic variability and in consequence of  
25 phenotypic variability. Most of the research on this topic has been focused on understanding the  
26 biological significance of gene duplication, and less attention has been given to the evolutionary  
27 role of gene loss. Gremlin 2 is a member of the DAN gene family and plays a significant role in  
28 tooth development by blocking the ligand-signaling pathway of BMP2 and BMP4. The goal of  
29 this study was to investigate the evolutionary history of gremlin 2 in cetartiodactyl mammals, a  
30 group that possesses highly divergent teeth morphology. Results from our analyses indicate that  
31 gremlin 2 has experienced a mixture of gene loss, gene duplication, and rate acceleration.  
32 Although the last common ancestor of cetartiodactyls possessed a single gene copy, pigs and  
33 camels are the only cetartiodactyl groups that have retained gremlin 2. According to the phyletic  
34 distribution of this gene and synteny analyses, we propose that gremlin 2 was lost in the common  
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36 chromosomal rearrangement. Our analyses also indicate that the rate of evolution of gremlin 2  
37 has been accelerated in the two groups that have retained this gene. Additionally, the lack of this  
38 gene could explain the high diversity of teeth among cetartiodactyl mammals; specifically, the  
39 presence of this gene could act as a biological constraint. Thus, our results support the notions  
40 that gene loss is a way to increase phenotypic diversity and that gremlin 2 is a dispensable gene,  
41 at least in cetartiodactyl mammals.

## 42 Introduction

43 One of the main goals of evolutionary biology is to understand the genetic basis of phenotypic  
44 diversity. To address this question, scientists have made efforts to identify genes that are linked  
45 to phenotypes and to explore the phenotypic consequences of genetic variability. With the  
46 availability of whole genome sequences, it has been possible to compare different forms of  
47 variability, and variation in gene copy number has been described as an important source of  
48 genetic variability. To date, most of the research on this topic has been focused towards  
49 understanding the biological significance of gene duplication, and less attention has been given  
50 to the evolutionary role of gene loss (Olson, 1999; Albalat and Cañestro, 2016). In the literature,  
51 there are examples of gene loss being associated with positive impacts on fitness. For example,  
52 the loss of the CCR5 gene in humans is associated with resistance to AIDS (Dean et al., 1996),  
53 and the loss of hair keratin genes in cetaceans is interpreted as an adaptation associated with the  
54 transition from terrestrial to aquatic life (Nery et al., 2014; Yim et al., 2014). Thus, evolutionary  
55 studies of genes that possess a clear link to a given phenotype represent an opportunity to  
56 understand the phenotypic effects of gene loss and gene dispensability.

57 Gremlin 2, previously known as a protein related to Dan and Cerberus (PRDC), is a  
58 member of the DAN gene family, a group of extracellular bone morphogenetic protein (BMP)  
59 inhibitors, which was originally identified in a gene trap screen for developmentally significant  
60 genes (Minabe-Saegusa et al., 1998). Gremlin 2, as an antagonist of BMPs (Kattamuri et al.,  
61 2012), plays a role in several developmental processes including organogenesis, body patterning,  
62 and tissue differentiation. In embryonic stages, this gene is expressed in the reproductive,  
63 nervous, respiratory, musculoskeletal, and integumentary systems (Müller et al., 2006).

64 Alternatively, during adulthood, it is a widely expressed gene found in high levels in ovaries,  
65 brain, and spleen (Sudo et al., 2004).

66 In the literature, it has been shown that gremlin 2 interacts with BMP2 and BMP4 by  
67 blocking their ligand-signaling pathway (Sudo et al., 2004). Human genetic studies have  
68 indicated that gremlin 2 variation can influence one's susceptibility of having a common tooth  
69 malformation (Kantaputra et al., 2015). Mutational analysis in seven out of 263 patients with  
70 different dental anomalies has revealed the presence of mutations predicted to cause disease.  
71 Five patients of this study carried the same heterozygous mutations (Ala13Val) while the other  
72 two were carriers of two different heterozygous missense mutations (Gln76Glu and Glu136Asp)  
73 (Kantaputra et al., 2015). This genetic study supports the notion that inheritance of hypodontia is  
74 autosomal dominant, and this is related to gremlin 2. Despite this, the study also gives evidence  
75 of incomplete penetrance and variable expressivity. Genetic experiments provide further support  
76 for the role of gremlin 2 in tooth development (Brommage et al., 2014); it has been shown that  
77 gremlin 2 deficient mice have upper and lower incisor teeth with markedly reduced breadth and  
78 depth, and the upper incisors are more severely affected than lower ones (Vogel et al., 2015).  
79 According to Voget et al. (2015) no other significant phenotypic effects have been observed in  
80  $gremlin2^{-/-}$  individuals, indicating that this gene could be dispensable. From a developmental  
81 perspective, it has been shown that the pathway that controls tooth differentiation is conserved in  
82 most mammals other than cetaceans, xenarthrans, and phocid seals (Armfield et al., 2013). In  
83 dolphins, it has been shown that expression of BMP4, which is one of the main targets of  
84 gremlin 2 (Sudo et al., 2004), is extended to the caudal region of the developing jaw, a region  
85 where the fibroblast growth factor 8 gene (FGF8) is expressed in most mammals (Armfield et al.,  
86 2013). This developmental difference could be related to the divergent dental phenotype of

87 dolphins. Similar results have been found during epibranchial placode development (Kriebitz et  
88 al., 2009). Within the same mammalian clade, other groups also have different dental  
89 morphologies. For example, ruminants do not possess incisors in the upper jaw; instead they  
90 possess a dental pad. Canines are also absent in most ruminant species with the exception of elk  
91 and red deer. This particular dental phenotype has consequences in the way these animals  
92 process food, which is different compared to related species (herbivores) that possess incisors in  
93 the upper jaw (e.g. horse).

94         The main goal of this study was to investigate the evolutionary history of gremlin 2, a  
95 gene that plays a significant role in the tooth development, in cetartiodactyl mammals a group  
96 that possesses divergent tooth morphologies. Results from our analyses show that gremlin 2 has  
97 experienced a mixture of gene loss, gene duplication, and rate acceleration. Although the last  
98 common ancestor of cetartiodactyls possessed a single gene copy, pigs and camels are the only  
99 cetartiodactyl groups that have retained gremlin 2. According to the phyletic distribution of this  
100 gene and synteny analyses, we propose that gremlin 2 was lost in the common ancestor of  
101 ruminants and cetaceans between 56.3 and 63.5 million years ago as a product of a chromosomal  
102 rearrangement. Our analyses also indicate that the rate of evolution of gremlin 2 in pigs and  
103 camels has been accelerated, and the possession of gremlin 2 clearly differentiates these groups  
104 from all other cetartiodactyl mammals.

