

1 **Convergent loss of the necroptosis pathway in disparate mammalian
2 lineages shapes virus countermeasures**

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18 Abstract

19 Programmed cell death is a vital process in the life cycle of an organism.
20 Necroptosis, an evolutionary restricted form of programmed necrosis, contributes to the
21 innate immune response by killing pathogen-infected cells. This virus-host interaction
22 pathway is organized around two key components: the receptor-interacting protein kinase
23 3 (RIPK3), which recruits and phosphorylates the mixed lineage kinase-like protein
24 (MLKL), thus inducing cellular plasma membrane rupture and cell death. Critically, the
25 presence of necroptotic inhibitors in viral genomes validates necroptosis as an important
26 host defense mechanism. Here, we show, counterintuitively, that in different mammalian
27 lineages of mammalian, central components of the necroptotic pathway, such as *RIPK3*
28 and *MLKL* genes, are deleted or display inactivating mutations. Frameshifts or premature
29 stop codons are observed in all the studied species of cetaceans and leporids. In
30 carnivores' genomes, the *MLKL* gene is deleted, while in a small number of species from
31 afrotheria and rodentia premature stop codons are observed in *RIPK3* and/or *MLKL*.
32 Interestingly, we also found a strong correlation between the disruption of necroptosis in
33 leporids and cetaceans and the absence of the C-terminal domain of E3-like homologs
34 (responsible for necroptosis inhibition) in their naturally infecting poxviruses. Overall, our
35 study provides the first comprehensive picture of the molecular evolution of necroptosis in
36 mammals. The loss of necroptosis multiple times during mammalian evolution highlights
37 the importance of gene/pathway loss for species adaptation and suggests that necroptosis
38 is not required for normal mammalian development. Moreover, this study highlights a co-
39 evolutionary relationship between poxviruses and their hosts, emphasizing the role of host
40 adaptation in shaping virus evolution.

41 **Introduction**

42 Sensing of viral pathogens by the host cells is critical for animal survival. Thus, a
43 variety of molecular responses, including the induction of inflammatory cytokines,
44 chemokines and interferons, as well as the activation of cell-death pathways that provide
45 clearance of pathogen-infected cells. Although apoptosis has long been considered a
46 critical clearance mechanism to control viral spread, caspase-independent cell death, or
47 programmed necrosis, has recently emerged as an alternative death pathway that
48 dominates under specific conditions (Xia et al. 2020).

49

50 Necroptosis is an inflammatory form of regulated necrosis that acts as an
51 alternative host defense pathway during some viral infections and plays a major role in the
52 killing and removal of pathogen-infected cells (Upton and Chan 2014; Nogusa et al. 2016;
53 Xia et al. 2020). Activation of necroptosis follows an intracellular signaling cascade that is
54 dependent on the receptor-interacting serine/threonine-protein kinase 3 (RIPK3) and its
55 substrate, the mixed lineage kinase like protein (MLKL) downstream of death receptors
56 (DRs) and pattern-recognition receptors (PPRs) (Fig. 1) (Sun et al. 2012; Moerke et al.
57 2019). Several pathway-specific adaptor proteins that contain a RIP homotypic interaction
58 motif (RHIM-domain) can activate RIPK3-induced necroptosis (Fig. 1). For example, when
59 there is an interference or loss of function of caspase-8, the induction of necroptosis
60 through the use of DRs results in the recruitment of RIPK1, which subsequently exposes
61 its RHIM-domain to recruit RIPK3 (He et al. 2009; Zhang et al. 2009; Upton et al. 2010).
62 Apart from RIPK1, the TIR-domain-containing adaptor-inducing IFN β (TRIF), an essential
63 protein downstream of Toll-like receptor (TLR)3/4 and the Z-DNA binding protein (ZBP1),
64 also directly activate RIPK3 (He et al. 2011; Upton et al. 2012) (Fig. 1). Exposure of RIPK3
65 to a RHIM adaptor (RIPK1, TRIF or ZBP1) is a crucial step in the initiation of necroptosis,
66 as these proteins activate the downstream executor of necroptosis MLKL that destabilizes
67 the plasma membrane integrity leading to cell swelling followed by membrane rupture of
68 infected cells and release of damage-associated molecular patterns (DAMPs) (Kaiser et al.
69 2013; Upton and Chan 2014). Thus, necroptosis provides a critical extra defense
70 mechanism against pathogen infection, facilitating the elimination of virus-infected cells
71 before the production of progeny virions. The importance of necroptosis for host defense is
72 further supported by the identification of viral inhibitors of necroptosis, like is the case of
73 Vaccinia virus (VACV) E3 protein and the murine cytomegalovirus (MCMV) M45 protein
74 (Upton et al. 2012; Kaiser et al. 2013).

75

Necroptosis has a major role in protecting cells against viral infection (Upton and Chan 2014; Nogusa et al. 2016; Xia et al. 2020). However, despite the recent advances to understand the molecular regulation of this unique pathway, it is still unclear whether the necroptotic cell death pathway acts as a universal cell death program in mammals. Previous studies suggested that components of necroptosis are absent in the genomes of extant birds and marsupials. Interestingly, within the Mammalia class, it was previously reported that order Carnivora lost the *MLKL* gene (Dondelinger et al. 2016). Taken together these reports suggest some degree of plasticity in the conservation of necroptosis responses. Here, we address the molecular evolution of the necroptotic pathway in multiple mammalian lineages. We show that during mammalian evolution, necroptosis was convergently inactivated several times in mammalian lineages. Remarkably, we also report that mammalian orders that lost the necroptotic pathway display infection episodes by poxviruses that have lost the ability to inhibit this pathway, showing a co-evolutionary relationship between host adaptation in shaping virus evolution.

90 **Results**

91 Necroptosis has a significant role in protecting cells against viral infection (Upton
92 and Chan 2014; Nogusa et al. 2016; Xia et al. 2020). However, despite recent advances to
93 understand the molecular regulation of this unique pathway, it is still unclear whether the
94 necroptotic cell death pathway acts as a universal cell death program in mammals. Here,
95 taking advantage of genomic collection databases and the use of Leporid samples (see
96 Methods section for more information), the goal is to better understand the molecular
97 evolution of the necroptotic pathway in different mammalian lineages.
98

99 **RIPK1 protein is under evolutionary conservation in mammals**

100 Human RIPK1 is a multidomain protein composed of an N-terminal Ser/Thr kinase,
101 a C-terminal death binding domain that mediates binding to DRs and an intermediate
102 domain that includes a K377 ubiquitination site and an RHIM motif that binds to other
103 RHIM-containing proteins (He and Wang 2018). Due to the importance of RIPK1 as an
104 adaptor molecule, previous phylogenetic analysis suggested that RIPK1 probably emerged
105 in the ancestor of vertebrates (Dondelinger et al. 2016). In accordance, our search
106 detected the presence of *RIPK1* homologues in all the studied mammalian lineages (Fig.
107 2A and S Appendix 1, 2 and 3).
108

109 To look for evidence of potential selection pressures acting on the different
110 domains of RIPK1 protein, we used the dataset of mammalian sequences mentioned
111 above and implemented an ML approach, by using the Datammonkey software (see
112 Methods section for more information). For most protein-coding genes, the rate between
113 nonsynonymous and synonymous substitutions (dN/dS) is a measure of natural selection,
114 with positive selection ($dN/dS > 1$) acting against the common genotype (Kosiol et al.
115 2008). In this study, we deduced ten sites that reflect strong positive selection pressure in
116 RIPK1, while more than 200 amino acids were under negative selection (Fig. 2B and S
117 Appendix 4). As seen in Fig. 2B, residues identified as being under positive selection fall
118 within or very close to the kinase domain (4 residues), the RHIM domain (5 residues) and
119 the death domain (1 residue) of RIPK1 (residues under positive selection are marked as
120 red circles). The N-terminal kinase domain is known to present several essential residues
121 for phosphorylation and ubiquitination (Ser14/15, 20, 161 and 166 and Lys115 and 163),
122 regulation of necroptosis and RIPK1-dependent apoptosis (Mifflin et al. 2020).
123 Interestingly, the codons under positive selection fall only at the end of the N-terminal

