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Poxviruses and the Evolution of Host Range and Virulence

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Abstract

Poxviruses as a group can infect a large number of animals. However, at the level of individual viruses, even closely related poxviruses display highly diverse host ranges and virulence. For example, variola virus, the causative agent of smallpox, is human-specific and highly virulent only to humans, whereas related cowpox viruses naturally infect a broad spectrum of animals and only cause relatively mild disease in humans. The successful replication of poxviruses depends on their effective manipulation of the host antiviral responses, at the cellular-, tissue- and species-specific levels, which constitutes a molecular basis for differences in poxvirus host range and virulence. A number of poxvirus genes have been identified that possess host range function in experimental settings, and many of these host range genes target specific antiviral host pathways. Herein, we review the biology of poxviruses with a focus on host range, zoonotic infections, virulence, genomics and host range genes as well as the current knowledge about the function of poxvirus host range factors and how their interaction with the host innate immune system contributes to poxvirus host range and virulence. We further discuss the evolution of host range and virulence in poxviruses as well as host switches and potential poxvirus threats for human and animal health.

Poxvirus host range factors

Poxviruses are large double-stranded DNA viruses, which exclusively replicate in the cytoplasm of their host cells. The genomes of currently sequenced poxviruses contain between 135 and 360 kb and contain up to 328 predicted open reading frames (ORFs). Poxviruses can be grouped into 2 subfamilies: *chordopoxvirinae*, which infect vertebrates and *entomopoxvirinae*, which infect insects. Among *chordopoxvirinae*, 10 genera are currently recognized: orthopoxviruses, yatapoxviruses, leporipoxviruses, capripoxviruses, cervidpoxviruses, suipoxviruses, parapoxviruses, molluscipoxviruses, crocodylipoxviruses and avipoxviruses. Because yatapoxviruses, leporipoxviruses, capripoxviruses, cervidpoxviruses and suipoxviruses form a sisterclade to orthopoxviruses in phylogenetic analyses, the former can be classified as “clade II” poxviruses (labeled in Figure 1) (Bratke and McLysaght, 2008; Hughes and Friedman, 2005). Approximately 50 relatively conserved genes are found in all sequenced poxviruses and another 40 genes are present in most chordopoxviruses (Lefkowitz et al., 2006). These genes are important for the general biology of poxviruses such as transcription, RNA processing, replication and virion

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assembly and are generally found in the central regions of the genomes. Genes that are involved in the interaction with the host map generally towards the terminal regions of the genomes and often exhibit lower sequence identity and lineage-specific distribution. Many but not all of these genes are dispensable for virus replication in cell culture, but viruses that have been engineered to lack them are usually attenuated in infection models. Therefore these genes are referred to as virulence genes, and their protein products as virulence factors. Inverted terminal repeats (ITRs) flank the ends of the genomes, which range in size from approximately 0.1 to 13 kb, and contain identical sequences at both ends (Table 1). In most viruses, the ITRs contain multiple examples of duplicated genes.

A striking feature of chordopoxviruses, is that the range of host species that can be productively infected by a given virus, referred to as host range, can vary drastically even between closely-related species within a single genus (McFadden, 2005). Among orthopoxviruses for example, variola virus, the causative agent of smallpox, was strictly human-specific before its eradication, whereas cowpox- and monkeypox viruses naturally infect a wide variety of mammalian species and therefore possess relatively broad host ranges. Remarkably, most poxviruses can enter a large variety of cells from many different animal species in a fashion that is mostly independent of species-specific receptors and involve virion proteins that are conserved in all poxviruses (Moss, 2006). Virus replication and establishment of permissive infection requires the successful manipulation of the host antiviral immune system, especially, the innate immune responses. Certain poxvirus genes have been identified, which are important for viral replication in a subset of cells or host animals, whereas they are dispensable in others. Genes that are important for poxvirus-specific differences in host range can be referred to as host range genes (McFadden, 2005). Until now, approximately 15 genes have been identified in different poxviruses that possess host range function (reviewed in (Werden et al., 2008)). Deletion of those genes leads to viral replication defects in a subset of normally permissive cells. Known poxvirus host range genes can be grouped into 12 distinct gene families. Some of these families contain only one member, whereas others contain many members, likely resulting from lineage-specific duplications (Bratke et al., 2013). Interestingly, not a single host range gene has been identified that is present in all poxviruses. On the contrary, the majority of host range genes show evidence for multiple independent lineage-specific inactivation events, which indicates that those genes might have been dispensable for virus replication in their respective hosts. Alternatively, gene inactivations might have provided the virus with a selective advantage, by causing its attenuation and making its infected host less sick. This could lead to better host survival and increased mobility, which could in some cases lead to better virus transmission.

The precise molecular mechanisms of how host range genes influence poxvirus host range are only partly understood. Different molecular mechanisms for host range function are possible (Figure 2). Since host range proteins often antagonize the host cell innate immune system, the mere presence or absence of host range genes can lead to successful replication or replication defects in different animals or their derived cells. Many host range genes have been identified by the analysis of spontaneously arisen mutants and/or targeted gene deletions that resulted in altered growth characteristics and host range, as compared to their progenitors. Examples of this include the spontaneous deletions comprising the serpin gene SPI-1 in rabbitpox virus (RPXV) and K1L in vaccinia virus (VACV) that were subsequently confirmed by targeted deletions (Ali et al., 1994; Gillard et al., 1986). All deletions of host range genes analyzed so far have resulted in the restriction of the virus host range, however theoretically, the deletion of at least certain genes could also result in extended host range, e.g. if a host-derived molecule targeted a viral gene product and thereby mediated the induction of an antiviral response only in cells possessing that factor. Another way to identify host range genes is by the complementation of host range genes phenotype in a one

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virus, which has restricted host range, with the addition of ectopic genes from another virus. This method was used in the identification of the ankyrin/F-box protein CP77 (CHOhr/CPXV-BR025), which enabled VACV replication in otherwise non-permissive CHO cells (Spehner et al., 1988). Additionally, differences in host range genes between poxviruses can influence host range. Such differences may result in different coding capacities that might directly influence interactions with host molecules or alter protein-stability. Moreover, variations between viruses can also lead to altered transcription or translation levels. It is also possible that the expression of viral host range factors is influenced by host- and cell-specific differences. On the side of the host, the presence or absence of antiviral genes or their expression levels might directly influence viral replication. It is also possible that some poxviruses that show species- or cell type specificity need certain host molecules for successful replication. Species-specific variations in host antiviral genes as well as differences in their expression might directly affect interaction with host range proteins.

The next section will introduce well-characterized chordopoxviruses and summarize our current knowledge about their host range and virulence and discuss notable examples of host range genes in those viruses.

Orthopoxviruses

The orthopoxvirus genus comprises some of the best-studied poxviruses. The genomes of many orthopoxvirus species and strains have been completely sequenced, which provides valuable insights into the evolution of orthopoxviruses. Orthopoxviruses are closely related to one another antigenetically, which results in cross-reactivity and cross-protection against recurrent orthopoxvirus infection. Among the orthopoxviruses are two of the most renowned viruses: Variola virus (VARV), which caused human smallpox, and vaccinia virus (VACV), which has been successfully used in the campaign for the eradication of VARV. Other completely sequenced orthopoxviruses include monkeypox virus (MPXV), ectromelia virus (ECTV), camelpox virus (CMLV), taterapox virus (TATV), rabbitpox virus (RPXV), horsepox virus (HPXV) and different cowpox virus (CPXV) species. Raccoonpox virus, volepox virus and skunkpox virus are North American (NA) orthopoxviruses, whose genomes are as of yet only partially available. Phylogenetic analysis of these sequences showed that NA orthopoxviruses formed a sister clade to the other “old world” orthopoxviruses in phylogenetic analyses. Interestingly, sequence diversity within NA orthopoxviruses is much higher than within “old world” orthopoxviruses (Emerson et al., 2009). The following chapters will focus on the genomics and host range of orthopoxviruses.

Variola virus

Variola virus (VARV), the causative agent of smallpox, is thought to have caused more human fatalities throughout history than all other infectious diseases combined (McFadden, 2005). It was likely the first pathogen used for immunization by a process known as variolation, and is the only human virus that has been successfully eliminated from nature through mass vaccinations. VARV was most frequently transmitted in nature by droplet infection, usually requiring close contact, and caused a systemic disease with variable clinical manifestations. These ranged from a mild form without any rash, to the classic and most frequent form, which caused a macular rash to appear that developed into papules, vesicles, pustules and finally crusts. Severe, highly lethal hemorrhagic forms also existed with persistent fever in affected patients. All lesions advanced simultaneously and were more numerous on the extremities and head, including the oral cavity, compared to the trunk in what is referred to as centrifugal distribution. Blindness was a common complication of smallpox and survivors were often severely disfigured due to extensive scarring. Multiple

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VARV strains existed that exhibited major differences in disease severity. Generally, strains that caused high case fatality rates (CFRs), usually ranging between 10-30%, were designated as VARV major strains, whereas strains that caused low CFRs of < 1% were referred to as VARV minor strains (reviewed in (Damon, 2007)). The natural occurrences of less virulent VARV strains were frequently observed. In his seminal work on the vaccination against smallpox, Edward Jenner described a “variety of the Small-pox” that was present in 1792 in Gloucestershire, England, a region that was usually afflicted by highly virulent strains, which was “ ... of so mild a nature, that a fatal instance was scarcely ever heard of ...” (Jenner, 1798). Similarly, these mild smallpox outbreaks occurred at various times in many different locations throughout the world (reviewed in (Riley, 2010)). Less virulent strains were frequently replaced by more virulent strains, indicating major fluctuations in VARV population structures. Phylogenetic analyses of various VARV strains isolated during the 20th century showed the existence of three major clades and revealed that VARV minor strains evolved independently at least twice (Babkina et al., 2004; Esposito et al., 2006). Genome sizes of these VARV strains range between 185-188 kb and overall show low sequence diversity (Esposito et al., 2006).

VARV is the only orthopoxvirus that exhibited complete human-specificity. No animals known have become naturally infected with VARV. In experimental settings, the non-human primate species *Macaca fascicularis* (Cynomolgous monkey), *Papio cynocephalus* (baboon) and *Pan troglodytes* (Chimpanzee) have been successfully infected with relatively high inoculation doses of VARV (Heberling et al., 1976; Kalter et al., 1979; Noble and Rich, 1969). However, pathogenesis observed in those models only partially resemble smallpox in humans. Moreover, very high doses of VARV had to be administered intravenously to achieve lethal infection (Jahrling et al., 2004).

Considering the devastating effect VARV had on humans, understanding its evolution is of high importance. Sequence comparison and phylogenetic analyses show that VARV is most closely related to CMLV and TATV, who very likely shared a common ancestor with VARV that might have evolved from a common rodent orthopoxvirus (Esposito et al., 2006; Li et al., 2007). Different methods used to calculate VARV mutation rates yielded similar results in the range of 1×10^{-6} to 9×10^{-6} substitutions per site (nucleotide) per year using modern virus isolates (Babkin and Shchelkunov, 2006, 2008; Firth et al., 2010; Hughes et al., 2010). Using different calibration methods, different dates for the divergence of VARV and CMLV/TATV clades have been calculated by different groups. Based on the substitution rates obtained from modern VARV isolates and the assumptions that VARV minor Alastrim strains diverged from West African VARV strains about 400 years ago and that a highly virulent disease such as smallpox required a relatively high population size of susceptible human hosts, which did not occur before 10 thousand years ago, divergence dates for the VARV and CMLV/TATV clades of about 3000 to 6000 years were calculated (Babkin and Shchelkunov, 2006, 2008; Hughes et al., 2010). In addition to using isolation dates for the calibration of their analyses, which yielded similar VARV and CMLV/TATV diversion dates as those described by Babkin and Shchelkunov and Hughes et al., Li et al., used two different smallpox historical records for the calibration of their analyses of VARV evolution and calculated a divergence date of VARV and CMLV/TATV clades of approximately 16,000 or 68,000 years ago, depending on the calibration method (Li et al., 2007). However, the caveats of this calibration method and its outcome have also been raised (Babkin and Babkina, 2011; Shchelkunov, 2009). One problem with calculating substitution rates from modern viral sequences is that they can be drastically higher than long term viral substitution rates, which might be due to a large proportion of slightly disadvantageous mutations in modern viruses, which have not yet been removed from the population through purifying selection (Feschotte and Gilbert, 2012). Whereas the short-term evolutionary rates for both VARV and myxoma virus (MYXV) are similar (Babkin and

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Shchelkunov, 2008; Firth et al., 2010; Kerr et al., 2012), the long-term evolutionary rates of poxviruses in general are currently unclear. The resolution of this important subject might have to wait for the discovery of an endogenized poxvirus in a vertebrate genome, or possibly the sequencing of an ancestral poxvirus genome from ancient tissues or fossils, which could then be utilized in an alternative calibration approach.

Since CMLV and TATV viruses are the closest identified relatives of VARV and also show extremely narrow host ranges (see below), a comparison of their host range genes can offer valuable insights about which genes were lost in a particular lineage and which genes were present in a common ancestor that likely possessed a broader host range. Only 11 host range family genes are found in all of these three viruses: K3L, E3L, C7L, B5R, F1L p28, SPI-1, SPI-2, SPI-3 and ANK/F-Box genes #5 and #6 (Bratke et al., 2013). VCP, CrmB and ANK/F-Box gene #1 are found in VARV and CMLV but not TATV, whereas an ortholog of the M-T4 gene from MYXV is only found in CMLV and TATV. The following host range genes are only found in one of the three viruses: ANK/F-Box gene #3 in VARV; C4L and ANK/F-Box gene #10 in CMLV; ANK/F-Box genes #7 and #9 in TATV. All three viruses lack functional CrmC, CrmD, CrmE and K1L orthologs. K1L orthologs are inactivated by unique mutations in each of the three viruses, indicating that their most recent ancestor still possessed a functional K1L and that subsequent inactivations occurred independently in each lineage (Bratke et al., 2013). Relatively recent inactivations in the K1L orthologs are further supported by the findings that the principle gene structures are still intact and no major deletions occurred. Overall, it appears that the most recent ancestor of VARV, CMLV and TATV possessed at least 21 host range family genes, only lacking CrmC, CrmD, CrmE and ANK/F-Box genes #2, #4 and #7 (Bratke et al., 2013). Such an ancestral virus likely possessed a wider host range, and a natural reservoir for it might have been rodents (Li et al., 2007).

Camelpox and Taterapox viruses

CMLV and TATV viruses are the closest identified extant relatives of VARV. CMLV is a widespread pathogen of old world camels and causes an infection that resembles smallpox in humans (reviewed in (Duraffour et al., 2011)). It is characterized by fever, rash, the formation of vesicles and pustules concentrated on the head, neck and extremities, and the infection often spreads to the respiratory system. Depending on strain-specific and regional differences, mortality can reach up to 50%. As in human smallpox, blinding of surviving animals is frequently observed (Kriz, 1982). Camelpox is a disease of great economic importance. Surviving CMLV infection and vaccination with an attenuated CMLV or VACV leads to long lasting immunity (Baxby et al., 1975; Hafez et al., 1992). Interestingly, camels that were inoculated intradermally with VARV showed only transient lesions at the inoculation sites without displaying any disease symptoms and no virus could be successfully isolated from these injection sites or from blood samples. Moreover, camels that had been previously inoculated with VARV were resistant to infection with CMLV at a dose that normally produced general infection in camels, indicating that VARV was able to induce immunity to CMLV infection (Baxby et al., 1975). Despite the close relationship of CMLV with VARV and the close contact of infected animals with their human handlers, CMLV infection of humans appears to be very rare and lead to a localized and self-limited infection (Bera et al., 2011; Jezek et al., 1983; Kriz, 1982).

The two CMLV strains CMS and M-96, which contain approximately 202 and 206 kb, respectively, have been completely sequenced (Afonso et al., 2002b; Gubser and Smith, 2002). Sequences of both strains are very similar, exhibiting 99.9% identity on the nucleotide level in alignable regions. Major differences are found in the ITRs, and concern direct repeats. CMLV and TATV host range genes are discussed in the VARV section.

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TATV is the most enigmatic completely sequenced orthopoxvirus. It was isolated from the liver/spleen of an apparently healthy Kemp's gerbil (*Tatera kempii*) in Benin in a survey for new viruses (Kemp et al., 1974). Because this was the only time TATV was isolated, there is no information about the actual host range of this virus, but it is suspected to be narrow. In experimental settings, infection of Mongolian gerbils (*Meriones unguiculatus*), rabbits (*Oryctolagus cuniculus*) and a rhesus monkey (*Macaca mulatta*) only lead to localized reactions or fever without successful virus isolation (Lourie et al., 1975). The genome of TATV contains approximately 198 kb and is most closely related to CMLV (Esposito et al., 2006). A retrotransposon belonging to the family of short interspersed elements (SINEs) was identified in the TATV genome. Amazingly, this element is most closely related (97% nucleotide identity) to SINEs found in the poisonous West African carpet viper (*Echis ocellatus*). It was suggested that the SINE was transferred from the snake to the virus and that the snake might be a host for TATV (Piskurek and Okada, 2007). This finding implies that VARV-related orthopoxviruses might actually infect snakes, which would be surprising, since mammals are the only identified hosts of orthopoxviruses. This highlights the importance to include non-mammalian species in the search for new poxviruses.

