A peer-reviewed version of this preprint was published in PeerJ on 26 January 2017.

View the peer-reviewed version (peerj.com/articles/2901), which is the preferred citable publication unless you specifically need to cite this preprint.

Opazo JC, Zavala K, Krall P, Arias RA. 2017. Evolution of gremlin 2 in cetartiodactyl mammals: gene loss coincides with lack of upper jaw incisors in ruminants. PeerJ 5:e2901 https://doi.org/10.7717/peerj.2901

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

Juan C Opazo Corresp., [1] , **Kattina Zavala** [1] , **Paola Krall** [2] , **Rodrigo Arias** [3]

[1] Instituto de Ciencias Ambientales y Evolutivas, Universidad Austral de Chile, Valdivia, Chile

[2] Unidad de Nefrología, Universidad Austral de Chile, Valdivia, Chile

[3] 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[1], Kattina Zavala[1], Paola Krall[2], Rodrigo A. Arias[3]

7 [1]Instituto de Ciencias Ambientales y Evolutivas, Facultad de Ciencias, Universidad Austral de

8 Chile, Valdivia, Chile. [2]Unidad de Nefrología, Facultad de Medicina, Universidad Austral de

9 Chile, Valdivia, Chile. [3]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

16

17

18

19

Abstract

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.

Introduction

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

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

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

In the literature, it has been shown that gremlin 2 interacts with BMP2 and BMP4 by blocking their ligand-signaling pathway (Sudo et al., 2004). Human genetic studies have indicated that gremlin 2 variation can influence one's susceptibility of having a common tooth malformation (Kantaputra et al., 2015). Mutational analysis in seven out of 263 patients with different dental anomalies has revealed the presence of mutations predicted to cause disease. Five patients of this study carried the same heterozygous mutations (Ala13Val) while the other two were carriers of two different heterozygous missense mutations (Gln76Glu and Glu136Asp) (Kantaputra et al., 2015). This genetic study supports the notion that inheritance of hypodontia is autosomal dominant, and this is related to gremlin 2. Despite this, the study also gives evidence of incomplete penetrance and variable expressivity. Genetic experiments provide further support for the role of gremlin 2 in tooth development (Brommage et al., 2014); it has been shown that gremlin 2 deficient mice have upper and lower incisor teeth with markedly reduced breadth and depth, and the upper incisors are more severely affected than lower ones (Vogel et al., 2015). According to Voget et al. (2015) no other significant phenotypic effects have been observed in grem2$^{-/-}$ individuals, indicating that this gene could be dispensable. From a developmental perspective, it has been shown that the pathway that controls tooth differentiation is conserved in most mammals other than cetaceans, xenarthrans, and phocid seals (Armfield et al., 2013). In dolphins, it has been shown that expression of BMP4, which is one of the main targets of gremlin 2 (Sudo et al., 2004), is extended to the caudal region of the developing jaw, a region where the fibroblast growth factor 8 gene (FGF8) is express in most mammals (Armfield et al., 2013). This developmental difference could be related to the divergent dental phenotype of

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

The main goal of this study was to investigate the evolutionary history of gremlin 2, a gene that plays a significant role in the tooth development, in cetartiodactyl mammals a group that possesses divergent tooth morphologies. Results from our analyses show 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 in pigs and camels has been accelerated, and the possession of gremlin 2 clearly differentiates these groups from all other cetartiodactyl mammals.

Materials and methods

DNA data collection and phylogenetic analyses

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

(*Panthera tigris*), dog (*Canis familiaris*), ferret (*Mustela putorius*), Weddell seal (*Leptonychotes weddellii*), Pacific walrus (*Odobenus rosmarus*), panda (*Ailuropoda melanoleuca*); Perissodactyla: Przewalski's horse (*Equus ferus*), horse (*Equus caballus*), donkey (*Equus asinus*), Eulipotyphla: European hedgehog (*Erinaceus europaeus*); Chiroptera: Black flying fox (*Pteropus alecto*), Megabat (*Pteropus vampyrus*), Egyptian fruit bat (*Rousettus aegyptiacus*); and Cetartiodactyla: pig (*Sus Scrofa*), alpaca (*Vicugna pacos*), dromedary (*Camelus dromedarius*) and Bactrian camel (*Camelus bactrianus*) (Supplementary Table S1). Mouse and kangaroo rat sequences were used as outgroups. Amino acid sequences were aligned using the L-INS-i strategy from MAFFT v.6 (Katoh and Standley, 2013). Nucleotide alignment was generated using the amino acid alignment as a template using the software PAL2NAL (Suyama et al., 2006). Phylogenetic relationships were estimated using maximum likelihood and Bayesian approaches. In both cases, second codon positions were excluded. We performed a maximum likelihood analysis to obtain the best tree using the program RAxML version 8 (Stamatakis, 2014) and assessed support for the nodes with 1,000 bootstrap pseudoreplicates. Bayesian searches were conducted in MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003); two independent runs of six simultaneous chains for $20x10^6$ generations were set, and every 2,500 generations were sampled using default priors. The run was considered to have reached convergence once the likelihood scores reached an asymptote and the average standard deviation of the split frequencies remained < 0.01. We discarded all trees that were sampled before convergence, and we evaluated support for the nodes and parameter estimates from a majority rule consensus of the last 4,000 trees.