105

## 106 **Materials and methods**

### 107 **DNA data collection and phylogenetic analyses**

108 We annotated gremlin 2 genes in representative species of laurasiatherian mammals. Our study  
109 included representative species from the orders Carnivora: cat (*Felis catus*), Siberian tiger

110 (*Panthera tigris*), dog (*Canis familiaris*), ferret (*Mustela putorius*), Weddell seal (*Leptonychotes*  
111 *weddellii*), Pacific walrus (*Odobenus rosmarus*), panda (*Ailuropoda melanoleuca*);  
112 Perissodactyla: Przewalski's horse (*Equus ferus*), horse (*Equus caballus*), donkey (*Equus*  
113 *asinus*), Eulipotyphla: European hedgehog (*Erinaceus europaeus*); Chiroptera: Black flying fox  
114 (*Pteropus alecto*), Megabat (*Pteropus vampyrus*), Egyptian fruit bat (*Rousettus aegyptiacus*); and  
115 Cetartiodactyla: pig (*Sus Scrofa*), alpaca (*Vicugna pacos*), dromedary (*Camelus dromedarius*)  
116 and Bactrian camel (*Camelus bactrianus*) (Supplementary Table S1). Mouse and kangaroo rat  
117 sequences were used as outgroups. Amino acid sequences were aligned using the L-INS-i  
118 strategy from MAFFT v.6 (Katoh and Standley, 2013). Nucleotide alignment was generated  
119 using the amino acid alignment as a template using the software PAL2NAL (Suyama et al.,  
120 2006). Phylogenetic relationships were estimated using maximum likelihood and Bayesian  
121 approaches. In both cases, second codon positions were excluded. We performed a maximum  
122 likelihood analysis to obtain the best tree using the program RAxML version 8 (Stamatakis,  
123 2014) and assessed support for the nodes with 1,000 bootstrap pseudoreplicates. Bayesian  
124 searches were conducted in MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003); two  
125 independent runs of six simultaneous chains for  $20 \times 10^6$  generations were set, and every 2,500  
126 generations were sampled using default priors. The run was considered to have reached  
127 convergence once the likelihood scores reached an asymptote and the average standard deviation  
128 of the split frequencies remained  $< 0.01$ . We discarded all trees that were sampled before  
129 convergence, and we evaluated support for the nodes and parameter estimates from a majority  
130 rule consensus of the last 4,000 trees.

131

### 132 **Assessments of Conserved Synteny**



133 We examined genes found up- and downstream of gremlin 2 in the laurasiatherian mammal  
134 representative species. Synteny analyses were conducted for dog (*Canis familiaris*), panda  
135 (*Ailuropoda melanoleuca*), horse (*Equus caballus*), donkey (*Equus asinus*), European hedgehog  
136 (*Erinaceus europaeus*), Megabat (*Pteropus vampyrus*), Egyptian fruit bat (*Rousettus*  
137 *aegyptiacus*), alpaca (*Vicugna pacos*), dromedary (*Camelus dromedarius*), pig (*Sus scrofa*),  
138 sheep (*Ovis aries*), goat (*Capra hircus*), cow (*Bos taurus*), minke whale (*Balaenoptera*  
139 *acutorostrata*), killer whale (*Orcinus orca*) and baiji (*Lipotes vexillifer*). Initial ortholog  
140 predictions were derived from the EnsemblCompara database (Herrero et al., 2016) and were  
141 visualized using the program Genomicus v85.01 (Muffato et al., 2010). In other cases, the  
142 genome data viewer platform from the National Center for Biotechnology information was used.

143

## 144 **Results and Discussion**

### 145 **Phylogenetic relationships**

146 We constructed a phylogenetic tree in which we included representative species of  
147 laurasiatherian mammals (Fig. 1). Our phylogenetic analysis recovered the monophyly of each  
148 laurasiatherian order included in our sampling (Fig. 1). Although the phylogenetic relationships  
149 among laurasiatherian mammals at the ordinal level are still a matter of debate, the most  
150 important departure from current hypotheses detected here was the sister group relationship  
151 between Eulipotyphla and Perissodactyla (Fig. 1); in most studies, eulipotyphlan species appear  
152 sister to all other laurasiatherian mammals (Nery et al., 2012; Foley et al., 2016). The synteny  
153 analysis provided further support for the identity of the gremlin 2 gene lineage in this group of  
154 mammals (Fig. 2); genes found downstream were well conserved in all examined species (Fig.  
155 2). According to our survey, most species included in this study possessed four downstream

156 genes (*RGS7*, *FH*, *KMO* and *OPN3*) that define the identity of this genomic region (Fig. 2).  
157 Although the genes found upstream were more variable, they were to some degree more  
158 conserved in the different groups (Fig. 2). For example, in both camelid species four upstream  
159 genes (*RNF2*, *TRMT1L*, *SWT1* and *IVNSIABP*) were detected that were well conserved (Fig. 2).  
160 Similar results were found for sheep, goat, cow, minke whale, killer whale, and baiji (Fig. 2).  
161

### 162 **Molecular rates and structural divergence in cetartiodactyls**

163 The rate of molecular evolution, as measured here using branch lengths, was variable (Fig. 1),  
164 though the most striking result was that of the accelerated evolution of the cetartiodactyl clade  
165 (Fig. 1; green clade). To test whether the rate of gremlin 2 evolution in this group of species is  
166 significantly higher, we performed a relative rate test (Tajima, 1989) using the software MEGA 7  
167 (Kumar et al., 2016). We compared the rate of evolution of the five cetartiodactyl sequences  
168 using the cat sequence as a reference and the mouse sequence as the outgroup. Results of this  
169 analysis confirmed what was observed in our phylogenetic tree, i.e. the rate of evolution of  
170 cetartiodactyls is significantly higher than that of other laurasiatherian mammals (Supplementary  
171 table S2). To further investigate the evolutionary pattern of gremlin 2 in cetartiodactyls, we made  
172 an amino acid alignment that included, in addition to the five cetartiodactyl sequences,  
173 representative species of the laurasiatherian orders Perissodactyla, Carnivora, Chiroptera, and  
174 Eulipotyphla. From this, we found that there are 13 synapomorphies that define the gremlin 2  
175 genes in cetartiodactyls (Fig. 3). Among these, we identified 11 amino acid changes and two  
176 deletions (Fig. 3). Of all of the amino acid substitutions, changes at positions 34 (Tyr to Arg),  
177 109 (His to Pro), 131 (Thr to Ala), and 132 (Ser to Ala) represent changes affecting  
178 hydrophobicity (Fig. 3). To determine whether or not the observed amino acid replacements in

179 cetartiodactyls would have structural consequences, we predicted the secondary protein structure  
180 using the EMBOSS garnier package (Rice et al., 2000) (Fig. 4). From this analysis, we identified  
181 three main regions in which the secondary structure of the proteins of cetartiodactyls would  
182 diverge compared to other laurasiatherian species and humans (Fig. 4). Interestingly, in these  
183 three regions, two structural patterns could be distinguished. The first pattern was found in the  
184 gremlin 2 sequences of pig, while the other pattern was identified in the three camelid species  
185 (alpaca, dromedary, and Bactrian camel)(Fig. 4). The first region spanned the alignment  
186 positions 23 to 61 and was characterized by changes from beta strands to coil structures at the N-  
187 terminal portion of the region and changes from alpha helices to coil and beta strand structures at  
188 the C-terminal portion of the region (Fig. 4). The second region was mapped between the  
189 residues 104 and 157 and was mainly characterized by changes from alpha helices to turn and  
190 coil structures at the N-terminal portion of the region, from beta strands to alpha helices in the  
191 middle portion of the region, and from alpha helices to turns and beta strands at the C-terminal  
192 portion of the region (Fig. 4). Finally, the third region where we found cetartiodactyls to be  
193 structurally divergent started at alignment position 166 and ended at position 170. In the three  
194 camelid species, this region was characterized by changes from turn structures to alpha helices,  
195 and in the two pig gremlin 2 sequences the turn structures changed to coil structures (Fig. 4).