124 domain. A considerable portion of the negatively selected sites fall in the critical regions for
125 phosphorylation and ubiquitination (S Appendix 4), suggesting that the beginning of the
126 RIPK1 protein is under strong purifying selection. The same was also observed for the
127 rapidly evolving sites of the RHIM domain, which were grouped only at the beginning of
128 this domain (Fig. 2B). It was previously shown that the RHIM domain has a crucial
129 conserved core motif of 12 amino acids that resides at the end of this domain (Sun et al.
130 2002; Li et al. 2012). Indeed, changing four consecutive amino acids to alanine within this
131 core region abrogates interaction between RHIM domains and, as a consequence,
132 necroptosis (Sun et al. 2002; Li et al. 2012). Our findings that the positively selected
133 residues did not overlap with the core motif of 12 amino acids further support the
134 importance of the conservation of this region. Interestingly, in the RIPK1 death domain that
135 is known to mediate homodimerization as well as heterodimerization with other DD-
136 containing proteins, such as FADD, TNFR1 and Fas (Meng et al. 2018; Mifflin et al. 2020),
137 no positively selected sites were found, with most of the domain being under negative
138 pressure (S Appendix 4). Collectively, our results show signatures of positive selection
139 occurring at the end of the N-terminal kinase domain and at the beginning of the RHIM
140 domain of RIPK1 proteins. Interestingly, these residues do not overlap with domains
141 known to be fundamental for RIPK1-dependent apoptosis and necroptosis, suggesting that
142 these domains might be under evolutionary conservation and possibly functional constraint
143 for the studied mammals.

144
145 **Convergent erosion of RIPK3 and MLKL in mammalian lineages**

146 RIPK3 and MLKL form the core of the necroptotic machinery and both are, as a
147 consequence, important for necroptosis induction in mice and humans downstream of
148 PRRs and DRs (Sun et al. 2012; Moerke et al. 2019; Xia et al. 2020). To further
149 understand the evolutionary history of the necroptotic pathway in mammals, we performed
150 detailed sequence and phylogenetic analyses for RIPK3 and MLKL homologous proteins
151 (S Appendix 3). Our screens for *RIPK3* and *MLKL* genes revealed evidence of
152 pseudogenization in five mammalian lineages: order rodentia, lagomorpha, carnivora,
153 cetacean and in the superorder afrotheria (Fig. 2A). Given the fact that *MLKL*
154 pseudogenes have been previously identified in carnivores, they will not be discussed in
155 detail here (Dondelinger et al. 2016).

156

157 **Variation of the Necroptotic pathway within Afrotherian and Rodent families**

158 In afrotherian and rodent lineages, we found that the necroptotic pathway was
159 missing in some species (Fig. 2A). In rodents, two species from the Bathyergidae family
160 and one species from the Octodontidae family presented early stop codons in both RIPK3
161 and MLKL, resulting in the disruption of necroptosis. In the naked mole-rat
162 (*Heterocephalus glaber*), RIPK3 presented a premature stop codon in exon 6 resulting in a
163 shorter version of this protein (S Appendix 5). In the common degu (*Octodon degus*) and
164 in the damaraland mole-rat (*Fukomys damarensis*), both RIPK3 and MLKL proteins
165 presented several premature stop codons (S Appendix 5). However, disruption of these
166 proteins appears to have occurred in an independent way, rather than in a common
167 ancestral. Given the fact that *RIPK3* and *MLKL* present signs of pseudogenization, it is
168 expected that in the naked mole-rat, common degu and damaraland mole-rat necroptosis
169 is disrupted. Our studies also revealed that species from Afrotherian families, including the
170 African bush elephant (*Loxodonta africana*, family Elephantidae) and the Cape golden
171 mole (*Chrysochloris asiatica*, family Chrysochloridae) presented early stop codons in
172 RIPK3 and MLKL, respectively, while the West Indian manatee (*Trichechus manatus*,
173 family Trichechidae) present early stop codons in both genes (S Appendix 5). However,
174 our results also show that the Cape elephant (*Elephantulus edwardii*, family
175 Macroscelididae) and the lesser hedgehog tenrec (*Echinops telfairi*, family Tenrecidae)
176 present intact copies of the *RIPK3* and *MLKL* genes, indicating that RIPK3 and MLKL were
177 present in early stages of Afrotheria evolution, but must have been lost later in specific
178 lineages, resulting in the existence of alternative modes of necroptosis inactivation.

179
180 **The Necroptotic pathway is disrupted in Lagomorphs**

181 The Order Lagomorpha is divided into two families, Ochotonidae and Leporidae,
182 which diverged around 30–35 Mya (Melo-Ferreira et al. 2015). While Ochotonidae is only
183 composed of one extant genus, *Ochotona*, the Leporidae family includes 11 genera,
184 including *Lepus*, *Sylvilagus* and *Oryctolagus* (Ge et al. 2013). Using the methods
185 described previously, we were only able to identify *RIPK3* and *MLKL* transcripts for plateau
186 Pika (*O. curzoniae*), while no *RIPK3* and *MLKL* transcripts were found for the European
187 rabbit (*O. cuniculus*). For the American Pika (*O. princeps*), incomplete genome assemblies
188 in the vicinity of the *RIPK3* and *MLKL* regions made retrieving the sequence of these
189 genes impossible. By evaluating *RIPK3* gene from human and mouse and its genomic
190 context, we were able derive a partial *RIPK3* nucleotide sequence from the European

191 rabbit genome, which displays a frameshift mutation caused by the insertion of a single
192 nucleotide. It is well known that accurately detecting gene-inactivation mutations in these
193 alignments poses a number of challenges like, for example, sequencing errors and cases
194 of assembly incompleteness. For this reason, we assessed the accuracy of our database
195 prediction by sequencing that same genomic region in different Leporid species,
196 representative of different genera (*Lepus*, *Sylvilagus* and *Oryctolagus*). From the obtained
197 results, we confirmed the insertion of 1 nucleotide (+G, exon 3) not only in the European
198 rabbit RIPK3, but also in species from genus *Lepus* and *Sylvilagus*, suggesting that
199 disruption of *RIPK3* gene occurred in a common ancestral and was maintained throughout
200 Leporid evolution (Fig. 3A). During necroptosis, activated RIPK3 phosphorylates and
201 activates MLKL, which results in its recruitment and oligomerization in the plasma
202 membrane leading to rupture and cell death (Sun et al. 2012; Xia et al. 2020).
203 Interestingly, and despite all of our efforts, no *MLKL* transcripts or MLKL protein
204 accumulation were found in any of the studied Lagomorphs (data not shown). Detailed
205 analysis of the upstream and downstream MLKL flanking genes in both human and mouse
206 genomes reveal that MLKL resides between the ring finger and WD repeat domain 3
207 protein (*RFWD3*) and fatty acid 2-hydroxylase (*FA2H*) genes (Fig. 4). Accordingly,
208 although there are no gaps or incomplete genomic assemblies surrounding that region in
209 the European rabbit genome, we were not able to retrieve a complete or partial *MLKL*
210 gene, suggesting once more that this gene is not present in these mammals. Together, our
211 results suggest that the studied leporid species are deficient in the core proteins of the
212 necroptotic pathway, and that RIPK3 inactivation occurred at the stem Leporid branch and
213 was maintained during evolution.

214

215 **Inactivation of necroptosis components in Cetacea**

216 Modern Cetacea comprises Mysticete (or baleen whales) and Odontocete (or
217 toothed whales) and are the most specialized and diversified group of mammals
218 (McGowen et al. 2020). Comparative analysis of cetacean genomes has already provided
219 important insights into the unique cetacean traits and aquatic specializations (Sharma et
220 al. 2018; Huelsmann et al. 2019; Kawasaki et al. 2020). For our screen, Odontocetes were
221 represented by 12 species belonging to five different families (Delphinidae, Phocoenidae,
222 Monodontidae, Lipotidae and Physeteridae), and Mysticetes were represented by the
223 common minke whale from Balaenopteridae family. In Cetacean species, the disruption of
224 *RIPK3* occurred at different positions depending on the studied species: a frameshift

225 mutation was identified in exon 6 of two Delphinidae species, as well as in exon 8 of two
226 Phocoenidae species, one Monodontidae species and one Balaenopteridae species.
227 There was also evidence of two species (one species from Monodontidae and another
228 from Lipotidae families) with RIPK3 pseudogenes based on the presence of a stop codon
229 located in exon 2 (S Appendix 6). Interestingly, while Cetacean RIPK3 inactivation appears
230 to be a result of different mutations depending on the studied species, our results show a
231 shared mutation in the exon 1 of *MLKL* in all cetacean species (Fig. 3B). Moreover, this
232 premature stop codon leads to the absence of exon 2, 3, 4 and 5 in most cetacean
233 species, which very likely results in this gene inactivation. Given the presence of an
234 inactivating mutation that is shared between mysticetes and odontocetes, the most
235 parsimonious hypothesis suggests that they occurred before the split of these two clades
236 in the common ancestral branch of Cetacea.