Assessments of Conserved Synteny

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

Results and Discussion

Phylogenetic relationships

We constructed a phylogenetic tree in which we included representative species of laurasiatherian mammals (Fig. 1). Our phylogenetic analysis recovered the monophyly of each laurasiatherian order included in our sampling (Fig. 1). Although the phylogenetic relationships among laurasiatherian mammals at the ordinal level are still a matter of debate, the most important departure from current hypotheses detected here was the sister group relationship between Eulipotyphla and Perissodactyla (Fig. 1); in most studies, eulipotyphlan species appear sister to all other laurasiatherian mammals (Nery et al., 2012; Foley et al., 2016). The synteny analysis provided further support for the identity of the gremlin 2 gene lineage in this group of mammals (Fig. 2); genes found downstream were well conserved in all examined species (Fig. 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 *IVNS1ABP*) 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,

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

Among cetartiodactyls, we observed that gremlin 2 was differentially retained (Fig. 2). 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 in which gremlin 2 was present (Fig. 2). In cetaceans and ruminants, gremlin 2 was not present. Thus, according to the phyletic distribution of gremlin 2 within the main groups of cetartiodactyls, the most likely scenario is that the deletion of the gene occurred between 56.3 and 63.5 million of years ago in the common ancestor of the clade that includes ruminants, hippopotamuses, and cetaceans (Fig. 6). However, until information regarding gremlin 2 in hippopotamuses is obtained, caution must be taken when interpreting these results. If, in the future, the hippopotamus genome is found to possess gremlin 2, we can determine that two independent gene losses occurred, one in the ancestor of ruminants and a second in the ancestor of cetaceans. For now, a single gene loss event is assumed.

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

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

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

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

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

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

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

Concluding remarks

Our results show that in cetartiodatyl mammals gremlin 2 has experienced a mixture of gene loss, gene duplication, and rate acceleration. Although 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) are the only groups that have retained gremlin 2 (Fig.6). These groups also experienced acceleration in the rate of evolution of this gene, and it is this that clearly differentiates them from all other laurasiatherians (Fig. 3). The fact that all amino acid changes that define the gremlin 2 gene in tylapoda and suiformes are present in both groups suggests that this gene and its corresponding protein were remodeled in the last common ancestor of cetartiodactyls and subsequently inherited by all descendant lineages (Fig. 6). After that, gremlin 2 was probably lost in the ancestor of ruminants, hippopotamuses, and cetaceans between 56.3 and 63.5 million of years ago (Fig. 6). By removing a biological constraint imposed by the presence of gremlin 2, the lack of this gene could explain teeth diversity in these groups of mammals. Thus, the results presented here support the argument that gene loss is a way to increase phenotypic diversity (Olson, 1999; Albalat and Cañestro, 2016) and that gremlin 2 is a dispensable gene at least in this group of mammals.

Acknowledgements

292 This work was funded by a grant from the Fondo Nacional de Desarrollo Científico y
293 Tecnológico (FONDECYT 1160627) to JCO.

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

335 **References**

336

337 Aberg, T., Wozney, J. and Thesleff, I., 1997. Expression patterns of bone morphogenetic
338 proteins (Bmps) in the developing mouse tooth suggest roles in morphogenesis and cell
339 differentiation. Dev Dyn 210, 383-96.

340 Albalat, R. and Cañestro, C., 2016. Evolution by gene loss. Nat Rev Genet 17, 379-91.

341 Albertson, R.C., Cresko, W., Detrich, H.W. and Postlethwait, J.H., 2009. Evolutionary mutant
342 models for human disease. Trends Genet 25, 74-81.

343 Armfield, B.A., Zheng, Z., Bajpai, S., Vinyard, C.J. and Thewissen, J., 2013. Development and
344 evolution of the unique cetacean dentition. PeerJ 1, e24.

345 Brommage, R., Liu, J., Hansen, G.M., Kirkpatrick, L.L., Potter, D.G., Sands, A.T., Zambrowicz,
346 B., Powell, D.R. and Vogel, P., 2014. High-throughput screening of mouse gene
347 knockouts identifies established and novel skeletal phenotypes. Bone Res 2, 14034.

348 Dean, M., Carrington, M., Winkler, C., Huttley, G.A., Smith, M.W., Allikmets, R., Goedert, J.J.,
349 Buchbinder, S.P., Vittinghoff, E., Gomperts, E., Donfield, S., Vlahov, D., Kaslow, R.,
350 Saah, A., Rinaldo, C., Detels, R. and O'Brien, S.J., 1996. Genetic restriction of HIV-1
351 infection and progression to AIDS by a deletion allele of the CKR5 structural gene.
352 Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study,
353 Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study.
354 Science 273, 1856-62.

355 Foley, N.M., Springer, M.S. and Teeling, E.C., 2016. Mammal madness: is the mammal tree of
356 life not yet resolved? Philos Trans R Soc Lond B Biol Sci 371.

357 Herrero, J., Muffato, M., Beal, K., Fitzgerald, S., Gordon, L., Pignatelli, M., Vilella, A.J., Searle,
358 S.M., Amode, R., Brent, S., Spooner, W., Kulesha, E., Yates, A. and Flicek, P., 2016.
359 Ensembl comparative genomics resources. Database (Oxford) 2016.
360 Kantaputra, P.N., Kaewgahya, M., Hatsadaloi, A., Vogel, P., Kawasaki, K., Ohazama, A. and
361 Ketudat Cairns, J.R., 2015. GREMLIN 2 Mutations and Dental Anomalies. J Dent Res
362 94, 1646-52.
363 Katoh, K. and Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7:
364 improvements in performance and usability. Mol Biol Evol 30, 772-80.
365 Kattamuri, C., Luedeke, D.M., Nolan, K., Rankin, S.A., Greis, K.D., Zorn, A.M. and Thompson,
366 T.B., 2012. Members of the DAN family are BMP antagonists that form highly stable
367 noncovalent dimers. J Mol Biol 424, 313-27.
368 Kriebitz, N.N., Kiecker, C., McCormick, L., Lumsden, A., Graham, A. and Bell, E., 2009. PRDC
369 regulates placode neurogenesis in chick by modulating BMP signalling. Dev Biol 336,
370 280-92.
371 Kumar, S., Stecher, G. and Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics
372 Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33, 1870-4.
373 Minabe-Saegusa, C., Saegusa, H., Tsukahara, M. and Noguchi, S., 1998. Sequence and
374 expression of a novel mouse gene PRDC (protein related to DAN and cerberus) identified
375 by a gene trap approach. Dev Growth Differ 40, 343-53.
376 Muffato, M., Louis, A., Poisnel, C.E. and Roest Crollius, H., 2010. Genomicus: a database and a
377 browser to study gene synteny in modern and ancestral genomes. Bioinformatics 26,
378 1119-21.

