

1 **Tracing the origin of a new organ by inferring the genetic basis of rumen evolution**

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36 **Abstract**

37 The rumen is the hallmark organ of ruminants and hosts a diverse ecosystem of
38 microorganisms that facilitates efficient digestion of plant fibers. We used 897
39 transcriptomes from three Cetartiodactyla lineages: ruminants, camels and cetaceans,
40 as well as data from ruminant comparative genomics and functional assays to explore
41 the genetic basis of rumen origin and evolution. Comparative analyses reveal that the
42 rumen and the first-chamber stomachs of camels and cetaceans shared a common
43 tissue origin from the esophagus. The rumen recruited genes from other tissues/organs
44 and up-regulated many esophagus genes to acquire functional innovations involving
45 epithelium absorption, improvement of the ketone body metabolism and regulation of
46 microbial community. These innovations involve such genetic changes as
47 ruminant-specific conserved elements, newly evolved genes and positively selected
48 genes. Our *in vitro* experiments validate the functions of one enhancer, one
49 positively selected gene and two newly evolved antibacterial genes. Our study
50 provides novel insights into the origin and evolution of a complex organ.

51 Evolutionary biology has a long history of trying to understand how complex organs
52 evolve¹. The origin of some notable organs has been central to animal evolution, e.g.
53 the eyes of animals^{2,3}, electric organs of fishes⁴, mammalian placenta^{5,6} and ruminant
54 headgear⁷. Another remarkable organ innovation found in mammals are the
55 multi-chambered stomachs found in the Cetartiodactyla lineages, including Tylopoda
56 (e.g. camels), Tayassuidae (e.g. peccaries), Hippopotamidae (e.g. hippos), Cetacea
57 (e.g. whales) and Ruminantia (**Fig. 1**). Among these, ruminants have the most complex
58 digestive system in herbivores, allowing efficient uptake of nutrients from plant
59 material by providing a microbial fermentation ecosystem in the highly specialized
60 rumen⁸. Camels (Tylopoda) have three-chambered stomachs and are also sometimes
61 called "pseudo-ruminants" due to their similar ruminating behavior and microbial
62 fermentation taking place in their first-chamber (FC) stomach⁹. The whales (Cetacea)
63 form the sister group of the Ruminantia¹⁰, however the FC of their four-chambered
64 stomach is mainly used as a temporary storage chamber for ingested food and for
65 mechanical grinding of food items¹¹. With the rumen, ruminants obtained a unique
66 evolutionary advantage through superior utilization of short chain fatty acids (SCFAs)
67 from microbial fermentation, which significantly promoted the expansion and
68 diversification of ruminant taxa¹². The evolutionary innovation of the rumen is
69 therefore interesting not only in its functional complexity and uniqueness, but also
70 because it has greatly benefited humans by providing high-quality nutrition in the shape
71 of highly productive ruminant livestock species^{13,14}.

72 The anatomical predecessor from which the rumen evolved has been proposed to

73 be the esophagus¹⁵, yet the two organs are highly divergent in morphology and
74 physiology. The stratified squamous epithelium of the esophagus is smooth and
75 non-keratinized, and mainly serves a barrier function, but in contrast the rumen
76 stratified squamous epithelium is keratinized and lined with papillae, which facilitates
77 nutrient uptake and antibacterial peptide production^{16,17}. These features allow the
78 absorption of SCFAs and sustain the homeostasis of microorganisms. The origin and
79 evolution of new organs involve structural and functional innovations that were
80 proposed to be driven by several types of genetic reprogramming: recruitment of
81 genes usually expressed in other organs, transformation of regulatory elements such
82 as promoters and enhancers, mutations in protein-coding genes and
83 post-transcriptional mechanisms^{1,5}. Given the substantial structural and physiological
84 changes involved in the transition from esophagus to rumen, significant genetic
85 reprogramming must have occurred during the process.

86 Usually, it is challenging to obtain detailed insights into the genetic
87 reprogramming associated with organ evolution due to the rarity of such occurrences
88 and the lack of intermediate evolutionary states⁵. However, in the case of the rumen,
89 we can take advantage of two important points allowing “triangulation” of the
90 changes leading to the rumen: the availability of synapomorphic stomach chambers in
91 Cetartiodactyla and the likely ancestral relation between the esophagus and the rumen.
92 Here, we conducted a comprehensive comparison using 897 transcriptomes of
93 different tissues from three Cetartiodactyla lineages and multiple genomes to
94 investigate the genetic basis of gene programming evolution and functional

95 innovations in rumen, together with validation of some cases using *in vitro*

96 experiments.

97 **Results**

98 **Gene expression features of the rumen**

99 We sequenced transcriptomes of 33 samples across 14 adult tissues from Bactrian
100 camels, eight adult tissues from one species in Mysticeti (Bryde's whale) and one
101 species in Odontoceti (Indo-Pacific Finless Porpoise) from Cetacea, 852 samples (210
102 sequenced in this study and 642 published in previous studies^{7,18,19}) from 50 tissues of
103 two representative ruminants (sheep and roe deer) within Ruminantia (**Supplementary**
104 **Table 1**). The global gene expression patterns of all the FC stomachs are consistently
105 most similar to the esophagus in all species (**Fig. 2a, Fig. S1**). To investigate the
106 specifically expressed genes in the three types of FC stomachs, we defined those that
107 the rank of expression is less than or equal to a E50 index threshold with type I error
108 less than 0.05 (**Supplementary Note**) in the FC stomachs of ruminants, camels, and
109 cetaceans compared to other conspecific tissues/organs. We identified 655, 593, and
110 375 such specifically expressed genes in the FC stomachs of ruminants, camels, and
111 cetaceans, respectively (**Supplementary Table 2-4; Supplementary Note**).

112 *Comparisons of gene expression profiles between rumen and the first-chamber stomach*
113 *of camels and cetaceans*

114 Among these FC-specific genes, the three FC stomachs shared 18 genes which are
115 co-expressed in the esophagus in all species (**Supplementary Table 5**). The 18 genes
116 were significantly enriched in keratinocyte differentiation (**Supplementary Table 6**,
117 Fisher's exact test, adjusted P value = 9.85×10^{-3}). This is consistent with the fact that
118 the FC stomachs all share a basic stratified squamous epithelium with the

119 esophagus²⁰⁻²², which is markedly different from other stomach chambers (e.g. the
120 abomasum of the ruminants, the third-chamber stomachs of camels and cetaceans).
121 Notably, *PAX9*²³, a known key transcription factor during esophagus differentiation, is
122 highly expressed in all three FC stomachs and may play a role in the origin of the FC
123 stomachs from their anatomic origin (**Supplementary Table 5**). Our results therefore
124 indicate that the FC stomachs in Cetartiodactyla share a common developmental origin
125 from the esophagus, and that changes in epidermis development may be an ancestral
126 feature in this proto-rumen.

