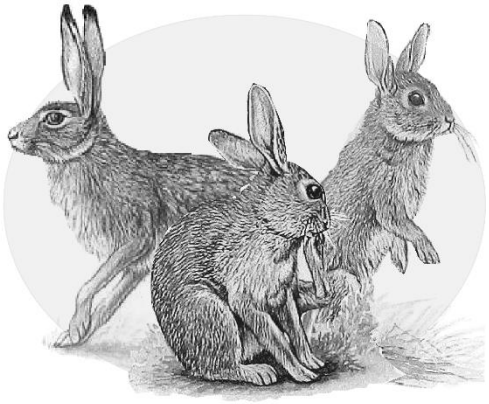

Insights about the evolutionary mechanisms of poxviruses: Myxoma virus in *Lepus*, a case study

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À minha mãe e à minha irmã

Statement

In compliance with the no. 2 of article 4 of the General Regulation of Third Cycles of the University of Porto and with article 31 of the Decree-Law no. 74/2006, of 24 March, with the alteration introduced by the Decree-Law no. 230/2009, on 14 September, the results of already published works were totally used and included in some of the chapters of this dissertation. As these works were performed in collaboration with other authors, the candidate clarifies that, in all these works, was the main participant in obtaining, interpreting, analyzing and discussing the results, as well as in the writing of the published forms.

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Abstract

Despite considerable progress over the last years, new and re-emerging pathogens continue to present significant challenges and are likely to be one of the main issues of medical and public health in the twenty-first century. Poxviruses are highly successful pathogens, known to infect humans, livestock and wild animals. The family *Poxviridae* includes Variola virus, the causative agent of smallpox, which throughout history has killed more human population than any other infectious disease. Despite the success of the World Health Organisation-led smallpox eradication, there remains considerable fear that variola virus, or other related poxviruses, could be acquired and used as deliberate agents of mass mortality. For example, monkeypox is a poxviral emerging infectious disease for which outbreak frequency has been steadily increasing. For this and other reasons, understanding poxviral evolution as well as uncovering the mechanisms that enable the cross-species jumps of viruses is essential in our understanding of the process of disease dynamics.

One of the most extensively documented models of poxviral evolution is the introduction of the lethal myxoma virus (MYXV) into the European rabbit (*Oryctolagus cuniculus*) populations as a biological control. The host-range genes evolved in MYXV natural *Sylvilagus* hosts yet, when MYXV encountered the naïve European rabbit, the virus caused an outbreak of a lethal disease called myxomatosis. Myxomatosis in the European rabbit is an exceptional example of host-virus arms race: the introduction of MYXV in Australia and Europe in the 1950s resulted in both the virus strains attenuation and the emergence of MYXV resistant European rabbit populations. Contrary to other poxviruses that have a broad host range, MYXV infection was only known to be restricted to *Sylvilagus* and *Oryctolagus* rabbits. However, in 2018, Iberian hares (*Lepus granatensis*) from the Toledo province of Spain were found dead with lesions consistent with those observed in myxomatosis, suggesting a cross-species jump of MYXV into Iberian hares. Examination of the deceased symptomatic Iberian hare samples resulted in the identification of a new recombinant MYXV, hereby referred as MYXV Toledo (MYXV-Tol). The genome of MYXV-Tol was ~99 % identical to the known MYXV Lausanne (MYXV-Lau) strain, known to cause myxomatosis in European rabbits, with the exception of the insertion of a new recombinant region (~2,800 bp) and three disrupted genes (*M009L*, *M036L* and *M152R*). Interestingly, the novel recombinant insert includes a new ortholog of a poxvirus host range gene, which is orthologous to the Vaccinia (VACV) C7L-like host range factor superfamily. Along with the presence of the

newly discovered MYXV strain in Iberian hare samples, we also found co-infections with a polyomavirus (family *Polyomaviridae*), representing a putative new species, and anelloviruses (family *Anelloviridae*) belonging to two new putative species.

In the past, three tandem C7L-like host range genes (*M062R*, *M063R*, and *M064R*) were identified in MYXV-Lau. In particular, M062R was identified as essential for MYXV-Lau pathogenesis and exhibits the ability to bind and antagonize host sterile α motif domain containing 9 (SAMD9), an innate antiviral host factor. As the new MYXV-Tol strain encodes a novel C7L host range protein, that we named M159, we hypothesized that this viral protein plays a role on how MYXV-Tol most effectively counteracts hare's immune defenses. To test this hypothesis, several recombinant viruses were constructed, including a MYXV-Tol fluorescently tagged (vMyxTol) and a M159-minus (vMyxTol-ΔM159) that were compared against a fluorescently tagged MYXV-Lau (vMyxLau) in cultured rabbit, hare and human cancer cells. While vMyxTol productively infected hare cells (hare peripheral blood mononuclear cells (PBMCs) and HN-R cell line) after 24 hours, vMyxLau did not result in a visible infection in these hare cells. Moreover, infection with vMyxTol-ΔM159 (deleted of *M159* gene) led to a drastic decrease of replication in hare PBMCs and HN-R cells, suggesting that this new host range factor is essential for productive replication of MYXV-Tol in the tested hare cells. Moreover, M159 expression was not required for MYXV binding, entry or early gene expression in hare HN-R cells, whereas DNA replication was aborted and late genes were not expressed in vMyxLau and vMyxTol-ΔM159. In accordance with its function in relieving an early block of viral replication, M159 was shown to be expressed as an early/late gene but was translocated into the nucleus at later time points. Finally, M159 protein did not contribute to increased replication in the tested permissive rabbit cells but did increase the virus replication efficiency in two non-permissive human cancer cell lines (PANC-1 and MDA-435), suggesting that M159 protein might target conserved intracellular signal transduction pathways in different cell types. Overall, these results show that M159 protein plays a critical role in determining the host specificity of MYXV-Tol in hare and human cells by imparting new host range functions.

Mechanisms of poxviral evolution may include nucleotide mutations, acquisition of new genes or the loss of genetic material. The consequences of these events can be highly variable, ranging from neutral changes, to loss of gene function or even changes in poxvirus host range. As exemplified by the remarkable case of MYXV-Tol, gene gain events through recombination between two poxviruses can lead to changes in poxvirus

host range by subversion of the new host intracellular signal transduction pathways. In fact, the gain of *M159* gene by MYXV-Tol, as a result of a recombinant event with an unsampled poxvirus, lead to the subversion of a novel host restriction and resulted in this virus ability to cross the barrier species and cause a myxomatosis-like disease for the first time in Iberian hares.

The innate immune system and several intracellular signal transduction pathways act to protect the host against invading pathogens. Necroptosis is a programmed form of necrosis, or inflammatory cell death that is mediated by RIPK3 and MLKL and protects the host during some viral infections, facilitating the elimination of virus-infected cells before the production of progeny virions. Given the importance of necroptosis as a host defense mechanism, poxviral genomes encode necroptotic inhibitors, like is the case of Vaccinia virus (VACV) E3 protein. In fact, E3L-encoded proteins are composed of an amino (N)-terminal Z-NA binding domain (zNA-BD), which in VACV is known to bind to VACV-induced Z-form nucleic acid and masking it, preventing RIPK3-mediated induction of necroptosis. Interestingly, we have shown a correlation between the loss of zNA-BD in the E3L orthologues from poxviruses like MYXV and cetaceanpoxviruses (CePV), and the absence of a functional necroptotic pathway in their natural infecting hosts: both Lagomorphs and Cetaceans presented frameshifts or premature stop codons that disrupted RIPK3 and MLKL, the key effectors of necroptosis. From the obtained results, it is possible that by being restricted to a small niche of hosts, both MYXV and CePV have suffered a gradual loss of genetic information that drove their evolution to a fitness peak that is most suited to their specific host niche. Moreover, by examining the core proteins of the necroptotic machinery (RIPK3 and MLKL), we also show that necroptosis might also be deleted in three more divergent mammalian lineages: in carnivores' genomes, the *MLKL* gene is deleted; while in a small number of species from afrotheria and rodentia premature stop codons can be observed in *RIPK3* and/or *MLKL* genes. The loss of function in *RIPK3* and/or *MLKL* genes in five divergent mammalian lineages (convergent loss) indicates that gene loss might be beneficial and that the deregulation of this pathway during mammalian evolution was detrimental for the organism by providing an evolutionary mechanism for adaptation. Moreover, the absence of this pathway in different mammalian lineages indicates that the necroptotic cell death pathway does not act as a universal cell death program against pathogens in mammals.

Overall, this work highlights the importance of events of gene gain in the evolution of poxviral host range, as well as the importance of a co-evolutionary relationship

between poxviruses and their hosts, emphasizing the role of host adaptation in shaping virus evolution. It also improves the current knowledge about poxviral host range and virulence and provides a unique platform for better understanding the pathogenicity and transmission success of emerging viruses like the new MYXV-Tol virus, rendering it capable of leaping into a new host species.

Keywords

Poxviruses; Co-infections; Myxoma virus; Leporidae; European rabbits, Iberian hare; Co-evolution; Host-virus interactions; Host range factors; VACV C7L Superfamily; M159 protein; Necroptosis; E3L host range proteins.

Resumo

Apesar de um progresso considerável nos últimos anos, os agentes patogénicos capazes de causar doenças infeciosas continuam a representar um grande desafio para a humanidade uma vez que novos e re-emergentes agentes patogénicos serão provavelmente um dos principais problemas de saúde médica e pública no século XXI. Os Poxvírus são uma família de vírus que apresentam um grande sucesso, sendo conhecidos por infectar humanos, animais domésticos e animais selvagens. A família *Poxviridae* inclui o vírus da Varíola que ao longo da história matou mais pessoas do que todas as outras doenças infeciosas juntas. Apesar do sucesso da erradicação da varíola liderada pela Organização Mundial da Saúde, existe ainda um medo considerável de que este vírus, ou outros poxvírus, possam vir a ser agentes biológicos responsáveis por mortalidades em massa. Por exemplo, o vírus monkeypox causa uma doença infeciosa para a qual a frequência de casos tem vindo a aumentar em todo o mundo. Por esta e outras razões, compreender a evolução viral, bem como descobrir os mecanismos que permitem os vírus infectarem outras espécies é essencial para a compreensão do processo da dinâmica das doenças infeciosas.

Os mecanismos pelos quais os poxvírus evoluem incluem mutações nucleotídicas, aquisição de novos genes ou perda de material genético. As consequências destes eventos podem ser neutras para o genoma do vírus ou então resultar na perda de função de genes. O ganho de novos genes pelo vírus pode resultar em alterações drásticas como a subversão de certas vias de restrição de hospedeiros e na sua consequente capacidade de infetar um novo hospedeiro. Um dos modelos mais utilizados para estudar a evolução de poxvírus é a introdução do vírus mixoma (MYXV) nas populações selvagens de coelho-bravo (*Oryctolagus cuniculus*) como controle biológico. O MYXV evoluiu em estrita associação com o seu hospedeiro natural *Sylvilagus*. No entanto, quando o MYXV foi inserido em populações de coelho-bravo, o vírus causou um surto de uma doença letal chamada mixomatose. De facto, a mixomatose no coelho-bravo é um exemplo clássico da luta constante entre vírus e novos hospedeiros: a introdução do MYXV na Austrália e na Europa na década de 1950 resultou na atenuação das estirpes do MYXV e no aparecimento de populações de coelho resistentes ao MYXV. Ao contrário de outros poxvírus que possuem a capacidade de infetar vários organismos, só se sabia que o MYXV causava letalmente mixomatose no coelho-bravo. No entanto, em 2018, lebres ibéricas (*Lepus granatensis*) da província de Toledo, em Espanha, foram encontradas mortas com lesões

compatíveis com as observadas na mixomatose, sugerindo que o MYXV foi capaz de infetar e causar pela primeira vez uma doença grave em lebres ibéricas. Ao analisar amostras sintomáticas de lebres ibéricas, identificamos uma nova estirpe de MYXV que sofreu um evento de recombinação e que irá ser referido como MYXV Toledo (MYXV-Tol). O genoma do MYXV-Tol era ~ 99% idêntico ao conhecido da estirpe que afeta o coelho-bravo do MYXV Lausanne (MYXV-Lau), com exceção da inserção de uma nova região recombinante (~ 2.800 bp) e de três genes não funcionais (*M009L*, *M036L* e *M152R*). Juntamente com a presença da nova estirpe de MYXV, também foram encontradas co-infecções com um poliomavírus (família *Polyomaviridae*), representando uma nova espécie, e anelovírus (família *Anelloviridae*) que pertencem a duas novas espécies. No total, dois novos genomas de poliomavírus foram identificados nas amostras analisadas (> 99% de identidade de todo o genoma), enquanto que 14 diferentes anelovírus foram identificados, partilhando entre 63-99% de identidade entre si em todo o genoma.

O gene *C7L* do vírus da Varíola é importante para a supressão do sistema imunitário dos hospedeiros. Três genes semelhantes aos do C7L foram identificados no MYXV-Lau (*M062R*, *M063R* e *M064R*). Em particular, M062R foi identificado como essencial para a patogenicidade do MYXV-Lau e tem a capacidade de se ligar e antagonizar a proteína SAMD9 (sterile α motif domain containing 9), uma proteína antiviral do sistema inato do hospedeiro. Como a nova estirpe do MYXV-Tol codifica uma nova proteína semelhante à C7L, à qual nós chamamos de M159, hipotetizamos que esta proteína desempenha um papel importante na neutralização das defesas imunológicas da lebre quando estas são infetadas pelo MYXV-Tol. Para testar esta hipótese foram construídos dois MYXV recombinantes: um MYXV-Tol intacto marcado com fluorescência (vMyxTol) e outro com o gene *M159* (vMyxTol-ΔM159) deletado. Estes vírus foram depois comparados com o MYXV-Lau (vMyxLau) em células de coelho, de lebre e linhas celulares humanas cancerígenas. Enquanto o MYXV-Tol intacto marcado com fluorescência é capaz de infectar células de lebre após 24 horas, a infecção pelo MYXV-Lau não resultou numa infecção visível nas mesmas células de lebre. Mais ainda, a infecção com o vírus MYXV-Tol sem o gene *M159* levou a uma diminuição drástica da replicação, uma vez que nenhum foco replicativo pôde ser observado em células de lebre, sugerindo assim que esta nova proteína identificada no MYXV-Tol é essencial para sua a replicação nestas células. A expressão de *M159* não foi necessária para a entrada e ligação do MYXV nem para a expressão genética inicial em células de lebre. No entanto, não ocorreu a replicação do DNA e genes tardios não

foram expressos na infeção com vMyxLau e vMyxTol-ΔM159. Curiosamente, a proteína M159 não contribuiu para um aumento da replicação viral nas células permissivas de coelho testadas, mas aumentou a eficiência da replicação do vírus em duas linhas celulares humanas cancerígenas não permissivas (PANC-1 e MDA-435). No geral, os dados apresentados mostram que a inserção do gene *M159* no MYXV-Tol devido a um evento de recombinação com um poxvírus ainda não identificado, resultou na capacidade do MYXV-Tol ultrapassar a barreira entre espécies e causar uma doença semelhante à mixomatose pela primeira vez em lebres ibéricas.

Como exemplificado pelo caso notável do MYXV-Tol, eventos que resultam no ganho de genes por meio de recombinação entre dois poxvírus podem levar a mudanças de hospedeiros e consequente subversão de certas vias de restrição do hospedeiro. Os genomas dos poxvirus também podem evoluir por meio da perda gradual de informação genética, o que pode desempenhar um papel crítico na especiação e evolução do vírus para um aumento de aptidão mais adequada a um determinado hospedeiro. Curiosamente, nós encontramos uma correlação entre a inativação da via da necroptose em leporídeos e cetáceos e a ausência da região N-terminal da proteína E3L nos poxvírus que infetam naturalmente estes organismos, uma região que é primariamente responsável pela inibição da necroptose. A necroptose é uma forma programada de necrose, ou morte celular inflamatória, mediada pelas proteínas RIPK3 e MLKL, e que protege o hospedeiro de infecções virais, facilitando assim a eliminação de células infectadas por vírus antes da sua replicação. A observação de que leporídeos e cetáceos não eram capazes de induzir a necroptosis, levou-nos a estudar a história evolutiva desta importante via de defesa nas principais linhagens de mamíferos. Ao examinar as proteínas chave da maquinaria necroptótica, RIPK3 e MLKL, mostramos que estas estão deletadas ou exibem mutações que as inativam em cinco linhagens de mamíferos: em todas as espécies estudadas de cetáceos e leporídeos foram observadas mudanças na sequência nucleotídica destes genes ou a presença de codões STOP prematuros; nos genomas dos carnívoros, o gene *MLKL* não está presente, enquanto que num pequeno número de espécies dos afrotheria e rodentia são observados codões STOP prematuros no *RIPK3* e/ou *MLKL*. No geral, foi demonstrada uma perda de genes em diferentes grupos de mamíferos, o que indica que esta via de defesa não atua como um programa universal de morte celular contra agentes patogênicos em mamíferos.

Este trabalho destaca a importância de eventos de ganho genético para a evolução dos poxvirus, bem como da importância de uma relação co-evolutiva entre os poxvírus e seus hospedeiros, realçando o papel da adaptação do hospedeiro para a evolução do vírus. Contribui ainda para um melhor conhecimento da virulência dos poxvírus. Assim, este trabalho reúne um conjunto de informações muito relevantes para a melhor compreensão da patogenicidade e do sucesso da transmissão de vírus emergentes como o novo MYXV-Tol.

Palavras chave

Poxvirus; Co-infeções; vírus do Mixoma; Leporídeos; Coelho-bravo, lebre ibérica; Co-evolução; interação entre hospedeiro e vírus; fatores que aumentam o tropismo viral; VACV C7L; proteína M159; Necroptosis; proteínas E3L.

Thesis framework

The work presented in this dissertation was integrated in a project supported by the Fundação para a Ciência e a Tecnologia entitled 'Myxoma virus leap into *Lepus*: insights behind a poxvirus cross species transmission' with the reference SFRH/BD/128752/2017. The thesis is organized into five chapters and a total of four scientific manuscripts that are published or in review in journals indexed in the Science Citation Index (SCI) are included.

To begin addressing the fundamental questions mediating virus cross-species transmission, it is crucial to study the biological and mechanistic underpinnings driving such events using a well-established model system. Myxoma virus (MYXV) is a Leporipoxvirus that naturally infects American rabbits (*Sylvilagus sp)*, resulting in a localized cutaneous fibroma. In European rabbits (*Oryctolagus cuniculus*), however, it causes a lethal disseminated disease known as myxomatosis. Due to its pathogenicity in European rabbits, the virus was used in the 1950s as a biological control agent against the introduced invasive rabbits into Australia and Europe. Until recently, it was only known that MYXV causes the lethal disease myxomatosis in the European rabbit.

A review of the current knowledge on MYXV and its encoded host range factors is presented in the first chapter, entitled "Chapter 1. General introduction." Aspects on poxviruses biology and species specificity as well as MYXV natural history, evolution and pathogenicity are comprehensively reviewed. Furthermore, the evolutionary aspects of MYXV encoded host range factors and their roles in the host immune system evasion are also reviewed in this chapter.

In 2018, Iberian hares (*Lepus granatensis*) from the Toledo province of Spain were found dead with lesions consistent with those observed in myxomatosis, suggesting a cross-species jump of MYXV into Iberian hares. In "Chapter 2. Genetic characterization of a novel MYXV strain and identification of viral co-infections in Iberian hares", the possibility of a new MYXV cross-species transmission was tested by analyzing collected samples from the deceased symptomatic Iberian hares. From these, a novel MYXV recombinant which we refer to as MYXV Toledo (MYXV-Tol) was isolated and characterized. The MYXV-Tol genome has three disrupted genes and a novel four genes "cassette" insertion of ~ 2,800 bp within the *M009L* gene, from unknown origin. Interestingly, the novel insert includes an orthologue of a poxvirus host range gene,

which is homologous to the MYXV C7L Superfamily of host range factors. Moreover, the high-throughput sequencing data from these samples also allowed the detection of other DNA viruses that were circulating in Iberian hare populations, providing additional insight into existing co-infections. These results are presented in two scientific research articles:

Research article 1: Genetic Characterization of a Recombinant Myxoma Virus in the Iberian Hare (*Lepus granatensis*) (2019). Ana Águeda-Pinto, Ana Lemos de Matos, Mário Abrantes, Simona Kraberger, Maria A. Risalde, Christian Gortázar, Grant McFadden, Arvind Varsani and Pedro J. Esteves, published in *Viruses;*

Research article 2: Coinfections of Novel Polyomavirus, Anelloviruses and a Recombinant Strain of Myxoma Virus-MYXV-Tol Identified in Iberian Hares (2020). Ana Águeda-Pinto, Simona Kraberger, Michael C. Lund, Christian Gortázar, Grant McFadden, Arvind Varsani and Pedro J. Esteves, published in *Viruses.*

In the third chapter, “Chapter 3: Characterization of M159, a novel host range factor found in MYXV-Tol that belongs to the VACV C7L superfamily”, we further investigate the role of the newly acquired “cassette” insertion of ~ 2,800 bp, particularly the role of the newly acquired host range gene (*M159*) in mediating MYXV species' jump from rabbits to hares. The MYXV-Tol M159-encoded protein is an ortholog of the Superfamily of C7-host range factors that are known for having major roles in pathogenicity and poxviral host tropism. Therefore, the main goal of the third chapter was to investigate whether this newly acquired viral protein has enabled MYXV to alter its host range from rabbits to hares and enabled the myxomatosis-like pathogenesis in hares. Most of the work was performed by constructing several MYXV recombinant viruses and testing them in hare and rabbit cell lines. Moreover, a recombinant MYXV-Tol virus expressing a V5-tagged M159 was used to study the M159 expression and cellular localization. This chapter allowed a better understanding of the mechanisms of how MYXV-Tol was able to cross the barrier species. The results are presented in one research article:

Research article 3: Identification of a novel Myxoma C7-like host range factor that enabled a species leap from rabbits to hares. Ana Águeda-Pinto, Simona Kraberger, Ami Gutierrez-Jensen, Honor L Gleen, Kevin P. Dalton, Ana Podadera, Francisco Parra, Arvind Varsani, Grant McFadden, Masmudur M. Rahmanc and Pedro J. Esteves, submitted for publication.

MYXV, like all poxviruses, encodes an extensive repertoire of virulence genes, which express an array of proteins responsible for the subversion and modulation of the host antiviral responses. A group of these genes, designated as host range genes, are essential for MYXV-specific tropism. Vaccinia E3 protein is a host range factor that has been shown to inhibit the IFN-primed virus induced necroptosis through its N-terminal zDNA binding domain. Necroptosis is a major form of programmed necrosis that contributes to the innate immune response by killing pathogen-infected cells and relies on the receptor-interacting serine/threonine-protein kinase 3 (RIPK3) and its substrate, the mixed lineage kinase like protein (MLKL). Interestingly, the MYXV E3-like ortholog (M029) lacks the N-terminal zDNA binding domain, suggesting that MYXV is not able to prevent the induction of Necroptosis. In “Chapter 4: Necroptosis, a lost pathway in different mammalian lineages”, the goal is to address the molecular evolution of the necroptotic pathway in multiple mammalian lineages, taking a closer look into the order Lagomorpha. At the same time, this chapter sheds some light on a co-evolutionary relationship between poxviruses and their hosts, emphasizing the role of host adaptation in shaping virus evolution. The results are presented in one research article:

Research article 4: Convergent loss of the necroptosis pathway in disparate mammalian lineages shapes viruses countermeasures (2021). Ana Águeda-Pinto, Luís Q. Alves, Fabiana Neves, Grant McFadden, Bertram L Jacobs, L. Filipe C. Castro, Masmudur M. Rahmand and Pedro J. Esteves, published in *Frontiers of immunology.*

Finally, in “Chapter 5. Final considerations”, the major conclusions of this dissertation and the implications of the results for future research are presented.

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Chapter 1

General Introduction

1.1. The Poxviruses

1.1.1. The Poxviruses taxonomy

Poxviruses are a highly diverse group of double-stranded DNA viruses with large genomes (130–360 kb in length), often containing more than 200 genes. Invertebrates, birds, reptiles and mammals can serve as natural hosts for these viruses [1]. Members of the poxviridae family are divided into two subfamilies: *Entomopoxviridae* that infect invertebrates; and *Chordopoxviridae* that infect vertebrates [2]. Among *Chordopoxvirinae*, 18 genera can be currently recognized by the International Committee on Taxonomy of Viruses: orthopoxvirus, capripoxvirus, cervidpoxvirus, suipoxvirus, leporipoxvirus, molluscipoxvirus, yatapoxvirus, avipoxvirus, crocodylidpoxvirus, parapoxvirus, centapoxvirus, macropopoxvirus, mustelpoxvirus, oryzopoxvirus, pteropopoxvirus, vespertilionpoxvirus, salmonpoxvirus and sciuripoxvirus (Table 1). However, as a result of advances in genome sequencing technologies, a considerable growing number of poxviruses are discovered every year. Yet, some still remain unassigned. This is the case, for example, of cetaceanpox viruses (CePVs) that infect cetaceans, both odontocetes and mysticetes species [3–5]. Over the years, detailed information on poxvirus bioinformatics has been collected and is currently available at www.poxvirus.org.

1.1.2. Poxviruses biology

Poxvirus members of the different genera are morphologically similar, sharing a characteristic pleomorphic, generally brick-shaped virion morphology (220-450 nm long x 140–260 nm wide x 140–260 nm thick) covered with a lipoprotein membrane [6]. The poxvirus genome is a double stranded DNA molecule with covalently closed hairpin termini flanked by terminal inverted repeat (TIR) regions, two identical DNA sequences oppositely oriented at each end of the genome [7]. Of the different open reading frames (ORFs) located throughout the genome, at least 90 are centrally located and are important for transcription, replication, virion assembly and cell entry and exit. These genes are usually highly conserved among the various poxviruses [8–10]. The remaining genes, located towards the ends of the genome, either within the TIR or near-terminal regions, encode proteins with greater diversity among the poxvirus family and are important for suppression and evasion of host innate and adaptive antiviral responses [8–10]. It is the specific repertoire of these genes that gives each poxvirus its unique characteristics of host range, immunomodulation and pathogenesis [11,12].

Table 1. Poxvirus classification and host range

Genus	Virus	Major hosts	Host range
Orthopoxviruses	Varíola virus (VARV) (smallpox)	Humans	Narrow
	Vaccinia vírus (VACV)	Numerous: humans, cattle, buffalo, swine, rabbits	Broad
	Cowpox virus (CPXV)	Numerous: rodents, domestic cats and large felines, cattle, humans, elephants, rhinoceros, okapi, mongoose, alpaca	Broad
	Camelpox virus (CMLV)	Camels	Narrow
	Ectromelia virus (ECTV)	Mice, voles	Narrow
	Monkeypox virus (MPXV)	Numerous: squirrels, monkeys, anteaters, great apes and humans	Broad
Capripoxviruses	Sheeppox virus (SPV)	Sheep, goats	Narrow
	Lumpy skin disease virus (LSDV)	Cattle, Cape buffalo	Narrow
	Goatpox virus (GTPV)	Goats, Sheep	Narrow
Cervidpoxvirus	Deerpox virus (DPV)	Deer including reindeer, gazelle	Broad
Suipoxvirus	Swinepox virus (SWPV)	Swine	Narrow
Leporipoxvirus	Myxoma virus (MYXV)	Rabbits (*Oryctolagus* and *Sylvilagus* spp.) and hares	Narrow
	Hare fibroma virus (SFV)	European hare (Lepus europaeus)	Narrow
	Squirrel fibroma virus (SQFV)	Eastern gray squirrel (Sciurus carolinensis),	Narrow
Molluscipoxvirus	Molluscum contagiosum virus (MOCV)	Humans	Narrow
Yatapoxvirus	Yabapox virus (YMTV) and tanapox virus (TPV)	Monkeys, humans	Narrow
Avipoxvirus	Fowlpox virus, canarypox, crowpox, juncopox, mynahpox, pigeonpox, psittacinepox, quailpox, etc	Chickens, turkeys, and many other bird species from different orders	Narrow
Crocodylidpoxvirus	Crocodilepox virus (CRV)	Crocodiles	Narrow
Parapoxviruses	Orf virus (ORFV)	Sheep, goats, humans (related viruses of camels and chamois)	Broad
	Bovine papular stomatitis virus (BPSV)	Cattle, humans	Narrow
	Red deerpox vírus (DPV)	Red deer	Narrow
Centapoxvirus	Yokapox virus	Unknown - Isolated from a mosquito pool	Unknown
Macropopoxvirus	Eastern and western kangaroopox virus	Kangaroos	Narrow
Mustelpoxvirus	Sea otterpox virus	Sea otter	Narrow

Oryzopoxvirus	Cotia virus (COTV)	Unknown - Isolated from sentinel suckling mice	Unknown
Pteropopoxvirus	Pteropox virus	Little red flying-fox	Narrow
Vespertilionpoxvirus	Eptesipox virus	Big brown bats	Narrow
Salmonpoxvirus	Salmon gillpox virus (SGPV)	Atlantic salmon	Narrow
Sciuripoxvirus	Squirrelpox virus (SQPV)	Grey squirrels	Narrow
Vespertilionpoxvirus	Eptesipox virus	Big brown bats	Narrow

1.1.3. Poxviruses-host spectrum

As a family, *Poxviridae* has a wide host range, being able to infect mammals, birds and reptiles [13]. However, when looking at a specific member of the *Poxviridae* family, the host spectrum is variable, with viruses infecting a large range of hosts [14–16], while others are restricted to only one host species [17,18]. The cowpox virus (CPXV) presents the widest host spectrum among the poxviruses, being able to infect more than 20 groups of hosts [13]. On the other hand, variola virus (VARV) and myxoma virus (MYXV) are very restricted, having only humans and leporids as their natural infecting hosts, respectively [13,19]. It is believed that such differences in the host range of each virus might lie in the diversity and abundance of their host range genes [16].

1.1.3.1. Human-specific poxviruses

Over the years, several outbreaks of poxviruses infecting humans have been reported worldwide. However, the only specific human orthopoxviruses are variola virus (smallpox) and molluscipox virus, which causes molluscum contagiosum (MC) [13]. MC is a viral cutaneous infection of the skin that spreads through person-to-person contact and contact with infected objects [20]. Though most common in children, MC can affect adults as well, particularly those with weakened immune systems [20,21]. The disease is chronic but usually resolves within a few months [21]. Smallpox, caused by VARV (orthopoxvirus genera), is highly contagious and the most deadly disease in human history, estimated to have caused over 300 million human deaths in the twentieth century alone [22,23]. Members of the *Orthopoxvirus* family have a high degree of antigenic similarity, which formed the basis of using different *Orthopoxviruses* species to protect against infection with another. This basic principle was first discovered by Edward Jenner when he used CPXV to protect humans against smallpox. Later, eradication of smallpox was achieved through the induction of protective immunity against the disease using vaccine strains of vaccinia virus (VACV) [24,25]. Following the promotion of a global

vaccination campaign led by the World Health Organization (WHO), the last naturally occurring case of this disease was in Ethiopia in 1977 [26]. Although VARV was eradicated as a naturally occurring human pathogen, there remains considerable fear that VARV could be acquired and used as deliberate agents of mass mortality [22]. In fact, the appearance of any pathogenic poxvirus that spreads efficiently to humans would be considered an immediate public health crisis.

1.1.3.2. Poxviruses with zoonotic potential

Besides the two known human-specific poxviruses, VARV and MCV, three genus of the subfamily *Chordopoxvirinae* have members that harbor zoonotic potential: orthopoxvirus (like VACV, CPXV, and monkeypox virus (MPXV)), parapoxvirus (like orf virus (ORFV)) and yatapoxvirus (namely tanapox virus) [27–30].

VACV, historically known as live attenuated virus used worldwide by the WHO in the smallpox vaccine, is the prototype species of the *Orthopoxvirus* genus. Different VACV isolates or sub-lineages have been consistently isolated in different countries and from a wide range of hosts (reviewed in [31]). As an example, VACV (also known as bovine VACV) causes a zoonotic disease that is primarily associated with the handling of infected dairy cattle in Brazil, causing economic losses as a result from compromised milking herds [32,33]. Since 1999, more than a thousand dairy cattle and up to 80% of their handlers have been affected by this infection [34,35].

It has been recognized that several orthopoxviruses that can infect both animals and humans, like CPXV and MPXV, are most likely acquired from rodent reservoir hosts. However, many of these rodent hosts have not been identified. A prime example is the 2003 USA outbreak of human monkeypox due to contact with pet prairie dogs that had been co-housed with MPXV-infected African rodents, imported from Ghana [29,36]. Luckily, at the time, no deaths were reported and no person-to-person transmission was proven (Centers for Disease Control and Prevention (CDC) Update: Multistate Outbreak of Monkeypox—Illinois, Indiana, Missouri, Ohio, and Wisconsin). In recent years (2019-2021), some African regions have continuously reported human cases of MPXV infection, including the Central African Republic with 27 confirmed cases and two deaths; the Democratic Republic of the Congo with more than 300 confirmed deaths between 2019-2021; and Nigeria with 139 confirmed cases and 7 deaths (WHO Weekly Bulletin on Outbreaks and Other Emergencies; week 22, 27 May-2 June 2019 and week 33, 9-15 August 2021). In July 2021, an imported case of human monkeypox was reported in

Dallas, Texas (USA), where the patient presented symptoms like fever, vomiting, mild cough and a painful genital rash (World Health Organization report). So far, no treatment for this infection has been shown to be effective or safe. A lack of understanding about this disease, associated with increased human cases of monkeypox over the last years raises serious concerns regarding MPXV emergence, surveillance, prevention, and control [37]. Regarding cowpox, and although it is present in a wide range of animals (reviewed in Silva et al. [31]), most infections are described in domestic cats probably due to their predatory behavior against rodents [38]. In cats, CPXV infection can cause multiple skin lesions through the body and, in the most severe cases, it can affect inner organs, with fatal outcomes being mostly associated with secondary bacterial infection [39]. The majority of human cases of infection by CPVX are traced to direct contact with infected domestic cats or rodents (such as *Rattus norvegicus*) [40] and the disease consists of localized pustular skin lesions on the hands or face [41]. Human cowpox cases are usually self-limiting and not lethal and fatal outcomes have only been associated with immune-compromised individuals [41].

Infections by parapoxviruses are mainly reported from people that are in close contact with sheep and cattle and are characterized by the appearance of local lesions on the hands [42,43]. The majority of human infections by Parapoxviruses are caused by ORFV [42,43]. In sheeps and goats, infection by ORFV can result in severe oral lesions or secondary infections that can lead to high mortality rates partially due to anorexia [44]. Yatapoxvirus infections are quite rare and only a small number of cases in humans have been reported [45,46].

The majority of these infections by poxviruses are mild and self-limiting, only causing widespread dissemination and death in immunosuppressed individuals. However, monkeypox infections in humans have been increasing worldwide and in some cases they can progress to a lethality of up to 10% [47]. These outbreak reports show how vulnerable the human population is to the emergence and reemergence of poxviral pathogens from unsuspected sources and indicate that poxviruses like VACV, MPXV, and CPXV pose a potential threat not only for humans, but for animals in different regions of the world.

1.1.3.3. Poxvirus with significant impact in livestock and wildlife

Different members of the Poxviridae family can infect animals, leading to significant impacts on economically important livestock and endangered wildlife.

Capripoxviruses are among the most serious of all animal poxviruses and cause serious skin disease, often fatal, in cattle (lumpy skin disease virus (LSDV)), sheeps (sheeppox virus (SPPV)) and goats (goatpox virus (GTPV)) [48]. Infection by these viruses cause negative economic consequences by damaging hides and wool and forcing the establishment of trade restrictions in response to an outbreak. The lumpy skin disease was first reported in southern and eastern Africa [21]. Probably as a result of increasing global trading, over the last decades it extended through many countries causing international concern [21]. Swinepox, caused by the swinepox virus (SWPV) is an acute, often mild, infectious disease characterized by skin eruptions that affects only pigs. On the other hand, myxomatosis is a lethal disease caused by myxoma virus (MYXV) that can kill up to 99 % when infecting naive populations of European rabbits (*Oryctolagus cuniculus*). Given its high mortality rates, MYXV was intentionally introduced in Australia, France, and England in the 1950s to control wild European rabbit populations [19,49]. In addition to mammals, avipoxviruses impact hundreds of domestic and wild bird species, including chickens, turkeys, penguins, and songbirds, risking economic loss and endangering wildlife [50,51].

1.2. The Myxoma virus

1.2.1. Natural history of Myxoma virus

1.2.1.1. Myxomatosis

MYXV is the prototypic member of the Leporipoxvirus genus (family *Poxviridae*, subfamily *Chordopoxvirinae*) [52,53]. MYXV was described for the first time in 1896 in South America (Uruguay) by Giuseppe Sanarelli following the emergence of a highly contagious and lethal disease affecting his imported European rabbits. This novel disease was characterized by swollen eyelids, ears, noses, and heads, mucopurulent discharge from the eyes and nose, together with the appearance of multiple cutaneous swellings (myxomas) through the body, leading to a rapid death of the infected European rabbits [54,55]. Sanarelli named this infectious agent as a “myxomatogene virus” and the disease was later named myxomatosis after the Greek muxa (meaning mucus) and oma (meaning tumor).

At the time, the source of this disease was not obvious, but subsequent work determined that the causative agent, MYXV, was a natural pathogen of the South American tapeti (*Sylvilagus brasiliensis*) (Figure 1). Although MYXV was initially

considered endemic to regions of South America, a second MYXV strain was later confirmed in brush rabbit (*S. bachmani*) populations from California and Oregon [56,57] (Figure 1). Therefore, two major geographic types of the virus were described: the South American or Brazilian (natural host: *S. brasiliensis*) and Californian (natural host: *S. bachmani*). MYXV infection of its long-term evolutionary hosts only causes a benign disease that rarely causes death [58]. On the other hand, in the European rabbit, which is exotic to the Americas, MYXV provides a powerful example of a pathogen that acquires virulence by switching hosts, causing a rapid systemic and lethal infection in European rabbits that has mortality rates at nearly 100% in naive rabbit populations [59]. Due to its pathogenicity in European rabbits, MYXV was the first viral agent used as a biological control to deliberately eradicate the introduced invasive European rabbits into Australia and Europe [53].

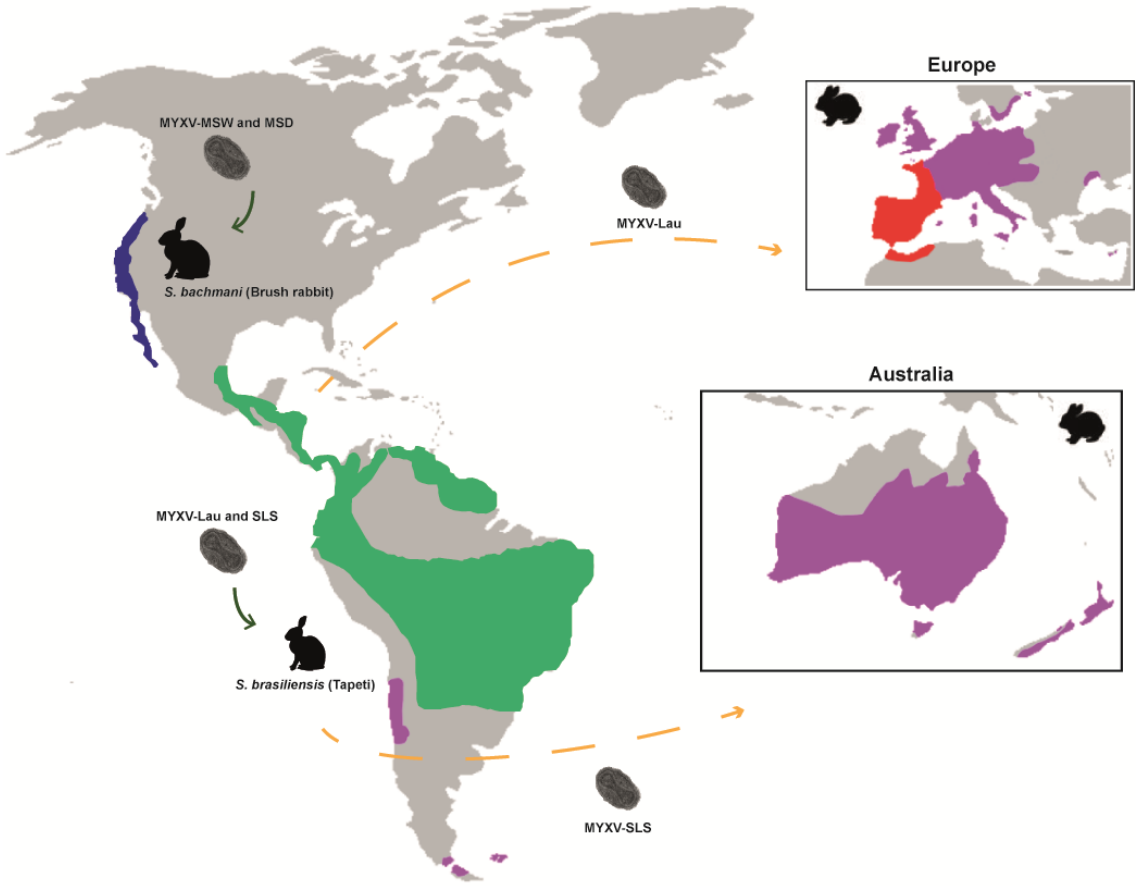

Figure 1. Distribution of different myxoma strains and their use for the biological control of European rabbits in different regions. Two distinct MYXV strains occur in *Sylvilagus* species: the Californian MYXV strains, which circulates in brush rabbit populations (blue in the map); whereas the South American MYXV strains circulates in tapeti populations (green in the map) of South America. European rabbits are natives to the Iberian Peninsula (redo on the map). However, the spread of the European rabbit to different countries of Europe and its introduction in Australia resulted in devastating effects on local biodiversity (purple on the map). In the 1950s, two different South American MYXV strains were introduced in Europe and Australia to control rabbit populations. This figure represents a simplified illustration of different Leporid species locations and not an extensive representation of their natural range.

1.2.1.2. Myxoma properties and strains

In 1911, Dr A. Moses recovered a MYXV strain from a naturally infected European rabbit [60] that, after being maintained for many years by serial passages in laboratory rabbits [59], was termed as the standard laboratory strain (SLS) [58]. Based on experiments in laboratory rabbits, it was expected that this strain killed 99.8 % of infected rabbits [59]. Another example of a South American strain is the Lausanne strain (Lau), which was isolated in Brazil in 1949 and also has a case fatality rate of essentially 100% in laboratory rabbits [58]. Fenner and Marshall [58] also characterized two isolates of Californian MYXV: MSD (San Diego 1949) and MSW (San Francisco 1950). In contrast, two highly attenuated strains were also described, the Hurst's 'neuromyxoma' and Berry's '80A mutant', both derived from the South American Moses strain that under different laboratory conditions lost part of their virulence [53,58].

Like other poxviruses, MYXV shares a characteristic large brick-shaped virion with dimensions of 286 x 230 x 75 nm, containing a double-stranded DNA genome with covalently closed hairpin loops and TIRs [19,61]. The MYXV Lau, the most often used strain for *in vitro* and *in vivo* studies, has a 161.8 kb genome with a total of 171 open reading frames (ORFs) with 12 being duplicated in each TIR [52]. The genes that are present at the central 120kb region of MYXV are known to be associated with the viral replicative machinery and structure, while genes that encode immunomodulatory proteins and host range proteins are located within the 15-25 kb at both ends of the genome [52]. Although the replicative and structural genes are strongly conserved, the virulence-related genes are specific and evolved in close association with its natural host [52,62]. The primary vector of MYXV transmission is the mosquito, although in some regions other biting arthropod vectors like fleas can also play an important role in this virus transmission [49,53]. In arthropod vectors, the virus particles adhere to the mouth parts of these vectors as they feed from virus-rich areas of the host for a blood meal [49,53]. While MYXV does not replicate in the mosquito (it is not an arbovirus), it is introduced in the dermal layer by biting insects at a subsequent feeding [63].

1.2.2. Myxoma and the leporipoxviruses

The leporipoxviruses are a genera within the *Chordopoxvirinae* subfamily of the *Poxviridae* and the name lepori- is derived from the latin *lepus* or *leporis* meaning hare [19]. This small group of viruses includes MYXV and three other species: the hare fibroma virus (FIBV), the Shope fibroma virus (SFV; or rabbit fibroma virus, RFV) and

the squirrel fibroma virus (SQFV) [19]. A recombinant between MYXV and SFV extracted from tumors of laboratory rabbits has also been described and named malignant rabbit virus (MRV); however, MRV has not been found in wild rabbit populations [64,65].

Leporipoxviruses have a host range restricted to Leporids (rabbits and hares) and squirrels [19]. Three of the four known Leporipoxviruses naturally infect Leporid species, namely *Sylvilagus* (MYXV and SFV) and *Lepus* (FIBV) [53,66,67]. Leporids include species from the family Leporidae (hares and rabbits), which have several extant genera and, together with Ochotonidae (pikas, monotypic), forms the order Lagomorpha [68]. Today, 11 extant Leporid genera are distributed globally and include: hares (genus *Lepus*) and rabbits (genera *Brachylagus*, *Bunolagus*, *Caprolagus*, *Nesolagus*, *Oryctolagus*, *Pentalagus*, *Poelagus*, *Pronolagus*, *Romerolagus* and *Sylvilagus*) [69,70]. It has been suggested that speciation of *Lepus* occurred ± 6-3 Mya, leading to the presence of different lineages across North America, Europe, Asia and Africa [70,71]. On the other hand, *Sylvilagus* species, which include the tapeti and the brush rabbit, are extensively distributed throughout North and Central America and the northern half of South America [69]. *Oryctolagus* species are native to the Iberian Peninsula and, as a monotypic genus, it includes the European rabbit as the only representative taxon [72]. While considered a key species in their native range, the introduction of the European rabbit in countries on all continents results in devastating effects on local biodiversity [73,74], in particular, European rabbits in Australia have had a dramatic negative effect on local biodiversity, ecosystems, and the economy, due in part to the lack of natural predators. The fourth member of the Leporipoxvirus genus, SQFV, is endemic in eastern grey squirrel (*Sciurus carolinensis*) populations native to North America [75,76], but is also known to infect western gray squirrels (*S. griseus*) [77] and American red squirrels (*Tamiascuirus hudsonicus*) [78,79].