196

### 197 **Gene copy number variation and differential retention in cetartiodactyls**

198 Most laurasiatherian species possess a single copy of the gene with the exception of pig (*Sus*  
199 *scrofa*) that has two copies on chromosome 10 (Fig. 5). As in all examined species, in pig, one of  
200 the copies (gremlin 2-T1) was found on the 5' side of the regulator of the G-protein signaling 7  
201 gene (RGS7) (Fig. 2). The second copy (gremlin 2-T2) was found within the RGS7 gene,

202 specifically between exons 13 and 14 (Fig. 5). At the amino acid level both copies differed in  
203 one amino acid (position 155); gremlin 2-T1 possessed an arginine, and gremlin 2-T2 possessed  
204 a lysine.

205         Among cetartiodactyls, we observed that gremlin 2 was differentially retained (Fig. 2).  
206 Species belonging to the suborders tylopoda (the group that includes camels, alpacas, vicuñas,  
207 and guanacos) and suiformes (the group that includes pigs and peccaries) were the only groups in  
208 which gremlin 2 was present (Fig. 2). In cetaceans and ruminants, gremlin 2 was not present.  
209 Thus, according to the phyletic distribution of gremlin 2 within the main groups of  
210 cetartiodactyls, the most likely scenario is that the deletion of the gene occurred between 56.3  
211 and 63.5 million of years ago in the common ancestor of the clade that includes ruminants,  
212 hippopotamuses, and cetaceans (Fig. 6). However, until information regarding gremlin 2 in  
213 hippopotamuses is obtained, caution must be taken when interpreting these results. If, in the  
214 future, the hippopotamus genome is found to possess gremlin 2, we can determine that two  
215 independent gene losses occurred, one in the ancestor of ruminants and a second in the ancestor  
216 of cetaceans. For now, a single gene loss event is assumed.

217         To gain insight into the genetic mechanisms that gave rise to the deletion of gremlin 2,  
218 we compared the chromosomal location of genes found up- and downstream of gremlin 2 in  
219 human, cow, and sheep (Fig. 7). We identified a chromosomal region of approximately 12Mb,  
220 which in human was on the 5' side of gremlin 2 (Fig. 7; pink box), while in cow and sheep it was  
221 found in a different chromosome in relation to other genes that are linked to gremlin 2 (Fig. 7;  
222 pink box). In cow, this region was moved from chromosome 16 to 28, while in sheep it was  
223 moved from chromosome 12 to 25 (Fig. 7). As a consequence of this chromosomal  
224 rearrangement, the regions that are located up- and downstream of the chromosome piece that

225 was moved are now located together in both cow and sheep (Fig. 7). Thus, in these species, the  
226 gene that is found on the 5' side of gremlin 2 (FMN2) was part of the chromosomal block that  
227 was moved to a different chromosome (Fig. 7) whereas the gene located on the 3' side (RGS7)  
228 was not. From this, we suggest that one of the break points that gave rise to the chromosomal  
229 rearrangement was the chromosomal region where gremlin 2 was located (Fig. 7).

230 From a biomedical perspective, the loss of gremlin 2 (e.g. in cow, sheep, goat, dolphins,  
231 whales) represents a natural gene knockout (evolutionary mutant models according to (Albertson  
232 et al., 2009)), thus presenting an outstanding opportunity to understand gremlin 2 biology. From  
233 a physiological standpoint, this phenomenon is interesting as gremlin 2 plays a role in several  
234 developmental processes, including organogenesis, body patterning, and tissue differentiation.  
235 Thus, several questions regarding the mode of action of this gene could be investigated  
236 considering the lack of this gene in certain species. For example, what happens with BMP2 and  
237 BMP4 in the absence of gremlin 2? Are these BMPs free of any antagonist action? Or does  
238 another member of the DAN gene family fulfill gremlin 2's molecular role? From a phenotypic  
239 perspective, it has been shown that BMP2 and BMP4 are involved in the signaling pathway that  
240 regulates tooth development (Aberg et al., 1997; Nadiri et al., 2004). Genetic manipulation  
241 experiments have shown that gremlin 2 deficient mice have upper and lower incisor teeth with  
242 markedly reduced breadth and depth, and the upper incisors are more severely affected than the  
243 lower ones (Kantaputra et al., 2015; Vogel et al., 2015). This supports the argument that the lack  
244 of gremlin 2 contributes to the divergent dental phenotype of ruminants and cetaceans.  
245 Ruminants do not have incisors in the upper jaw; instead they have a dental pad. With the  
246 exception of elk and red deer, canines are also absent in most species. This particular dental  
247 phenotype affects how ruminants eat, which differ from phylogenetically related species that

248 have incisors in the upper jaw (e.g. horse). For example, cows use their tongue to wrap and pull  
249 leaves into their mouths between the incisors of the lower jaw and the dental pad; thus, plants are  
250 not clearly cut during feeding. This contrasts with the feeding method of phylogenetically related  
251 species that have upper and lower incisors; these species cut plants and graze deeply. Once the  
252 food is in their mouths, cows swing their heads to chew the food slightly and mix it with saliva  
253 before swallowing. This lateral chewing action is required to cut plant tissues because molars  
254 and premolars of the maxillary jaw are wider than those located on the mandibular jaw.  
255 Conversely, sheep use their lips and teeth as their primary tools for food prehension. Their lips  
256 are used to bring food into their mouths, and the incisors of the lower jaw in combination with  
257 the dental pad allow them to cut leaves. As a consequence, sheep can bite closer to the ground  
258 and have the ability to be more selective.

259         The loss of gremlin 2 in cetaceans is more complicated to interpret considering that one  
260 subgroup (toothed whales) has teeth while another subgroup (baleen whales) does not. To further  
261 complicate this scenario, it has been argued that it is impossible to define teeth homology  
262 between toothed whales and non-cetacean mammals (Armfield et al., 2013). From a  
263 developmental perspective, it has been demonstrated that the pathway that controls tooth  
264 differentiation and number in cetaceans is different from the typical mammalian pattern  
265 (Armfield et al., 2013). Particularly interesting is that the expression pattern of BMP4, one of the  
266 main targets of gremlin 2, differs between cetaceans and non-cetacean mammals (Sudo et al.,  
267 2004).

268         Finally, the case of hippopotamuses remains an open question until the genome is  
269 sequenced. However, we can speculate that, as has been shown in cetaceans, the tooth

270 morphology of this group could be related to different regulatory pathways controlling teeth  
271 development as a consequence of the absence gremlin 2.