127 Despite the shared features of epithelial histology found in all Cetartiodactyla FC
128 stomachs, the rumen also has a series of unique structural and functional innovations.
129 Among the 655 rumen specifically expressed genes, we identified 448 up-regulated and
130 79 down-regulated genes when compared to the FC stomachs of camels (**Fig. 2b;**
131 **Supplementary Table 7**), and 563 up-regulated and 29 down-regulated genes when
132 compared to the FC stomachs of cetaceans (**Fig. 2b; Supplementary Table 8;**
133 **Supplementary Note**). Among these, the majority (427, 65.2%) are up-regulated in
134 rumen relative to both the FC stomach of camels and cetaceans (**Fig. 2b;**
135 **Supplementary Table 9**). These exclusively rumen-specific (i.e., not specifically
136 expressed in other FC stomachs) genes are significantly associated with the synthesis
137 and degradation of ketone bodies (Fisher's exact test, adjusted P value = 1.21×10^{-3})
138 (**Fig. 2c; Supplementary Table 10**). Unlike monogastric animals, in which
139 ketogenesis mainly occurs in the liver and the intestinal tract^{24,25}, the rumen is the main
140 site of ketogenesis in adult ruminants, and the occurrence of ketogenesis is regarded as

141 a diagnostic feature of rumen maturity²⁶. In addition to ketogenesis genes , seven genes
142 from the KEGG pathway *Staphylococcus aureus* infection were also highly expressed
143 in the rumen compared to the FC stomachs of camels and cetaceans (Fisher's exact test
144 for KEGG pathway enrichment, adjusted P value = 1.35×10^{-2}) (**Supplementary Table**
145 **10**). These results indicate that improved ketone body metabolism and microbial
146 regulation were important features in the evolution of the rumen from a proto-rumen
147 origin shared with other Cetartiodactyls.

148 *Gene recruitment by the rumen*

149 Among the 655 rumen specifically expressed genes, the rumen co-expressed 96
150 (14.7%) genes with the esophagus (**Fig. 2d; Supplementary Table 2**). The 96 genes
151 were enriched in the cornified envelope (adjusted $P = 4.11 \times 10^{-14}$) and epidermal cell
152 differentiation processes (adjusted $P = 3.77 \times 10^{-25}$) (**Supplementary Table 11**).
153 Meanwhile, we also found that the rumen recruited genes from a range of other tissues
154 and biological pathways (**Fig. 2d**), e.g. keratinocyte differentiation (**Supplementary**
155 **Table 12**, 88 genes co-expressed with keratinization-associated tissues), urea cycle
156 (**Supplementary Table 13**, 24 genes co-expressed with liver), monocarboxylic acid
157 transport (**Supplementary Table 14**, 61 genes co-expressed with intestine), skeletal
158 muscle contraction (**Supplementary Table 15**, 23 genes co-expressed with muscle),
159 urea transport (**Supplementary Table 16**, 19 genes co-expressed with kidney) and
160 saliva secretion (**Supplementary Table 17**, 10 genes co-expressed with salivary
161 gland). These pathways are all strongly associated with known rumen functions. For
162 instance, enhanced urea recycling is an important characteristic of the rumen leading to

163 increased nitrogen utilization for ruminants²⁷. Collectively, these results suggest that
164 the rumen—in addition to up-regulating genes expressed in the esophagus—recruited
165 genes from different tissues to evolve its unique structure and complex functions.

166 *Identification of genes functioning in early rumen development*

167 The above rumen specifically expressed genes are identified in postnatal rumen,
168 but the development of the rumen structure mainly occurs during early embryo
169 stages^{28,29}. In order to identify genes functioning in this critical stage, we performed
170 five RNA sequencing from the ruminal and esophageal epithelium cells of four 60
171 days' sheep embryos, the stage at which the ruminal epithelium starts to
172 differentiate^{28,29} (**Supplementary Table 1**). We identified 285 rumen up-regulated
173 differentially expressed genes (DEGs) compared to the esophagus (**Supplementary**
174 **Table 18**). These are enriched in cell-cell junction (adjusted P value = 8.33×10^{-3}) and
175 desmosome organization (adjusted P value = 1.47×10^{-3}) (**Supplementary Table 19**).
176 We also found 1,840 rumen down-regulated DEGs which are enriched in anatomical
177 structure morphogenesis (adjusted P value = 1.39×10^{-15}) (**Supplementary Table 18,**
178 **20**). These results indicate that the specific epithelial histology of the rumen wall
179 constitutes the most significant developmental genetic reprogramming as the organ
180 forms and grows in the embryo. After filtering redundancy, we combined the 655
181 rumen specifically expressed genes with the 285 rumen up-regulated DEGs compared
182 to the esophagus at the key development stage and eventually obtain 846 rumen key
183 genes which we consider crucial for rumen development and evolution.

184

185 **Evolutionary analyses on the rumen key genes**

186 Based on the data from ruminant comparative genomics³⁰, we employed evolutionary
187 genomic analyses on the 846 rumen key genes in the evolutionary context of 51
188 ruminants and 12 other mammals, by identifying ruminant-specific conserved
189 nonexonic elements (RSCNEs) (≥ 20 bp), newly evolved genes and positively selected
190 genes (PSGs) to systematically investigate the genetic changes associated with these
191 rumen key genes. In the common ancestor of Ruminantia, we identified 657 genes with
192 RSCNEs (**Supplementary Table 21**), two newly evolved genes and 28 PSGs
193 (**Supplementary Table 22**) among the 846 rumen key genes. They are mainly
194 involved in keratin filament binding, serine-type peptidase activity, ketone body
195 metabolism and detection of bacterium.

196 *Improved ketone body synthesis in rumen*

197 In the pathway of synthesis and degradation of ketone bodies, *HMGCS2* and
198 *SLC16A1* were under positive selection in the common ancestor of ruminants (**Fig. 2c**,
199 **3a; Supplementary Table 9, 10, 22**), and had ruminant-specific mutations when
200 compared to non-ruminant mammals (**Fig. 3b**). Of the five ruminant-specific amino
201 acid changes in the HMGCS2 protein, four are located in the HMG-CoA synthase
202 domain (PF01154) (**Fig. 3b**). To further examine the effects of these mutations on the
203 enzyme structure, we conducted three-dimensional (3D) structure simulations, and
204 found that mutations in HMG-CoA synthase domain could induce a change of the
205 protein 3D structure when compared to the human HMGCS2 protein (**Fig. 3c**). We also
206 noted that the *SLC16A1* gene, which participates in the transportation of ketone bodies

207 into the blood²⁴, exhibited seven ruminant-specific mutations, six of which are located
208 in the MFS_1 domain (PF07690), resulting in a domain structure change as revealed by
209 protein structure homology-modeling (**Fig. S2, S3**). We therefore hypothesized that the
210 changes in *HMGCS2* and *SLC16A1* may result in a more efficient ketone body
211 metabolism in ruminants. This is supported by *HMGCS2* being the key rate-limiting
212 enzyme in the ketogenesis pathway²⁴. To explore the functional relevance of these
213 mutations, we synthesized sheep and human *HMGCS2* orthologs *in vitro* and tested
214 their enzyme synthetic activities by measuring the activities in a reconstituted system
215 consisting of the enzyme and substrate (**Supplementary Note**). The sheep HMGCS2
216 (S) protein variant exhibits significantly higher metabolic efficiency than human
217 proteins (H) (~2-fold increase, t-test, $P < 0.001$) (**Fig. 3d**). The enzyme activity of
218 human HMGCS2 containing the five ruminant-specific amino acids replacements
219 (H-5R) is also significantly higher than the regular human protein (~1.5-fold increase,
220 $P < 0.01$), while sheep HMGCS2 with the corresponding five human amino acid
221 replacements (S-5H) exhibits significantly lower enzymatic activities than the sheep
222 protein (~2-fold decrease, $P < 0.001$) (**Fig. 3d**). These results confirm that ruminants
223 have evolved a more efficient ketogenesis than that of other mammals.