1.2.2.1. Leporipoxviruses and their natural evolutionary hosts

As previously mentioned, two distinct MYXV strains occur in *Sylvilagus* species: the MYXV South American strain, which circulates in tapeti populations (*S. brasiliensis*) of South America; whereas the Californian MYXV strain circulates in brush rabbit populations (*S. bachmani*) from California and Oregon (USA) (Figure 1) [56,57]. Infection by Brazilian MYXV in tapeti produces cutaneous fibromas that can persist for some weeks before regression [54,55]. Similarly, in brush rabbits, infection by the Californian MYXV strain results in fibromes that generally heal within four weeks [54,55]. This benign

host-pathogen coexistence has been presented as a textbook example of an evolutionary climax in the long-term coevolution of host and pathogen [80].

The SFV has evolved with the cottontail rabbit (*S. floridanus*) of East and Central America and was the first DNA virus associated with transmissible tumors [81,82]. Infection of *Sylvilagus* species by SFV does not cause severe disease, causing only a skin fibromes at the primary site that can last several months [82,83]. SFV is genetically and antigenically related to MYXV [84]. However, infection by this virus in *Oryctolagus* rabbits, only results in the presence of fibromas at the inoculation site [84]. Hare Fibromatosis is a disease caused by FIBV, which has as its natural host the European hare (*L. europaeus*) [19]. Hare Fibromatosis was first described in Italy and France [85] and the disease is characterized by the presence of solid tumors that usually occur in the legs and ears of infected European hares. Nevertheless, mortality by this virus is low and animals usually recover within one to three months after infection. Reports of small dermal tumors on three African hares (*L. capensis*) in Kenya suggest that this virus might extend its range to African hares [67]. Squirrel fibromatosis is caused by SQFV and it has been reported in eastern gray squirrels (*Sciurus carolinensis*) [76,79], a western gray squirrel (*S. griseus*) [77] and in American red squirrels (*Tamiascuirus hudsonicus*) [78,79]. Like in other infections by Leporipoxviruses, this virus is transmitted by biting insects, including mosquitoes and the squirrel flea *Orchopeus howardi* [86], and disease usually regresses spontaneously.

1.2.2.2. Myxoma host-tropism

Over the years, the susceptibility of different *Sylvilagus* species to MYXV strains has been tested in controlled experiments. However, it was soon proven that there appear to have been specific adaptations of each of these viruses to their natural hosts. For example, mosquitoes can not transmit the Californian MYXV strain to the tapeti species (*S. brasiliensis*). The same way, mosquitoes fed on brush rabbits (*S. bachmani*) that were infected with the Brazilian MYXV strain could not transmit the virus [87,88]. Experimentally, when infected with a MYXV Californian strain, four different North American *Sylvilagus* species (*S. audubonii*, *S. floridanus*, *S. idahoensis*, and *S. nuttallii*) developed visible fibromes, but these failed to be mosquito-infective lesions and no progression of the disease was observed [87]. When infected with the Brazilian strain, three species of North American cottontails (*S. audubonii*, *S. floridanus* and *S. nuttallii*) were susceptible to experimental infection, but again mosquitoes failed to transmit the virus [87,88]. Overall, it may be concluded that MYXV strains are highly specific and

have evolved in close association with their respective natural hosts, with only closely related species being susceptible to infection. Moreover, transmission is generally even more restricted, with infected animals not reaching sufficient titers to be further transmitted by mosquitoes.

Regarding controlled experiments in hares, black-tailed jackrabbits (*L. californicus*) were inoculated with a Californian MYXV and did not form tumors [87]. In the wild, cases of myxomatosis in hares have been reported sporadically. In 2006, a few reports in Spain suggested that a myxomatosis-like disease was circulating in Iberian hares (*L. granatensis*) (Personal communication). However, no studies were conducted at the time. In 2014, a European hare (*L. europaeus*) was found dead with clinical signs of myxomatosis in Great Britain and infection by MYXV was confirmed by electron microscopy and PCR of a skin lesion [89]. However, no genetic characterization of the circulating MYXV strain was obtained. More recently, in 2019, several reports identified Iberian hare carcasses exhibiting classical symptoms of myxomatosis in the Toledo province Spain. The study of diseased Iberian hares from this region identified a novel recombinant MYXV strain (MYXV-Tol; GenBank Accession MK836424) showing, for the first time, that a pathogenic myxoma virus could also infect and cause myxomatosis in species of the *Lepus* genus [90] (described in detail in Chapter 2). Since its first report, outbreaks of the MYXV-Tol were detected in most of the Spanish regions where the Iberian hare is present as well as in some regions of Portugal [91,92]. As expected, MYXV-Tol was also found infecting wild and farming *Oryctolagus* rabbits [93,94]. These recent cases of myxomatosis outbreaks in Iberian hares seem to indicate that MYXV is evolving towards more pathogenic forms for hares, raising concerns for the future of wild leporid species.

1.2.3. Historic context: Myxoma introduction in Australia and Europe

Fossil and archaeological records show that European rabbits are native to the Iberian Peninsula and Southwestern of France. European rabbit domestication was started by the Romans who used these animals as a source of food, but due to continuous human action and a highly adaptability of this species, European rabbits today exist in the wild on almost every continent [95,96]. By 1859, feral European rabbits were introduced into the Australian wild by a Victorian landowner for sport hunting. With no natural predators and litter of five or more baby bunnies seven times a year, it took only 60 years for the European rabbits to spread across the Australian continent [97,98], which caused widespread damage to crops and contributed to the decline of native plant

and animal species [99]. Initial attempts to keep rabbits in the eastern parts of Australia from invading the western regions were largely ineffective and relied on the use of rabbit-proof fences, trapping and rabbit warren ripping and fumigation. It was not until the mid-twentieth century that the Australian government turned to biocontrol in an attempt to control the rabbit populations. Being a rabbit-specific disease and given the severity of the disease, the use of myxomatosis to control European rabbit populations in Australia was proposed as early as 1919 by Dr. Aragão [56]. Initial trials on the use of myxomatosis showed that the disease was not able to be established in arid parts of Australia, although they proved possible to eliminate rabbits in warrens in which the virus had been introduced [100]. By the late 1940s, due to a combination of favourable seasons and reduced manpower for trapping and fence maintenance (Second World War), the rabbit population rapidly reached record levels, leading to pressure for further trials of myxomatosis. A new trial conducted on the Murray River in Victoria looked to be a failure until the heavy rains of December 1950 (summer in Australia) produced a massive build-up of mosquito numbers (*Culex annulirostri*s and *Anopheles annulipes* mosquitoes) along river systems, thus providing the perfect conditions for the first epidemic spread of myxomatosis [100] (Figure 1). The released MYXV-SLS strain [60] spread like wildfire and, although no accurate counts were made of the mortality rates during the 1950-1951 epidemic, casual observations suggested that they were extremely high [100]. Following the next 2 years, aided by wet summers and inoculation campaigns, MYXV spread throughout the areas of southeastern and western Australia.

Myxomatosis also has a well-documented course in Europe. In June 1952, Dr. P. F. Armand Delille deliberately inoculated wild rabbits of his property with the MYXV-Lau strain, a lethal strain isolated in Brazil [100] (Figure 1). Within the next few years, myxomatosis had wiped out between 90-98 % of France's rabbit population. From France, the disease spread naturally into all areas of Europe where rabbits were present and reached Britain by 1953, where once again it was used as a biocontrol agent, reducing the European rabbit population by greater than 98% [101]. While in Australia the wild rabbits were seen as a plague, in Europe, they occupied several sociological and ecological niches. MYXV infections affected not only wildlife conservation and biodiversity but also affected the health of farmed rabbits, causing severe economic losses. This was particularly evident in Portugal and Spain, where rabbit farming is an active livestock production industry and rabbits are keystone species for the survival of top predators like the imperial eagle (*Aquila adalberti*), Bonelli's eagle (*Aquila fasciata*) and the Iberian lynx (*Lynx pardinus*).

1.2.4. Host–pathogen coevolution: Changes in virulence and increased resistance of European rabbits to Myxoma

1.2.4.1. Pathogenesis of Myxoma in laboratory rabbits

At the time of the first release in Australia, experimental tests in laboratory rabbits estimated the death of 99.8% of infected rabbits with the SLS and Lau strains, which was confirmed in the early epizootics [58]. Virulent SLS and Lau strains initially replicate in MHC-II+ at the dermal/epidermal interface of the inoculation site and then spread to the closest draining lymph node [102] (Figure 2). From the lymph node, MYXV disseminates to distal tissues and mucocutaneous sites such as the eyelids, anogenital region and upper nasal passages, carried in lymphocytes and possibly monocytes since there is little or no free virus in the bloodstream [102,103]. The first sign of acute myxomatosis is a large red lesion at the inoculation site that appears after 2-4 days. Swollen secondary lesions (myxomas) appear 4 days after infection over the entire body (Figure 2). Within 6-8 days, very high titres are reached in secondary cutaneous lesions and in swollen eyelids and ears, which are key sites for mosquito transmission of MYXV [103]. At this stage, conjunctival inflammation (blepharoconjunctivitis) and mucopurulent discharge from the nose and eyes occur, allowing MYXV transmission by contact. As the disease progresses, generalized immunosuppression occurs [104,105], allowing the development of severe bacterial infections that contribute to the lethality of the disease [106,107]. Infected animals often survive no more than 2 weeks before succumbing to the disease (Figure 2).

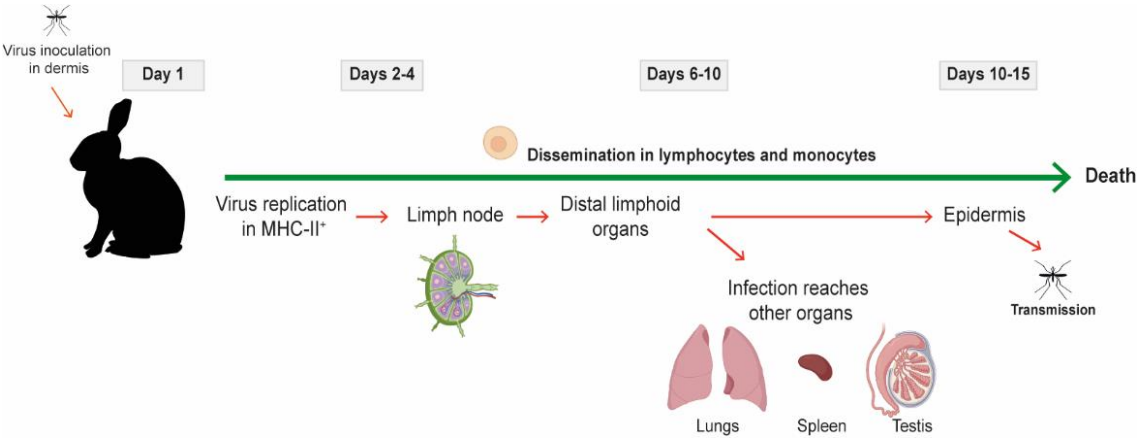

Figure 2. Pathogenesis of virulent MYXV in susceptible European rabbits. Virus inoculated intradermally by mosquitoes initially replicates in MHC-II+ cells at the dermal/epidermal junction and spreads to the overlying epidermal cells and to the draining lymph node. Replication in the epidermis induces cell proliferation and destruction with the formation of a raised primary lesion or myxoma. In the draining lymph node, MYXV induces massive loss of lymphocytes. Then, MYXV disseminates in infected lymphocytes and possibly monocytes to distal tissues including. Clinical signs become apparent from days 5–6 along with respiratory distress. Death normally occurs between days 9–12.

Infection of European rabbits with the Californian MSW strain causes a more rapid disease compared with South American strains, without showing clinical signs of myxomatosis; however, the average survival time of rabbits infected with MSW is less than that for SLS and Lau, suggesting a higher innate virulence [108]. Initial findings suggested that this strain was neurotropic, as some laboratory rabbits developed some type of central nervous system dysfunction [58]. However, later studies have shown that dysfunction of the central nervous system was not due to replication of the virus in the brain, being more likely associated with a metabolic dysfunction induced by the host response to infection [108]. In contrast to MSW, infection of laboratory rabbits with the Californian MSD strain did not cause any signs of central nervous system malfunction, resembling more the clinical signs of myxomatosis of moderately attenuated South American virus strains of MYXV [106,108].

1.2.4.2. Attenuation of myxoma strains following its introduction

One of the classic natural experiments in host-pathogen co-evolution was provided by the release of MYXV in Australia in 1950 as a biological control for the European rabbit. Following the first epizootic event of myxomatosis in Australia, the MYXV-SLS was again released in the summer of 1952/1953 with case fatality rates calculated at 99.8% [100]. During the following winter and spring season, the greatly reduced rabbit population was again increasing until another outbreak occurred. However, despite ideal conditions for transmission, the case fatality rate had dropped to 90% and rabbit survival times were higher compared to the previous release of MYXV-SLS [109], suggesting that during the interval of just 1 year the virus had changed its virulence. In an attempt to standardize virulence among the circulating MYXV strains, Fenner and Marshall (1957) grouped strains into five virulence grades defined by mortality rate, average survival times (AST) and clinical signs in small groups of laboratory rabbits [58] (Table 2). In this classification, Grade I MYXV strains were 99.5% lethal with an AST ≤ 13 days, Grade II viruses were 99% lethal with an AST between 13 and 16 days, Grade III viruses were 90% lethal with an AST between 16 and 28 days, Grade IV viruses were 60–70% lethal with an AST that ranged from 28 to 50 days, while Grade V viruses killed <30% of infected European rabbits [58]. Despite subsequent criticisms of the methods and statistical approaches [110,111], this methodology allowed the classification of hundreds of MYXV strains collected in the field and clearly showed the evolution pattern of this virus.

Table 2. Virulence grades of myxoma strains[(a)]

Grades	Mortality rate	AST days	ST range	Examples of strains
I	>99	≤ 13	8–15	South American strains Lau and SLS MSW strain
II	95–99	13 and 16	---	---
III	70–95	16 and 28	13-S	KM13 (Aust /Dec 1952)
IV	60–70%	28 to 50	15-S	Uriarra (Aust/Feb 1953)
V	N/A	N/A	15-S	Neuromyxoma Nottingham (UK/April 1955)

(a) Descriptions from Fenner and Marshall (1957). With the xception of MSW strain, all viruses are derived from Lau and SLS. AST – average survival time; ST – survival time.

The initial interaction between MYXV and European rabbits was extensively modelled based on data obtained in Australia [100,112,113]. However, despite the starting MYXV strain, vectors and environment, the overall evolution of MYXV in both continents was broadly similar, with the emergence of moderately attenuated MYXV strains and selection for genetic resistance in the European rabbit populations. Observations between 1950 and 1965 showed that in both Australia and Europe a variety of strains had emerged, with the grade III strains starting to dominate in Australia and strains of grade II virulence dominating in Britain [100]. More attenuated grade IV and V viruses were less common, while highly virulent grade I viruses became relatively rare, despite the ongoing release of virulent MYXV in the field [100]. Laboratory experiments showed that efficiency of transmission by mosquitoes/fleas was correlated with the titer of virus in rabbit lesions and the duration for which the infected animals are able to transmit the virus. For example, strains of very high virulence kill rabbits quickly, giving mosquitoes just a few days to spread the virus. On the other hand, more attenuated field strains (virulence grades IV and V) produce small viral titers and lesions that regress quickly. This is the case for infections with neuromyxoma and field isolates such as Nottingham, both highly attenuated MYXV strains that can be quickly resolved by the infected rabbit [58]. Therefore, there was a rapid natural selection for slightly attenuated MYXV strains (virulence grades II and III) which, by allowing longer survival of the infected rabbit, were more efficiently transmitted by the mosquito vectors [114].

1.2.4.3. Genetic resistance of European rabbits to Myxoma

At the same time that MYXV was evolving for less virulent strains, there was also a strong selection for resistance to myxomatosis in the wild rabbit population. When less

virulent MYXV strains appeared, higher percentages of infected rabbits survived the infection and it was clear that selection for resistance to MYXV was occurring. In England, initial tests performed in 1966 showed that resistance to grade III virus was higher in wild populations of rabbits compared to control laboratory rabbits, with wild rabbits having longer average survival times (29.8 vs. 20.2 days, respectively) and lower mortality rates (90% vs 99%, respectively) [115]. Further testing between 1978 and 1980 showed that different rabbit populations had acquired even higher resistance to the previous grade III strain, showing mortality rates of 45% compared to almost 99% for laboratory rabbits [116]. In Australia, genetic resistance to myxomatosis developed much earlier. Only eight years after the introduction of myxomatosis in Australia, when inoculated with a grade III strain, the mortality rate in wild rabbits decreased from the expected 90% to only 20% [117]. These early studies clearly showed that the proportion of rabbits surviving a MYXV infection was increasing each year, suggesting that genetic resistance to myxomatosis was increasing in wild rabbit populations. Nearly seventy years after myxomatosis decimated rabbit populations of Australia, Britain and France, a study showed that resistance of the European rabbit populations against MYXV evolved by a strong pattern of parallel evolution, with selection on immunity-related genes favoring the same alleles in Australia, France, and the United Kingdom [118]. Results from DNA extracted from nearly 200 rabbits dating from 1865 to 2013 showed that the evolution of genetic resistance to MYXV was a polygenic trait that was associated with multiple alleles shifting in frequency across the genome (homologs of CD96, FCRL, CD200-R and IFN-α), which enhanced innate antiviral immunity of rabbits against MYXV [118].

1.2.4.4. The ongoing coevolution between virus and host: evolution of the phenotype of myxomatosis

In the early 1960s, Fenner's work helped categorize hundreds of field isolates [58,117]. Despite the critical work of Fenner to the study of the early evolution of MYXV, a big part about the understanding of MYXV evolution occurred in the pre-genomic era. Genome scale analysis of MYXV evolution in both Australia and Europe revealed that there are multiple routes to viral virulence or attenuation, and that there is convergence for phenotype but not genotype among the studied MYXV strains [119,120]. Indeed, despite the large number of complete sequenced genomes, there are almost no shared mutations between viruses from the two radiations [121]. It is expected that mutations that affect genes involved in immunomodulation or host-range functions have a direct

consequence in virulence [122]. However, there are many reports of hypervirulent strains with mutations that disrupt multiple virulence genes [120,121]. Accordingly, the MSW strain, which is known to be the most virulent strain of MYXV described [58,108] has multiple virulence genes that are disrupted [123]. These comparative genomic analysis suggest that disruptions to major virulence genes are not necessarily associated with attenuation, which might be explained by: (i) multiple epistatic mutations playing a major role in virulence; (ii) a sophisticate mechanism that allow suppression of open reading frames disruptions; or (iii) acquisition of novel functions by truncated proteins/retained functional activity [120].

Apart from the different trends in genetic virulence, changes were also observed in the clinical outcome of myxomatosis. From 1979 onwards, outbreaks of a “respiratory” or “amyxomatous” form of myxomatosis have occurred in Europe [124,125]. Contrary to the typical myxomatosis form, rabbits infected by this new form do not present cutaneous tumours [126,127]. However, they maintain many of the clinical signs of myxomatosis such swollen eyelids, heads and ears, serous/purulent rhinitis and blepharoconjunctivitis [126,127]. This amyxomatous form had a high prevalence in farmed rabbits, which can be probably explained by its spread being under-diagnosed and confused with bacterial respiratory infections, allowing its ongoing transmission [128,129]. In Australia, grade I-III strains collected in the 1990s induced lethal immune collapse syndrome in laboratory rabbits with no resistance [121]. Unlike the classical cutaneous myxomatosis, infected rabbits died between days 10–15, with no clear delineation of the primary lesion at the inoculation site and rarely developed secondary cutaneous lesions [121]. Death of rabbits by acute collapse phenotype was associated with a form of septic or toxic shock characterized by an absence of cellular inflammatory, near or complete depletion of lymphocytes from lymphoid tissues and frequently the presence of bacteria throughout tissues, probably as a result of the loss of neutrophils [121]. According to the authors, these Australian isolates from the 1990s are more virulent than the parental strain SLS, suggesting that MYXV is evolving for higher virulence in the face of widespread host resistance.

The most extensively documented field model of pathogen evolution following a species jump is the introduction of the lethal MYXV into the European rabbit populations as a biological control, revealing much about the relationship between virulence and transmissibility [119–121,130]. Over time, this natural “experiment” became the bedrock theory of virus evolution, where after the initial radiation of MYXV in both continents a

reduction in virulence was selectively favoured because, by killing hosts so rapidly, highly virulent viruses had shorter infectious periods and hence lower fitness. In turn, the emergence of genetic resistance in the wild rabbit populations likely drove the evolution to more virulent MYXV, setting up an arms race between host and the virus [113,131]. Sampling of 49 years of MYXV evolution provided strong evidence that MYXV is evolving with a nucleotide substitution rate at $\sim 1\times10^{-5}$ subs/site/year, one of the highest ever reported for a dsDNA virus and similar to the one reported for Variola (VARV) virus [132]. Maybe due to strong selection for enhanced transmissibility and a variety of phenotypic traits over time, the large and complex MYXV genome provides the genetic flexibility for multiple routes of virulence/attenuation that can not be seen in other viruses. So far, it is not possible to understand the contribution of each individual mutation to the virulence and attenuation in such evolutionary complexity. Nevertheless, these detailed studies about the genomics and disease phenotypes of recent isolates show that MYXV is evolving to clinically distinct forms than the one caused by progenitors MYXV-Lau and SLS, showing once more that MYXV is able to explore many pathways to find evolutionary success.

1.3. Molecular basics of Myxoma tropism

1.3.1. Poxvirus infection

The most intensively studied poxviruses, such as VACV and MYXV, have proven to be excellent research models to study host innate recognition and virus–host protein interactions and have provided important insights into the fields of virology and immunology [133,134]. However, despite considerable advances in the comprehension of poxvirus infection, the fundamental mechanisms by which zoonotic infections occur or how poxviruses occasionally leap into novel host species are still poorly understood.

On the basis of the current knowledge, it is believed that poxviruses do not require specific host-cell receptors for virus fusion events and initiation of early transcription, being able to bind and enter mammalian cells promiscuously [16]. However, after entry and initiation of early transcription, poxviruses might not be able to complete the complex cytoplasmic replication cycle that is needed to generate progeny virus [16]. Rather, poxviral infection in host cells is highly regulated by the activation of mechanisms of defense by the cell such as apoptosis, necroptosis and the interferon pathway; and the ability of a given virus to inhibit these cellular antiviral responses [16]. Therefore, the

ultimate outcome of a poxviral infection is highly influenced by the unique repertoire of immunomodulatory and host-range genes that give each poxvirus unique properties of host range, pathogenesis and the potential for host-to-host spread [1,13,135]. It is known that the non-conserved genes located at the TIRs give each poxvirus its unique characteristics of host range, immunomodulation and pathogenesis [11]. Among these, poxviral virulence genes affect viral pathogenesis, allowing the virus to evade the host immune system and establish a successful infection [12,136]. Targeted gene-knockout studies in MYXV have been showing that deletion of virulence genes results in attenuation of the myxomatosis disease progression in rabbits, thus demonstrating their importance in mediating MYXV evasion of the host immune system [137,138]. On the other hand, proteins that specifically mediate poxvirus tropism in specific cell types or tissues are referred to as host-range factors [13,135]. These proteins are known to interact with and antagonize the activated intracellular signal pathways allowing the virus entry and spread in cells that are normally non-permissive for the wild-type virus [13,135]. For example, it is believed that *Orthopoxvirus* members are capable of replicating in a wider array of cell types compared with leporipoxviruses because of their wider spectrum of host-range factors that manipulate host signaling pathways, establishing cellular conditions that are more favorable for viral replication [13].

Next, we describe the main antiviral responses induced by the host in response to infection and give examples of known MYXV host range factors that are able to overcome the activation of the different pathways (Table 3). For a descriptive and broad example of host range genes in poxviruses, we would like to refer to other recent reviews [12,139].

Table 3. MYXV host range factors

MYXV host range factors	Protein type	Function/Targets	Cells with defects in virus host range	Main references
MT-2	TNF receptor	Apoptosis- block of TNF-mediated apoptosis	Rabbit T cells	[140,141]
MT-4	ER-localized	Apoptosis - not defined	Rabbit T cells	[142]
MT-5	Ankyrin-repeats	Cell cycle progression, phosphorylation of AKT	Rabbit T cells, human tumour cells	[143,144]
M11L	Mitochondrial	Apoptosis - mimicking of pro-survival Bcl-2 proteins	Rabbit T cells and monocytes	[142,145,146]
M13L	Pyrin domain	Inhibition of the inflammasome	Rabbit T cells, primary blood monocytes	[147,148]

M029	E3-like protein	Antiviral responses activated by dsRNA - PKR (and maybe DHX9)	Human cell lines, human tumour cells	Reviewed in [149]
M062, M063 and M064	C7L-like genes	Antagonize host restriction factor SAMD9	Human cell lines	[150]

1.3.2. Host innate immune defense mechanisms and myxoma antagonists

1.3.2.1. Host innate immune defense responses

During early stages of infection, the innate immune responses play a critical role in the host defense and recognition of viral pathogens by limiting virus multiplication, providing temporary protection against the virus onslaught. Upon entry into host cells, pathogen-associated molecular patterns (PAMPs) from poxviruses, such as DNA and RNA, as well as envelope or core proteins are recognized by several pattern recognition receptors (PRRs) that initiate the first line of defense against invading pathogens - the innate immune responses [12,151–153]. Different PPRs are known to detect poxviral infection, retinoid acid-inducible gene-I (RIG-I)-like receptors (RLRs), many RNA and DNA sensors, multiple Toll-like receptors (TLRs), and components of the inflammasome [154,155]. These antiviral proteins mediate direct antiviral effects or induce the expression of type I interferons (IFN-I), which are produced and released from cells. IFN-I then bind to specific IFN-receptors on cells, which can trigger signaling pathways resulting in the expression of genes called IFN-stimulated genes (ISGs) [156,157]. Poxviral infection can also induce multiple cytokines, such interleukin-6 (IL-6), IL-12, and tumor necrosis factor-alpha (TNF) and chemokines, which then act systemically to induce immune responses [158,159].

1.3.2.2. Myxoma evasion of PKR

Poxviral replication involves synthesis of double-stranded RNA (dsRNA) in the infected cells as a result of overlapping transcripts, which function as PAMPs and trigger the host antiviral pathways [160]. The IFN-induced dsRNA dependent protein kinase R (PKR) is expressed in most cells and is responsible for recognizing dsRNA via its two N-terminal dsRNA binding domains (dsRBDs) [161,162]. This event leads to PKR activation and autophosphorylation that results in the phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2), a product that shuts down protein synthesis and induces apoptosis [163] (Figure 3).

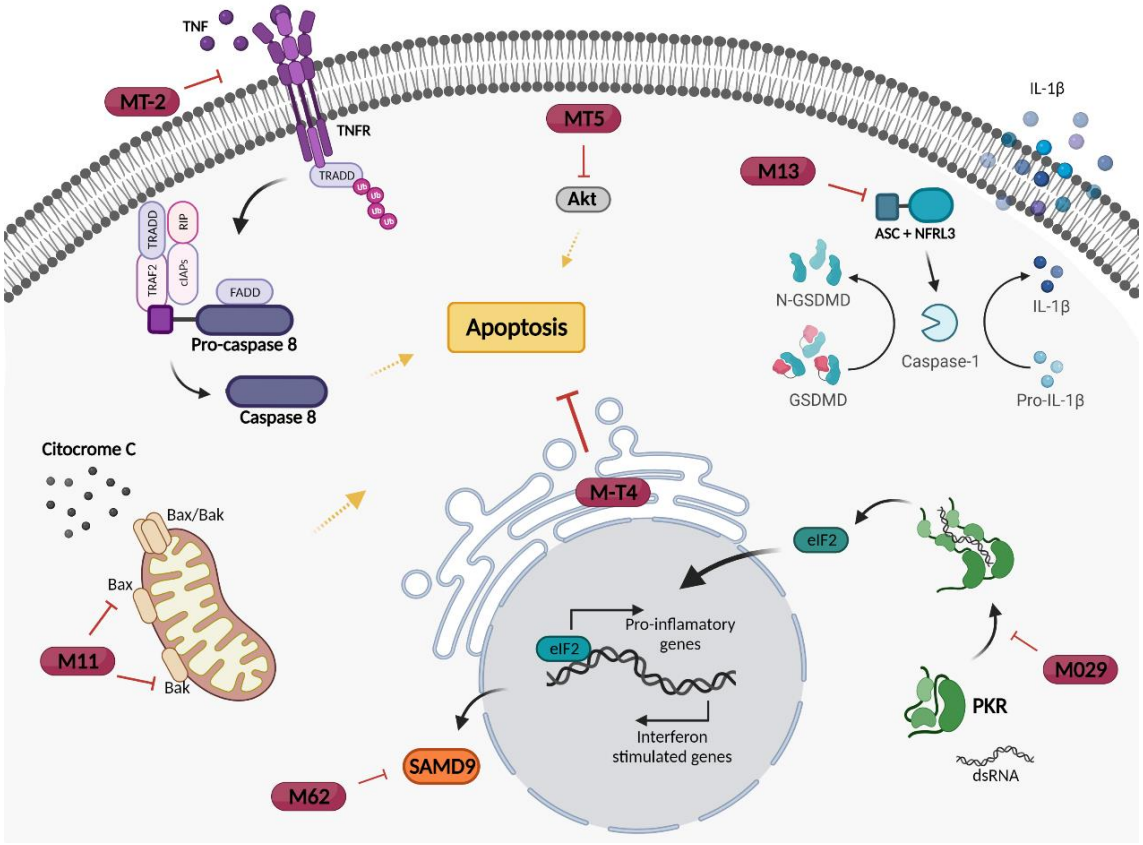

Figure 3. Molecular interactions of MYXV host-range factors with host proteins. The host range function of proteins encoded by MYXV can be attributed to several interactions with specific host proteins. Some MYXV encoded proteins encode inhibitors of proteins involved in antiviral responses, such as apoptosis, pyroptosis or translational shut-off, ultimately reducing the inflammatory or interferon response mediated by the host cell. The specificity of these interactions with their host targets can either restrict or expand the range of hosts in which MYXV can replicate. Abbreviations: TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; Ub, ubiquitin; TRADD = TNFR-1 associated Death domain protein; FADD = Fas-associated Death domain protein; cIAPs, Inhibitors of apoptosis); RIP, receptor interacting protein; SAMD9, sterile α motif domain containing 9; ASC, apoptosis-associated speck-like protein; GSDMD, effector protein gasdermin D; eIF2, eukaryotic translation initiation factor 2; PKR, protein kinase R. Organelles and proteins not drawn to scale

It is known that poxviruses encode different PKR antagonists that directly or indirectly inhibit this pathway, like is the case of poxvirus-encoded E3 proteins [164,165]. The E3-like (E3L) encoded proteins are composed by two domains: an amino (N)-terminal Z-nucleic acid-binding domain (zNABD) and a carboxy (C)-terminal dsRNABD discovered by homology to the PKR [166,167]. The VACV E3 protein is expressed early in infection and interacts with dsRNA at its C-terminal binding domain, suppressing the activation of PKR and preventing eIF2 phosphorylation and subsequent dsRNA induced apoptosis [167,168] (Figure 3). VACV with the *E3L* gene deleted displays a different growth phenotype from the WT-VACV in cell culture, a fact that can be rescued using cell lines with reduced or no PKR expression [169]. MYXV also encodes a *E3L* gene, M029, which inhibits dsRNA induced PKR activation: PKR knock-down or knockout

restored MYXV replication in human cell lines in the absence of M029 [170]. In fact, M029 is a pivotal host range protein required for MYXV replication in different cultured mammalian cell lines and its function is mediated by the inhibition of PKR activation/phosphorylation in response to virus infection [170]. Although it is clear that PKR inhibition by M029 is critical for MYXV tropism, M029 also plays a key role in the inhibition of the antiviral state induced by IFN in rabbit cells [170]. A recent study showed that M029KO virus remains sensitive to IFN-I-induced responses even in the absence of PKR and that specific ISG(s) might also be targeted by MYXV M029 for the inhibition of the IFN-I antiviral state [171]. Apart from M029, the MYXV-encoded protein M156, ortholog of the VACV K3, is a eIF2 homolog that acts as a species-specific pseudosubstrate for PKR, inhibiting its activation [122] (Figure 3).

1.3.2.3. Myxoma evasion of SAMD9 and the VACV C7-like superfamily of host range genes

One of the most critical host-range factors of poxviruses belongs to the family of host interactive viral proteins that share sequence similarity with the VACV C7 protein, known to have major roles in pathogenicity [172]. The sterile alpha motif domain-containing 9 (SAMD9), a IFN-stimulated antiviral factor, is an element that can respond to both viral stimuli (antiviral granules) and environmental stimuli (stress granules) in both eIF2α-dependent and -independent manners [173]. SAMD9 was found to be the target of MYXV-M062 protein, and knocking down SAMD9 in human cells rescued the replication defect of M062-deleted MYXV [150] (Figure 3). In most mammalian poxviruses, at least one copy of a member from the C7L host range superfamily can be found encoded in the genome [174]. However, MYXV carries three tandem C7L proteins that were probably derived by two distinct duplication events: M062, M063 and M064 [172]. Investigations on the roles of these host range genes have shown that only M062 can be a substitute for VACV C7 in overcoming host range restriction by binding directly to SAMD9, while M063 only facilitates the latter interaction [150,175]. On the other hand, M064 does not exhibit any known host range properties, but acts as a virulence factor that controls the kinetics of MYXV infection both *in vitro* and *in vivo [176]*. Interestingly, the MYXV-Tol strain, able to cause a myxomatosis-like disease for the first time in Iberian hares, acquired a recombinant region of ~2.8kb encoding for several new genes, including a novel host range gene (*M159*) that is an ortholog member of the VACV C7 host range factor family [90]. Recent observations demonstrate that M159 protein plays

a critical role in determining the host specificity of MYXV-Tol in hare and human cells by imparting new host range functions (see Chapter 5 for more information).

1.3.2.4. Superfamily of DExD/H-box helicases

Over the recent years, the cellular superfamily of RNA helicases has been identified as having an increasing number of cellular functions including oncogenesis and inflammatory responses [177]. RNA helicases also play important roles in viral infection by sensing foreign RNAs and mediating the antiviral immune response [178]. However, the influence of RNA helicases in poxvirus replication has not yet been studied in great detail. In VACV infection, it is known that the VACV K7 protein forms a complex with the DNA helicase DDX3 and antagonizes interferon-beta promoter induction [179,180]. It is also known that knockdown of DDX41 expression, a cytosolic sensor of PAMPs, blocked the production of IFN-I and IL-6 in response to VACV [181]. In MYXV infection of primary human macrophages, the cells are nonpermissive to MYXV replication because the virus is rapidly sensed by the PRR retinoic acid-inducible gene-I (RIG-I), a DEAD box RNA helicase, that then triggers induction of IFN-I and TNF [182]. A recent study also showed that in human cancer cells, the knockdown of DDX3X, DDX5, DHX9, DHX37 or DDX52 increased MYXV replication, suggesting a potential role in the regulation of either the antiviral mechanisms or the innate immune responses. Accordingly, cellular DHX9 was previously identified as a binding partner of M029 in human cancer cells [170]. On the other hand, knocking down DHX29, DHX35 or RIG-I reduced MYXV replication, which likely suggests a proviral role for these helicases [183]. While there is still much to learn about the impact of RNA helicases in poxviral infection, these early studies suggest that replication of poxviruses can be positively and negatively affected by multiple host RNA helicases.

1.3.3. Activation of Apoptosis by host cells and myxoma antagonists

1.3.3.1. Apoptosis, a crucial frontline defense mechanism for host cells

Cytokines and chemokines have a vital role in the host reaction to viral infection and their corresponding receptors are known to be important regulators of cell death. Apoptosis is a form of cellular suicide initiated either by extracellular (death receptor pathway of apoptosis) or intracellular (mitochondrial/intrinsic pathway of apoptosis) signalling that is a major defense mechanism against pathogens [184]. The death receptor pathway of apoptosis is engaged when TNF-related apoptosis-inducing ligands

(TRAIL) bind and oligomerize to a subset of TNF receptors [185]. A simplified death receptor pathway started by the death receptor TNFR-1 is shown in Figure 3. Binding of death-inducing ligands to a death receptor triggers a rearrangement in the receptor, which initiates the activation of pro-caspase-8 through interactions with the death receptor recruits FAS-associated death-domain protein (FADD) and, in some cases TNFR-associated death-domain (TRADD) [186]. Then, by interaction with different proteins, inactive pro-caspase-8 suffers homodimerization, a key step in forming active caspase which leads to cell death [186]. The mitochondrial pathway of apoptosis is often activated when there is an alteration in the expression and/or function of proteins of the Bcl-2 family [187]. The Bcl-2 family is divided into pro- and anti-apoptotic Bcl-2 members that share one or more Bcl-2 homology (BH) regions [188]. Three Bcl-2 proteins, Bax, Bak and Bok, directly cause mitochondrial outer membrane permeabilization (MOMP), which releases cytochrome c and other apoptosis inducing factors that lead to cell death [188]. Other Bcl-2 proteins, including Bcl-2, Bcl-xL, Mcl-1, among others, inhibit the action of Bax and Bak and are, therefore, named anti-apoptotic Bcl-2 proteins. A third group, the BH3-only proteins, regulates the other two, leading to the inhibition of anti-apoptotic Bcl-2 proteins and activating Bax and Bak to activate MOMP [188].

1.3.3.2. Myxoma direct evasion of apoptosis

Poxviruses have been shown to encode multiple inhibitors of extrinsic and intrinsic apoptosis and this includes viral Bcl-2 (vBcl-2) homologs, caspase inhibitors, tumor necrosis factor (TNF) homologs, and death effector proteins to evade the host immune system (reviewed in [184,189,190]).

It is known that poxviruses encode secreted versions of cytokine receptors as a unique strategy to sequester cytokines, thereby evading the host immune response [191]. The TNF receptor has a major role in initiation of the extrinsic apoptosis pathway [185]. Therefore, to avoid TNF receptors induced apoptosis, poxviruses encode anti-apoptotic proteins that mimic the action of TNF receptors or bind TNF receptors to inhibit their function [190,192]. MYXV M-T2 and SFV virus S-T2 were the first described poxviral viroreceptors and share sequence similarity with the N-terminal ligand-binding domain of TNF receptors [193]. In MYXV, M-T2 exhibits two different functions: the secreted M-T2 specifically binds and neutralizes rabbit TNF; whereas the intracellular form is not rabbit specific and has the ability to bind cellular TNF receptors, blocking the induction of TNF-mediated apoptosis [140] (Figure 3). Initial studies showed that when M-T2 was deleted from the MYXV genome (vMyx-ΔM-T2), MYXV was unable to start a successful

infection in rabbit T lymphocytes (RL-5 cell line), demonstrating the importance of this to infect lymphocytes and expand MYXV cell host range [141]. Moreover, when European rabbits were infected with vMyx-ΔM-T2, myxomatosis was observed to be significantly attenuated, showing that besides its role as a host range factor, MYXV M-T2 is also a virulence factor for myxomatosis in European rabbits [194].

Poxviruses can also block apoptosis by selectively mimicking and supplementing the action of particular pro-survival Bcl-2 proteins, which are important during viral infection and proliferation [184,189,190]. VACV F1L was the first identified Bcl-2 like protein linked to mitochondria apoptosis inhibition in VACV [195]. MYXV also encodes a vBcl-2 like protein, M11L, which is localized to the MOMP via its hydrophobic C-terminal region [142]. M11L displays high affinity toward the BH3-only protein Bim [145] but, unlike VACV F1L, M11L subverts host cell apoptosis by primarily sequestering cellular pro-apoptotic proteins Bak and Bax [142,145,146], but not Bim [196] (Figure 3). MYXV replication was severely attenuated in rabbits infected with MYXV deficient in the *M11L* gene (vMyx-ΔM11L), showing that M11L-coding protein is an important virulence factor [197]. In T lymphocytes and monocytes, infection by vMyx-ΔM11L was impaired and could not inhibit apoptosis in response to viral infection [197], also showing the importance of M11L as a host range factor by preventing apoptosis of leukocytes during host infection. Interestingly, M11L also showed ability to inhibit Fas-ligand induced apoptosis in HEK293 cells, suggesting that MYXV M11L may inhibit host apoptosis through both the intrinsic and extrinsic pathways [145].

1.3.3.3. Myxoma indirect evasion of apoptosis

Overall, the discovery of specific poxvirus host-range genes and their roles in inhibiting the host immune system has been the result of targeted gene-knockout studies, especially in VACV and MYXV [16,135]. This is the case, for example, of MYXV *M-T4* and *M-T5*-encoded proteins that are known to block apoptosis induction when the host cells sense MYXV infection. MYXV *M-T4* gene encodes a 25 kDa protein that localizes in the endoplasmic reticulum (ER) of infected cells and has no known cellular homologue [198]. When *M-T4* was deleted from MYXV genome (vMyx-ΔM-T4), cultured rabbit T lymphocytes underwent extensive cellular apoptotic response, resulting in an abortion of MYXV replication [198]. *In vivo*, infection with vMyx-ΔM-T4 resulted in myxomatosis attenuation [199]. In European rabbits, MYXV pathogenesis is dependent on the migration of infected leukocytes from the primary site to distal tissues [102,103]. Therefore, the ability of MYXV to infect lymphocytes is a critical step for progression of

myxomatosis and might explain the attenuated phenotype of vMyx-ΔM-T4 infected rabbits. Despite being suggested that M-T4 protein subverts the apoptotic pathway by interaction with ER proteins [199], the mechanisms by which M-T4 coordinates MYXV virulence and tropism are still poorly understood.

The ankyrin repeat (ANK) is a 33-residue motif that was first identified in yeast cell cycle regulators and subsequently shown to be a feature of many proteins involved in regulation of the cell cycle, transcription, development, and cytoskeletal organization [200,201]. Most of the poxviral ANK-containing proteins contain at their carboxy-termini F-box-like domains, which are also known as Pox protein repeats of ankyrin C-terminal (PRANC) domains [202,203]. However, only two ANK/F-box proteins have been identified as host range genes: CP77 in CPXV [204] and M-T5 in MYXV [143]. *In vitro*, MYXV M-T5 protein is required for its replication in T lymphocytes and primary rabbit peripheral mononuclear cells due to the shut-down of host and viral protein synthesis that induces apoptosis [143]. Moreover, MYXV lacking the *M-T5* gene (vMyx-ΔM-T5) showed a replication defect in different human tumor cells that were permissive for the wild type virus [144]. Several studies showed that cell death was increased in vvMyx-ΔM-T5-infected cells because it was not able to overcome the G0/G1 arrest induced by the viral infection, suggesting that M-T5 protein is able to manipulate the cell cycle progression at the G0/G1 checkpoint. Thus, control of the cell cycle in both rabbit lymphocytes and transformed human cells might protect infected cells from diverse innate host antiviral responses normally triggered by G0/G1 cell cycle arrest [205].

1.3.4. Pyroptosis in response to poxviral infections and antagonists

1.3.4.1. Pyroptosis, an inflammatory form of lytic cell death

Pyroptosis, a form of regulated necrosis, is activated when the effector protein gasdermin D (GSDMD) is cleaved by the action of different caspases, which promotes its oligomerization to form large pores in the plasma membrane and cell death [187,206]. To cleave GSDMD, Caspase-1 must first be engaged by a large caspase activation platform, called the inflammasome [187] (Figure 3). To date, four distinct caspase-1-dependent inflammasomes have been identified: RIG-I inflammasome, the absent in melanoma 2 (AIM2) inflammasome, the interferon-gamma inducible protein 16 (IFI16) inflammasome and the NLR family pyrin domain-containing 3 (NLRP3) inflammasome [207]. In response to viral infection, the oligomerization of RIG-I, NLRP3, AIM2 or IFI16 recruits the apoptosis-associated speck-like protein (ASC) through homotypic PYD–PYD

interactions, which then activates caspase-1 and pro-inflammatory cytokines (IL-1β and IL-18) [187,206]. Pyroptosis is associated with a strong inflammatory response because following rupture of the cell membrane by GSDMD, large amounts of inflammatory cytokines and DAMPs are released into the extracellular environment [207,208].

1.3.4.2. MYXV M13L allows evasion of the NLRP3 inflammasome

To avoid induction of apoptosis through activation of the inflammasome NLRP3 protein, MYXV encodes an inhibitor of both the inflammasome and the NF-κB pathway, the M13L protein [147,209]. MYXV M13L protein is a small 127 aa protein that contains a pyrin domain (PYD/PAAD) that can directly interact with ASC to inhibit the interaction between NLRP3 and ASC (Figure 3), resulting in the inhibition of activated caspase-1 and consequent secretion of IL-1β and IL-18 [148]. MYXV with a deletion of the *M13L* showed normal replication in rabbit RK13 cells, whereas in RL5 cells and in primary blood monocytes the virus was not able to replicate [147]. Moreover, this virus was attenuated in a rabbit model for MYXV infection [147]. In a similar way, the gp013 protein from RFV also interacts with ASC to interfere with PYD-mediated activation of caspase-1 [210].

1.3.5. Poxviral evasion of necroptosis

1.3.5.1. The necroptotic pathway

The innate immune response not only restricts viral replication, but also serves to promote anti-viral inflammation through cell death-associated release of damage-associated molecular patterns (DAMPs). In recent years, necroptosis has been recognized as an important response against many viruses [211–213].