237

238 **Diversity among the poxvirus encoded E3-like necroptosis antagonists**

239 Previously, it was shown that the N-terminus of VACV E3 competes with ZBP1 for
240 binding to virus-induced Z-nucleic acid, being a key component to inhibit the action of IFN
241 and induction of necroptosis (Koehler et al. 2017) (Fig. 1). E3-like encoded proteins are
242 composed of a carboxy (C)-terminal double-stranded RNA binding domain (dsRNA-BD)
243 and an amino (N)-terminal Z-nucleic acid-binding domain (zNA-BD) (Kim et al. 2003; Kim
244 et al. 2004) (Fig. 5A). Given the importance of the N-terminus region from VACV E3
245 protein against virus infection, we hypothesize that poxviruses that lack this region in their
246 E3 homologs can still successfully replicate in their natural host because of a
247 compromised necroptotic pathway. Among the E3L proteins, E3 from VACV is the best
248 studied protein. However, E3 homologs can be found in orthopoxviruses, clade II
249 poxviruses and parapoxviruses (Kim et al. 2003; Kim et al. 2004). Recently, the genome
250 characterization of CePV-TA identified two novel E3L homologs: CePV-TA-20 and CePV-
251 TA-21 (Rodrigues et al. 2020).

252

253 Our analysis on 11 different E3 homologues revealed that these are highly
254 divergent: while CPXV 069 (Cowpoxvirus E3 homolog) and TATV 060 (Tateropoxvirus E3
255 homolog) presented identities of >90% to VACV E3, E3L homologs from poxviruses like
256 the Deerpoxvirus, Sheppoxvirus and Yaba monkey poxvirus presented less than 40%
257 identity (Fig. 5B). Analysis of the two newly identified E3 homologs from CePV-TA shows
258 that both present low identity to VACV E3, with CePV-TA-20 and CePV-TA-21 proteins

259 only presenting sequence identity of 37 % and 34 %, respectively (Fig. 5B). It is known
260 that at the amino acid level, the C-terminal of E3-like proteins display a higher level of
261 sequence similarity than the N-terminal domain (Rahman and McFadden 2020).
262 Accordingly, the dsRNA-BD domain from CePV-TA-20 and CePV-TA-21 proteins also
263 display a higher level of sequence similarity compared to other E3 homologs (Fig. 5C),
264 suggesting that in CeTV this domain might also target conserved antiviral dsRNA-activated
265 pathways. Similar to what is observed for MPXV and MYXV E3 homologues, both E3
266 homologs from CePV-TA present incomplete or disrupted N-terminal zNA-BDs. As shown
267 in Fig. 5C, CePV-TA-20 is missing 20 amino acids in its N-terminal domain. However, this
268 region still retains the conserved LY and PPXW motifs, as well the basic KKCINR motif
269 (Fig. 5C), residues known to contact with Z-NA (Kim et al. 2003). Interestingly, MPXV F3
270 protein, also lacking 37 amino acids from the N-terminal domain, is not able to compete
271 with ZBP1 and inhibit sensing (unpublished data), even though it retains the key residues
272 important for binding to Z-NA (Fig. 5C). While CePV-TA-20 and F3 proteins contain an
273 incomplete zNA-BD, M029 and CePV-TA-21 proteins are missing most of their N-terminal
274 zNA-BD (Fig. 5B and C), suggesting a total inactivation of this domain and a loss of
275 function regarding the inhibition of ZBP1-dependent necroptosis. Overall, our results show
276 that the novel CePV-TA presents two E3L homologues that, like E3L homologues from
277 MPXV and MYXV, present incomplete or disrupted N-terminal zNA-BD. The presence of
278 an incomplete or disrupted zNA-BD in E3L homologues highly suggests that these
279 proteins cannot fully compete with ZBP1 to inhibit necroptosis induction. However, further
280 studies will be necessary to fully comprehend the action of these proteins regarding
281 complete necroptosis inhibition.

282 **Discussion**

283 Necroptosis is an inflammatory form of cell death that is mediated by RIPK3 and
284 MLKL and provides an extra defense mechanism against pathogen infection, facilitating
285 the elimination of virus-infected cells before the production of progeny virions (Upton and
286 Chan 2014; Nogusa et al. 2016; Xia et al. 2020). Given the crucial role of necroptosis in
287 the innate immune response of humans and mice (Orzalli and Kagan 2017; Nailwal and
288 Chan 2019), it was broadly accepted that this pathway was ubiquitous in mammals. Our
289 results from 67 species across nine mammalian lineages provides the first comprehensive
290 picture of the molecular evolution of necroptosis in mammals. We show that while RIPK1
291 is under evolutionary conservation, RIPK3 and MLKL are poorly conserved in lineages that
292 evolved separately over the course of evolution.

293

294 A detailed analysis of RIPK3 and MLKL in mammals reveals a complex pattern
295 where lagomorphs, cetaceans, carnivores and species from rodent and afrotheria lineages
296 separately lost key components of the necroptotic pathway (Fig. 2A). The order
297 lagomorpha includes two big families, Ochotonidae and Leporidae (Melo-Ferreira et al.
298 2015). The presence of the same frameshift mutation in Leporid species (+G, Fig. 3A)
299 suggests that the disruption of the necroptotic pathway occurred early, but only after the
300 bifurcation between Ochotona and Leporid given that the plateau Pika presents intact
301 RIPK3 and MLKL proteins (Fig. 2A). Despite all efforts, and despite the complete genome
302 assembly surrounding the MLKL flanking genes in the European rabbit genome, no partial
303 or complete MLKL gene was found, indicating that this gene is deleted in the European
304 rabbit genome and possibly in the remaining Leporid species (Fig. 4). The core genes of
305 the necroptotic pathway also presented premature stop codons in cetaceans. In the
306 studied cetaceans, the MLKL gene presented a common stop codon in the first exon,
307 resulting in the inactivation of this gene (Fig. 3B). Again, the presence of similar patterns of
308 pseudogenization in RIPK3 or MLKL genes within species of the same order infer that
309 disruption of these genes occurred before their diversification and was maintained
310 throughout evolution. On the other hand, *RIPK3* disruption in cetaceans appears to be the
311 result of insertions or deletions that are not shared between closely related species, but
312 rather specific to each species (S Appendix 6), suggesting that these disrupting mutations
313 occurred later in evolution when compared to MLKL. The addition of some rodents as well
314 as afrotheria species to the list of mammals that have disrupted necroptotic pathways,
315 raises the possibility that other closely related species might have lost this pathway after

316 the diversification of these lineages. It is currently believed that activation of the RIPK3 and
317 recruitment of MLKL are critical steps during necroptosis. For example, deleting either
318 RIPK3 or MLKL can lead to the suppression of skin and liver inflammation in mice
319 (Dannappel et al. 2014; Rickard et al. 2014). Moreover, when mice are treated
320 intravenously with a high-doses of TNF, there appears to be no differences between
321 RIPK3-deficient and MLKL-deficient mice (Moerke et al. 2019), substantiating the premise
322 that MLKL follows RIPK3 in the necroptotic signalling. However, this appears not to be the
323 case for all necroptotic cell death responses, as different studies revealed alternative
324 pathways for MLKL and RIPK3-dependent programmed necrosis that are executed in the
325 absence of RIPK3 or MLKL, respectively (Günther et al. 2016; Zhang et al. 2016). To date,
326 there are no studies suggesting that RIPK3/MLKL double-knockout mice are still able to
327 induce necroptosis, which indicates that species that have disrupted RIPK3 and MLKL lost
328 the necroptotic pathway throughout evolution.
329