224 *Immune system and microbial regulation*

225 We identified one PSG (*NOD2*) (**Supplementary Table 22**) and two newly
226 evolved genes (*DEFB1* and *LYZI*) in the rumen key gene list that are involved in
227 immune functions. Among these, our transcriptomic data show that *NOD2* was
228 co-expressed with the macrophage cells, and highly expressed in the rumen compared

229 to both the FC stomachs of camels and cetaceans (**Supplementary Table 2, 9**). We
230 detected 11 ruminant-unique amino acid changes in NOD2, resulting in domain
231 structure changes as revealed by protein structure homology-modeling (**Fig. S4, S5**).
232 This gene functions in the upstream part of IL17 signaling pathway, activating the
233 Th17 cells to produce IL17F as part of the gastrointestinal immune system³¹ (**Fig. 4a**).
234 The IL17 signaling pathway protects the host against extracellular pathogens via
235 activating downstream pathways to induce the expression of antimicrobial peptides³².

236 Among the newly evolved genes in the ancestor of ruminants, we identified a
237 rumen key gene, *DEFB1*, which belongs to the beta-defensin family that have
238 important roles as antimicrobial peptides in the resistance of epithelial surfaces to
239 microbial colonization (**Supplementary Table 2**). In addition, we identified one
240 newly evolved rumen key gene *LYZ1* in the lysozyme *c* family (**Supplementary Table**
241 **2**), which may protect the rumen epithelium from the activity of pathogenic bacteria¹⁸.
242 We predicted that the *LYZ1* contains a ruminant-specific 20 amino-acid-chain that
243 encodes a probable transmembrane anchor (**Fig. S6, S7**), suggesting that the *LYZ1* gene
244 encodes a secreted membrane-anchored protein, which may act on the rumen
245 environment.

246 To validate the functions of these two newly evolved genes, we synthesized
247 *DEFB1* and *LYZ1* *in vitro* and tested their antibacterial ability by performing an
248 inhibition zone assay on agarose plates with *Escherichia coli* (American Type Culture
249 Collection, ATCC 25922) and *Staphylococcus aureus* (ATCC 29213) as representative
250 of Gram-negative and -positive bacteria (**Supplementary Note**). The *DEFB1* (**Fig. 4b**)

251 and LYZ1 (**Fig. 4c**) protein both showed antibacterial activity to *S. aureus*, but not *E.*
252 *coli*. This characteristic of selective inhibition of Gram-positive bacteria is similar to
253 that of monensin, which is commonly used as an antibiotic drug that regulates the
254 microbiome and increases ruminant feed conversion efficiency^{33,34}. Taken together,
255 these results highlight that several important antibacterial functions are uniquely
256 evolved in the rumen relative to other similar organs, and that some of these may
257 work by specifically managing the microbiome composition.

258 *New regulatory elements related to rumen epithelium absorption function*

259 We searched among 221,166 RSCNEs to identify candidate regulatory regions in
260 the vicinity of rumen key genes. We found that 657 of the 846 rumen key genes have
261 nearby RSCNEs (**Supplementary Table 21**). To assess the regulatory role of these
262 RSCNEs in the recruitment of increased gene expression in the rumen, we performed
263 eight ATAC-seq libraries of the ruminal and esophageal epithelium cells from four 60
264 days' sheep embryos (**Supplementary Table 23; Supplementary Note**). Our analysis
265 indicates that 243 rumen key genes have nearby RSCNEs overlapping with identified
266 open accessible peaks (**Supplementary Table 24**), and these genes are enriched in
267 epidermal cell differentiation (adjusted P value = 4.82×10^{-19}) (**Supplementary Table**
268 **25**). In the comparison of ATAC-seq between the rumen and esophagus, we identified
269 3,904 rumen-specific and 5,531 esophagus-specific open differentially accessible
270 peaks (DAPs) (**Fig. S8; Supplementary Table 26**). Interestingly, we found 267 and
271 478 RSCNEs (≥ 20 bp) overlapping with rumen-specific and esophagus-specific
272 DAPs, which is highly statistically significant (Fisher's exact test, both P value = 0.00).

273 Rumen-specific DAP-associated RSCNEs are physically near 22 rumen key genes
274 (**Supplementary Table 27**). Among these genes, *CRNN* is one of the genes in the
275 epidermal differentiation complex (EDC) locus, which is essential for the cornified cell
276 envelope in rumen¹⁵, and is implicated in several epithelial malignancies in human³⁵. A
277 rumen-specific DAP-associated RSCNE with six ruminant-specific mutations was
278 found at the 5' upstream of *CRNN* of ruminants, which might play a role in regulating
279 its expression in rumen. Concordantly, *DMRT2* is a key transcriptional factor in the
280 dermomyotome organization and *DMRT2*-deficient mice have epithelial morphology
281 abnormalities³⁶. We observed that *DMRT2* has five rumen-specific DAP-associated
282 RSCNEs in its 3' downstream region, potentially causing high *DMRT2* expression in
283 rumen.

284 Interestingly, *WDR66* is not only highly expressed in the rumen compared with
285 both the FC stomachs of camels and cetaceans but also under positive selection in the
286 common ancestor of Ruminantia (**Fig. 5a; Supplementary Table 9, 22**). It regulates
287 the expression of occludin, which tightens the intercellular space and enables epithelial
288 permeability³⁷. We observed 10 ruminant-specific non-synonymous mutations and one
289 rumen-specific DAP-associated RSCNE in the intronic region of *WDR66* (**Fig. 5b; Fig.**
290 **S9; Supplementary Table 27**). In order to assess the regulatory activity of this
291 particular RSCNE, we cloned it into a luciferase reporter vector (pGL3-Promoter) and
292 transfected it into both sheep and goat fibroblasts *in vitro*. The RSCNE showed
293 significantly higher luciferase transcriptional activation compared to the
294 pGL3-Promoter control (t-test, $P < 0.05$) (**Fig. 5c**), confirming that it acts as an

295 enhancer. Therefore, these DAP-associated RSCNEs might plausibly have exerted
296 novel *cis*-regulation of the rumen key genes, thus providing a mechanistic explanation
297 of how the rumen might have recruited these genes from other tissues. Hence, we
298 propose a central role of such regulatory elements in the development and evolution of
299 rumen structure and function.