Necroptosis is an inflammatory form of regulated necrosis that acts as an alternative host defense pathway during the course of some viral infections and is known to play a major role in the killing and removal of pathogen-infected cells [211–213]. Activation of necroptosis follows an intracellular signaling cascade that is dependent on the receptor-interacting serine/threonine-protein kinase 3 (RIPK3) and its substrate, the mixed lineage kinase like protein (MLKL) downstream of death receptors (DRs) and PPRs (Figure 4) [214,215]. Nevertheless, several pathway-specific adaptor proteins that contain a RIP homotypic interaction motif (RHIM-domain) are also required for necroptosis induction. When there is an interference or loss of function of caspase-8, the induction of necroptosis through the use of DRs results in the recruitment of RIPK1,

which subsequently exposes its RHIM-domain to recruit RIPK3 (Figure 4) [216–218]. The TIR-domain-containing adaptor-inducing IFN β (TRIF) is an essential protein downstream of Toll-like receptor (TLR)3/4, while the Z-DNA binding protein 1 (ZBP1) also contains two RHIM-like domains, which allows it to directly activate RIPK3 (Figure 4) [219,220]. Exposure of RIPK3 to a RHIM adaptor (RIPK1, TRIF or ZBP1) is a key step in the initiation of necroptosis, as these proteins activate the downstream executor or necroptosis MLKL that destabilizes the plasma membrane integrity leading to cell swelling followed by membrane rupture of infected cells and release of DAMPs [212,221]. This way, necroptosis provides an extra defense mechanism against pathogen infection, facilitating the elimination of virus-infected cells before the production of progeny virions. The importance of necroptosis for host defense is further supported by the identification of viral inhibitors of necroptosis, like is the case of VACV E3 protein [221].

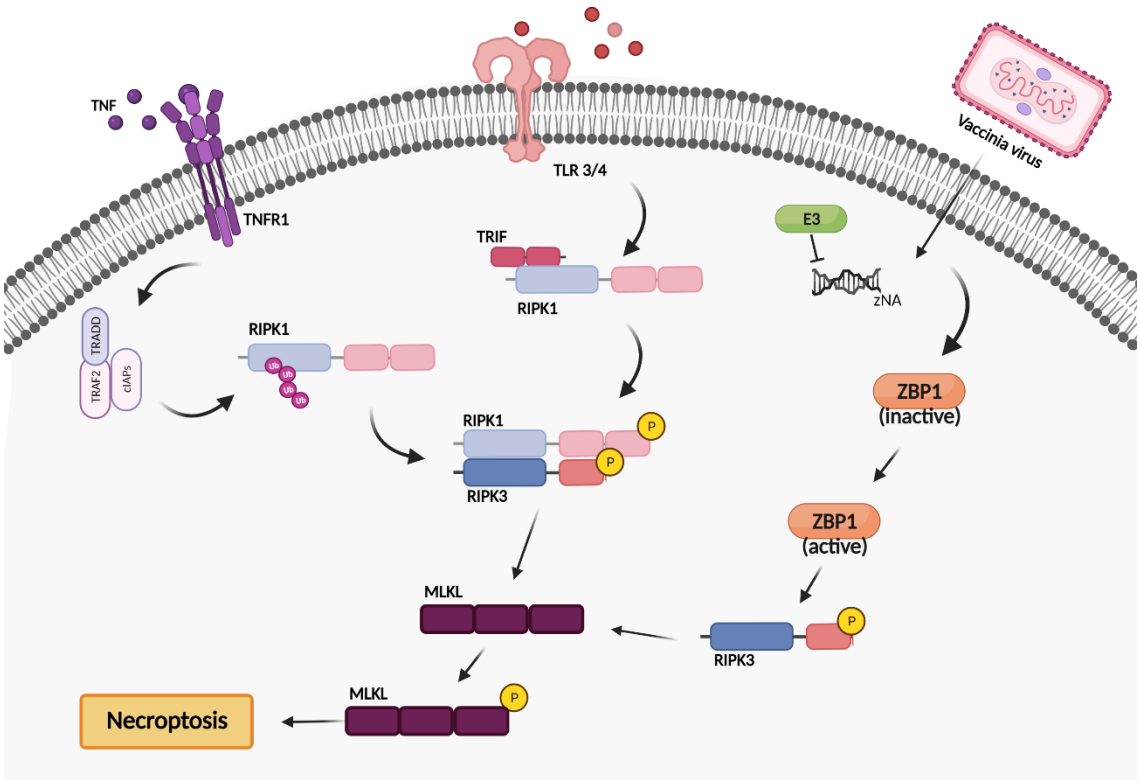

Figure 4. The necroptotic signaling pathway. Simplified schematic representation of necroptosis upon stimulation of the TNFR, TRF3/4 and infection by Vaccinia virus (VACV). All of these necroptosis-inducing signals converge on the kinase RIPK3, which is activated through the homotypic interaction with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

1.3.5.2. Poxviral evasion of necroptosis

The ZBP1, also known as a DNA-dependent activator of IFN-regulatory factor (DAI), is a key innate sensor that recognizes and binds Z-DNA structures (Figure 4). Upon binding to Z-DNA, ZBP1 dimerizes and recruits several proteins that induce IFN-I and the factor nuclear kappa B (NF-kB) pathway [222,223]. Activated ZBP1 also interacts with RIPK3 via RHIM that leads to the activation of MLKL [219,220]. E3L proteins not only inhibit the IFN-induced PKR, but also sequester dsRNA limiting the activation of the innate immune system against the virus infection (Figure 4). In fact, strong evidence showed that the VACV E3 N-terminal domain competes with ZBP1 to prevent ZBP1-dependent activation of RIPK3 and consequent necroptosis [224,225]. The model proposed by the authors suggests that during WT-VACV infection, the zNA-BD of E3 binds to VACV-induced Z-form nucleic acid and masks it, preventing sensing by ZBP1 and further RIPK3 necroptosis induction (Figure 4) [224,225].

Of the poxviruses that contain a VACV E3 homologue, it is well established that MPXV and the leporipoxviruses MYXV and SFV lack a full-length homologue [52,190,226]. The genomic sequence of the E3L orthologue from MPXV (F3L) shows that this protein has a complete and functional dsRNA-BD, while the N-terminal region has a truncation of the first 37 amino acids [227]. Leporipoxviruses E3-like-encoded proteins (M029 in MYXV) lack the entire N-terminal zDNA-BD while having a complete C-terminal domain [149,171]. Recently, the genome characterization of a CePV from a bottlenose dolphin (*Tursiops aduncus*) predicted the presence of two VACV E3L homologues: while the CePV-TA-21 protein was shown to have a shorter N-terminal zDNA-BD domain and a complete C-terminal dsRNA-BD, the *CePV-TA-20* gene was predicted to encode a truncated E3L homolog with only the C-terminal dsRNA-BD [3]. Given the importance of the necroptotic pathway for virus infected cells, an intriguing question emerges as how these viruses are able to replicate in their natural host even in the absence of a complete zDNA-BD (studied in detail in Chapter 5).

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Chapter 2

Genetic characterization of a novel MYXV strain and identification of viral co-infections in Iberian hares

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Genetic Characterization of a Recombinant Myxoma Virus in the Iberian Hare (*Lepus granatensis*)

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Abstract

Myxomatosis is a lethal disease of wild European and domestic rabbits (*Oryctolagus cuniculus*) caused by a Myxoma virus (MYXV) infection, a leporipoxvirus that is found naturally in some *Sylvilagus* rabbit species in South America and California. The introduction of MYXV in the early 1950s into feral European rabbit populations in Australia and Europe demonstrate the best documented field example of host-virus coevolution following a cross-species transmission. Recently, a new cross-species jump of MYXV has been suggested in both Great Britain and Spain, where European brown hares (*Lepus europaeus*) and Iberian hares (*Lepus granatensis*) were found dead with lesions consistent with those observed in myxomatosis. To investigate the possibility of a new cross-species transmission event by MYXV, tissue samples collected from a wild Iberian hare found dead in Spain (Toledo region) were analyzed and deep sequenced. Our results report a new MYXV isolate (MYXV Toledo) in the tissues of this species. The genome of this new virus encodes three disrupted genes (*M009L*, *M036L* and *M152R*) and a novel 2.8kb recombinant region that resulted from an insertion of four novel poxviral genes towards the 3' end of the negative strand of its genome. From the open reading frames inserted into the MYXV Toledo virus, a new orthologue of a poxvirus host range gene family member was identified which is related to the MYXV gene *M064R*. Overall, we confirmed the identity of a new MYXV isolate in Iberian hares that we hypothesize was able to more effectively counteract the host defenses in hares and start an infectious process in this new host.

Keywords: poxvirus; Myxoma virus; recombinant virus; *Lepus granatensis*

1. Introduction

Myxoma virus (MYXV), a poxvirus belonging to the *Leporipoxvirus* genus, is the etiological agent of myxomatosis which is a highly lethal viral disease of wild and domestic European rabbits (*Oryctolagus cuniculus*) [1]. The classical form of the disease is characterized by systemic spread of the virus, overwhelming the immune system, and the development of secondary skin lesions called 'myxomas' [2, 3]. Mortality rate varies between 20-100%, according to the grade of virulence of the MYXV strain [3]. The virus has its natural host in the South American tapeti, or forest rabbit (*Sylvilagus brasiliensis*), where it causes an innocuous and localized cutaneous fibroma at the inoculation site [2]. Related poxviruses to MYXV are found in other *Sylvilagus* species in North America: the Californian MYXV strains, for which the natural host is *Sylvilagus bachmani* (brush rabbit), and rabbit fibroma virus (RFV) found in *Sylvilagus floridanus* (eastern cottontail) [2, 4]. MYXV not appearing to cause significant clinical disease in the natural *Sylvilagus* hosts, though being highly pathogenic to the naive *Oryctolagus* host, made it a classic example of a pathogen that is highly virulent in a new host species with no evolutionary history of adaptation to that pathogen.

In 1950, with the urge of controlling the infesting population of European rabbits in Australia, a MYXV strain originally isolated in Brazil (standard laboratory strain [SLS]) was used as a biological agent [1]. The release in France in 1952 of a different Brazilian isolate of MYXV (Lausanne [Lu] strain) resulted in the establishment and spread of MYXV in Europe, including the United Kingdom (UK) [5]. After an initial massive reduction of the wild rabbit populations (>99%) in both Continents, a substantial decline in the case fatality rates occurred as a result of natural selection for slightly attenuated viruses, but also due to an increased resistance to myxomatosis in the rabbit populations [4, 6, 7]. It has been recently shown that the convergent phenotype of viral resistance observed in Australia, France and UK rabbit populations was followed by a strong pattern of parallel evolution, a consequence of selection acting on standing genetic variation that was present in the ancestral rabbit populations in continental Europe [8].

The susceptibility of other leporids species to MYXV has been tested in controlled experiments, while evidence of myxomatosis in wild leporid populations have been seldom reported. Using a California MYXV strain four different North American *Sylvilagus* species (*S. audubonii*, *S. floridanus*, *S. idahoensis* [now *Brachylagus idahoensis*] and *S. nuttallii*) developed tumors following mosquito transfers, but these failed to be mosquito-infective lesions [9]. Three of these *Sylvilagus* species (*S. audubonii*, *S. floridanus* and

S. nuttallii) when infected with the Brazilian Lu strain also developed prominent tumors, however this time the South American strain produced mosquito-infective lesions [10]. On the other hand, black-tailed jackrabbits (*Lepus californicus*) inoculated with Californian MYXV did not form tumors [9]. In wild populations of European hare (*Lepus europaeus*) cases of myxomatosis have been reported sporadically and in small number. In the past, the confirmation of the disease arose from injecting rabbits with tissues from dead hares and replicating its typical clinical symptoms [11]. Most recently, in 2014, for the first time a case of myxomatosis in a European brown hare in Great Britain was confirmed using electron microscopy and a PCR of a skin lesion [12].

Recently, in late summer-fall of 2018, the first cases of myxomatosis in Spanish wild Iberian hare (*Lepus granatensis*) populations were reported, mainly in the Andalusia and Castilla-La Mancha regions. The Spanish Ministry of Agriculture, Fisheries and Food, and the Institute for Game and Wildlife Research identified what appeared to be a cross-species transmission into a new leporid species. Iberian hares were found in moribund state, with signs of blindness, weakness and disorientation, and consequently analyzed in different laboratories. Here, using culturing and deep sequencing, we genetically characterize for the first time a recombinant MYXV isolated from an Iberian hare carcass exhibiting classical symptoms of myxomatosis collected in Toledo province, Spain during the 2018 outbreak (referred to as MYXV Toledo).

2. Methods

2.1. Sampling and pathology

An adult Iberian hare (*L. granatensis*) female, was found dead on 21st of August 2018 in La Villa de Don Fabrique municipality in the Toledo province of Spain. The hare manifested lesions compatible with myxomatosis in European rabbits (Figure 1) and was completely emaciated (kidney fat index = 0). On arrival at the laboratory, duplicate samples (4mm diameter) were taken from eyelid, ear and vulva and stored in RNAlater and without preservative, at -80ºC. For the histopathological study, representative samples of the main organs and tissues, were fixed in 10% buffered formalin for 48-72 hours at 22±2ºC, and then, dehydrated in a graded series of ethanol, immersed in xylol, and embedded in paraffin wax using an automatic processor. Sections were cut at 4 µm and stained with hematoxylin and eosin (H&E), following standard procedures.

2.2. Cell lines

European rabbit RK13 kidney epithelial cells (Millipore Sigma, USA) were maintained in Dulbecco's modified Eagle medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml of penicillin/streptomycin. Cells were maintained at 37°C in a humidified 5% CO_2 incubator.

2.2.1. Isolation, replication and purification of the new Myxoma virus (MYXV Toledo)

Samples from lesions of the eyelid of an Iberian hare (*L. granatensis*) specimen were manually homogenized. A small volume (5-10 µl) of the processed tissues was used to inoculate confluent RK13 cells monolayers in a 6-well plate and allowed to incubate at 37ºC. At 2 days after infection, distinctive MYXV foci were visualized using a Leica DMI6000 B inverted microscope (Supplementary Figure 1). To proceed with the virus isolation, infected cells were harvested, freeze-thawed at −80°C and 37°C for three times and sonicated for one minute to release the viruses from infected cells. The virus was inoculated back onto a confluent RK13 cells monolayer in a 150 mm dish and incubated at 37ºC for 48 hours. Cells were collected to perform a serial dilution and the one with the best individualized foci (dilution 10^{-5}) was used for inoculating a new 150 mm dish. After 2 days of infection, a last round of cell harvest, freeze-thaw cycles and sonication was done before proceeding to virus amplification into twenty 150 mm dishes. Purification of the virus through a 36% sucrose cushion was performed as described before [13]. Titration of the number of plaque forming unitsof virus was determined by crystal violet foci staining of the infected RK13 cell monolayers, while the total number of viral particles was counted using the NanoSight NS300 instrument (Malvern Panalytical, USA).

2.2.2. Viral nucleic acid extraction, Illumina sequencing and de novo assembly of the genome

Total viral nucleic acid was extracted from 200 µl of the viral preparation using a phenol-chloroform extraction protocol as previously described [14]. The viral DNA was used to generate a 2×100 bp Illumina sequencing library and this was sequenced on a Illumina HiSeq4000 (Illumina, USA) at Macrogen Inc. (Korea). The paired-end raw reads (40,730,938 reads) were *de novo* assembled using metaSPAdes v3.12.0 [15] with kmer of 33, 55 and 77. The *de novo* assembled contigs then assembled into a genome length contigs using MYXV-Lu (GenBank accession # MK836424) as a scaffold, primarily to

resolve the terminals redundancy. The quality of the final assembly was verified by mapping the raw reads back to the genome using BBMap [16] (Supplementary Figure 2).

2.3. Genome analysis

All MYXV and poxvirus RefSeqs were downloaded from GenBank on the April 9, 2019. Global alignments of the MYXV with the genome determined in this study were carried out using MAFFT [17]. ORFs in the genome were determined with ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) coupled with a local MYXV ORF database generated from the MYXV genomes. ORFs that did not have any similarity to MYXV ORFs were analyzed using BLASTn and BLASTx sequence queries [18]. All pairwise identities (nucleotide and protein) were calculated using SDV v1.2 [19].

Nucleotide sequence and protein sequence aligns were undertaken using MAFFT [20]. The nucleotides alignments of the genomes, and the recombinant gene 'cassette' –like sequences used to infer maximum likelihood phylogenetic trees using PHYML 3.0 [21] with substitution model GTR+G+I.Amino acid alignments of the newly derived poxvirus virion protein, thymidine kinase, host range protein and poly(A) Polymerase subunit were used to inferred maximum likelihood phylogenetic trees using PHYML 3.0 [21] with substitution models JTT+G, WAG+G+F, JTT+G+F and JTT+G+F respectively, determined using ProtTest [22]. Branches with aLRT support of <0.8 were collapsed using TreeGraph2 [23].

3. Results and Discussion

The natural host for MYXV is the South American tapeti (South American strains) [2, 4]. As expected from predictions of long-term virus/host co-evolution, MYXV strains are highly adapted to their natural hosts, causing only benign cutaneous fibromas [4]. However, when another susceptible host becomes available to the virus transmission system, in this case the European rabbit (*Oryctolagus cuniculis*), a successful cross-species transmission can occur. Indeed, when MYXV first entered the European rabbit host, it was immediately pathogenic and caused close to 100% mortality. After the use of MYXV in the 1950s to control feral rabbit populations in Australia and Europe, rapid co-evolutionary changes occurred in both rabbit host and virus, due to increased resistance of rabbit populations and the appearance of less virulent virus strains [8, 24]. In 2014, a study reported the presence of a myxomatosis-like disease in the European

brown hare (*Lepus europaeus*) [12]. However, a MYXV virus capable of infecting hares has not been previously genetically characterized. More recently, reports of abnormal mortalities in Iberian hares were described in the Spanish regions of Andalucía, Castilla-La Mancha, Extremadura, Madrid and Murcia. The animals found in the hunting grounds presented with inflammation of the eyelids, conjunctivitis and also inflammation of the perianal area, symptoms consistent with classic rabbit myxomatosis.

In this study, a new MYXV virus (MYXV Toledo) was isolated and sequenced from an Iberian hare found in Toledo province that presented the classical lesions of myxomatosis, including a bilateral blepharitis and conjunctivitis, and a swollen vulvar and anal region (Figure 1A, 1B). The basal third of the left ear presented two myxoma-like lesions of 5 mm diameter (Figure 1B). Moreover, epistaxis and strong congestion of the trachea were observed, whereas the lung was swollen and presented few petechial hemorrhages. Histopathology analysis of the eyelid skin revealed the typical proliferation and ballooning degeneration of the epidermal cells, containing single large rare, intracytoplasmic, round and eosinophilic inclusion bodies (Figure 1C). In this tissue, a severe acanthosis with erosion and ulceration was observed. Blepharoconjunctivitis lesions were also associated with an inflammatory cell response in the underlying dermis, with infiltration of large macrophage-like cells, diffuse edema and fibrin deposition (Figure 1D). In the lung, mild congestion, alveolar edema and hemorrhages were observed. These vascular lesions were also recorded in the liver and kidneys.

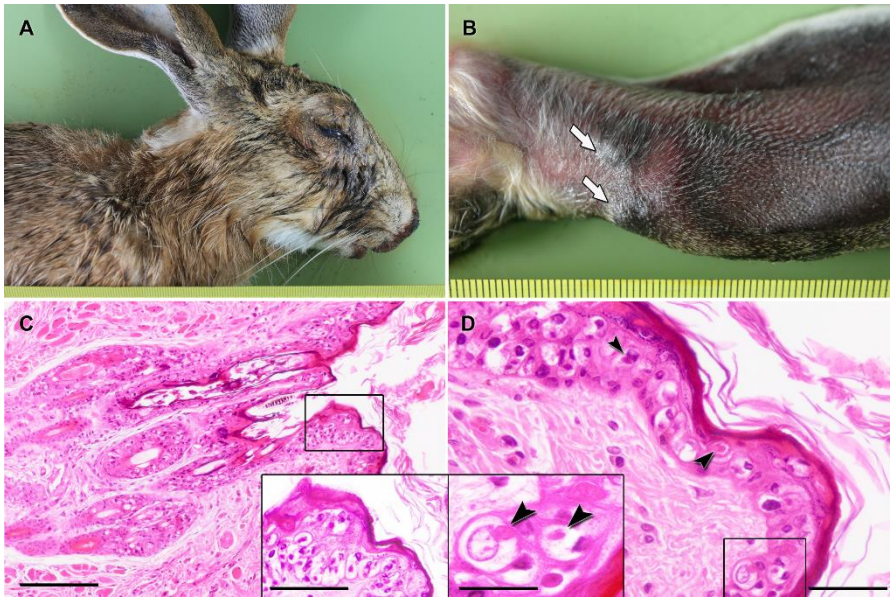

Figure 1. Iberian hare with myxomatosis-compatible lesions. A) Blepharitis and conjunctivitis with seropurulent discharge. B) Myxomas at the base of the left ear (arrows). C) Severe acanthosis of the eyelid skin, with hyperkeratosis. D) Ballooning degeneration of the epidermal cells, and intracytoplasmic eosinophilic inclusion bodies in the eyelid skin (arrowheads). Bars: 100 µm (C), 25 µm (inset in C, D), 10 µm (inset in D).

3.1. Comparison of MYXV Lausanne variant with the newly discovered MYXV Toledo variant and has of RFV Kasza

From the collected samples, a new MYXV was isolated which we have named MYXV Toledo (MYXV-To). The *de novo* assembled genome is 164,579 bps (Sup Figure 2). This genome was aligned to MYXV-Lu (GenBank accession # AF170726) and RFV-Ka (GenBank accession # AF170722) for preliminary analysis (Figure 2). The MYXV-Tol genome (GenBank accession # MK836424) was found to be ~2,800 bp longer than the one reported for the MYXV-Lu strain (161,777 bp) [25].

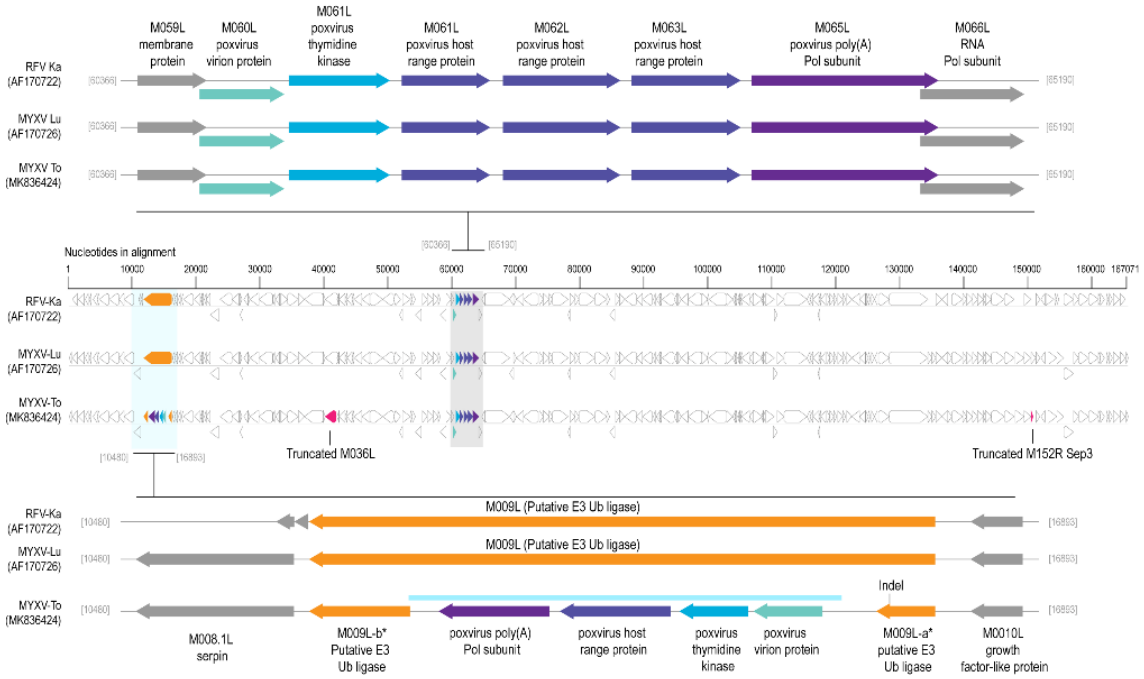

Figure 2. Representation of the aligned genome organization of both RFV-Ka (AF170722), MYXV-Lu (AF170726) and MYXV-To (MK836424): blue ORF illustrations represent truncated genes; purple show the location of M060R, M061R, M062R, M063R, M064R and M065R genes in both MYXV virus, orange shows the M009L gene (intact in RFV-Ka, MYXV-Lu and disrupted in MYXV-To) and shades of blue represent the new gene 'cassette' identified in MYXV-To that is highly likely derived from a recombinant event with an unsampled poxvirus.

Based on the published genome sequence, the MYXV-Lu strain has a total of 171 genes (12 of which are duplicated in the terminal inverted repeats (TIRs) regions) [3, 25] and can be divided into three regions: the terminals, 14.1 kb extending from the left TIR (*M0005.1L* to *M011L*) and 23.1 kb extending from the right TIR (*M143R* to *M000.5L*) mostly contain genes involved in the MYXV virulence and host subversion, while the central 124.5 kb region (*M012L* to *M142R*) includes a mixture of virulence genes and essential viral genes conserved across all poxviruses [2, 26]. Around ~99 % of the encoded gene products of MYXV-To were identical to those of MYXV-Lu, with the exception the ORFs *M009L*, *M152R* and *M036L*. Phylogenetically, MYXV-To clusters with majority of the MYXV genomes (Sup Figure 3; Sup Data 2). Furthermore, we

identified a novel insertion of ~ 2,800 bp within the *M009L* gene that spans the 12,236 to the 15,082-bp region (i.e. within the 10,480-16,893 nts alignment position in Sup Data 1) of the left end of the MYXV-To genome (Figure 2).

3.2. Viral genes disrupted in the new MYXV-To isolate

As previously reported for MYXV isolates from feral rabbits in Australia and Great Britain, single or multiple indels that result in the disruption of ORFs are relatively common [27-29]. In the Lausanne strain, *M009L* encodes a putative E3 ubiquitin (Ub) ligase of 509 aa with a N-terminal BTB-BACK domain followed by 4 Kelch motifs [30]. Our genomic analysis reveals that ORF *M009L* of MYXV-To is disrupted by an insertion of four nucleotides (+TATA, at position 15,586-bp), causing a frameshift mutation. This indel results in a smaller truncated M009L predicted protein of 148 aa. Several reports show that this same gene is also disrupted in multiple Australian MYXV strains [28], as well as in the Californian MSW strain [16], which suggest that the disruption of this gene does not abrogate MYXV survival in the wild. Four additional nucleotides were also found in the *M036L* gene (+TTTT, position 42,007 bp), thereby creating a premature stop codon in frame within this gene. M036L is an orthologue of the O1 protein that is found in the orthopoxvirus vaccinia virus (VACV) [29]. However, the function of M036L in the MYXV virus is not reported. A previous study showed that certain MYXV field isolates carry a deletion of 89 nt in this gene [31]. However, this indel appear to have no major effects in the survival and spread of MYXV in rabbits [31]. In the MYXV-Lu, ORF M152R encodes a serine proteinase inhibitor (Serp3) of 266 aa [32]. In the MYXV-To virus, this gene is disrupted as a result of an insertion of a single nucleotide (+C, at position 150,688 bp), resulting in the appearance of an early stop codon. The exact biological function of Serp3 is not known in MYXV. To date, two other serpins have been identified in MYXV, Serp1 and Serp2 [33], both of which are implicated in the modulation of host inflammatory responses [34-36]. Phenotypically, the deletion of specific host range proteins inevitably results in the reduced ability of the resulting virus to infect cells or tissues of species for which the parental virus was adapted. For this reason, we consider it less likely that the truncation of *M152R* contributes to the observed virulence of MYXV-To in Iberian hares.

3.3. Analyses of the new recombinant region of the MYXV-To isolate

Analyses of the MYXV-To genome sequence revealed an insertion of ~2,800 bp in the left side of the genome (Figure 2). This new recombinant region encodes at least four genes that are predicted to encode four viral proteins that are homologous, but not

identical, to the poxvirus gene families exemplified by the *M060R*, *M061R*, *M064R* and *M065R* genes from MYXV. We exploited sequence similarity searches to predict the functions of these new MYXV-To proteins. According to the obtained results, the recombinant region encodes a known virion protein (rPox-virion protein), followed by a thymidine kinase (Recombinant pox virus thymidine kinase; rPox-thymidine kinase), a C7L-like host range protein (rPox-host range protein) and a poly A polymerase subunit (rPox-poly(A) Pol subunit) (Figure 2). In the MYXV-Lu genome, the region that spans the locus at ~57,500 bp include a set of six genes that are present in all MYXV (*M060R* to *M065R*) [25, 37]. The predicted functions for the proteins found in the recombinant region are in accordance to those found in the ~57,500 bp region of other MYXV virus [37]. However, it should be noted that the *M062R* and the *M063R* genes that are present in all MYXV virus are not present in the new recombinant insertion region at the left end (encoded on the minus strand) of MYXV-To (Figure 2). A BLASTn-based coupled with complete genome searches of all poxviruses for the complete recombinant region with the new four gene “cassette” revealed that this virus gene arrangement is only found in genomes (encoded on the positive strand) of capripoxviruses, cervidpoxviruses, suipoxviruses, yatapoxviruses and three unclassified poxviruses (BeAn 58058 virus, cotia virus and eptesipoxvirus; Figure 3) sharing 69-73% nucleotide identity (Figure 4). These results suggest that the recombinant region is derived from a new still-unreported or unsampled poxvirus that shares a common ancestral origin with capripoxviruses, cervidpoxviruses, suipoxviruses, yatapoxviruses and three unclassified poxviruses (BeAn 58058 virus, cotia virus and eptesipoxvirus).

Occurrences of recombination between leporipoxviruses have been described before. In fact, it was established that the malignant rabbit fibroma virus (RFV) is a result of a recombination event between two other leporipoxviruses, the Shope rabbit fibroma virus (SRFV) and MYXV [24, 25]. The recombinant MRV was capable of immunosuppression and fatal malignancy in a broader host range unlike the case of SFV but more like MYXV [26-29].

Figure 3. Illustration of the 'four gene cassette' sequences identified in the genomes of representative capripoxviruses, cervidpoxviruses, suipoxviruses, yatapoxviruses and three unclassified poxviruses (BeAn 58058 virus, cotia virus and eptesipoxvirus) that share similarity to the one found encoded on the negative strand of MYXV-To genome sequence.

The rPox-thymidine kinase predicted protein sequence shares ~70% identity to its homologous protein from leporipoxviruses and 60-65% identity to that of capripoxviruses and cervidpoxviruses (Figure 5). The rPox-virion protein predicted protein sequence shares 73% amino acid identity to those of leporipoxviruses and 63-70% with those of centapoxviruses, capripoxviruses and orthopoxviruses (Figure 6). rPox-poly(A) Pol subunit shares the highest amino acid pairwise identity (80-86%) with those from capripoxviruses, cervidpoxviruses and leporipoxviruses (Figure 7). On the other hand, the newly identified rPox-host range protein of MYXV-To is the least conserved among the proteins found in the recombinant region, sharing only 35-40% amino acid identity with M064R protein family of centapoxviruses, cervidpoxviruses and leporipoxviruses (Figure 8). Moreover, it should be noticed that the new rPox-host range protein also shares ~40% identity to the M062R protein found in MYXV strains and RFV and ~28% amino acid pairwise identity to the M063R protein, also found in MYXV and SRFV. Although the proteins found in the new recombinant region of MYXV-To share higher

pairwise identity to their homologous versions found in leporipoxviruses, it should be noted that in most cases a small difference (~5% pairwise identity) segregate them from, for example, centapoxviruses and cervidpoxviruses. Moreover, and as mentioned before, the new recombinant region only presents one member of the C7L-like host range gene superfamily. In fact, leporipoxviruses constitute a unique example in the evolution of this gene family, since they encode three related C7L-like gene members in tandem, *M062R* and *M063R* and *M064R* [30]. It is suggested that the emergence of these three C7L-like gene copies in MYXV arose after two events of gene duplication [30]. In our results, we report that the new recombinant insertion region of MYXV-To only contains one predicted host range protein (Figure 2 and 3), which reinforces our hypothesis that this new gene insertion region found at the left end of the MYXV-To genome is probably not a result of a recombinant event between two leporipoxviruses, but rather between MYXV and a still-unidentified poxvirus.

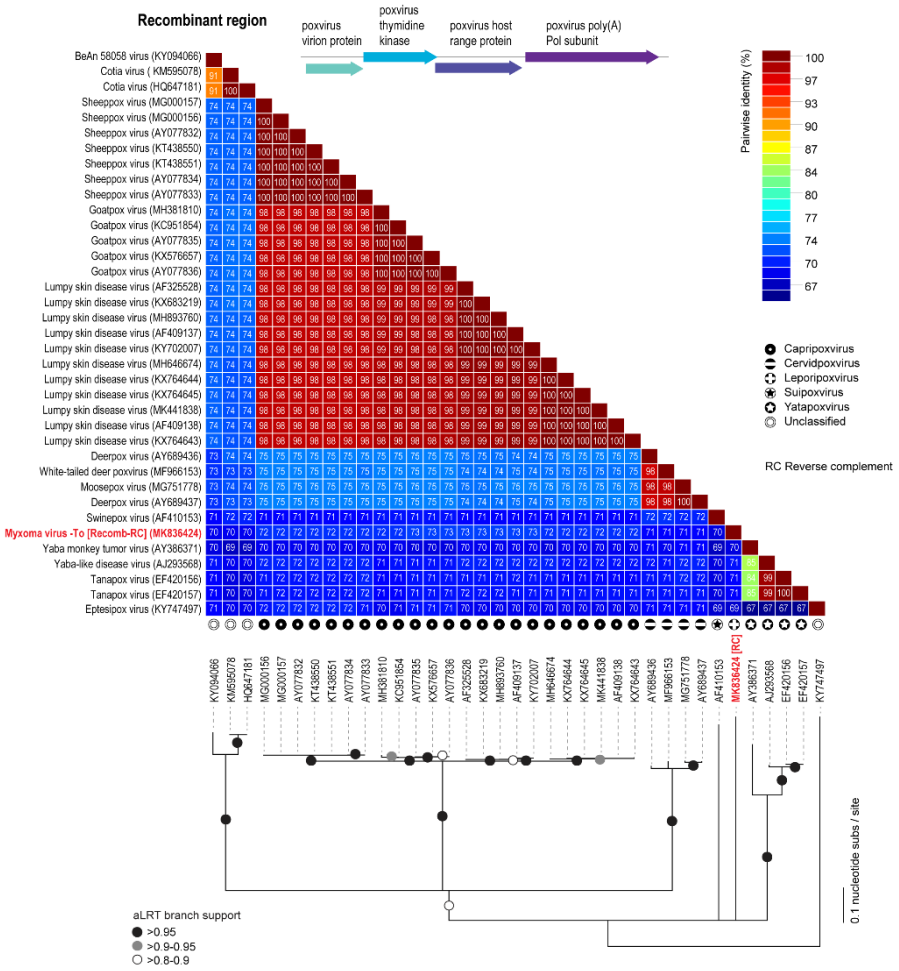

Figure 4. Pairwise nucleotide identity matrix (upper image) and maximum-likelihood phylogenetic tree (model GTR+G+I) showing the relationships of the MYXV-To recombinant 'four gene cassette' to similar sequences in genomes of genomes of capripoxviruses, cervidpoxviruses, suipoxviruses, yatapoxviruses and three unclassified poxviruses (BeAn 58058 virus, cotia virus and eptesipoxvirus). Branches with aLRT support 0.95 are indicated with black circles whereas branches exhibiting 0.9-0.95 and 0.8-0.9 are indicated with grey and white circles, respectively.

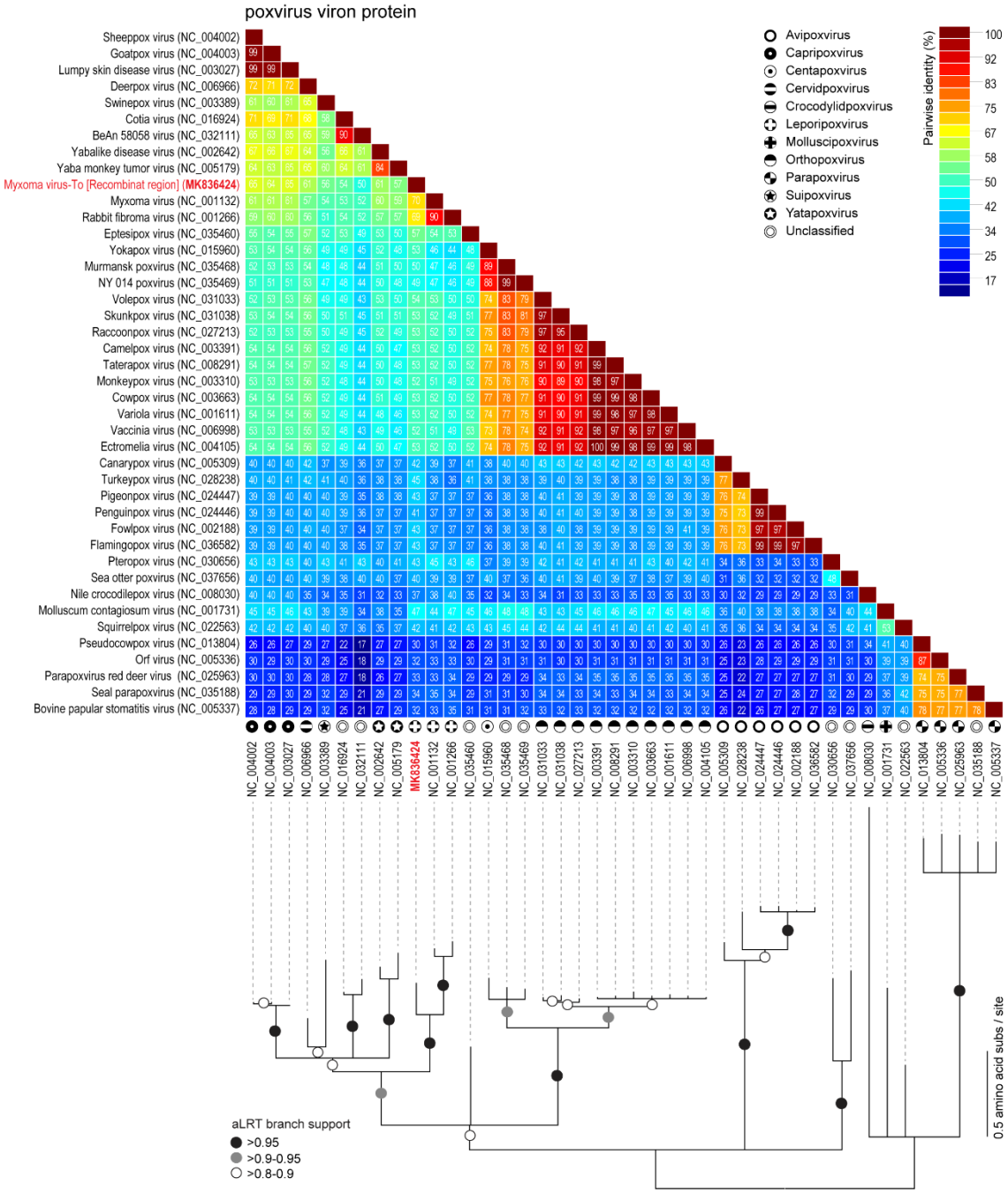

Figure 5. Pairwise amino acid identity matrix (upper image) and maximum-likelihood phylogenetic tree (model JTT+G) showing the relationships of the rPox-virion protein (highlighted in red) found in the recombinant region of MYXV-To and its homologous proteins found in representative sequences (NCBI RefSeq) of poxvirus. Branches with aLRT support 0.95 are indicated with black circles whereas branches exhibiting 0.9-0.95 and 0.8-0.9 are indicated with grey and white circles, respectively.

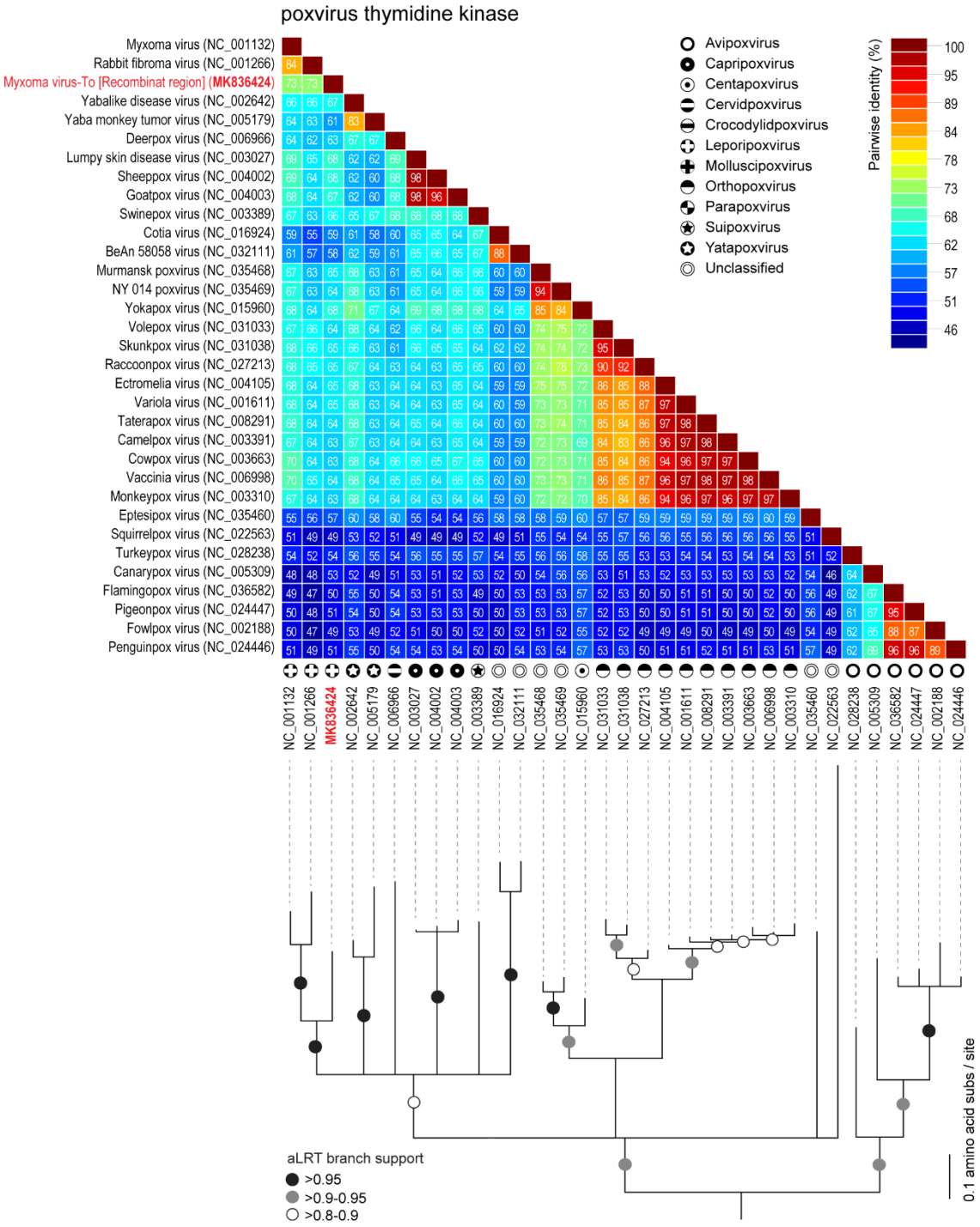

Figure 6. Pairwise amino acid identity matrix (upper image) and maximum-likelihood phylogenetic tree (model WAG+G+F) showing the relationships of the rPox-thymidine kinase (highlighted in red) found in the recombinant region of MYXV-To and its homologous proteins found in representative sequences (NCBI RefSeq) of poxvirus. Branches with aLRT support 0.95 are indicated with black circles whereas branches exhibiting 0.9-0.95 and 0.8-0.9 are indicated with grey and white circles, respectively.