Vaccinia virus, Horsepox virus and Rabbitpox virus

Vaccinia virus (VACV) is the best studied of the poxviruses. It has been studied extensively as a model to understand the basic biology, virulence, and host range of poxviruses as well as its induction of host immune responses. Due to the fact that it usually causes only mild infections in humans and can induce protective immunity against other orthopoxviruses, it was used successfully in the campaign to eliminate smallpox (reviewed in (Smith, 2007)).

Currently available VACV strains have been propagated in animals and cell culture for many decades and the primary origins of these strains are not clear (Smith, 2007). Edward Jenner suspected that the viruses he used for his immunization experiments were initially transmitted from horses, suffering from a once common disease called grease, to their human handlers. The handlers, he reasoned, subsequently transferred it to the udders of cows, which then infected the milkmaids. Since the milkmaids who provided the infectious material for his initial experiments acquired it from cows, Jenner called the infectious entity “cow pox” (Jenner, 1798). It should not be confused with current cowpox viruses, which form phylogenetically distinct clades from VACV (Bratke et al., 2013). In later experiments Jenner showed that material directly extracted from the grease of horses, directly provided protective immunity against smallpox infection (Jenner, 1798). Due to a long passage history and selection for cell-culture adapted and less virulent VACV strains, the genomes of VACV strains are highly heterogeneous, due mainly to large deletions. Experimental infections showed that several VACV strains can productively infect many hosts, including primates, rodents, lagomorphs and ungulates. VACV has also been successfully used to immunize zoo and circus animals against other orthopoxviruses (reviewed in (Essbauer et al., 2010)). VACV can occasionally also be transmitted from recently vaccinated humans to other human contacts and cause serious illness, including death, especially in immunocompromised people. Moreover, transmission from a mother to her fetus can result in rare, but often lethal, fetal vaccinia virus infection (reviewed in (Cono et al., 2003; Sepkowitz, 2003)). Orthopoxviruses that are closely related to VACV have repeatedly caused severe disease in Asian buffalos (*Bubalus bubalis*) and are therefore sometimes referred to as buffalopox viruses (BPXV), which occasionally cause zoonotic infections in humans (reviewed in (Essbauer et al., 2010; Moussatche et al., 2008)). Phylogenetic analyses of a small number of genes from these viruses show a close relationship of BPXV to VACV strains, which makes it likely that they represent descendants of escaped now-feral VACV (Yadav et al., 2010). Similarly, VACV-related viruses have caused disease outbreaks in Brazilian cattle and in human farmers (reviewed in (Essbauer et al., 2010; Moussatche et

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al., 2008)). Closely related viruses, which are most closely related to VACV-like strains that caused outbreaks in cattle and humans, were also detected in capuchin monkeys (*Cebus apella*) and black-howling monkeys (*Allouata caraya*) by PCR and isolated from a wild house mouse (*Mus musculus*) in Brazil (Abrahao et al., 2009; Abrahao et al., 2010). These findings indicate natural reservoirs for Brazilian VACV-like viruses that might have escaped during VACV vaccinations. However, more sequence data and additional phylogenetic analyses are necessary to confirm the notion that BPXV and Brazilian VACV strains isolated from wild or domesticated animals indeed represent descendants of escaped vaccine-derived VACVs.

Two other VACV-related orthopoxviruses, horsepox virus (HSPV) and rabbitpox virus (RPXV) have been completely sequenced (Li et al., 2005; Tulman et al., 2006). Horsepox, a systemic febrile disease, often accompanied by pox-like skin and mucosa eruptions in horses, was once prevalent in parts of Europe and North America and caused infections in humans (Jenner, 1798; Tizard, 1999). HSPV, the infective agent was considered to be extinct until it was recovered during a disease outbreak in horses in Mongolia in 1976 (Tulman et al., 2006). Since sequences of historic isolates of HSPV are unknown, it is impossible to determine the exact relationship between them and the isolate from Mongolia. RPXVs were originally isolated from laboratory rabbit colonies in New York City and the Netherlands (Utrecht) in the 1930's and 1940's, respectively. RPXVs have only been isolated from laboratory colonies but have not been isolated from wild rabbits. It is therefore unknown, if rabbits constitute the natural host of RPXV (reviewed in (Chapman et al., 2010)). Most subsequent RPXV work has been conducted with the Utrecht strain, which is highly virulent in rabbits after intradermal infection and can be transmitted by aerosols between animals (Adams et al., 2007). RPXV has also been shown to infect mice (reviewed in (Chapman et al., 2010)). Earlier phylogenetic trees showed that HSPV and RPXV formed a well-supported monophyletic clade with VACV, but did not resolve the exact relationship between those viruses (Li et al., 2005; Tulman et al., 2006). A recent phylogenetic analyses, which included sequences of 100 conserved orthopoxvirus genes and included 11 completely sequenced VACV strains confirmed those findings, and additionally showed that HSPV and RPXV are well-separated from two major VACV clades (Bratke et al., 2013). This indicates that HSPV, RPXV and all VACV strains shared a common ancestor but that the former ones are not direct descendants from the latter. This is further supported by the analyses of host range genes present in these viruses. The total number of predicted full-length host range family genes in HSPV, RPXV and VACV-WR is 17, 15 and 13, respectively and each show unique distribution patterns, e.g. HSPV is the only of those viruses that contains an intact TNFR-2 homolog (CrmB ortholog) and ANK-Box genes #5 and #8 (Bratke et al., 2013). Our current knowledge about the actual host range of extant HSPV (historic HSPV infected horses, cows and humans) and RPXV is limited. The presence of more host range family genes in HSPV and RPXV than in VACV and a close phylogenetic relationship with each other, indicates that HSPV and RPXV might actually possess a comparable or even broader host range than VACV.

Cowpox viruses

Historically, the designation cowpox virus (CPXV) was used to describe orthopoxviruses that infect cows and were occasionally transmitted to humans who had close contact with infected cows. Characteristic of CPXV, although not specific, are the formation of cytoplasmic A-type inclusions, which contain mature virions embedded in a matrix formed by the late viral protein ATI, and the formation of large hemorrhagic pocks on the chorioallantoic membrane (reviewed in (Essbauer and Meyer, 2007)). CPXVs have the broadest host-range among known poxviruses. Their reservoir hosts are likely rodents including many vole and mouse species. CPXVs naturally infect many other species

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including cats, dogs, cows, horses, primates, including humans, and a diverse set of zoo animals such as elephants, rhinoceroses and big cats. Most human CPXV infections are caused by contact with infected cats or pet rats, through skin lesions. Whereas infections in immunocompetent individuals are usually localized, immune-compromised patients, including those suffering from atopic skin diseases are at higher risk for systematic and even fatal infections (reviewed in (Essbauer and Meyer, 2007; Essbauer et al., 2010).

The complete genomes of 12 CPXVs are currently available, including CPXV-Brighton Red (BR), CPXV-Germany 91-3 (GER) and CPXV- GRI-90 (GRI) which contain approximately 224, 228 and 224 kb, respectively, thus possessing the largest of all orthopoxvirus genomes (Carroll et al., 2011). Phylogenetic analyses using concatenated protein alignments from conserved genes showed that CPXVs form clearly distinct clades. Whereas CPXV strains BR and GER form a clade with ECTV, CPXV-GRI forms a clade with VACV, RPXV and HPXV (Bratke et al., 2013). A phylogenetic analyses including sequences from all 12 CPXVs that used ECTV as outgroup showed 5 distinct CPXV clades (Carroll et al., 2011). This confirms and extends previous analyses that implied the existence of at least two CPXV species (Gubser et al., 2004; Lefkowitz et al., 2006).

In agreement with their wide host-range, CPXVs possess by far the largest number of intact host range gene homologs among orthopoxviruses: 27 in CPXV-GRI, and 26 in CPXV-BR and CPXV-GER (Bratke et al., 2013). The only host range family gene that is missing in CPXV-BR is CrmE. CrmE in CPXV-BR is inactivated by stop-codons and deletions, some of which are shared with CrmE pseudogenes in ECTV and MPXV. Interestingly, CrmE is intact in CPXV-GER, indicating a recombination event may have occurred. The only host range gene that is inactivated in CPXV-GER is CrmD (Bratke et al., 2013).

Monkeypox virus

Human monkeypox is an emerging zoonotic disease in humans, which clinically resembles smallpox. It is caused by monkeypox virus (MPXV), which is endemic to central and western African countries and possesses one of the broadest host ranges among poxviruses. The natural host reservoirs of MPXV are probably rodents including rope squirrels (*Funisciurus sp.*), sun squirrels (*Heliosciurus sp.*) and Gambian giant rats (*Cricetomys sp.*), as indicated by the isolation of virus from these species and the high seroprevalence. Additionally, many other species can be naturally infected by MPXV including many primate species. MPXV constitutes a human health threat. After the eradication of smallpox and the cessation of mass vaccinations against it, an increasing number of humans have become susceptible for infection by other orthopoxviruses, including MPXV. Humans usually get infected with MPXV by direct contact with infected animals. Additionally, in some cases human-to-human transmission has been reported, which might be increasing (reviewed in (Essbauer et al., 2010; Parker et al., 2007)). If MPXV would become more adapted to humans leading to increased virulence and/or human-to-human transmission and at the same time retain its ability to infect multiple animal species, controlling such virus would pose a major challenge.

The complete genomes of 13 MPXV strains, which contain between 196-206 kb, are currently available (Chen et al., 2005; Likos et al., 2005; Nakazawa et al., 2013; Shchelkunov et al., 2002). Phylogenetic analyses show the existence of two MPXV clades, which are associated with their geographic origin: the Congo Basin clade, which we refer to as clade I, and the West African-derived clade, which we refer to as clade II. Importantly, MPXV clade I and clade II exhibit differences in virulence and transmissibility. Whereas infections with MPXV clade I strains cause higher lethality (approximately 10%) in humans and experimentally infected animals, clade II strains cause milder diseases (reviewed in

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(Essbauer et al., 2010; Parker et al., 2007)). Interestingly, major genomic differences distinguish strains belonging to the two clades, which include known poxvirus virulence and host range genes. This includes the ortholog of MYXV host range gene T4 (B10R in MPXV ZAI96), which contains deletions leading to premature stop-codons in all type II strains (Bratke et al., 2013; Chen et al., 2005; Likos et al., 2005). Another candidate for the distinct virulence of MPXV strains is the ortholog of VACV VCP/C3L, which is found in clade I strains but deleted in clade II strains (Uvarova et al., 2005). In clade I strains the ORF terminates due to a premature stop-codon in short complement-like repeat domain 4, and has been shown to be at least partially functional (Liszewski et al., 2006). In addition to genomic differences due to major deletions, there are hundreds of SNPs and small insertions/deletion (indels), which affect the amino acid sequence of proteins and might also contribute to distinct biological properties of MPXV strains.

One of the most striking differences between MPXVs and other orthopoxviruses are gene- or domain-inactivating mutations in both MPXV orthologs of VACV protein kinase R (PKR) inhibitors K3L and E3L, respectively. The K3L ortholog in MPXV is inactivated by a stop-codon and deletions comprising key residues for PKR inhibition. In the E3L ortholog the Z-DNA binding domain (Zα) is inactivated by mutations of the start-codon and two 2 bp deletions. Translation of the E3L ortholog is probably initiated within the Zα domain, which is predicted to lack Z-form binding activity (Bratke et al., 2013). The Zα domain of VACV E3L has been shown to be a virulence factor for infections in mice (Brandt and Jacobs, 2001). The partial or complete inactivation of E3L and K3L orthologs might have led to an attenuation of MPXV in many different hosts. Such “attenuation by gene inactivation” might result in a better transmissibility by making the infected hosts less sick and more mobile. Acquisition of complete E3L and K3L orthologs by MPXV through recombination with other poxviruses might yield MPXV strains that are even more virulent and have larger host ranges.

Both VARV and MPXV cause clinically similar and potentially lethal infections in humans, and yet each exhibit profound differences in host range, with VARV being human-specific and MPXV showing a broad host range. For this reason, a comparison of host range family genes between both viruses might provide clues to explain these different biological properties. Genes that are found in VARV but are absent or inactivated in MPXV include VACV orthologs of K3L and ANK/F-box gene #3. Orthologs of VACV E3L and VCP show partial deletions in MPXV, but might maintain some activity. Those differences might contribute to a higher virulence and transmissibility of VARV in humans. Host range genes that are present in MPXV but absent or inactivated in VARV are orthologs of VACV K1L, MYXV M-T4 (Complete ORF only found in MPXV clade I strains) and ANK/F-box genes #1, #7, #8 and #9 (Bratke et al., 2013). Those genes might contribute to the broad host range of MPXV.

Ectromelia virus

Ectromelia virus (ECTV) causes mousepox in laboratory mice (*Mus musculus*) (reviewed in (Esteban and Buller, 2005)). The complete genome of two ECTV strains is available: ECTV-Mos and ECTV-Nav, which contain 210 and 208 kb, respectively (Chen et al., 2003) (complete sequence of ECTV-Nav can be found at: http://www.sanger.ac.uk/Projects/Ectromelia_virus/EV.seq). Sequence identity between the two strains is approximately 99.5%, with only three ORFs showing disruptions between strains (Esteban and Buller, 2005). In phylogenetic analyses using concatenated alignments of conserved poxvirus proteins, ECTV formed a monophyletic clade with CPXV-BR and CPXV-GER strains (Bratke et al., 2013; Lefkowitz et al., 2006). Many genes that are important for host-virus interactions that are found in other orthopoxviruses contain disruptions in the ORFs or are

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deleted in ECTV (Chen et al., 2003). Among genes that contain premature stop-codons are host range family orthologs of K3L, CrmB, CrmC, CrmE and ANK/F-Box genes #1, #4, #7, #8, #9 and #10. C4L and T4 orthologs are completely missing from the ECTV genome (Bratke et al., 2013). Since ECTV infections have only been observed in laboratory mouse colonies, its natural host is unknown. However, it is believed that wild mice or other rodents are natural hosts of ECTV. Experimental infection studies indicate a narrow host range for ECTV (reviewed in (Buller and Palumbo, 1991)). Intriguingly, there are large differences in disease severity of ECTV infections in different mouse strains. Whereas ECTV is highly lethal in some strains, e.g. BALB/c and A/J mice, it causes only mild or subclinical diseases in other strains such as C57BL/6. Variable susceptibility to ECTV infection was also observed in wild mice (reviewed in (Buller and Palumbo, 1991; Esteban and Buller, 2005). A possible explanation for the differential sensitivity to ECTV is a polarized type I cytokine response in more resistant mice. This leads to the production of Interferon-γ, tumor necrosis factor and Interleukin-2, and an associated strong cytotoxic T lymphocyte (CTL) response, as opposed to a type 2 cytokine response in susceptible strains with the production of Interleukin-4, which is associated with a weak CTL response (Chaudhri et al., 2004). It is noteworthy that, while being only weakly susceptible to ECTV administered in footpad infections, intranasal inoculation of ECTV at low virus doses can cause lethal infections in C57BL/6 mice, highlighting that the route of infection plays an important role in poxvirus infections (Gratz et al., 2011; Kremer et al., 2012; Paran et al., 2009).

North American orthopoxviruses

North American (NA) orthopoxviruses, including raccoonpox virus (RACV), skunkpox virus (SKPV) and volepox virus (VPXV), are presumed to be endemic to North America and can be described as “new world” orthopoxviruses as opposed to the previously characterized “old world” orthopoxviruses (Emerson et al., 2009). The characteristics of these viruses, such as geographic distribution, host range and taxonomic position have not yet been determined in detail. Phylogenetic analysis with selected genes, which are fairly conserved among poxviruses showed that NA orthopoxviruses formed a monophyletic sister group to all other formerly described OPXV species (Emerson et al., 2009).