272

### 273 **Concluding remarks**

274 Our results show that in cetartiodactyl mammals gremlin 2 has experienced a mixture of gene  
275 loss, gene duplication, and rate acceleration. Although the last common ancestor of  
276 cetartiodactyls possessed a single copy of the gene, species belonging to the suborders tylopoda  
277 (the group that includes camels, alpacas, vicuñas, and guanacos) and suiformes (the group that  
278 includes pigs and peccaries) are the only groups that have retained gremlin 2 (Fig.6). These  
279 groups also experienced acceleration in the rate of evolution of this gene, and it is this that  
280 clearly differentiates them from all other laurasiatherians (Fig. 3). The fact that all amino acid  
281 changes that define the gremlin 2 gene in tylopoda and suiformes are present in both groups  
282 suggests that this gene and its corresponding protein were remodeled in the last common  
283 ancestor of cetartiodactyls and subsequently inherited by all descendant lineages (Fig. 6). After  
284 that, gremlin 2 was probably lost in the ancestor of ruminants, hippopotamuses, and cetaceans  
285 between 56.3 and 63.5 million of years ago (Fig. 6). By removing a biological constraint  
286 imposed by the presence of gremlin 2, the lack of this gene could explain teeth diversity in these  
287 groups of mammals. Thus, the results presented here support the argument that gene loss is a  
288 way to increase phenotypic diversity (Olson, 1999; Albalat and Cañestro, 2016) and that gremlin  
289 2 is a dispensable gene at least in this group of mammals.

290

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294



295 **Figure legends**

296

297 **Figure 1.** Maximum likelihood phylogenetic tree depicting relationships among gremlin 2 genes  
298 in laurasiatherian mammals. Numbers on the nodes correspond to Bayesian posterior  
299 probabilities and maximum likelihood bootstrap support values. Sequences of mouse and  
300 kangaroo rat were used as outgroups.

301

302 **Figure 2.** Patterns of conserved synteny in the genomic regions that harbor gremlin 2 genes in  
303 laurasiatherian mammals. Upper panel: genomic region that harbors gremlin 2 genes. Lower  
304 panel: conserved synteny in the genomic region that would be the putative location of the  
305 gremlin 2 gene in ruminants and cetaceans.

306

307 **Figure 3.** An alignment of gremlin 2 amino acid sequences from laurasiatherian mammals.  
308 Amino acid positions in bold denote the 11 amino acid synapomorphies that define the sequences  
309 of pigs and camels.

310

311 **Figure 4.** An alignment of the predicted gremlin 2 secondary structure in laurasiatherian  
312 mammals and humans.

313

314 **Figure 5.** Schematic representation of the gremlin 2 syntenic region in pigs. One of the copies  
315 (gremlin 2-T1) is located on the 5' side of the regulator of the G-protein signaling 7 gene (RGS7)  
316 whereas the second copy (gremlin 2-T2) is located within the RGS7 gene, specifically between  
317 exons 13 and 14.

318

319 **Figure 6.** An evolutionary hypothesis regarding the evolution of the gremlin 2 gene in  
320 cetartiodactyl mammals. According to this model, the last common ancestor of cetartiodactyls  
321 possessed a single copy of the gene. Species belonging to the suborders tylopoda, the group that  
322 includes camels, alpacas, vicuñas and guanacos, and suiformes, the group that includes pigs and  
323 peccaries, were the only groups that retained gremlin 2. According to the phyletic distribution of  
324 gremlin 2, we propose that this gene was lost in the common ancestor of ruminants,  
325 hippopotamuses, and cetaceans between 56.3 and 63.5 million of years ago as a product of a  
326 chromosomal rearrangement.

327

328 **Figure 7.** Schematic representation of the chromosomal regions that harbor genes located up-  
329 and downstream of gremlin 2. Upper panel: chromosomal region that harbors genes that are up-  
330 and downstream of gremlin 2 in humans. Middle panel: Chromosomal regions (chrs 16 and 28)  
331 that harbor genes that are located up- and downstream of the putative location of gremlin 2 in  
332 cow. Lower panel: Chromosomal regions (chrs 25 and 12) that harbor genes that are located up-  
333 and downstream of the putative location of gremlin 2 in sheep.

334

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336

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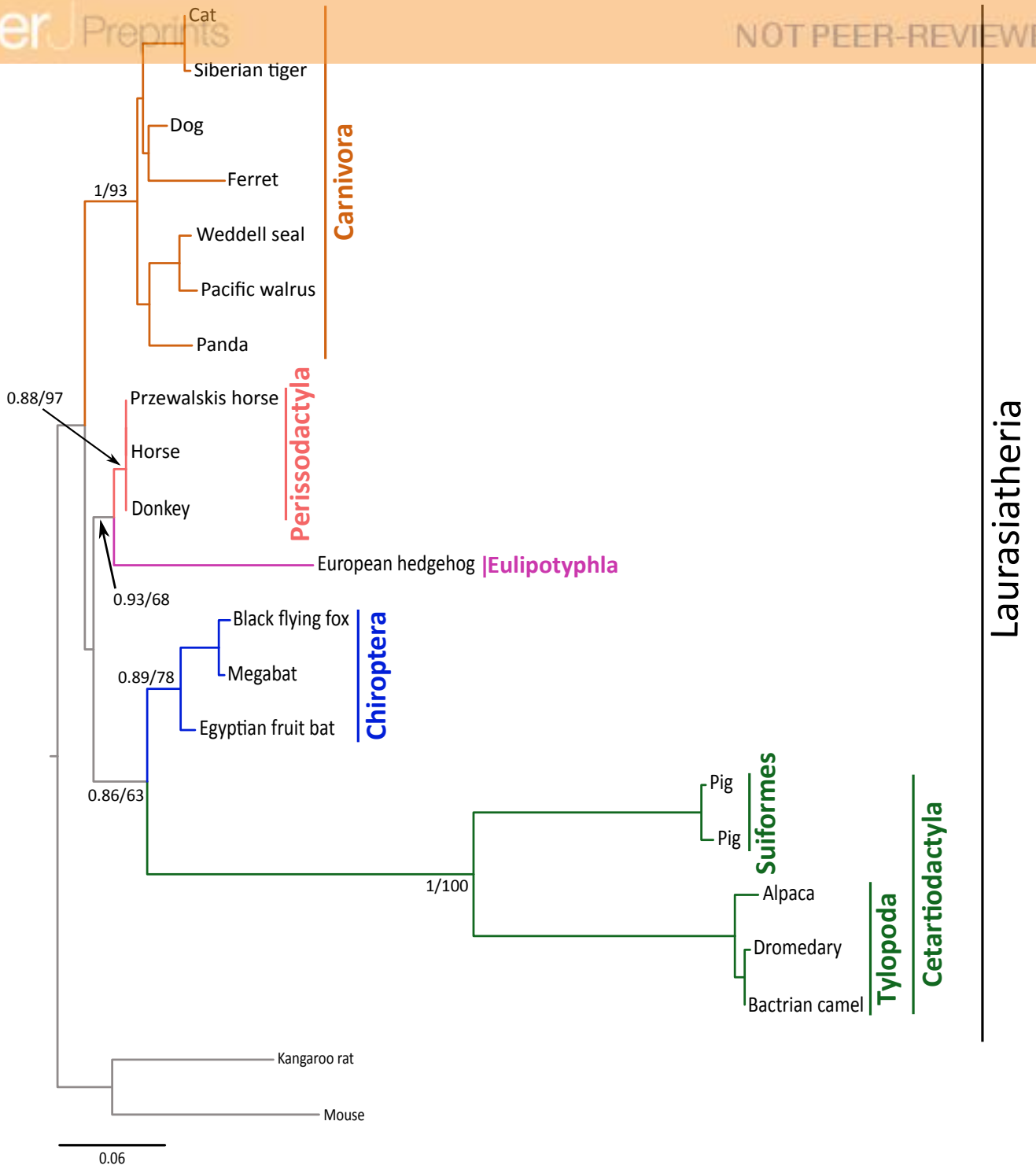
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**Figure 1** (on next page)