330 The loss of function of *RIPK3* and *MLKL* in independently evolving lineages
331 (convergent evolution) indicates that gene loss is an important evolutionary mechanism for
332 phenotypic change in these animals and may contribute to similar adaptations. Even
333 though it would be expected that loss of genes is maladaptive, gene loss can be beneficial
334 by providing an evolutionary mechanism for adaptations. In fact, if the loss of an existing
335 gene would increase fitness by making a species better adapted to the environment that
336 surrounds it, then gene loss would be an easy solution to an evolutionary problem.
337 Necroptosis contributes to innate immunity as a pathogen clearance mechanism (Xia et al.
338 2020). However, contrary to apoptosis, in which several highly immunogenic intracellular
339 proteins are sequestered in the dead cell, necroptosis releases DAMPs in the surrounding
340 tissue that promote strong inflammatory responses and result in the attraction of different
341 types of immune cells to the site of infection (Kaczmarek et al. 2013). Studies in mouse
342 models have provided strong evidence that necroptosis is implicated in several
343 inflammatory neurodegenerative diseases, including multiple sclerosis and amyotrophic
344 lateral sclerosis (Ofengeim et al. 2015; Ito et al. 2016). Mouse-model experiments
345 identified keratinocyte necroptosis as a trigger of skin inflammation (Bonnet et al. 2011)
346 and a correlation between necroptosis and intestinal inflammation has also been
347 established (Welz et al. 2011; Pierdomenico et al. 2014). Thus, while necroptosis might
348 mediate host defense, its inhibition in certain contexts may lessen disease severity. It is
349 known that excessive inflammation can promote cancer cell growth and metastasis

350 (Najafov et al. 2017). Thus, it is possible that a pro-inflammatory cell death like necroptosis
351 might promote metastasis and thus, inhibition of this pathway might represent an
352 advantage for regulation of cancer cell growth. Intriguingly, some of the species that are
353 lacking the core necroptotic machinery are known to resist cancer. That is the case for
354 cetaceans, the naked mole-rat and african elephants (Liang et al. 2010; Abegglen et al.
355 2015; Tejada-Martinez et al. 2021). It is also possible that selection against necroptosis in
356 different mammalian lineages could have been driven by different factors depending on
357 the environment or conditions. For example, it was previously suggested that the absence
358 of MLKL in Carnivores reflected a microbe-rich and virus-containing diet of raw meat,
359 causing evolutionary counter-selection against necroptosis (Dondelinger et al. 2016).
360 Nevertheless, the absence of the necroptotic pathway in independently evolving lineages
361 suggest that the deregulation of this pathway was detrimental for the host organism, which
362 ultimately drove selection against the presence of RIPK3 and MLKL.

363

364 As many other viruses, poxviruses express immunomodulatory and host-range
365 factors important for the suppression and evasion of the host innate and adaptive antiviral
366 responses (Werden et al. 2008; Oliveira et al. 2017). VACV protein E3 not only sequester
367 dsRNA through their dsRNA-BD limiting the activation of the innate immune system
368 against the virus infection, but also inhibit the IFN-induced dsRNA dependent protein
369 kinase (PKR), known to be a crucial component of the host innate immunity against viral
370 infection, replication, and spread (Davies et al. 1993; Sharp et al. 1998). Our results show
371 that the dsRNA-BD of distant E3L proteins present high levels of sequence similarity (Fig.
372 5B and C), which is consistent with the ability of this domain to target conserved pathways
373 present in different hosts. Although the dsRNA sequestration functions of the E3 C-
374 terminal have been clear for decades (Chang and Jacobs 1993; Thompson et al. 1994),
375 the IFN sensitivity of VACV E3 N-terminal deletion mutants remained unresolved for a long
376 time. Recently, strong evidence showed that the E3 N-terminal domain competes with
377 ZBP1 to prevent ZBP1-dependent activation of RIPK3 and consequent necroptosis
378 (Koehler et al. 2017; Koehler et al. 2020). The model proposed by the authors suggests
379 that during WT-VACV infection, the zNA-BD of E3 binds to VACV-induced Z-form nucleic
380 acid and masks it, preventing sensing by ZBP1 and further RIPK3 necroptosis induction
381 (Koehler et al. 2017; Koehler et al. 2020). However, it is interesting that poxviruses like
382 MPXV, MYXV and CePV-TA have E3L homologs that present a complete dsRNA-BD but
383 not zNA-BD (Fig. 5B). In VACV-E3 Δ83N–infected cells (mutant lacking the first 83 aa

384 corresponding to the zNA-BD), the absence of the zNA-BD facilitates ZBP1 to sense
385 VACV-induced PAMPs and initiate necroptosis induction (Koehler et al. 2017). Therefore,
386 it is expected that E3L homologues that lack N-terminal zNA domains, like CePV-TA-21
387 and M029, cannot prevent Z-form nucleic acid sensing triggering necroptosis induction and
388 early abortion of viral replication. Like CePV-TA-20, F3 protein is also missing several
389 amino acids in the N-terminal region and presents high conservation in areas that are
390 known to bind to zNA (Fig. 5C). Nevertheless, F3 protein seems to have lost the ability to
391 compete with ZBP1 and inhibit sensing (unpublished data). It was previously shown that
392 the N-terminus of VACV E3 is necessary for IFN resistance in JC cells since VACV-
393 E3Δ37N (mutant mimicking MPXV E3 zNA-BD) did not initiate DNA replication (Arndt et al.
394 2015). However, MPXV was able to replicate efficiently in the same cells, despite having a
395 partial N-terminal zNA-BD, suggesting that the predicted binding to z-form nucleic acid
396 was intragenic and downstream of z-NA sensing, rather than related to the ability of F3
397 zNA-BD to mask z-form nucleic acid (Arndt et al. 2015).

398

399 Interestingly, inactivation of necroptosis in Lagomorphs and Cetaceans seems to
400 correlate with the absence of the E3L zNA-BD in their naturally infecting poxviruses,
401 namely leporipoxviruses (MYXV and SFV) and cetaceanpoxviruses (CePV-TA),
402 respectively. Monkeypox is a viral zoonosis endemic to central and western Africa areas
403 where African rope squirrels and other rodents are likely reservoir hosts (Essbauer et al.
404 2010). Interestingly, the absence of a functional N-terminus in MPXV F3 protein also
405 seems to correlate with the fact that some rodents appear incapable of undergoing
406 necroptosis. Like MPXV, leporipoxviruses and CePV-TA pathogenesis are restricted to
407 only certain species and have little or no pathogenesis capability in all others (Rahman et
408 al. 2013; Arndt et al. 2015; Rodrigues et al. 2020). Infection of the same host over
409 hundreds of years or even millennia may drive the evolution of each virus to rapidly evolve
410 to a fitness peak in a given host environment. Previous niche-filling models (Holt 2009;
411 Cooper et al. 2010; Simmonds et al. 2019) emphasize the role of host interactions in
412 shaping virus evolution. According to these models, as hosts diversify and speciate over
413 longer evolutionary periods, viral host factors that aim to counter the host antiviral
414 functions are subject to continuous changes. Indeed, it is known that genes associated
415 with host antiviral mechanisms present high evolutionary rates and are often under
416 positive selection (Münk et al. 2012; van der Lee et al. 2017; Águeda-Pinto, Lemos de
417 Matos, et al. 2019). Here, we suggest that during the evolution of these poxviruses, the

418 loss of the zNA-BD did not present a disadvantage in the host organism; therefore, this
419 trait was maintained, which reflects how these viruses adapt as their niche changed.