RACV was isolated from the respiratory tract of a raccoon in a forest and swamp area in Maryland. A study in the 1970s in the same area reported a seroprevalence of 23% in wild raccoons, indicating a wide endemic spread of the virus (Alexander et al., 1972). Interestingly, a virus that is closely related to RACV was isolated from a localized skin lesion on the paw of a cat in Canada. The partial sequence that was amplified from the lesion showed 97% sequence identity to the RACV hemagglutinin gene (Yager et al., 2006). SKPV is the only NA orthopoxvirus that was initially isolated from a sick animal. However, there was no evidence to prove that SKPV was the cause of the illness (Emerson et al., 2009). VPXV was isolated from a healthy California vole (*Microtus californicus*) and a pinyon mouse (*Peromyscus truei*) in California, and serological studies indicate that VPXV is endemic to the region where the viruses were isolated (Knight et al., 1992; Regnery, 1987). Experimental intranasal infection of California mice (*Peromyscus californicus*) with VPXV led to severe morbidity, a high lethality rate of more than 50% and high viral loads (Gallardo-Romero et al., 2012). Overall, the biology of NA orthopoxviruses including prevalence, geographic distribution, host range and virulence is only poorly understood and deserves additional studies.

Yoka poxvirus

Yoka poxvirus (YKV) was isolated in 1972 from *Aedes simpsoni* mosquitoes in the Central African Republic. It was identified as a poxvirus in 1989 by electron microscopy (Zeller et

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al., 1989), however, it was not until recently that its genetic and evolutionary relationship to other poxviruses has been investigated (Zhao et al., 2011). The host range of this virus is currently unknown and there has been very little work to explore the host range function of its genes. It has been shown to cause a cytopathic effect in Vero, CER, PS and BHK-21 cells without producing distinguishable plaques (Zeller et al., 1989). Suckling mice inoculated intracranially with the virus subsequently died within six days. The natural host for YKV is unknown at this point, but a close phylogenetic relationship to mammalian orthopoxviruses and the fact that it was isloated from mosquitos indicates that (a) mammal(s) is/are the natural host(s) for YKV and that it might be transmitted via arthropod vectors. The YKV genome has been recently sequenced. Phylogenetic analyses from this study indicated a close relationship to orthopoxviruses. The formation of a separate branch in the poxvirus phylogenetic tree suggests that it constitutes a distinct genus of poxvirus, which was proposed to be called centapoxvirus (Zhao et al., 2011). The YKV genome contains all of the 90 conserved core poxvirus genes common to all chordopoxviruses as well as several orthologs to known host range genes, all of which share high sequence identity with other orthopoxvirus genes. In support of a mammalian host for YKV, a gene predicted to encode an MHC-I-like protein with highest sequence identity to those found in mammals was identified in the YKV genome, which may have been transferred to the virus via horizontal gene transfer (Zhao et al., 2011).

YKV contain three genes with homology to poxvirus clade II serpins: YKV165 is the ortholog of orthopoxvirus SPI-2/CrmA, whereas YKV177 and YKV182 are most closely related to one another and are orthologs of SPI-1. Because orthopoxvirus SPI-1 and SPI-2 originated from a gene duplication after the split from clade II poxviruses, this indicates that this duplication occurred in an ancestor to both YKV and orthopoxviruses. YKV182 contains a premature stop codon that is due to a short out-of frame deletion in the middle of the gene. It is interesting that a SPI-3 ortholog, that is present in orthopoxviruses and some clade II poxviruses is missing in YKV. YKV contains two TNFR-II-related genes: YKV181 is most closely related to CrmB. However it lacks the predicted C-terminal domain due to disruptions of the ORF. YKV178 is most closely related to CrmE. Several other proteins are encoded within the genome that could potentially serve a host range function as inhibitors of NF-κB activation and interferon signaling, however, the role these genes play in controlling the host range of YKV has yet to be experimentally investigated.

Yatapoxviruses

The genus yatapoxvirus comprises three viruses that have been completely sequenced: Yaba-like disease virus (YLDV), Tanapox virus (TPV) and Yaba monkey tumor virus (YMTV), the genomes of which comprise 145 kb (YLDV and TPV) 135 kb (YMTV) (Brunetti et al., 2003; Lee et al., 2001; Nazarian et al., 2007). All of these viruses infect primate species. YLDV and TPV are closely related to one another. A pairwise comparison of their genomes using blastn shows 98.6 % identity on the nucleotide level. YMTV is more distantly related to YLDV and TPV with a nucleotide comparison of alignable regions showing approximately 81% sequence identity between them. YLDV and TPV are sometimes viewed as members of the same species. Two TPV strains have been completely sequenced that were isolated 50 years apart from distinct geographical regions (Nazarian et al., 2007): TPV-Kenya was isolated near the Tana River in Kenya in 1957 and TPV-RoC, which was isoloated from a student who contracted the disease while working with chimpanzees in the Republic of Congo in 2002 (Dhar et al., 2004). Remarkably, both isolates were very similar, differing in only 35 positions, despite their different origins (more than 2500 km apart) and isolations dates, indicating the low mutation rate in this virus (Nazarian et al., 2007).

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YMTV was identified, when it was isolated as an agent that induced subcutaneous histiocytic tumor formation in a laboratory colony of rhesus monkeys (*Macaca mulatta*) in Yaba, Nigeria (Bearcroft and Jamieson, 1958; Niven et al., 1961). The disease was apparently transmitted to a Guinea baboon (*Papio papio*), which was housed in the same facility and developed a tumor on the nose. During this outbreak, the formation of tumors, which grew to 2-4 cm in size, was restricted to the limbs and face, sparing the trunk. Affected animals recovered spontaneously and the affected lesions healed by granulation. The disease could be experimentally transmitted by subcutaneous inoculation to rhesus and tantalus (*Cercopithecus tantalus*) monkeys. Experimental inoculation of red-capped mangabeys (*Cercocebus torquatus torquatus*) and patas monkeys (*Erythrocebus patas patas*) failed to produce disease symptoms (Bearcroft and Jamieson, 1958). Interestingly, African monkeys born in the USA were susceptible to YMTV infections, whereas all individuals from the corresponding species that were imported from Africa were all resistant, indicating that the African monkeys acquired immunity to YMTV and that this or a closely related virus was widespread in Africa (Ambrus and Strandstrom, 1966). In agreement with this, high titer neutralizing antibodies were described in 76% of African green monkeys (*Chlorocebus aethiops*) tested, whereas the incidence of the same antibodies in different Asian macaques ranged between 0 and 20%. Thus, YMTV seems to be able to infect a large variety of primates and the resistance of some species to infection might rather reflect previous contact with the virus and subsequent immunity than resistance of those species per se. Humans are also susceptible to YMTV infection. Patients with terminal cancer developed lesions similar to those seen in monkeys after experimental infection (Grace et al., 1962). Moreover, a healthy lab worker became infected with YMTV after an accidental needle-stick injury and developed both antibodies and a tumor at the site of the needle puncture (Grace and Mirand, 1963). Infection of rabbits with YMTV resulted in mild disease but failed to produce transmissible virus as tested by inoculating rhesus monkeys (Niven et al., 1961). Attempts to infect mice have been unsuccessful (Bearcroft and Jamieson, 1958; Niven et al., 1961). Interestingly, YMTV also failed to replicate in a large number of primate and rodent cell lines (Grace and Mirand, 1963).

Outbreaks of TPV infections were first recognized in different locations close to the Tana River in Kenya, in 1957 and 1962. During this period, extensive flooding led to the isolation of humans along with their domestic animals as well as wild animals on islands within floodplains (Downie et al., 1971). Experimental inoculation of animals with TPV produced productive infection with the formation of papules in rhesus and vervet (*Chlorocebus pygerythrus*) monkeys, whereas no signs of infections were recognized in a calf, a goat, a lamb, two pigs, a guinea pig or in mice. Inoculation of a human volunteer resulted in productive infection with papule formation around the 7^{th} day post inoculation, and viral particles were successfully isolated from the lesions (Downie et al., 1971). From 1979-1983, 264 laboratory-confirmed cases of TPV were reported from the Democratic Republic of the Congo (DRC, previously known as Zaire). The majority of cases occurred during the months of November to March, with a peak of infections in the second half of 1980 (Jezek et al., 1985). It has been suggested that TPV was mechanically transmitted to humans by mosquitos after feeding on infected animals. Humans lived in close proximity to many animals at the time of infection and were frequently bitten by mosquitos (Downie et al., 1971). Such a mechanism of transmission would also explain the seasonal clustering of TPV cases in DRC, which coincides with increased mosquito activity (Jezek et al., 1985). During the TPV outbreaks in Kenya and DRC, there was no evidence for direct human-to-human transmission (Downie et al., 1971; Jezek et al., 1985). Infection with TPVs or closely related viruses were also described in travelers returning from Congo, Tanzania and Sierra Leone (Croitoru et al., 2002; Dhar et al., 2004; Stich et al., 2002). Although the natural host(s) of TPVs, are unknown, African primates might be the natural hosts.

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YLDV was discovered in 1965 and 1966, when it caused outbreaks in rhesus monkeys and Japanese macaques in three primate centers in the USA (Casey et al., 1967; Hall and McNulty, 1967). Infection was also detected in humans handling the monkeys (Crandell et al., 1969; McNulty et al., 1968). Experimental infection of rabbits resulted in productive infections in German checker rabbits but not in New Zealand white rabbits. Mice, hamsters, guinea pigs and chicken showed no detectable lesions or any other signs of infection (Crandell et al., 1969). The genome of the YLDV Davies strain has been completely sequenced (Lee et al., 2001).

YLDVs and TPVs are indistinguishable by examination of disease symptoms and serological tests but can be differentiated by restriction fragment length polymorphism (RFLP), which also showed that TPV isolates from Zaire were indistinguishable from those obtained near the Tana River (Knight et al., 1989). However, rigorous tests to distinguish YLDV and TPV on the molecular level have not been applied in all cases. In comparison to YLDV and TPV, YMTV has lost 13 ORFs (Brunetti et al., 2003). Gene losses include host range family genes serpin group 3 (149R) and MYXV 013L orthologs (Bratke et al., 2013; Brunetti et al., 2003). Interestingly, ORF 11L, which encodes ANK/F-box group 6 gene, is disrupted by a stop-codon in TPV-Ken by a C to A substitution at nucleotide position 706 but is intact in TPV-RoC, YLDV, YMT, DPV and orthopoxviruses.

Overall, the biology of yatapoxviruses is inadequately understood. Yatapoxviruses are able to infect many different primates, but experimental infection of non-primate species has been largely unsuccessful. Since yatapoxviruses can infect humans, they pose a health threat, especially if they would become more adapted to humans. A better understanding of their biology, including natural reservoirs, host range and the molecular basis for differences in disease progression in YMTV versus TPV/YLDV infection is needed.

Leporipoxviruses

Leporipoxviruses include the completely sequenced Myxoma virus (MXYV) and rabbit fibroma virus (RFV, also known as Shope fibroma virus) (Cameron et al., 1999; Willer et al., 1999). The natural hosts of MXYV and RFV are rabbits of the *Sylvilagus* species in America. MYXV infections appear to be limited to lagomorphs with two major groups currently recognized: Californian and South American strains. The natural host of Californian strains of MYXV is the brush rabbit (*S. bachmani*), which is endemic to the Pacific coastal regions of North America. The South American strains of MYXV naturally infect the Tapeti (forest rabbit, *S. brasiliensis*), which inhabits large parts of South and Central America. MYXV infections usually result in mild and self-limited diseases in their natural hosts causing cutaneous fibroma (reviewed in (Barrett and McFadden, 2007; Stanford et al., 2007)).

MYXV is highly pathogenic and causes myxomatosis in the European rabbit (*Oryctolagus cuniculus*) with lethality rates approaching 100% (reviewed in (Kerr, 2012)). *Sylvilagus* and *Oryctolagus* lineages diverged approximately 10 million years ago (mya) (Matthee et al., 2004). In *O. cuniculus*, California MYXV strains are generally more virulent, with most animals dying within the first week, usually without showing extensive clinical signs of the Brazilian form of myxomatosis. In contrast, South American strains induce classical myxomatosis in these rabbits, with numerous tumors developing all over the body and death usually occurring within two weeks. Reasons for the pronounced virulence of MYXV in *O. cuniculus* might be due to the efficient viral replication in migratory leukocytes and/or manipulation of the host immune response (reviewed in (Barrett and McFadden, 2007; Stanford et al., 2007)). The deliberate introduction of MYXV into Australia (SLS strain) and Europe (Lu strain) had devastating effects on *O. cuniculus* populations there and led to

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fascinating insights into the complex mechanisms of host-pathogen evolution. Initially, CFRs of the released virus were higher than 99%, as tested under laboratory conditions. However the MYXVs became attenuated over time, in terms of virulence against the extant feral strains of *O. cuniculus*. Concomitantly, these wild European rabbit populations became more resistant to MYXV infection, even to the original SLS strain of MYXV (reviewed in (Fenner, 2000; Kerr, 2012)). The complete genome of a naturally attenuated MYXV strain that was isolated in 1995, 43 years after the release of its parental Lausanne strain, was recently published. Overall, 73 differences in comparison with its parental strain were identified, of which most (n=67) represent single nucleotide substitutions (Morales et al., 2009). More recently, twenty-two MYXV isolates collected from the field in Australia and UK between the years 1950 to 1999 were fully sequenced and published in 2012, which greatly aided the understanding of the evolution of MYXV virulence after its introduction into the European rabbit populations. Compared to the parental SLS strain, samples collected at different time points revealed a mean evolutionary rate of 9.6×10^{-6} substitutions/site/year, rendering MYXV the fastest-evolving dsDNA virus ever recorded. In addition, mutations in the ITR, where most of the immune evasion and host-range genes are located, were more frequently found than that on the central part of the genome (Kerr et al., 2012). This high mutation rate may be driven by the active arms race between the virus and the immune responses of the host and might be influenced by the host switch from *Sylvilagus* to *Oryctolagus* genera.

RFV naturally infects Eastern cottontail rabbits (*S. floridanus*), which are found in large parts of the USA, Central America, and northern South America. RFV infection causes cutaneous fibroma that can persist for many months. It is interesting to note that RFV only poorly infects the natural hosts of MYXV *S. bachmani* and *S. brasiliensis*, and that *Oryctolagus* species can recover from RFV infection within a few weeks (reviewed in (Barrett and McFadden, 2007)).

Two additional leporipoxviruses have been described, but they are poorly characterized on the molecular level: hare fibroma virus (FIBV) which infects hares (*Lepus* species) (Barrett and McFadden, 2007) and squirrel fibroma virus (SQFV), which can infect squirrels (*Sciurus* species and *Tamiasciurus hudsonicus*) (as referenced in (Bangari et al., 2009).

Host range and immune evasion genes have been well characterized in MYXV. Six host range genes have been identified in MYXV (reviewed in (Werden et al., 2008)). A recent survey of poxvirus host range genes revealed several interesting aspects in leporipoxviruses: 1. The VACV E3L orthologs of both MYXV and RFV lack the N-terminal Z-DNA binding domain (Zα) found in E3L and only contain the C-terminal dsRNA-binding domain (dsRBD). As orthologs of all other clade II poxviruses contain a Zα domain, it is likely that it was lost in an ancestral leporipoxvirus and might contribute to host range restriction (as discussed below). 2. Whereas MYXV contains three serpin genes, RFV possesses only one (SERP-2). Because MYXV SERP1 orthologs are present in YLDV, TPV and DPV, it too was apparently lost during RFV evolution. 3. Leporipoxviruses possess three copies of M63R/C7L family genes, which are nested with one another in phylogenetic analyses, indicating two duplication events in leporipoxvirus evolution. All other clade II poxviruses contain only one copy. 4. MYXV and RFV also contain five and four ANK/F-box genes, respectively. According to our classification, MYXV contains two copies of group 14 ANK/F-box genes (T5 and 150R), whereas RFV as well as all other clade II poxviruses contain only one copy. Moreover, MYXV and RFV lack group 11 orthologs, which are found in all other clade II poxviruses (Bratke et al., 2013).

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Capripoxviruses

The genus *Capripoxvirus* contains three closely related members: Sheeppox virus (SPPV), Goatpox (GTPV) and Lumpy skin disease virus (LSDV), which naturally infect sheep, goats and cattle, respectively. The currently known capripoxviruses cannot be distinguished by serological tests. Survival of infection or vaccination with one capripoxvirus species usually confers resistance to the others. Species distinction is made according to the infected host species from which the virus was isolated and has been complemented by sequencing and phylogenetic analyses. In general, capripoxviruses show preference for their respective hosts, but some SPPV and GTPV isolates can infect both sheep and goats (reviewed in (Babiuk et al., 2008; Diallo and Viljoen, 2007). The genomes of SPPV (150 kb), GTPV (150 kb) and LSDV (151 kb) have been sequenced and display approximately 96 % nucleotide sequence identity with one another (Tulman et al., 2001; Tulman et al., 2002).

