Towards vaccine development against African swine fever virus in Eastern and Southern Africa

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Tonny Kabuuka

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Dekan: Prof. Dr. Werner Weitschies

1. Gutachter: Prof. Dr. Dr. h.c. Thomas C. Mettenleiter

2. Gutachter: Priv.-Doz. Dr. Asisa Volz

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1 CHAPTER ONE: INTRODUCTION

1.1 Taxonomy of African swine fever virus and related viruses

African swine fever (ASF) is caused by the African swine fever virus (ASFV) which belongs to the family *Asfarviridae* and the genus Asfivirus where it is the sole species (Alonso et al., 2018; Andreani et al., 2017; https://talk.ictvonline.org/taxonomy/). The name *Asfarviridae* is derived from *A*frican *s*wine *f*ever *a*nd *r*elated viruses. However, although various sequences exhibiting some similarities to ASFV have been identified in virus fractions from sewage, human serum, and oceans in metagenomics sequencing projects (Loh et al., 2009), no closely related viruses could be identified up to now. ASFV was initially assigned to the virus family *Iridoviridae* but with in-flow of more information on replication strategies and genes encoded, it was classified as a separate single-species family within the nucleo-cytoplasmic large DNA virus (NCLDV) superfamily, which additionally includes *Iridoviridae, Poxviridae, Mimiviridae* and *Phycodnaviridae* (Dixon et al., 2013; Loh et al., 2009; Yanez et al., 1995). Some viruses like faustoviruses, kaumoebavirus and pacmanvirus that are still unclassified and have considerably larger genomes (about 400 kbp) than ASFV (170 - 190 kbp) contain about 30 genes similar to those of ASF (Andreani et al., 2017).

1.2 Virion structure and properties of African swine fever virus

Morphologically, the extracellular ASFV virions vary in diameter from 175 – 251nm (Dixon et al., 2013). Each virion consists of an icosahedral capsid with an outer lipid-containing envelope and an internal lipid membrane which encases a core that ranges from 70-100 nm in diameter. The outer envelope is not required for infectivity, and missing in intracellular particles and virions released by cell lysis (Alonso et al., 2018; Dixon et al., 2013; Salas & Andres, 2013). The icosahedral (T=189-217) capsid contains 1892-2172 capsomers assumed to be organized as 12 pentamers and 2160 to 1880 hexamers depending on the T value (Alonso et al., 2018; Carrascosa et al., 1984). Glycolipids and phospholipids like phosphatidylinositol,

phopshatidlycholine and phosphatidylethanolamine are found in the enveloped virion, together with the virus encoded glycoprotein pEP402R or CD2v (Dixon et al., 2013). Virions are sensitive to compounds like chloroform, deoxycholate and ether, and are inactivated by incubation at 60°C for 30 min. Virions survive at 4°C or 20°C for several years, and may endure pH values from 4 to 13. Irradiation destroys infectious virus, and paraphenylphenolic disinfectants are very effective for disinfection. Two percent NaOH for 1 day or 1% formaldehyde for 6 days can also be used (Dixon et al., 2013).

1.3 Genome structure and proteins of African swine fever virus

ASFV contains a large, linear, double-stranded DNA genome that varies in length from 170 – 193 kbp depending on the isolate, and encodes 150 - 167 genes which are located on both DNA strands. The genome contains terminal inverted repeats of approximately 2.1 kbp at both ends that are covalently linked (Alonso et al., 2018; Dixon et al., 2013; Yanez et al., 1995). The inverted repeat sequences are further characterized by numerous tandem repeat arrays (Chapman et al., 2008). Fifty four structural and more than 100 infected cell proteins encoded by ASFV have been identified (Alonso et al., 2018; Arias et al., 2017; Kessler et al., 2018; Yanez et al., 1995). Several viral gene products have been functionally characterized, and identified as structural proteins, or enzymes involved in nucleotide metabolism, DNA replication and repair, and transcription. The virus also encodes proteins that modulate the response of the host to virus infection (Dixon et al., 2013). Table 1 is a summary of the currently known or putative ASFV proteins and their functions (Alonso et al., 2018). In the present study, 14 ASFV genes and/or proteins were investigated as will be described in the subsequent chapters: the viral thymidine kinase (TK, K196R), dUTPase (E165R), ribonucleotide reductase (F334L and F778R) , the predicted redox pathway enzyme 9GL (B119L), and the structural proteins p11.5 (A137R), p12 (O61R), p30 (CP204L), p54 (E183L), p72 (B646L), pA104R,

pE199L, pK145R, and p285L (Alejo et al., 2003; Carrascosa et al., 1993; Yanez et al., 1995; Arias et al., 2017).