420

421 **Concluding remarks**

422 The disruption of necroptosis in independently evolving lineages suggests a
423 convergent evolutionary loss of this pathway, probably reflecting an important selective
424 mechanism for phenotypic change. Interestingly, we also found a strong correlation
425 between the disruption of necroptosis in leporids and cetaceans and the absence of the
426 E3L zNA-BD (responsible for necroptosis inhibition) in their naturally infecting poxviruses
427 as in the case of MYXV and CePV-TA, respectively. Overall, our study provides the first
428 comprehensive picture of the molecular evolution of necroptosis in mammals, highlighting
429 the importance of gene/pathway loss for the process of species adaptation and suggesting
430 that it is a true pathogen-response pathway that is not required for normal mammalian
431 development. Moreover, this study sheds some light on a co-evolutionary relationship
432 between poxviruses and their hosts, emphasizing the role of host adaptation in shaping
433 virus evolution.

434 **Materials and Methods**

435 **Genomic approach to detect genes associated with the necroptosis pathway**

436 To detect intact and inactivated genes, we first identified the key genes of the
437 necroptotic pathway (i.e., *RIPK1*, *RIPK3* and *MLKL*) in the human (*H. sapiens*) and mouse
438 (*Mus musculus*) reference genomes and looked for the presence of orthologues in existing
439 genome sequence databases from 67 different species that belong to the 9 main
440 mammalian orders/superorders: primates, rodents, lagomorpha, chiroptera, carnivora,
441 perissodactyla, artiodactyla, cetacea, and afrotheria (S Appendix 1). We did not only
442 search for the complete loss of exons or entire genes, but also searched for insertions and
443 deletions that shift the reading frame, frame-preserving insertions that create a premature
444 stop codon and substitutions that create an in-frame stop codon. The respective search
445 methodology had been previously applied to the identification of different homologues of
446 different annotated mammalian genomes (Sharma et al. 2018; Águeda-Pinto, Castro, et al.
447 2019). To further ensure that all gene loss events discussed in this study are real and not
448 due to sequencing errors, we validated them either by sequencing of samples or by using
449 a curated bioinformatic pipeline (see below).

450

451 **Amplification and sequencing of *RIPK1* and *RIPK3* nucleic acid sequences from
452 Lagomorpha species**

453 In contrast to the majority of mammalian orders, lagomorpha only presents three
454 annotated genomes: the European rabbit (*Oryctolagus cuniculus*, accession #
455 GCA_000003625.1), the American Pika genome (*Ochotona princeps*, accession #
456 GCA_014633375.1) and the plateau pika (*O. curzoniae*, accession # GCA_017591425.1).
457 Given the importance of lagomorphs for this study, samples from different lagomorpha
458 species were used to obtain the nucleic coding sequence from *RIPK1*. For that, RNA was
459 extracted from tissues of *O. cuniculus cuniculus*, *O. cuniculus algirus*, *Lepus americanus*,
460 *L. europaeus*, *L. timidus*, *L. granatensis*, *Sylvilagus floridanus*, *S. bachmanis*, *O. princeps*
461 and *O. collaris* samples, using the Qiagen DNeasy Blood & Tissue kit (Qiagen, USA)
462 following manufacturer's instructions. Synthesis of cDNA was achieved by using
463 SuperScript III Reverse Transcriptase (Invitrogen, USA). Primers were designed according
464 to the *RIPK1* transcript from *O. cuniculus* [Accession # XM_017350509.1] (Forward 5'-
465 ATGTCTTGATGACATTAAATG-3' and Reverse 5'-CTACTTCTGGCTGAGCTGTATC-
466 3') and used to amplify the samples mentioned before. Phusion® High-Fidelity DNA
467 Polymerase (Finnzymes, Espoo, Finland) was used in the PCR amplification and the

468 conditions included an initial denaturation (98°C for 3min), 35 cycles of denaturation (98°C
469 for 30s), annealing (60°C for 15s) and extension (72°C for 30s) followed a final extension
470 (72°C for 5 min).

471

472 From our initial search, the *RIPK3* gene was not annotated in the European rabbit.
473 However, after mapping the location of RIPK3 based on its location in *H. sapiens*, *M.*
474 *musculus* and *O. curzoniae*, we were able to identify a partial RIPK3 sequence in the
475 European rabbit genome that presented an early stop codon. To exclude potential artifacts
476 that can mimic real gene-inactivating mutations, a forward (5'-
477 ATGTCTTCTGTCAAATTGTGG-3') and a reverse (5'-ACTGCCTGCATCAGGATC-3')
478 primer were designed based on the parcial *RIPK3* sequence and were used to amplify the
479 same region in the genomes from *O. cuniculus cuniculus*, *S. floridanus*, *L. americanus*, *L.*
480 *europaeus* and *L. saxatilis*. For that, genomic DNA was extracted using the Qiagen
481 DNeasy Blood & Tissue kit (Qiagen, USA) according to the manufacturer's instructions.
482 Phusion® High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) was used in the
483 PCR amplification and the conditions included an initial denaturation (98°C for 3min), 9
484 cycles of denaturation (98°C for 30s), annealing (66°C for 15s) and extension (72°C for
485 30s) followed by more 25 cycles of denaturation (98°C for 30s), annealing (61°C for 15s)
486 and extension (72°C for 30s) and a final extension (72°C for 5min).

487

488 Amplicons sequencing from RIPK1 and RIPK3 was performed with the ABI PRISM
489 BigDye Terminator v3.1 Cycle Sequencing Kit and according to manufacturer's protocol;
490 reactions were cleaned with Sephadex™ (GE Healthcare Life Sciences, UK) and applied
491 on an ABI PRISM 310 Genetic Analyser (Life Technologies, Applied Biosystems,
492 Carlsbad, CA, USA). The obtained RIPK1 coding sequences and the partial RIPK3
493 sequences from the different Lagomorphs have been deposited in the GenBank database
494 under the accession numbers that are shown in S Appendix 2. All samples were supplied
495 by CIBIO/InBIO, Vairão, Portugal and used in previous studies (de Matos et al. 2011;
496 Águeda-Pinto, Lemos de Matos, et al. 2019). No animals were captured, handled, or killed
497 specifically for the purpose of this study.

498

499 **Detailed analysis on the cetacean genomes**

500 Briefly, NCBI gene annotations for the gene orthologues of *MLKL* and *RIPK3* were
501 initially screened via PseudoChecker (pseudochecker.ciimar.up.pt), which evaluates the

502 coding condition of a gene (Ranwez et al. 2018; Alves et al. 2020). For each gene, a
503 PseudoChecker analysis was run (default parameters), using the *Bos taurus* (cow) gene
504 orthologue as a comparative input (NCBI Accession ID regarding cow MLKL:
505 XM_002694707.6; RIPK3: XM_024997365.1), as well as the genomic sequences
506 encompassing the putative ORF of the orthologous counterpart of each target species,
507 directly exported from the NCBI genome browser. Through PseudolIndex, a built-in
508 assistant metric, we quickly assessed the erosion status of the tested genes on a discrete
509 scale ranging from 0 (coding) to 5 (pseudogenized) (Alves et al. 2020). Subsequent
510 manual annotation was performed by importing the previously collected genomic
511 sequences into Geneious Prime 2020 software (www.geneious.com) (Kearse et al. 2012)
512 and determining each gene's CDS using as reference cow's *MLKL* and *RIPK3* orthologues
513 sequences. In detail, per gene and species, using the built-in map to reference tool
514 (highest sensitivity parameter selected), each (3' and 5' untranslated region-flanked)
515 reference coding-exon was mapped against each target genomic sequence. Exons
516 alignments were further screened for gene disruptive mutations, including in-frame
517 premature stop codons, frameshift, and splice site mutations (any deviation from the
518 consensus donor splice site GT/GC or the consensus acceptor splice site AG).

519

520 To inspect if the identified genetic lesions were not rendered as result of
521 sequencing and/or genome assembly artifacts, we performed mutational validation (one
522 per gene and species), resorting of raw genomic sequencing reads, retrieved from two
523 independent genomic projects from the NCBI sequence read archive (SRA), when
524 available. Explicitly, blastn searches were directed to the selected SRA projects, using the
525 nucleotide sequence portion containing the selected mutation(s) as a query. The matching
526 sequencing reads were downloaded into Geneious Prime 2020 (Kearse et al. 2012)
527 software and mapped against the manually annotated mutation (highest sensibility
528 parameter selected), confirming, or not, the presence of the identified mutation.