SPPV and GTPV are prevalent in north equatorial Africa and in large parts of Asia, whereas LSDV is restricted to Africa and the Middle East. However, capripoxviruses are sometimes exported into other countries through animal trade (reviewed in (Babiuk et al., 2008)). Capripoxvirus infections have led to serious economic losses. Mortality rates for SPPV and GTPV reach up to 100%. Mortality rates in LSDV infections are usually much lower (about 1 %) but can occasionally reach 75% (reviewed in (Babiuk et al., 2008)). SPPV and GTPV are primarily transmitted through aerosols and droplets, direct animal-animal contact and contaminated objects. Additionally, blood-feeding stable flies (*Stomoxys calcitrans)* have been implicated in infections via mechanical virus transmission. Mechanical transmission by insects is believed to be the general route for LSDV infection (reviewed in (Babiuk et al., 2008; Bhanuprakash et al., 2006).

Artificial inoculation of African buffalos, gnus, impalas and giraffes with LSDV led only to a detectable infection in the latter two species, which succumbed to a disease like that seen in LSDV infections (Young et al., 1970). In a survey of sera from 3445 individuals from 44 African wildlife species, neutralizing antibodies against LSDV were detected in six species at a low prevalence: the Greater Kudu (*Tragelaphus strepsiceros*; 1.1%), waterbucks (*Kobus ellipsiprymnus and K. defassa*; 16%), reedbuck (*Redunca arundinum*; 10%), impala (*Aepyceros melampus*; 4.5%), springbok (*Antidorcas marsupialis*; 1.8%) and giraffe (*Giraffa camelopardalis*; 2.1%), which all belong to the suborder *Ruminantia*. Many individuals from the remaining 38 species lacking detectable antibodies to capripoxviruses included both *Ruminantia* and *Nonruminantia* as well as some distantly related mammals (Hedger and Hamblin, 1983). These data indicate a low prevalence of capripoxviruses in African wildlife. Since the assay could not discriminate between antibodies to the different capripoxviruses, the species to which the present capripoxviruses belong is currently unclear. Prevalence of capripoxviruses in wildlife could potentially pose risks for successful vaccination campaigns of livestock against capripoxviruses, because unvaccinated animals could become infected after transmission from wildlife after initial successful eradication of capripoxviruses from livestock. In addition to cattle, LSDV has been also isolated from springboks in South Africa (Lamien et al., 2011). The current data indicate a relatively narrow host range for capripoxviruses. Infection of humans with capripoxviruses has not been reported.

SPPV, GTPV and LSDV all possess the same set of known host range family genes. Interestingly, capripoxviruses lack an ortholog of the MYXV host range gene 13L, which is found in all other clade II poxviruses except for YMTV (Bratke et al., 2013). Deletion of a 13L ortholog likely occurred in an ancestor of extant capripoxviruses and might contribute to their host range restriction. Remarkably, the SPPV vaccine strain NK contains gene-inactivating mutations of ANK/F-box encoding ORF138 (ANK/F-Box group 12) and

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ORF141 (group 11). Homologs of these genes have been shown to confer host range function, and might therefore contribute to viral attenuation (Tulman et al., 2002).

Deerpox virus

Deerpoxvirus (DPV) is the sole recognized member of the genus *Cervidpoxvirus*. Two strains have been completely sequenced W-848-83 (DPV-W83) and W-1170-84 (DPV-W83) and contain 166 and 171 kb, respectively. W83 and W84 strains show 95 % nucleotide identity (Afonso et al., 2005). They were isolated from free ranging mule deer *(Odocoileus hemionus)* fawns in Wyoming in 1983 and 1984, respectively. Both fawns were seriously ill (one died spontaneously and the other had to be euthanized) and displayed severe keratoconjunctivitis and crusty skin lesions (Williams et al., 1985). Four related viruses have recently been described that infected mule deer and black-tailed deer (*Odocoileus hemionus* and *O. h. columbianus*, a mule deer subspecies) in Oregon and California, and reindeer (*Rangifer tarandus tarandus*) in the Toronto Zoo (Ontario, Canada). Phylogenetic analyses using three predicted proteins showed a close relationship to W83 and W84 and indicated that they are cervidpoxviruses (Moerdyk-Schauwecker et al., 2009). The currently available data is insufficient to answer the question whether the new isolates represent new cervidpoxvirus species or should be considered as DPV strains. Current knowledge indicates a relatively narrow host range of cervidpoxviruses. In a recent serosurvey of five cervid species in Oregon, USA, a high prevalence of DPV antibodies was detected in three species from the genus *Odocoileus*,whereas none were detected in two elk species (*Cervus spp.*) (Jin et al., 2013). Only two genera were examined in this study, however, the absence of DPV antibodies in the elk species suggests this virus has a restricted host range. The high sero-prevalence in *Odocoileus* species also suggests this virus may be more widespread in deer populations of North America than previously thought and less virulent, only causing disease symptoms occasionally.

Swinepox virus

Swinepox virus (SWPV) is the only member of the genus *Suipoxvirus* and infection is entirely restricted to pigs (Datt, 1964; Delhon et al., 2007). Swinepox occurs worldwide and is a common skin disease in pigs. Piglets are usually more severely affected. SWPV is thought to be predominantly transmitted mechanically by the hog louse, *Haematopinus suis*, but it can also be transmitted through direct contact and congenitally (House and House, 1992; Thibault et al., 1998). The genome of SWPV comprises 146 kb (Afonso et al., 2002a).

Host range genes in SWPV largely overlap with those found in other clade II poxviruses, but have a unique profile of genes, with the notable absence of orthologs of T4, ANK/F-box group 6 and TNFR-2 related genes found in other clade II poxviruses (Bratke et al., 2013). In comparison with the related LSDV, 13 genes (including the T4 ortholog) are missing in SWPV (Afonso et al., 2002a). It is possible that the absence of those genes contribute to the narrow host range of SWPV. Four short 63-73 amino acids encoding ORFs (SPV 018, 019, 020 and 026) appear to be unique for SWPV and might therefore contribute to pathogenesis in pigs (Afonso et al., 2002a). Work on SWPV host range genes is very limited. SWPV C8L (ORF 010) shows 43% amino acid sequence identity to VACV K3L and was shown to inhibit human PKR in a yeast assay (Kawagishi-Kobayashi et al., 2000). A cell culture-based assay for the inhibition of PKR revealed that mouse and human PKRs were moderately sensitive to C8L inhibition. In the same assay, human PKR was moderately inhibited by VACV K3L, whereas mouse PKR was strongly inhibited. Interestingly, mutations in human and mouse PKR in the putative binding interface of PKR with K3L/C8L changed the sensitivity of both PKRs to K3L but not to C8L (Rothenburg et al., 2009). Those data indicate a specific interaction between PKR of a given host and its

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pseudosubstrate inhibitor. In another study, the function of SWPV 064, an ortholog of VACV C7L, was investigated. SWPV 064 was able to rescue the replication of K1L/C7L double deficient VACV in both HeLa and murine 3T3 and LA-4 cell lines. In addition, the successful replication of this SWPV 064 reconstituted VACV was independent of the treatment of IFN-β, which was also observed when VACV C7L was reinserted, indicating that SWPV 064 can substitute the host range function of C7L in the replication of VACV in human and mouse cell lines (Meng et al., 2012).

Cotia virus

Cotia virus (COTV) was isolated in 1961 during an arbovirus surveillance program at Cotia field station near São Paulo, Brazil, which used sentinel mice to detect novel viruses in the environment (Lopesode et al., 1965). Although there was initially some controversy over the classification of COTV, a comprehensive study of the fully sequenced genome including a phylogenetic analysis along with an examination of replication kinetics in different cell lines has indicated that COTV represents a distinct lineage with a well-supported separation from orthopoxviruses (Afonso et al., 2012). The COTV genome encodes all 90 core genes conserved across chordopoxviruses, which show the highest identity with clade II poxviruses, whereas some of its immunomodulatory genes share a greater identity with orthopoxvirus genes (Afonso et al., 2012), which is indicative of recombination events. A cell culture adapted COTV was able to infect cells from African green monkey (BSC-40, Vero), rat (C6), rabbit (RK-13), mouse (L929), human (Hep-2), and chicken (CEF) cells with the highest titers being produced in BSC-40 and C6 cells (Afonso et al., 2012). Interestingly, in this same study, COTV was unable to replicate in another rat cell line (Rat-2), which is normally permissive to VACV. The natural host for COTV is unknown and the original prototype strain, SPAn232 has never been re-isolated from the wild. It has been suggested that an asymptomatic host may be present in the wild, but there is no evidence that COTV is currently circulating throughout Brazil.

It is noteworthy that COTV is the only clade II poxvirus known to date that possesses a direct K1L ortholog. It is also interesting that two C7L homologs are found in COTV. In a phylogenetic analysis COTV232 was nested within C7L homologs of clade II poxviruses in phylogenetic analysis, whereas COTV024 was found in the orthopoxvirus C7L clade (Liu et al., 2012a). Presence of host range and virulence genes that are most closely related to those of orthopoxviruses in COTV probably resulted from recombination events between a COTV and orthopoxvirus ancestors and may contribute to the relatively wide range of cell lines that can be infected by COTV and may allow for an extended host range. Interestingly, COTV lacks a B5R-related gene that is predicted to contain a transmembrane domain. Transmembrane-containing B5R homologs are found in all other sequenced ortho- and clade II poxviruses. However, COTV encodes for a B5R-related gene that is most closely related to VCP/C3L of orthopoxviruses. The lack of transmembrane-containing B5R homolog may explain the small plaque size observed by COTV relative to VACV in the tested cell lines as B5R is important for cell-to-cell spread by interacting with actin tails previously shown to be important for viral dissemination. Three COTV genes show homology with TNFR-II homologs from other poxviruses: the COTV008/178 is predicted to encode for a TNF-binding domain most closely related to CrmE, whereas COTV 007/179 and COTV 168 lack predicted TNF-binding domains but are homologous to the C-terminal domains of CrmB and CrmD and may have other functions than cytokine binding.

Parapoxviruses

Parapoxviruses are widespread pathogens that can infect a variety of different mammals including humans. Prototypic members of this genus are Orf virus (ORFV), Bovine papular

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stomatitis virus (BPSV) and Pseudocowpoxvirus (PCPV), the genomes of which have been completely sequenced and contain 134 to 140 kb (four ORFV strains), 134 kb and 135-145 kb (PCPV), respectively (Delhon et al., 2004; Hautaniemi et al., 2010). In phylogenetic analyses parapoxviruses form a sister-clade to orthopoxviruses and clade II poxviruses (Bratke and McLysaght, 2008; Bratke et al., 2013). Parapoxviruses genomes are GC rich (> 60%), a feature shared with Molluscum contagiosum virus and Crocodilepox virus (Lefkowitz et al., 2006).

Parapoxviruses are thought to establish infection after entry through small skin lesions and infection is usually restricted to the epidermis and oral mucosa (reviewed in (Fleming and Mercer, 2007)). ORFV causes serious infections in sheep and goats worldwide and poses a significant economic burden. Other animals that can be infected include camels, musk ox, cats, reindeer and humans. Interestingly, ORFV can reinfect its host, although reinfection usually results in milder pathogenicity (reviewed in (Fleming and Mercer, 2007; Hosamani et al., 2009).

BPSV and PCPV cause skin diseases in cattle worldwide, but usually induce milder diseases than ORFV in sheep and goats. Transmission of BPSV and PCPV from cattle to humans can also occur (reviewed in (Fleming and Mercer, 2007; Hosamani et al., 2009). Phylogenetic analysis indicated that PCPV is more closely related to ORFV than to BPSV, which might explain why both of the former viruses can infect reindeer (Hautaniemi et al., 2010). ORFV, BPSV and PCPV cause indistinguishable skin infections, known as Milker's nodules, in humans that have had close contact with infected animals. Lesions are generally localized and resolve after 4-6 weeks. However, serious complications can occur, such as the development of large tumor-like lesions and erythema multiforme, especially in immunodeficient individuals. Remarkably, immunity against PPV infections in humans appears to be incomplete, as reinfections can occur (reviewed in (Fleming and Mercer, 2007)).

Infection with different parapoxviruses is also widespread in many different seal and sea lion species, which can be transmitted to humans from them (Nollens et al., 2006). Another parapoxvirus has been described in red deer (*Cervus elaphus*) in New Zealand (reviewed in (Fleming and Mercer, 2007)). Viruses, which are closely related to PCPV and are sometimes called Azudyk virus or Camel contagious ecthyma virus, have been isolated from camels and might represent a PCPV subclade that has been adapted to camels (Abubakr et al., 2007).

Studies on parapoxvirus host range genes are scarce. Completed sequences of parapoxviruses contain only two known host range gene members: orthologs of VACV E3L and ANK/F-box genes (Figure 1). Parapoxviruses and squirrelpoxvirus are the only non-orthopoxvirus/ non-clade II poxviruses that contain E3L orthologs, indicating that E3L evolved in a common ancestor (Bratke et al., 2013). A VACV strain, in which E3L was substituted with its ORFV ortholog 20L, which shows 31% amino acid sequence identity to E3L, replicated equally well as wild type VACV in human HeLa and rabbit RK13 cells. However, pathogenicity of the recombinant virus after intranasal and intracranial infection of C57BL/6 mice was vastly reduced. Experiments with chimeric viruses indicate that differences in the double-stranded RNA binding domain of E3L/20.L are responsible for the differences observed during infections in mice (Vijaysri et al., 2003). ORFV and BPSV contain five and seven ANK/F-box genes respectively, which likely arose through duplications of a single gene in a common ancestor of those viruses (Bratke et al., 2013; Hughes et al., 2010; Sonnberg et al., 2011).

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Squirrelpox virus

Squirrelpox virus (SQPV) was identified as the causative agent of a lethal disease of red squirrels (*Sciurus vulgaris*) in the United Kingdom. It was initially described as a parapoxvirus due to similar morphology observed by electron microscopy (Scott et al., 1981; Thomas et al., 2003). However, phylogenetic analyses with sequences from the partially sequenced SQPV genome showed that the SQPV branch was well separated from that formed by parapoxviruses. This finding together with relatively low sequence identity levels between SQPV and parapoxvirus proteins indicates that SQPV might belong to a new poxvirus genus (McInnes et al., 2006; Thomas et al., 2003). Squirrelpoxvirus causes erythematous exudative dermatitis on the skin around the eyes, nose, and lips of infected red squirrels that later spreads to the chest and feet. The origin of the virus is unknown, but it is likely that the natural host is the American grey squirrel (*Sciurus carolinensis*) as the disease was not seen in red squirrel populations until after the introduction of the American grey squirrel to these areas. The grey squirrels are almost entirely asymptomatic, however, many produce antibodies against SQPV indicating a previous exposure (Tompkins et al., 2002). In fact 60-70% of healthy grey squirrels were found to be seropositive for SQPV compared to only 2-3% of red squirrels tested, which were either dead or dying (Sainsbury et al., 2000). Populations of red squirrels that are in contact with seropositive grey squirrels have a greater disease incidence and show a dramatic decline in numbers. Whether SQPV is transmitted directly between the squirrel species or via a vector is currently unknown. The host range of SQPV appears to be restricted. Serum analysis of various rodents from the UK failed to find evidence of infection in wood mice or bank voles (McInnes et al., 2006), suggesting it is specific to squirrels with severe infection only observed in red squirrels. At present there are a handful of immunomodulatory genes within the SQPV genome that could potentially have a role in the restricted host range of the virus, but a more in-depth analysis awaits the publishing of the fully sequenced genome. Among the available putative host range SQPV genes is H1L, an ortholog of VACV E3L. The predicted gene product of H1L contains an unusually long linker region between the Z-DNA- and dsRNA-binding domains of about 230 highly acidic amino acids, instead of 30 amino acids linking both domain in other E3L orthologs.