Maximum likelihood phylogenetic tree depicting relationships among gremlin 2 genes in laurasiatherian mammals

**Figure 1.** Maximum likelihood phylogenetic tree depicting relationships among gremlin 2 genes in laurasiatherian mammals. Numbers on the nodes correspond to Bayesian posterior probabilities and maximum likelihood bootstrap support values. Sequences of mouse and kangaroo rat were used as outgroups.



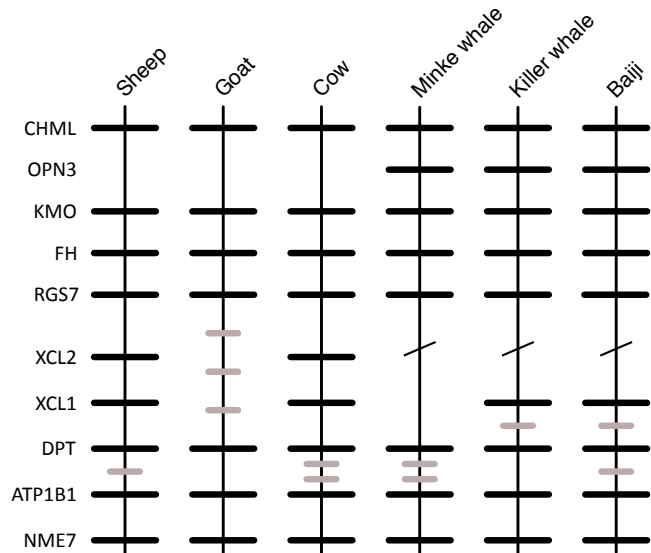
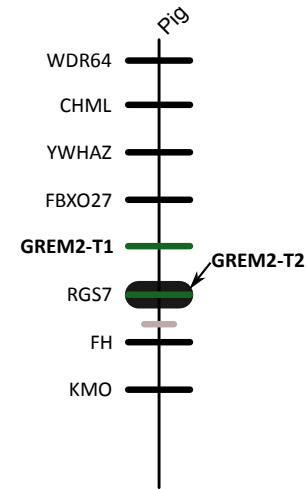
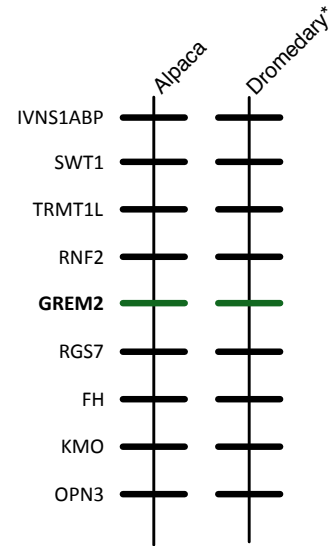
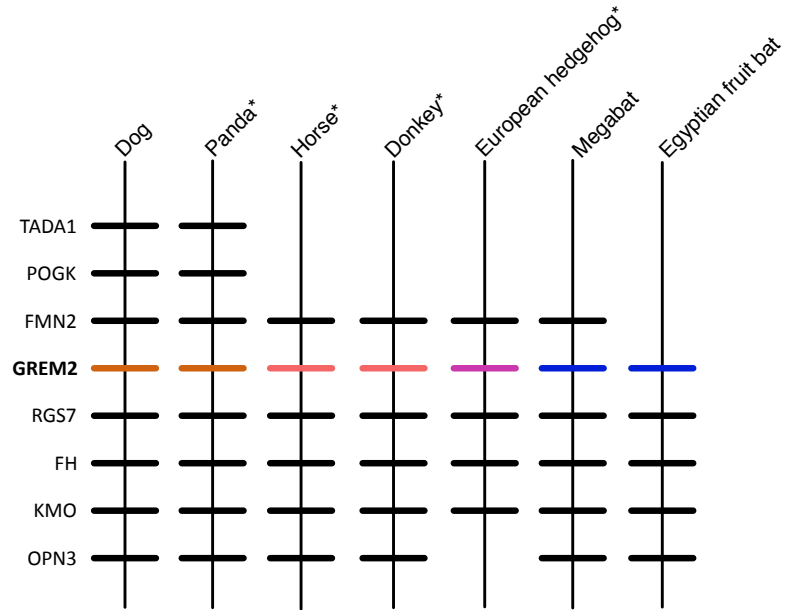
Laurasiatheria



**Figure 2**(on next page)

Patterns of conserved synteny in the genomic regions that harbor gremlin 2 genes in laurasiatherian mammals

**Figure 2.** Patterns of conserved synteny in the genomic regions that harbor gremlin 2 genes in laurasiatherian mammals. Upper panel: genomic region that harbors gremlin 2 genes. Lower panel: conserved synteny in the genomic region that would be the putative location of the gremlin 2 gene in ruminants and cetaceans.



**Figure 3**(on next page)

An alignment of gremlin 2 amino acid sequences from laurasiatherian mammals

**Figure 3.** An alignment of gremlin 2 amino acid sequences from laurasiatherian mammals. Amino acid positions in bold denote the 11 amino acid synapomorphies that define the sequences of pigs and camels.