1.4 Replication cycle of African swine fever virus

Infection with ASFV is initiated either through ingestion, by contact or tick transmission (Alonso et al., 2018). ASFV replicates in both, *Ornithodoros* soft ticks and vertebrates of the family *Suidae* where it mainly occurs in cell types of the mononuclear-phagocytic system, including macrophages (Basto et al., 2006; Cuesta-Geijo et al., 2012). Other species are apparently resistant against ASFV infection. In the vertebrate host, the replication of ASFV DNA in the cytoplasm resembles that of members of the family *Poxviridae* (Dixon et al., 2013; Brookes et al., 1996). The mechanism of viral entry into the host is not fully understood and different mechanisms are discussed (Andrés & Tsai, 2017; Sanchez et al., 2017). Host cell entry is pH-, temperature-, and energy-dependent, and involves the cellular lipid cholesterol (Cuesta-Geijo et al., 2016; Dixon et al., 2013; Sanchez et al., 2017). It is presumed that the virus enters the cell through clathrin-mediated dynamin-dependent endocytosis or actin-driven macropinocytosis followed by uncoating through the early endosomal and macropinosomal pathways resulting into late multivesicular endosomes (Andrés & Tsai, 2017; Cuesta-Geijo et al., 2012). Host factors are utilized for viral uncoating in the endosome, and the acidic pH in the endosomal lumen facilitates disassembly of the capsid (Barrado-Gil et al., 2017) in a stepwise process (Andrés & Tsai, 2017). The viral cores eventually exit the endosome after degradation of the capsids by fusion of the vesicle membranes with the inner virus envelopes. Final degradation of the viral cores is supported by the ubiquitin-protease system, and leads to release of replication-competent viral DNA (Barrado-Gil et al., 2017). The microtubular motor protein dynein transports the DNA to the perinuclear region, where viral factories are formed (Jouvenet et al., 2004). Although ASFV DNA replicates in the cytoplasm, an early nuclear phase whose role is still unknown, has been also documented. Transcription of viral genes and

RNA processing is executed by viral enzymes, and regulated in a cascade-like fashion resulting in 4 classes of mRNAs (Dixon et al., 2013). Protein translation is initiated from mRNAs possessing 5'-caps and 3'-polyadenylation (Alonso et al., 2018). Genome replication by viral DNA polymerase occurs approximately 6 hours post infection, and leads to head-to-head concatemeric replicative intermediates (Dixon et al., 2013). After initiation of DNA replication, intermediate and late viral proteins like the major capsid protein p72 are synthesized in an expression pattern similar to that of poxviruses (Barrado-Gil et al., 2017), and new virus particles containing an inner lipid envelope derived from the rough endoplasmic reticulum are assembled (Andrés & Tsai, 2017). The virions leave the perinuclear factories to the plasma membrane via kinesin-mediated transport (Jouvenet et al., 2004) followed by budding to acquire the outer envelope from the plasma membrane, which, however, is not essential for infectivity (Andrés & Tsai, 2017; Jouvenet et al., 2004).