Avipoxviruses

Avipoxviruses are common bird pathogens. Bolte et al. summarized avipoxvirus infections in 232 bird species, in which they can cause serious infections (Bolte et al., 1999). The genomes of two the two fowlpoxvirus (FWPV) strains Iowa (I) and HP-438/Munich (M) and one Canarypoxvirus (CNPV) have been completely sequenced and contain 289, 266 and 360 kb respectively (Afonso et al., 2000; Laidlaw and Skinner, 2004; Tulman et al., 2004). The large genome sizes of avipoxviruses can be attributed to multiple duplications of 3 gene families: the ANK family and N1R/p28 and B22R-related genes, which together comprise 36% of the CNPV genome (Tulman et al., 2004). In phylogenetic analyses, FWPV and CNPV form a monophyletic clade, which is well separated from other chordopoxviruses (Bratke and McLysaght, 2008; Bratke et al., 2013). High sequence divergence between avipoxviruses as well as high variability in the genomic organization between FWPV and CNPV led to the suggestion that avipoxviruses might form a subfamily of chordopoxviruses (Boyle, 2007; Jarmin et al., 2006; Tulman et al., 2004). Avipoxvirus species are distinguished by a combination of biological and genetic characteristics. Currently, 10 established APV species are recognized (Boyle, 2007). This list will likely be extended with the ongoing and improved characterization of avipoxviruses. PCR amplification of the conserved P4b gene has been used for diagnostic and classification purposes. Phylogenetic analyses based on those sequences indicate the existence of 3 major avipoxvirus clades: A, B and C, as well as several A and B subclades. Interestingly, some viruses that have been

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isolated from the same bird species were grouped into different clades, indicating that some birds can be infected by multiple avipoxvirus species (Jarmin et al., 2006; Manarolla et al., 2010). The host range of avipoxviruses appears to be broad, although detailed information is limited and complicated by the existence of many avipoxvirus species. CNPV preferentially infects passerine bird species and causes more serious, and often lethal infection in them. Experimental infection with the same CNPV strain, led to 100% lethality in crowned sparrows *(Zonotrichia sp.*), house sparrows *(Passer domesticus)* and canaries (*Fringillidae*), whereas only self-limited, localized reactions were observed in Leghorn chickens and turkeys (both *Galliormes*) and pigeons (*Columbiformes*) (Giddens et al., 1971). FWPV is thought to predominantly infect chickens and turkeys, in which it causes slow-spreading skin lesions and affects growth rates and egg production, however mortality rates are low for these infections (reviewed in (Boyle, 2007)). It is interesting to note that in phylogenetic analyses using P4b sequences, sequences obtained from an infected sparrow and canary were nested within the FWPV A1 clade (Jarmin et al., 2006; Manarolla et al., 2010). This might indicate an extended spectrum of FWPV-related viruses. Another more recent phylogenetic analysis using the DNA polymerase gene as well as the 4b core protein gene to look at a larger group of bird species from a wider range of geographic locations supported the clade classification set up by Jarmin et al., but also identified three novel subclades within clade A to include poxviruses of waterfowl, pigeons, and raptors. This study found that the viruses grouped mainly on the basis of the family and order of the host species they were isolated from, however, they also found some evidence for cross-species infections within predator-prey systems (Gyuranecz et al., 2013). This suggests that these viruses, while most likely being restricted to avian hosts, can infect a diverse range of bird species if they are within a close enough proximity to each other. More genomic and epidemiological data are needed to clarify this. The mode of avipoxvirus transmission is not completely understood, but mechanical transmission by mosquitos and other biting insects likely plays a prominent role (reviewed in (Boyle, 2007)).

An interesting observation is that most FWPV isolates contain sequences that are identical or nearly identical to reticuloendotheliosis virus (REV), an avian retrovirus (Hertig et al., 1997). There is considerable variation in the sequence length of the retroviral insertion, ranging from nearly complete to absent. In FWPV vaccine strains, only the long-terminal repeat regions are present (Davidson et al., 2008). Infection with a FWPV isolate, which contains the near-full-length provirus elicited a strong humoral immune response against REV and let to the detection of proviral REV DNA in blood cells of infected birds (Hauck et al., 2009). Infection with REV-containing FWPV likely increases its virulence (Singh et al., 2005).

Avipoxviruses cannot replicate successfully in non-avian species but can infect cells, initiate protein expression and induce an immune response in those species. This has been harnessed for the construction of live recombinant FWPV and CNPV vaccines, which express foreign antigens and which are being tested as vaccines against many infections including HIV and malaria (reviewed in (Boyle, 2007)).

FWPV and CNPV contain three of the identified host range gene families: ANK/F-Box, Serpin and p28/N1R family genes. ANK/F-Box genes constitute the largest gene family comprising 35, 20 and 19 intact genes in CNPV, FWPV-I and FWPV-M, respectively. Moreover, several copies are found in these genomes that contain ankyrin repeats without F-Boxes and in which the ORF is interrupted due to mutations. Phylogenetic analyses including all chordopoxvirus ANK/F-Box genes, all avipoxvirus genes formed a monophyletic clade that was well separated from those of other chordopoxviruses, indicating independent gene duplications in various lineages. Four serpin genes are found in CNPV and FWPV, which are nested with each other in phylogenetic analyses and thus

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likely originated from three independent duplications during avipoxvirus evolution (Bratke et al., 2013). Two closely related p28/N1R orthologs, combining both KilA-N and RING domains, are found in FWPV (150 and 157) and CNPV (197 and 205). These also likely arose through duplication in avipoxvirus evolution. Avipoxviruses lack many host range genes found in other chordopoxviruses, including orthologs of VACV E3L and K3L. In an attempt to boost protein expression in a CNPV-based HIV vaccine, the recombinant expression of both E3L and K3L resulted in the inhibition of PKR activation and the concomitant elevation of protein production of HIV antigens after infection of human cell lines (Fang et al., 2001).

Molluscum contagiosum virus

Molluscum contagiosum virus (MOCV) is the only identified member of the genus *Molluscipoxvirus* and displays the narrowest host range and tissue tropism of all known poxviruses. It is specific to humans and is only known to replicate in basal keratinocytes. MOCV causes molluscum contagiosum, which is a common skin disease characterized by wart-like skin lesions that affects mainly children and immunocompromised people (reviewed in (Bugert, 2007)). The genome of MOCV comprises 190 kb, codes for an estimated 182 proteins, and lacks many genes commonly found in other poxviruses (Senkevich et al., 1996; Senkevich et al., 1997). Indeed, none of the previously characterized host range family genes is present in MOCV (Bratke et al., 2013). On the other hand, MOCV possesses many genes that are absent in most other poxviruses, some of which are thought to be important for subverting host defenses and are homologous to cellular genes (Moss et al., 2000; Senkevich et al., 1997). Because MOCV cannot be readily grown in cell culture or animals, elucidation of the biological functions of its genes is complicated (Moss et al., 2000). However, it can be assumed that many of the unique genes of MOCV are important for antagonizing the host immune defense and that genes that were superfluous for MOCV infection of basal keratinocytes were lost during evolution, thus contributing to the restricted host range and tissue tropism (Senkevich et al., 1997).

Crocodilepox virus

Poxvirus infections have also been reported from reptiles, including many crocodilian species. Poxviruses can cause serious infections in young farmed crocodiles and caimans, whereas adults remain largely asymptomatic (as referenced in (Afonso et al., 2006; Huchzermeyer, 2002)). The genome of a crocodilepox virus (CRV) isolated from a Nile crocodile (*Crocodylus niloticus*) was completely sequenced. The 190 kb genome encodes for 173 predicted proteins, of which 62 do not contain recognizable homologues in other poxviruses (Afonso et al., 2006). Interestingly, CRV lacks homologues of most virulence genes found in other poxviruses, as well as all characterized poxviruses host range genes (Afonso et al., 2006; Bratke et al., 2013). This indicates that CRV evolved unique strategies to counteract the host response and might be influenced by differences in the immune system of crocodilians and other poxvirus hosts. The findings of high sequence divergence between CRV and other chordopoxviruses, as well as a well-supported separation of the CRV clade from other poxvirus clades in phylogenetic analyses indicate that CRV is a member of a novel poxvirus genus, which is now called crocodylipoxvirus (Afonso et al., 2006). Since epidemiological studies of crocodilian poxvirus infections that utilize genetic information are lacking, it is currently unknown if they are caused by CRV or other poxvirus species. This lack of knowledge, as well as lack of systematic infection studies using CRV, impedes the exact assessment of CRV host range.

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Entomopoxviruses

Entomopoxvirinae constitute a subfamily of Poxviridae, which specifically infect insects. Based on the host species, morphology and genomic characteristics, three entomopoxvirus genera are recognized: alphaentomopoxviruses, which infect beetles, betaentomopoxviruses, which infect butterflies and moths, and gammaentomopoxviruses, which infect flies and mosquitos. Additionally, some entomopoxviruses have not yet been classified (reviewed in (Becker and Moyer, 2007)). The genomes of six entomopoxviruses have been completely sequenced: Amsacta moorei entomopoxvirus (AMEV) (Bawden et al., 2000), as well as Adoxophyes honmai entomopoxvirus, Choristoneura biennis entomopoxvirus, Choristoneura rosaceana entomopoxvirus and Mythimna separata entomopoxvirus (Theze et al., 2013), which all belong to Betaentomopoxviruses and Melanoplus sanguinipes entomopoxvirus (MSEV), which is currently not assigned to a genus (Afonso et al., 1999). AMEV naturally infects the red hairy caterpillar (*Amsacta moorei*) and can also infect the salt marsh moth (*Estigmene acrea*) and the gypsy moth (*Lymantria dispar*) (as referenced in (Bawden et al., 2000)). MSEV was isolated from a lesser migratory grasshopper (*Melanoplus sanguinipes*) and can also infect other grasshopper and locust species (M. differentialis, M. packardii, Schistocerca gregaria and *Locusta migratoria*) (as referenced in (Afonso et al., 1999)). The genomes of AMEV and MSEV contain 232 and 236 kb, respectively (Afonso et al., 1999; Bawden et al., 2000). Forty-nine genes are shared between entomopoxviruses and chordopoxviruses and thus define components of a minimal poxvirus genome (Gubser et al., 2004; Upton et al., 2003). It is noteworthy that the gene order of those genes, which is generally conserved among chordopoxviruses, is not conserved between entomopoxviruses and chordopoxviruses and even divergent between AMEV and MSEV. This non-conserved gene order together with relatively low overall sequence identities between AMEV and MSEV led to the removal of MSEV from the betaentomopoxviruses and indicate that entomopoxviruses and chordopoxviruses diverged a long time ago (Becker and Moyer, 2007). In phylogenetic analyses using concatenated multiple sequence alignments of conserved poxvirus proteins, AMEV and MSEV form monophyletic clades that are well-separated from the chordopoxviruses (Bratke and McLysaght, 2008; Bratke et al., 2013; Hughes et al., 2010). Considering the large biological differences between entomopoxviruses and chordopoxviruses hosts and the consequential difference in requirements for the successful manipulation of the host response, it is not surprising that neither chordopoxvirus host range genes nor other chordopoxvirus immuomodulators are found in entomopoxviruses (Afonso et al., 1999; Bawden et al., 2000; Bratke et al., 2013). It can be expected that entomopoxviruses possess unique host range genes and immuomodulators that are tailored to inhibit the host response of a given host, which may differ considerably between different entomopoxviruses.

Poxvirus host range gene families

PKR inhibitors K3L and E3L

The host double-stranded (ds) RNA-activated protein kinase PKR is activated upon infection by of many viruses and initiates an antiviral response by phosphorylating the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α). Many viruses in turn have evolved mechanisms that subvert this antiviral response (reviewed in (Langland et al., 2006)). As a consequence, PKR has evolved rapidly throughout the vertebrate lineage due to positive selection (Elde et al., 2009; Rothenburg et al., 2009). VACV possesses two genes encoding PKR inhibitors: K3L and E3L, which have orthologs in many poxviruses. K3 is a pseudosubstrate inhibitor of PKR. It is homologous to the N-terminal S1 domain of eIF2α and binds to the catalytic domain of activated PKR, which is phosphorylated on serine 446 (human PKR) and thereby inhibits access of PKR to its target and also prevents secondary PKR authophosphorylation events (Figure 3) (Dar and Sicheri, 2002; Rothenburg et al.,

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2011). Replication of K3L-deleted VACV strains was not impaired in human HeLa cells, but was impaired in Syrian hamster BHK cells and in murine L929 cells (Beattie et al., 1991; Langland and Jacobs, 2002). A likely explanation for these observed differences in host range is that human PKR was only weakly inhibited by K3 in a cell culture-based assay, whereas mouse PKR was highly sensitive to inhibition (Rothenburg et al., 2009). Similarly, it was shown that PKRs from hominidae primates were resistant to inhibition by K3 in a yeast-based assay, whereas PKRs from other primates were sensitive (Elde et al., 2009). Differential sensitivity of PKRs to K3 inhibition could be mapped to the helix-αG region of PKR. Swapping of positively selected residues in helix-αG between PKRs resulted in altered sensitivity to K3 inhibition (Elde et al., 2009; Rothenburg et al., 2009). Intact K3L orthologs are found in most orthopoxviruses and all clade II poxviruses, which are remarkably divergent. The SWPV K3L ortholog has also been shown to weakly inhibit human and mouse PKR at levels comparable to VACV K3L inhibition of human PKR (Rothenburg et al., 2009). K3L orthologs might have evolved to specifically inhibit PKR of their respective hosts, but this has yet to be determined experimentally. Interestingly, K3L orthologs are found in the genomes of MPXV and ECTV, which are inactivated by stop-codons and short deletions (Bratke et al., 2013).

VACV E3L contains an N-terminal Z-DNA binding domain (Zα) and a C-terminal dsRBD. E3L is important for VACV replication in a wide variety of cells, e.g. it is required in human HeLa cells, but it is dispensable for replication in BHK cells (Langland and Jacobs, 2002). Interestingly, an E3L deleted virus replicated almost as well as wild type VACV in HeLa cells in which PKR was stably knocked-down, indicating that PKR is the major target of E3 in those cells (Figure 3)(Zhang et al., 2008). The molecular basis for the host range function of E3L is unknown, although different amounts of viral dsRNA and PKR in different cells has been suggested as an explanation (Langland and Jacobs, 2002). In addition to inhibition of PKR activation, E3 was also shown to inhibit other dsRNA-binding proteins and cytolpasmatic DNA sensors including RIG-I, 2'-5' OAS/RNase L and ZBP1/DLM-1 (Figure 3) (reviewed in (Perdiguero and Esteban, 2009))

E3L orthologs are found in orthopoxviruses, clade II poxviruses and parapoxviruses. The ORFV E3L ortholog was able to rescue VACV replication when it replaced E3L in cell culture, but showed greatly impaired virulence in a mouse model (Vijaysri et al., 2003). In MPXV, the first start-codon of the E3L ortholog is mutated, which likely leads to translation initiation at the second start-codon within the Zα domain and probably results in a loss of function of this domain. In leporipoxviruses, the part encoding these dsRBD is present, whereas the Zα domain is completely lost from the E3L orthologs (Bratke et al., 2013). Whereas, the Zα domain was dispensable for VACV replication in cells in culture cells, it was important for pathogenesis in mice. For this reason it can thus be considered a virulence factor (Brandt and Jacobs, 2001). By analogy, the mutations in MPXV and leporipoxviruses might also reduce the virulence of these viruses within their hosts. M029, the MYXV ortholog of E3L, was recently shown to be essential for MYXV replication in a wide variety of mammalian cell lines, and was only able to replicate in the presence of ectopically expressed VACV PKR inhibitors. Moreover, M029 deficient MYXV was severely attenuated in European rabbits. The M029 protein binds and inhibits PKR via dsRNA bridging but directly binds and activates the host cell DHX9 RNA helicase to mediate its cell tropism properties (Rahman et al., 2013).

Serine protease inhibitors

Serpins (serine protease inhibitors) belong to a multi-gene family, which play important regulatory roles in many physiological processes, including blood coagulation, angiogenesis, inflammation and apoptosis and often display cell-specific expression. Serpins that counteract antiviral proteases are also found in poxviruses (reviewed in (Silverman et al.,

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2001; Turner and Moyer, 2002; van Gent et al., 2003)). Serpins are found in orthopoxviruses, clade II poxviruses and avipoxviruses. Three serpin genes are present in all orthopoxviruses: SPI-1, SPI-2 (also called CrmA) and SPI-3. Phylogenetic analyses of poxvirus serpin genes revealed that SPI-1 and SPI-2 form a monophyletic clade and likely arose via a duplication event in orthopoxvirus evolution. They form a sister-clade to serpins of clade II poxviruses, whose prototypic member is MYXV SERP-2 (151R), whereas orthopoxvirus SPI-3 genes form a monophyletic clade with the orthologs of MYXV SERP-1, YLDV, TPV and DPV. Well-separated from orthopoxviruses and clade II poxviruses serpins were four serpins of avipoxviruses, which likely resulted from independent duplication events within this lineage (Bratke et al., 2013).

Host range function has been described for RPXV and VACV SPI-1 (reviewed in (Werden et al., 2008)). Targeted deletion of RPXV SPI-1 resulted in replication defects in PK-15 and A549 cells but not in RK13, QT-6, CEF, CV-1, or RAT-2 cells. In contrast, a SPI-2-deleted virus showed no replication defects in those same cell lines. Interestingly, no replication defects in those cells were observed in CPXV-BR when SPI-1, which is 95% identical to RPXV SPI-1, was deleted compared to the wild type virus. The role of CPXV-BR SPI-1 for infection of PK-15 cells could not be determined because neither the wild type nor the mutant virus replicated in those cells (Ali et al., 1994). These findings indicate that either differences in SPI-1 between RPXV and CPXV-BR contribute to a distinct host range function in A549 cells or that CPXV-BR contains other genes that make SPI-1 dispensable for replication in those cells. Deletion of SPI-1 in VACV led to deficient replication in primary human keratinocytes and A549 cells but not in BS-C-1 cells (Shisler et al., 1999). The molecular mechanisms of serpin host range function are not fully understood and are complicated by the fact that that they can inhibit a range of proteases. However, a screen for the identification of suppressors of a host range mutation in RPXV SPI-1 link host range function to viral replication (Luttge and Moyer, 2005). Host range function for other serpins has not been demonstrated, but it seems possible given the high sequence diversity among serpins and the selected presence or absence of some serpins in certain lineages.