1 10 20 30 40 50 60 70 80

Pig MFWKLSVSLLLMAALEKVEDAQRARPAGSIPSP**PK**DGSPSTGRWQ-LIKEVLASSQEALVVTER**RY**LRS~~LD~~WCKTQPLRQT**V**HEE

Pig MFWKLSVSLLLMAALEKVEDAQRARPAGSIPSP**PK**DGSPSTGRWQ-LIKEVLASSQEALVVTER**RY**LRS~~LD~~WCKTQPLRQT**V**HEE

Alpaca MFWKISMSLLLVAALGKLEEQGARPAGAIIPSP**PK**DRGTNNSQNWQ-HIREVLSSSQEALVVTER**RY**LRRD~~LD~~WCKTQRLRQT**V**REE

Bactrian camel MFWKISMSLLLVAALGKLEEQGARPAGAIIPSP**PK**DRGTNNSQNWQ-HIREVLSSSQEALVVTER**RY**LRRD~~LD~~WCKTQRLRQT**V**REE

Dromedary MFWKISMSLLLVAALGKLEEQGARPAGAIIPSP**PK**DRGTNNSQNWQ-HIREVLSSSQEALVVTER**RY**LRRD~~LD~~WCKTQRLRQT**V**REE

Donkey MLWKLSLSLFLVAVLVKVAEARKNRPAAGAIIPSP**Y**KDGSSNTSERWQHQIKEVLASSQEALVVTER**KY**LKSDWCKTQPLRQT**V**SEE

Horse MLWKLSLSLFLVAVLVKVAEARKNRPAAGAIIPSP**Y**KDGSSNTSERWQHQIKEVLASSQEALVVTER**KY**LKSDWCKTQPLRQT**V**SEE

Przewalskis horse MLWKLSLSLFLVAVLVKVAEARKNRPAAGAIIPSP**Y**KDGSSNTSERWQHQIKEVLASSQEALVVTER**KY**LKSDWCKTQPLRQT**V**SEE

Cat MFWKLSLSLCLVAVLVKVAEARKNRPAAGAIIPSP**Y**KDGSSNSHSEWQHQIKEVLASSQEALVVTER**KY**LKSDWCKTQPLRQT**V**SEE

Siberian tiger MFWKLSLSLCLVAVLVKAAEARKNRPAAGAIIPSP**Y**KDGSSNSHSEWQHQIKEVLASSQEALVVTER**KY**LKSDWCKTQPLRQT**V**SEE

Ferret MFWKLSLSLCLVAVLVKAAEARKNRPAAGAIIPSP**Y**K-GSSNSHSEWQHQIKEVLASSQEALVVTER**KY**LKSDWCKTQPLRQT**V**SED

Dog MFWKLSLSLCLVAVLVKVAEARKNRPAAGAIIPSP**Y**KDGSSNSHSEWQHQIKEVLASSQEALVVTER**KY**LKSDWCKTQPLRQT**V**SEE

Pacific walrus MFWKLSLSLCLVAVLVKVAEARKNRPAAGAIIPSP**Y**KDGSSNSHSEWQHQIKEVLASSQEALVVTER**KY**LKSDWCKTQPLRQT**V**SEE

Weddell seal MFWKLSLSLCLVAVLVKVAEARKNRPAAGAIIPSP**Y**KDGSSNSHSEWQHQIKEVLASSQEALVVTER**KY**LKSDWCKTQPLRQT**V**SEE

Panda MFWKLSLSLCLVAVLVKVAEARKNRPAAGAIIPSP**Y**KDGSSNSHSEWQQQIKEVLASSQEALVVTER**KY**LKSDWCKTQPLRQT**V**SEE

Megabat MFWKLSLSLLLVAVLVKGVDARKNRPAAGAIIPSP**Y**KDGSSNTSERWQHQIKEVLASSQEALVVTER**KY**LKSDWCKTQPLRQT**V**GEE

Egyptian rousette MFWKLSLSLLLVAVLVKGVDARKNRPAAGAIIPSP**Y**KDGSSNTSERWQHQIKEVLASSQEALVVTER**KY**LKSDWCKTQPLRQT**V**GEE

Black flying fox MFWKLSLSLLLVAVLVKGVDARKNRPAAGAIIPSP**Y**KDGSSNSHSEWQHQIKEVLASSQEALVVTER**KY**LKSDWCKTQPLRQT**V**GEE

European hedgehog MFWKLSLSLFLVAVLVKVAEGRKNRPAAGAIIPSP**Y**KDGSSNSHSEWQHQIKEVLASSQEALVVTER**KY**LKSDWCKTQPLRQT**V**SEE

90 100 110 120 130 140 150 160 170

Pig GC**HS**R~~T~~VLNRF~~CY~~GCNSFFI**PR**GGGSW--GSFQSCAF**CR**PQRA**AA**LLVELQCPGRDPP**FL**RK**IK**QKV**KQ**CR**CM**SV**TL**GSD**EP**-

Pig GC**HS**R~~T~~VLNRF~~CY~~GCNSFFI**PR**GGGSW--GSFQSCAF**CR**PQRA**AA**LLVELQCPGRDPP**FL**RK**IK**QKV**KQ**CR**CM**SV**TL**GSD**EP**-

Alpaca GC**HS**R~~T~~VLNRF~~CY~~GCNSFYI**PR**GGEGEGSGSFQSCAF**CR**PQRA**AA**LLVELQCPSRDPP**V**RL**RK**IK**QK**V**KQ**CR**CM**SV**NL**G**AD**ES-

Bactrian camel GC**HS**R~~T~~VLNRF~~CY~~GCNSFYI**PR**GGEGEGSGSFQSCAF**CR**PQRA**AA**LLVELQCPSRDPP**V**RL**RK**IK**QK**V**KQ**CR**CM**SV**NL**G**AD**ES-

Dromedary GC**HS**R~~T~~VLNRF~~CY~~GCNTSS**TP**RGEGEGSGSFQSCAF**CR**PQRA**AA**LLVELQCPSRDPP**V**RL**RK**IK**QK**V**KQ**CR**CM**SV**NL**G**AD**ES-

Donkey GC**RS**R~~T~~ILNRF~~CY~~GCNSFYI**PR**HVKKEE--ESFQSCAF**CP**Q**RV****TSV**LVELECPGLDPP**F**RL**KK**IK**QK**V**KQ**CR**CM**SV**NL**S**D**SD**KQ**

Horse GC**RS**R~~T~~ILNRF~~CY~~GCNSFYI**PR**HVKKEE--ESFQSCAF**CP**Q**RV****TSV**LVELECPGLDPP**F**RL**KK**IK**QK**V**KQ**CR**CM**SV**NL**S**D**SD**KQ**

Przewalskis horse GC**RS**R~~T~~ILNRF~~CY~~GCNSFYI**PR**HVKKEE--ESFQSCAF**CP**Q**RV****TSV**LVELECPGLDPP**F**RL**KK**IK**QK**V**KQ**CR**CM**SV**NL**S**D**SD**KQ**

Cat GC**RS**R~~T~~ILNRF~~CY~~GCNSFYI**PR**HVRKEE--ESFQSCAF**CP**Q**RV****TSV**LVELECPGLDPP**F**RL**KK**IK**QK**V**KQ**CR**CM**SV**NL**S**D**SD**KQ**

Siberian tiger GC**RS**R~~T~~ILNRF~~CY~~GCNSFYI**PR**HVRKEE--ESFQSCAF**CP**Q**RV****TSV**LVELECPGLDPP**F**RL**KK**IK**QK**V**KQ**CR**CM**SV**NL**S**D**SD**KQ**

Ferret GC**RS**R~~T~~VLNRF~~CY~~GCNSFYI**PR**HVRKDE--ESFQSCAF**CP**Q**RV****TSV**LVELECPGMDPP**F**RL**KK**IK**QK**V**KQ**CR**CM**SV**NL**S**D**SD**KQ**