379 Müller, I.I., Knapik, E.W. and Hatzopoulos, A.K., 2006. Expression of the protein related to Dan
380 and Cerberus gene--prdc--During eye, pharyngeal arch, somite, and swim bladder
381 development in zebrafish. Dev Dyn 235, 2881-8.
382 Nadiri, A., Kuchler-Bopp, S., Haikel, Y. and Lesot, H., 2004. Immunolocalization of BMP-2/-4,
383 FGF-4, and WNT10b in the developing mouse first lower molar. J Histochem Cytochem
384 52, 103-12.
385 Nery, M.F., Arroyo, J.I. and Opazo, J.C., 2014. Increased rate of hair keratin gene loss in the
386 cetacean lineage. BMC Genomics 15, 869.
387 Nery, M.F., Gonzalez, D.J., Hoffmann, F.G. and Opazo, J.C., 2012. Resolution of the
388 laurasiatherian phylogeny: Evidence from genomic data. Molecular Phylogenetics and
389 Evolution 64, 685-689.
390 Olson, M.V., 1999. When less is more: gene loss as an engine of evolutionary change. Am J
391 Hum Genet 64, 18-23.
392 Rice, P., Longden, I. and Bleasby, A., 2000. EMBOSS: the European Molecular Biology Open
393 Software Suite. Trends Genet 16, 276-7.
394 Ronquist, F. and Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under
395 mixed models. Bioinformatics 19, 1572-1574.
396 Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of
397 large phylogenies. Bioinformatics 30, 1312-3.
398 Sudo, S., Avsian-Kretchmer, O., Wang, L.S. and Hsueh, A.J., 2004. Protein related to DAN and
399 cerberus is a bone morphogenetic protein antagonist that participates in ovarian paracrine
400 regulation. J Biol Chem 279, 23134-41.

401 Suyama, M., Torrents, D. and Bork, P., 2006. PAL2NAL: robust conversion of protein sequence
402 alignments into the corresponding codon alignments. Nucleic Acids Res 34, W609-12.
403 Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA
404 polymorphism. Genetics 123, 585-95.
405 Vogel, P., Liu, J., Platt, K.A., Read, R.W., Thiel, M., Vance, R.B. and Brommage, R., 2015.
406 Malformation of incisor teeth in Grem2$^{-/-}$ mice. Vet Pathol 52, 224-9.
407 Yim, H.S., Cho, Y.S., Guang, X., Kang, S.G., Jeong, J.Y., Cha, S.S., Oh, H.M., Lee, J.H., Yang,
408 E.C., Kwon, K.K., Kim, Y.J., Kim, T.W., Kim, W., Jeon, J.H., Kim, S.J., Choi, D.H.,
409 Jho, S., Kim, H.M., Ko, J., Kim, H., Shin, Y.A., Jung, H.J., Zheng, Y., Wang, Z., Chen,
410 Y., Chen, M., Jiang, A., Li, E., Zhang, S., Hou, H., Kim, T.H., Yu, L., Liu, S., Ahn, K.,
411 Cooper, J., Park, S.G., Hong, C.P., Jin, W., Kim, H.S., Park, C., Lee, K., Chun, S.,
412 Morin, P.A., O'Brien, S.J., Lee, H., Kimura, J., Moon, D.Y., Manica, A., Edwards, J.,
413 Kim, B.C., Kim, S., Wang, J., Bhak, J. and Lee, H.S., 2014. Minke whale genome and
414 aquatic adaptation in cetaceans. Nat Genet 46, 88-92.
415

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.