Ankyrin repeat domain-containing host range genes CP77, MT5 and K1L

The ankyrin repeat (ANK) domain is commonly found in proteins and is involved in protein-protein interactions (Mosavi et al., 2004). Proteins containing ANK domains fulfill a diverse set of biological functions within the cell, often acting as adaptor proteins within larger complexes. ANK-containing proteins form the largest family of poxvirus proteins, with the vast majority of poxviruses encoding a large repertoire of them. However, they are absent from the genomes of entomopoxviruses, MOCV and CRV (Bratke et al., 2013). The largest number of ANK-containing proteins have been identified in avipoxviruses, in which multiple gene duplications have created a large expansion of this gene family within their genomes (Afonso et al., 2000; Bratke et al., 2013; Tulman et al., 2004). 51 copies of ANK-containing proteins are encoded by CNPV and represent nearly 21% of its genome (Tulman et al., 2004). Most of these virally encoded ANK-containing proteins also have a C-terminal F-box domain, also called a PRANC domain (Mercer et al., 2005; Sonnberg et al., 2008). The ANK/F-box poxvirus proteins have been shown to interact with Skp1 or Cullin-1, members of the SCF ubiquitin ligase complex (Johnston et al., 2005; Sonnberg et al., 2008; van Buuren et al., 2008).

Two ANK/F-box proteins have been identified as host range factors: CP77 (CPXV-BR025/CHOhr) in CPXV-BR (Spehner et al., 1988) and M-T5 in MYXV (Mossman et al., 1996). Another ANK-containing protein with host range function is encoded by VACV K1L, which lacks an F-Box.

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CP77 was discovered as a host range protein because it enabled VACV replication in CHO cells, in which VACV usually causes an abortive infection (Spehner et al., 1988). Similarly, insertion of CP77/CPXV025 into ECTV allowed for efficient virus replication in hamster and rabbit cell lines that are normally only weakly permissive to the virus (CHO, CCL 14, CCL 16, CCL 39, SIRC, and RK13) (Chen et al., 1992). On the molecular level, it was shown that CP77 suppressed TNF-α mediated NF-κB activation, bound the p65 NF-κB subunit by its N-terminal ANK domains, and interacted with components of the SCF ubiquitin ligase complex via its F-box (Figure 3). Surprisingly, the F-box domain was not required for NF-κB inhibition and suppression of NF-κB was independent of the host range function of CP77, indicating that CP77 performs a yet unidentified function to allow viral replication in specific hosts (Chang et al., 2009).

M-T5 (encoded by 005L/005R) is found in the ITR region of the MYXV genome and is essential for the virus to cause myxomatosis in infected rabbits. Its deletion resulted in deficient MYXV replication in RL5 cells and primary rabbit peripheral mononuclear cells, which underwent apoptotic death, whereas replication in RK13 cells was not impaired (Mossman et al., 1996). M-T5-deficient MYXV also showed defective replication in a subset of human tumor cells. Interestingly, the permissibility of those cell lines to MYXV was correlated with the activation state of the protein kinase Akt, which was shown to interact with M-T5 in MYXV infected cells and prevent apoptosis (Figure 3) (Wang et al., 2006). Moreover, pharmacological manipulation of the Akt pathway altered the permissibility of cancer cells for MYXV (Werden and McFadden, 2010).

Phylogenetic analyses of poxvirus ANK/F-box genes indicate that distinct lineages of these genes arose by independent gene duplications in different clades of poxviruses and suggest that only a few ANK/F-box genes were present in the common ancestors of extant poxviruses (Bratke et al., 2013; Sonnberg et al., 2011). In phylogenetic analyses using avipoxvirus ANK/F-box genes as outgroup, parapoxvirus and orthopoxvirus/clade II poxvirus genes formed different clades. Within the orthopoxvirus/clade II poxvirus clade, 14 subclades were formed, which were arbitrarily classified into groups 1-14 (Bratke et al., 2013). Up to 10 ANK/F-box genes can be found in orthopoxviruses, with only CPXVs containing all 10 intact genes (groups 1 -10). Group 6 genes are unique because they represent the only ANK/F-box genes that are intact in all orthopoxviruses and because members of this group are also found in clade II poxviruses (yatapoxviruses and DPV). The other clade II poxvirus ANK/F-box genes belong to groups 11-14. The ANK/F-box genes with identified host range gene function belong to group 7 (CP77/CPXV025) and group 14 (M-T5). In most orthopoxviruses, some ANK/F-box genes have been either deleted or inactivated by short indels and incorporation of premature stop-codons (Bratke et al., 2013). The presence of several ANK/F-box genes in most poxviruses indicates that they perform distinct functions and may be important for the infection of different hosts. For this reason, they are good candidates for host range genes, in addition to CP77 and M-T5.

VACV K1L was identified as a host range gene because its insertion into a VACV mutant, which was replication deficient in human cells but replication competent in other cells and that contained 18 kb comprising the K1L locus, was able to rescue VACV replication in human HepII cells (Drillien et al., 1981; Gillard et al., 1986). Deletion of K1L from the VACV Copenhagen strain caused a severe replication defect in RK13 cells, but had only minor effects on replication in MRC-5, Vero, or LLC-PK1 cells (Perkus et al., 1990). In agreement with that finding, K1L insertion into the MVA genome, which contains a deletion comprising most of the K1L locus, rescued viral replication in RK13 cells and equine E-derm cells, but had little or no effect in other cell lines tested (Meyer et al., 1991). Moreover, the stable expression of K1L in RK13 cells enabled both MVA and a VACV K1L deletion mutant to replicate (Sutter et al., 1994). The viral replication defect observed

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in RK13 cells has been associated with the rapid shutdown of viral and host protein synthesis (Ramsey-Ewing and Moss, 1996). K1L has been shown to inhibit NF-κB activation, probably by inhibiting IκBα degradation (Shisler and Jin, 2004), and it has also been shown to inhibit the accumulation of dsRNA levels at early and intermediate stages of VACV infection (Figure 3). This might directly inhibit the activation of PKR and prevent its downstream effects (Willis et al., 2011).

The K1L gene of VACV and its orthologs in other orthopoxviruses are the only genes encoding ankyrin repeat domains within this family that do not also encode an F-box domain. Intact K1L orthologs that are not disrupted by inactivating mutations are found in YKV, COTV and most orthopoxviruses. In VARV, CMLV, and TATV, however, the ORF of each K1L ortholog is disrupted by a unique premature stop-codon or frameshift-causing indels, which indicates the independent inactivation of the K1L orthologs in these closely related viruses. The retention of inactivated K1L orthologs in these viruses further indicates their inactivation occurred relatively recently. Interestingly, in all available VARV genomes, the putative promoter element for K1L is deleted, whereas it is present in CMLV and TATV (Bratke et al., 2013). In COTV, the K1L ortholog clusters with other orthopoxvirus genes, e.g. orthologs of VACV host range genes K3L, C3L and C7L, while COTV is overall more closely related to other clade II poxviruses (Afonso et al., 2012). This might be the result of a recombination event between the genomes of COTV and an OPV ancestor. Apparent K1L orthologs are missing in all other poxviruses (Bratke et al., 2013). It is possible that some of the ankyrin repeat proteins in other poxvirus families that either lack or contain the F-box domain perform similar functions. This notion is supported by the observation that the function of K1L can be partially complemented by CP77/CPXV025 (Ramsey-Ewing and Moss, 1996).

M-T4-like apoptosis inhibitors

MYXV T4 is an endoplasmic reticulum-localized protein, which shows no apparent sequence homology with non-poxvirus proteins. A T4-deficient MYXV was replication competent in RK13 cells, but it showed replication defects in rabbit RL5 and peripheral blood lymphocytes due to the induction of apoptosis and was severely attenuated in a rabbit infection model (Figure 3) (Barry et al., 1997). The localization of M-T4 to the ER was independent of its C-terminal RDEL motif. Infection of RL5 cells with a MYXV missing that motif in T4 showed a rate of replication intermediate to that of wild type and T4-deficient viruses and showed an exacerbated inflammatory response at secondary sites of infections, which indicated a role for the RDEL motif in infection (Hnatiuk et al., 1999). Additionally, the T4 ortholog from CPXV-BR (ORF203) was found to be disrupted in an attenuated CPXV due to a recombination event (Pickup et al., 1984). CPXV-BR203 was shown to block antigen presentation by binding to MHC I proteins and down-regulating their expression (Byun et al., 2007). CPXV-BR203 was recently shown to adopt a β-sandwich topology, which is shared with poxvirus chemokine-binding proteins, and to bind both classical and non-classical MHC I proteins (McCoy et al., 2012).

MT4 orthologs are present in the ITRs of RFV, DPV, capripoxviruses and YKV but are missing in yatapoxviruses, COTV and SWPV (Figure 1). In orthopoxviruses, intact T4 orthologs are found in CPXVs, CMLV, TATV, and clade I MPXV. A striking observation is that these orthologs form two distinct groups: group I orthologs from CMLV, TATV, CPXV-BR, CPXV-GER1990, CPXV- FRA2001, CPXV-GER91, CPXV-UK2000, CPXV-GER1998, CPXV-GER2002 and CPXV-GER1980 and group II orthologs from MPXV, CPXV-GRI, CPXV-AUS1999 and CPXV-FIN2000. T4 orthologs within each group share about 98-95% amino acid sequence identity with other group members; however, sequence identities between members of the two groups is only about 65%. Truncated T4 orthologs

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are found in MPXV clade II strains, VACV, HSPV and RPXV, whereas no traces could be found in VARV (Bratke et al., 2013).

Bcl-2-like apoptosis inhibitors M11L and F1L

MYXV M11L and VACV F1L encoded proteins are structurally related to the antiapoptotic Bcl-2 protein family, despite displaying little if any sequence homology (Douglas et al., 2007; Kvansakul et al., 2007; Kvansakul et al., 2008). M11 and F1 can both block apoptosis by binding to Bax and Bak (Figure 3) (Douglas et al., 2007; Everett et al., 2000; Postigo et al., 2006; Su et al., 2006; Wang et al., 2004). Deletion of MYXV 11L resulted in drastically reduced virulence in rabbits and a replication defect in primary mixed rabbit spleen cells, whereas replication in RK13 was not impaired (Opgenorth et al., 1992).

M11L and F1L are probably true orthologs, despite having low amino acid sequence identities of around 14% because they can be connected by PSI-BLAST searches and because they map to syntenic regions in orthopoxvirus and clade II poxviruses (Bratke et al., 2013). M11L/F1L orthologs are found in all orthopoxvirus and clade II poxviruses, YKV and SQPV, but are absent in other poxviruses. Among these orthologs DPV022 and SPPV14 have been shown to interact with Bax and Bak and to complement F1L in VACV infection of human Jurkat cells (Banadyga et al., 2011; Okamoto et al., 2012). Interestingly, SPPV14 and M11 showed similar affinity to some BH3 ligands, whereas their binding affinity to Hrk and Puma was markedly different, indicating species-specific differences (Okamoto et al., 2012). Many poxviruses, including VACV, contain additional Bcl-2-related proteins that adopt a Bcl-2 fold, which include VACV N1, A52 and B14. However, they don't have Bax binding activities and likely possess other functions (Aoyagi et al., 2007; Graham et al., 2008; Postigo and Way, 2012). However, some Bcl-2-like proteins that are not M11L/F1L orthologs like FWPV039 can interact with Bak and Bax and complement F1L in VACV infection (Banadyga et al., 2007; Banadyga et al., 2009).

Short complement-like repeats containing proteins B5 and VCP

VACV contains the two short complement-like repeats (SCRs) encoding genes B5R and C3L that encode for B5 and VCP proteins, respectively. SCRs are also found in protein of the host complement system (Engelstad et al., 1992; Takahashi-Nishimaki et al., 1991). In the attenuated VACV LC16m8 strain, the B5R encoded protein contains a premature stop codon at amino acid position 93, which is caused by a single nucleotide deletion and a subsequent shift of the reading frame. VACV LC16m8 is replication deficient in Vero cells but replication competent in rabbit RK13 cells. Complementation of the defective B5R in VACV LC16m8 resulted in host range and plaque size comparable to that of the parental LC16m0 strain (Takahashi-Nishimaki et al., 1991). A B5R deficient RPXV strain showed reduced plaque sizes in RK13, CV1 and Rat2 cells, whereas no plaques were observed in pig PK15, CEFs and quail QT6 cells. Interestingly, B5R deficient RPXV was still able to replicate in Vero cells, despite its inability to form plaques, whereas replication in CEFs was severely impaired (Martinez-Pomares et al., 1993). B5R deficient VACV and RPXV strains are severely attenuated in mouse and rabbit infection models (Engelstad and Smith, 1993; Stern et al., 1997; Wolffe et al., 1993). VACV B5 contains four SCRs and a transmembrane domain. It is a component of the extracellular enveloped virus (EEV) and is required for EEV and intracellular actin tail formation (Engelstad et al., 1992; Engelstad and Smith, 1993; Herrera et al., 1998; Isaacs et al., 1992b; Mathew et al., 1998; Wolffe et al., 1993). B5 is involved in activating the cellular Src kinase, which phosphorylates another VACV protein, A36, resulting in actin tail formation (Figure 3) (Newsome et al., 2004). VCP, the second SCRs-containing protein in VACV, also contains four SCRs but lacks a transmembrane domain. It is a secreted protein that binds to complement factors (Isaacs et al., 1992a; Kotwal et al., 1990). In addition, VCP was also found to be membrane-associated

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where it is bound to VACV A56 via intermolecular disulfide bridging (Figure 3) (DeHaven et al., 2010; Girgis et al., 2008). VCP-deficient VACV caused smaller lesions in rabbits, guinea pigs and mice, demonstrating that it can act as a virulence factor (Girgis et al., 2011; Isaacs et al., 1992a). All orthopoxviruses and clade II poxviruses contain one SCRs encoding gene with a predicted transmembrane domain. However, the number of SCRs varies in clade II poxviruses and differs from the four found in orthopoxviruses: three predicted SCRs are found in yatapoxviruses, leporipoxviruses and DPV, whereas only two are present in capripoxviruses and in SWPV (Bratke et al., 2013). Most orthopoxviruses, with the exception of MPXV and TATV contain genes that encode for proteins containing all four SCRs and lacking a transmembrane region, which are direct orthologs of VACV C3L/VCP. In MPXV clade II strains, the C3L ortholog D4L contains a premature stop codon in the beginning of SCR4, whereas the gene is absent from MPXV clade I strains (Likos et al., 2005). In TATV, the predicted ORF is terminated within SCR3 and a hexanucleotide expansion in SCR1 make it unlikely that this repeat is functional (Bratke et al., 2013). Phylogenetic analysis indicates that a SCRs- and transmembrane-containing protein was present in an orthopoxvirus/clade II poxvirus ancestor and that a gene duplication during orthopoxvirus evolution led to the emergence of B5R-like and C3L-like genes (Bratke et al., 2013). Sequence identity among VCP orthologs in orthopoxvirus proteins ranges between 97 and 87%. Interestingly, different VCP orthologs were shown to inhibit complement in a virus and host-species specific fashion. In a comparison between VACV VCP and its VARV ortholog, SPICE/D15L, VCP inhibited guinea bovine, porcine cat and dog complement more efficiently than SPICE, whereas the latter was a better inhibitor of human and baboon complement. Similar species specificity was also shown for the inhibitory potential of VCP and SPICE on bovine and human C3b (Rosengard et al., 2002; Yadav et al., 2012). The MPXV ortholog D4L/MOPICE showed binding to human C3b that was intermediate to that of SPICE and VCP, but in contrast to SPICE and VCP, showed no decay-accelerating activity for C3 and C5 convertases (Liszewski et al., 2006). These results highlight the potential for SCR-containing poxvirus proteins to act in a species-specific manner.

p28/N1R-related KilA-N and RING domain containing proteins

ECTV p28 (012) contains an N-terminal KilA-N and a C-terminal RING domain, a combination that is unique to poxviruses (Iyer et al., 2002; Senkevich et al., 1994). A p28-deficient ECTV showed a reduced virulence in mice and a replication defect in mouse peritoneal macrophages, but replicated as efficiently as the wild type ECTV in BS-C-1 cells and RAW 264.7 cells as well as in primary mouse fibroblasts and ovary cells (Senkevich et al., 1994; Senkevich et al., 1995). ECTV p28 and its orthologs from VARV, VACV-IHD and MYXV display E3 ubiquitin ligase activity, which is dependent on the RING domain (Huang et al., 2004; Nerenberg et al., 2005). ECTV p28 and its RFV ortholog, N1R, have also been also implicated to be inhibitors of apoptosis (Figure 3) (Brick et al., 2000; Brick et al., 1998).