1.5 Host range and tick transmission

ASFV is transmitted through contact, ingestion, or bite of *Ornithodoros* soft ticks and infects all investigated members of the family *Suidae* including domestic pigs, wild boar, warthogs and bush pigs (Alonso et al., 2018; Correia et al., 2013). Pigs get infected mainly through the oro-nasal route following contact with infected animals or after feeding on swill that contains virus (Bellini et al., 2016). The course of ASFV infection is markedly different in various pig species, and ranges from persistent asymptomatic infections in African wild swine, to fatal clinical disease in domestic and feral pigs and wild boar (Costard et al., 2013). Wild suids are known to be ancestral host species at least since the 1700s in Africa (Alkhamis et al., 2018), and up to now bush pigs and warthogs act as reservoirs of the virus (Escribano et al., 2013). Furthermore, ASFV is the only known DNA arbovirus, since it can be transmitted by soft ticks of the family *Argasidae*. The soft tick species *Ornithodoros erraticus* in Portugal and Spain, and *Ornithodoros moubata* in Africa have been implicated in ASF spread in these regions

(Alkhamis et al., 2018; Bellini et al., 2016; Boinas et al., 2011; Costard et al., 2013). In these ticks, the virus has been shown to replicate productively for several weeks, followed by viral clearance in both experimental and natural infections (Basto et al., 2006). However, persistent infection of soft ticks for more than five years after removal of the infectious hosts has been also reported (Boinas et al., 2011). So far, other haematophagous invertebrates serving as biological vectors for ASFV were not recognized, but the biting fly *Stomoxys calcitrans* showed the possibility of passive transmission (Costard et al., 2013). In the domestic pig population, new virus introductions result in high morbidity and mortality rates close to 100%, while in endemic regions lower mortality rates and more frequent chronic infections are observed (Costard et al., 2013). Among wild swine, warthogs (*Phacochoerus africanus*) are obviously the natural vertebrate hosts of ASFV, which, together with ticks of the *O. moubata* complex, are involved in a sylvatic replication cycle (Correia et al., 2013). (Forth et al., unpublished results) have found integrations of ASFV-like sequences that encode a viral histone-like protein pA104R in certain tick genomes. Bush pigs (*Potamochoerus larvatus*) and red river hogs (*Potamochoerus porcus*) have a significant though still not well defined role in ASF transmission especially to other hosts. The giant forest hog (*Hylochoerus meinertzhageni)* may occasionally get infected with ASFV with negligible epidemiological effects (Costard et al., 2013). In Europe and Asia, feral pigs and wild boar exhibit the same susceptibility to ASFV infection and mortality as domestic pigs (Costard et al., 2013). ASFV is maintained within the wild boar population through a wild boar-habitat cycle characterized by both direct transmission between infected and susceptible wild boar, and indirect transmission from littered carcasses (Chenais et al., 2018).

1.6 African swine fever – history and epidemiology

Eustace Montgomery first reported ASF as a new form of swine fever observed in June 1910 in British East Africa (Montgomery, 1921), which is present day Kenya. He also noted that

apparently healthy warthogs (Phacocoerus) were near farms where the infections had been diagnosed. The affected domestic pigs had been imported into the Kenya protectorate from Europe. Wild bush pigs obtained from Jinja, Uganda, did not succumb to infection when infected experimentally. Obviously, ASF persisted in a sylvatic cycle between warthogs and *Ornithodoros* long before a disease was observed in the imported swine species *Sus scrofa* (Montgomery, 1921; Mulumba-Mfumu et al., 2019). Today, African swine fever is endemic in 28 countries of Sub-Saharan Africa (Arias et al., 2017) with periodic introductions into other parts of the world (Alonso et al., 2018). A significantly high evolutionary rate of the ASFV derived from p72-central variable region (CVR) gene sequences that were isolated from outbreaks in Africa and Eurasia between 1960 and 2015 has been shown to occur since its divergence in the 1700s from East Africa. This surge in genetic diversity points to a genetic drift and relates to increased pig trade across continents in the 19th Century (Alkhamis et al., 2018).