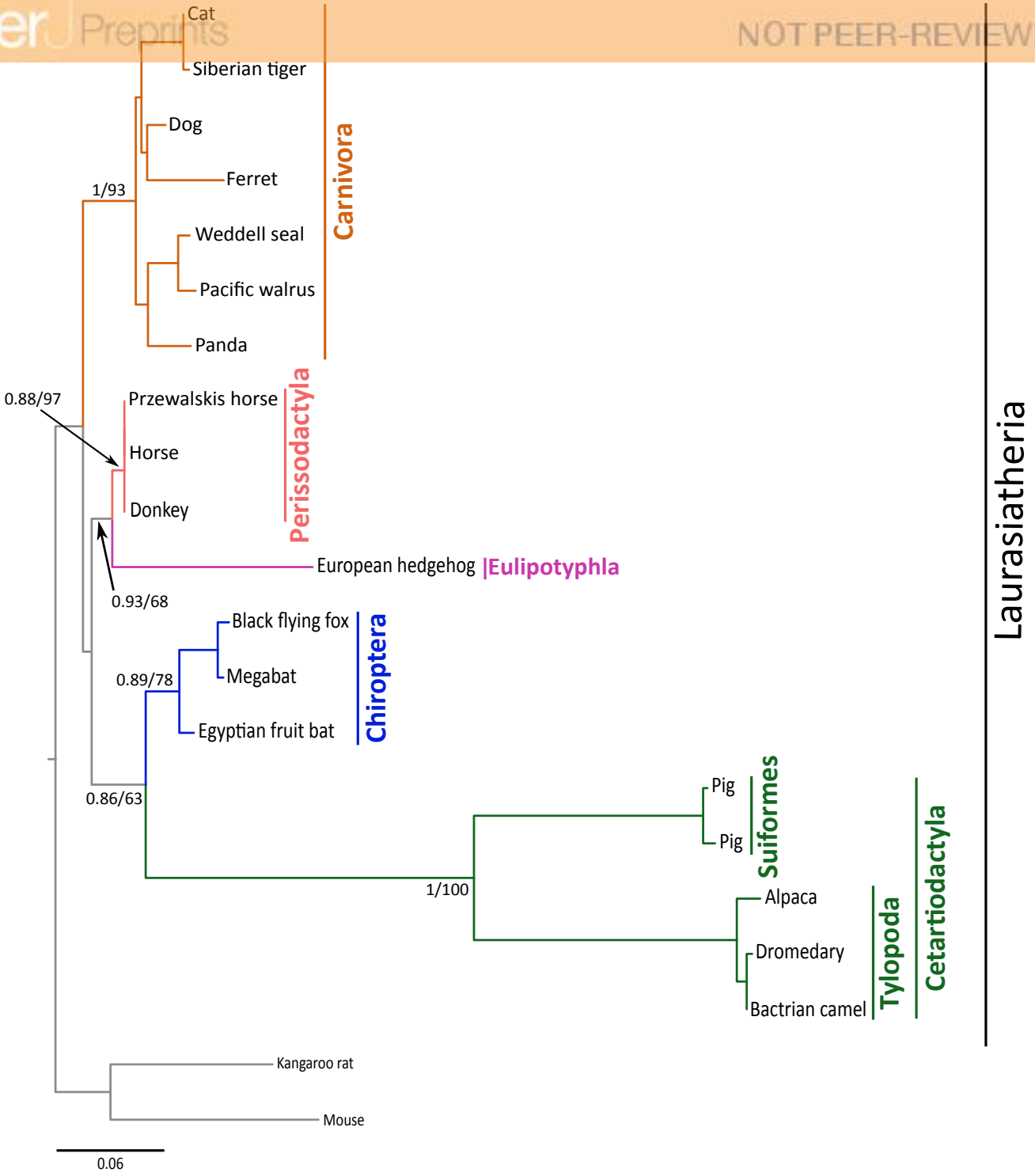


Figure 2(on next page)

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.

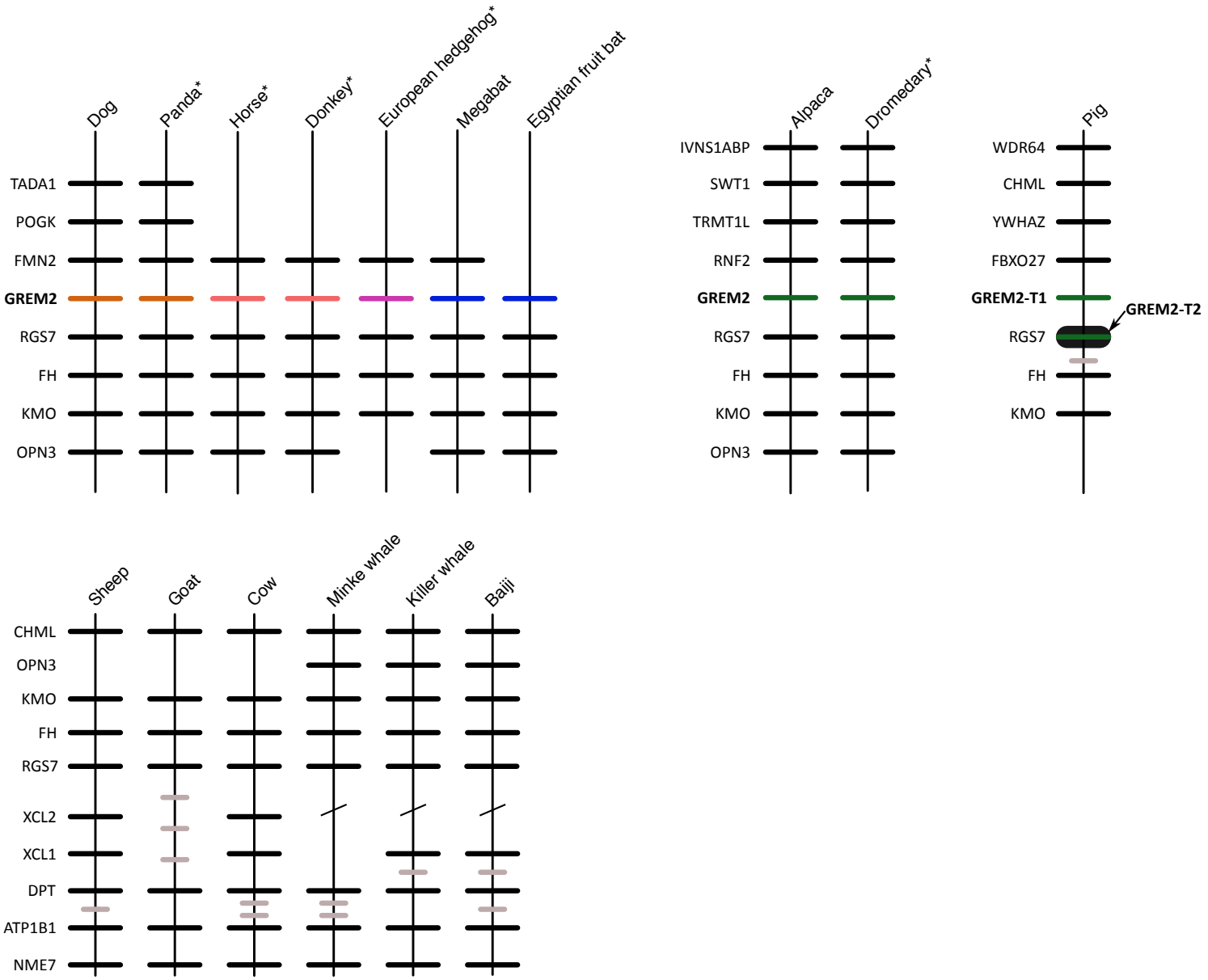


Figure 3(on next page)