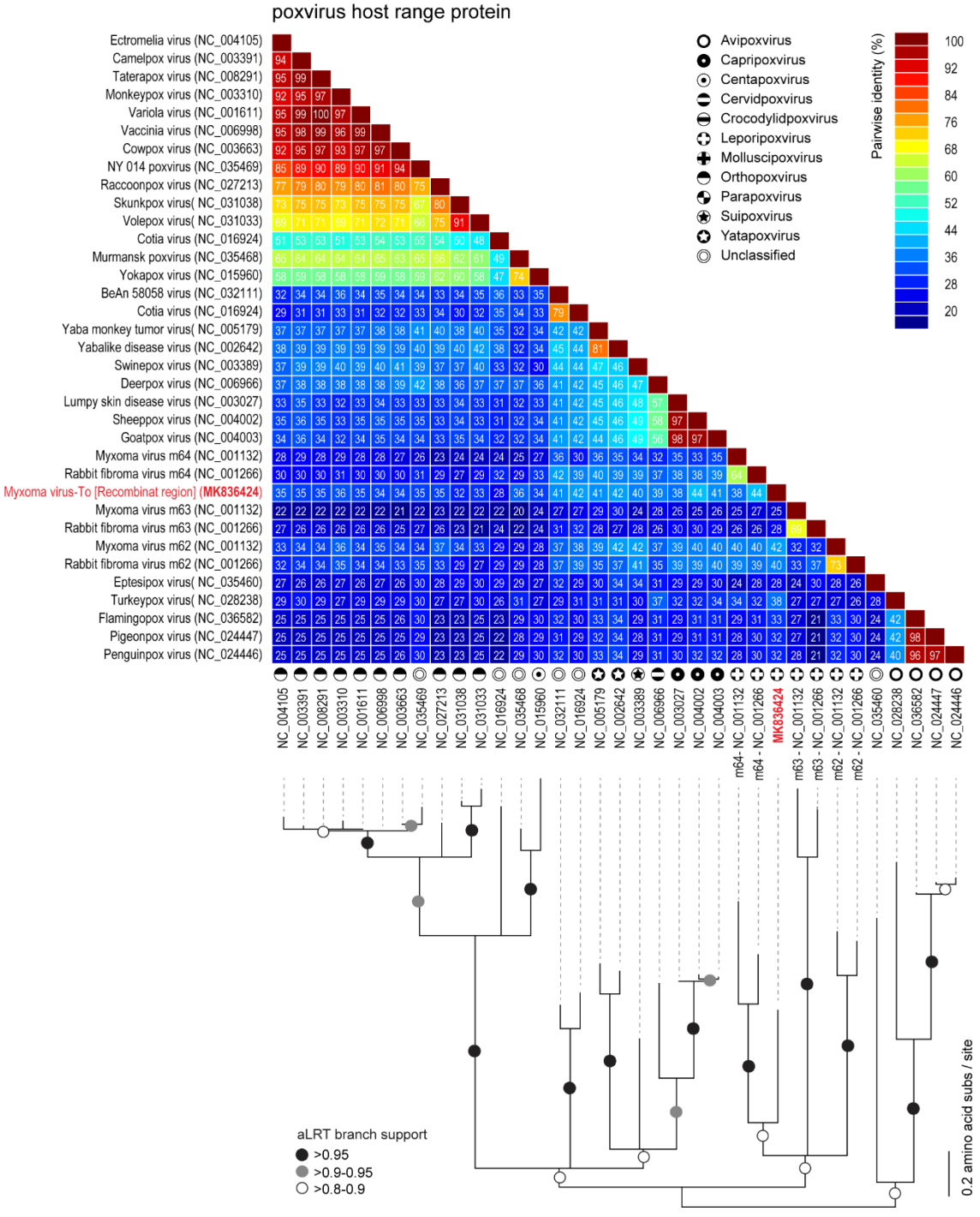

Figure 7. Pairwise amino acid identity matrix (upper image) and maximum-likelihood phylogenetic tree (JTT+G+F) showing the relationships of the rPox-host range protein (highlighted in red) found in the recombinant region of MYXV-To and its homologous proteins found in representative sequences (NCBI RefSeq) of poxvirus. Branches with aLRT support 0.95 are indicated with black circles whereas branches exhibiting 0.9-0.95 and 0.8-0.9 are indicated with grey and white circles, respectively

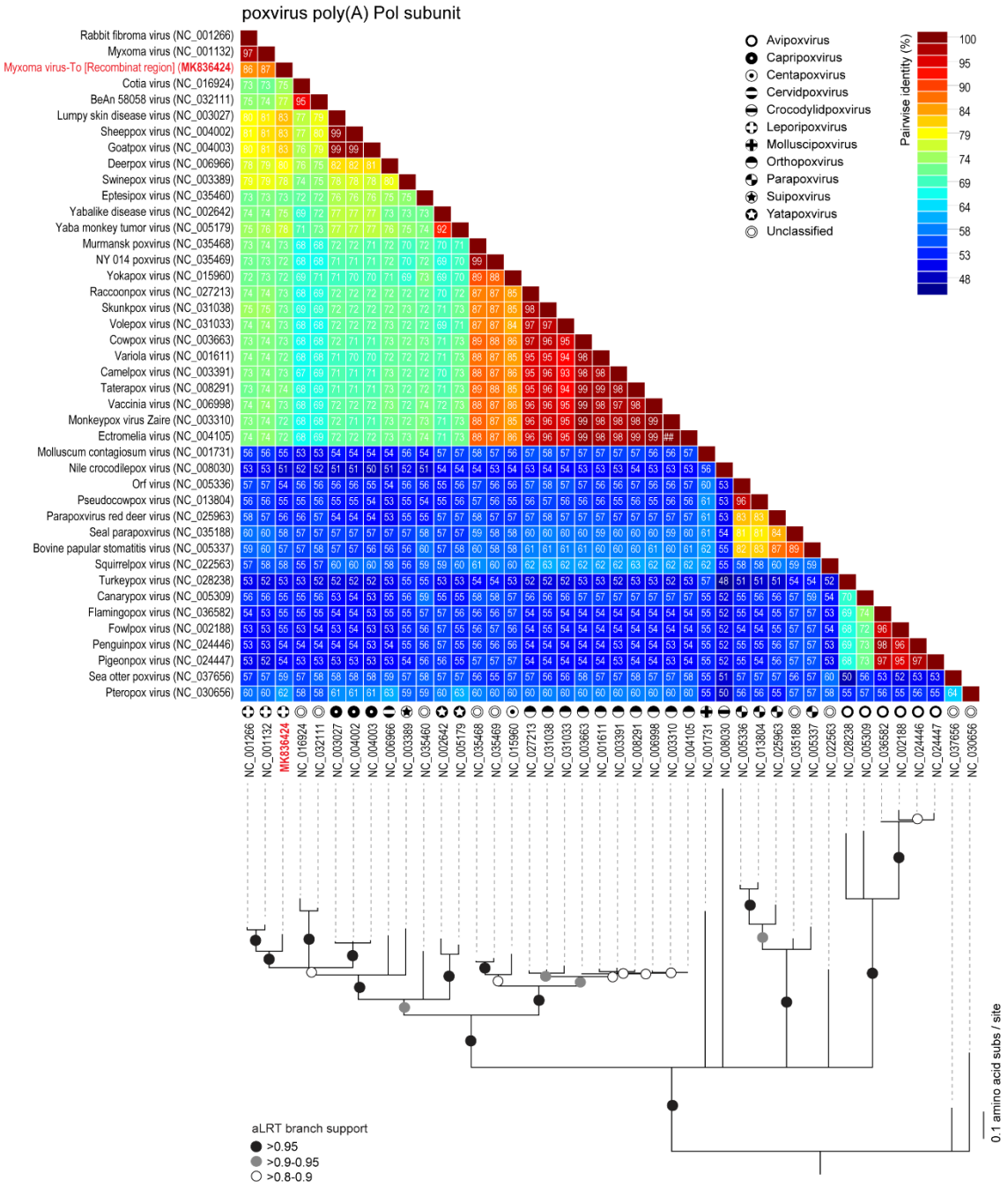

Figure 8. Pairwise amino acid identity matrix (upper image) and maximum-likelihood phylogenetic tree (model JTT+G+F) showing the relationships of the rPox-poly(A) Pol subunit (highlighted in red) found in the recombinant region of MYXV-To and its homologous proteins found in representative sequences (NCBI RefSeq) of poxvirus. Branches with aLRT support 0.95 are indicated with black circles whereas branches exhibiting 0.9-0.95 and 0.8-0.9 are indicated with grey and white circles, respectively.

4. Concluding remarks

Other than the disruption of *M009L*, *M036L* and *M152R*, the MYXV-To has a full complement of genes present in other MYXV isolates and strains. So the question arises

of how MYXV-To, with a new recombination insertion region derived from an unreported poxvirus with a common origin to capripoxviruses, cervidpoxviruses, suipoxviruses, yatapoxviruses and three unclassified poxviruses (BeAn 58058 virus, cotia virus and eptesipoxvirus), likely became pathogenic in Iberian hares. Examination of the four new poxvirus genes found in the recombinant "cassette" at the left end of the MYXV-To genome (encoded on the negative strand), that may induce factor(s) that mediate host range and/or immunosuppression in hares, allowing the increased infection and propagation of this new virus in hares. Regarding virulence in hares, it is likely that acquisition of new genes involved in immunosuppression and/or host-range functions in specific cell types might have a preponderant role in this apparent species leaping of MYXV-To [38]. From the four new genes present in the recombinant insertion region, rPoxhost range protein is the clear candidate that suggests a possible function in novel host interactions for this new recombinant poxvirus. As mentioned before, in Lausanne strain M064R belong to the C7L-like host range factor superfamily that are known to be important for MYXV pathogenesis [39-41]. However, since this *M064R-like* gene of MYXV-To shares relatively low similarity (~40%) to its orthologous proteins found in other leporipoxviruses, it is likely that this new protein has acquired new roles, perhaps reflected by alternative host targets, compared to those in known MYXV strains. Host range proteins are defined as a group of virus-produced proteins important for the capacity of virus to infect cells or tissues of certain species [42]. The capacity of direct engagement and modulation of the host antiviral responses highlight the constant pressures exerted by the co-evolutionary arms race between host and viral pathogens [42, 43]. In fact, the high divergence observed in the new rPox-host range protein in MYXV-To might suggest that the parental virus from which this recombinant region was donated is able to replicate within a completely different host species that has a unique repertoire of anti-viral response pathways. This might ultimately result in a new poxvirus capable of differentially modulating the anti-viral responses of hare cells compared to MYXV, playing a critical role in species leaping and virus pathogenicity in the new host. Nevertheless, the biological implications of the new genes found in the recombination "cassette" still need to be experimentally addressed.

The data presented in this paper report that MYXV-To is a result of a recombinant event between a MYXV virus and a still-unreported poxvirus that shares common ancestral sequences to that of capripoxviruses, cervidpoxviruses, suipoxviruses, yatapoxviruses and three unclassified poxviruses (BeAn 58058 virus, cotia virus and eptesipoxvirus). Recent reports genetically characterized a large number of European

and Australian strain MYXV genomic sequences [4, 27] and haplotypes are usually suitable for tracking the spread of MYXV virus [44]. However, the discrimination of alterations in MXYV genomes that are responsible for increased virulence grades or attenuated phenotypes is still a complicated task. While it is not yet understood what precise mechanisms allowed the MYXV-To apparently acquired virulence and species leap into Iberian hares, the genetic characterization of this novel MYXV-To virus, in combination with further studies of the proteins found in the new recombinant insertion region, will provide the foundation to a better understanding of this cross-species transmission.

Supplementary Material

Figure S1. After performing a serial dilution of the purified MYXV-To virus, RK13 cells were infected and incubated for 48 hours at 37ºC. At 2 days post infection, a typical MYXV cytopathic effect (foci formation) was visualized using a Leica DMI6000 B inverted microscope at 10x (A) and 20x (B).

Figure S2. Posterior mapping of the Illumina sequencing read to the genome of MYXV-To using BBmap [16].

Figure S3. Maximum likelihood phylogentic tree of the aligned genomes of MYXV and RFV. Branches with aLRT support 0.95 are indicated with black circles whereas branches exhibiting 0.9-0.95 and 0.8-0.9 are indicated with grey and white circles, respectively.

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Supplementary data

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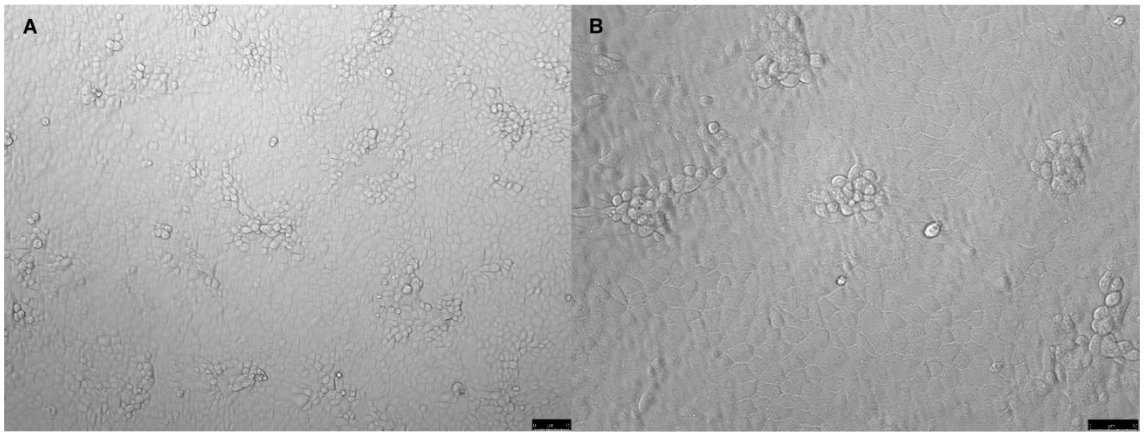


Figure S2: Posterior mapping of the Illumina sequencing read to the genome of MYXV-To using BBmap [16].

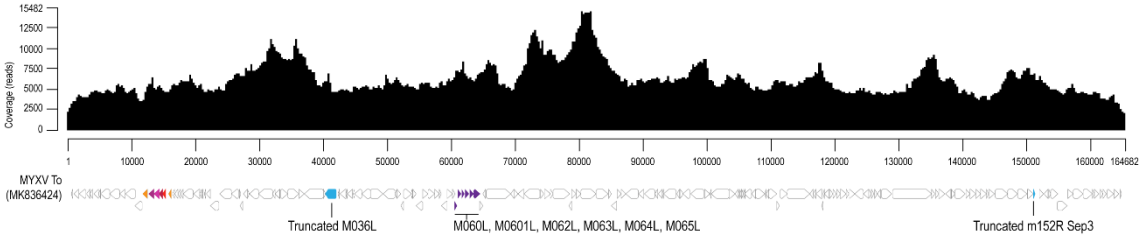
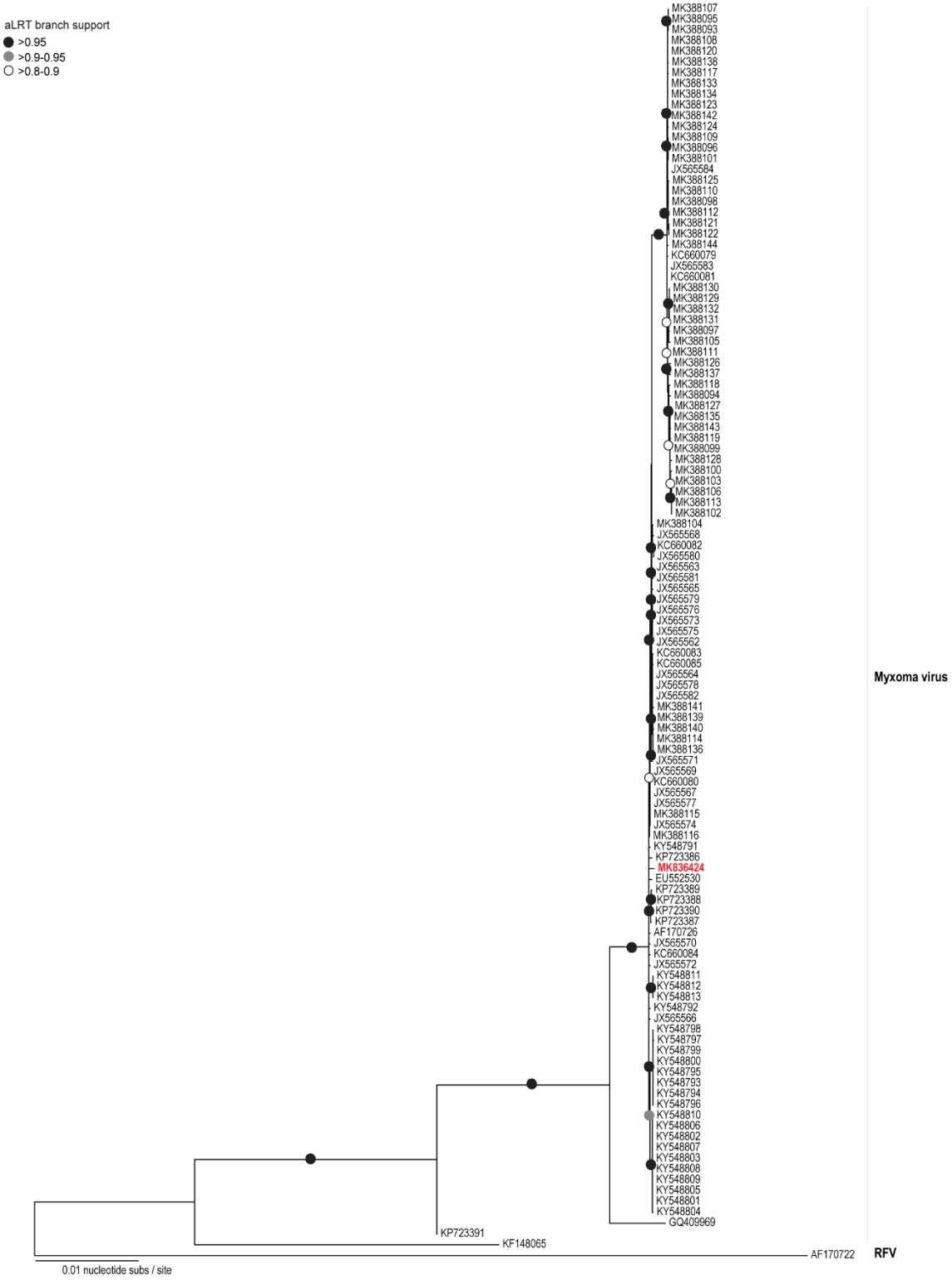


Figure S3: Maximum likelihood phylogentic tree of the aligned genomes of MYXV and RFV. Branches with aLRT support 0.95 are indicated with black circles whereas branches exhibiting 0.9-0.95 and 0.8-0.9 are indicated with grey and white circles, respectively.



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Coinfections of Novel Polyomavirus, Anelloviruses and a Recombinant Strain of Myxoma Virus-MYXV-Tol Identified in Iberian Hares

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Abstract

Viruses are ubiquitous in nature; however, very few have been identified in the Leporid species. In the fall of 2018, an outbreak of myxomatosis in Iberian hares *(Lepus granatensis*) was reported in Spain and a novel recombinant myxoma virus strain (MYXV-Tol) was identified. To investigate variability within the recombinant region of the MYXV-Tol and identify any potential viral coinfections, samples (ear, eyelid or vaginal) of Iberian hares were collected from Spain and analyzed. The presence of the recombinant region of the MYXV-Tol was confirmed in six out of eleven samples analyzed. Additionally, a polyomavirus (family *Polyomaviridae*), representing a putative new species, and anelloviruses (family *Anelloviridae*) belonging to two putative species were identified, some as coinfection with the recombinant MYXV-Tol. The two polyomavirus genomes were identified in two hares and share >99% genome-wide identity. Based on the analysis of their large T-antigen, the new polyomavirus clusters in a distant clade from other mammals sharing <64% amino acid identity. A total of 14 anelloviruses were identified, which share 63–99% genome-wide identity. Overall, our results show a coinfection of different DNA viruses in the studied samples and raise awareness regarding the extensive unsampled diversity of viruses in hares.

Keywords: Leporidae; Iberian hare; *Lepus granatensis*; myxoma virus; *Anelloviridae*; *Polyomaviridae*; coinfection; Spain

1. Introduction

New molecular tools and sequencing technologies have revolutionized viral detection, enabling a better exploration of viral diversity within various organisms. However, most studies have focused on viruses that are strongly associated with disease. The bias toward the study of viruses associated with disease is clear in the Leporidae family (Lagomorpha order). In fact, most of the research undertaken has been directed toward the highly pathogenic lagoviruses (family *Caliciviridae*), such as the rabbit hemorrhagic disease virus [1–3], and the leporipoxviruses (family *Poxviridae*) like the myxoma virus (MYXV) [4–7]. In a recent study of diseased Iberian hares (*Lepus granatensis*) from the Toledo province in Spain, a novel recombinant MYXV strain was identified (MYXV-Tol; GenBank Accession MK836424) showing, for the first time, that a pathogenic myxoma virus could also infect and cause myxomatosis in species of the *Lepus* genus [8]. Historically, MYXV has evolved in natural *Sylvilagus* hosts, like the South American tapeti and the North American brush rabbit. Nonetheless, when MYXV encounters naïve European *Oryctolagus* rabbits, the virus causes the lethal myxomatosis disease. The genome of the recombinant MYXV-Tol identified in Iberian hares is ~99% similar to MYXV variants/strains previously reported circulating in European rabbits, with the exception of a recombinant region ~2800 bp in length and three disrupted genes (*M009L*, *M036L* and *M152R*). The recombinant region encodes four additional open reading frames (ORFs), which were more closely related, but not identical, to the MYXV proteins encoded by *M060R*, *M061R*, *M064R* and *M065R* genes [8–10].

Besides the rabbit hemorrhagic disease virus and MYXV, there is limited information on viruses associated with Leporids. A recent study has reported the presence of a novel herpesvirus (*Herpesvirida*e family) in Iberian hare samples collected during the myxomatosis outbreak in Spain [11]. Metagenomics studies targeting hare fecal samples found circular replication-associated proteins encoding single-stranded (CRESS) DNA viruses in the families *Genomoviridae* and *Smacoviridae* as well as unclassified groups associated with the European hare (*Lepus europaeus*) and Snowshoe hare (*Lepus americanus*) [12,13].

To determine whether the Iberian hares found dead in Spain harbor viruses in addition to the MYXV-Tol strain, a metagenomic approach was used to identify circular DNA viruses. Using this approach, we identified circular DNA viruses for which we designed specific abutting primers to screen and recover full genomes from various

Iberian hare samples collected in Spain following a myxomatosis outbreak. In these samples, virus genomes belonging to the *Anelloviridae* and *Polyomaviridae* families were identified and recovered. Neither of these have been previously reported in Leporids. The *Anelloviridae* family is a group of highly diverse circular single-stranded DNA viruses [14–18] with genome sizes ranging from ~2–4 kb. First identified in humans in 1997 [15], anelloviruses have been found in a wide range of animals, including pigs, non-human primates, seals, bats and horses [16,18–21], and are largely host species-specific. Polyomaviruses on the other hand are circular double-stranded DNA viruses of ~4–7kb that, like anelloviruses, are host specific and have been identified in various animals [22–24]. In our analysis of various sample types from 11 hares, we identified 14 viruses belonging to two new species of anelloviruses and two viruses belonging to a new species of polyomavirus. Furthermore, we highlighted that three of the hares were coinfected with the MYXV-Tol variant, and either both or one of the novel anelloviruses and/or polyomaviruses.

2. Material and Methods

2.1. Sample Collection and Viral Nucleic Acid Extraction

Samples from eleven symptomatic Iberian hares (19 samples in total from either ear, eyelid or vaginal sample) found dead in the fall of 2018 were collected and stored in RNAlater® (Sigma, St. Louis, MO, USA) at −80°C, as shown in Table 1. These samples were collected from different regions of Spain: Toledo, Cuenca, Ciudad Real and Madrid. Viral nucleic acid was extracted using 5 g of each sample according to a modified phenol–chloroform extraction protocol [25] described in Águeda-Pinto et al. [8].

2.2. Detection of the MYXV-Tol

To detect the presence of the new MYXV-Tol, primers flanking the unique recombinant region (~2800 bp) were designed based on the previously described MYXV-Tol sequence (GenBank Accession MK836424), shown in Table S1, that yielded a ~4.8 kb amplicon. Viral nucleic acid from each individual sample was used as a template for PCR amplification using Kapa HiFi HotStart DNA polymerase (Kapa Biosystems, Wilmington, MA, USA) following manufactures' recommendations at an annealing temperature of 60°C. The amplicons were resolved on a 0.7% agarose gel stained with SYBR Safe (ThermoFisher Scientific, Waltham, MA, USA) and ~4.8 kb size fragments were excised, gel purified and cloned into pJET1.2 plasmid vector (ThermoFisher Scientific, Waltham, MA, USA). The resulting recombinant plasmids were Sanger

sequenced by primer walking at Macrogen Inc. (Seoul, Korea). The sequence contigs were assembled using Geneious 11.1 [26].

2.3. Detection and Recovery of Anelloviruses and Polyomavirus Genomes

Previously, the MYXV-Tol variant from sample Lag01_EL, shown in Table 1, was cultured in permissive rabbit cells (RK13, ATCC # CCL-37), purified and sequenced using high-throughput sequencing, which led to the characterization of the full genome of a new myxoma strain, MYXV-Tol, GenBank Accession MK836424 [8].

Table 1. Sample information of the hare samples analyzed in this study including geographic origin, tissue type and GenBank accession numbers of the viral sequences.

			Poxvirus	**Anellovirus**		**Polyomavirus**
Animal ID	**Location**	**Tissue type**	**MYXV-Tol**	**LepTTV1**	**LepTTV2**	**LepPyV1**
Lag01	Spain: Toledo	Eyelid	MK836424	MN994854	MN994855	MN994868
Lag02	Spain: Cuenca	Ear	-	-	-	-
Lag03	Spain: Ciudad Real	Ear	-	-	-	-
Lag04	Spain: Ciudad Real	Vaginal	MT072323	-	-	-
Lag05	Spain: Ciudad Real	Ear	-	-	-	-
		Eyelid		-	-	-
Lag06	Spain: Ciudad Real	Ear	MT072322	-	-	-
		Eyelid	MT072321	-	MN994856	-
		Vaginal	MT072320	-	-	-
Lag07	Spain: Ciudad Real	Ear	MT072319	-	-	-
		Eyelid	MT072318	-	-	-
Lag10	Spain	Eyelid	-	MN994857	MN994858	MN994869
		Vaginal	-	MN994859	MN994860 MN994861	-
Lag11	Spain: Toledo	Eyelid	-	-	MN994862	-
		Vaginal	-	-	-	-
Lag12	Spain: Ciudad Real	Ear	-	-	-	-
		Eyelid	-	-	-	-
Lag14	Spain: Madrid	Eyelid	-	-	MN994863 MN994864	-
		Vaginal	MT072317	MN994865	MN994866 MN994867	-

In this study, viral DNA from the sample Lag01_EL was extracted using the Roche High Pure Viral nucleic acid kit (Roche Diagnostics, Indianapolis, IN, USA). Circular molecules were amplified using rolling circle amplification (RCA) with the Illustra TempliPhi 100 Amplification Kit (GE Healthcare, Chicago, IL, USA). The RCA amplicons were used to generate a 2 × 100 bp Illumina sequencing library and sequenced on an

Illumina HiSeq4000 (Illumina, San Diego, CA, USA) at Macrogen Inc. (Seoul, Korea). The paired-end reads were de novo assembled using metaSPAdes 3.12.0 [27]. Contigs with terminal sequence redundancy were assumed to represent circular molecules and all contigs >750 nucleotides (nt) were analyzed using BLASTx [28] against a viral GenBank RefSeq protein database.

Based on these contigs, abutting primers were designed, shown in Table S1, and these primers were used to screen and recover full genomes from the 19 individual samples of 11 animals by PCR. The total DNA extracted from each of the 19 tissue samples was subjected to RCA to amplify circular molecules and 0.5 µl of this was used as a template with Kapa HiFi HotStart DNA polymerase (Kapa Biosystems, Wilmington, MA, USA) following manufactures' recommendations at an annealing temperature of 60°C with specific primers for the amplification of the anellovirus and polyomavirus genomes. The amplicons were resolved on a 0.7% agarose gel stained with SYBR Safe (ThermoFisher Scientific, Waltham, MA, USA). Amplicons (~2.5 kb for anelloviruses and ~5.4 kb for polyomaviruses) were excised, gel purified and cloned into pJET1.2 plasmid vector (ThermoFisher Scientific, Waltham, MA, USA). The resulting recombinant plasmids were Sanger sequenced by primer walking at Macrogen Inc. (Seoul, Korea). The sequence contigs were assembled using Geneious 11.1 [26].

2.4. Sequence Analysis of MYXV-Tol Regions, Anelloviruses and Polyomaviruses

2.4.1. MYXV-Tol Recombinant Region Sequences

A dataset of MYXV-Tol recombinant region sequences was assembled with sequences from this study (n = 7) and the two available in GenBank (GenBank accession # MK836424 and MK340973). These were aligned using MUSCLE [29] and any polymorphism, insertions or deletions were identified manually. The nucleotide sequence pairwise identities were determined using SDT [30].

2.4.2. Anellovirus Sequences Analyses

A dataset of the most closely related anelloviruses (n = 4) was created. The ORF1 sequences were extracted and an alignment of the ORF1 amino acid sequences was used to infer a maximum likelihood phylogenetic tree using PHYML [31] with the WAG+G substitution model, determined as the best fit model using ProtTest [32]. Branches with

<0.8 aLRT support were collapsed using TreeGraph2 [33] and the resulting phylogenetic tree was midpoint rooted.

2.4.3. Polyomavirus Sequences Analyses

A dataset of representative polyomaviruses (*n* = 125) was downloaded from GenBank. From these, the large T-antigen and VP1 sequences were extracted. The aligned amino acid sequences of the large T-antigen were used to infer a maximum likelihood phylogenetic tree using PHYML [31] with the rtREV+G+I substitution model, determined as the best fit model using ProtTest [32]. Branches with <0.8 aLRT support were collapsed using TreeGraph2 [33] and the resulting phylogenetic tree was rooted with large T-antigen sequences of fish polyomaviruses [22]. The genome-wide nucleotide, the large T-antigen and the VP1 amino acid sequence pairwise identities were determined using SDT v1.2 [30].

3. Results and Discussion

After the outbreak of myxomatosis in Iberian hares, samples from Spain were collected to evaluate the presence of novel DNA viruses. Previously, the MYXV from one of the Iberian hare samples (Lag01; eyelid) was cultured in permissive rabbit cells (RK13, ATCC # CCL-37). Following purification, the genome of the MYXV was sequenced using high-throughput sequencing, which led to the identification of the recombinant region (~2.8kb) derived from an unknown poxvirus in the MYXV genome and thus the recombinant strain, MYXV-Tol [8]. Following this report, other studies [9,10] also identified the MYXV containing the same novel ~2.8 kb recombinant region.

In this study, we identify the recombinant region of MYXV-Tol in seven of the 18 Iberian hare tissue samples from four hares (not including sample Lag01 eyelid from which the full MYXV was isolated). Sequence analyses of the recombinant region show that the obtained amplicons are >99% similar to the recombinant region in the MYXV-Tol (GenBank accession # MK836424) strain previously reported [8]. Among these sequences, three single nucleotide polymorphisms (SNPs) and two deletions were identified, shown in Figure 1. All the SNPs resulted in non-synonymous changes. One deletion of three nucleotides (ATC) located in the pox virus host range gene (GenBank accession # MT072320) resulted in the deletion of an amino acid (D) near the C terminus. In the sequence of the recombinant region of the MYXV-Tol from Lag04 V sample

(GenBank accession # MT072323) there was a four nucleotide (TATA) deletion in the m009L gene resulting in a frame shift and extension of the open reading frame.

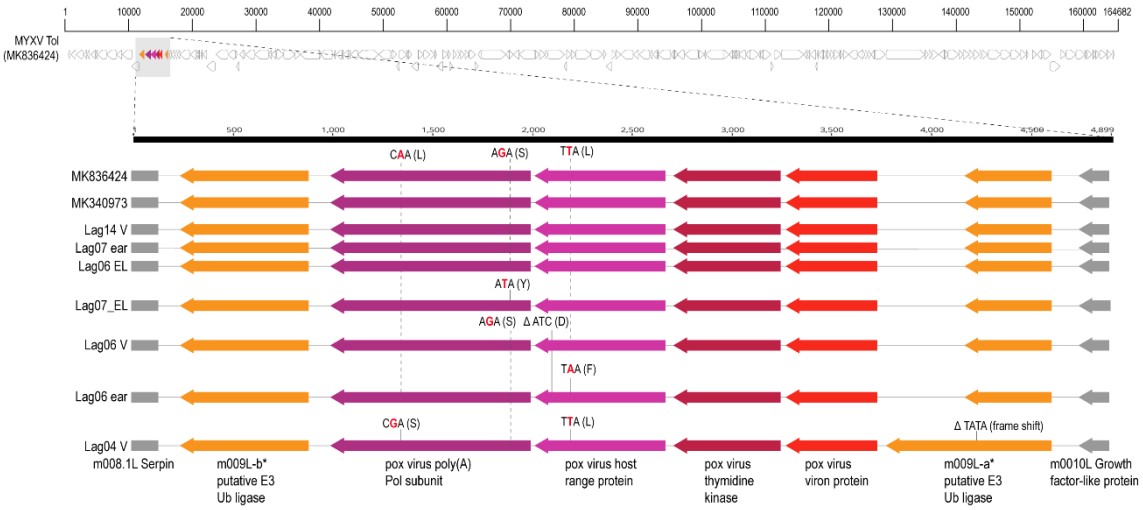

Figure 1. Myxoma virus genome schematic with recombinant region highlighted. The variations identified in the recombinant gene cassette of the sequenced MYXV-Tol variants from various samples are summarized in reference to the MYXV-Tol GenBank accession. SNPs are shown with red highlighted nucleotide and the encoded amino acid in brackets. Deletions are shown with Δ symbol and the deleted amino acid in brackets.

In the 19 Iberian hare tissue samples, a diverse range of unique anelloviruses (*n* = 14) were recovered, as seen in Table 1, suggesting that these viruses are common in Iberian hares. The hare- derived anelloviruses have a genome organization that has at least three ORFs and a conserved untranslated region (UTR). The hare anelloviruses range in size from 2490 to 2529 nt. Based on the last report of the International Committee on Taxonomy of Viruses (ICTV) [34], the classification of viruses in the family *Anelloviridae* is based on a global alignment pairwise identity of the ORF1 gene nucleotide sequence, with the cut-off values of >35% divergence for species and >56% divergence for genera. To facilitate the assignment of the anelloviruses at species and genera levels, a nucleotide pairwise comparison was generated, shown in Supplementary Data S1, and based on this, the anelloviruses genomes could be tentatively classified into two species which we refer to as Lepus torque teno virus (LepTTV) 1 and 2 from here on. Of the 14 genomes identified, four form a single LepTTV 1 supported clade, and 10 sequences form a second supported clade comprising of LepTTV 2s, shown in Figure 2. The closest relatives of the anelloviruses identified from hares are those identified in rodents (GenBank accession # MF684738 and MF684741) [35] and mosquitoes (GenBank accession # HQ335083 and HQ335082) [36]. The analysis of the pairwise identity for the ORF1 nucleotide, amino acid and full genome revealed that the LepTTVs share >63%, >56% and >70% pairwise identity with each

other, respectively, shown in Figure 2 and Supplementary Data S1. When compared to anelloviruses previously identified in other organisms, LepTTVs share 61%–67% (nt), 48%–53% (aa), and 61%–64% (full genome) pairwise identity. LepTTV 1 was identified in the eyelid (*n* = 2) and vaginal (*n* = 2) samples from three individual hares, shown in Table 1. LepTTV 2 was identified in the ear, eyelid (*n* = 5) and vaginal (*n* = 2) samples from three individual hares, shown in Table 1.

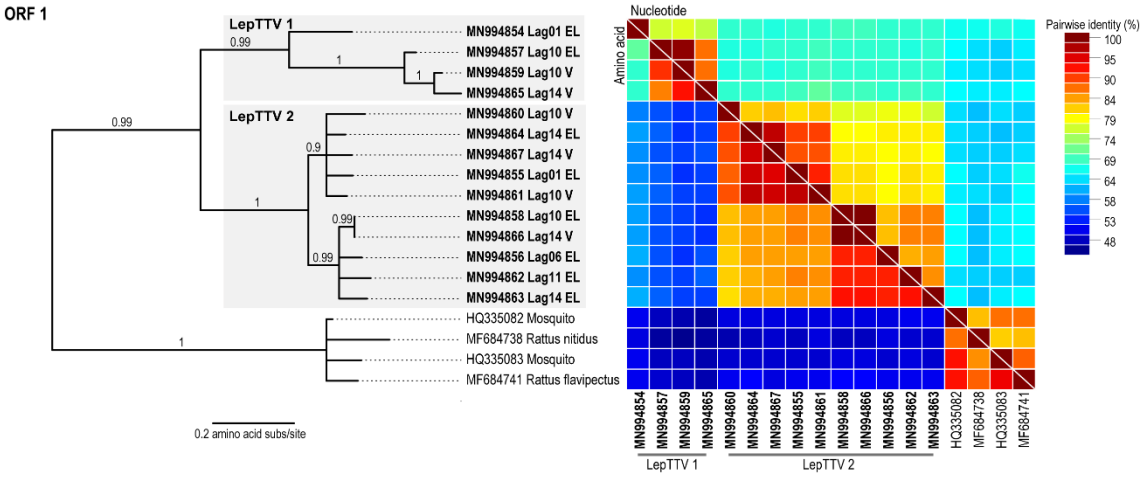

Figure 2. Maximum likelihood phylogenetic tree of the anelloviruses ORF1 protein sequences of those recovered from hares together with those most closely related, available in GenBank. A pairwise comparison color matrix of the ORF1 amino acid and nucleotide sequence is shown to the right of the phylogenetic tree.

The polyomaviruses (5386 bp) were detected in two samples, the eyelid of Lag01 and Lag10, shown in Table 1. Typically, polyomavirus genomes are divided into three different regions: a non-coding region that regulates the expression of early and late genes; an early region that encodes the large T-antigen and the small T-antigen; and a late gene region that encodes the capsid proteins VP1, VP2, and VP3 [37,38]. In the genome of the polyomavirus recovered from the Iberian hare samples, we identified all the polyomavirus ORFs. The large T-antigen, VP1 and genome-wide pairwise analysis of the *Lepus* polyomavirus (LepPyV) and those most closely related revealed that these two LepPyV isolates share >99% identity and are most closely related to the *Glis glis* polyomavirus (GenBank accession # MG701352) [39] sharing 60% (large T-antigen), 67% (VP1) and 64% (full genome) pairwise identity, shown in Figure 3. The large T-antigen of the hare-derived polyomaviruses recovered in this study is nested within a larger mammal clade but forms a distinct sub-clade. The species demarcation for polyomaviruses is based on >15% divergence of the large T-antigen, thus the hare-derived polyomavirus represents a new species and we tentatively refer to this

polyomavirus as Lepus polyomavirus 1 (LepPyV 1) and this would be part of the *Betapolyomavirus* genus [24,40].

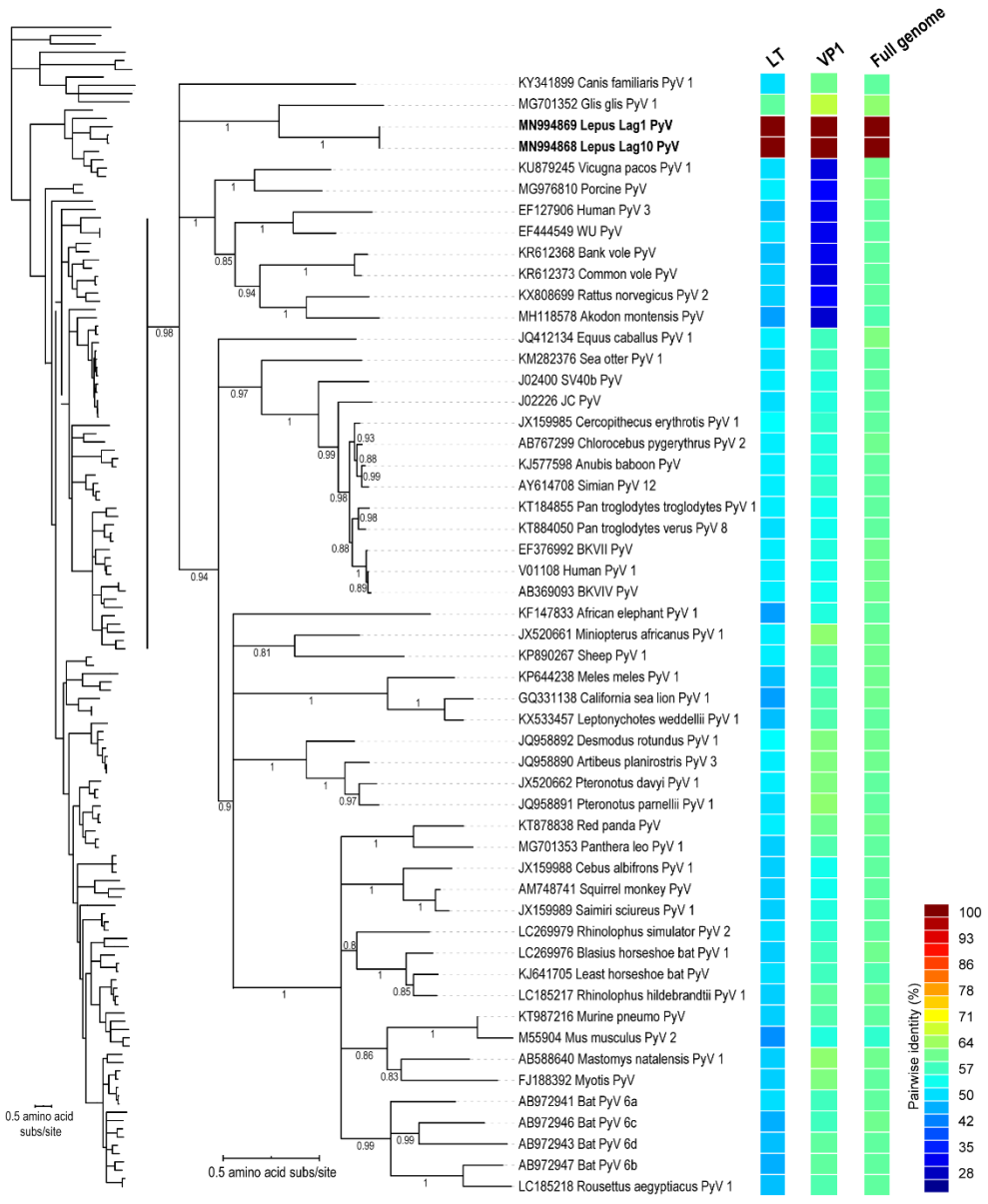

Figure 3. Maximum likelihood phylogenetic tree of the polyomavirus large T-antigen amino acid sequences of polyomaviruses identified in hares and representative sequences of polyomavirus species available in GenBank. The enlarged clade shows those most closely related to the hare polyomavirus. A pairwise comparison color matrix of the large-T protein, VP1 protein and the full genome is provided to the right of the phylogenetic tree.

Anelloviruses have a high degree of genetic variability between genotypes and strains and can establish persistent infections in their hosts, with no clear associated pathology [41]. However, it is still poorly understood how the host's immune system is affected by the continuous exposure to anelloviruses [15,42]. Reports suggest that anelloviruses have a negative impact in several clinically relevant infections by modulating the baseline level of inflammation and interference with molecules of different

signal pathways (reviewed in Maggi and Bendinelli [43]). For example, anellovirus ORF2 protein suppresses the NF-κB pathway, a key regulatory element that participates in the synthesis of proinflammatory cytokines like IL-6, IL-8, and cyclo-oxygenase-2 [44]. It was also found that anelloviruses encode miRNAs that could be involved in viral immune evasion and regulation of IFN signaling [45,46]. Studies have shown that the polyomavirus large T-antigen is capable of inactivating some host proteins such as p53, which is responsible for the control of cell-cycle inducing apoptosis [47,48]. Polyomaviruses are also able to prevent lysis of infected cells and initiate oncogenic transformation [38,49,50]. However, without further studies it is not possible to determine whether the coinfections of MYXV-Tol with LepTTV or LepPyV, present in Iberian hares, have any effect on the myxomatosis disease manifestation.

4. Concluding remarks

In this study, we provide the first report of anelloviruses and polyomaviruses associated with hares. In addition, we identify the presence of the MYXV-Tol variant in four hares, and reveal coinfections in three hares, of either both or one of the novel anelloviruses and/or polyomaviruses. Hare populations have declined since the late 1960s which is thought to be in part due to disease [7,51,52]. The recent documentation of an outbreak of myxoma virus in hares caused by a prevalent recombinant MYXV-Tol [8–10] highlights another threat to the species. As an important food source for many predator species, such as the endangered Iberian lynx (*Lynx pardinus*), evaluating circulating pathogen populations and monitoring disease spread as well as spillover of pathogens is crucial for the survival of the hare and other species higher on the trophic cascade. Overall, this study expands our knowledge of DNA viruses associated with hares and also highlights the unsampled diversity of viruses, and the importance of investigating pathogen complexes within a host to further understand disease dynamics.

Supplementary material

Table S1: Summary of primers used to amplify the MYXV-Tol recombinant cassette sequences, and the anellovirus and polyomavirus genomes.

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Supplementary data

Table S1: Summary of primers used to amplify the MYXV-Tol recombinant cassette sequences, and the anellovirus and polyomavirus genomes.

Virus group	Accession #	Fwd 5'-3'	Rev 5'-3'
MYXV-Tol	MK836424	TTAAACATAAGACGAGGACCAGATACTTCA	GTAGCATTAAACAATGTTTCACTTAACCC
LepPyV1	MN994868 MN994869	AGACCAAGGGGAAAAGGAAAGTTTCTCTTG	TGTTATTGGTCCAGCCTCAAGAGATCTAGT
LepTTV1	MN994854	GTACTCTGCATCTACATATACCAGGTAGTC	AGAGACTTTAATCTGTGGATGAAACAGTG
	MN994857 MN994859 MN994865	ATACTGCAAACTGGCCTATGGACCTTTTAT	CTGTTATTGTTTTGAAAGTCACCTGGTGGC
LepTTV2	MN994855	CTATATAGTCTAACTGTGGATGTGGCCA	TATACTTCGATCCAGAATACAGGGATTTCG
	MN994856 MN994860 MN994864 MN994867	GTGCTTGGAGGTGGTATAAAGATTTTATGT	AATGAATAGTGGCTGGAATTGGATGAAAGA
	MN994858 MN994862 MN994863 MN994866	TATGAACCTGTTGTTTTTGTACCAGTCTG	CACCTGGGATATGGACCATTCATTTATAAA
	MN994861	GTTATGGGCCGTTTGTCTATAAAAACAAG	CCAGTTTGATGAATCTGTTGTTCTTATACC

Chapter 3

Characterization of M159, a novel host range factor found in MYXV-Tol that belongs to the VACV C7L superfamily

Article 3

Identification of a novel Myxoma C7-like host range factor that enabled a species leap from rabbits to hares

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Abstract

Myxoma virus (MYXV) is naturally found in rabbit *Sylvilagus* species, and is known to cause the lethal myxomatosis in European rabbits (*Oryctolagus cuniculus*). In 2019, a MYXV strain (MYXV-Tol) causing a myxomatosis-like disease in Iberian hares (*Lepus granatensis*) was identified. MYXV-Tol acquired a recombinant region of ~2.8kb encoding for several new genes, including a novel host range gene (*M159*) that we show to be an orthologous member of the vaccinia C7 host range family. Here, to test whether M159 alone has enabled MYXV to alter its host range to Iberian hares, several recombinant viruses were generated, including a MYXV-Tol ΔM159 (knockout). While MYXV-Tol underwent fully productive infection in hare cells, neither wild-type MYXV-Lau strain (lacking M159) or vMyxTol-ΔM159 (deleted for M159) were able to infect and replicate, showing that the ability of MYXV-Tol to infect hare cells and replicate depends on the presence of *M159* gene. Similar to other C7L family members, M159 was shown to be expressed as an early/late gene but was translocated into the nucleus at later time points, indicating further studies are needed to elucidate its role in the nucleus. Finally, in rabbit cells, M159 protein did not contribute to increased replication, but was able to upregulate the replication levels of MYXV in non-permissive and semi-permissive human cancer cells, suggesting that the M159 targeted pathway is conserved across mammalian species. Altogether, these observations demonstrate that M159 protein plays a critical role in determining the host specificity of MYXV-Tol in hare and human cells by imparting new host range functions.