300 *Positively selected genes involved in rumen epithelium absorption*

301 We observed that eight rumen key genes involved in the cell junction biological
302 process (*WDR66*, *COL7A1*, *EVPL*, *KRT14*, *CLDN23*, *F2RL1*, *TMPRSS13* and
303 *TMPRSS11A*) were under positive selection in ruminants (**Fig. 5a; Fig. S9-S16;**
304 **Supplementary Table 22**). Non-synonymous changes in these genes may result in the
305 change of cell junctions, which may break the epithelium barrier and increase the
306 epithelium absorption properties³⁸⁻⁴². *COL7A1* is highly expressed in the rumen of fetal
307 sheep, but not in the esophagus (**Supplementary Table 18**). We detected 17 unique
308 amino acid (aa) changes in *COL7A1* in ruminants (**Fig. S10**). *COL7A1* is an anchoring
309 fibril between the external epithelia and the underlying basal lamina³⁹. Amino acid
310 mutations in this gene are associated with epidermolysis bullosa, a condition in which
311 tissue fluid diffuses through the intercellular space into the epidermis³⁹. In addition,
312 *TMPRSS13*, a membrane-anchored serine protease gene⁴¹, is highly expressed in rumen
313 compared to esophagus (**Supplementary Table 18**). Interestingly, we identified five
314 ruminant-specific aa changes in *TMPRSS13*, four of which are located in the
315 trypsin-like serine protease domain (**Fig. S15**). It is reported that the deficiency of
316 *TMPRSS13* in mice impairs stratum corneum formation and epidermal barrier

317 acquisition, accompanied by trans-epidermal fluid loss⁴¹. In normal epithelium cells
318 (e.g., epithelium cells of skin), the epithelium barrier is produced by strong intracellular
319 protein filaments crossing the cytoplasm and attaching to specialized junctions, which
320 in turn ties the surfaces of adjacent cells either to each other or to the underlying basal
321 lamina⁴³ (**Fig. 5a**). Given that the epithelium transportation and absorption functions
322 are affected by the epithelium barrier, mutations in these cell junction-related genes
323 may be related to metabolite uptaking function of the rumen.

324 **Discussion**

325 Our large quantity of transcriptomic data in adults and an early embryo rumen
326 development stage provide a detailed comparative insight into the distinct gene
327 expression profile of the rumen. Although there has been no consensus about the
328 evolutionary relationship between the FC stomachs of camels, peccaries, cetaceans and
329 ruminants^{21,44}, it is unlikely that the multi-chambered stomach evolved independently
330 four times in Cetartiodactyla exclusively. Therefore, the most parsimonious
331 explanation is that they may have a single evolutionary origin, followed by
332 specialization in the different lineages of the Cetartiodactyla due to their specific diets
333 and niches. For instance, the FC stomachs of camels have evolved the ability to store
334 water^{21,45}, the FC stomachs of cetaceans has the capacity to mechanically grind food¹¹,
335 and the rumen provides efficient fermentation and metabolism of plant material. The
336 gene expression profiles of the FC stomachs in ruminants, camels and cetaceans show
337 that they are all highly similar to the esophagus, suggesting these organs share an
338 anatomical origin from the esophagus (**Fig. 2a; Fig. S1**).

339 Based on our comparative genomic and functional data, we outline the genetic
340 mechanisms underlying the origin, development and evolution of the rumen from the
341 ancestral esophagus tissue. These genetic innovations are mainly related to epithelium
342 absorption, ketone body metabolism and microbial regulation. Among the 846 rumen
343 key genes (**Supplementary Table 2, 18**), we found that 657 (77.7%) genes have nearby
344 RSCNEs (**Supplementary Table 21**), 28 genes are under positive selection
345 (**Supplementary Table 22**) and two genes newly evolved in the common ancestor of

346 ruminants, suggesting these three types of genetic reprogramming all contributed to the
347 structural and functional evolution of rumen. Notably, the majority of rumen key genes
348 have RSCNEs nearby and our ATAC-seq validated that 243 rumen key genes had
349 nearby RSCNEs overlapping with highly accessible chromatin (**Supplementary Table**
350 **24**), suggesting the RSCNEs as regulatory elements may play a crucial role in rumen
351 gene recruitment. The highly significant association between RSCNEs, rumen key
352 genes and open accessible peaks is a strong indication of this, although there were also
353 many RSCNEs that did not overlap with open accessible peaks in our ATAC-seq
354 analysis. While this suggests that RSCNEs play other roles besides being regulatory
355 elements, it is also possible that some were false negatives due to the limitations of
356 development stages sampled in this study, which might have omitted some associations
357 between rumen key genes and regulatory RSCNEs. Hence, a denser sampling of
358 different developmental time points might expand the rumen key gene list and reveal
359 novel regulatory roles of RSCNEs. Nevertheless, our study has revealed the important
360 genetic mechanisms underlying the key evolutionary innovations of the rumen. The
361 identified rumen key genes and their specific mutations provide a starting point for
362 future studies of rumen development, and for understanding the interactions between
363 rumen and microbiota. This will be key to further improvement of ruminant livestock,
364 e.g. by providing a framework for manipulating the rumen fermentation process.

365 **Data availability**

366 The raw reads for all RNA-seq data, the ATAC-seq data from the rumen and the
367 esophagus have been deposited at the Sequence Read Archive (SRA) under project
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379 **Author contributions**

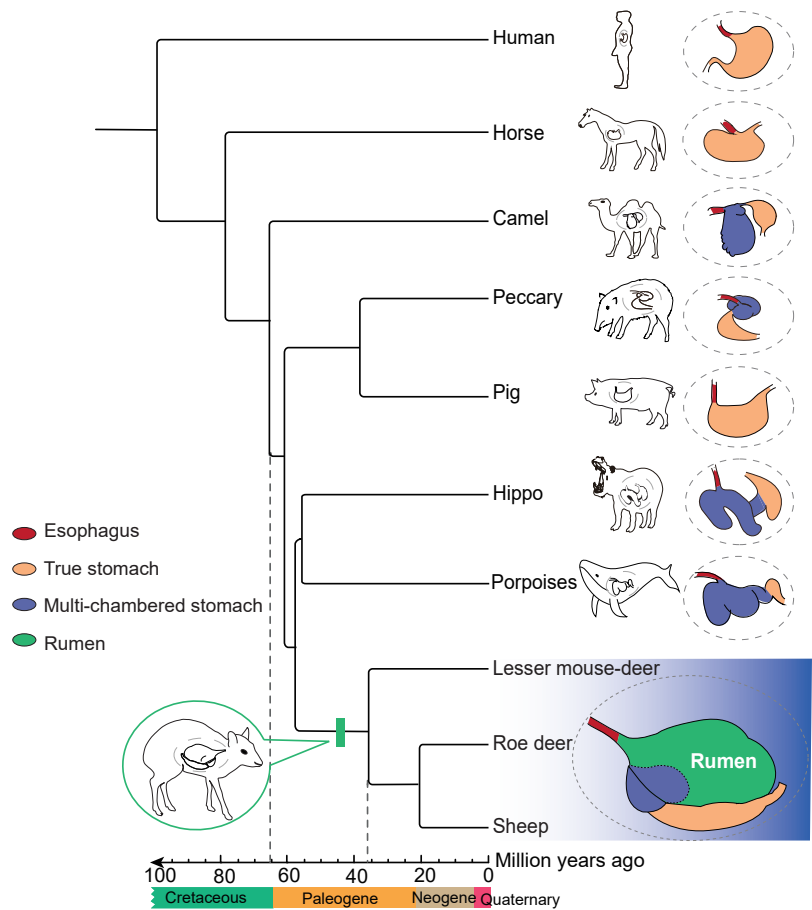
380 Y.J. and W.W. conceived the project and designed the research. X.P., Y.C., N.W., C.
381 Z., and X.H. performed the majority of analysis with contributions from K.W., L.C.,
382 Z.L., Z.Z., B.W., S.H.; Q.Q., S.M., X.L., W.F., L.L., Y.L., W.S., W.L., T.Z., J.H.,
383 M.L., S.L., S.H., M.L., C.L., and Y.C. prepared the sheep, camels and cetaceans
384 samples for transcriptomics and rumen and esophagus epithelium cells for ATAC-seq.
385 H.L. performed the luciferase reporter assay. X.C., Y.Y. and Z.H. performed the
386 inhibition zone assay and the enzyme synthetic activities assay. X.P., Z.L. and Y.C.