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                       MFWKLSVSLLLMAALEKVEDAQRARPAGSIPSPRKDGSPESTGRWQ-LIKEVLASSQEALVVTERRYLRSDWCKTQPLRQTVHEE
Pig                       MFWKLSVSLLLMAALEKVEDAQRARPAGSIPSPRKDGSPESTGRWQ-LIKEVLASSQEALVVTERRYLRSDWCKTQPLRQTVHEE
Alpaca                    MFWKISMSLLLVAALGKLEEIQGARPAGAIPSPRKDRGTNNSQNWQ-HIREVLSSSQEALVVTERRYLRRDWCKTQRLRQTVREE
Bactrian camel            MFWKISMSLLLVAALGKLEEIQGARPAGAIPSPRKDRGTNNSQNWQ-HIREVLSSSQEALVVTERRYLRRDWCKTQRLRQTVREE
Dromedary                 MFWKISMSLLLVAALGKLEEIQGARPAGAIPSPRKDRGTNNSQNWQ-HIREVLSSSQEALVVTERRYLRRDWCKTQRLRQTVREE
Donkey                    MLWKLSLSLFLVAVLVKVAEARKNRPAGAIPSPYKDGSSNTSERWQHQIKEVLASSQEALVVTERKYLKSDWCKTQPLRQTVSEE
Horse                     MLWKLSLSLFLVAVLVKVAEARKNRPAGAIPSPYKDGSSNTSERWQHQIKEVLASSQEALVVTERKYLKSDWCKTQPLRQTVSEE
Przewalskis horse         MLWKLSLSLFLVAVLVKVAEARKNRPAGAIPSPYKDGSSNTSERWQHQIKEVLASSQEALVVTERKYLKSDWCKTQPLRQTVSEE
Cat                       MFWKLSLSLCLVAVLVKVAEARKNRPAGAIPSPYKDGSSNHSERWQHQIKEVLASSQEALVVTERKYLKSDWCKTQPLRQTVSEE
Siberian tiger            MFWKLSLSLCLVAVLVKAAEARKNRPAGAIPSPYKDGSSNHSERWQHQIKEVLASSQEALVVTERKYLKSDWCKTQPLRQTVSEE
Ferret                    MFWKLSLSLCLVAVLVKAAEARKNRPAGAIPSPYK-GSSNHSERWQHQIKEVLASSQEALVVTERKYLKSDWCKTQPLRQTVSED
Dog                       MFWKLSLSLCLVAVLVKVAEARKNRPAGAIPSPYKDGSSNHSERWQHQIKEVLASSQEALVVTERKYLKSDWCKTQPLRQTVSEE
Pacific walrus            MFWKLSLSLCLVAVLVKVAEARKNRPAGAIPSPYKDGSSNHSERWQHQIKEVLASSQEALVVTERKYLKSDWCKTQPLRQTVSEE
Weddell seal              MFWKLSLSLCLVAVLVKVAEARKNRPAGAIPSPYKDGSSNHSERWQHQIKEVLASSQEALVVTERKYLKSDWCKTQPLRQTVSEE
Panda                     MFWKLSLSLCLVAVLVKVAEARKNRPAGAIPSPYKDGSSNHSERWQQQIKEVLASSQEALVVTERKYLKSDWCKTQPLRQTVSEE
Megabat                   MFWKLSLSLLLVAVLVKVGDARKNRPAGAIPSPYKDGSSNTSERWQHQIKEVLASSQEALVVTERKYLKSDWCKTQPLRQTVGEE
Egyptian rousette         MFWKLSLSLLLVAVLVKVVDARKNRPAGAIPSPYKDGSSNTSERWQHQIKEVLASSQEALVVTERKYLKSDWCKTQPLRQTVGEE
Black flying fox          MFWKLSLSLLLVAVLLKVGDARKNRPAGAIPSPYKDGSSNTSERWQHQIKEVLASSQEALVVTERKYLKSDWCKTQPLRQTVGEE
European hedgehog         MFWKLSLSLFLVAVLVKVAEGRKNRPAGAIPSPYKDGSSNNSERWQHQIKEVLASSQEALVVTERKYLKSDWCKTQPLRQTVSEE