ASF spread from Africa to Europe, in particular Spain and Portugal, in 1957 and 1960, respectively. During the 1960s ASF from West Africa became endemic for 30 years in Europe and South America. In the mid-1990s, ASF was eradicated from all countries outside Africa, except the Italian island of Sardinia where it is endemic up to now. However, another transcontinental ASF spread was reported in June 2007 in the Republic of Georgia in the Caucasus region and eventually spilled over to neighboring countries of Armenia, Azerbaijan and the Russian Federation (Sanchez-Cordon et al., 2018; Boinas et al., 2011; Chapman et al., 2011; Costard et al., 2013; Escribano et al., 2013). During 2014, ASF was introduced into the Baltic states and Poland, with continued spread within the wild boar population of these regions, and an increasing risk of spread to Western Europe (Sargsyan et al., 2018; Olesen et al., 2018). In 2019 ASFV was also detected in Belgian wild boars, and has now been reported in sixteen European countries namely Georgia, Azerbaijan, Armenia, the Russian Federation, Ukraine, Belarus, Estonia, Latvia, Lithuania, Poland, Moldova, Czech Republic, Romania,

Hungary, Bulgaria, and Belgium (Gallardo et al., 2018; Jurado et al., 2018; https://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/WI). The latest incursion of ASF to a new geographical location happened during August 2018 in China with disease spread observed in 8 provinces (Wang, Sun, & Qiu, 2018). In the meantime, also neighboring Asian countries like Vietnam, Cambodia, and Laos became affected (Gaudreault & Richt, 2019). ASF is moderately contagious and in previously unexposed herds up to 100% morbidity rates are observed, with varying mortalities between 0 and 100% depending on the host, the virus, the dose, and exposure route (Costard et al., 2013). In Africa, two epidemiological cycles are thought to keep the virus in circulation with one occurring within domestic pigs and another involving the soft tick vector and the warthog reservoir (Basto et al., 2006). In West Africa, the regional ASF risk is mainly determined by the domestic cycle, while the sylvatic cycle may influence the regional ASF risk in East Africa (Huang et al., 2017). In the domestic pig cycle, ASFV is transmitted via direct contact and fomites like infected meat and blood, supported by pig trade and movement coupled with lack of biosecurity barriers (Costard et al., 2013). It is also thought that an ASF carrier status within domestic pigs may lead to continued viral spread in East Africa (Abworo et al., 2017). In Uganda for instance, several ASFV-surviving domestic pigs were identified during outbreaks (Kabuuka et al., 2014), and healthy pigs carrying ASFV were found in Kenya and Uganda without an obvious link to outbreaks (Abworo et al., 2017). In Nigeria, a mild form of ASF was circulating in the field, and led to increased numbers of persistently infected domestic pigs (Fasina et al., 2010). In Western Kenya, where no outbreaks had ever occurred, ASFV DNA was detected using PCR in visually asymptomatic domestic pigs presented for slaughter (Thomas et al., 2016). Recent studies showed that some highly virulent strains may become naturally attenuated (Gallardo et al., 2018). In East Africa, ASFV seems to exhibit intra-genotypic variations correlating with different clinical outcomes in the field (Kabuuka et al., in prep, unpublished data, conference proceedings; Wambura et al., 2006). Some genotype II viruses induced unusual forms of ASF with a possibility of conversion

from the sub-acute to a chronic form of the disease (Sargsyan et al., 2018). Furthermore, ASFV with considerable sequence identity to highly virulent ASFV genotype II (Georgia 2007/1) was isolated in Tanzania from asymptomatic European type cross bred pigs (Uttenthal et al., 2013). In several areas of Europe, more and more wild boars are becoming seropositive indicating that they survived ASFV infection (Gallardo et al., 2018). Within the Russian Federation, numerous investigations of ASFV infection in wild boar raised fears that the virus will be perpetually established in the population (Chapman et al., 2011). As mentioned, the sylvatic cycle of ASFV occurring in eastern and southern Africa involves warthogs and ticks of the *O. moubata* complex (Costard et al., 2013; Jori & Bastos, 2009). Soft ticks that infest the warthog burrows feed on and infect young suckling warthogs, which then remain asymptomatically infected for life. Even in the absence of blood meals, tick colonies can maintain ASFV infection for at least 15 months, allowing virus transmission to warthogs at their next farrowing season (Costard et al., 2013). The co-existence of soft ticks, warthogs and domestic pigs carrying different ASFV genotypes in the same locality has been documented. ASFV viruses isolated from ticks and domestic pigs aligned within genotype IX while those from warthogs belonged to genotype X within the same location in Kenya (Gallardo et al., 2011). In Madagascar antibodies against salivary gland proteins of the soft tick *O. moubata* have been found in domestic pigs, also indicating a possible role of the arthropod vector in the domestic cycle of ASFV (Ravaomanana et al., 2011).