387 drafted the manuscript with input from all authors, whereas Y.J., W.W., R.H., B.P.D.,

388 G.Z., X.W. and Y.W. revised the manuscript.

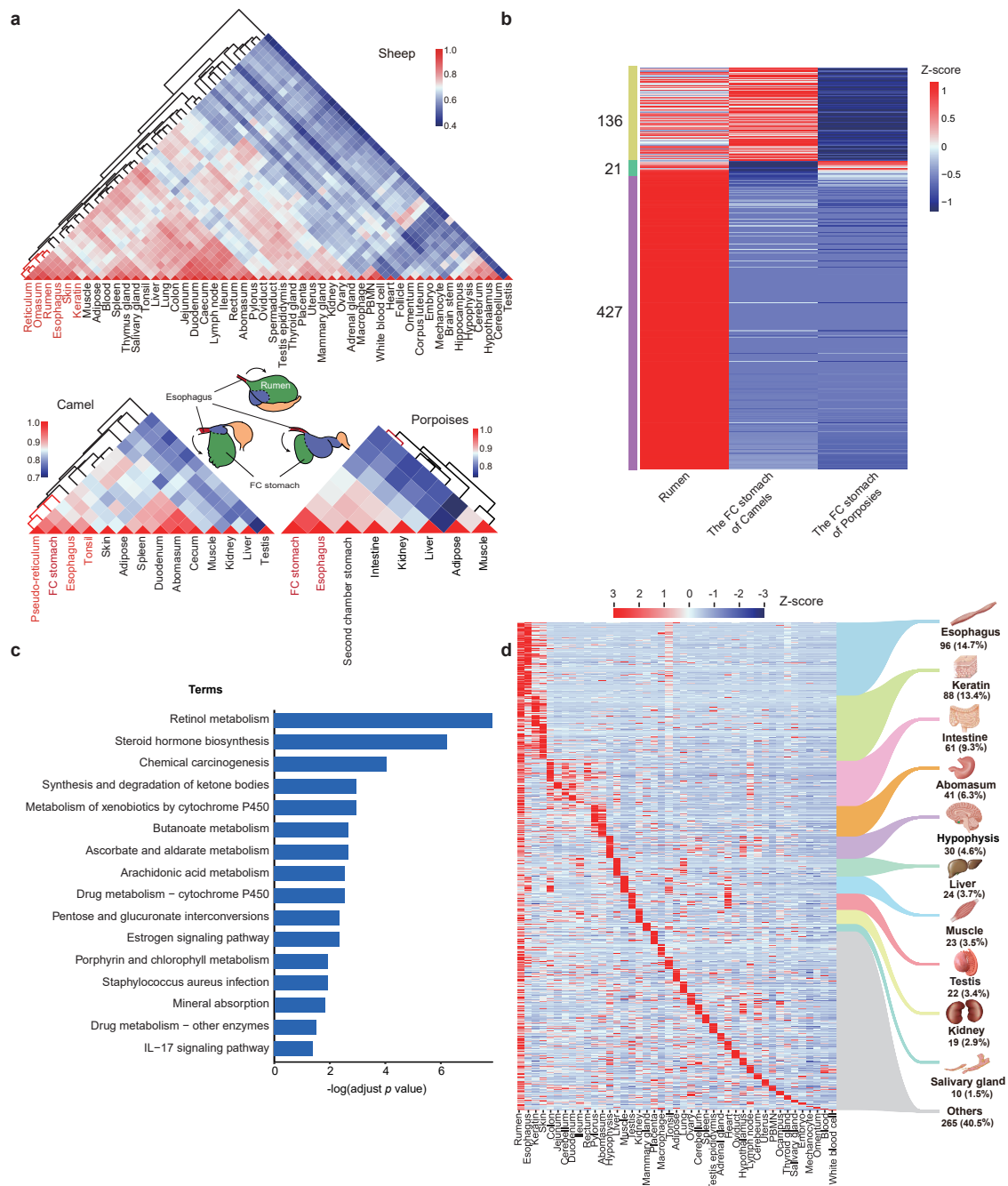
389 **Competing interests**

390 Two provisional Chinese patent applications on potential application in the antimicrobial and antibiotic substitute by way of the *DEFBI* gene and *LYZI* gene have been filed by Northwest A&F University (application number 202010100677.8 and 202010097562.8), where Y.J., X.P., X.C, and W.W. are listed as inventors. The authors declare no competing interests.



391

392 **Fig. 1 | Origin of the rumen.** Maximum-likelihood (ML) tree generated using 3,316,385 four-fold degenerate
 393 sites with 11,567 single-copy orthologous genes. Dates for major events are taken from the TimeTree Database⁴⁶
 394 and Chen *et al.*,³⁰. The green rectangular block indicates the Ruminantia. Dotted lines link to the detailed
 395 divergence times of the two taxa. The esophagus is colored red, the additional stomach chambers in the
 396 multi-stomach lineages purple, the rumen green, and the true stomach/abomasum orange.



397

398 **Fig. 2 | Comparisons of gene expression profile among rumen and other tissues. a,**