                          90        100       110       120       130       140       150       160       170
Pig                       GCHSRTVLNRFCYGQCNSFFIPRPGGGSW--GSFQSCAFCRPQRAAALLVELQCPGRDPPFHLRKIQKVKQCRCMSVTLGSDEP-
Pig                       GCHSRTVLNRFCYGQCNSFFIPRPGGGSW--GSFQSCAFCRPQRAAALLVELQCPGRDPPFHLRKIQKVKQCKCMSVTLGSDEP-
Alpaca                    GCHSRTVLNHFCYGQCNSFYIPRPGGEGEGSGSFQSCAFCRPQRAAALLVELQCPSRDPPVRLRKIQKVKQCRCMSVNLGADES-
Bactrian camel            GCHSRTVLNHFCYGQCNSFYIPRPGGEGEGSGSFQSCAFCRPQRAAALLVELQCPSRDPPVRLRKIQKVKQCRCMSVNLGADES-
Dromedary                 GCHSRTVLNHFCYGQCTSSHTPRPGGEGEGSGSFQSCAFCRPQRAAALLVELQCPSRDPPVRLRKIQKVKQCRCMSVNLGADES-
Donkey                    GCRSRTILNRFCYGQCNSFYIPRHVKKEE--ESFQSCAFCKPQRVTSVLVELECPGLDPPFRLKKIQKVKQCRCMSVNLSDSDKQ
Horse                     GCRSRTILNRFCYGQCNSFYIPRHVKKEE--ESFQSCAFCKPQRVTSVLVELDCPGLDPPFRLKKIQKVKQCRCMSVNLSDSDKQ