Dog GC**RS**R~~T~~ILNRF~~CY~~GCNSFYI**PR**HVRKEE--ESFQSCAF**CP**Q**RV****TSV**LVELECPGLDPP**F**RL**KK**IK**QK**V**KQ**CR**CM**SV**NL**S**D**SD**KQ**

Pacific walrus GC**RS**R~~T~~ILNRF~~CY~~GCNSFYI**PR**HVRKEE--ESFQSCAF**CP**Q**RV****TSV**LVELECPGMDPP**F**RL**KK**IK**QK**V**KQ**CR**CM**SV**NL**S**D**SD**KQ**

Weddell seal GC**RS**R~~T~~ILNRF~~CY~~GCNSFYI**PR**HVRKEE--ESFQSCAF**CP**Q**RV****TSV**LVELECPGMDPP**F**RL**KK**IK**QK**V**KQ**CR**CM**SV**NL**S**D**SD**KQ**

Panda GC**RS**R~~T~~ILNRF~~CY~~GCNSFYI**PR**HVRKEE--ESFQSCAF**CP**Q**RV****TSV**LVELECPGLDPP**F**RL**KK**IK**QK**V**KQ**CR**CM**SV**NL**S**D**SD**KQ**

Megabat GC**RS**R~~T~~VLNRF~~CY~~GCNSFYI**PR**HVKKEE--ESFQSCAF**CP**Q**RV****TSV**LVELECPGLDPP**F**RL**KK**IK**QK**V**KQ**CR**CM**SV**NL**S**D**SD**KQ**

Egyptian rousette GC**RS**R~~T~~VLNRF~~CY~~GCNSFYI**PR**HVKKEQ--ESFQSCAF**CP**Q**RV****TSV**LVELECPGLDPP**F**RL**KK**IK**QK**V**KQ**CR**CM**SV**NL**S**D**SD**KQ**

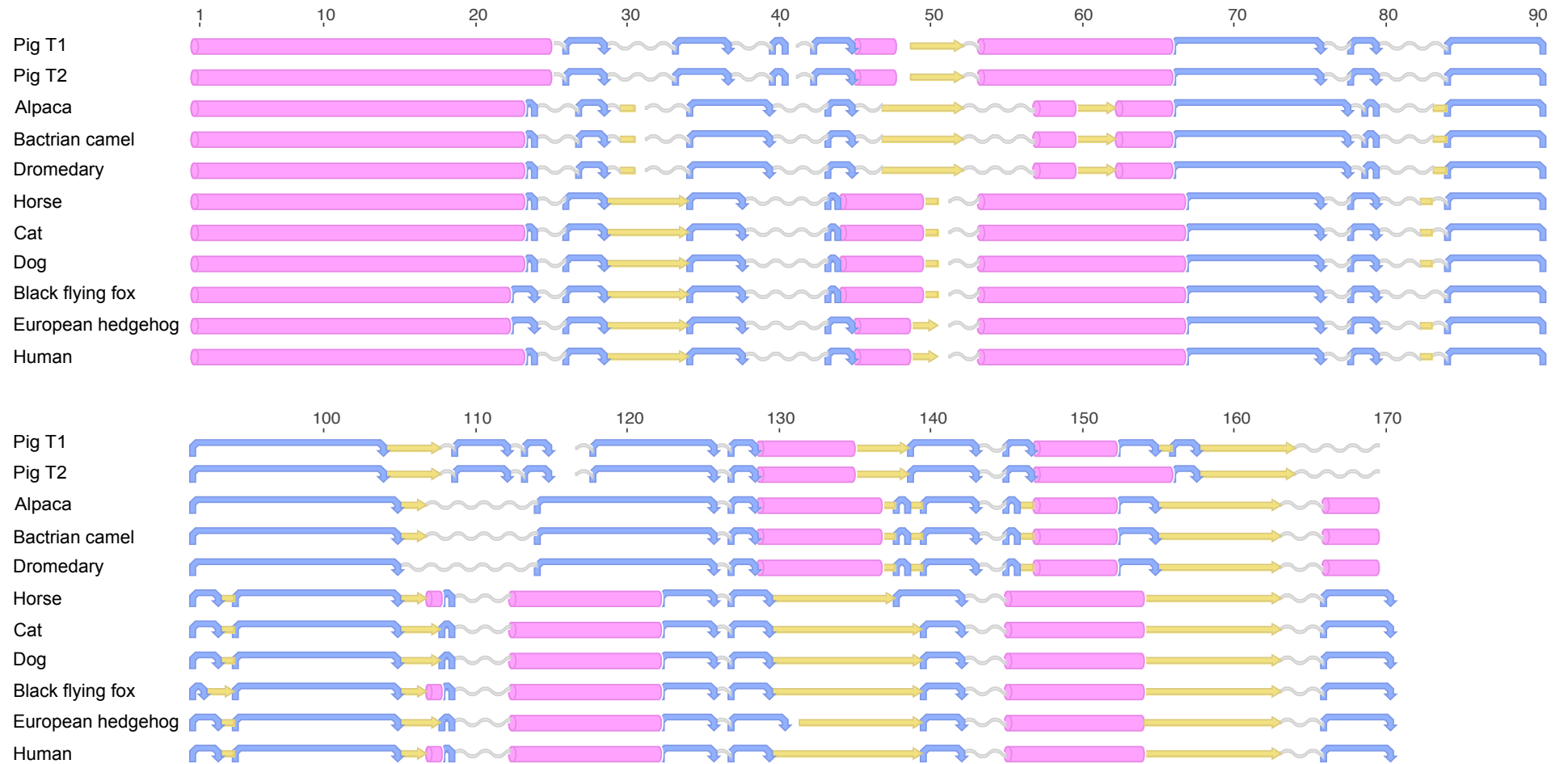
Black flying fox GC**RS**R~~T~~VLNRF~~CY~~GCNSFYI**PR**HVKKEE--ESFQSCAF**CP**Q**RV****TSV**LVELECPGLDPP**F**RL**KK**IK**QK**V**KQ**CR**CM**SV**NL**S**D**SD**KQ**

European hedgehog GC**RS**R~~T~~ILNRF~~CY~~GCNSFYI**PR**HVRKEE--ESFQSCAF**CP**Q**RA****TSV**LVELECPGLDPP**F**RL**KK**IK**QK**V**KQ**CR**CM**SV**NL**S**D**SD**KQ**

**Figure 4**(on next page)

An alignment of the predicted gremlin 2 secondary structure in laurasiatherian mammals and humans