399 Hierarchical clustering results showing the relationships among 50 tissues of sheep and a heatmap

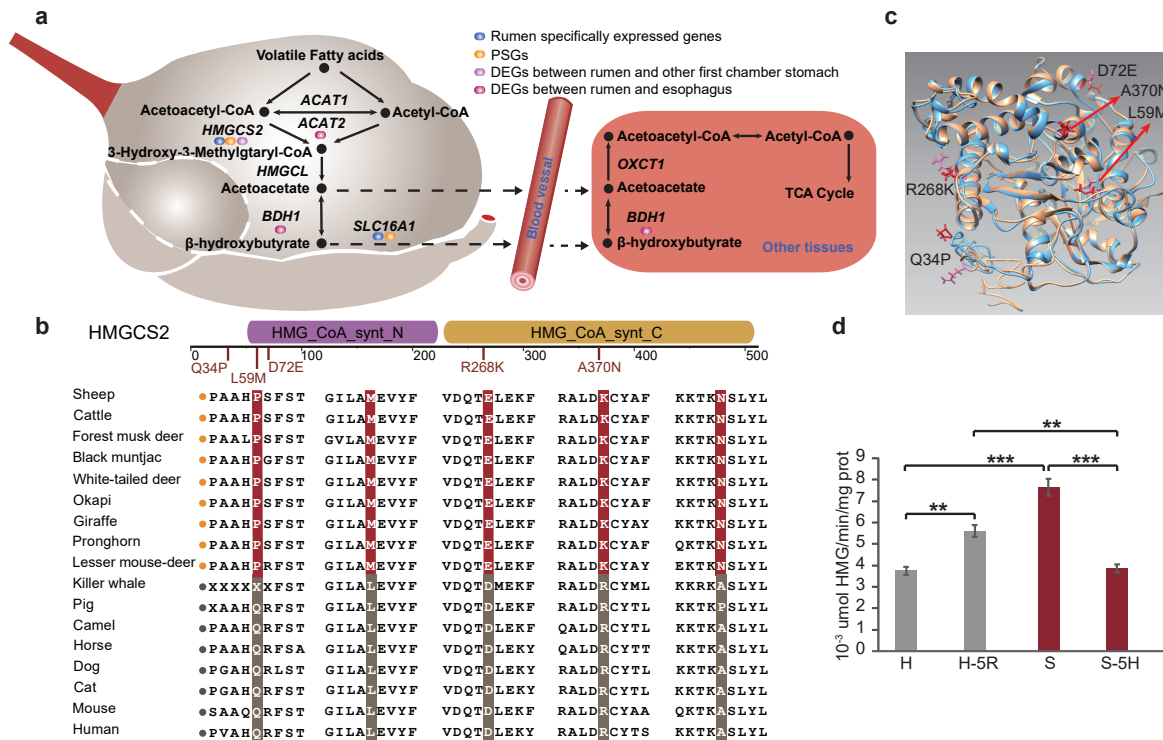
400 showing the pairwise Spearman correlations between sheep tissues(the top triangle), between 14

401 tissues of camels (lower left triangle) and between eight tissues of two cetaceans (lower right

402 triangle). **b,** Heatmap of differentially expressed rumen specifically expressed genes among the

403 rumen and other FC stomachs. The color bars on the left present 136 DEGs of the rumen relative to

404 the FC stomach of cetaceans (yellow), 21 DEGs relative to the FC stomach of camels (green), and
405 427 DEGs relative to the FC stomach of both species (purple). The expression levels were
406 normalized by Z-scores. **c**, KEGG pathway analysis of 427 rumen up-regulated DEGs relative to
407 both the FC stomach of camels and cetaceans. **d**, Heatmap showing the gene expression profiles of
408 all 655 rumen specifically expressed genes across 43 tissues of sheep. Different colored lines
409 represent the tissues from which the rumen specifically expressed genes were recruited. Number of
410 genes from each tissue is shown below the tissue name with the percentage of total genes recruited
411 in parentheses.



412

413 **Fig. 3 | Genetic changes in the rumen ketone body metabolism genes and pathways. a, Genes**

414 annotated in the ketone body metabolism are labeled with different color to indicate rumen

415 specifically expressed genes (blue), positively selected genes in ruminant (orange) and

416 differentially expressed genes between rumen and other FC stomachs (purple). The solid arrows

417 represent ketone body metabolism pathways. The dashed arrows indicate the process of material

418 transport from rumen to other tissues. **b, Top panels:** Structural domains of the HMGCS2 protein

419 and the location of the ruminant specific mutations. Lower panel: Peptide sequence alignment of

420 HMGCS2. The species is followed a yellow circle belonging to the ruminant. The red highlighting

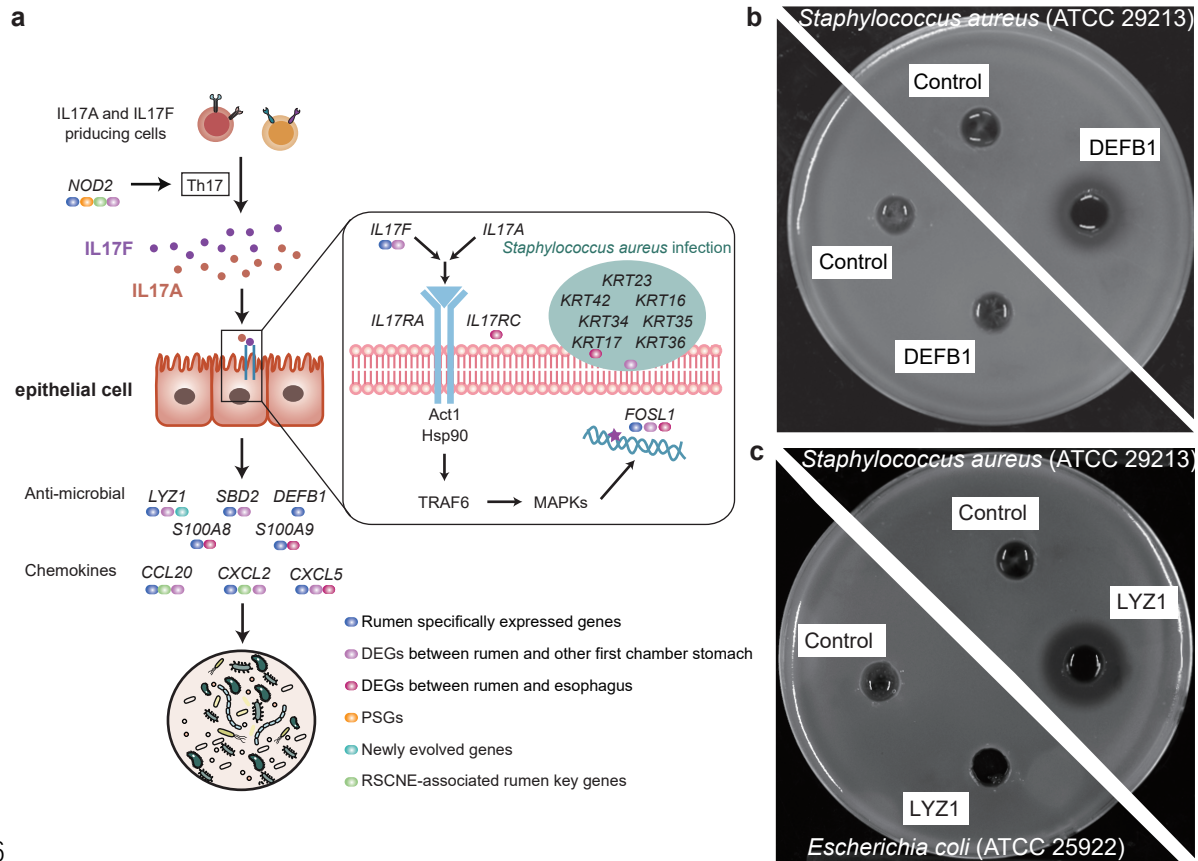
421 indicates ruminant-specific amino acid mutations. **c, Predicted tertiary structures of the HMGCS2**

422 of ruminant (blue) and other mammals (orange), respectively. **d, Enzyme activities of HMGCS2**

423 compared with those of sheep and human in vitro. H: human, H-5R: human HMGCS2 with five