Keywords: Myxoma virus, Leporids, *Poxviridae*, Species leap, Host range, M159 protein, C7 family

1. Introduction

The ability of any virus to jump into a new host, successfully replicate, cause overt disease, and be transmitted to another individual of the new host species, is largely dependent on two factors: 1) receptor specificity (if any), and 2) the ability to modulate the host innate immune response to the viral infection. To begin addressing the fundamental questions mediating virus cross-species transmission, it is crucial to study the biological and mechanistic underpinnings driving such events using a well-established model system. The most extensively documented field model of pathogen evolution following a species jump is the introduction of the lethal myxoma virus (MYXV) into the European rabbit (*Oryctolagus cuniculus*) populations in Australia and Europe as a biological control agent (1). The host-range genes of MYXV evolved in the natural *Sylvilagus* hosts, yet when MYXV first encountered naïve European rabbit populations, the virus immediately (i.e., with no required adaptation in the new host) caused an outbreak of a lethal disease called myxomatosis. In fact, when the highly virulent Standard Laboratory strain (SLS) was introduced in Australia and the South American strain of MYXV (Lausanne strain; Lau) was introduced in France, the case fatality rates of infected rabbits were estimated at ~ 99% (2). In both cases, attenuated MYXV strains came to dominate the field populations within a few years, allowing infected rabbits to survive longer, increasing the probability of transmission from skin lesions by arthropod vectors like mosquitoes (3, 4). The importance of MYXV and European rabbit interaction is transversal to the field of emerging infectious diseases, since it provides an outstanding model to study dynamic host-pathogen interactions in the wild.

Recently, we characterized for the first time the full genome of recombinant MYXV variants (MYXV-Tol, also previously described as ha-MYXV Tol08-18) capable of causing myxomatosis in a *Lepus* species (5–7). MYXV-Tol shares high similarity with the MYXV-Lau strain previously released in Europe, with the exception of three disrupted genes (*M009L*, *M036L, M152R*) plus a recombinantly-derived cassette (recTol) of ~ 2,800 bp from an unknown/unsampled poxvirus donor genome, which based on the arrangement of the gene sequences most closely resembles that from ungulate-associated poxviruses. The new recombinant cassette found in MYXV-Tol encodes a known poxviral structural protein (M157), a thymidine kinase (M158), a C7-like (C7L) host range protein (M159) and a poly A polymerase subunit (M160), which are most closely similar, but not identical, to the MYXV *M060R*, *M061R*, *M064R* and *M065R* genes, respectively (5, 6). Analyses shows that the newly discovered M159 protein encoded by MYXV-Tol shares a higher pairwise identity to the previously described

MYXV host range proteins M064R and M062R, that belong to the C7L family of host range factors (5, 7).

One of the most critical poxvirus host-range factors belongs to the family of host interactive viral proteins that share sequence similarity with the vaccinia virus (VACV) C7 protein, known to have major roles in pathogenicity (8). The Sterile Alpha Motif Domain-containing 9 (SAMD9), a large cytoplasmic protein with diverse functions including antiviral, antineoplastic, and stress-responsive properties, is a key host target of many C7L viral proteins (8). In most mammalian poxviruses, at least one copy of a member from the C7L host range superfamily can be found encoded in the genome (9). The wild type MYXV carries three tandem C7L genes that were probably derived by two distinct duplication events: *M062*, *M063* and *M064* (8). Investigations on the roles of these host range genes have shown that only *M062* can be a substitute for VACV C7 in overcoming host range restriction by binding directly to SAMD9, while M063 only facilitates the latter interaction (10, 11). On the other hand, M064 does not exhibit any known host range properties, but acts as a virulence factor that controls the kinetics of MYXV infection both *in vitro* and *in vivo* (12). The presence of this C7L protein in the recombinant region of MYXV-Tol prompted us to hypothesize that this newly acquired viral factor has enabled MYXV to alter its host range from rabbits to hares and caused the myxomatosis-like pathogenesis in hares.

Here, we report our study of the role of the M159-encoded protein as a MYXV-Tol virulence factor in cultured cells from hare, rabbit and human origin by examining the phenotype of different recombinant MYXVs *in vitro*, including a targeted MYXV-Tol M159-knockout (vMyxTol-ΔM159) and a MYXV-Lau strain expressing the novel M159 (vMyxLau-V5M159) alone. Moreover, a V5-tagged M159 was constructed to characterize the expression patterns and localization of this viral host range protein in cultured cells. The data show that M159 protein is required for productive MYXV replication in hare cells, and improves the replication of MYXV in non-permissive or semi-permissive human cancer cells, thus providing a platform for a better understanding of poxvirus host range and cross-species transmission.

2. Results and Discussion

Since the autumn of 2018, hundreds of Iberian hares from the Iberian Peninsula, Europe, have died as a result of a viral infection with a newly discovered MYXV strain, hereby named MYXV-Tol (5, 13). This MYXV strain shares high nucleotide identity to the

previously described strains such as MYXV-Lau with the exception of a unique “cassette” of genes. Interestingly, some studies highlight that Iberian hares have been in contact with MYXV (or an antigenically similar virus) at least since the 1990s (14) without an outbreak occurring, suggesting that the recent acquisition of this unique “cassette” of genes (recombinant event between MYXV and a unknown poxvirus) allowed MYXV to cross the species barrier, causing disease in these animals. The predicted functions of the four newly acquired viral proteins (virion structural protein, thymidine kinase, C7L host range member and poly A polymerase subunit) found in the recombinant region are in accordance with the gene order arrangement found in the ~57,500 bp region of other poxviruses (5, 7). However, given the fact that this cassette is apparently inverted in MYXV-Tol and that the predicted encoded proteins show relatively low similarity to the existing gene members in MYXV-Lau, we have assigned new viral ORF numbers, which we refer as M157 for the virion structural protein, M158 for the thymidine kinase, M159 for the C7L host range factor and M160 for the poly A polymerase subunit (Figure 1A).

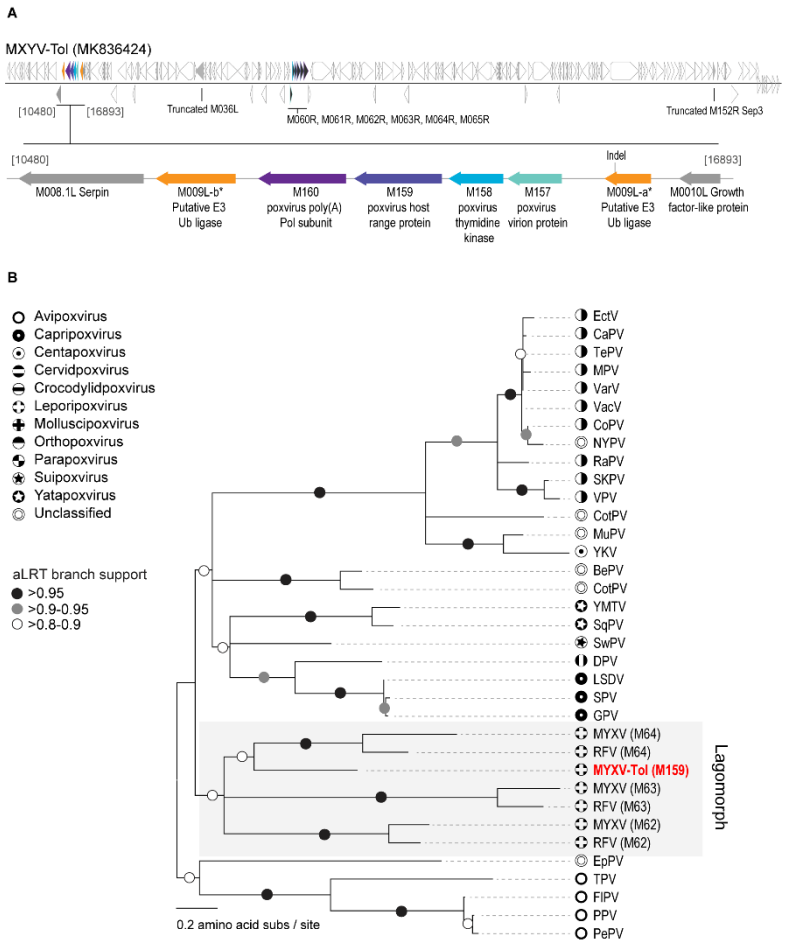

Figure 1. The newly discovered M159 from MYXV-Tol belongs to the C7 Superfamily of host range proteins. A) Representation of the genome organization of MYXV-Tol (MK836424). Orange shows the disrupted *M009L* gene. Blue and purple ORFs show the new gene “cassette” identified and the location of *M157*, *M158*, *M159* and *M160* genes in MYXV-Tol **B)** Phylogenetic analysis of C7 like members from 23 different Poxviruses.

2.1. M159 is a new member of the C7L family of poxviral host range genes

VACV C7 family of proteins are present in almost all poxviruses that infect mammalian species, including MYXV, Yaba-like diseases virus (YLDV), swinepox virus (SWPV), and sheeppox virus (SPPV) (8, 9, 15). As can be seen in Figure 1B, the new MYXV-Tol *M159* gene found in the recombinantly-derived region encodes a predicted member of the C7L family of host range factors. Previous reports show that the recombinant cassette that was inserted within the *M009L* gene of MYXV-Tol was originated from a still-unknown donor poxvirus that shares a common ancestral lineage of capripoxviruses, cervidpoxviruses, suipoxviruses and yatapoxviruses. Yet, it is interesting to observe that the newly identified M159 protein shares a higher pairwise identity to the C7L family of host range proteins that are found in leporipoxviruses. In fact, and as can be seen in Figure 1B, the M159 protein is more closely related to the M064 protein from MYXV and rabbit fibroma virus (RFV), sharing 41.8% and 39.9% pairwise amino acid identity, respectively. M159 also shares a high degree of similarity to M063 and M062 from MYXV and RFV, with pairwise identities that range from 25.3 to 44.3% (Figure 1B).

Despite the fact that C7L family members frequently share low sequence identity to each other, their tertiary structures tend to be relatively conserved (16). This family of proteins has adopted a compact β-sandwich fold with an approximate dimension of 45 × 35 × 30 Å that consist mainly of two curved layers, each comprising a six-stranded antiparallel β sheet (16). Therefore, we carried out homology modeling of the newly identified M159 structure using MYXV M064 and VACV C7 as templates (Protein Data Bank database, accession no. 5cz3B and 5cywB, respectively). The M159 protein homology model shows the conserved structure of the C7L protein family with a modelled 12-stranded antiparallel β-sandwich wrapped in two short α helices (Figure 2B). On one side, M159 protein has a layer of strand β1, β2, β4, β7, β8, and β12 and a second layer of strands β3, β5, β6, β9, β10, and β11. Moreover, the M159 model also includes two α helices (α1 and α2), with helix α1 linking strands β9 and β10 and α2 being located at the C-terminus (Figure 2B). Altogether, these results clearly show that M159 protein belongs to the C7L family of host range genes. We predict that, like other C7L members, M159 functions via host-derived binding partners but the sequence divergence within the C7L

family is sufficiently large that it is difficult to model how different the putative host target(s) of M159 might be, in terms of its closest know poxvirus gene relatives.

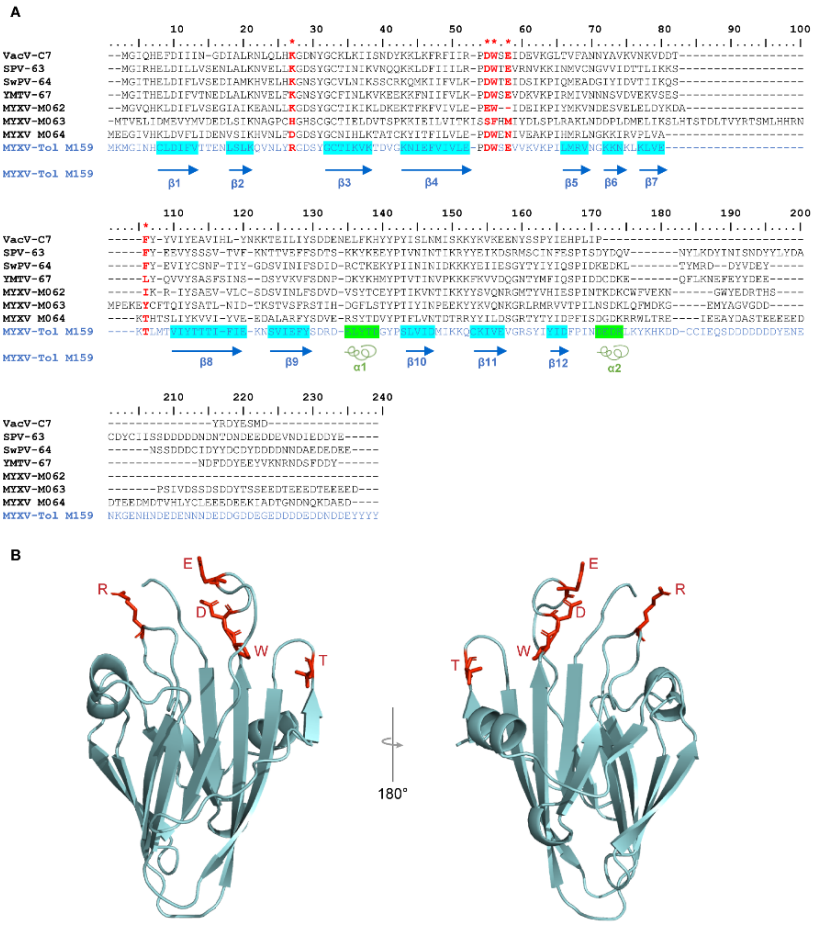

Figure 2. M159 adopts the conserved structure of the C7 protein family. A) Amino acid sequence comparison of different members of the C7 like superfamily of host range genes including VACV C7, SPPV-63, SWPV-64, YLDV-67, MYXV-M062, M063, M064 and MYXV-Tol M159. The 12-stranded antiparallel β-sandwich (blue boxes) and the two short α helices (green boxes) from MYXV-Tol M159 are highlighted. The five VACV C7 conserved residues (K24, D51, W52, E54, and F79) critical for binding to SAMD9 are indicated by a red asterisk (*). **B)** Homology modelled structure of the M159 protein. The 12 stranded antiparallel β-sandwich and two α helices are shown in light green. The five VACV C7 conserved residues (K24, D51, W52, E54, and F79) are shown in red.

Reports suggest that the functional core for the C7L family is the conserved β-sandwich (8). Structure-guided mutagenesis studies on the VACV C7, identified residues K24, D51, W52, E54, and F79 as being critical for binding to SAMD9 and therefore viral replication (16). These residues are located in three loops clustered on one edge of the β sandwich and are characterized with functionally important negatively charged, positively charged, and hydrophobic residues, forming a unique “three-fingered molecular claw”. Interestingly, this claw is not conserved in the MYXV M063 and M064 proteins, two C7L family members that do not antagonize SAMD9 (16). Analysis of the five conserved C7L residues at the corresponding positions of the M159 protein show that only D51, W52 and E54 are conserved (Figure 2A). Interestingly, in the

corresponding position of K24, M159 protein has an arginine (R) that, like lysine (K), is positively charged. However, in the corresponding position of F79, the M159 protein has a threonine (T), which contrarily to phenylalanine (F) is not a hydrophobic residue (Figure 2A). The replacement of a hydrophobic residue for a polar residue (T) in the "three-fingered molecular claw" responsible for binding to SAMD9 might suggest a lower affinity for SAMD9 or a different conformation of this claw in the M159 protein and, therefore, a different binding partner or function compared to the ones observed for VACV C7 and MYXV M062.

2.2. M159 is essential for the replication of MYXV-Tol in cultured hare cells

Regarding the expansion of host range, it is likely that the acquisition of new genes involved in immunomodulation or host-range functions in specific cell types would have a preponderant role in this apparent species leaping of MYXV-Tol. Moreover, given that the *M159* gene was acquired from a currently-unknown "donor" poxvirus with a different repertoire of antiviral response pathways, we speculated that this new host range protein is capable of differentially modulating the antiviral response of hare vs rabbit cells.

To elucidate the role of *M159* gene and/or gene product during MYXV infection, we constructed a knockout virus from the wild type MYXV-Tol backbone in which only the *M159* gene was disrupted by a gene cassette having reporter GFP and TdT genes under the control of a synE/L promoter and a p11 late promoter, respectively (vMyxTol-Δ159). To understand the importance of the *M159* gene alone, a V5-tagged *M159* gene (controlled under its own native promoter) was inserted between the *M135L* and *M136L* gene loci of MYXV-Lau backbone along with GFP gene under the control of a synE/L promoter (vMyxLau-V5M159). In addition, to understand the importance of the remaining genes of the recTol region relative to the *M159* gene alone, we constructed two recombinant viruses where the entire 2.7 kb cassette was inserted in two different regions of the MYXV-Lau: one between the *M135L* and *M136L* gene loci (vMyxLau-recTol) and another one within the *M009L* gene (Δ*M009L*), replicating the position of the recTol region in the wild type MYXV-Tol (vMyxLau-Δ*M009L*+recTol). All these viruses were studied and compared against wild-type MYXV-Lau and MYXV-Tol, both expressing a GFP and TdT gene cassette under the control of a synE/L promoter and a p11 late promoter, respectively (vMyxLau and vMyxTol). This reporter gene cassette was

inserted between the conserved intergenic regions of the *M135* and *M136* genes. Table 1 illustrates the characteristics of all recombinant viruses used in this study.

Table 1. Summary of the recombinant viruses generated of this study

Recombinant virus	Backbone	Insertion	Disrupted genes	Virus abbreviation
vMyxTol-TdT-GFP	MYXV-Tol	TdT GFP	---	vMyxTol
vMyxTol-ΔrecTol-GFP	MYXV-Tol	GFP	ΔrecTol region	vMyxTol-ΔrecTol
vMyxTol-ΔM159-TdT-GFP	MYXV-Tol	TdT GFP	ΔM159	vMyxTol-ΔM159
vMyxTol-V5tagM159-GFP	MYXV-Tol	V5tag M159 GFP	---	vMyxTol-V5M159
vMyxLau-TdT-GFP	MYXV-Lau	TdT GFP	---	vMyxvLau
vMyxLau-ΔM009-tdT-recTol-GFP	MYXV-Lau	TdT GFP RecTol region	ΔM009	vMyxLau-ΔM009+recTol
vMyxLau-recTol-tdT-GFP	MYXV-Lau	TdT GFP RecTol region	---	vMyxLau-recTol
vMyxLau-V5tagM159-GFP	MYXV-Lau	V5tag M159 GFP	---	vMyxLau-V5M159

When MYXV recombinant viruses were tested in two different hare cell types: hare HN-R cell line and primary hare PBMCs; these test cells engaged similarly in supporting dramatically different levels of viral infection that reflect the essential nature of the M159 gene. As expected, viral infection by MYXV-Tol (vMyxTol) was fully productive in both cell types (Figure 3A and C), whereas infection was blocked in the wild type MYXV-Lau (vMyxLau) (Figure 3A and C). Remarkably, knockout of the M159 gene alone in the MYXV-Tol backbone (vMyxTol-Δ159) resulted in an abortive replication cycle in the hare HN-R cell line and hare PBMCs, as there was no increase in vMyxTol-Δ159 titer after 24 hpi (Figure 3B and D). Since both hare cell types reached higher progeny virus titers after 24 hpi with vMyxTol, and disruption of M159 in the MYXV-Tol backbone resulted in an abortive replication, it highly suggests that M159 gene is critical for MYXV-Tol infection. Interestingly, while insertion of the *M159* gene in the MYXV-Lau backbone (vMyxLau-V5M159) enabled infection in hare HN-R cells, as vMyxLau-V5M159 showed efficient virus infection and an increase in virus titer after 24 hpi (Figure 3A and B); infection of hare PBMCs with vMyxLau-V5M159 did not lead to an increase in virus titer after 24 h.p.i (Figure 3D). In fact, infection of hare PBMCs by vMyxLau-V5M159 led to an early viral gene expression (GFP expression in Figure 3C) but late viral expression

was absent (not shown), suggesting that in hare PBMCs, M159 alone is not enough to rescue vMyxLau replication to the level of vMyxTol and we conclude that primary PBMCs might have a different way of sensing vMyxLau-V5M159 compared to the immortalized hare HN-R cell line. Interestingly, in the hare HN-R cell line, the three viruses that have MYXV-Lau as backbone (vMyxLau-V5M159, vMyxLau-recTol and vMyxLau-ΔM009+recTol) exhibited similar replication and progeny virus yelds (Fig. S1), suggesting that the disruption of the *M009L* gene with the recTol region does not alter MYXV replication and that the position of the recTol cassette does not affect MYXV infection of hare HN-R cells.

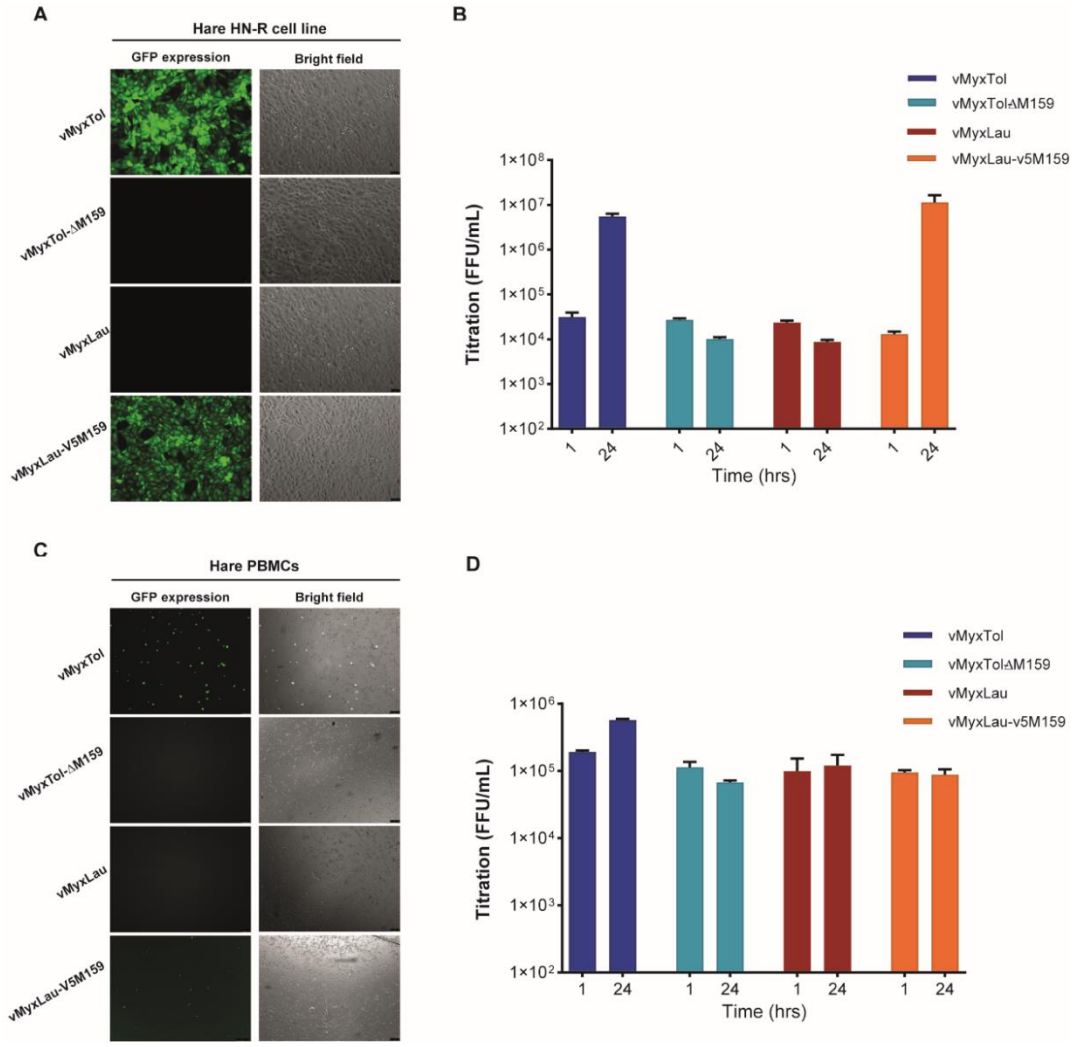

Figure 3. M159 is required for MYXV-Tol replication in hare PBMCs and hare HN-R cell line. A) Fluorescence microscope images of infected hare HN-R cells. The cells were infected with vMyxTol, vMyxTol-Δ159, vMyxLau and vMyxLau-V5M159 at a MOI of 1 and the images were taken using an inverted fluorescence microscope with 5x magnification at 24 hpi. **B)** Proliferation of different recombinant MYXVs in hare HN-R cell line. The cells were infected with the indicated viruses at an MOI of 1 for 1h and 24h. The virus titers from three independent experiments were investigated in triplicate following serial dilutions onto rabbit RK13 cells. Differences at $P<0.05$ were considered significant. **C)** Fluorescence microscope images of infected hare PBMCs. The cells were infected with vMyxTol, vMyxTol-Δ159, vMyxLau and vMyxLau-V5M159 at an MOI of 10 and the images were taken using an inverted fluorescence microscope with 5x magnification at 24 hpi. **D)** Proliferation of different recombinant MYXVs in hare PBMCs. The cells were infected with the indicated viruses at an MOI of 10 for 1h and 24h. The virus titers from three independent experiments were investigated in triplicate following serial dilutions onto rabbit RK13 cells. Differences at $P<0.05$ were considered significant.

2.3. M159 is essential for viral late protein synthesis in hare cells

The microscopy analyses of infected cultures show that, vMyxLau or vMyxTol-ΔM159 only induced a barely detectable level of GFP expression and no TdT expression in hare HN-R cells (Fig. S1). In contrast, vMyxTol, vMyxLau-V5M159, vMyxLau-recTol and vMyxLau-ΔM009+recTol productively infected HN-R hare cells after 24 hpi, as can be seen by the robust expression of the late reporter protein TdT (Figure 3A and Fig. S1). We further confirmed the importance of the M159 protein for MYXV replication in the hare HN-R cell line by monitoring the viral genomic copy number after infection at different time points (Figure 4A). The qPCR results show that both vMyxLau and vMyxTol-ΔM159 genomic DNA is detectable in hare cells at 8 hpi; however, at later time points viral genome amplification was significantly reduced compared to the vMyxTol. Again, the presence of the complete recTol region and/or the *M159* gene locus in the MYXV-Lau backbone show the same trend for viral replication as vMyxTol (Figure 4A).

In contrast to many other DNA viruses, poxvirus DNA replication takes place in cytoplasmic viral factories (17). Upon infection, poxviruses exhibit a tight regulation of a gene expression cascade that results from the genes being regulated by promoters that are transcribed at early, intermediate, and/or late times of infection (17, 18). In infection by VACV, following virion binding to the cell surface and internalization, there is a release of the virion core into the cytoplasm followed by transcription of early viral mRNA that typically encode products required for immune evasion, core uncoating, release of genomic DNA and initiation of DNA synthesis (19). The beginning of viral DNA replication coincides with a switch to transcription of intermediate and then late mRNAs whose products are required for virion assembly (17, 18). Finally, viral genome replication and assembly of progeny virus particles occur in cytoplasmic viral factories (20, 21). In this study, we have shown that a block in wild-type MYXV-Lau replication exists in hare HN-R cells, when M159 protein is not present (i.e. cells infected with vMyxvTol-ΔM159 and vMyxvLau) (Figure 4A). To evaluate when this block occurs in hare HN-R cells, the expression of known early and late MYXV proteins was tested by western blot (22, 23). We infected hare HN-R cells with vMyxTol, vMyxvTol-ΔM159, vMyxLau and vMyxvLau-V5M159 at a high MOI (MOI=5) and left the cells untreated or added AraC (40 µg/ml), an inhibitor of poxviral DNA replication and late protein synthesis for 1 hr following virus adsorption. Infected cell extracts were then immunoblotted using anti-M-T7, a strongly expressed MYXV early gene product and with anti-Serp1, a MYXV late gene product (22, 23). The early M-T7 protein was detectable as early as 3 hpi and at 24 hpi regardless

of the virus used, in the presence or ansence of AraC (Figure 4B). Late Serp1 was detectable at 24 hpi in hare HN-R cells infected by vMyxTol and vMyxLau-V5M159, except when DNA synthesis was blocked by the addition of AraC. On the other hand, Serp1 was not detectable at any time point collected for vMyxTol-ΔM159 or vMyxLau infected hare cells (Figure 4B). These results reinforce the earlier demonstration that infection by vMyxTol-Δ159 and vMyxLau undergo an early block in viral replication, with M159 playing a critical role for the transition into late stages of infection and replication in hare HN-R cells. Moreover, while there was evidence for the existence of viral factories in hare HN-R cells infected by vMyxTol and vMyxLau-V5M159 (arrows in Fig. S2), the same was not observed after infection by vMyxTol-Δ159 or vMyxLau (Fig. S2).

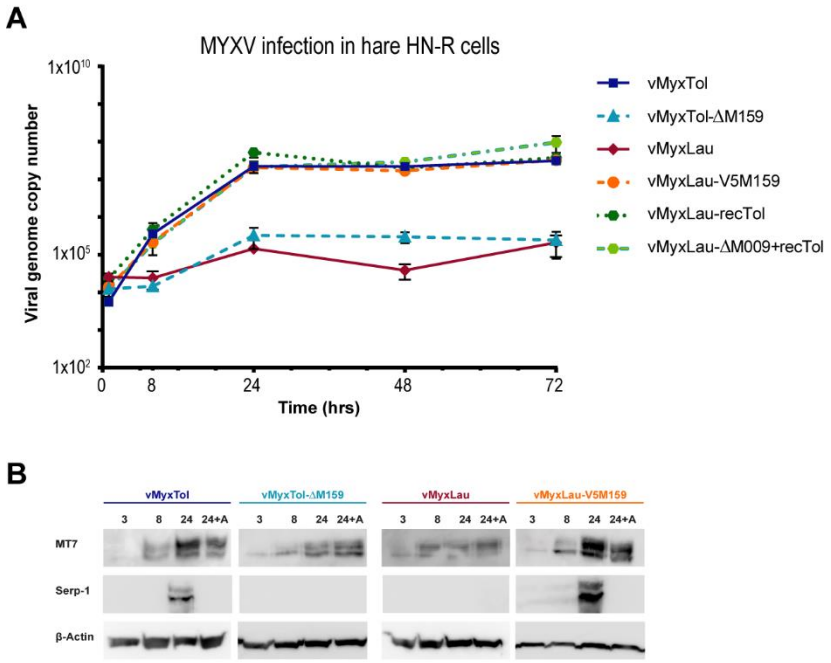

Figure 4. M159 is essential for MYXV transition into late expression in hare HN-R cells. A) Infection with vMyxLau and M159 knockout virus (vMyxTol-Δ159) in hare HN-R cells. HN-R cells were infected with vMyxTol, vMyxTol-Δ159, vMyxLau, vMyxLau-V5M159, vMyxLau-ΔM009+recTol and vMyxLau-recTol at an MOI of 1. At given time points (0, 8, 24 and 48 hpi), cell lysates were harvested for DNA extraction, followed by qPCR targeting the MYXV *M053* gene. Differences among means for three independent experiments were investigated using a two-way analysis of variance (ANOVA) followed by Dunnett's test using Prism® 6 (GraphPad Software, Inc.). Differences at $P<0.05$ were considered significant. **B)** Western-blot analyses of early and late viral gene expression after infection with vMyxTol, vMyxTol-Δ159, vMyxLau, vMyxLau-V5M159. HN-R cells were pretreated for 1 hr with AraC (40 µg/ml) or not pretreated, followed by infection at a MOI of 5. At given time points (3, 8 and 24 hpi), cell lysates were harvested for Western blotting. Early/late gene expression (M-T7) and late gene expression (SERP-1) were compared between the viruses with Actin used as control. The molecular size of M-T7 is 35 kDa, 55 kDa for Serp-1 and 42 kDa for Actin. The results shown are representative of two independent experiments.

Overall, while early gene expression remained unaltered in cell-infections by all recombinant MYXVs tested, the absence of M159 in MYXV prevented viral factory formation in hare HN-R cells and thus culminated in defective late protein production and

viral DNA replication. Altogether, these results confirm that the *M159* gene product is necessary and sufficient for the successful replication of MYXV in hare HN-R cells.

2.4. M159 is expressed as an early/late product that is translocated into the nucleus at late time points in virus-infected hare cells

Previous studies indicate that the other MYXV C7L members are expressed as early/late genes (10–12). Given the higher similarity of *M159* to MYXV C7L genes (Figure 1B), we tested whether M159 is also expressed as an early/late product. For that, a recombinant MYXV-Tol was constructed bearing V5-tagged M159 (vMyxTol-V5M159) inserted between the *M135L* and *M136L* gene loci and expressed GFP under a poxvirus early/late promoter (Table 1). Given that vMyxTol-V5M159 has two *M159* gene copies, virus replication was tested in the hare HN-R cell line and compared with the wild type MYXV-Tol (vMyxvTol). Both viruses show similar transmission kinetics and progeny virus titers after 96 hpi in HN-R cells (Fig. S3). A time course study of M159 protein synthesis was conducted by infecting HN-R cells at a MOI of 5 in the presence or absence of AraC. Similar studies were also conducted with the vMyxLau-V5M159 described previously (Fig. S4).

Contrary to what is observed for the three MYXV C7L members (10–12), M159 protein was only detected at high concentrations at 8 hpi (Figure 5A) and its levels were maintained until 48 hpi (Figure 5A). However, following treatment with AraC, M159 protein levels were drastically reduced, with only a small amount of M159 being detected at 24 hpi (Figure 5A). Given that AraC acts to block DNA synthesis, these results suggest that the *M159* gene does have an early/late promoter, but with the bulk of M159 being expressed as a late gene. To confirm these results, *M159* gene expression was analyzed by qPCR using primers and a probe that specifically targets the mRNA product of this gene. Despite M159 protein levels only starting to be easily detectable at 8 hpi (Figure 5A), *M159* mRNA can be detected at 3 hpi and increases over time with maximum expression levels at 24 hpi (Figure 5B). This difference between protein vs mRNA levels of *M159* could be due to lower the sensitivity of protein detection by Western blotting, or the co-incident early transcription of the M159 gene from an upstream promoter in the vMyx-Tol genome. The expression of *M159* and Serp-1 mRNA was noticeably lower following the treatment with AraC (Figure 5B and C), suggesting once more that *M159* is expressed as an early/late gene, which would be consistent with its function in relieving an early block of viral replication.

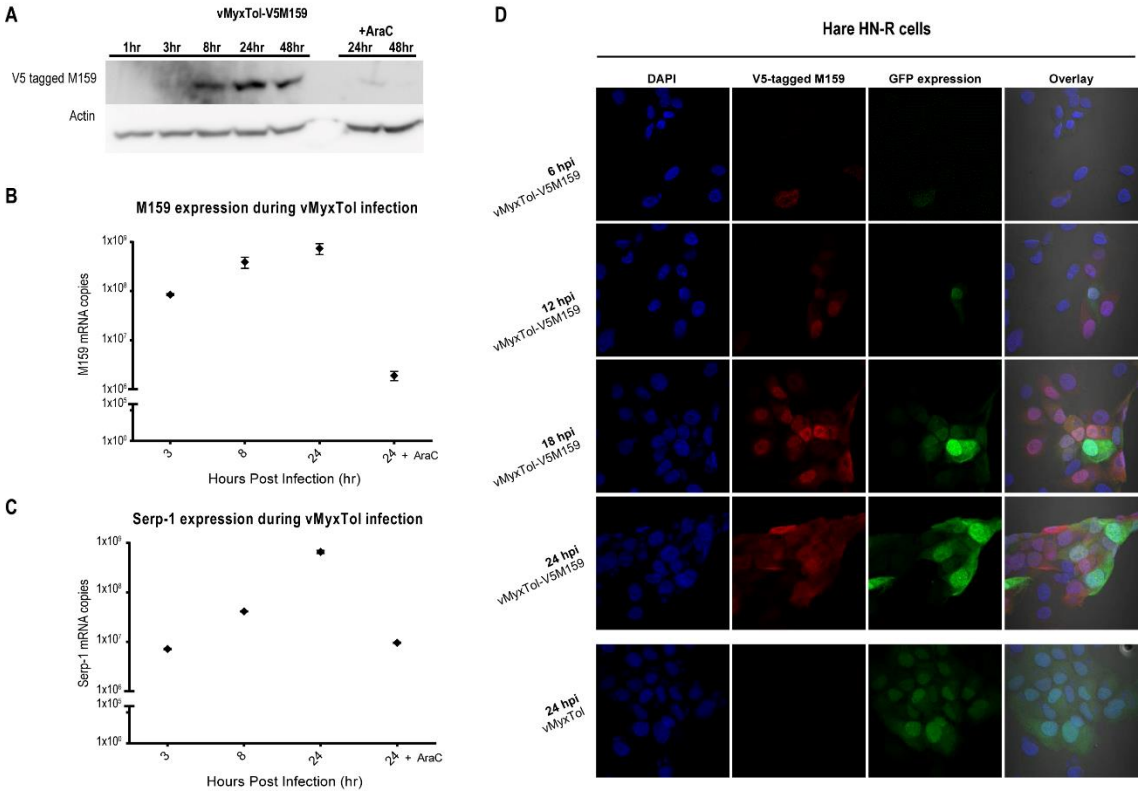

Figure 5. M159 is expressed as a late gene and moves into the nucleus at later time points of infection. A) Western-blot analysis of M159 expression during viral infection. HN-R cells were pretreated or not with AraC (40 µg/ml), followed by infection with vMyxTol-V5M159 at an MOI of 5. At given time points (1, 3, 8, 24 and 48 hpi), cell lysates were harvested for Western blotting. V5-tagged M159 was detected with the anti-V5 antibody. The molecular mass of actin is 42 kDa and the expected size for the V5 tagged product is ~25 kDa. Three independent experiments were made to confirm the obtained results. RT-qPCR was used to investigate expression of two MYXV genes: **B)** *M159* and **C)** *Serp1*. The comparative CT method was used to calculate and compare the relative levels of RNA. A control of HN-R cells pretreated with AraC for 1h before infection and collected 24 hpi was also used in the same conditions. The results of the two biological replicates are shown. Significant differences among means for experiments in B) and C) were analyzed using a two-way ANOVA followed by Dunnett's test using Prism® 6 (GraphPad Software, Inc.). Differences at $P<0.05$ were considered significant. **D)** M159 localizes to the nuclear and cytoplasmic compartments. HN-R cells grown on glass coverslips were infected with vMyxTol-V5M159 and vMyxTol (negative control) at a MOI of 1. After 6, 12, 18 and 24 hpi, cells were fixed, permeabilized, stained and observed using confocal microscopy. DNA in the nucleus and viral factories were labeled with DAPI (blue). Infection was tracked by GFP expression (green) and V5-tagged M159 (red) using immunolabelling.

We next examined the localization of M159 protein in virus-infected hare HN-R cells using confocal microscopy (Figure 5D). When hare HN-R cells were infected with vMyxTol-V5M159, expression of the V5-tagged M159 protein was detected after 6 hpi (Figure 5D, row 1). At later time points (12, 18 and 24 hpi), M159 protein started to be detected in both the cytoplasmic and nuclear compartments of hare HN-R cells (Figure 5D, rows 2, 3 and 4). Interestingly, throughout the time course of the experiment, no V5-tagged M159 was detected in the viral factories. C7L family protein localization is significantly dependent upon interaction with host binding partners. The VACV C7 protein and some of its related host range proteins use their “three-fingered molecular claw” to bind and counteract cellular SAMD9. This cytoplasmic host cell protein is known to act

as a major restriction factor in poxvirus infection, triggering an early block in infection and is involved in inhibition of viral mRNA translation (16, 24). The same has been shown for the MYXV M062 protein, which also binds and counteracts the action of SAMD9 (10). The data in this work indicate that like other C7L members, M159 protein is also located in the cytoplasmic compartment during infection, suggesting a possible interaction with SAMD9. However, given the presence of M159 in the nucleus at later time points, it is possible that this protein might also have a specific function in this compartment. Nevertheless, the ability of M159 to bind to SAMD9 remains to be tested.

Over the past years, proteomic analyses, such as co-immunoprecipitation (co-IP) and mass spectrometry, have been foundational in identifying host factors that interact with C7L family members. However, as some interactions can be transient or cellular localization dependent, co-IP techniques may not always be ideal for interaction analyses. In this study, co-IP techniques that successfully identified the host binding partners of different MYXV proteins like M029 and M062 were used to try to identify V5-tagged M159 binding partners (10, 25). However, we were unable to identify long-lived direct binding partner/s for the M159 protein in hare HN-R cells. Further studies of transitory protein-protein interactions will be needed to elucidate the mechanism of M159 action involved in MYXV-Tol host range in hares.

2.5. M159 is not essential for MYXV infection and replication in rabbit cells

Recently, MYXV-Tol was also reported to be capable of causing myxomatosis in European rabbits (13). However, these reported cases of pathogenesis were less frequent in rabbits, suggesting a different susceptibility to MYXV-Tol amongst the Lagomorphs. To compare infectivity of the MYXV-Lau and MYXV-Tol and to examine whether *M159* gene expression also has an effect in replication and spread of MYXV in rabbit cells, the several recombinant viruses were evaluated in two different rabbit cell lines: RK13, a rabbit kidney derived cell line and RL-5, a rabbit CD4+ T cell line. These two rabbit cell lines were infected with the six types of recombinant viruses described above (Table 1) at a MOI 1 (one-step) or MOI 0.1 (multi-step) (Fig. S5). In contrast to what was observed in hare PBMCs and HN-R cells (Figure 3A and B), all tested viruses/viral constructs replicated efficiently and to similar levels in the permissive rabbit RK13 cell line, regardless of the MOI used (Fig. S5, panel A and B). A similar scenario was observed for the rabbit RL-5 cell line, where all viruses exhibited identical

transmission kinetics and reached similar titers at 72 hpi (Fig. S5, panel C and D). These results suggest that in the rabbit cell lines tested, neither the expression of *M159* gene or the different MYXV backbones have detectable effects in the overall replication efficiency of these recombinant viruses in rabbit cells.

Since the 1950s, MYXV has evolved rapidly and converged to less pathogenic phenotypes in the feral European rabbit populations in both Australia and Europe (26). More recently, Alves et al. (27) also showed that resistance of the European rabbit populations to MYXV evolved by a strong pattern of parallel evolution, with selection on immunity-related genes favoring the same alleles in Australia, France, and the United Kingdom. As mentioned before, apart from the inserted recombinant region of unknown origin, MYXV-Tol backbone is ~ 99% similar to the MYXV-Lau strain and, therefore, it is possible that the European rabbit populations in Spain and Portugal have undergone a long-term adaptation to MYXV strains. Moreover, our results show that M159 does not contribute to MYXV host range functions in the tested rabbit cells, which might indicate that in the native environment there is no difference in the pathogenicity of MYXV-Tol, compared to other MYXV strains in European rabbit populations. Nevertheless, future *in vivo* experimentation in rabbits will need to be conducted to understand the clinical outcome of myxomatosis for the different MYXV constructs.

2.6. M159 upregulates the replication of MYXV-Lau and MYXV-Tol in selected human cancer cells.

Despite MYXV being able to only infect Leporids in nature, MYXV can selectively enter and kill cancer cells from different non-rabbit species, including mice and humans (28). For this reason, different multi-transgene-armed recombinant MYXV constructs are currently being developed as oncolytic therapeutics for human cancer (29). Based on our observations that the *M159* gene plays a major role in the ability of MYXV to replicate in hare cells (Figure 3), we tested infection and replication of different recombinant MYXVs (Table 1) in a variety of human cancer cell lines that are considered to be non-permissive or semi-permissive to the parental MYXV-Lau strain (Figure 6). The parental virus (vMyxLau) replicates poorly in the human pancreatic cancer cell line PANC-1 and human melanoma cancer cell line MDA-435 (30). As can be seen in Figure 6, the presence of *M159* in the MYXV-Lau backbone significantly increased MYXV yield in both PANC-1 and MDA435 cell lines after 48 and 72 hpi, suggesting that expression of M159 can, to some extent, confer replication competence of MYXV-Lau in these non-

permissive human cell lines. Interestingly, cell viability was unchanged in both cell lines when infected with the different recombinant viruses (data not shown). When the MDA435 cell line was infected with wild type vMyxTol, higher transmission kinetics and higher progeny virus titers were also observed when compared with vMyxLau infection (Figure 6A and C). Interestingly, in PANC-1 cancer cells, infection by vMyxTol reached similar transmission kinetics to vMyxLau (Figure 6B). The fact that *Serp3* is disrupted in the MYXV-Tol backbone might partially explain the similar titers obtained for infection with vMyxLau (5). However, it is also possible that other mutations in the MYXV-Tol backbone might lower the overall infectivity of this virus in this human cell line, even with expression of the *M159* gene. Given that M159 exhibits host range properties in both hare and human cells, we suggest that the host target pathway of M159 might be conserved across species, which will be of extreme importance for future studies on M159 targeted pathways.