529

530 **Phylogenetic and molecular evolutionary analyses**

531 The complete dataset of RIPK1, RIPK3 and MLKL proteins was aligned in BioEdit
532 Sequence Alignment Editor using Clustal W (Thompson et al. 1994), followed by manual
533 corrections when necessary. Amino acid alignments were then used to infer Maximum
534 Likelihood (ML) phylogenetic trees using MEGA X (Kumar et al. 2018), with the

535 substitution models JTT+G+F, JTT+G and HKY+G+I, respectively; determined using
536 ProtTest (Darriba et al. 2011).

537
538 Given the fact that RIPK3 and MLKL proteins are highly divergent across the
539 studied mammalian species, we decided not to perform any evolutionary analysis using
540 these alignments. To look for signatures of natural selection operating in the RIPK1
541 alignment, we used HyPhy software implemented in the Datammonkey Web server (Pond
542 and Frost 2005), to detect codons under selection: the Single Likelihood Ancestor
543 Counting (SLAC) model, the Fixed Effect Likelihood (FEL) method (Kosakovsky Pond and
544 Frost 2005), the Random Effect Likelihood, the Mixed Effects Model of Evolution (MEME)
545 (Murrell et al. 2012) and Fast Unbiased Bayesian AppRoximation (FUBAR) (Murrell et al.
546 2013) methods were used. To avoid a high false positive rate, codons with p-values <0.05
547 for SLAC, FEL and MEME models and a posterior probability >0.95 for FUBAR were
548 accepted as candidates for selection. For a more conservative approach, only residues
549 identified as being under positive selection in three or more ML methods were considered.

550
551 **Analysis of VACV E3 homologues**

552 VACV E3 homologues encoded by different poxviruses (=11) were retrieved from
553 the NCBI database (<https://www.ncbi.nlm.nih.gov/>) and aligned in the BioEdit Sequence
554 Alignment Editor using Clustal W (Thompson et al. 1994), followed by manual corrections
555 when necessary. Amino acid alignments of the representative E3-like proteins were used
556 to generate schematic diagrams using the COBALT program from the NCBI database.

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769 **Figure captions**

770 **Fig. 1 The necroptosis signaling pathway.** Simplified schematic representation of the
771 necroptosis signaling pathway upon stimulation of the TNFR, TRF3/4 and infection by
772 poxviruses. All of these necroptosis-inducing signals converge on the kinase RIPK3, which
773 is activated through the homotypic interaction with RIPK1 or other RHIM-containing
774 proteins, such as TRIF and DAI. When the activity of caspase-8 is inhibited, binding of
775 TNF to TNFR1 leads to the phosphorylation and activation of RIPK1 that binds to RIPK3
776 through their RHIM domains to form a protein complex (necrosome). Activated RIPK3
777 recruits MLKL that oligomerizes and translocates to the plasma membrane to cause
778 necroptosis. In TLR3- and TLR4-induced necroptosis, TRIF is required for the activation of
779 RIPK3. ZBP1 is required for the activation of RIPK3 in response to the presence of Z-form
780 nucleic acids. In VACV-infected cells, the poxviral E3 protein binds to VACV-induced Z-
781 form nucleic acid, preventing RIPK3-induced necroptosis. Abbreviations: TNFR, tumor
782 necrosis factor receptor; TLR 3/4, toll-like receptor; TRIF, TIR-domain-containing adaptor-
783 inducing IFN β ; RIP, receptor-interacting protein kinase; ZBP1, Z-DNA binding protein;
784 MLKL, mixed-lineage kinase domain like.

785 **Fig. 2 Evolution of RIPK1, RIPK3 and MLKL in different mammalian lineages. A)**
786 Phylogenetic tree showing the independent lineages that lost necroptotic core proteins
787 (RIPK3 and MLKL) during evolution. Green circles represent genes that are present in the
788 studied species, red circles represent genes that are disrupted, yellow circles represent
789 genes that have incomplete assemblies and grey rectangles indicate that genes were not
790 found in those species genomes. **B)** A schematic diagram of RIPK1, RIPK3 and MLKL
791 domains. RIPK1 contains an N-terminal kinase domain (KD), an intermediate domain with
792 a RIP homotypic interaction motif (RHIM), and a C-terminal death domain (DD). The
793 phosphorylation and ubiquitination sites are indicated above the RIPK1 domains. Red
794 circles represent residues that are under positive selection. RIPK3 contains a KD and a
795 RHIM domain. MLKL is composed of an N-terminal bundle four-helix bundle (4HD) domain
796 that is regulated by the C-terminal pseudokinase domain (PKD).

797 **Fig. 3 Loss of RIPK3 and MLKL genes in the steam lineage of Leporids and**
798 **Cetaceans. A)** Genomic analysis of the first tree exons from Leporids (marked as dark
799 green). In Leporids, RIPK3 was lost as a result of a shared insertion (+G) in the third exon
800 that resulted in the appearance of several premature stop codons. **B)** A point mutation (C

801 to T) in all the studied cetacean species indicates that MLKL inactivation occurred in
802 Cetacea steam lineage. Moreover, 9 out of the 11 studied species (excluding *B.*
803 *acutorostrata* and *M. Monoceros*) lost exon 2, 3, 4 and 5 throughout evolution (represented
804 by faint yellow). Premature stop codons are represented by an asterisk (*).

805 **Fig. 4 Gene synteny of the genome regions containing MLKL gene in different**
806 **mammals.** Genomic regions containing the *MLKL* gene or its flanking genes in *H. sapiens*,
807 *M. musculus*, *O. curzoniae*, *O. cuniculus*, *P. vampyrus*, *C. lupus* and *B. taurus*. Horizontal
808 lines indicate chromosome fragments and coloured arrows identify genes and their
809 orientation in the genome. Orthologous genes are indicated in the same colour and their
810 names are indicated above/below. Black arrows indicate the presence of pseudogenes.
811 Abbreviations: RFWD3, ring finger and WD repeat domain 3; MLKL, mixed-lineage kinase
812 domain like; FA2H, fatty acid 2-hydroxylase; GLG1, golgi glycoprotein 1; WDR59, WD
813 repeat domain 59; TMPOP2, thymopoietin pseudogene; GM6014, ubiquitin-40S ribosomal
814 protein S27a pseudogene; LOC788457, translationally-controlled 1 pseudogene.

815 **Fig. 5 Protein sequence alignment of E3L proteins. A)** Schematic diagram of VACV E3
816 protein binding domains: yellow box represents the zNA-BD and blue box represents the
817 dsRNA-BD. The same color scheme is used in B and C. **B)** E3L homologues from 11
818 poxviruses (VACV E3, Cowpoxvirus (CPXV) 069, Tateropoxvirus (TATV) 060, Yaba
819 monkey tumor virus (YMTV) 034, swinepoxvirus (SWPV) 34L, Deerpoxvirus (DPV) 042,
820 Sheeppoxvirus (SPPV) 034, Monkeypoxvirus (MPXV) F3, Myxoma virus (MYXV) M029
821 and Cetaceanpoxvirus (CePV) CePV-TA-20 and 21) were used to perform a schematic
822 alignment using COBALT program from the NCBI platform. Length of each E3L
823 homologue as well as their identity to VACV E3 proteins are shown in the column to the
824 right. **C)** Amino acid sequence comparison of 11 different members of the E3L family
825 including VACV E3, TATV 060, YMTV 034, SWPV 34L, DPV 042, SSPPV 034, MPXV F3
826 and MYXV M029 and CePV-TA 20 and 21. Conserved areas known to bind to zNA are
827 shown in grey boxes.

828 **Supporting information**

829 **S. Appendix 1** Accession numbers for *RIPK1*, *RIPK3* and *MLKL* genes found in different
830 mammalian lineages.