Intact ECTV p28 orthologs are found in all clade II poxviruses, YKV and most orthopoxviruses with the exception of HSPV, most VACV strains (only IHD-W, LC16m8 and LC16mO contain intact genes) and four VARV isolates. In HSPV and most VACV strains, independent deletions lead to the incorporation of premature stop-codons and thus predicted C-terminally truncated proteins. In VACV-Cop and MVA strains, the p28 genes are either completely or largely deleted from the genomes, respectively (Bratke et al., 2013). Two p28-related genes that contain KilA-N and RING domains are foun in both FWPV and CNPV. Additionally, avipoxviruses contain many proteins that contain only the KilA-N domain, which are also classified as p28-like or p28-family proteins (Afonso et al., 2000; Tulman et al., 2004).

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C7L family

VACV C7L encodes an 18kDa protein that shows no apparent sequence homology with non-poxviral proteins. C7L is the prototypic member of the C7L-related poxvirus host range gene family (reviewed in (Liu et al., 2012a)). Host range function for C7L was identified because its insertion into a VACV-WR strain, which contains a 21 kb deletion comprising the C7L and K1L locus and shows a host range defect, rescued VACV replication in MRC-5 and LCC-PK1 cells but not in RK13 cells. C7L has been shown to be partially functionally redundant with K1L because targeted deletions in both C7L and K1L were necessary to abort VACV-WR replication in MRC-5 and LCC-PK1 cells, whereas K1L deletion on its own abolished replication in RK13 cells (Perkus et al., 1990). Insertion of C7L into a K1L/C7L-deleted VACV-WR strain also allowed replication in human A431and HeLa cells and in murine 3T3 and LA-4 cells (Meng et al., 2008; Meng et al., 2012).

Orthopoxviruses, YKV and clade II poxviruses contain one to three copies of C7L homologs, whereas they are absent from other poxviruses. Some C7L homologs show less then 20% sequence identity with one another. In addition to direct C7L orthologs, which are found in all orthopoxviruses, CPXVs and CMLV possess a second C7L homolog, which is called C4L/020 in CPXV. C4L orthologs that are predicted to be inactivated or truncated C4L orthologs are present in VARV, TATP, MPXV and HSPV, whereas they are missing in all VACV strains, RPXV and ECTV. Whereas most clade II poxviruses contain one C7L homolog, MYXV and RFV virus contain three tandemly arranged copies (062R, 063R and 064R). Two C7L homologs are also found in COTV. Phylogenetically, orthopoxvirus C7L and C4L orthologs are more closely related to one another than to the C7L homologs from clade II poxviruses (Bratke et al., 2013; Liu et al., 2012a). Several other C7L homologs were also shown to possess host range function. A MYXV 063R-deficient virus was severely attenuated in rabbits and displayed a severe replication defect in different rabbit cells lines, but only a mild (HOS and 3T3 cells) or no (BS-C-40 and BGMK cells) replication defect in other cell lines (Barrett et al., 2007). Deletion of 062R from MYXV led to replication defects in BGMK, RK13, RL-5 and in 18 of 21 tested human cell lines, whereas it replicated well in BS-C-40 cells (Liu et al., 2011). In contrast a 062R-deficient MYXV showed no altered host range in cell culture but acted as a virulence factor in infection (Liu et al., 2012b). In addition to C7L, MYXV 062R and YLDV 67R allowed replication in the background of K1L/C7L double deleted VACV in 3T3, HeLa and A431 cells, whereas MYXV-063R, MYXV-064R and CPXV-020, the latter of which was expressed poorly, were unable to rescue replication (Meng et al., 2008). C7L homologs from SWPV (064) and SPPV (063) were able to rescue VACV replication in HeLa cells, whereas only SWPV-064, but not SPPV-063, allowed replication in 3T3 and LA-4 cells. These differences in host range function between SWPV and SPPV could be attributed two amino acid residues. Introduction of these two SWPV-064 residues into SPPV-063 allowed efficient VACV replication in 3T3 and LA-4 cells (Meng et al., 2012). The observed virus species-specific function of C7L homologs in conferring differences in host cell tropism highlights the importance of this family as host range factors. Some C7L homologs were shown to antagonize an interferon-β induced antiviral effect in Huh7 cells, which correlated with their host range function in human cells (Meng et al., 2012). MYXV-062 was shown to interact with the interferon-regulated SAMD9 protein in human cell lines and SAMD9 knock-down was shown to partially rescue M062-deficient MYXV (Figure 3) (Liu et al., 2011). However, the exact molecular mechanisms how C7L homologs contribute to host range have still to be characterized.

Tumor necrosis factor receptor II-related poxvirus genes

An abundantly used strategy for poxviruses evasion of the host immune response is to target cytokines and chemokines, direct mediators of the innate immune response, through the

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expression of decoy receptors, that have been coined viroreceptors (Johnston and McFadden, 2003; Upton et al., 1991). One family of these viroreceptors that comprises MYXV and RFV T2, contains homologs of the cellular tumor necrosis factor receptor (TNFR) 2 (Smith et al., 1991; Upton et al., 1991). T2 proteins are TNFR-2 homologs that contain four N-terminal cysteine rich domains (CRDs) that show highest sequence identity with cellular TNFR-2, and a C-terminal domain (CTD), which shows no apparent sequence similarities with non-poxvirus proteins and plays a role in T2 secretion (Schreiber and McFadden, 1996). A T2-defient MYXV replicated well in RK13 and BGMK cells but showed a replication defect in RL-5 cells and was also severely attenuated in infected rabbits (Macen et al., 1996; Upton et al., 1991).

Four TNFR-2-related genes are found in CPXV that are named cytokine response modifier (Crm) B, C, D and E, which can be classified into two groups: one group (CrmB and CrmD) contains both CRDs and the CTD, whereas the other group members (CrmC and CrmE) only contain the CRDs (Hu et al., 1994; Loparev et al., 1998; Saraiva and Alcami, 2001; Smith et al., 1996). Among the surveyed orthopoxviruses, only CPXV-GRI-90 contained all four TNFR-2-related genes (Figure 1). Full-length CrmB orthologs are the most widespread and are found in CPXV-BR, MPXV, CMLV, VARV and HSPV. C-terminally truncated CrmB orthologs are predicted for TATV, RPXV and VACV strains (Figure 1). CrmC, D and E orthologs are either disrupted by indels and/or premature stop-codons or completely missing in most other orthopoxviruses, indicating multiple lineage-dependent gene losses (Bratke et al., 2013). Two TNFR-2-related genes are found in YKV, which lack the CTD. COTV is the only clade II poxvirus, in addition to MYXV and RFV, that contains a TNFR-2-related gene (COTV008/178) (Afonso et al., 2012). This gene is more closely related to orthopoxvirus CrmE genes than to leporipoxvirus T2 genes and lacks an CTD. Interestingly, some poxviruses (including CPXVs, ECTV, DPV and COTV) contain genes encoding for proteins that only contain the CTD but lack the CRDs (Afonso et al., 2012; Bratke et al., 2013). Those genes are sometimes annotated as coding for TNF receptor-like proteins despite their lack of homology with TNFR (Afonso et al., 2012). However the presence of these genes in more distantly related poxviruses indicates functions that are independent from TNF binding. Indeed, it was shown that the CTD of the VARV CrmB ortholog can bind chemokines independent of the CRDs and was thus named smallpox virus-encoded chemokine receptor (SECRET) domain (Alejo et al., 2006). Interestingly, the crystal structure of the SECRET domain of ECTV CrmD showed similar topology to the poxvirus CC chemokine inhibitor and A41 (Xue et al., 2011). Viroreceptors might have a more widespread host range function because their ligands from different species can display considerable sequence differences. Along this line, MYXV T2 was shown to specifically interact with rabbit TNF but not with that of human or mouse origin (Schreiber and McFadden, 1994) and VARV CrmB showed better inhibition of human TNF than of mouse and rat TNF (Alejo et al., 2006), which correlates with the host range of these viruses.

Poxvirus host switches

Our current knowledge is insufficient to accurately predict the outcomes and consequences of poxvirus host switches such as virulence, transmissibility and whether persistence within a new host species can be established. This is complicated by the problem that for many poxviruses, the reservoir host-species have not been unequivocally identified. Many poxviruses cause only mild disease in their respective reservoir hosts, probably reflecting sufficient time for host-virus coevolution. Notable exceptions for this include VARV infection of humans and CMLV infections in camels. However certain patterns for host switches can be observed that are helpful for a better understanding of the effects of this important subject: host switches involving more distantly related species tend to cause

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relatively milder disease, whereas those involving more closely related species can lead to disastrous outcomes. Examples for the latter are MYXV and SQPV. It is interesting that MYXV infection of the natural *Sylvilagus* rabbit hosts results usually in mild disease, whereas infection of European rabbits is highly lethal. In contrast, RFV, which causes a mild disease in its natural host *Sylvilagus floridanus*, only causes mild disease in European rabbits. It is noteworthy that both MYXV and RFV infect closely related *Sylvilagus* species, and probably coevolved with their hosts, but display striking differences in their virulence for European rabbits (Barrett and McFadden, 2007), which shared a common ancestor about 10 mya (Matthee et al., 2004). This indicates that chance is involved in determining disease severity when a poxvirus is transmitted from one related species to another. The high rate of nucleotide substitution estimated for MYXV field strains might have been influenced by the host-switch and subsequent altered selection pressure (Kerr et al., 2012). Another striking example where a host switch between related species results in markedly increased virulence is SQPV. SQPV appears largely avirulent for its presumptive host *Sciurus carolinensis* but is highly virulent for *Sciurus vulgaris* and probably contributes to the demise of this species the United Kingdom (Rushton et al., 2006).

Poxviruses from the three genera yatapoxvirus, parapoxvirus and orthopoxvirus can cause zoonotic infections in humans. In most cases those infections are relatively mild, localized and poorly transmissible among humans, with the notable exception of MPXV Congo basin strains, which can cause mortality rates of approximately 10% (Essbauer et al., 2010). However, compared to the cases of MYXV in European rabbits and SQPV in red squirrels, pathogenicity of MPXV is lower and more importantly transmissibility among humans is low. Additionally, many poxviruses can infect other animal species in addition to their natural host(s). This includes CPXVs, which probably infect rodents as their reservoir hosts but have been isolated from many animals belonging to various mammalian orders (Essbauer et al., 2010).

Eminent poxvirus threats

Poxviruses face stiff inter-species competition. Because VARV was so prevalent in human populations and smallpox survivors gained immunity against other orthopoxviruses, the latter likely never had a chance to establish themselves in the human population. However, with the eradication of VARV from nature and the cessation of mass vaccinations with vaccinia virus, we are left vulnerable to infection by other orthopoxviruses. One major threat would be the emergence of a human-adapted MPXV with increased virulence and/or human-to-human transmission while at the same time retaining its ability to infect multiple animal species. The control of such a virus would pose a major challenge. Other threats to humans are CPXVs, which have a very broad host range and repeatedly cause infections in humans. Because CPXVs contain the largest sets of immune-regulatory and host range genes of all orthopoxviruses, the evolvability of CPXVs into a more virulent pathogen might be especially high. In addition other poxviruses, especially parapoxviruses and yatapoxviruses, have the potential to evolve into potent human pathogens. Moreover, the high recombinogenic potential of poxviruses makes the emergence of hybrid poxviruses possible that have increased virulence and/or host ranges possible. Evidence for such events can be found in orthopoxvirus orthologs of the MYXV apoptosis inhibitor T4 (Bratke et al., 2013) and in COTV, which contains many potential immune-regulatory genes that are most closely related to orthopoxvirus genes, despite a close relationship of COTV with clade II poxviruses in the core genes (Afonso et al., 2012). Poxviruses can also endanger animal species, especially when they face tough ecological competition as seen with European red squirrels and cause deleterious effects on whole ecosystems, as seem with the introduction of MYXV on the Iberian Peninsula and the caused decline of European rabbit populations (Kerr, 2012; Rushton et al., 2006).

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Because genetic manipulation of poxviruses is relatively easy and because of advancements in gene synthesis, another danger is the accidental or deliberate release of poxviruses with increased virulence and host range or viruses that might cause disease even in immunized people/animals. Another concern is the release of viruses, such as VARV, that were developed as bio-weapons (Barrett and McFadden, 2007; McFadden, 2010; Shoham and Wolfson, 2004).

Evolution of poxvirus virulence

Viruses can have intricate relationships with their hosts that can lead to the coevolution of virus and host genes, which can influence the virulence and transmissibility of the viruses. Evolution of virulence for a certain host is probably influenced by the effectiveness and route of transmission of the virus, the immune status of host species, the availability of additional reservoir hosts, intra- and inter-species competition with other viruses and the positive selection for hosts that are more resistant to virus infection. One of the best-studied examples of virus-host coevolution is MYXV infection of European rabbits in Australia and Europe. In these examples the CFR dropped from near 100% initially, to below 50%. The drop in CFR correlated with a longer survival time of the infected rabbits, which probably allowed for a more efficient and prolonged transmission of the virus. The milder courses of MYXV infection over time were due to both the attenuation of the MYXV as well as increased resistance of the host (reviewed in (Kerr, 2012)). The situation is different for SQPV in Great Britain. There SQPV causes little harm to the natural host (grey squirrel; invasive species), in which SQPV causes little harm, but is highly lethal in the new host (red squirrel; native species). This scenario might not put selective pressure on the virus to attenuate, because viruses can be transmitted through the natural host. It is possible that red squirrels will evolve partial resistance against SQPV over time, but the strong inter-species competition for resources with the grey squirrel might not allow for high enough population sizes or enough time for resistance to evolve.

Human CFRs after VARV infections with different strains varied remarkably between less than 1% and 30% and differences in CFRs evolved independently multiple times in various VARV lineages (Esposito et al., 2006; Fenner et al., 1988; Jenner, 1798; Riley, 2010). It is often assumed that VARV was always highly virulent and infectious in humans throughout history and that large populations sizes were necessary to sustain VARV in the human population (Fenner et al., 1988; Gubser and Smith, 2002; Shchelkunov, 2009). If we take into account the relatively low virulence or transmissibility of other orthopoxviruses in humans as well as their host range, the evolution of gene content and phylogeny of poxviruses, it appears most likely that an ancestor of VARV (aVARV) was transmitted to humans from a more distantly related mammal (possibly a rodent as suggested (Li et al., 2007)) and that this virus had a higher gene content. It is further possible that the initial virulence of aVARV in humans was not as high as seen for modern VARV strains and might have been more comparable to that of CPXV in humans, and that aVARV initially retained an animal reservoir. In this scenario aVARV could have existed despite a relatively low population size and become more virulent over time, reducing its gene content and becoming human-specific.

The example of differential sensitivity of European rabbits to the closely related MYXV (very severe infection) and RFV (mild infection), which both cause mild infection in their natural *Sylvilagus spe.* hosts highlights that the establishment of infection in other species is influenced in part by chance and may be hard to predict without detailed molecular knowledge about host-virus interactions. In general, it appears that transfer between relatively closely related species as seen for MYXV and SQPV hosts, can cause more severe

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disease, whereas virus transfer between more distantly related species, might result in relatively less disease severity and/or transmissibility.

The molecular basis for the high virulence of VARV to humans, the human-specificity and the attenuation of some VARV strains is not directly apparent from sequence comparisons (Esposito et al., 2006). Similarly, the molecular mechanisms responsible for the high MYXV virulence in European rabbits as opposed to in American rabbits as well as for the attenuation of MYXV field strains are unsatisfactorily understood, despite the availability of whole genome sequences of many MYXV isolates (Kerr et al., 2012). An interesting example of how host range genes could evolve was recently reported for VACV K3L (Elde et al., 2012). K3 is only a weak inhibitor for human PKR (Elde et al., 2009; Rothenburg et al., 2009). In a E3L deleted VACV strain, serial passaging in human HeLa cells resulted in tandem amplification of the K3L locus and overexpression of K3, which was accompanied by higher virus replication rates (Elde et al., 2012). Interestingly, K3L copy number was reduced after passaging in BHK cells, which are permissive for the parental strain (Langland and Jacobs, 2002). Moreover, a mutation in K3L (H47R) was identified in some plaque-purified viruses, that was originally identified as a better inhibitor of human PKR in a yeast screen (Kawagishi-Kobayashi et al., 1997). These findings have interesting implications for the evolution of poxvirus host range and virulence genes. After initial host changes, copy number of genes that are sub-optimally adapted for their new hosts, might initially expand and increase their chance of acquiring beneficial mutations for replication in their new host. After acquisition of such mutations, the copy number might collapse. Additional copies might be retained if additional mutations evolve that provide a selective advantage for the virus, e.g. by targeting a different host protein or if the virus cycles in different hosts. An example of a host range gene family that likely arose from tandem amplification comprises MYXV and RFV 062R, 063R and 064R, which play different roles in the infection of rabbits (reviewed in (Liu et al., 2012a)).