Przewalskis horse         GCRSRTILNRFCYGQCNSFYIPRHVKKEE--ESFQSCAFCKPQRVTSVLVELDCPGLDPPFRLKKIQKVKQCRCMSVNLSDSDKQ
Cat                       GCRSRTILNRFCYGQCNSFYIPRHVRKEE--ESFQSCAFCKPQRVTSVLVELECPGLDPPFRLKKIQKVKQCRCMSVNLSDSDKQ
Siberian tiger            GCRSRTILNRFCYGQCNSFYIPRHVRKEE--ESFQSCAFCKPQRVTSVLVELECPGLDPPFRLKKIQKVKQCRCMSVNLSDSDKQ
Ferret                    GCRSRTVLNRFCYGQCNSFYIPRHVRKDE--ESFQSCAFCKPQRVTSVLVELECPGMDPPFRLKKIQKVKQCRCMSVNLSDSDKQ
Dog                       GCRSRTILNRFCYGQCNSFYIPRHVRKEE--ESFQSCAFCKPQRVTSVLVELECPGLDPPFRLKKIQKVKQCRCMSVNLSDSDKQ
Pacific walrus            GCRSRTILNRFCYGQCNSFYIPRHVRKEE--ESFQSCAFCKPQRVTSVLVELECPGMDPPFRLKKIQKVKQCRCMSVNLSDSDKQ
Weddell seal              GCRSRTILNRFCYGQCNSFYIPRHVRKEE--ESFQSCAFCKPQRVTSVLVELECPGMDPPFRLKKIQKVKQCRCMSVNLSDSDKQ
Panda                     GCRSRTILNRFCYGQCNSFYIPRHVRKEE--ESFQSCAFCKPQRVTSVLVELECPGLDPPFRLKKIQKVKQCRCMSVNLSDSDKQ
Megabat                   GCRSRTVLNRFCYGQCNSFYIPRHVKKEE--ESFQSCAFCKPQRVTSVLVELECPGLDPPFRLKKIQKVKQCRCMSVNLSDSDKQ