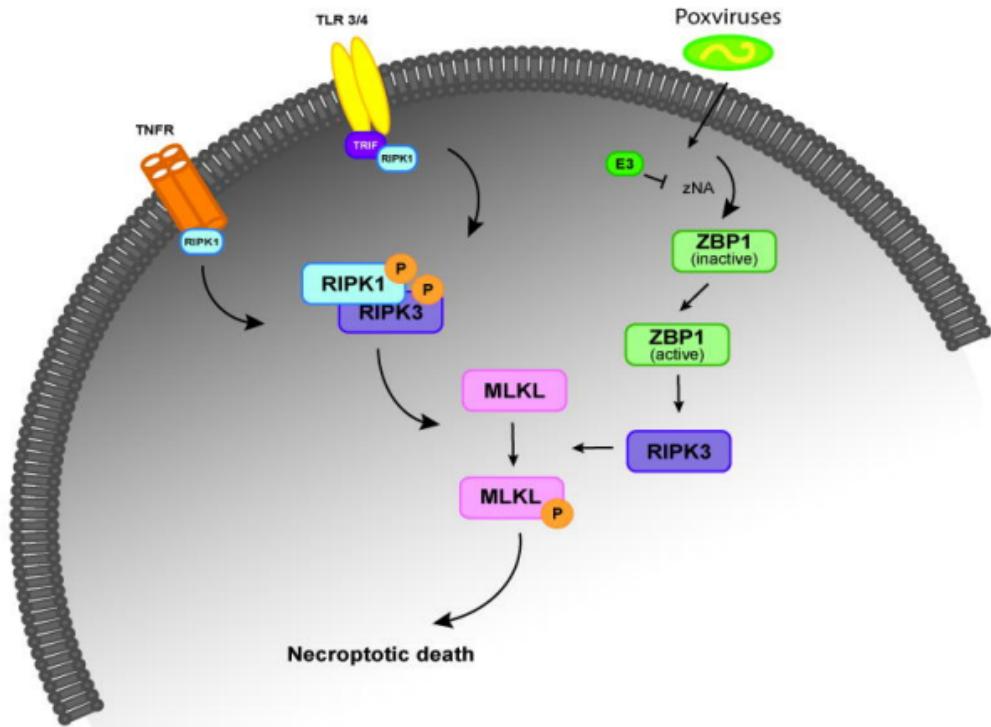
831 **S. Appendix 2** Accession numbers for *RIPK1*, *RIPK3* obtained from different Leporid
832 samples.

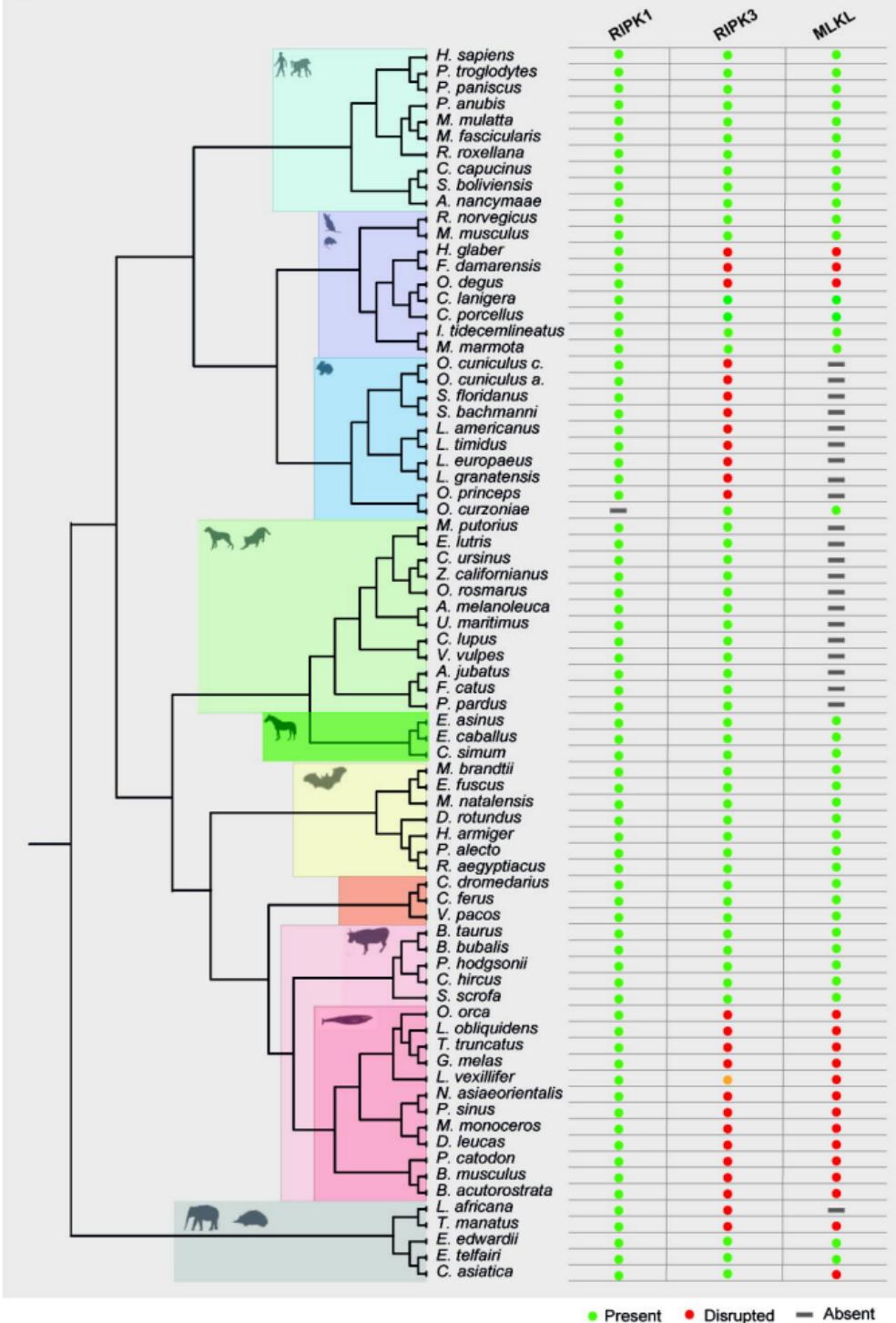
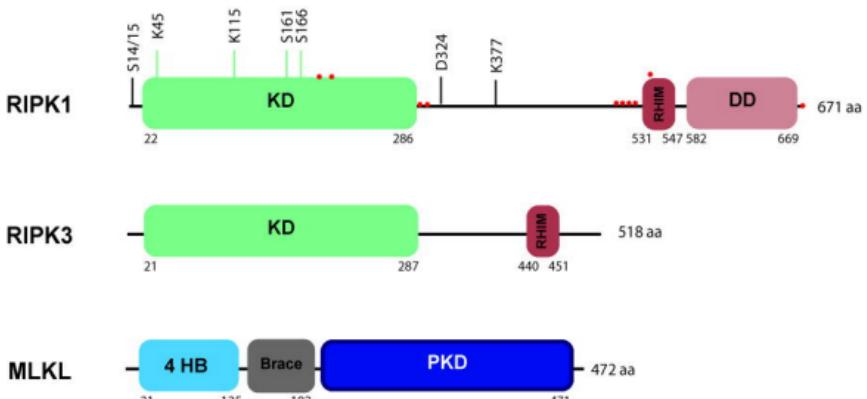
833 **S. Appendix 3** Phylogenetic analysis for RIPK1, RIPK3 and MLKL proteins from different
834 mammalian lineages.