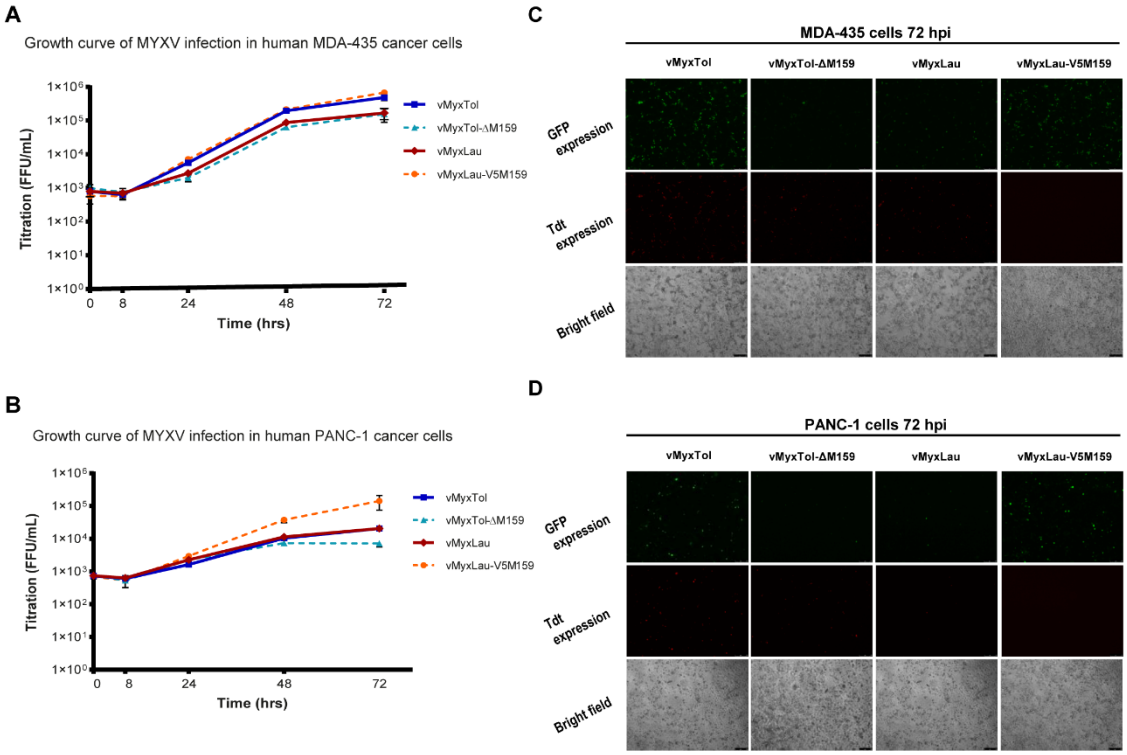

Figure 6. M159 alters the replication of MYXV in human cancer cells. Single step growth curves of MYXV in **A)** MDA-435 and **B)** PANC-1 human cancer cell lines. The cultures were infected with vMyxTol, vMyxTol-Δ159, vMyxLau and vMyxLau-V5M159 at an MOI of 1, and then cells were collected at 1, 8, 24, 48 and 72 hpi. Virus titers are representative of three independent experiments and were determined in triplicate following serial dilutions onto RK13 cells. Significant differences among means for experiments in A) and B) were determined using a two-way ANOVA followed by Dunnett's test using Prism® 6 (GraphPad Software, Inc.). Differences at $P<0.05$ were considered significant. **C)** Fluorescence microscope images of infected MDA-435 cancer cells. The cells were infected with vMyxTol, vMyxTol-Δ159, vMyxLau and vMyxLau-V5M159 at a MOI of 1 and the images were taken using an inverted fluorescence microscope with 5x magnification at 72 hpi. **D)** Fluorescence microscope images of infected PANC-1 cancer cells. The cells were infected with vMyxTol, vMyxTol-Δ159, vMyxLau and vMyxLau-V5M159 at an MOI of 1 and the images were taken using an inverted fluorescence microscope with 5x magnification at 72 hpi.

3. Concluding Remarks

The presence of a novel C7L host range gene family members in a MYXV strain (Toledo) capable of crossing the species barrier and causing myxomatosis in *Lepus granatensis*, raises some relevant questions regarding the host range abilities of this new virus. In this study, we have shown that the ability of MYXV-Tol to infect hare cells (and to a lesser extent, normally nonpermissive human PANC-1 and MDA-435 cancer cells) and replicate, ultimately depends on the M159 protein alone. Interestingly, the M159 protein did not contribute to increased replication in the tested permissive rabbit cells. Our studies with wild-type MYXV-Lau or vMyxTol-ΔM159, showed that both were unable to detectably replicate in hare cells, while MYXV-Tol virus that expresses M159 underwent a fully productive replication. Interestingly, in primary hare PBMCs, insertion of the *M159* gene into the MYXV-Lau backbone was not enough to rescue MYXV-Lau infection in these cells, raising awareness for the need of additional replication requirements in this hare cell type. From the results shown, M159 expression was not required for MYXV binding, entry or early gene expression, but it was necessary for relieving an early block of viral replication and late protein synthesis in hare HN-R cells.

Our 3D modelling analyses show that the predicted M159 protein has the common protein scaffold that characterizes other C7L family members, but presents amino acid differences in its sequence at the three-fingered molecular claw, which is responsible for the ability of some C7L family members to bind to the host restriction factor SAMD9. Moreover, while the results show that *M159* is an early/late gene, the M159 protein can only accumulate at high levels at later time points. Considering the M159 migration to the nucleus from the cytoplasmic compartment it is possible that, besides its ability to bind the hare version of SAMD9, M159 might also bind or induce cellular factor(s) that mediate host range or immunosuppression at later time points from the nuclear compartment of hare cells. M159 was derived from an unknown donor poxvirus that in terms of gene cassette order resembles members from the ungulate poxvirus family. For this reason, it is possible that in its “natural” host the M159 protein acts by regulating a unique repertoire of anti-viral response pathways that confer advantage in engaging and modulating the hare’s antiviral responses. Nevertheless, although we have been unable to demonstrate a stable protein:protein interaction complex between M159 and SAMD9 in hare cells, a putative transitory interaction with the hare version of SAMD9 cannot be discarded.

Several relevant technical and logistical limitations exist to establish hare colonies to MYXV pathogenicity. Nevertheless, our results provide strong evidence that the M159 protein found in the recombinant region of MYXV-Tol is the prime candidate enabling this recombinant MYXV-Tol to infect hare cells, which ultimately might culminate in myxomatosis in Iberian hares. We believe that these data have the potential to improve the current knowledge about the host range and virulence of poxviruses in general and provide a platform for better understanding the pathogenicity and transmission success of emerging viruses like the new MYXV-Tol virus, rendering it capable of leaping into a new host species. Moreover, the presence of this novel host range factor in a MYXV strain capable of crossing the species barrier, suggests that the acquisition of this gene had a preponderant role in this species leap and highlights that homologous recombination could also be a feature of gene gain that might play a major role in poxvirus evolution.

4. Material and Methods

4.1. Phylogenetic analysis and homology modelling of C7-like proteins of MXYV

The M159 amino acid sequence and all other representative related protein sequences (n=34) encoded by poxviruses were downloaded and aligned using MAFFT. The resulting alignment was used to infer a maximum likelihood phylogenetic tree using PHYML (31) with JTT+G+F used as the amino acid substitution model, determined by ProtTest (32). Branches with <0.8 aLRT branch support were collapsed using TreeGraph2 (33).

The amino acid sequence of C7L proteins (M159, M64, M63 and M62) were used for modelling with protein data bank ID 5cz3B (crystal structure of myxoma virus m64) and 5cywB (crystal structure of vaccinia virus C7 protein) structures using Phyre2 (34). The structures were aligned and visualized in PyMOL Molecular Graphics System v2.4 (Schrödinger, LLC).

4.2. Construction of recombinant viruses

Based on our findings that MYXV-Tol variant contains a new genomic cassette insertion including a tentative candidate host range factor (M159), we constructed several MYXV recombinants to assess whether M159 protein influences MYXV-Tol

infectivity in cells from different Leporids. For this, we constructed two different sets of recombinant viruses: viruses with MYXV-Lau as backbone and viruses based on MYXV-Tol as backbone (5) (Table 1).

In the recombinant virus vMyxTol-ΔrecTol-GFP, the entire 2.7 kb recombinant cassette (recTol) from MYXV-Tol was removed and replaced with *GFP* gene under the control of a poxvirus p11 late promoter. In the wild-type MYXV-Tol, a reporter cassette that expresses TdTomato protein (TdT) (36) under a poxvirus late p11 promoter and green fluorescent protein (GFP) gene under a poxvirus early-late promoter was inserted between the *M135R* and *M136R* genes (vMyxTol-TdT-GFP). The same reporter cassette was also inserted within the *M159* locus of MYXV-Tol, which results in a *M159* gene knock out recombinant MYXV-Tol (vMyxTol-ΔM159-TdT-GFP). In the MYXV-Lau backbone, the new recTol cassette was inserted within the *M009L* locus (vMyxLau-ΔM009-TdT-recTol-GFP) along with TdT gene (late p11 promotor) and GFP gene (early-late promoter), which disrupts the *M009L* and allow the expression of all the viral proteins from the novel 2.8 kb recombinant cassette derived from MYXV-Tol. In another construct, which acts as a control, the full recTol cassette was inserted between *M135* and *M136* gene loci of MYXV-Lau (vMyxLau-recTol-tdT-GFP). To characterize M159 expression in hare cells, recombinant MYXVs were constructed expressing V5-tagged *M159* gene under its own promoter and GFP gene under early-late promoter, which were inserted between the *M135L* and *M136L* gene locus of both MYXV backbones (vMyxLau-V5tagM159-GFP and vMyxTol-V5tagM159-GFP). The wild-type MYXV-Lau expressing TdT (late p11 promotor) and GFP (early-late promoter) between the *M135L* and *M136L* loci (vMyxLau-TdT-GFP) was used for comparison (37).

For all recombinant viruses, recombinant plasmids were first constructed using the MultiSite Gateway Pro (Invitrogen) system, as described before (25, 38, 39) or the Gibson cloning method (New England Biolabs, USA). Upstream and downstream hybridizing sequences were amplified by PCR using specific primers to create entry clones by Gateway BP recombination system with appropriate pDONR vectors. The final recombination plasmids were obtained by recombination of the three pDONR entry vectors with a destination vector (pDEST40; Invitrogen), using Gateway LR recombination system. Subconfluent monolayers of RK13 cells were then infected with the appropriate MYXV backbone and transfected with the final recombination plasmids using Effectene Transfection Reagent (Qiagen, USA) or Lipofectamine 2000 (Thermofisher, USA) following the manufacturer instructions. Confirmation of a

successful recombinant virus clone was achieved by PCR amplification, Sanger sequencing and subsequent foci purification. Purification of the recombinant virus stocks was carried out using 36% w/v sucrose cushions. All recombinant viruses showed fully permissive replication in the RK13 cell line. The viral titers were quantified by counting foci (focus-forming units per mL, FFU/mL), areas of virus-infected cells, under a fluorescence microscope.

4.3. Cell lines and cell culture

Rabbit cells (cell line RK13; ATCC# CCL-37) and the human cancer cells MDA-435 (cell line MDA-MB-435; ATCC# HTB-129) and PANC-1 (30) were cultured in Dulbecco minimum essential medium (DMEM; Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 2 mM glutamine (Invitrogen, USA) and 100 µg/ml of penicillin-streptomycin (pen/strep; Invitrogen, USA). Hare cells (cell line HN-R; Friedrich-Loeffler-Institut, Germany), an spontaneously immortalized cell line derived from kidney (35), were cultured in 50 % Iscove's Modified Dulbecco's media (IMDM) and 50 % Ham's F-12 supplemented with 10% FBS, 2 mM glutamine and 100 µg of pen/strep per ml. All cultures were maintained at 37°C in a humidified 5% CO_2 incubator (Thermo Fisher, USA). The RL-5 rabbit CD4+ T cell line was cultured in RPMI 1640 (obtained from Gibco), also supplemented with 10% FBS, 2 mM glutamine and 100 g/ml of pen/strep.

4.4. Isolation of hare PBMCs and infection with viruses

Blood samples were obtained during the sanitary management of Iberian hare individuals from approved breeding hares. Blood was collected from healthy unvaccinated (namely juveniles) and vaccinated individuals (adults) of Iberian hare. Animals were caught with hare-specific nests and handled in dark conditions to reduce stress. Blood was collected by puncture from the marginal vein of the ear and immediately transferred in sodium citrate vacutainers or mixed with equal volume of 6.4% of anticoagulant sodium citrate. Blood sample was diluted with the same volume of sterile 1xPBS + 5% FBS and mixed thoroughly. Then, diluted blood was carefully layered in sterile falcon tubes with Lypmphopred^{TM} and centrifuged at 1,200 × g for 10 min at 20°C, with breaks turned off. Layers corresponding to plasma cells, mononuclear cells, granulocytes and the Lymphoprep TM were transferred to a sterile centrifuge tube, mixed with the same volume of 1xPBS+5% FBS and centrifuged at 400 × g for 10 minutes at 25°C. Virus infections were performed in complete RPMI 1640 culture media. For virus

infections, PBMCs were incubated with the test recombinant MYXVs at an MOI of 10 at 37°C for 1 h to allow virus adsorption, washed twice with complete RPMI 1640 to remove unbound virus and then cells were resuspended in a desired volume of media, incubated at 37°C for virus infection. Cells were monitored under a fluorescence microscope or used for virus titration. Virus titration of the samples from hare PBMCs were performed using RK13 cells, where all the test viruses demonstrated permissive replication.

4.5. One-step and multiple-step viral growth curves

One-step growth curves were made by quantifying viral yields at various time points from HN-R or RK13 cells infected with virus at various multiplicities of infection (MOIs). After 1 hr of incubation with virus inoculum, the cells were washed with phosphate-buffered saline (PBS), and the medium was replaced with the appropriate growth medium. Cells were then collected at the desired time points. Multiple-step growth curves were made by titrating virus yields from cell lysates harvested at various time points after infection at a MOI of 0.1. Titrations were conducted using HN-R or RK13 cells, as described previously (Liu et al. 2008). Cytosine arabinoside (AraC; Sigma Life Science) was used to pretreat cells (40 μg/ml) for 1 hr before viral infection.

4.6. Microscopy

To process the hare HN-R cells for immunofluorescence microscopy, cells were washed twice with PBS and fixed with 2% formaldehyde, prepared fresh in PBS from paraformaldehyde, for 12 min at room temperature (RT). The cells were then permeabilized for 1 min at RT in PBS containing 0.1% Triton X-100, washed twice with PBS, and blocked with 3% BSA in PBS for 30 min at 37°C. The cells were then incubated with V5 primary antibody (Invitrogen, USA) at 4°C overnight, washed six times with PBS, and incubated with Alexa Fluor 633 anti-goat secondary antibody (Thermo Fisher, USA) for 30 minutes at 37°C. Nuclei and viral factories were then stained with 4′,6-diamidino-2-phenylindole (DAPI) and the coverslips sealed with nail polish. Samples were imaged with a Nikon C2 scanning confocal on a Nikon Ti microscope, using a 60X plan apo, water immersion lens with numerical aperture of 1.2. Excitation lasers of 405 nm, 488 nm, and 640 nm paired with blue, green, and far-red detectors, were used respectively to detect DAPI, GFP, and immunolabeled V5-tagged M159. Images were post-processed using the Nikon Elements software.

4.6. Western blot analysis

The mock and infected cells were harvested at different time points after infection with viruses, washed with PBS and stored at −80°C or processed immediately with RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 1 mM PMSF, and protease inhibitor cocktail; Roche, USA). The amount of total proteins was estimated by Bradford assay (Bio-Rad, USA) and equal amounts of total protein was used for Western blot analysis as described previously (40). Commercial antibodies targeting anti-V5 (Invitrogen, USA), anti-actin (Ambion, USA), goat anti-mouse IgG conjugated with peroxidase (Thermo Scientific, USA) and goat anti-rabbit IgG conjugated with peroxidase (Santa Cruz Biotechnology, USA) were used for this study. Antibodies against MYXV SERP-1 and MT-7 were previously described (12). Samples were analyzed on 10% SDS-PAGE gels and transferred to PVDF membrane (GE Healthcare, USA) using a wet transfer apparatus (Invitrogen, USA). The membranes were blocked with 5% non-fat milk in TBS buffer (20 mM Tris, 150 mM NaCl, pH 7.6) containing 0.1% Tween-20 (Fisher Scientific, USA) for 1 hr at room temperature. Following this, membranes were incubated at 4°C overnight with primary antibody at the appropriate dilution, washed three times with TBST, and incubated with the secondary antibody conjugated to horseradish peroxidase (HRP) for 1 hr at room temperature. After washing, proteins of interest were detected using Immobilon Western HRP substrate (Millipore) on Kodak BioMax film (Carestream Health, Inc.).

4.7. DNA and RNA extraction and quantitative PCR

To quantify viral load and gene expression, nucleic acid was extracted from RK13 and HNR cell lines using the Qiagen DNeasy Blood & Tissue kit (Qiagen, USA). Quantitative (q)Real-time PCR was set up using SsoAdvanced Universal SYBR Green Supermix (BioRad, USA) targeting a region in the M53 myxoma virus gene (M53F- 5'-CTATCGCGTCGTTAAACAAATTGC-3' and M53R - 5'-GTTGTCGTCCTTATCCATGATAATTCG-'3) that is conserved in MYXV-Tol and -Lau. Each reaction was as follows: 10 µl SsoAdvanced Universal SYBR Green Supermix (BioRad, USA), 0.4 µl (10µM) forward and reverse primers, 5.2 µl water and 4 µl of DNA. pJET 1.2 vector containing target m53 region was used as a standard. Assay was run on a Biorad CFX96 real time thermal cycler using cycling conditions as per SsoAdvanced Universal SYBR Green Supermix manufacturer's recommendations, a melt curve included.

RNA was extracted from HN-R infected cells using Qiagen RNeasy Mini Kit (Qiagen, USA). Reverse transcription (RT) qPCR for assessing gene expression of *M159* and *Serp-1* in vMyxTol infected HN-R cells was undertaken using SuperScript® III First-Strand Synthesis SuperMix (ThermoFisher Scientific, USA). Primer and probe targeting *M159* gene region (M159F-5'-GGTTTTACTTTTACCACTTCACTCC-3', M159R- 5'-GAGGGGACAGTTATGGATGTAC and M159Probe - 5'FAM-CCGGTTCCAACACAATAACG-3'BHQ1) and Serp-1 gene regions (Serp-1F-5'-CAATAGCAGCATTTTAGTATCTCGGTCTAG3', Serp-1R- 5'-GTCATTAACTCGTACGTTAAGGATAAGACG-3' and Serp-1Probe -5'FAM-GTCCAATACGCGTGGGACGTCTC-3'BHQ1) were used in separate assays. The assays consisted of the following master mix per reaction: 12.5 µl 2x RT reaction mix, 0.5 µl SuperScript® III First-Strand Synthesis SuperMix (ThermoFisher Scientific, USA), 0.5 µl (10µM) forward and reverse primers, 0.25 µl (10µM) probe, 6.75 µl water and 4 µl of RNA. Standards for both target regions, M159 and Serp-1, were generated using pJET 1.2 vector. qPCR was performed on a Biorad CFX96 real time thermal cycler as per the cycling conditions recommended by Thermo Fisher Scientific for the SuperScript® III First-Strand Synthesis SuperMix

Supplemenatry Material

Supplementary Material 1. M159 is required for MYXV replication in hare HN-R cells. A) Single step growth curve of MYXV infection in hare HN-R cells. The cells were infected with vMyxTol, vMyxTol-Δ159, vMyxLau, vMyxLau-V5M159, vMyxLau-ΔM009+recTol and vMyxLau-recTol at an MOI of 1, and then collected at 0, 8, 24, 48 and 72 hpi. The virus titers are representatives of three independent experiments and were determined in triplicate following serial dilution onto rabbit RK13 cells. Differences at $P<0.05$ were considered significant. **B)** Fluorescence microscope images of infected HN-R cells. The cells were infected with vMyxTol, vMyxTol-Δ159, vMyxLau, vMyxLau-V5M159, vMyxLau-ΔM009+recTol and vMyxLau-recTol at an MOI of 1 and the images were taken using an inverted fluorescence microscope using a lens with 5x magnification at 24 hpi.

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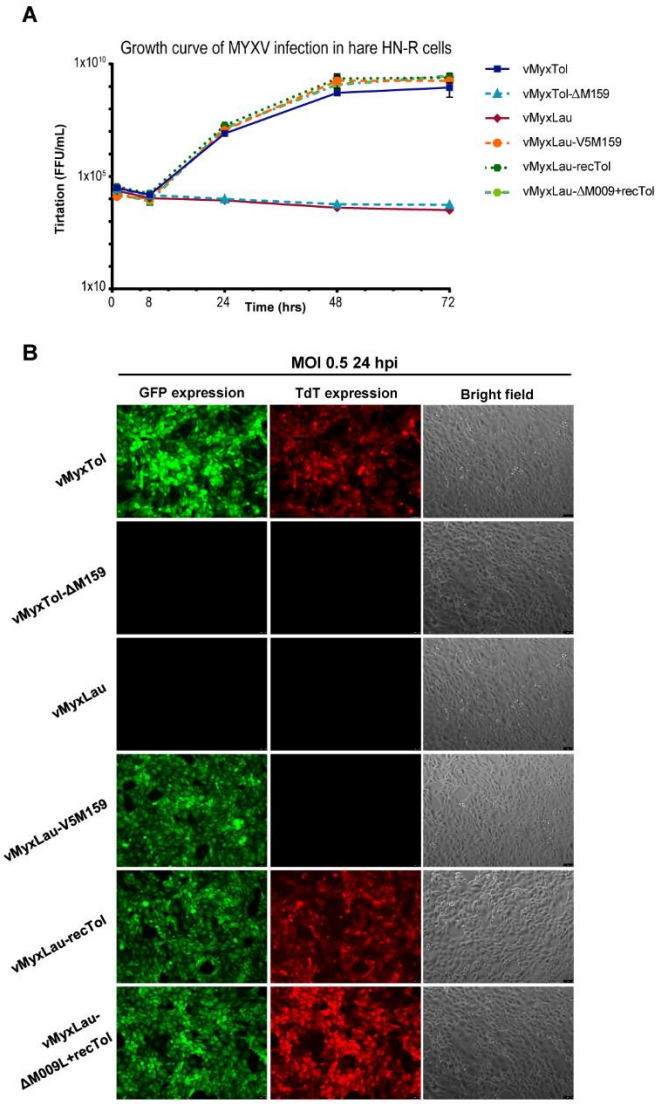

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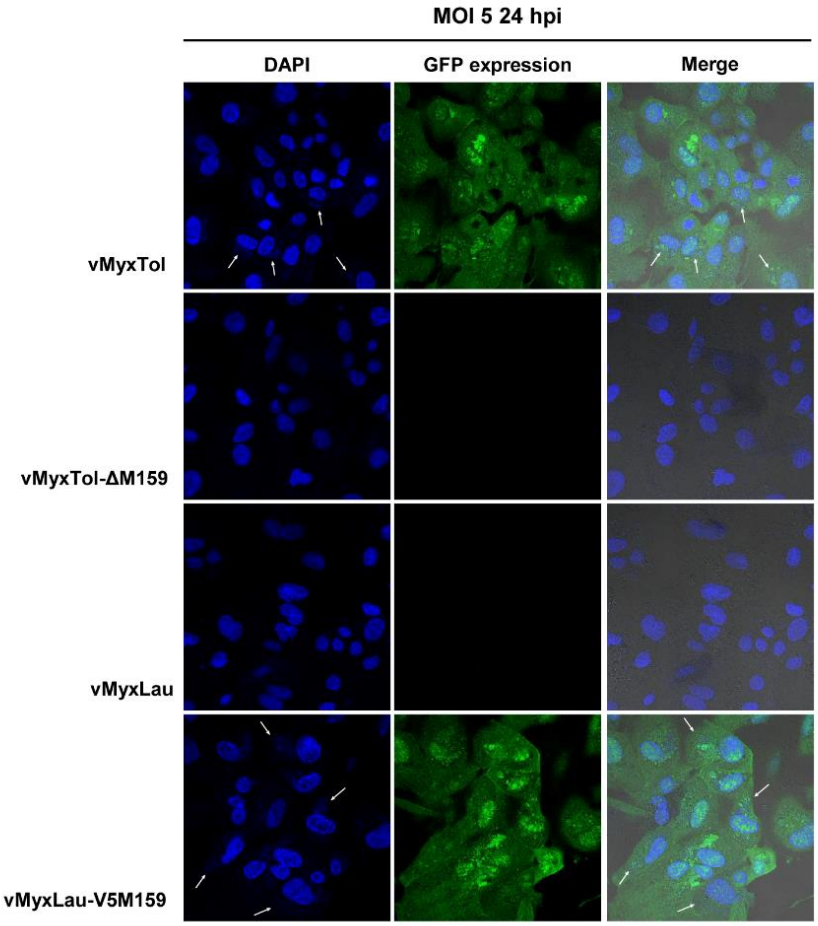

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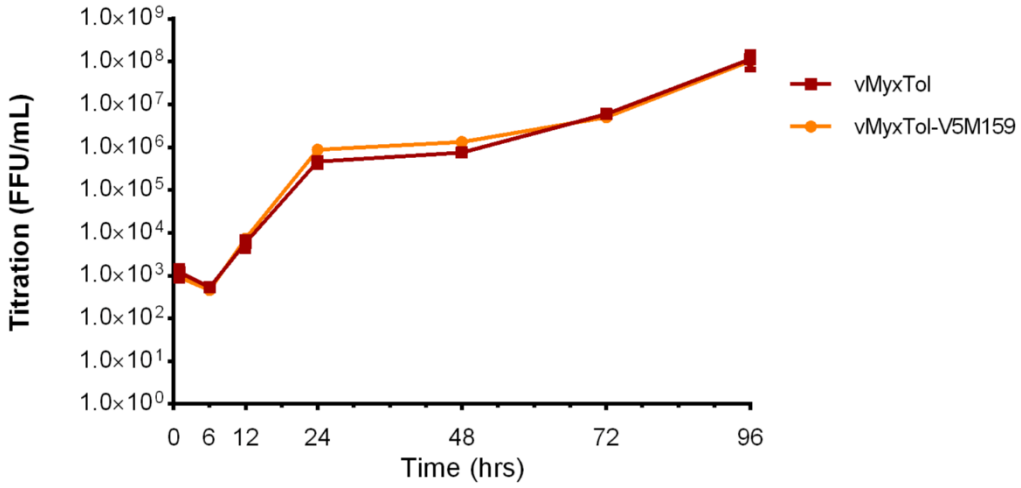

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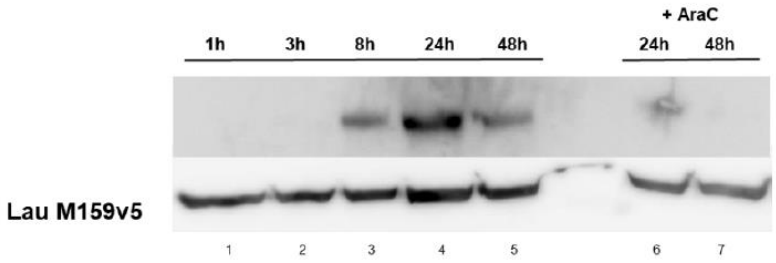

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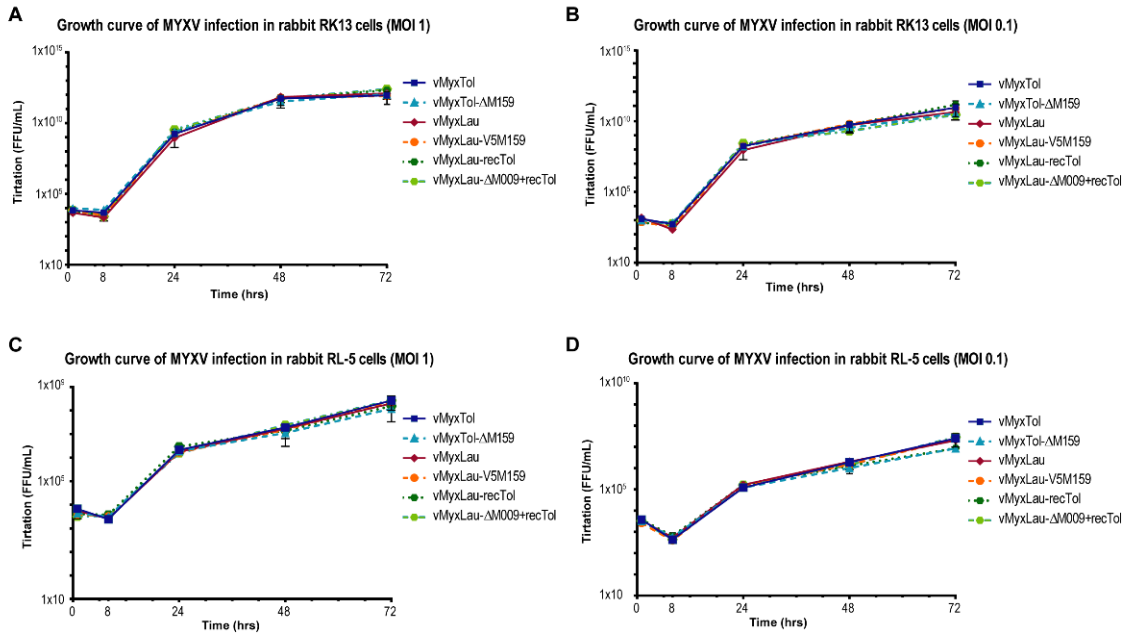

Chapter 4

Necroptosis: a lost pathway in different mammalian lineages

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Convergent loss of the necroptosis pathway in disparate mammalian lineages shapes viruses countermeasures

Ana Águeda-Pinto, Luís Q. Alves, Fabiana Neves, Grant McFadden, Bertram L Jacobs, L. Filipe C. Castro, Masmudur M. Rahman and Pedro J. Esteves

Abstract

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

1. Introduction

Sensing of viral pathogens by the host cells is critical for animal survival. Thus, a variety of molecular responses, including the induction of inflammatory cytokines, chemokines and interferons, as well as the activation of cell-death pathways that provide clearance of pathogen-infected cells, require sensing of pathogen associated molecular patterns for activation. Although apoptosis has long been considered a critical clearance mechanism to control viral spread, caspase-independent cell death, or programmed necrosis, has recently emerged as an alternative death pathway that dominates under specific conditions (1).

Necroptosis is an inflammatory form of regulated necrosis that acts as an alternative host defense pathway during some viral infections and plays a major role in the killing and removal of pathogen-infected cells (1–3). Activation of necroptosis follows an intracellular signaling cascade that is dependent on the receptor-interacting serine/threonine-protein kinase 3 (RIPK3) and its substrate, the mixed lineage kinase like protein (MLKL) downstream of death receptors (DRs) and pattern-recognition receptors (PPRs) (Fig. 1) (4,5). Several pathway-specific adaptor proteins that contain a RIP homotypic interaction motif (RHIM-domain) can activate RIPK3-induced necroptosis (Fig. 1). For example, when there is an interference or loss of function of caspase-8, the induction of necroptosis through the use of DRs results in the recruitment of RIPK1, which subsequently exposes its RHIM-domain to recruit RIPK3 (6–8). Apart from RIPK1, the TIR-domain-containing adaptor-inducing IFN β (TRIF), an essential protein downstream of Toll-like receptor (TLR)3/4 and the Z-DNA binding protein (ZBP1), also directly activate RIPK3 (9,10) (Fig. 1). Exposure of RIPK3 to a RHIM adaptor (RIPK1, TRIF or ZBP1) is a crucial step in the initiation of necroptosis, as these proteins activate the downstream executor of necroptosis, MLKL, that destabilizes the plasma membrane integrity leading to cell swelling followed by membrane rupture of infected cells and release of damage-associated molecular patterns (DAMPs) (3,11). Thus, necroptosis provides a critical extra defense mechanism against pathogen infection, facilitating the elimination of virus-infected cells before the production of progeny virions. The importance of necroptosis for host defense is further supported by the identification of viral inhibitors of necroptosis, like is the case of Vaccinia virus (VACV) E3 protein and the murine cytomegalovirus (MCMV) M45 protein (9,11).

Necroptosis has a major role in protecting cells against viral infection (1–3). 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 (12). 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.

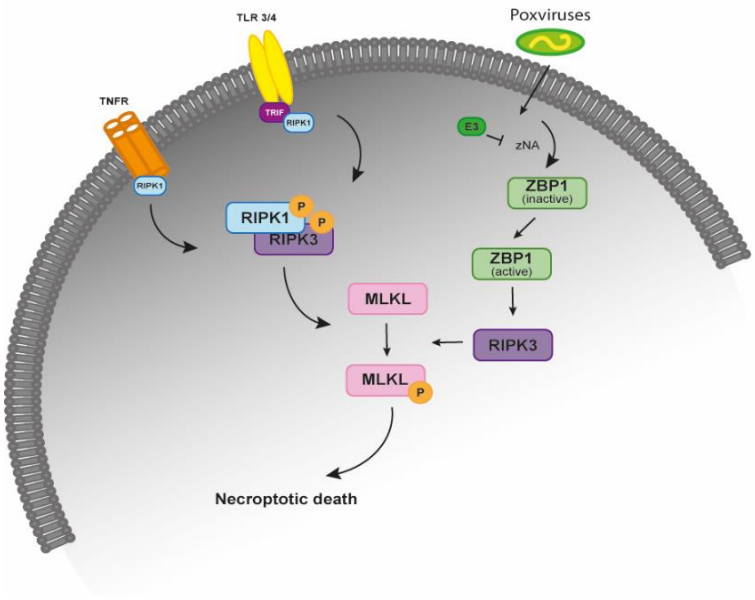

Figure 1. The necroptosis signaling pathway. Simplified schematic representation of the necroptosis signaling pathway upon stimulation of the TNFR, TRF3/4 and infection by poxviruses. All of these necroptosis-inducing signals converge on the kinase RIPK3, which is activated through the homotypic interaction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

2. Results

Necroptosis has a significant role in protecting cells against viral infection (1–3). However, despite 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. Here, taking advantage of genomic collection databases and the use of Leporid samples (see Methods section for more information), the goal is to better understand the molecular evolution of the necroptotic pathway in different mammalian lineages.

2.1. RIPK1 protein is under evolutionary conservation in mammals

Human RIPK1 is a multidomain protein composed of an N-terminal Ser/Thr kinase, a C-terminal death binding domain that mediates binding to DRs and an intermediate domain that includes a K377 ubiquitination site and an RHIM motif that binds to other RHIM-containing proteins (13). Due to the importance of RIPK1 as an adaptor molecule, previous phylogenetic analysis suggested that RIPK1 probably emerged in the ancestor of vertebrates (12). In accordance, our search detected the presence of RIPK1 homologues in all the studied mammalian lineages (Fig. 2A and Supplementary file 1, 2 and 3).

To look for evidence of potential selection pressures acting on the different domains of RIPK1 protein, we used the dataset of mammalian sequences mentioned above and implemented an ML approach, by using the Datamonkey software (see Methods section for more information). For most protein-coding genes, the rate between nonsynonymous and synonymous substitutions (dN/dS) is a measure of natural selection, with positive selection (dN/dS > 1) acting against the common genotype (14). In this study, we deduced ten sites that reflect strong positive selection pressure in RIPK1, while more than 200 amino acids were under negative selection (Fig. 2B and Supplementary file 4). As seen in Fig. 2B, residues identified as being under positive selection fall within or very close to the kinase domain (4 residues), the RHIM domain (5 residues) and the death domain (1 residue) of RIPK1 (residues under positive selection are marked as red circles). The N-terminal kinase domain is known to present several essential residues for phosphorylation and ubiquitination (Ser14/15, 20, 161 and 166 and Lys115 and 163), regulation of necroptosis and RIPK1-dependent apoptosis (15). Interestingly, the codons under positive selection fall only at the end of the N-terminal domain. A considerable portion of the negatively selected sites fall in the critical regions for phosphorylation and ubiquitination (Supplementary file 4), suggesting that the beginning of the RIPK1 protein is under strong purifying selection. The same was also observed for the rapidly evolving sites of the RHIM domain, which were grouped only at the beginning of this domain (Fig. 2B). It was previously shown that the RHIM domain has a crucial conserved core motif of 12 amino acids that resides at the end of this

domain (16,17). Indeed, changing four consecutive amino acids to alanine within this core region abrogates interaction between RHIM domains and, as a consequence, necroptosis (16,17). Our findings that the positively selected residues did not overlap with the core motif of 12 amino acids further support the importance of the conservation of this region. Interestingly, in the RIPK1 death domain that is known to mediate homodimerization as well as heterodimerization with other DD-containing proteins, such as FADD, TNFR1 and Fas (15,18), no positively selected sites were found, with most of the domain being under negative pressure (Supplementary file 4). Collectively, our results show signatures of positive selection occurring at the end of the N-terminal kinase domain and at the beginning of the RHIM domain of RIPK1 proteins. Interestingly, these residues do not overlap with domains known to be fundamental for RIPK1-dependent apoptosis and necroptosis, suggesting that these domains might be under evolutionary conservation and possibly functional constraint for the studied mammals.

2.2. Convergent erosion of RIPK3 and MLKL in mammalian lineages

RIPK3 and MLKL form the core of the necroptotic machinery and both are, as a consequence, important for necroptosis induction in mice and humans downstream of PRRs and DRs (1,4,5). To further understand the evolutionary history of the necroptotic pathway in mammals, we performed detailed sequence and phylogenetic analyses for RIPK3 and MLKL homologous proteins (Supplementary file 3). Our screens for *RIPK3* and *MLKL* genes revealed evidence of pseudogenization in five mammalian lineages: order rodentia, lagomorpha, carnivora, cetacean and in the superorder afrotheria (Fig. 2A). Given the fact that *MLKL* pseudogenes have been previously identified in carnivores, they will not be discussed in detail here (12).

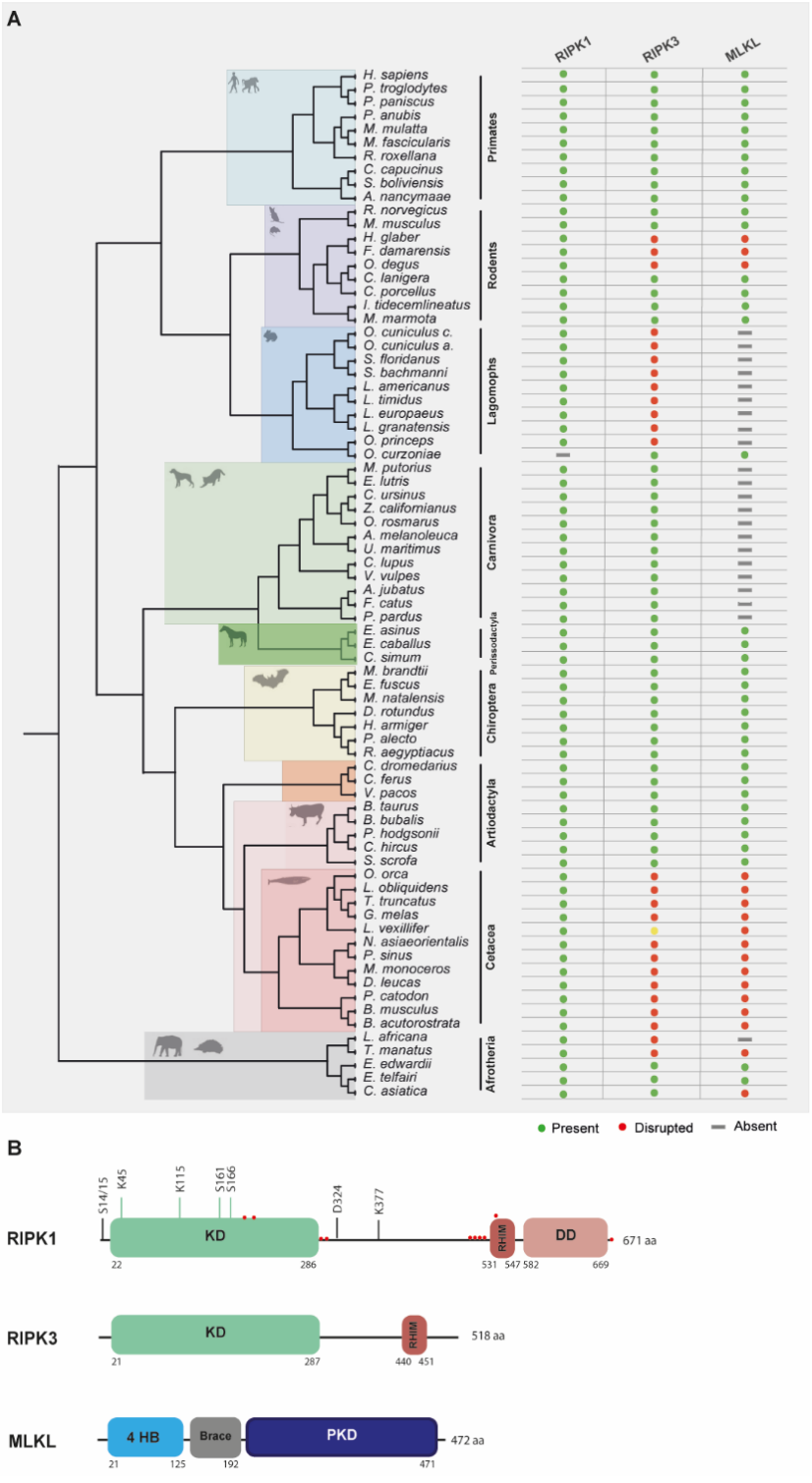

Figure 2. Evolution of RIPK1, RIPK3 and MLKL in different mammalian lineages. **A**) Phylogenetic tree showing the independent lineages that lost necroptotic core proteins (RIPK3 and MLKL) during evolution. Green circles represent genes that are present in the studied species, red circles represent genes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

2.3. Variation of the Necroptotic pathway within Afrotherian and Rodent families

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

2.4. The Necroptotic pathway is disrupted in Lagomorphs

The Order Lagomorpha is divided into two families, Ochotonidae and Leporidae, which diverged around 30–35 Mya (19). While Ochotonidae is only composed of one extant genus, *Ochotona*, the Leporidae family includes 11 genera, including *Lepus*, *Sylvilagus* and *Oryctolagus* (20). Using the methods described previously, we were only able to identify *RIPK3* and *MLKL* transcripts for plateau Pika (*O. curzoniae*), while no *RIPK3* and *MLKL* transcripts were found for the European rabbit (*O. cuniculus*). For the American Pika (*O. princeps*), incomplete genome assemblies in the vicinity of the *RIPK3* and *MLKL* regions made retrieving the sequence of these genes impossible. By evaluating *RIPK3* gene from human and mouse and its genomic context, we were able

derive a partial *RIPK3* nucleotide sequence from the European rabbit genome, which displays a frameshift mutation caused by the insertion of a single nucleotide. It is well known that accurately detecting gene-inactivation mutations in these alignments poses a number of challenges like, for example, sequencing errors and cases of assembly incompleteness. For this reason, we assessed the accuracy of our database prediction by sequencing that same genomic region in different Leporid species, representative of different genera (*Lepus*, *Sylvilagus* and *Oryctolagus*). From the obtained results, we confirmed the insertion of 1 nucleotide (+G, exon 3) not only in the European rabbit RIPK3, but also in species from genus *Lepus* and *Sylvilagus*, suggesting that disruption of *RIPK3* gene occurred in a common ancestral and was maintained throughout Leporid evolution (Fig. 3A).

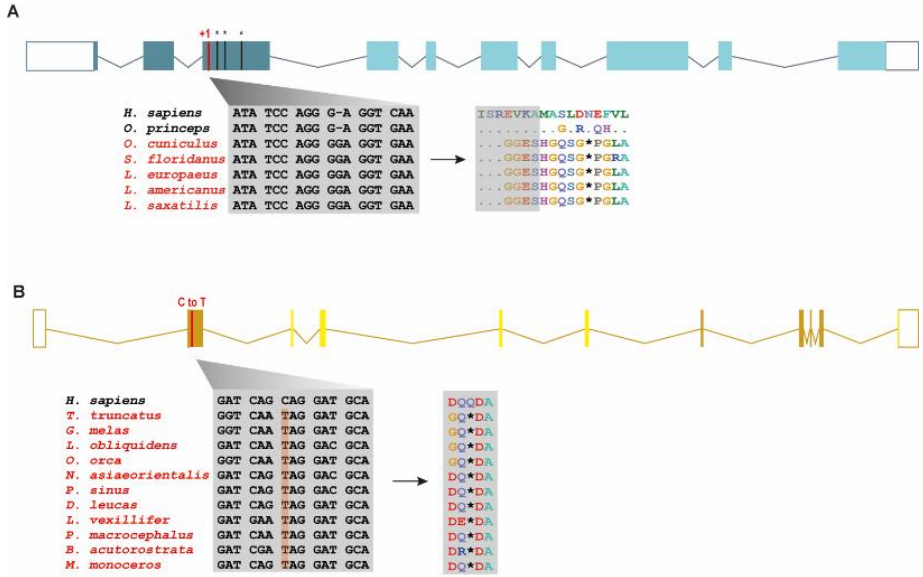

Figure 3. **Loss of RIPK3 and MLKL genes in the steam lineage of Leporids and Cetaceans. A)** Genomic analysis of the first tree exons from Leporids. In Leporids, RIPK3 was lost as a result of a shared insertion (+G) in the third exon that resulted in the appearance of several premature stop codons. **B)** A point mutation (C to T) in all the studied cetacean species indicates that MLKL inactivation occurred in Cetacea steam lineage. Moreover, 9 out of the 11 studied species (excluding B. acutorostrata and M. Monoceros) lost exon 2, 3, 4 and 5 throughout evolution (represented by faint yellow). Premature stop codons are represented by an asterisk (*).