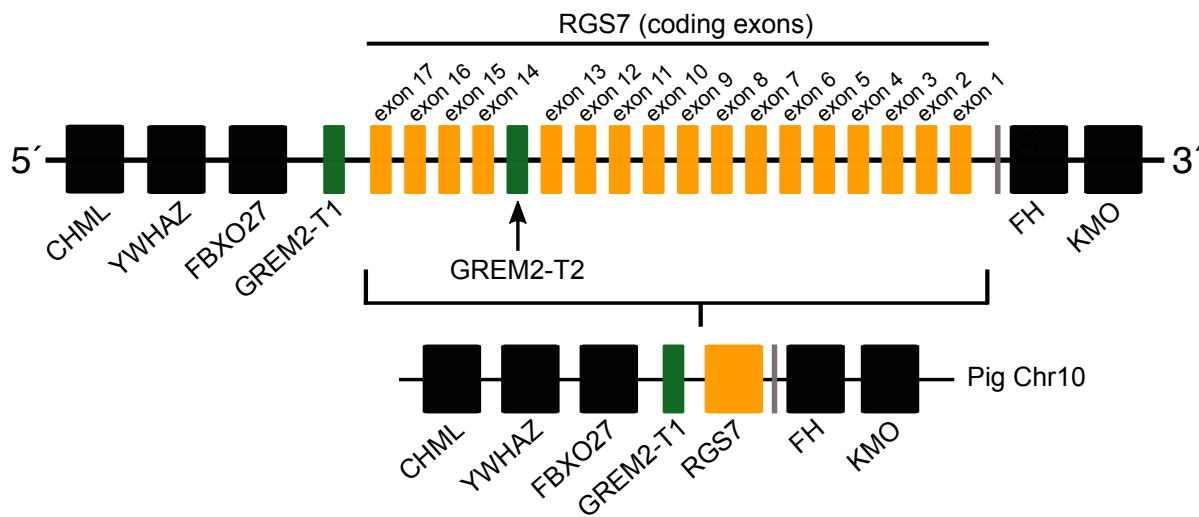
**Figure 4.** An alignment of the predicted gremlin 2 secondary structure in laurasiatherian mammals and humans.



**Figure 5**(on next page)

Schematic representation of the gremlin 2 syntenic region in pigs

**Figure 5.** Schematic representation of the gremlin 2 syntenic region in pigs. One of the copies (gremlin 2-T1) is located on the 5' side of the regulator of the G-protein signaling 7 gene (RGS7) whereas the second copy (gremlin 2-T2) is located within the RGS7 gene, specifically between exons 13 and 14.

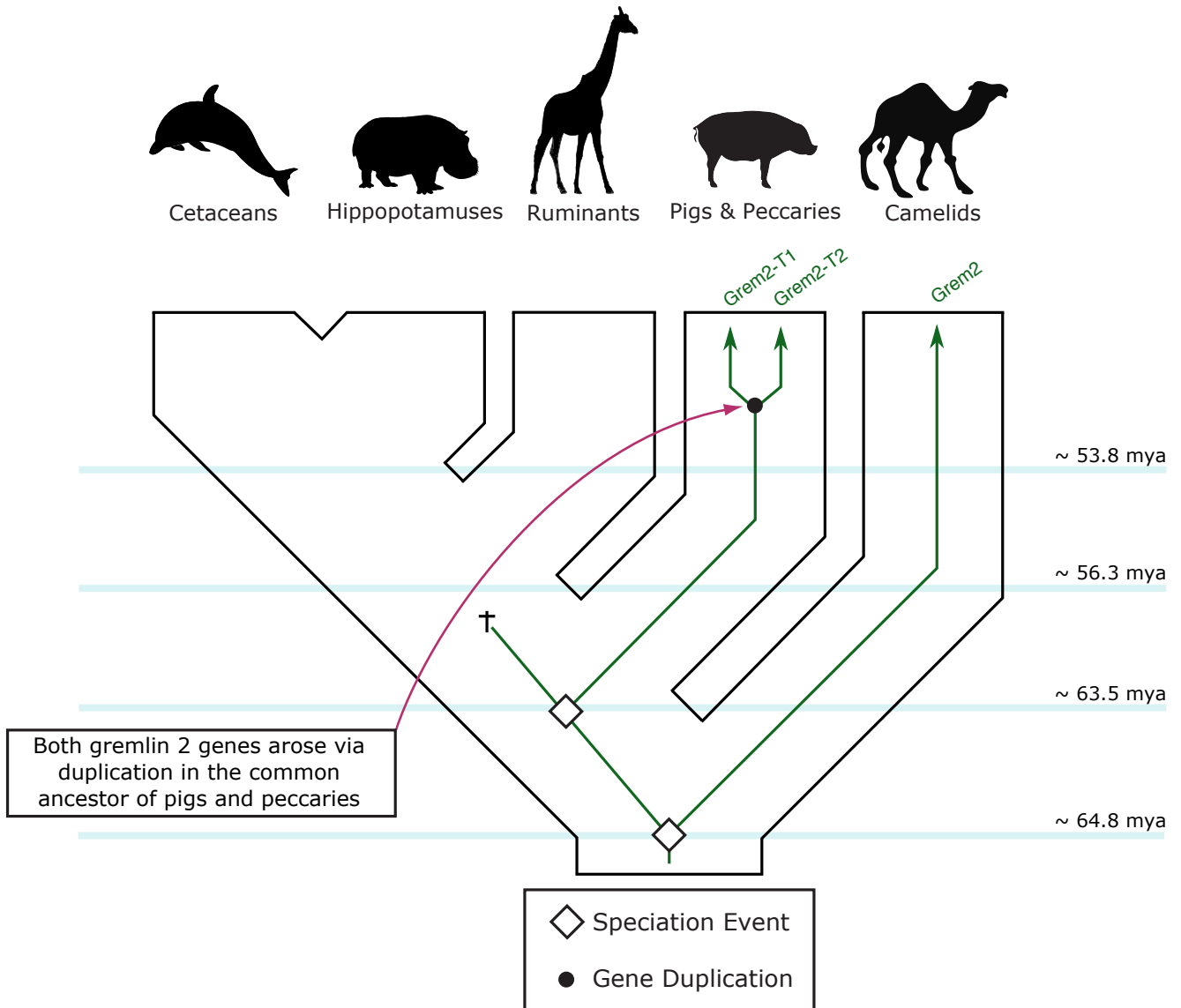




**Figure 6**(on next page)

An evolutionary hypothesis regarding the evolution of the gremlin 2 gene in cetartiodactyl mammals

**Figure 6.** An evolutionary hypothesis regarding the evolution of the gremlin 2 gene in cetartiodactyl mammals. According to this model, the last common ancestor of cetartiodactyls possessed a single copy of the gene. Species belonging to the suborders tylopoda, the group that includes camels, alpacas, vicuñas and guanacos, and suiformes, the group that includes pigs and peccaries, were the only groups that retained gremlin 2. According to the phyletic distribution of gremlin 2, we propose that this gene was lost in the common ancestor of ruminants, hippopotamuses, and cetaceans between 56.3 and 63.5 million of years ago as a product of a chromosomal rearrangement.



**Figure 7** (on next page)

Schematic representation of the chromosomal regions that harbor genes located up- and downstream of gremlin 2

**Figure 7.** Schematic representation of the chromosomal regions that harbor genes located up- and downstream of gremlin 2. Upper panel: chromosomal region that harbors genes that are up- and downstream of gremlin 2 in humans. Middle panel: Chromosomal regions (chrs 16 and 28) that harbor genes that are located up- and downstream of the putative location of gremlin 2 in cow. Lower panel: Chromosomal regions (chrs 25 and 12) that harbor genes that are located up- and downstream of the putative location of gremlin 2 in sheep.