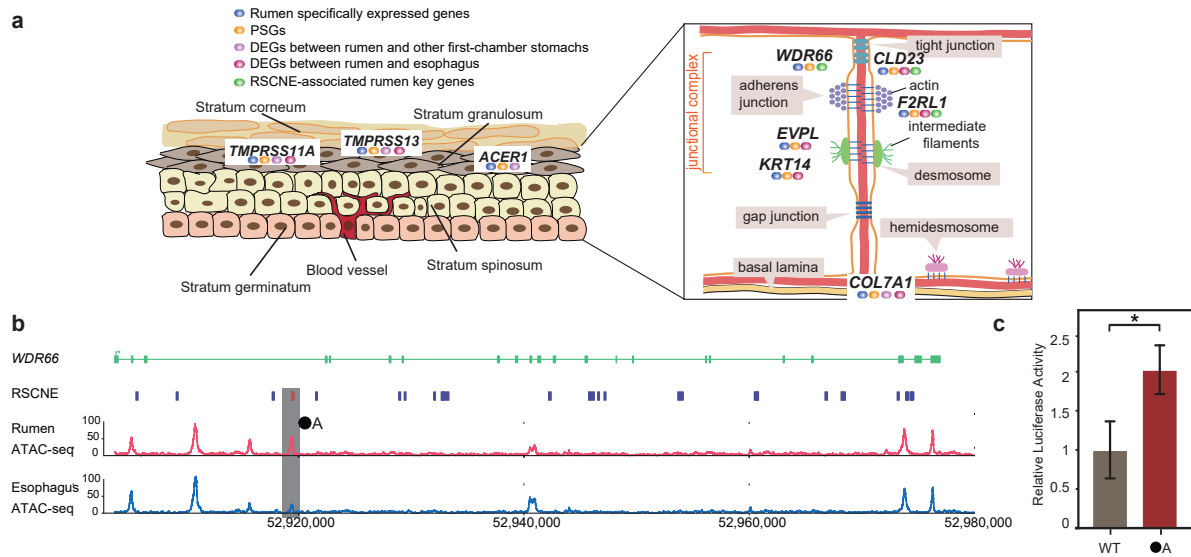
424 ruminant aa replacements, S: sheep, S-5H: sheep HMGCS2 with five human aa replacements. **

425 *p* value < 0.01, *** *p* value < 0.001 calculated from the t test. Data are shown as mean±s.d.



426

427 **Fig. 4 | Microbial management of the rumen.** **a**, Rumen specifically expressed genes (blue),
 428 differentially expressed genes between rumen and other FC stomachs (purple), positively selected
 429 genes in ruminant (orange), differentially expressed genes between rumen and esophagus (red),
 430 newly evolved genes (cyan) and RSCNE-associated rumen key genes (green) involved in IL17
 431 signaling pathway and *Staphylococcus aureus* infection. The antibacterial ability of **(b)**, DEFB1
 432 and **(c)**, LYZ1. Inhibition zone assays on agarose plates with *Escherichia coli* (ATCC 25922) and
 433 *Staphylococcus aureus* (ATCC 29213).



434

435 **Fig. 5 | Genetic changes related to rumen epithelium transportation and absorption. a,**

436 Diagram of rumen epithelial cell proteins involved in epithelium permeability identified in the

437 common ancestor of the ruminants. Rumen specifically expressed genes (blue), positively selected

438 genes in ruminant (orange), differentially expressed genes between rumen and other FC stomachs

439 (purple), differentially expressed genes between rumen and esophagus (red), and

440 RSCNE-associated rumen key genes (green). Note the junction structure (desmosome) between

441 keratinocytes of the ruminal epithelium has been degraded, instead the enlarged intercellular space

442 with copious blood supply enables metabolites absorption in the ruminal epithelium⁴⁷. **b,** Gene

443 structure of *WDR66* based on the NCBI Oar_v4.0 annotation shown above. Green boxes represent

444 exons. Purple bars indicate ruminant-specific conserved non-exonic elements (RSCNEs). Red and

445 blue bars indicate ATAC-seq peaks of the ruminal and esophageal epithelium cell, respectively.

446 The grey rectangle box is the overlapping element of RSCNE and ATAC-seq which is located in

447 the intron region. **c,** The luciferase activity of the pGL3-Promoter (WT) and the pGL3-Promoter

448 with the RSCNE (●A). * p value < 0.05 calculated from the t test. Data are shown as mean \pm s.d.

449 References

- 450 1. Gregory, T. R., The evolution of complex organs. *Evo. Edu. Outreach* **1**, 358-389 (2008).
- 451 2. Land, M. F. & Fernald, R. D., The evolution of eyes. *Annu. Rev. Neurosci.* **15**, 1-29 (1992).
- 452 3. Land, M. F., The optics of animal eyes. *Contemp. Phys.* **29**, 435-455 (1988).
- 453 4. Gallant, J. R. *et al.*, Genomic basis for the convergent evolution of electric organs. *Science* **344**,
- 454 1522-1525 (2014).
- 455 5. Griffith, O. W. & Wagner, G. P., The placenta as a model for understanding the origin and
- 456 evolution of vertebrate organs. *Nat. Ecol. Evol.* **1**, 0072 (2017).
- 457 6. Lynch, V. J., Leclerc, R. D., May, G. & Wagner, G. P., Transposon-mediated rewiring of gene
- 458 regulatory networks contributed to the evolution of pregnancy in mammals. *Nat. Genet.* **43**,
- 459 1154-1159 (2011).
- 460 7. Wang, Y. *et al.*, Genetic basis of ruminant headgear and rapid antler regeneration. *Science* **364**,
- 461 v6335 (2019).
- 462 8. Janis, C., The evolutionary strategy of the equidae and the origins of rumen and cecal digestion.
- 463 *Evolution* **30**, 757-774 (1976).
- 464 9. Dehority, B. A., Gastrointestinal tracts of herbivores, particularly the ruminant: Anatomy,
- 465 physiology and microbial digestion of plants. *J. Appl. Anim. Res.* **21**, 145-160 (2002).
- 466 10. Novacek, M. J., Mammalian phylogeny: Shaking the tree. *Nature* **356**, 121-125 (1992).
- 467 11. Mathiesen, S. D. *et al.*, Digestive physiology of minke whales. *Developments in Marine Biology* **4**,
- 468 351-359 (1995).
- 469 12. Cantalapiedra, J. L. *et al.*, Dietary innovations spurred the diversification of ruminants during the
- 470 Caenozoic. *Proc. Biol. Sci.* **281**, 20132746 (2014).
- 471 13. De Tarso, S. G. D. S., Oliveira, D. & Afonso, J. A. B., Ruminants as part of the global food
- 472 system: How evolutionary adaptation and diversity of the digestive system brought them to the
- 473 future. *J. Dairy Vet. Anim. Res.* **3**, 171-176 (2016).
- 474 14. Zeder, M. A., Domestication and early agriculture in the Mediterranean Basin: Origins, diffusion,
- 475 and impact. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 11597-11604 (2008).
- 476 15. Xiang, R., Oddy, V. H., Archibald, A. L., Vercoe, P. E. & Dalrymple, B. P., Epithelial, metabolic
- 477 and innate immunity transcriptomic signatures differentiating the rumen from other sheep and
- 478 mammalian gastrointestinal tract tissues. *PeerJ* **4**, e1762 (2016).
- 479 16. Millen, D. D., Mario, D. B. A. & Rodrigo, D. L. P., *Rumenology*. (Springer, Switzerland
- 480 , 2016).
- 481 17. Huttner, K. M., Brezinski-Caliguri, D. J., Mahoney, M. M. & Diamond, G., in *Molecular and*
- 482 *Cellular Studies of Rumen Epithelial Metabolism* (1998).
- 483 18. Yu, J. *et al.*, The sheep genome illuminates biology of the rumen and lipid metabolism. *Science*
- 484 **344**, 1164-1168 (2014).
- 485 19. Clark, E. L. *et al.*, A high resolution atlas of gene expression in the domestic sheep (*Ovis aries*).
- 486 *Plos Genet.* **13**, e1006997 (2017).
- 487 20. Stevens, C. E. & Hume, I. D., *Comparative physiology of the vertebrate digestive system*.
- 488 (Cambridge University Press, 2004)
- 489 21. Vallenias, A., Cummings, J. F. & Munnell, J. F., A gross study of the compartmentalized stomach
- 490 of two new-world camelids, the llama and guanaco. *J. Morphol.* **134**, 399-423 (1971).
- 491 22. Tarpley, R. J., Sis, R. F., Albert, T. F., Dalton, L. M. & George, J. C., Observations on the