During necroptosis, activated RIPK3 phosphorylates and activates MLKL, which results in its recruitment and oligomerization in the plasma membrane leading to rupture and cell death (1,4). Interestingly, and despite all of our efforts, no *MLKL* transcripts or MLKL protein accumulation were found in any of the studied Lagomorphs (data not shown). Detailed analysis of the upstream and downstream MLKL flanking genes in both human and mouse genomes reveal that MLKL resides between the ring finger and WD repeat domain 3 protein (*RFWD3*) and fatty acid 2-hydroxylase (*FA2H*) genes (Fig. 4). Accordingly, although there are no gaps or incomplete genomic assemblies surrounding

that region in the European rabbit genome, we were not able to retrieve a complete or partial *MLKL* gene, suggesting once more that this gene is not present in these mammals. Together, our results suggest that the studied leporid species are deficient in the core proteins of the necroptotic pathway, and that RIPK3 inactivation occurred at the stem Leporid branch and was maintained during evolution.

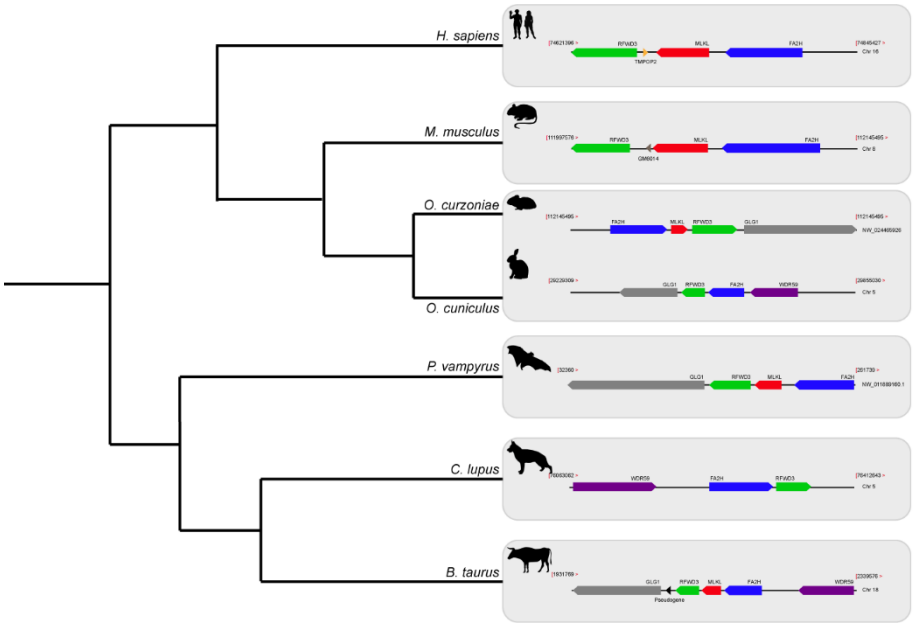

Figure 4. Gene synteny of the genome regions containing MLKL gene in different mammals. Genomic regions containing the MLKL gene or its flanking genes in H. sapiens, M. musculus, O. curzoniae, O. cuniculus, P. vampyrus, C. lupus and B. taurus. Horizontal lines indicate chromosome fragments and coloured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

2.5. Inactivation of necroptosis components in Cetacea

Modern Cetacea comprises Mysticete (or baleen whales) and Odontocete (or toothed whales) and are the most specialized and diversified group of mammals (21). Comparative analysis of cetacean genomes has already provided important insights into the unique cetacean traits and aquatic specializations (22–24). For our screen, Odontocetes were represented by 12 species belonging to five different families (Delphinidae, Phocoenidae, Monodontidae, Lipotidae and Physeteridae), and Mysticetes were represented by the common minke whale from Balaenopteridae family. In Cetacean species, the disruption of *RIPK3* occurred at different positions depending on the studied species: a frameshift mutation was identified in exon 6 of two Delphinidae species, as well as in exon 8 of two Phocoenidae species, one Monodontidae species and one Balaenopteridae species. There was also evidence of two species (one species from Monodontidae and another from Lipotidae families) with RIPK3 pseudogenes based on

the presence of a stop codon located in exon 2 (Supplementary file 6). Interestingly, while Cetacean RIPK3 inactivation appears to be a result of different mutations depending on the studied species, our results show a shared mutation in the exon 1 of *MLKL* in all cetacean species (Fig. 3B). Moreover, this premature stop codon leads to the absence of exon 2, 3, 4 and 5 in most cetacean species, which very likely results in this gene inactivation. Given the presence of an inactivating mutation that is shared between mysticetes and odontocetes, the most parsimonious hypothesis suggests that they occurred before the split of these two clades in the common ancestral branch of Cetacea.

2.6. Diversity among the poxvirus encoded E3-like necroptosis antagonists

Previously, it was shown that the N-terminus of VACV E3 competes with ZBP1 for binding to virus-induced Z-nucleic acid, being a key component to inhibit the action of IFN and induction of necroptosis (25) (Fig. 1). E3-like encoded proteins are composed of a carboxy (C)-terminal double-stranded RNA binding domain (dsRNA-BD) and an amino (N)-terminal Z-nucleic acid-binding domain (zNA-BD) (26,27) (Fig. 5A). Given the importance of the N-terminus region from VACV E3 protein against virus infection, we hypothesize that poxviruses that lack this region in their E3 homologs can still successfully replicate in their natural host because of a compromised necroptotic pathway. Among the E3L proteins, E3 from VACV is the best studied protein. However, E3 homologs can be found in orthopoxviruses, clade II poxviruses and parapoxviruses (26,27). Recently, the genome characterization of CePV-TA identified two novel E3L homologs: CePV-TA-20 and CePV-TA-21 (28).

Our analysis on 11 different E3 homologues revealed that these are highly divergent: while CPXV 069 (Cowpoxvirus E3 homolog) and TATV 060 (Tateropoxvirus E3 homolog) presented identities of >90% to VACV E3, E3L homologs from poxviruses like the Deerpoxvirus, Sheeppoxvirus and Yaba monkey poxvirus presented less than 40% identity (Fig. 5B). Analysis of the two newly identified E3 homologs from CePV-TA shows that both present low identity to VACV E3, with CePV-TA-20 and CePV-TA-21 proteins only presenting sequence identity of 37 % and 34 %, respectively (Fig. 5B). It is known that at the amino acid level, the C-terminal of E3-like proteins display a higher level of sequence similarity than the N-terminal domain (29). Accordingly, the dsRNA-BD domain from CePV-TA-20 and CePV-TA-21 proteins also display a higher level of

sequence similarity compared to other E3 homologs (Fig. 5C), suggesting that in CeTV this domain might also target conserved antiviral dsRNA-activated pathways. Similar to what is observed for Monkeypox virus (MPXV) and Myxoma virus (MYXV) E3 homologues, both E3 homologs from CePV-TA present incomplete or disrupted N-terminal zNA-BDs. As shown in Fig. 5C, CePV-TA-20 is missing 20 amino acids in its N-terminal domain. However, this region still retains the conserved LY and PPXW motifs, as well the basic KKCINR motif (Fig. 5C) that are known to bind with Z-NA (27). Interestingly, MPXV F3 protein, lacking 37 amino acids from the N-terminal domain, is not able to compete with ZBP1 and inhibit sensing (unpublished data), even though it retains the key residues important for binding to Z-NA (Fig. 5C). While CePV-TA-20 and F3 proteins contain an incomplete zNA-BD, M029 and CePV-TA-21 proteins are missing most of their N-terminal zNA-BD (Fig. 5B and C), suggesting a total inactivation of this domain and a loss of function regarding the inhibition of ZBP1-dependent necroptosis.

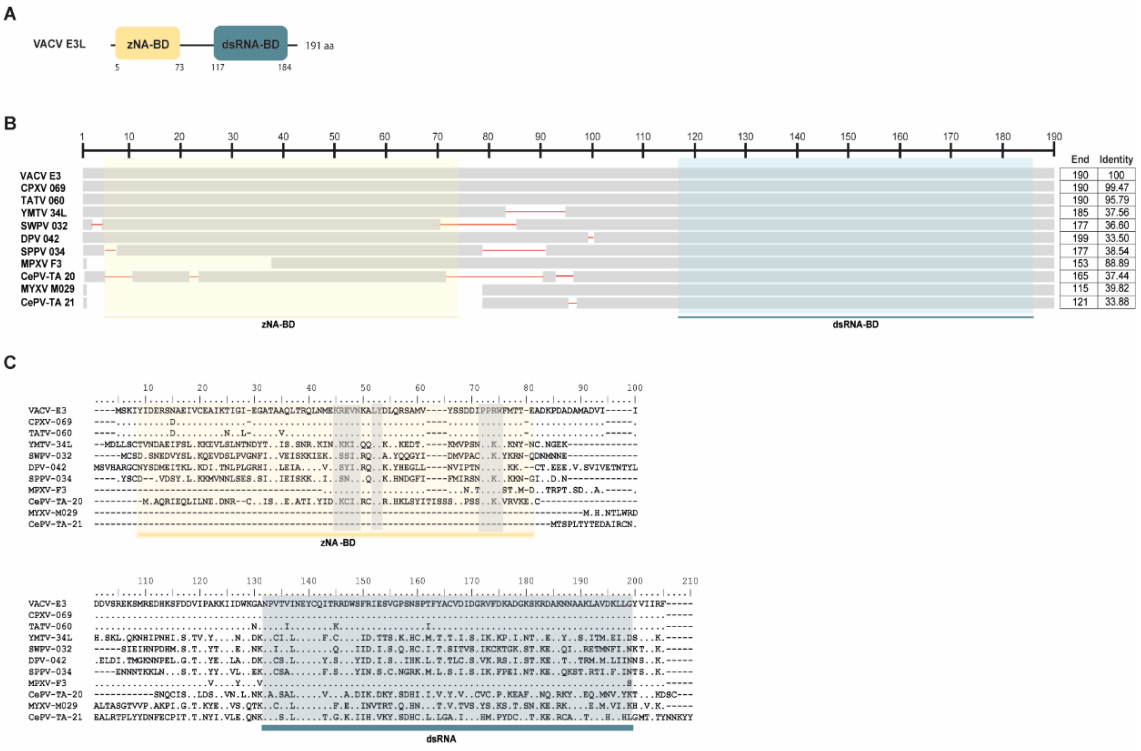

Figure 5. Protein sequence alignment of E3L proteins. A) Schematic diagram of VACV E3 protein binding domains: yellow box represents the zNA-BD and blue box represents the dsRNA-BD. The same color scheme is used in B and C. **B)** E3L homologues from 11 poxviruses (VACV E3, Cowpoxvirus (CPXV) 069, Tateropoxvirus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

Overall, our results show that the novel CePV-TA presents two E3L homologues that, like E3L homologues from MPXV and MYXV, present incomplete or disrupted N-

terminal zNA-BD. The presence of an incomplete or disrupted zNA-BD in E3L homologues highly suggests that these proteins cannot fully compete with ZBP1 to inhibit necroptosis induction. However, further studies will be necessary to fully comprehend the action of these proteins regarding complete necroptosis inhibition.

3. Discussion

Necroptosis is an inflammatory form of cell death that is mediated by RIPK3 and MLKL and provides an extra defense mechanism against pathogen infection, facilitating the elimination of virus-infected cells before the production of progeny virions (1–3). Given the crucial role of necroptosis in the innate immune response of humans and mice (30,31), it was broadly accepted that this pathway was ubiquitous in mammals. Our results from 67 species across nine mammalian lineages provides the first comprehensive picture of the molecular evolution of necroptosis in mammals. We show that while RIPK1 is under evolutionary conservation, RIPK3 and MLKL are poorly conserved in lineages that evolved separately over the course of evolution.

A detailed analysis of RIPK3 and MLKL in mammals reveals a complex pattern where lagomorphs, cetaceans, carnivores and species from rodent and afrotheria lineages separately lost key components of the necroptotic pathway (Fig. 2A). The order lagomorpha includes two big families, Ochotonidae and Leporidae (19). The presence of the same frameshift mutation in Leporid species (+G, Fig. 3A) suggests that the disruption of the necroptotic pathway occurred early, but only after the bifurcation between Ochotona and Leporid given that the plateau Pika presents intact RIPK3 and MLKL proteins (Fig. 2A). Despite all efforts, and despite the complete genome assembly surrounding the MLKL flanking genes in the European rabbit genome, no partial or complete MLKL gene was found, indicating that this gene is deleted in the European rabbit genome and possibly in the remaining Leporid species (Fig. 4). The core genes of the necroptotic pathway also presented premature stop codons in cetaceans. In the studied cetaceans, the MLKL gene presented a common stop codon in the first exon, resulting in the inactivation of this gene (Fig. 3B). Again, the presence of similar patterns of pseudogenization in RIPK3 or MLKL genes within species of the same order infer that disruption of these genes occurred before their diversification and was maintained throughout evolution. On the other hand, *RIPK3* disruption in cetaceans appears to be the result of insertions or deletions that are not shared between closely related species, but rather specific to each species (Supplementary file 6), suggesting that these disrupting mutations occurred later in evolution when compared to MLKL. The addition

of some rodents as well as afrotheria species to the list of mammals that have disrupted necroptotic pathways, raises the possibility that other closely related species might have lost this pathway after the diversification of these lineages. It is currently believed that activation of the RIPK3 and recruitment of MLKL are critical steps during necroptosis. For example, deleting either RIPK3 or MLKL can lead to the suppression of skin and liver inflammation in mice (32,33). Moreover, when mice are treated intravenously with a high-doses of TNF, there appears to be no differences between RIPK3-deficient and MLKL-deficient mice (5), substantiating the premise that MLKL follows RIPK3 in the necroptotic signalling. However, this appears not to be the case for all necroptotic cell death responses, as different studies revealed alternative pathways for MLKL and RIPK3-dependent programmed necrosis that are executed in the absence of RIPK3 or MLKL, respectively (34,35). To date, there are no studies suggesting that RIPK3/MLKL double-knockout mice are still able to induce necroptosis, which indicates that species that have disrupted RIPK3 and MLKL lost the necroptotic pathway throughout evolution.

The loss of function of *RIPK3* and *MLKL* in independently evolving lineages (convergent evolution) indicates that gene loss is an important evolutionary mechanism for phenotypic change in these animals and may contribute to similar adaptations. Even though it would be expected that loss of genes is maladaptive, gene loss can be beneficial by providing an evolutionary mechanism for adaptations (36). In fact, if the loss of an existing gene would increase fitness by making a species better adapted to the environment that surrounds it, then gene loss would be an easy solution to an evolutionary problem (36). Necroptosis contributes to innate immunity as a pathogen clearance mechanism (1). However, contrary to apoptosis, in which several highly immunogenic intracellular proteins are sequestered in the dead cell, necroptosis releases DAMPs in the surrounding tissue that promote strong inflammatory responses and result in the attraction of different types of immune cells to the site of infection (37). Studies in mouse models have provided strong evidence that necroptosis is implicated in several inflammatory neurodegenerative diseases, including multiple sclerosis and amyotrophic lateral sclerosis (38,39). Mouse-model experiments identified keratinocyte necroptosis as a trigger of skin inflammation (40) and a correlation between necroptosis and intestinal inflammation has also been established (41,42). Thus, while necroptosis might mediate host defense, its inhibition in certain contexts may lessen disease severity. It is known that excessive inflammation can promote cancer cell growth and metastasis (43). Thus, it is possible that a pro-inflammatory cell death like necroptosis might promote metastasis and thus, inhibition of this pathway might represent an advantage for

regulation of cancer cell growth. Intriguingly, some of the species that are lacking the core necroptotic machinery are known to resist cancer. That is the case for cetaceans, the naked mole-rat and african elephants (44–46). It is also possible that selection against necroptosis in different mammalian lineages could have been driven by different factors depending on the environment or conditions. For example, it was previously suggested that the absence of MLKL in Carnivores reflected a microbe-rich and virus-containing diet of raw meat, causing evolutionary counter-selection against necroptosis (12). Nevertheless, the absence of the necroptotic pathway in independently evolving lineages suggest that the deregulation of this pathway was detrimental for the host organism, which ultimately drove selection against the presence of RIPK3 and MLKL.

As many other viruses, poxviruses express immunomodulatory and host-range factors important for the suppression and evasion of the host innate and adaptive antiviral responses (47,48). VACV protein E3 not only sequester dsRNA through their dsRNA-BD limiting the activation of the innate immune system against the virus infection, but also inhibit the IFN-induced dsRNA dependent protein kinase (PKR), known to be a crucial component of the host innate immunity against viral infection, replication, and spread (49,50). Our results show that the dsRNA-BD of distant E3L proteins present high levels of sequence similarity (Fig. 5B and C), which is consistent with the ability of this domain to target conserved pathways present in different hosts. Although the dsRNA sequestration functions of the E3 C-terminal have been clear for decades (51,52), the IFN sensitivity of VACV E3 N-terminal deletion mutants remained unresolved for a long time. Recently, strong evidence showed that the E3 N-terminal domain competes with ZBP1 to prevent ZBP1-dependent activation of RIPK3 and consequent necroptosis (25,53). The model proposed by the authors suggests that during WT-VACV infection, the zNA-BD of E3 binds to VACV-induced Z-form nucleic acid and masks it, preventing sensing by ZBP1 and further RIPK3 necroptosis induction (25,53). However, it is interesting that poxviruses like MPXV, MYXV and CePV-TA have E3L homologs that present a complete dsRNA-BD but not zNA-BD (Fig. 5B). In VACV-E3 Δ83N–infected cells (mutant lacking the first 83 aa corresponding to the zNA-BD), the absence of the zNA-BD facilitates ZBP1 to sense VACV-induced PAMPs and initiate necroptosis induction (25). Therefore, it is expected that E3L homologues that lack N-terminal zNA domains, like CePV-TA-21 and M029, cannot prevent Z-form nucleic acid sensing triggering necroptosis induction and early abortion of viral replication. Like CePV-TA-20, F3 protein is also missing several amino acids in the N-terminal region and presents high conservation in areas that are known to bind to zNA (Fig. 5C). Nevertheless, F3 protein

seems to have lost the ability to compete with ZBP1 and inhibit sensing (unpublished data). It was previously shown that the N-terminus of VACV E3 is necessary for IFN resistance in JC cells since VACV-E3Δ37N (mutant mimicking MPXV E3 zNA-BD) did not initiate DNA replication (54). However, MPXV was able to replicate efficiently in the same cells, despite having a partial N-terminal zNA-BD, suggesting that the predicted binding to z-form nucleic acid was intragenic and downstream of z-NA sensing, rather than related to the ability of F3 zNA-BD to mask z-form nucleic acid (54).

Interestingly, inactivation of necroptosis in Lagomorphs and Cetaceans seems to correlate with the absence of the E3L zNA-BD in their naturally infecting poxviruses, namely leporipoxviruses (MYXV and SFV) and cetaceanpoxviruses (CePV-TA), respectively. Monkeypox is a viral zoonosis endemic to central and western Africa areas where African rope squirrels and other rodents are likely reservoir hosts (55). Interestingly, the absence of a functional N-terminus in MPXV F3 protein also seems to correlate with the fact that some rodents appear incapable of undergoing necroptosis. Like MPXV, leporipoxviruses and CePV-TA pathogenesis are restricted to only certain species and have little or no pathogenesis capability in all others (28,54,56,57). Infection of the same host over hundreds of years or even millennia may drive the evolution of each virus to rapidly evolve to a fitness peak in a given host environment. Previous niche-filling models (58–60) emphasize the role of host interactions in shaping virus evolution. According to these models, as hosts diversify and speciate over longer evolutionary periods, viral host factors that aim to counter the host antiviral functions are subject to continuous changes. Indeed, it is known that genes associated with host antiviral mechanisms present high evolutionary rates and are often under positive selection (61–63). Here, we suggest that during the evolution of these poxviruses, the loss of the zNA-BD did not present a disadvantage in the host organism; therefore, this trait was maintained, which reflects how these viruses adapt as their niche changed.

4. Concluding Remarks

The disruption of necroptosis in independently evolving lineages suggests a convergent evolutionary loss of this pathway, probably reflecting an important selective mechanism for phenotypic change. Interestingly, we also found a strong correlation between the disruption of necroptosis in leporids and cetaceans and the absence of the E3L zNA-BD (responsible for necroptosis inhibition) in their naturally infecting poxviruses as in the case of MYXV and CePV-TA, respectively. Overall, our study provides the first comprehensive picture of the molecular evolution of necroptosis in mammals,

highlighting the importance of gene/pathway loss for the process of species adaptation and suggesting that it is a true pathogen-response pathway that is not required for normal mammalian development. Moreover, this study sheds some light on a co-evolutionary relationship between poxviruses and their hosts, emphasizing the role of host adaptation in shaping virus evolution.

5. Material ans Methods

5.1. Genomic approach to detect genes associated with the necroptosis pathway

To detect intact and inactivated genes, we first identified the key genes of the necroptotic pathway (i.e., *RIPK1*, *RIPK3* and *MLKL*) in the human (*H. sapiens*) and mouse (*Mus musculus*) reference genomes and looked for the presence of orthologues in existing genome sequence databases from 67 different species that belong to the 9 main mammalian orders/superorders: primates, rodents, lagomorpha, chiroptera, carnivora, perissodactyla, artiodactyla, cetacea, and afrotheria (Supplementary file 1). We did not only search for the complete loss of exons or entire genes, but also searched for insertions and deletions that shift the reading frame, frame-preserving insertions that create a premature stop codon and substitutions that create an in-frame stop codon. The respective search methodology had been previously applied to the identification of different homologues of different annotated mammalian genomes (23,64). To further ensure that all gene loss events discussed in this study are real and not due to sequencing errors, we validated them either by sequencing of samples or by using a curated bioinformatic pipeline (see below).

5.2. Amplification and sequencing of *RIPK1* and *RIPK3* nucleic acid sequences from Lagomorpha species

In contrast to the majority of mammalian orders, lagomorpha only presents three annotated genomes: the European rabbit (*Oryctolagus cuniculus*, accession # GCA_000003625.1), the American Pika genome (*Ochotona princeps*, accession # GCA_014633375.1) and the plateau pika (*O. curzoniae*, accession # GCA_017591425.1). Given the importance of lagomorphs for this study, samples from different lagomorpha species were used to obtain the nucleic coding sequence from RIPK1. For that, RNA was extracted from tissues of *O. cuniculus cuniculus*, *O. cuniculus algirus*, *Lepus americanus*, *L. europaeus*, *L. timidus*, *L. granatensis*, *Sylvilagus floridanus*, *S. bachmanis*, *O. princeps* and *O. collaris* samples, using the Qiagen DNeasy

Blood & Tissue kit (Qiagen, USA) following manufacturer's instructions. Synthesis of cDNA was achieved by using SuperScript III Reverse Transcriptase (Invitrogen, USA). Primers were designed according to the *RIPK1* transcript from *O. cuniculus* [Accession # XM_017350509.1] (Forward 5'-ATGTCTTTGGATGACATTAAAATG-3' and Reverse 5'-CTACTTCTGGCTGAGCTGTATC-3') and used to amplify the samples mentioned before. Phusion® High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) was used in the PCR amplification and the conditions included an initial denaturation (98ºC for 3min), 35 cycles of denaturation (98ºC for 30s), annealing (60ºC for 15s) and extension (72ºC for 30s) followed a final extension (72ºC for 5 min).

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

Amplicons sequencing from RIPK1 and RIPK3 was performed with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit and according to manufacturer's protocol; reactions were cleaned with Sephadex™ (GE Healthcare Life Sciences, UK) and applied on an ABI PRISM 310 Genetic Analyser (Life Technologies, Applied Biosystems, Carlsbad, CA, USA). The obtained RIPK1 coding sequences and the partial RIPK3 sequences from the different Lagomorphs have been deposited in the GenBank database under the accession numbers that are shown in Supplementary file 2. All samples were supplied by CIBIO/InBIO, Vairão, Portugal and used in previous studies (62,65). No animals were captured, handled, or killed specifically for the purpose of this study.

5.3. Detailed analysis on the cetacean genomes

Briefly, NCBI gene annotations for the gene orthologues of *MLKL* and *RIPK3* were initially screened via PseudoChecker (pseudochecker.ciimar.up.pt), which evaluates the coding condition of a gene (66,67). For each gene, a PseudoChecker analysis was run (default parameters), using the *Bos taurus* (cow) gene orthologue as a comparative input (NCBI Accession ID regarding cow MLKL: XM_002694707.6; RIPK3: XM_024997365.1), as well as the genomic sequences encompassing the putative ORF of the orthologous counterpart of each target species, directly exported from the NCBI genome browser. Through PseudoIndex, a built-in assistant metric, we quickly assessed the erosion status of the tested genes on a discrete scale ranging from 0 (coding) to 5 (pseudogenized) (66). Subsequent manual annotation was performed by importing the previously collected genomic sequences into Geneious Prime 2020 software (www.geneious.com) (68) and determining each gene's CDS using as reference cow's *MLKL* and *RIPK3* orthologues sequences. In detail, per gene and species, using the built-in map to reference tool (highest sensitivity parameter selected), each (3' and 5' untranslated region-flanked) reference coding-exon was mapped against each target genomic sequence. Exons alignments were further screened for gene disruptive mutations, including in-frame premature stop codons, frameshift, and splice site mutations (any deviation from the consensus donor splice site GT/GC or the consensus acceptor splice site AG).

To inspect if the identified genetic lesions were not rendered as result of sequencing and/or genome assembly artifacts, we performed mutational validation (one per gene and species), resorting of raw genomic sequencing reads, retrieved from two independent genomic projects from the NCBI sequence read archive (SRA), when available. Explicitly, blastn searches were directed to the selected SRA projects, using the nucleotide sequence portion containing the selected mutation(s) as a query. The matching sequencing reads were downloaded into Geneious Prime 2020 (68) software and mapped against the manually annotated mutation (highest sensibility parameter selected), confirming, or not, the presence of the identified mutation.

5.4. Phylogenetic and molecular evolutionary analyses

The complete dataset of RIPK1, RIPK3 and MLKL proteins was aligned in BioEdit Sequence Alignment Editor using Clustal W (52), followed by manual corrections when

necessary. Amino acid alignments were then used to infer Maximum Likelihood (ML) phylogenetic trees using MEGA X (69), with the substitution models JTT+G+F, JTT+G and HKY+G+I, respectively; determined using ProtTest (70).

Given the fact that RIPK3 and MLKL proteins are highly divergent across the studied mammalian species, we decided not to perform any evolutionary analysis using these alignments. To look for signatures of natural selection operating in the RIPK1 alignment, we used HyPhy software implemented in the Datamonkey Web server (71), to detect codons under selection: the Single Likelihood Ancestor Counting (SLAC) model, the Fixed Effect Likelihood (FEL) method (72), the Random Effect Likelihood, the Mixed Effects Model of Evolution (MEME) (73) and Fast Unbiased Bayesian AppRoximation (FUBAR) (74) methods were used. To avoid a high false positive rate, codons with p-values <0.05 for SLAC, FEL and MEME models and a posterior probability >0.95 for FUBAR were accepted as candidates for selection. For a more conservative approach, only residues identified as being under positive selection in three or more ML methods were considered.

5.5. Analysis of VACV E3 homologues

VACV E3 homologues encoded by different poxviruses (=11) were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/) and aligned in the BioEdit Sequence Alignment Editor using Clustal W (52), followed by manual corrections when necessary. Amino acid alignments of the representative E3-like proteins were used to generate schematic diagrams using the COBALT program from the NCBI database.

Supplementary material

Supplementary file 1. Accession numbers for *RIPK1*, *RIPK3* and *MLKL* genes found in different mammalian lineages.

Supplementary file 2. Accession numbers for *RIPK1*, *RIPK3* and *MLKL* genes found in different Lagomorpha species.

Supplementary file 4. Positive and negative selection analyses for RIPK1.

Supplementary file 5. RIPK3 and MLKL protein alignment from species from rodent and afrotheria lineages.

Supplementary file 6. Tables identifying RIPK3 and MLKL mutations and premature stop codons in Cetacea order.

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Supplementary material

Supplementary file 1. Accession numbers for *RIPK1*, *RIPK3* and *MLKL* genes found in different mammalian lineages.

Superorder/ Order	Superfamily/Family	Species name	*RIPK1*	*RIPK3*	*MLKL*
Primates	**Hominidae**	*Homo sapiens*	NM 001354930.1	NM_006871.4	NM_152649.4
		Pan troglodytes	XM 016954824.1	XM 001169864.3	XM_016930166.2
		Pan paniscus	XM 003805555.3	XM 003809099.1	XM_024925971.1
	Cercopithecidae	*Papio anubis*	XM 003896962.4	XM 003901654.4	XM_017953558.2
		Macaca mulatta	XM 001091986.3	XM 001114079.3	XM_015126622.1
		M. fascicularis	XM 015449962.1	XM 005560982.2	XM_015443138.1
		Rhinopithecus roxellana	XM 010361800.1	XM 010363712.1	XM_010382960.1
	Cebidae	*Cebus capucinus*	XM 017497471.1	XM 017520641.1	XM_017530561.1
		Aotus nancymaae	XM 021672660.1	XM 012468022.1	XM_012437528.1
		Saimiri boliviensis	XM 010338093.1	XM 003924268.2	XM_010350629.1
Rodents	**Muroidea**	*Rattus norvegenicus*	NM 001107350.1	NM 139342.1	XM_008772572.2
		Mus musculus	NM 001359997.1	AF178953.1	BC023755.1
	Chinchillidae	*Chinchilla lanigera*	XM 005398899.2	XM 013510393.1	XM_013503862.1
	Caviidae	*Cavia porcellus*	XM 013151465.1	XM 003474285.4	XM_013154995.2
	Octodontidae	*Octodon degus*	XM_004628459.2	**STOP***	**STOP***
	Bathyergidae	*Heterocephalus glaber*	XM_013073264.2	**STOP***	**STOP***
		Fukomys damarensis	XM_010622756.3	**STOP***	**STOP***
	Sciuridae	*Ictidomys tridecemlineatus*	XM 021727876.1	XM 013364463.2	XM_013365168.2
		Marmota marmota	XM 015493957.1	XM 015491924.1	XM_015496076.1
Lagomorpha	**Ochotona**	*Ochotona princeps*	XM_004596516.1	**INC****	**---**
		Ochotona curzoniae	---	XM_040969693.1	XM_040982371.1
	Leporidae	*Oryctolagus cuniculus*	XM_017350507.1	**STOP***	**---**
Chiroptera	**Vespertilionidae**	*Myotis brandtii*	XM 005869628.2	XM 005859571.2	XM_014536746.1
		Eptesicus fuscus	XM 028136859.1	XM 008158442.2	XM_028142801.1
	Miniopteridae	*Miniopterus natalensis*	XM 016220367.1	XM 016205787.1	XM_016197763.1
	Phyllostomidae	*Desmodus rotundus*	XM 024552320.1	XM 024556248.1	XM_024555929.1
	Hipposidenidae	*Hipposideros armiger*	XM 019668516.1	XM 019663032.1	XM_019646402.1
		Pteropus alecto	XM 025044923.1	XM 006913529.3	XM_025044559.1
		Rousettus aegyptiacus	XM 016166677.1	XM 016120560.1	XM_016143898.1
Carnivora	**Mustelidae**	*Mustela putorius*	XM 004753625.2	XM 004755192.2	**---**
		Enhydra lutris	XM 022517756.1	XM 022523405.1	**---**
	Otoriidae	*Callorhinus ursinus*	XM 025891041.1	XM 025870243.1	**---**
		Zalophus californianus	XM 027602761.1	XM 027569817.1	**---**
	Odobenidae	*Odobenus rosmarus*	XM 004408406.2	XM 004402153.1	**---**
	Ursidae	*Ailuropoda melanoleuca*	XM 002924876.3	XM 011227269.2	**---**

		Ursus maritimus	XM 008692863.1	XM 008708807.1	---
	Canidae	*Canis lupus*	XM 022414382.1	XM 025442787.1	---
		Vulpes vulpes	XM 025982629.1	XM 026007627.1	---
	Felidae	*Acinonyx jubatus*	XM 027040185.1	XM 015086216.1	---
		Felis catus	XM 023253722.1	XM 003987566.5	---
		Panthera pardus	XM 019419890.1	XM 019429283.1	---
Perissodactyla	**Equidae**	*Equus asinus*	XM 014867439.1	XM 014868621.1	XM_014850092.1
		Equus caballus	XM 023624365.1	XM 005603254.3	XM_005608429.3
		Ceratotherium simum	XM 004432163.2	XM 004421921.2	XM_014792683.1
Artiodactyla	**Bovidae**	*Bos taurus*	NM 001035012.1	XM 005211281.3	XM_024978879.1
		Bubalus bubalis	XM 025264923.1	XM 006061455.1	XM_025268400.1
		Pantholops hodgsonii	XM 005971736.1	XM 005985345.1	XM_005980145.1
		Capra hircus	XM 018038999.1	XM 013966825.2	XM_013970972.2
	Suidae	*Sus scrofa*	MG586799.1	XM 001927424.4	MG543991.1
	Camelidae	*Camelus dromedarius*	XM 010978020	XM 010995799.1	XM_010987761.1
		Camelus ferus	XM 006175577.2	XM 006172835.2	XM_014554645.1
		Vicugna pacos	XM 006198664.2	XM 006217294.2	XM_015241915.1
Cetacea	**Delphinidae**	*Orcinus orca*	XM 004281071.2	**STOP***	**STOP***
		Lagenorhynchus obliquidens	XM 027128236.1	**STOP***	**STOP***
		Tursiops truncatus	XM_019945096.2	**STOP***	**STOP***
		Globicephala melas	XM_030881534.1	**STOP***	**STOP***
	Phocoenidae	*Neophocaena asiaeorientalis*	XM_024738443.1	**STOP***	**STOP***
		Phocoena sinus	XM_032650323.1	**STOP***	**STOP***
	Monodontidae	*Monodon monoceros*	XM 007452108.1	**STOP***	**STOP***
		Delphinapterus leucas	XM_022586536.2	**STOP***	**STOP***
	Lipotidae	*Lipotes vexillifer*	XM 028478546.1	**STOP***	**STOP***
	Physeteridae	*Physeter catodon*	XM 028164463.1	**STOP***	**STOP***
	Balaenopteridae	*Balaenoptera acutorostrata*	XM 004281071.2	**STOP***	**STOP***
Afrotheria	**Elephantidae**	*Loxodonta africana*	XM_010597547.2	**STOP***	**----**
	Trichechidae	*Trichechus manatus*	XM_023741740.1	**STOP***	**STOP***
		Elephantulus edwardii	XM 006888715.1	XM 006903266.1	XM_006878861.1
		Echinops telfairi	XM 004711762.1	XM 004698921.1	XM_004704794.1
		Chrysochloris asiatica	XM 006870555.1	XM 006835518.1	**STOP***

* STOP – Presence of an early STOP codon that disrupts the protein.
** INC – Incomplete sequence.

Supplementary file 2. Accession numbers for *RIPK1*, *RIPK3* and *MLKL* genes found in different Lagomorpha species.

Superorder/Order	Genus	Species name	*RIPK1*	*RIPK3*	*MLKL*
Lagomorpha	***Oryctolagus***	*Oryctolagus cuniculus*	XM_017350507.1	STOP*	---
		Oryctolagus cuniculus algirus	MZ913427	STOP*	---
	Sylvilagus	*Sylvilagus floridanus*	MZ913428	STOP*	---
		Sylvilagus bachmani	MZ913429	STOP*	---
	Lepus	*Lepus americanus*	MZ913430	STOP*	---
		Lepus timidus	MZ913431	STOP*	---
		Lepus europaeus	MZ913432	STOP*	---
		Lepus granatensis	MZ913433	STOP*	---
	Ochotona	*Ochotona collaris*	MZ913434	---	---
		Ochotona princeps	XM_004596516.1	INC**	---

* STOP – Presence of an early STOP codon that disrupts the protein.
** INC – Incomplete sequence.

Supplementary file 4. Positive and negative selection analyses for RIPK1.

Supplementary file 4.1. Positive selection using SLAC, FEL, MEME and FUBAR methods.

Gene	Amino acids under Positive Selection in the alignment				Total of sites*
	SLAC[a]	FEL[b]	MEME[b]	FUBAR[c]	
RIPK1	183, 204, 258, 293, 294, 465, 501, 517, 518, 537, 539, 541, 543, 552, 689	183, 204, 258, 293, 294, 465, 501, 518, 537, 541, 543, 552, 597, 605, 689	19, 35, 54, 181, 184, 200, 204, 235, 236, 254, 265, 290, 293, 294, 315, 377, 490, 492, 518, 531, 537, 540, 541, 543, 546, 552, 579, 597, 626, 656, 679, 680, 679, 680, 681, 682, 683, 689, 691	204, 293, 294, 518, 543, 689	**10**

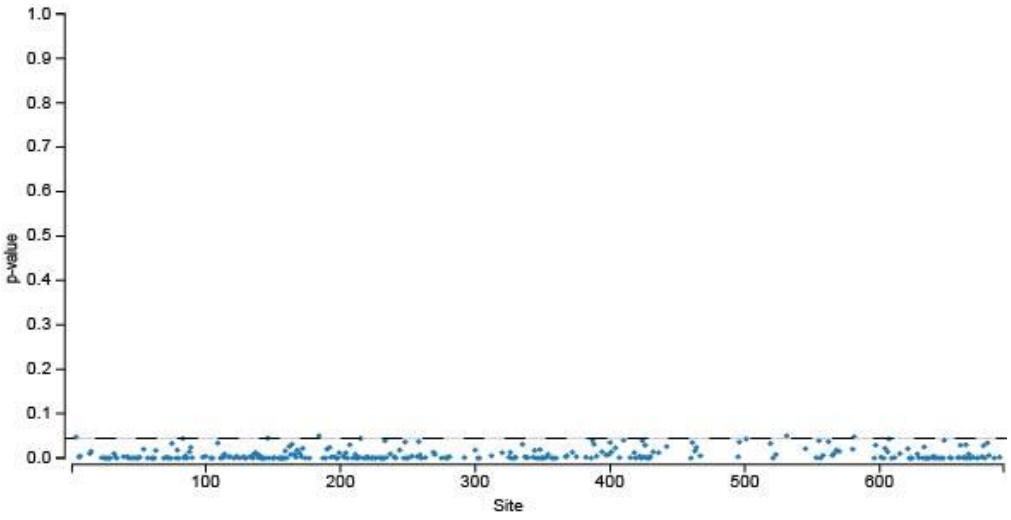
a) Codons with significance level <0.1

b) Codons with significance level <0.05

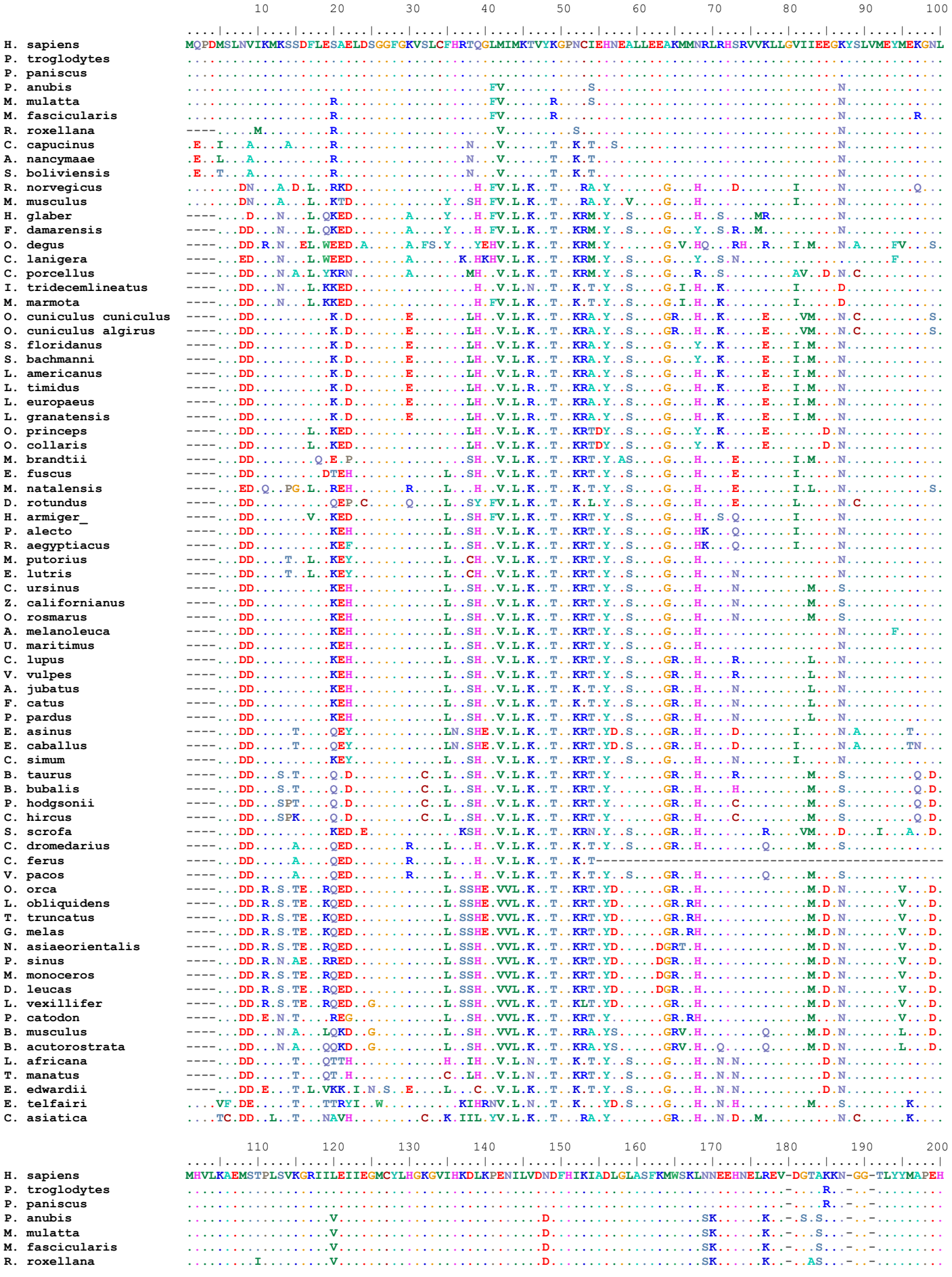
c) Codons with posterior probabilities >0.90

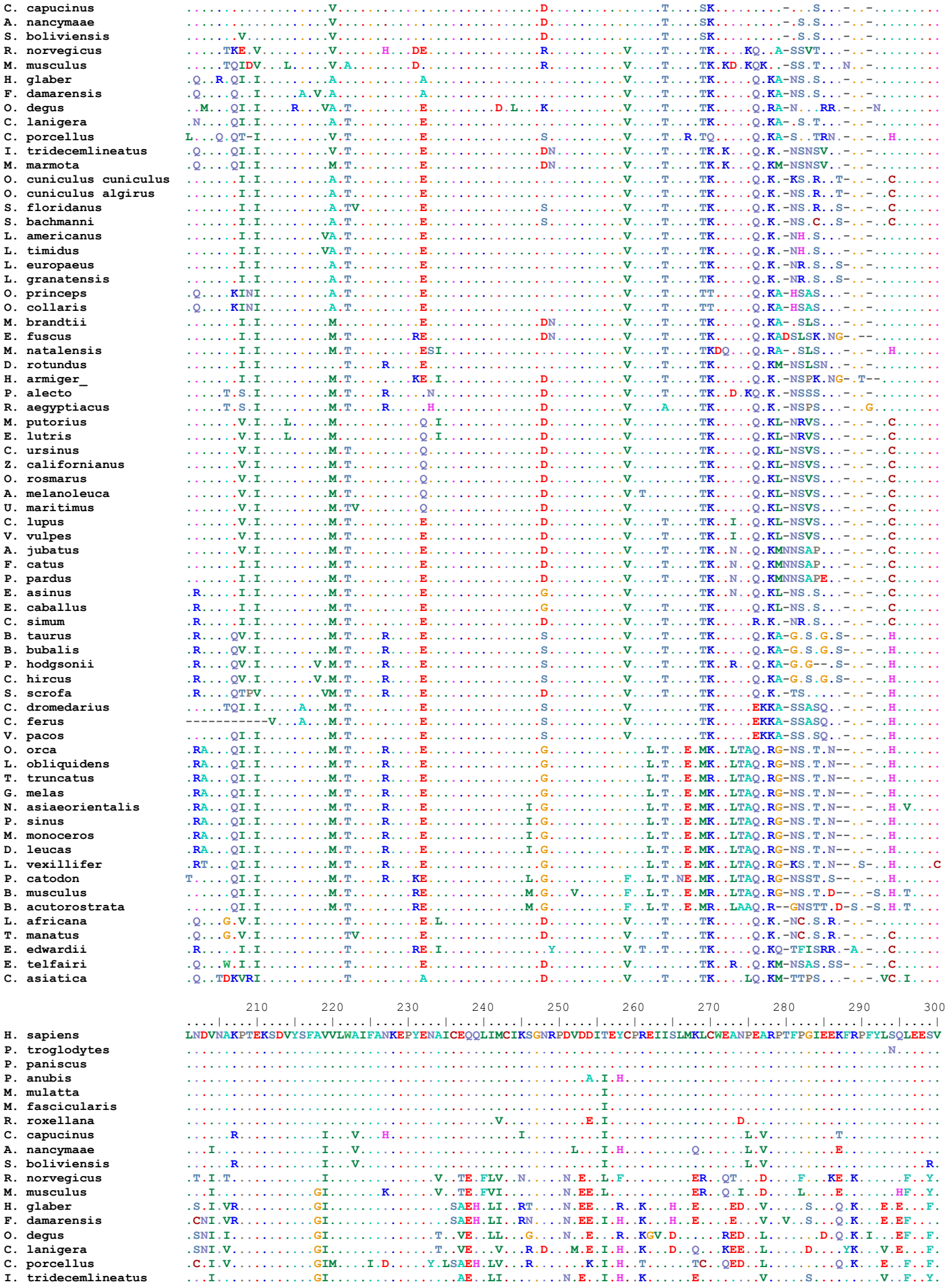
*) Selected sites in three out of the four methods

Supplementary file 4.2. Negative selection analysis using FEL method with negative residues selected at P > 0.5. Majority of negative selected residues fall in the first 300 aa and at the end of RIPK1 protein.