Egyptian rousette         GCRSRTVLNRFCYGQCNSFYIPRHVKKEQ--ESFQSCAFCKPQRVTSVLVELECPGLDPPFRLKKIQKVKQCRCMSVNLSDSDKQ
Black flying fox          GCRSRTVLNRFCYGQCNSFYIPRHVKKEE--ESFQSCAFCKPQRVTSVLVELECPGLDPPFRLKKIQKVKQCRCMSVNLSDSDKQ
European hedgehog         GCRSRTILNRFCYGQCNSFYIPRHVRKEE--ESFQSCAFCKPQRATSVLVELECPGLDPPFRLKKIQKVKQCRCMSVNLSDSDKQ
```

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.

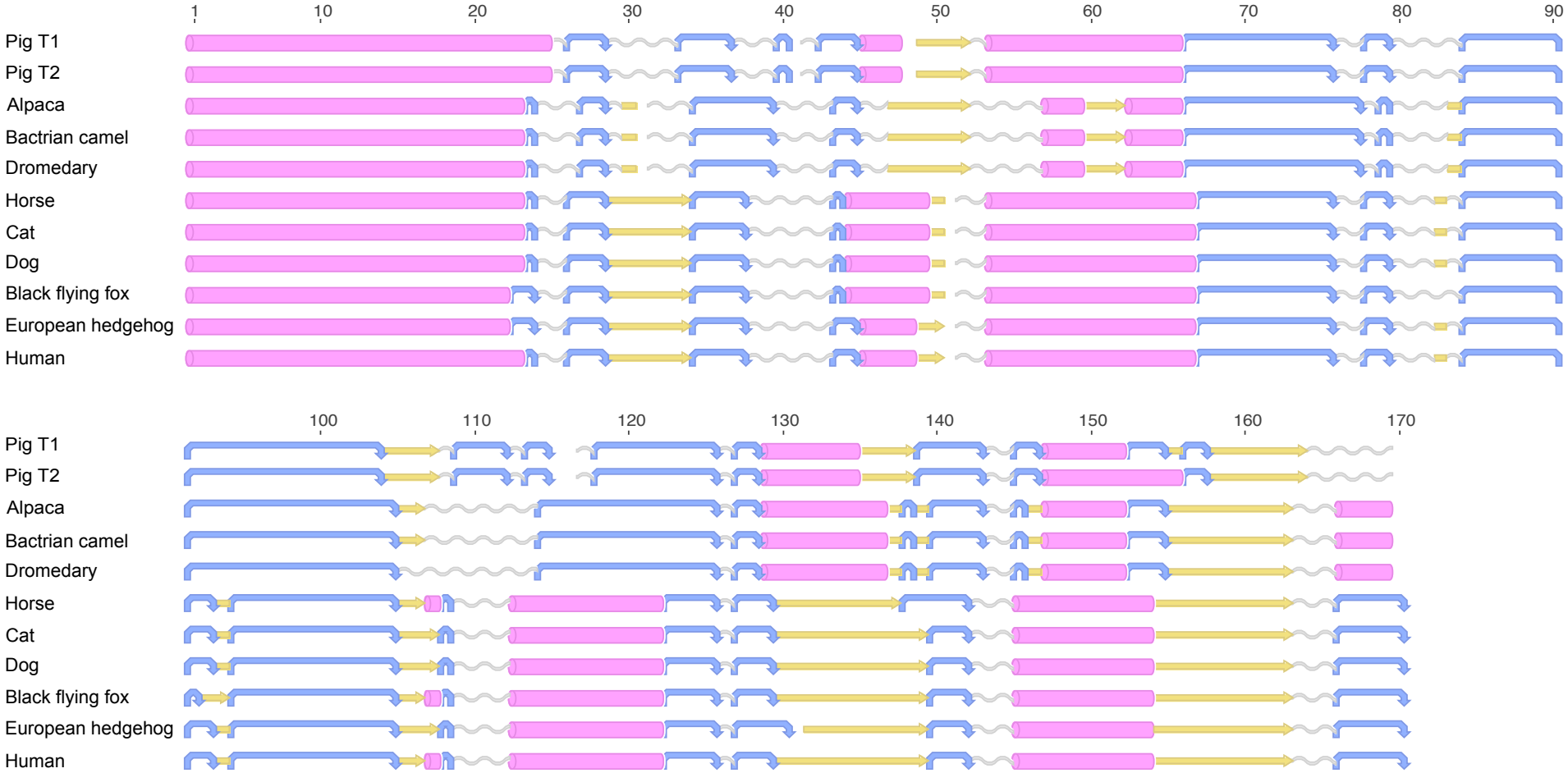


Figure 5(on next page)

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.

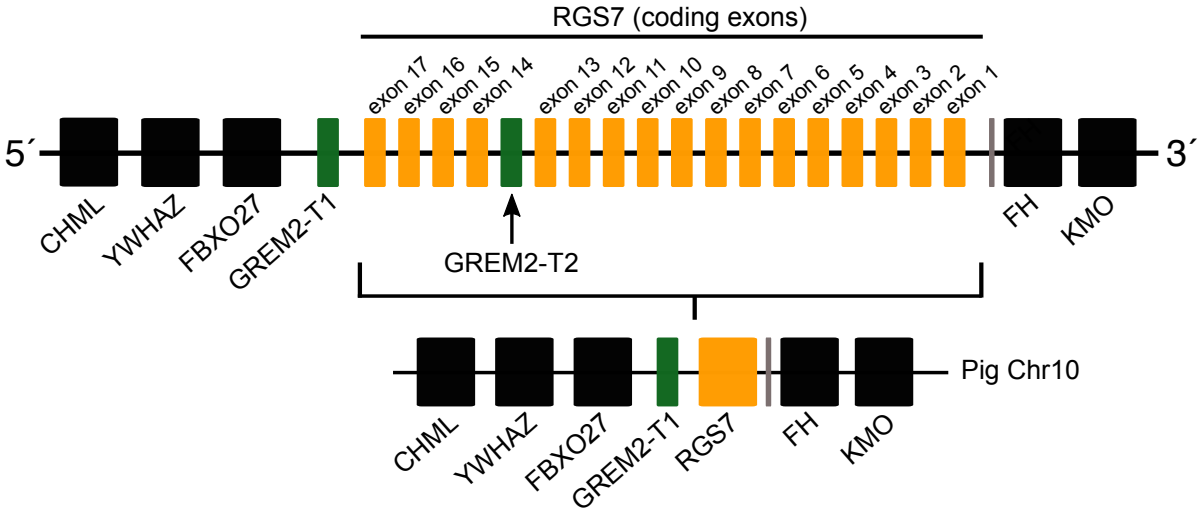


Figure 6(on next page)

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.

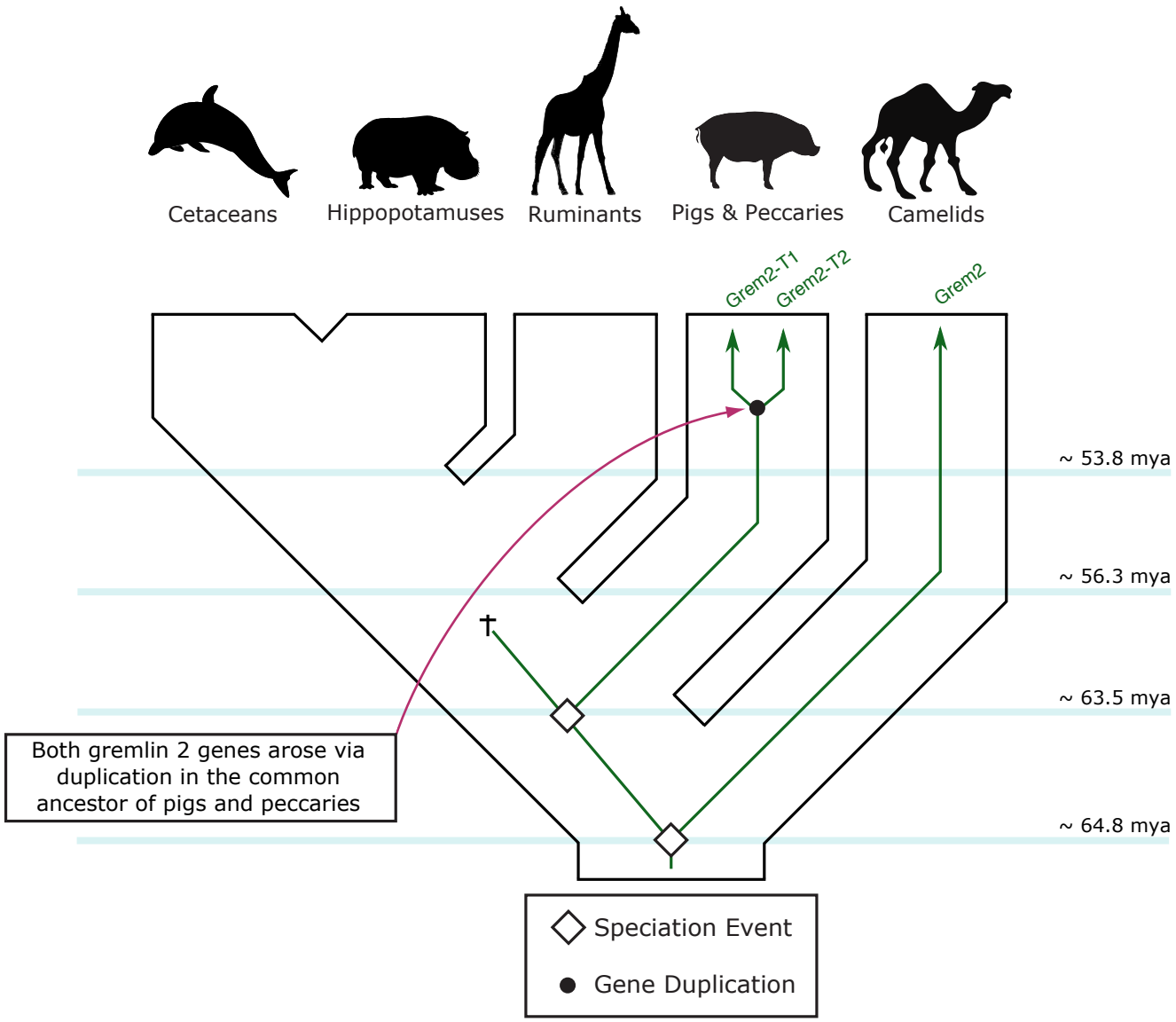
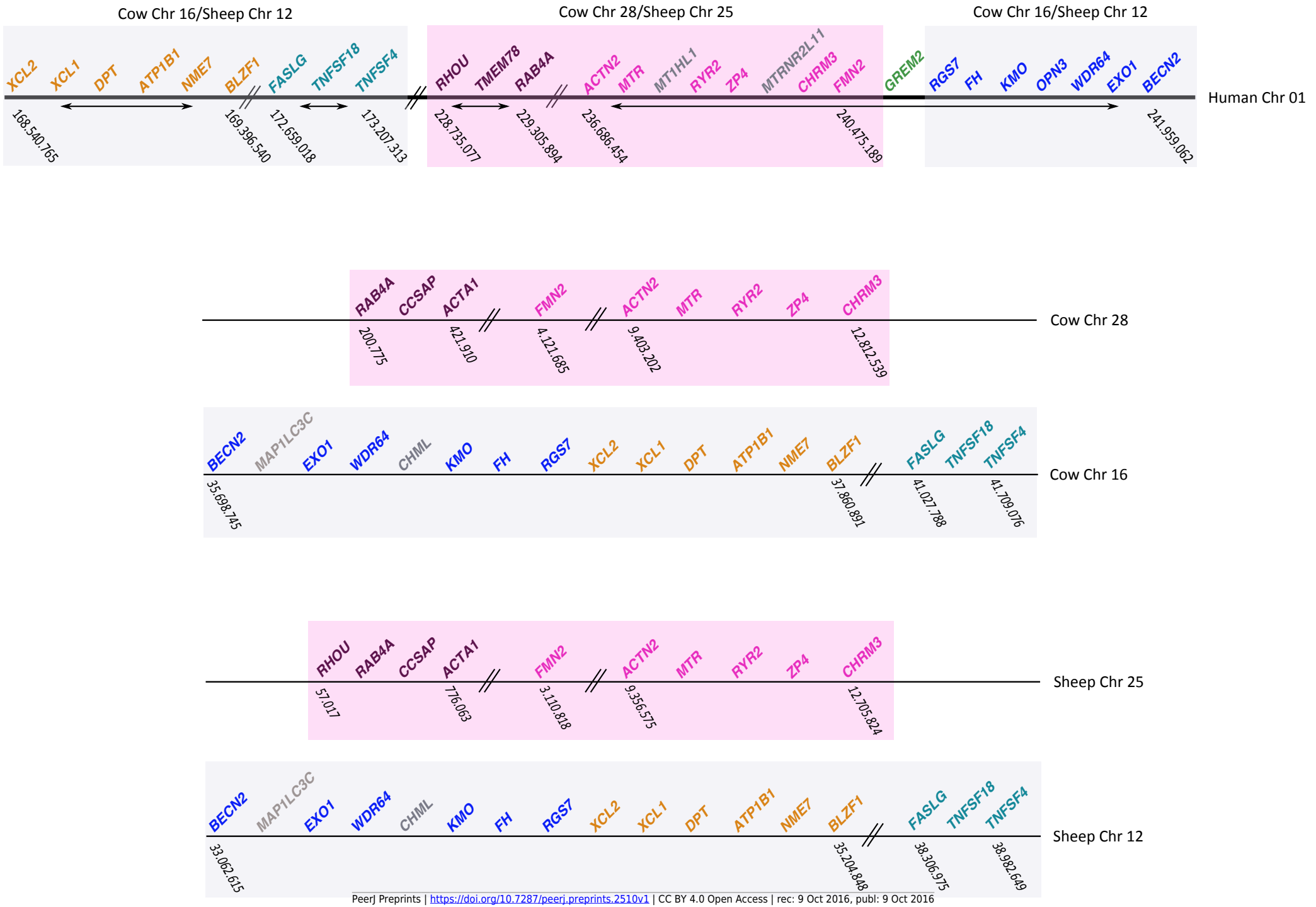


Figure 7 (on next page)

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.



PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2510v1 | CC BY 4.0 Open Access | rec: 9 Oct 2016, publ: 9 Oct 2016