Conclusions

Our current knowledge is insufficient to predict the outcomes of poxvirus host-switches. Every animal or human that comes into contact with poxvirus-infected animals or arthropod vectors of poxviruses is at risk for productive infection. Whether productive infection can be established depends on the effective manipulation of the host immune response by the querying virus. It is likely that both gene loss and the acquisition of new genes, through gene duplication, recombination, horizontal transfer and sequence- and expression differences in viral proteins, as well as species-specific interactions with host proteins play prominent roles in determining virus host range. The availability of so much new poxvirus genomic sequence information is providing a treasure trove for future research on host-virus interaction that will lead to a better understanding of virus host range. Much research on poxvirus host range genes has been performed in heterologous systems that don't represent natural host-virus relationships or with viruses without a well-defined host range. Detailed analyses of natural host-virus interactions, facilitated through technological advances and the development of new assay systems, will extend our knowledge about this important subject and will provide us with valuable information for risk assessment of possible threats and management of newly emerging viruses.

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Highlights

1. Review of poxvirus families and poxvirus genes that influence poxvirus host range.
2. Molecular mechanisms poxviruses use to subvert the host innate immune system.
3. Evolution of poxvirus host range genes and their role in the virulence of poxviruses.

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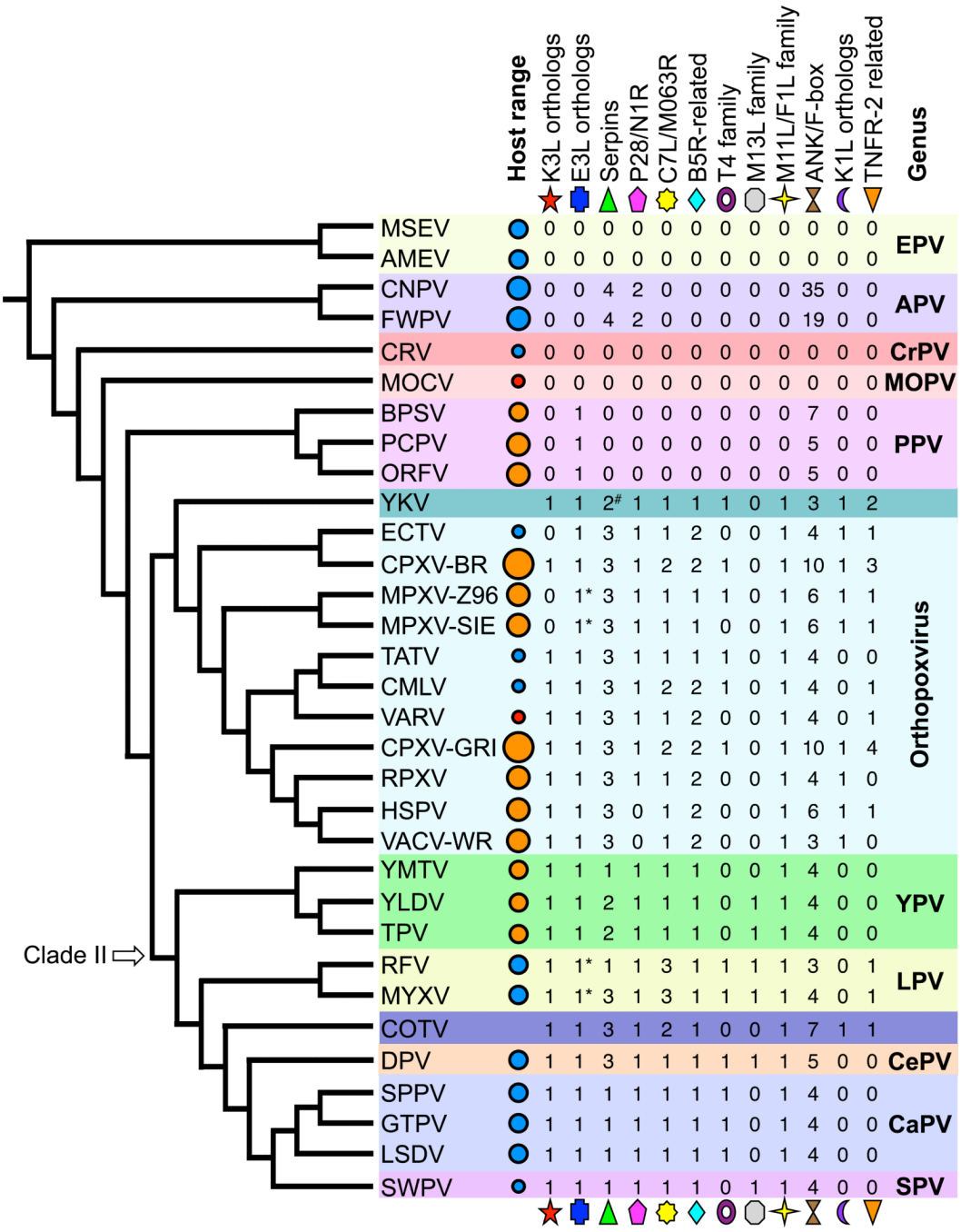

Figure 1. Presence of host range gene families in poxviruses

The shown schematic poxvirus phylogeny is based on a recently published phylogenetic analysis (Bratke et al., 2013) and incorporates the phylogenetic positions of PCPV, YKV and COTV as published (Afonso et al., 2012; Hautaniemi et al., 2010; Zhao et al., 2011). Branch lengths are not drawn to scale. Approximate extent of virus host range, as discussed in this manuscript, is indicated by circles. A small circle indicates a very limited host range. Large circles indicate a broad host range. No circle indicates insufficient data to project host range. Human-specific viruses are marked by red circles. Viruses that can cause zoonotic infections in humans are marked by orange circles. Presence of likely functional host range genes or gene families are indicated by numbers (see (Bratke et al., 2013) and this

manuscript). Asterisks (*) mark E3L orthologs that don't encode for a functional Zα domain. The number (#) sign denotes an additional serpin gene in YKV that is probably non-functional (see YKV section). Abreviations for poxvirus genera are: EPV = Entomopoxvirus B; APV = Avipoxvirus; CrPV = Crocodylidpoxvirus; MOPV = Molluscipoxvirus; PPV = Parapoxvirus; YPV = Yatapoxvirus; LPV = Leporipoxvirus; CePV = Cervidpoxvirus; CaPV = Capripoxvirus; SPV = Suipoxvirus.

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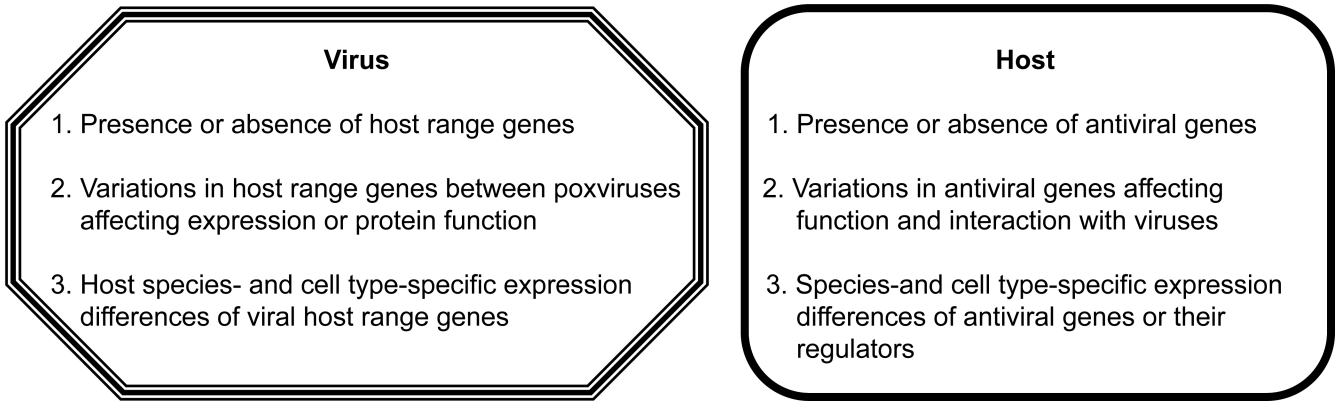

Figure 2. Molecular basis for poxvirus host range

Potential molecular mechanisms of how genetic differences of both the virus (left) and host (right) contribute to the host range of poxviruses.

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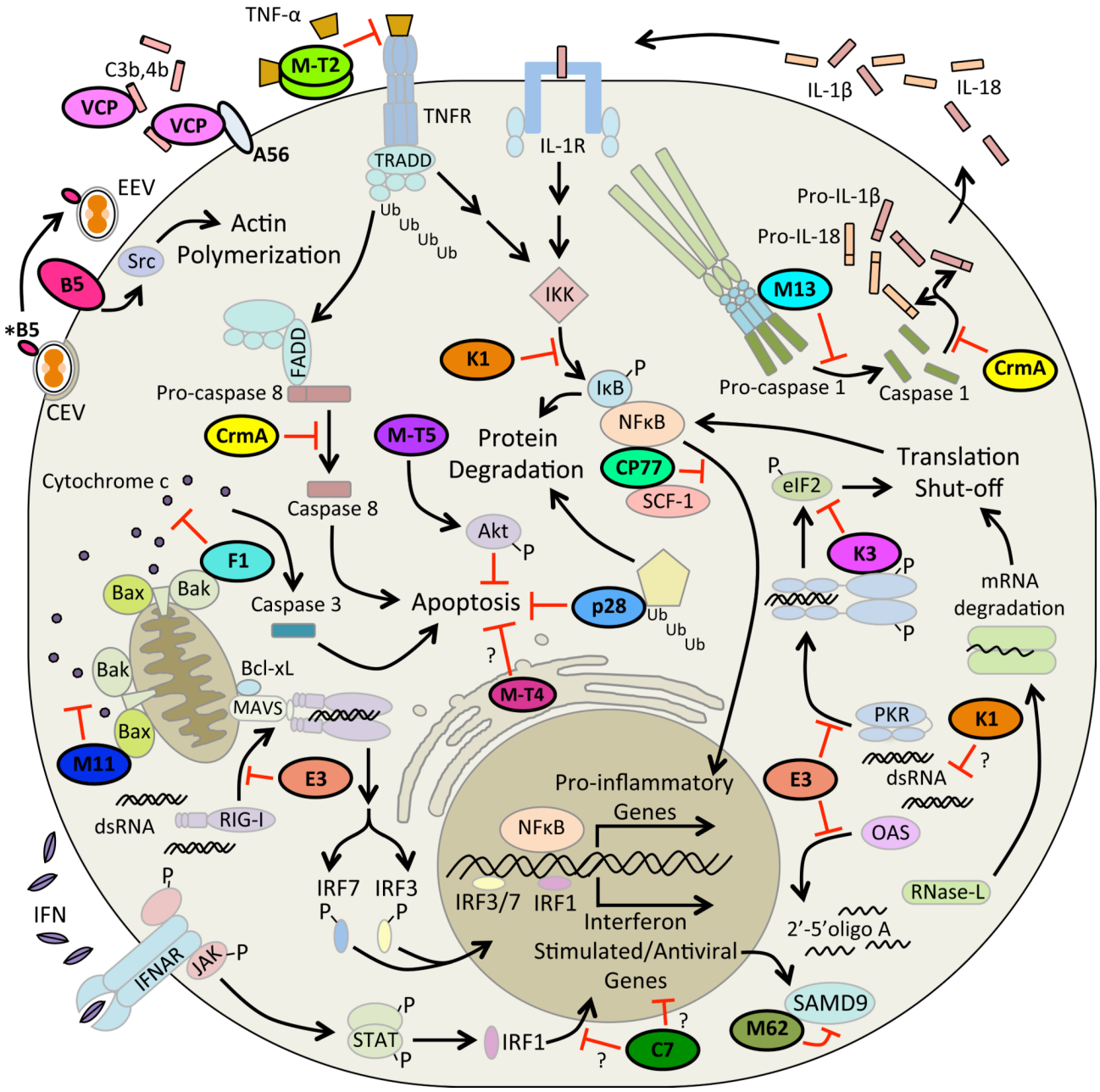

Figure 3. Molecular interactions of poxvirus host-range factors with host proteins

The host range function of proteins encoded by poxviruses can be attributed to several interactions with specific host proteins. Some poxviruses encode proteins that use the host's machinery to their own benefit to promote viral spread or degrade unwanted host proteins. Many also encode inhibitors of proteins involved in antiviral responses, such as apoptosis or translational shut-off, ultimately reducing the inflammatory or interferon response mediated by the cell to promote viral replication. The specificity of these interactions with their host targets can either restrict or expand the range of hosts in which the virus can replicate. Abbreviations used: dsRNA = double-stranded RNA; MAVS = mitochondrial antiviral signaling protein (also known as IPS-1/Cardiff/VISA); TNFR = tumor necrosis factor receptor; IRF (1,3,7) = interferon response factor; CEV = cell associated enveloped virus; EEV = extracellular enveloped virus; Ub = ubiquitin; SCF-1 = Skp1:Cullin-1:F-box

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ubiquitin ligase complex; eIF2 = eukaryotic translation initiation factor 2; IFNAR = interferon receptor; IFN = interferon; Bcl-xL = B-cell lymphoma extra large; FADD = Fas-associated Death domain protein; TRADD = TNFR-1 associated Death domain protein; Organelles and proteins not drawn to scale. * B5 is present on both cellular and EEV membranes (here shown in two sizes) and promotes the transition from CEV to EEV during viral replication.

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Table 1

Names, abbreviations, genome sizes and lengths of inverted terminal repeats of poxviruses discussed in this review.

Poxvirus Name	Abbreviation	Genome size (in kb)	ITR size (in kb)	Genus
Amsacta moorei entomopoxvirus	AMEV	232	9.4	Entomopoxvirus B
Melanoplus sanguinipes entomopoxvirus	MSEV	236	7	Unassigned
Canarypox virus	CNPV	360	6.5	Avipoxvirus
Fowlpox virus	FWPV	266-289	9.5-10.1	Avipoxvirus
Crocodilepox virus	CRV	190	1.7	Crocodylidpoxvirus
Molluscum contagiosum virus subtype 1	MOCV	190	4.7	Molluscipoxvirus
Bovine papular stomatitis virus	BPSV	134	1.1	Parapoxvirus
Orf virus	ORFV	137-140	3.1-3.9	Parapoxvirus
Pseudocowpox virus	PCPV	135-145	2.8-14.9	Parapoxvirus
Ectromelia virus	ECTV	210	9.4	Orthopoxvirus
Cowpox virus -Brighton Red	CPXV-BR	224	9.7	Orthopoxvirus
Monkeypox virus	MPXV	196-206	6.4-10.8	Orthopoxvirus
Camelpox virus	CMLV	202-206	6.1-7.7	Orthopoxvirus
Taterapox virus	TATV	198	4.8	Orthopoxvirus
Variola virus	VARV	185-188	0.1-1.2	Orthopoxvirus
Cowpox virus - GRI-90	CPXV-GRI	224	8.3	Orthopoxvirus
Rabbitpox virus	RPXV	198	10	Orthopoxvirus
Horsepox virus	HSPV	212	7.5	Orthopoxvirus
Vaccinia virus	VACV	165-200	3.4-16.4	Orthopoxvirus
Yoka poxvirus	YKV	175	2.3	Unassigned
Yaba monkey tumor virus	YMTV	135	2	Yatapoxvirus
Yaba-like disease virus	YLDV	145	1.8	Yatapoxvirus
Tanapox virus	TPV	145	1.9	Yatapoxvirus
Myxoma virus	MYXV	162	11.5	Leporipoxvirus
Rabbit fibroma virus	RFV	160	12.4	Leporipoxvirus
Deerpox virus W-848-83	DPV-W83	166	5	Cervidpoxvirus
Deerpox virus W-1170-84	DPV-W84	171	7	Cervidpoxvirus
Sheeppox virus	SPPV	150	2.2	Capripoxvirus
Goatpox virus	GTPV	150	2.3	Capripoxvirus
Lumpy skin disease virus	LSDV	151	2.3-2.4	Capripoxvirus
Swinepox virus	SWPV	146	3.7	Suipoxvirus
Cotia virus	COTV	185	13.7	Unassigned