- 492 anatomy of the stomach and duodenum of the bowhead whale, *Balaena mysticetus*. *Am. J. Anat.*
493 **180**, 295-322 (1987).
- 494 23. Xiong, Z. *et al.*, PAX9 regulates squamous cell differentiation and carcinogenesis in the
495 oro-oesophageal epithelium. *J. Pathol.* **244**, 164-175 (2018).
- 496 24. Newman, J. C. & Verdin, E., Ketone bodies as signaling metabolites. *Trends in Endocrinology &*
497 *Metabolism* **25**, 42-52 (2014).
- 498 25. Thumelin, S., Forestier, M., Girard, J. & Pegorier, J. P., Developmental changes in mitochondrial
499 3-hydroxy-3-methylglutaryl-CoA synthase gene expression in rat liver, intestine and kidney.
500 *Biochem. J.* **292**, 493-496 (1993).
- 501 26. Baldwin, R. L., McLeod, K. R., Klotz, J. L. & Heitmann, R. N., Rumen development, intestinal
502 growth and hepatic metabolism in the pre- and postweaning ruminant. *J. Dairy Sci.* **87**, E55-E65
503 (2004).
- 504 27. Leng, R. A. & Nolan, J. V., Nitrogen metabolism in the rumen. *J. Dairy Sci.* **67**, 1072-1089
505 (1984).
- 506 28. Wardrop, I. D., Some preliminary observations on the histological development of the
507 fore-stomachs of the lamb I. Histological changes due to age in the period from 46 days of foetal
508 life to 77 days of post-natal life. *J. Agric. Sci.* **57**, 335-341 (1961).
- 509 29. Fath, E. M., Schwarz, R. & Ali, A. M., Micromorphological studies on the stomach of sheep
510 during prenatal life. *Anat. Histol. Embryol.* **12**, 139-153 (1983).
- 511 30. Chen, L. *et al.*, Large-scale ruminant genome sequencing provides insights into their evolution
512 and distinct traits. *Science* **364**, (2019).
- 513 31. Gaffen, S. L., An overview of IL-17 function and signaling. *Cytokine* **43**, 402-407 (2008).
- 514 32. van Beelen, A. J. *et al.*, Stimulation of the intracellular bacterial sensor NOD2 programs dendritic
515 cells to promote interleukin-17 production in human memory T cells. *Immunity* **27**, 660-669
516 (2007).
- 517 33. Thornton, J. H. & Owens, F. N., Monensin supplementation and in vivo methane production by
518 steers. *J. Anim. Sci.* **52**, 628-634 (1981).
- 519 34. Duffield, T. F., Merrill, J. K. & Bagg, R. N., Meta-analysis of the effects of monensin in beef
520 cattle on feed efficiency, body weight gain, and dry matter intake. *J. Anim. Sci.* **90**, 4583-4592
521 (2012).
- 522 35. Kyriiotou, M., Huber, M. & Hohl, D., The human epidermal differentiation complex: Cornified
523 envelope precursors, S100 proteins and the 'fused genes' family. *Exp. Dermatol.* **21**, 643-649
524 (2012).
- 525 36. Seo, K. W. *et al.*, Targeted disruption of the DM domain containing transcription factor *Dmrt2*
526 reveals an essential role in somite patterning. *Dev. Biol.* **290**, 200-210 (2006).
- 527 37. Wang, Q., Ma, C. & Kemmner, W., *Wdr66* is a novel marker for risk stratification and involved
528 in epithelial-mesenchymal transition of esophageal squamous cell carcinoma. *Bmc Cancer* **13**,
529 137 (2013).
- 530 38. Risk, J. M. *et al.*, *Envoplakin*, a possible candidate gene for focal NEPPK/esophageal cancer
531 (TOC): The integration of genetic and physical maps of the TOC region on 17q25. *Genomics* **59**,
532 234-242 (1999).
- 533 39. Sheth, J., Mistri, M., Patel, H., Ankleshwaria, C. & Parikh, A., Autosomal dominant mutation in
534 *COL7A1* gene causing epidermolysis bullosa dystrophica. *Mol. Cytogenet.* **7**, P58 (2014).
- 535 40. Bousquet, O. *et al.*, The nonhelical tail domain of keratin 14 promotes filament bundling and

- 536 enhances the mechanical properties of keratin intermediate filaments in vitro. *J. Cell Biol.* **155**,
537 747-754 (2001).
- 538 41. Madsen, D. H., Szabo, R., Molinolo, A. A. & Bugge, T. H., Tmprss13 deficiency impairs
539 stratum corneum formation and epidermal barrier acquisition. *Biochem. J.* **461**, 487-495 (2014).
- 540 42. Gareus, R. *et al.*, Normal epidermal differentiation but impaired skin-barrier formation upon
541 keratinocyte-restricted IKK1 ablation. *Nat. Cell Biol.* **9**, 461-469 (2007).
- 542 43. Alberts, B., Johnson, A. & Lewis, J., *Molecular biology of the cell*. (Garland Pub., 1983).
- 543 44. Mead, J. G., in *Encyclopedia of Marine Mammals (Second Edition)* (Academic Press, 2009).
- 544 45. von Engelhardt, W., Dycker, C. & Lechner-Doll, M., Absorption of short-chain fatty acids,
545 sodium and water from the forestomach of camels. *J. Comp. Physiol. B* **177**, 631-640 (2007).
- 546 46. Kumar, S., Stecher, G., Suleski, M. & Hedges, S. B., TimeTree: A resource for timelines,
547 timetrees, and divergence times. *Mol. Biol. Evol.* **34**, 1812-1819 (2017).
- 548 47. Phillipson, A. T., *Physiology of digestion and metabolism in the ruminant*. (Oriel Press Ltd.,
549 1970).