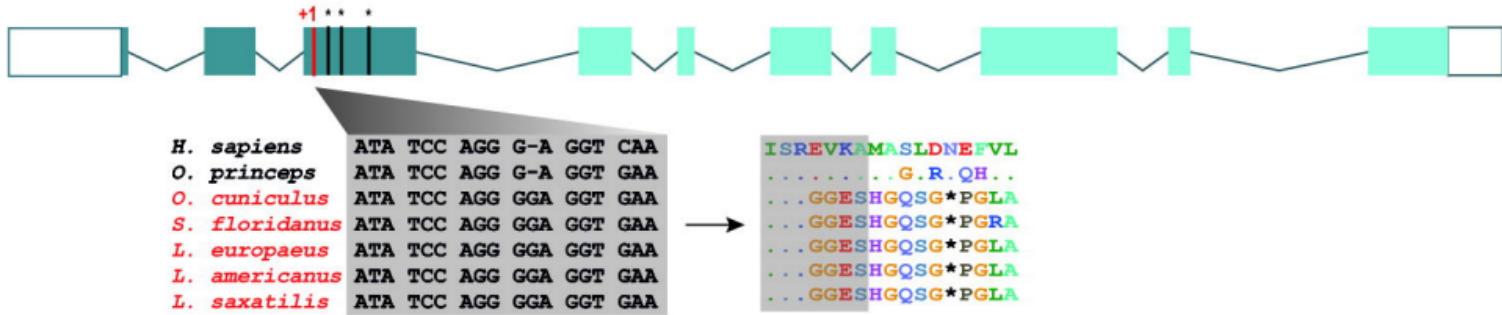
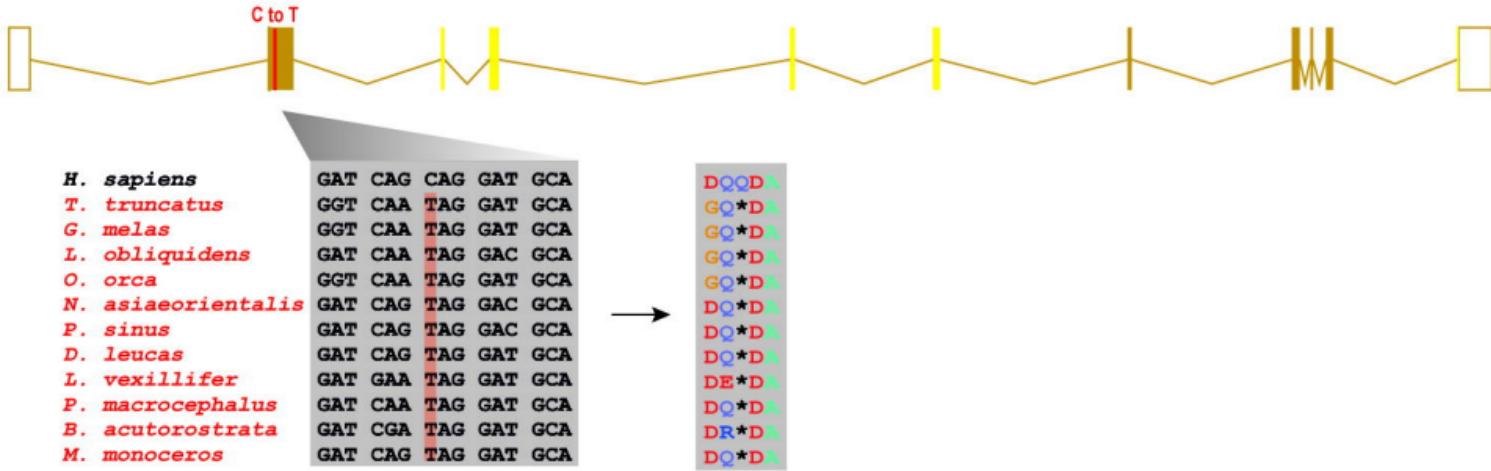
835 **S. Appendix 4** Positive and negative selection analyses for RIPK1 protein.

836 **S. Appendix 5** RIPK3 and MLKL protein alignment from species from rodent and
837 afrotheria lineages.

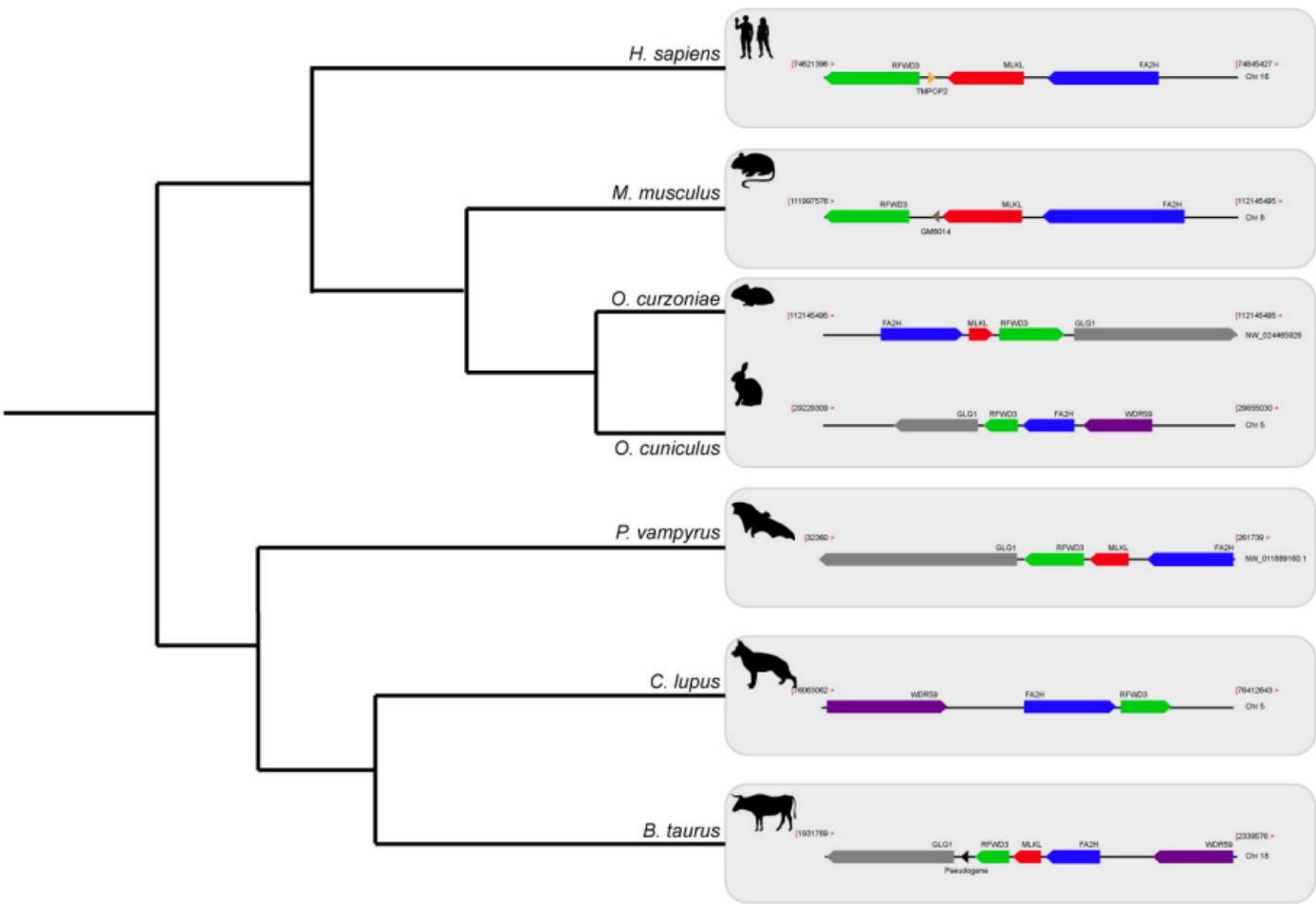
838 **S. Appendix 6** Tables identifying RIPK3 and MLKL mutations and premature stop codons
839 in Cetacea order.



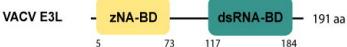
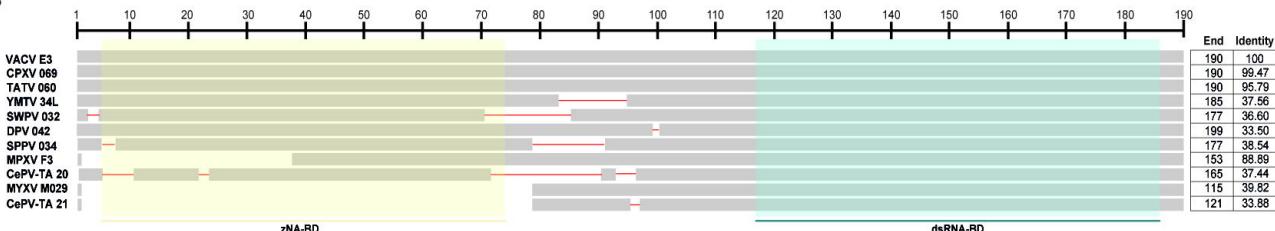
A**B**

A**B**

Águeda-Pinto et al. Fig 4



Águeda-Pinto et al. Fig 5

A**B****C**