Supplementary file 4.3. RIPK1 protein alignment used for positive and negative analysis.

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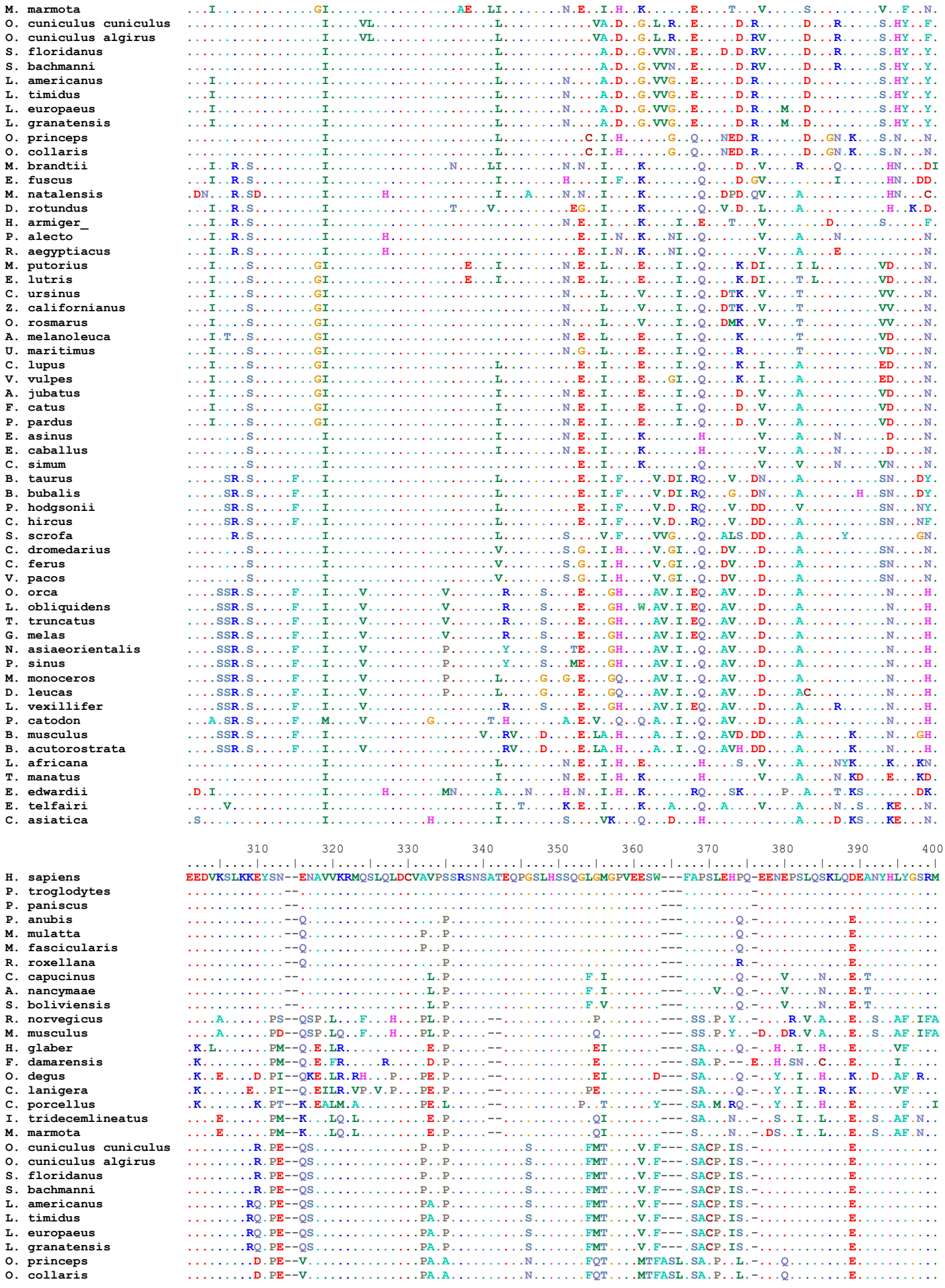
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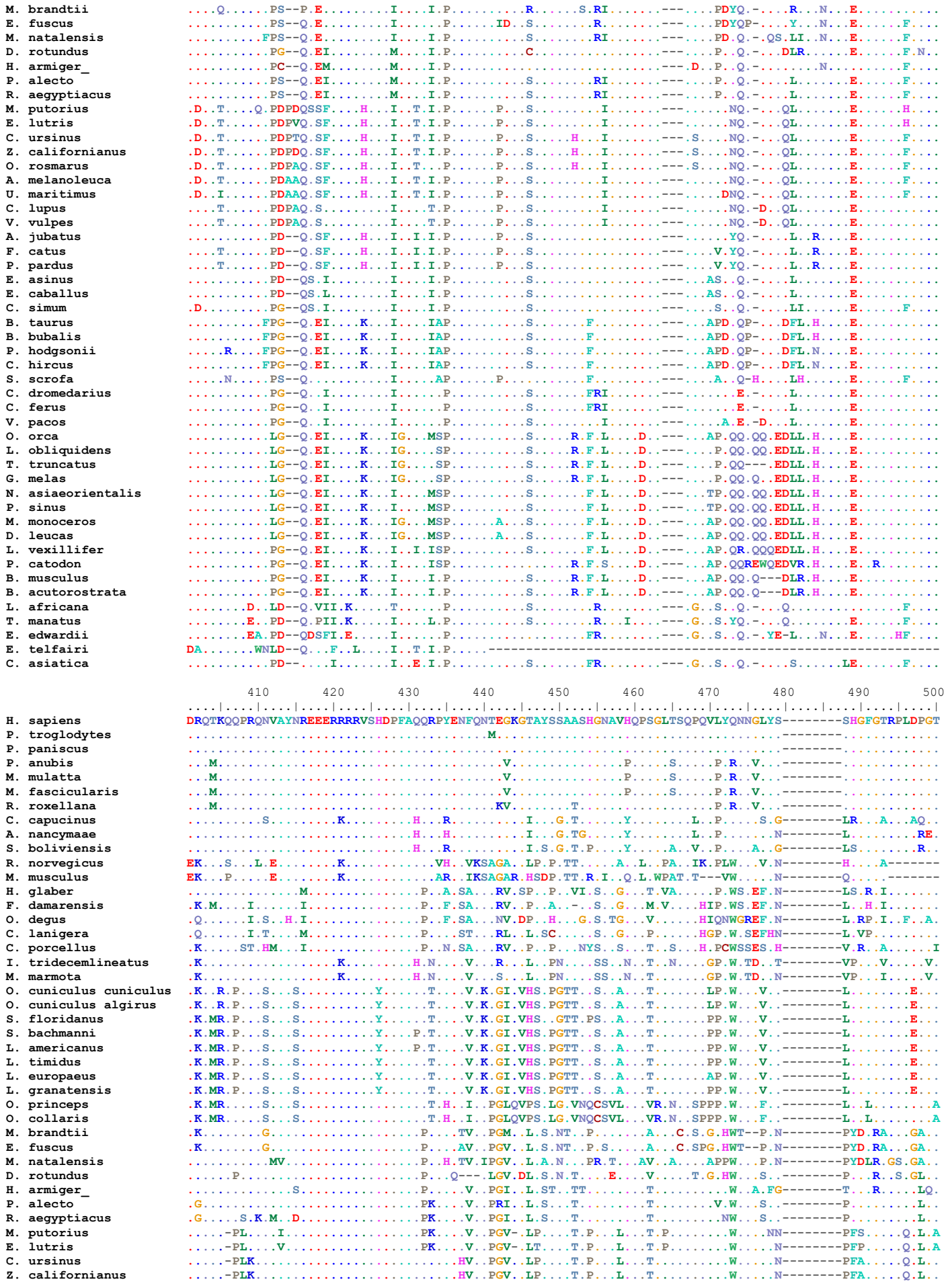
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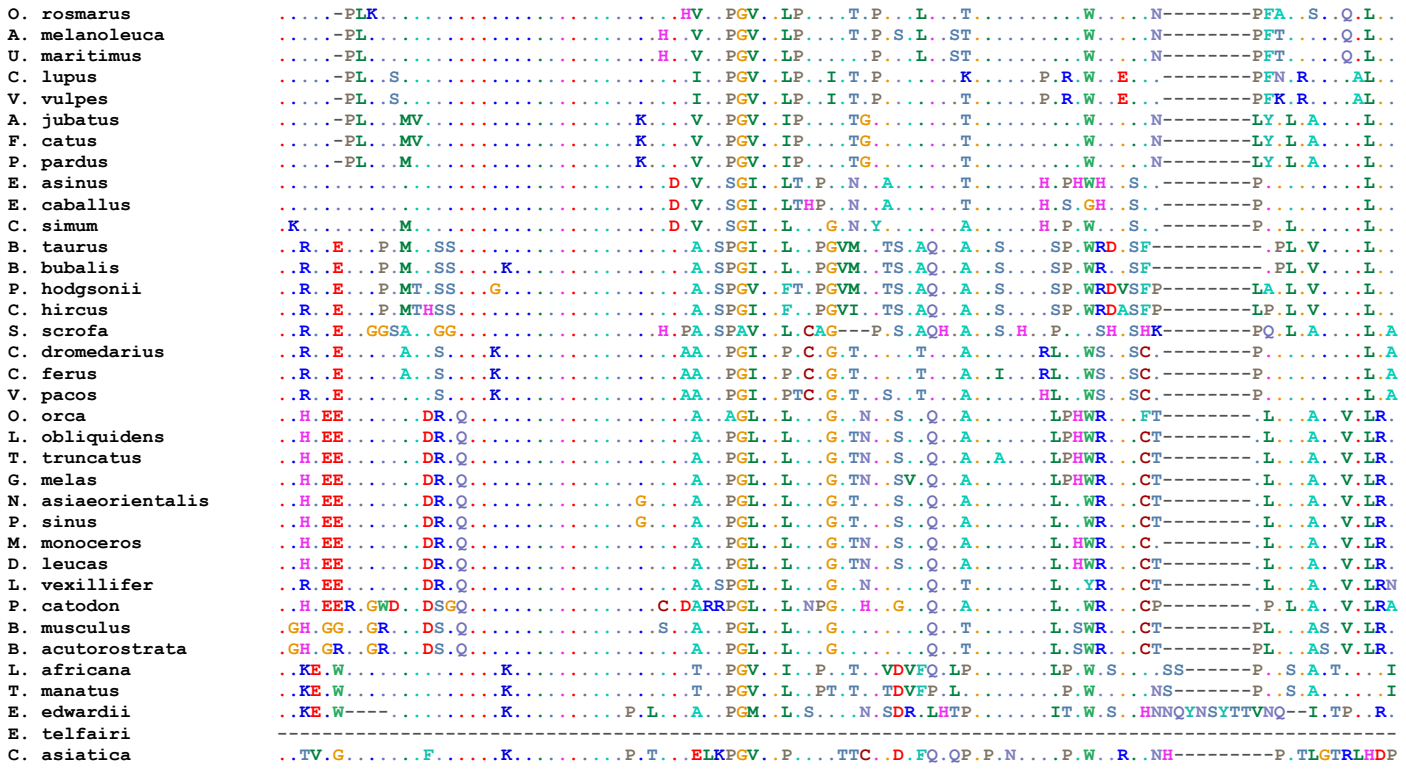
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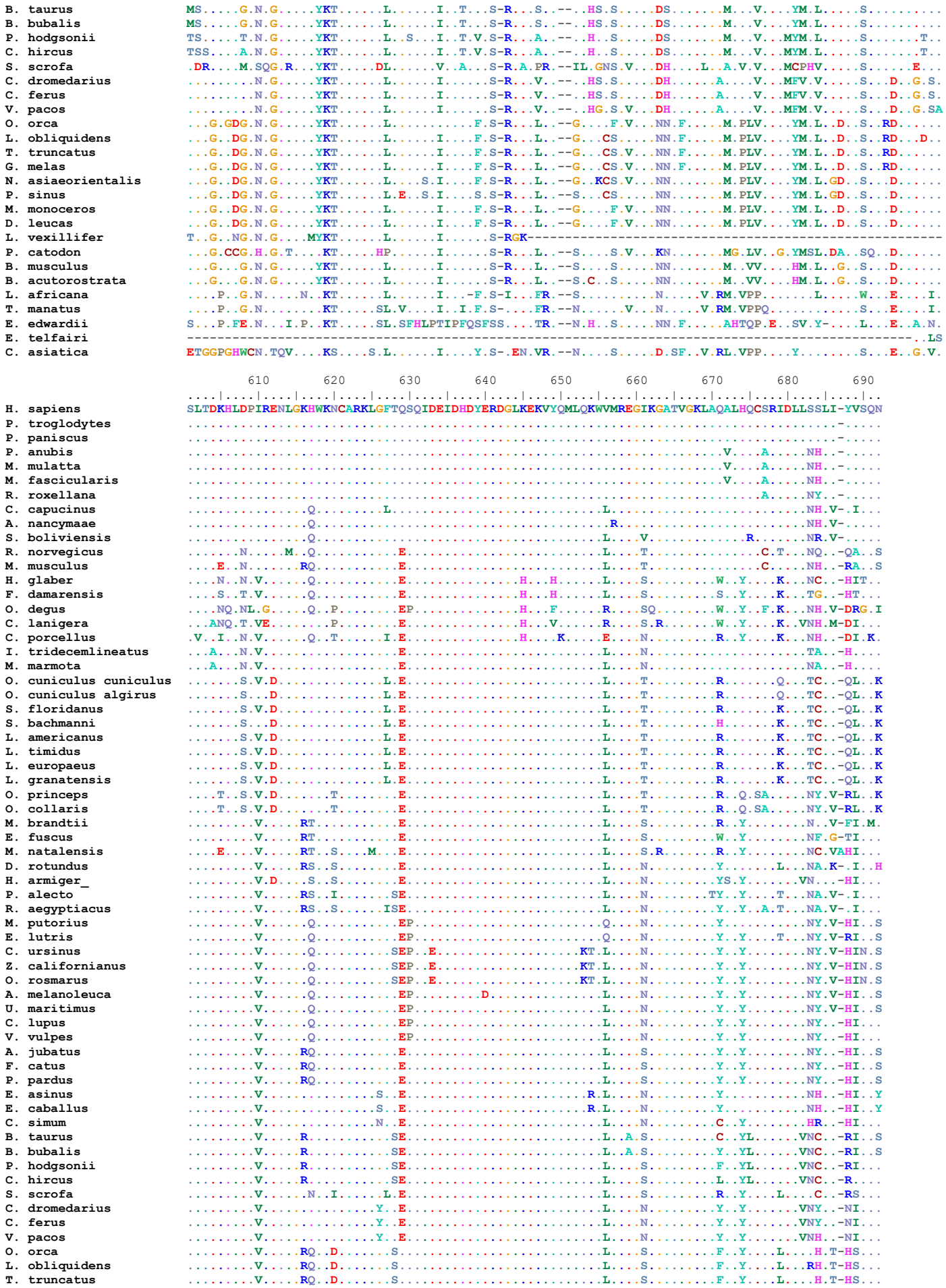
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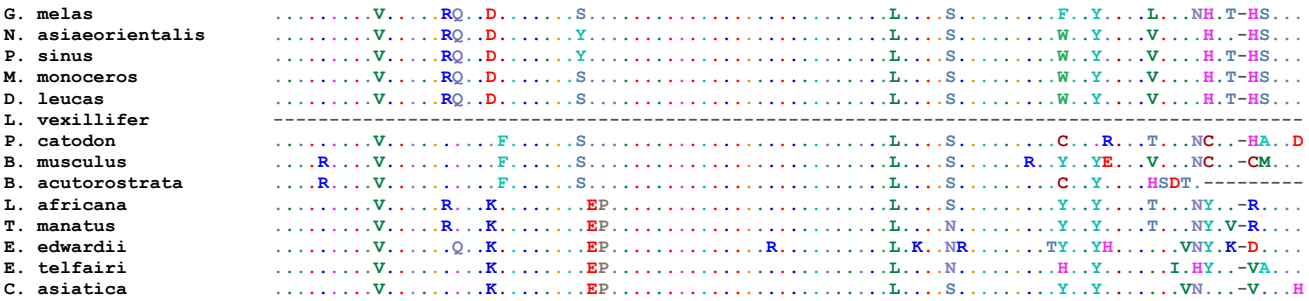
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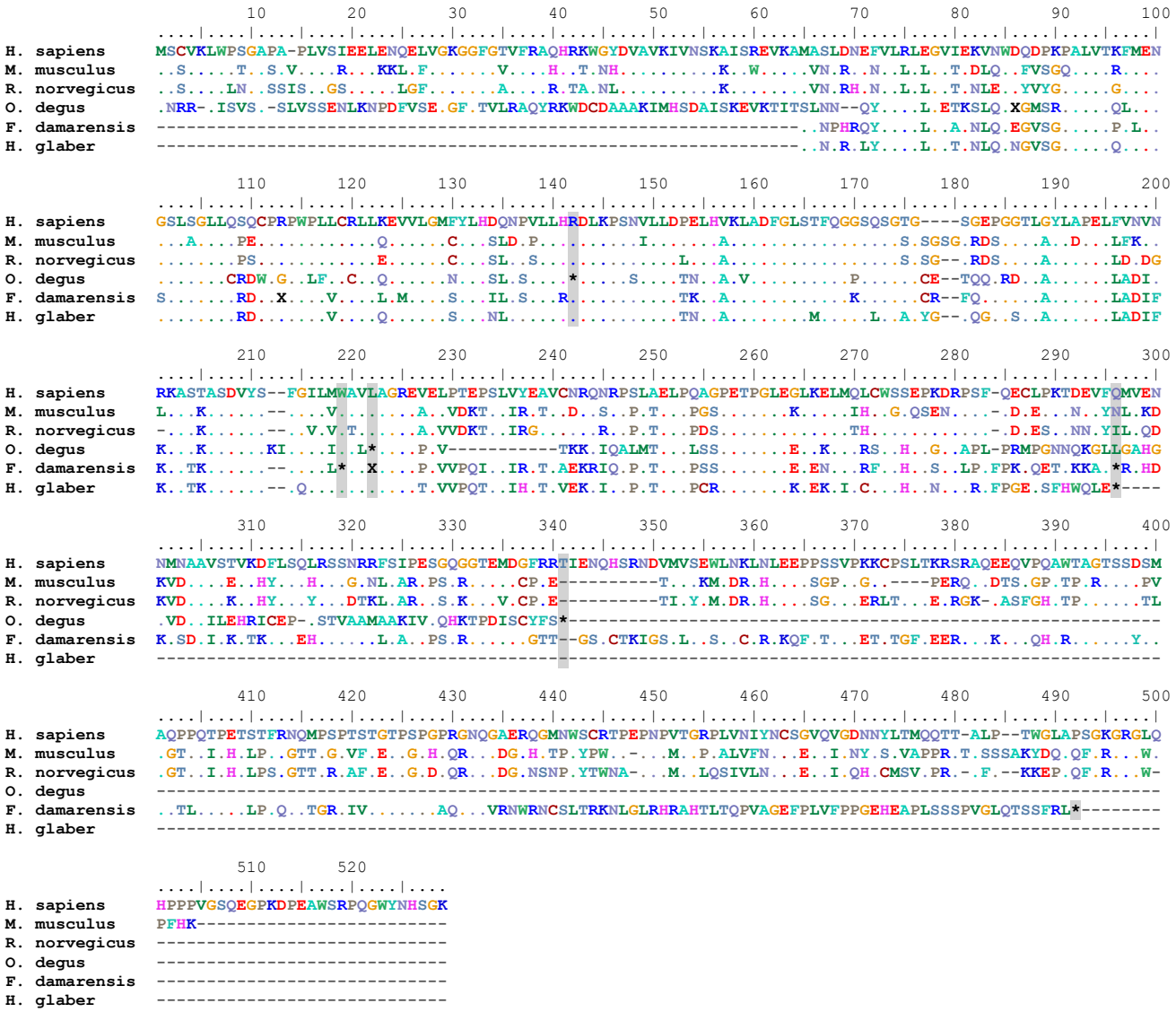
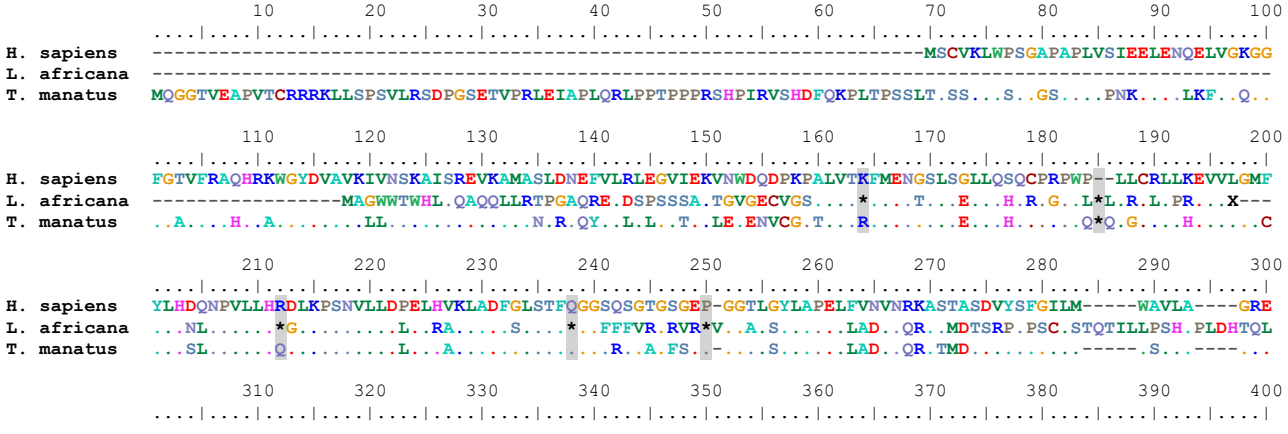
E. edwardii
E. telfairi
C. asiatica

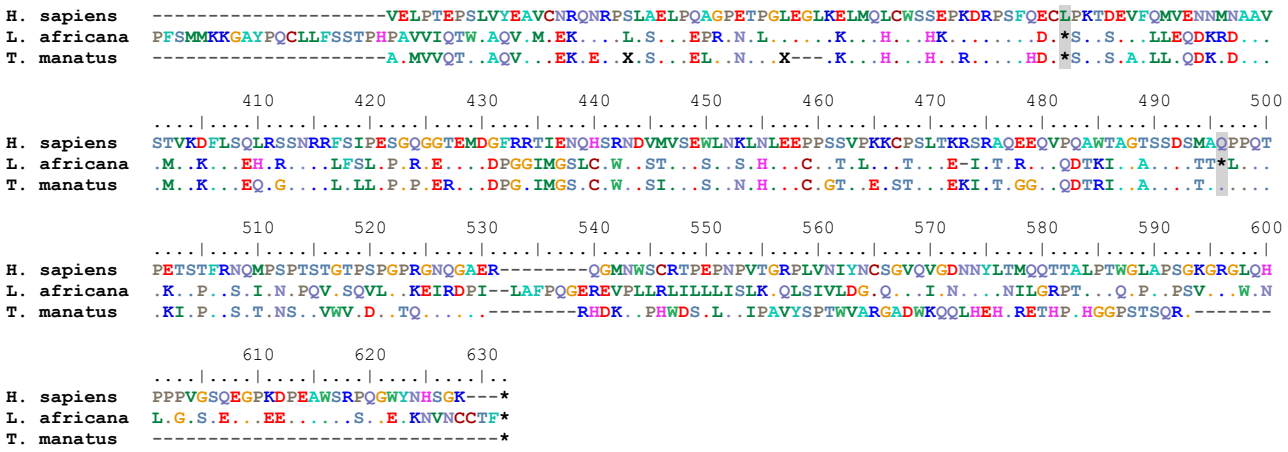
Supplementary file 5. RIPK3 and MLKL protein alignment from species from rodent and afrotheria lineages.

Supplementary file 5.1. Rodent RIPK3 protein alignment. RIPK3 protein alignment from human and 5 rodent genomes. Stop codons are indicated by an asterisk (*) and grey boxes.

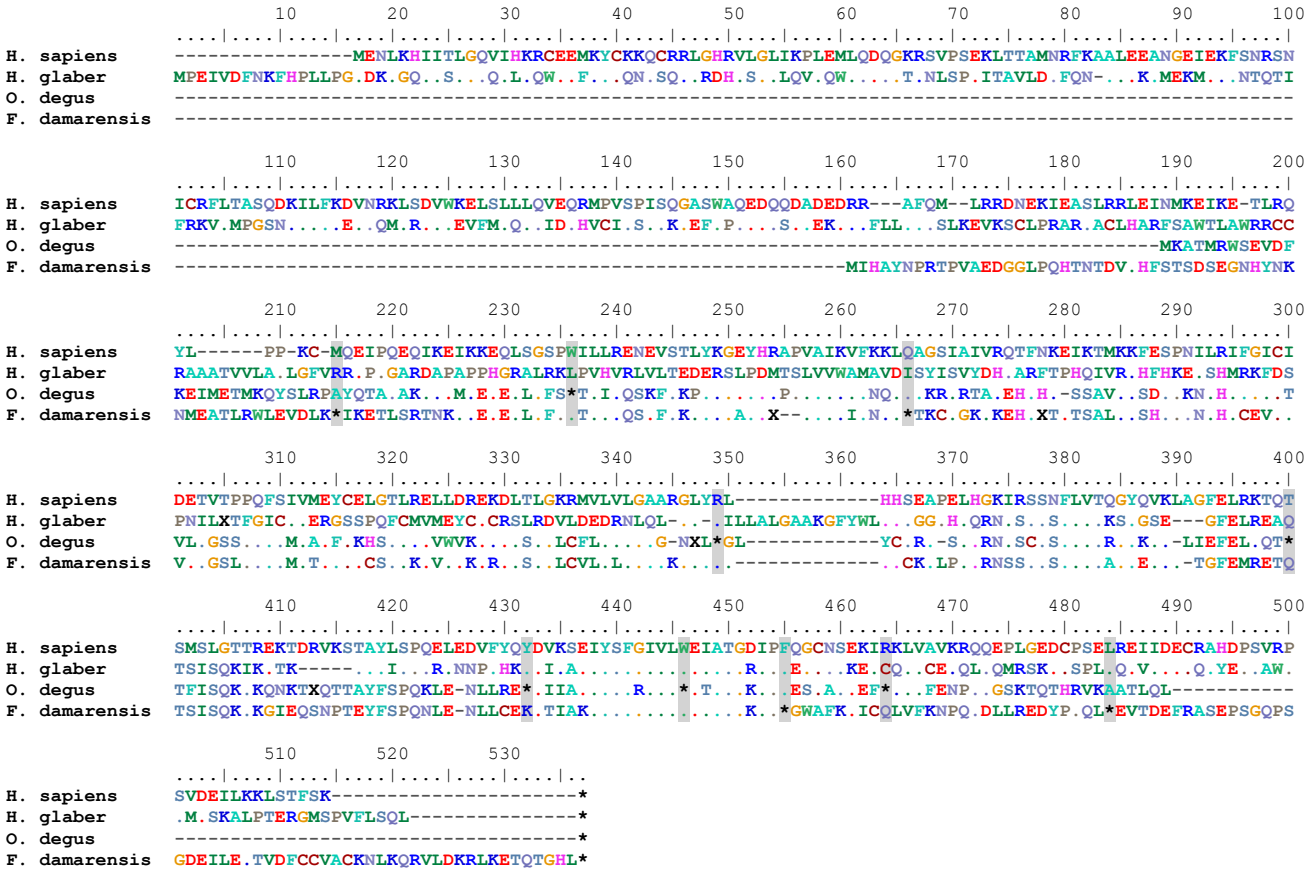


Supplementary file 5.2. Afrotheria RIPK3 protein alignment. RIPK3 protein alignment from human and 2 afrotheria genomes. Stop codons are indicated by an asterisk (*) and grey boxes.

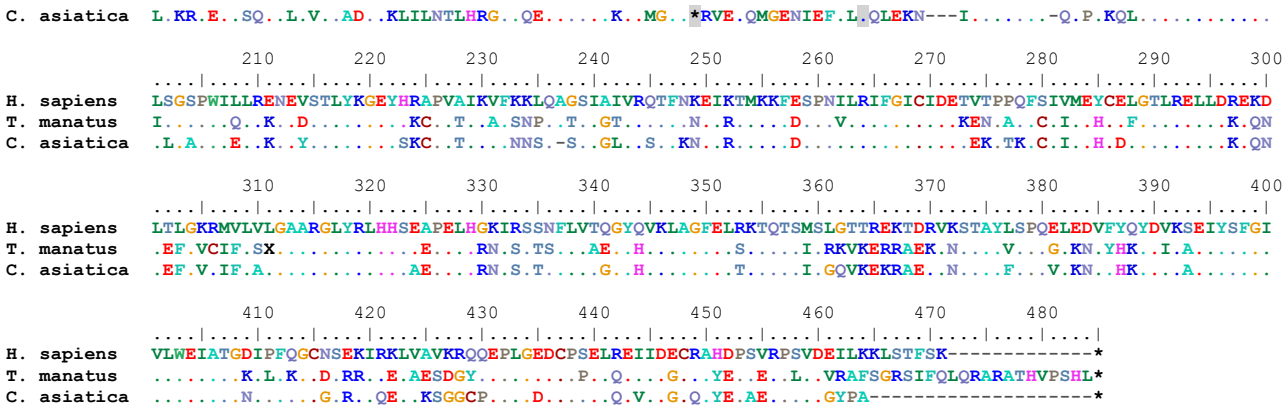




Supplementary file 5.3. Rodent MLKL protein alignment. RIPK3 protein alignment from human and 3 rodent genomes. Stop codons are indicated by an asterisk (*) and grey boxes.



Supplementary file 5.4. Afrotheria MLKL protein alignment. MLKL protein alignment from human and 2 afrotheria genomes. Stop codons are indicated by an asterisk (*) and grey boxes.


C. asiatica L.KR.E..SQ..L.V..AD..KLILNTLHRG..QE.....K..MG..*RVE.QMGENIEF.L.QLEKN---I.....-Q.P.KQL.....
210 220 230 240 250 260 270 280 290 300
H. sapiens LSGSPWILLRENEVSTLYKGEYHRAPVAIKVFKKLQAGSIAIVRQTFNKEIKTMKKFESPNILRIFGICIDETVTPPQFSIVMEYCELGTLRELLDREKD
T. manatus I.....Q..K..D.....KC..T..A.SNP..T..GT.....N..R.....D...V.....KEN.A..C.I..H..F.....K.QN
C. asiatica .L.A...E..K..Y.....SKC..T....NNS.-S..GL..S..KN..R.....D.....EK.TK.C.I..H.D.....K.QN
310 320 330 340 350 360 370 380 390 400
H. sapiens LTLGKRMVLVLGAARGLYRLHHSEAPELHGKIRSSNFLVTQGYQVKLAGFELRKTQTSMSLGTTREKTDRVKSTAYLSPQELEDVFYQYDVKSEIYSFGI
T. manatus .EF.VCIF.SX.....E....RN.S.TS...AE..H.....S.....I.RKVKERRAEK.N....V...G.KN.YHK..I.A.....
C. asiatica .EF.V.IF.A.....AE....RN.S.T.....G..H.....T.....I.GQVKEKRAE..N....F...V.KN..HK....A.....
410 420 430 440 450 460 470 480
H. sapiens VLWEIATGDIPFQGCNSEKIRKLVAVKRQQEPLGEDCPSELREIIDECRAHDPSVRPSVDEILKKLSTFSK----------*
T. manatus .....K.L.K..D.RR..E.AESDGY.....P..Q....G...YE..E..L..VRAFSGRSIFQLQRARATHVPSHL*
C. asiatica .....N.....G.R..QE..KSGGCP....D.....Q.V..G.Q.YE.AE.....GYPA----------*

Supplementary file 6. Tables identifying RIPK3 and MLKL mutations and premature stop codons in Cetacea order.

Supplementary file 6.1. Table identifying RIPK3 mutations and premature stop codons in Cetacea order.

	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8	Exon 9	Exon 10
Tursiops truncatus	7nt del (242); Stop (411)	Exon Not Found	Exon Not Found	Exon Not Found	Exon Not Found	OK	1nt del (20); Stop (42)	OK	OK	1nt del (12)
Globicephala melas	7nt Del (242); Stop (411)	Exon Not Found	Exon Not Found	Exon Not Found	Exon Not Found	OK	1nt del (20); Stop (42)	OK	OK	Exon Not Found
Lagenorhynchus obliquidens	7nt Del (242); Stop (411)	Exon Not Found	Exon Not Found	Exon Not Found	Exon Not Found	OK	1nt del (20); Stop (42)	OK	OK	Exon Not Found
Orcinus orca	7nt Del (242); Stop (411)	Exon Not Found	Exon Not Found	Exon Not Found	Exon Not Found	OK	1nt del (20); Stop (42)	OK	OK	1nt del (12)
Neophocaena asiaeorientalis asiaeorientalis	Stop (84); 7nt Del (242); Stop (411)	Exon Not Found	Exon Not Found	Exon Not Found	Exon Not Found	OK	Stop (42)	OK	OK	1nt del (12)
Phocoena sinus	Stop (84); 7nt Del (242); Stop (411)	Exon Not Found	Exon Not Found	Exon Not Found	Exon Not Found	OK	Stop (42)	OK	OK	OK
Monodon monoceros	Stop (84); 7nt Del (242); Stop (411)	Exon Not Found	Exon Not Found	Exon Not Found	Exon Not Found	OK	Stop (42)	OK	OK	Exon Not Found
Delphinapterus leucas	Stop (84); 7nt Del (242); Stop (411)	Exon Not Found	Exon Not Found	Exon Not Found	Exon Not Found	OK	Stop (42)	OK	OK	Exon Not Found
Lipotes vexillifer	Stop (411)	Exon Not Found	Exon Not Found	Exon Not Found	Exon Not Found	OK	Stop (42)	OK	OK	Exon Not Found
Physeter macrocephalus	Stop (411); 1nt del (445)	Unknown (Fragmented Genomic Region, N's)	Stop (110)	OK	Ok	OK	Stop (42); 2nt del (71)	OK	OK	OK
Balaenoptera acutorostrata scammoni	Stop (228); Stop (411)	2nt ins (5);	Stop (110)	OK	OK	OK	Stop (42)	OK	OK	OK

Supplementary file 6.2. Table identifying MLKL mutations and premature stop codons in Cetacea order.

	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8	Exon 9
Tursiops truncates	Exon Not Found	1nt ins (116)	1nt del (138)	1nt del (11)	OK	1nt del (87); Stop (161)	2nt del (53)	1nt ins (213)	OK
Globicephala melas	Exon Not Found	1nt ins (116)	1nt del (138)	1nt del (11)	OK	1nt del (87); Stop (161)	2nt del (53)	1nt ins (213)	OK
Lagenorhynchus obliquidens	Exon Not Found	1nt ins (116)	1nt del (138)	OK	OK	1nt del (87)	2nt del (53)	1nt ins (213)	OK
Orcinus orca	Exon Not Found	1nt ins (116)	1nt del (138)	OK	OK	1nt del (87)	Stop (5); 2nt del (53)	1nt ins (213)	OK
Neophocaena asiaeorientalis asiaeorientalis	Exon Not Found	1nt ins (116)	1nt del (138)	OK	OK	1nt del (87)	2nt del (53)	Stop (39); 1nt ins (213)	OK
Phocoena sinus	Exon Not Found	1nt ins (116)	1nt del (138)	OK	OK	1nt del (87)	2nt del (53)	Stop (39); 1nt ins (213)	OK
Monodon Monoceros	Exon Not Found	1nt ins (116)	1nt del (142)	OK	OK	1nt del (87)	2nt del (53)	Stop (39); 1nt ins (213)	OK
Delphinapterus leucas	Exon Not Found	Stop (51); 1nt ins (116)	1nt del (121); 1nt del (138); 1nt del (182)	OK	OK	1nt del (87)	2nt del (53)	1nt ins (213)	OK
Lipotes vexillifer	Exon Not Found	Stop (51); 1nt ins (116)	1nt del (121); 1nt del (138); 1nt del (182)	OK	OK	1nt del (87)	2nt del (53)	1nt ins (213)	OK
Physeter microcephalus	Unknown (Incomplete Genomic Sequence)	Unknown (Incomplete Genomic Sequence)	Unknown (Incomplete Genomic Sequence)	Unknown (Incomplete Genomic Sequence)	Unknown (Incomplete Genomic Sequence)	1nt del (87)	OK	Stop (246)	OK
Balaenoptera acutorostrata scammoni	Exon Not Found	OK	2nt del (10)	OK	OK	1nt del (87); 1nt del (134)	OK	Stop (246)	OK

Legend:
ins – insertion
del – deletion
stop – in-frame premature stop codon
(xxx) – (coordinate of the annotated deleterious mutation relative to the reference exon)

Chapter 5

Final Considerations and Future Perspectives

Final Considerations

Myxoma species jump into *Lepus*: a model for poxviruses evolution

Among their many functions, the innate immunity and intracellular signal transduction pathways, such as the apoptotic and necroptotic responses, act to protect the host against hostile assaults like in the case of invading pathogens. Their importance for the host defense is further supported by the identification of several poxviral gene products that systematically sabotage key components of the immune system [1,2]. Over the last couple of years, the study of MYXV infection in rabbits presented a unique way to explore viral immunomodulation on the outcome of infection in the MYXV susceptive host, the European rabbit, where it causes a devastating disease known as myxomatosis [3]. MYXV evolved in close association with its natural evolutionary host, the *Sylvilagus* species. However, successful MYXV infection of novel hosts requires the evasion and manipulation of the potent and complex host immune defenses by a remarkable repertoire of viral proteins known as host range factors [4,5].

The carefully documented emergence of European rabbits with genetic resistance to myxomatosis and attenuated strains of virus is the paradigm for pathogen virulence and host-pathogen co-evolution. When the highly virulent MYXV strains were introduced in Australia and Europe, the case fatality rate was estimated at 99.8% [6]. However, a few years after its introduction, slightly attenuated MYXV strains came to dominate the field populations allowing infected rabbits to survive longer, increasing the probability of transmission from skin lesions by mosquito vectors [7]. These natural experiments have become a textbook example of virulence evolution, revealing much about the correlation between virulence and transmission. Several decades after the discovery of MYXV, the natural arms race between MYXV and Leporids continues to escalate, highlighting the constant pressures exerted by this co-evolutionary battle. Reports of Iberian hares with symptoms of a myxomatosis-like disease started to emerge from Spain in the late summer–fall of 2018. Sequence studies provided the basis for understanding the molecular changes that led a myxoma virus strain, MYXV-Tol, to infect a new host [8]. Other than the disruption of *M009L*, *M036L*, and *M152R*, the MYXV-Tol presented a new recombinant insertion region inserted at the left end of the MYXV-Tol genome, which was derived from an unreported poxvirus with a common origin to capripoxviruses, cervidpoxviruses, suipoxviruses, yatapoxviruses and three unclassified poxviruses - BeAn 58058 virus, cotia virus and eptesipoxvirus [8]. The discovery of this novel MYXV strain in Iberian hare samples along with many reports showing its

successful spread through different areas of Spain and Portugal [9–11], suggests that MYXV is evolving towards a more lethal phenotype capable of infection and spread in Iberian hares.

There are different features that characterize poxvirus genome evolution. Despite varying among different gene families, gene gain events have been consistent characteristics of pox genome evolution and these events have led to changes in poxvirus host range and subversion of host restriction [12–14]. For example, capture of host genes by horizontal gene transfer (HGT) has been a recurrent feature of poxvirus evolution and include many gene families that are known to improve survival of the virus in a given host, such as the MHC class I, the interferon gamma receptor and tumor necrosis factor receptor II, or against environmental damage like glutaredoxin and glutathione peroxidase [12–14]. Given the remarkable case of MYXV-Tol and the high level of sequence similarity between poxvirus genomes, we highlight that homologous recombination could also be a feature of gene gain that might play a major role in poxviral evolution. Previously, events of recombination between orthopoxviruses and also between orthopoxviruses and centapoxviruses have also been reported [15–18]. The MRV (malignant rabbit virus), which belongs to the leporipoxviruses, is a virus that resulted from a recombinant event between MYXV and SFV that was extracted from tumors of laboratory rabbits [19,20]. A recombinant event between SFV and an unknown orthopoxvirus was also suggested in 1982, when two different strains of SFV presented striking differences in their biological and molecular properties [21]. Recombination between viruses occurs when at least two viral genomes co-infect the same host cell and exchange genetic segments [22]. Considering the known host range of MYXV, the recombinant event detected in MYXV-Tol probably occurred in a Leporid and it was dependent on the hare or rabbit being able to be infected by both MYXV and the unknown poxvirus that donated the “cassette”. For this to occur, the two circulating poxviruses must have overlapped geographically. Similarly, it is difficult to know whether this was a recent recombinant event or if this recombinant virus was maintained by circulating in rabbits for several months or years until it found the perfect conditions to spread in Iberian hares. Further studies will be necessary to fully understand the origin of this recombinant virus and to uncover the extensive unsampled diversity of viruses circulating in Leporids.

Examination of the newly acquired “cassette” from MYXV-Tol revealed the presence of a novel host range gene (named *M159*) that was most closely related with members of the VACV C7 superfamily of host range factors [8]. The presence of this

novel host range factor in a MYXV strain capable of crossing the species barrier and causing myxomatosis in *Lepus* species, suggested that the acquisition of this gene had a preponderant role in this species leaping. Our studies using wild-type MYXV-Lau and MYXV-Tol, as well as several recombinant viruses including a MYXV-Tol deleted of M159 protein (vMyxTol-ΔM159) and a MYXV-Lau expression M159 (vMyxLau-v5M159), showed that recombinant MYXVs that did not express *M159* were unable to detectably replicate in hare cells. Interestingly, M159 protein did not contribute to increased replication in the tested permissive rabbit cells (see results from Chapter 3). From the obtained results, M159 expression was not required for MYXV binding, entry or early gene expression, but it was necessary for relieving an early block of viral replication and late protein synthesis in hare HN-R cells. Consitently, and similar to other *C7-like* genes, *M159* was shown to be expressed as an early/late product. However, M159 protein can only accumulate at high levels at later time points. Taking in consideration the amino acid differences in its sequence at the three-fingered molecular claw, region responsible for binding to SAMD9 protein (3D modeling analysis), and M159 migration to the nucleus at late time points it is possible that, besides its ability to bind the hare version of SAMD9, M159 might also bind or induce cellular factor(s) that mediate host range or immunosuppression at later time points from the nuclear compartment of hare cells. While there is still much to learn regarding the ability of M159 protein to engage and modulate the hare's antiviral responses, it is obvious that the gain of this gene by MYXV resulted in its ability to infect hare cells and possibly cause a myxomatosis-like disease for the first time in Iberian hares.

As exemplified by the case of MYXV-Tol *M159*, genes acquired during poxvirus evolution are likely to have host-specific effects. Another major mechanism of poxviral evolution is the gradual loss of genetic information and coding genes through progressive deletion of DNA content. While loss of genetic information is a random process that influences virus biology and evolution, it must provide a selective advantage to be fixated within the virus genome. Accordingly, we have shown a correlation between the loss of E3L zNABD in poxviruses like MYXV and CePV, a domain known to inhibit RIPK3-induced necroptosis, and the absence of necroptosis in their natural infecting hosts: both Lagomorphs and Cetaceans presented frameshifts or premature stop codons that disrupted *RIPK3* and *MLKL*, the key effectors of necroptosis (see results from Chapter 4). Probably as a result of being restricted to a small niche of hosts, the interaction between MYXV and CePV and their natural hosts may have driven their evolution to a fitness peak that is most suited for the host environment.

Overall, the study of MYXV and its co-evolution with Leporids continues to provide valuable information about the evolution of poxviral virulence. The emergence of MYXV-Tol and its negative impact in Iberian hare populations emphasizes the role of recombination in the evolution of poxviral host range, revealing how limited is our knowledge about the possible emergence of harmful zoonotic poxviruses with increased range potential. In the future, the role of recombination in poxvirus evolution should be comprehensively characterized by phylogenomic analysis. Moreover, while viruses that jump hosts evolve rapidly as a result of gene gain, whether by HGT or recombination, this study also emphasizes that long-term evolution of viruses are ultimately shaped by host interactions that lead to fitness optimization and co-adapted virus-host relationships.

Future Perspectives

Poxvirus host range factors are powerful tools to uncover novel functions of uncharacterized host factors and signaling pathways. Therefore, further studies regarding MYXV-Tol will present a unique opportunity to elucidate how poxviral gene gain or loss could affect host range of poxviruses and enable species jump.

First, further systematic studies of Leporids and their infecting viruses are needed to fully understand how this complex story started and to understand the biological and molecular mechanisms of this evolution. As shown before for the relationship between MYXV strains and European rabbits, it is possible that a reduction in virulence will also be favored in MYXV-Tol over time through a combination of natural selection and co-evolution/adaptation. Therefore, we see this event as a unique opportunity to characterize the evolution of the novel MYXV-Tol since its outbreak in Iberian hares and European rabbits to current periods. Given the importance of mosquitos for the transmission of this disease, these insects should also be taken into consideration. This study also shows that M159 protein from MYXV-Tol is a key obligatory component to infect hare cells, expanding MYXV host range. In the future, it will be of the utmost importance to identify possible binding partners of the M159 protein, helping us to gain important insights on which pathways this novel MYXV-Tol was able to counteract to cause disease in the Iberian hares.

Given our previous results regarding the characterization of novel anelloviruses and polyomaviruses and the possible existence of an unsampled poxvirus circulating in Leporids, we believe that studies targeting Leporid samples will allow the detection of other DNA/RNA viruses that are circulating in these populations and thus will provide additional insight into existing co-infections and possible new species of viruses. The description of new poxvirus species not only in Leporid samples, but also in a wide range of mammalian hosts, can bring new aspects of poxvirus biology and new host range genes. Like in the case of M159, other genes in host range families are likely to possess host range functions that might impact the host spectrum of viruses at the organism level. We believe that these analyses could also provide valuable insights about the risk assessment for poxvirus emergence.

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