1.7 African swine fever – differential diagnosis, disease and pathology

Several diagnostic methods are used to detect specific antibodies against ASFV, viral antigens, genomic DNA, or to isolate infectious virus (Zsak et al., 2005; OIE, 2019). ASFV isolates are currently classified using either genotyping based on DNA sequencing and molecular phylogeny analysis (Gallardo et al., 2009; Bastos et al., 2003; Boshoff et al., 2007) or serotyping based on the haemagglutination inhibition (HAI) assay (Malogolovkin et al., 2015). Molecular diagnosis of ASFV is done by PCR amplification of genomic DNA fragments followed by

either full gene or partial gene sequencing including analysis of variable repeat regions. Partial p72 gene sequencing revealed 22 long-known and two new genotypes (Mulumba-Mfumu et al., 2019). Sequencing of the complete p54 gene permitted further sub-typing (Arias et al., 2017; Gallardo et al., 2009; Bastos et al., 2003; Boshoff et al., 2007). In Europe only two genotype clusters exist, moderately virulent genotype I strains in Sardinia, and, at least originally, highly virulent genotype II strains in Eastern and Central Europe (Arias et al., 2017; Bellini et al., 2016). In Africa, all 24 genotypes exist, and often several different genotypes are found in the same region (Arias et al., 2017; Boshoff et al., 2007; Carmina et al., 2011). Alternatively, ASFV can be classified into serogroups based on the HAI. Eight ASFV HAI serogroups were identified that clustered some viruses from different p72 phylogenetic classes together (Malogolovkin et al., 2015), and correlation between cross-reactive serogroups and sequence-based genotypes seems to be limited (Dixon et al., 2013). ASFV causes mostly lethal infections with characteristics of a hemorrhagic fever in domestic pigs and wild boar (Fig 1-1), but some strains may also lead to sub-acute, chronic or subclinical infections. In contrast, bush pigs and African warthogs show generally unapparent persistent infections (Arias et al., 2017; Escribano et al., 2013).



Figure 1-1: *Clinical and pathological signs of African swine fever, which causes almost 100 % lethality of infected pigs. Hemorrhagic gastrohepatic and renal lymph nodes, and petechiae in kidneys are frequently observed lesions (Blome et al., 2013; Virus Res 173: 122-130; Jolene Carlson Plum Island Animal Disease Center).*

The per-acute form of ASF elicits 100% mortality in domestic pigs around 4-7 days after infection preceded by hemorrhagic symptoms, high fever, appetite loss, lethargy and

hyperpnoea. In the acute form of ASF similar signs except hyperpnoea are observed, and instead gastro-intestinal signs are evident. Asymptomatic forms are less common in the species *Sus scrofa*, but chronic forms characterized by respiratory signs and lameness can be seen in carriers (Arias et al., 2017; Sanchez-Vizcaino et al., 2012; Schulz et al., 2017). Pathological signs of the per-acute and acute forms of ASF are erythema with petechial hemorrhages in several organs, lung edema and abortions. A similar pathology is evident in the sub-acute form but with more hemorrhage found in the lymph nodes. In the chronic form of ASF, there are arthritis, pneumonia, necrotic skin lesions, pericarditis and also abortion (Schulz et al., 2017).

1.8 African swine fever – host immune response and immune evasion

An infection with ASFV results in host immune responses that vary depending on the virus strain, and if protection ensues at all, it is frequently strain-specific (Correia et al., 2013). This correlates with considerable variations between different ASFV isolates (Dixon et al., 2013), which is particularly due to gain or loss of proteins encoded by the multigene families (MGFs) 100, 110, 300, 360, 505/530 and the p22 family encoded within the approx. 40 kbp -left and 20kbp-right terminal genome regions. Furthermore, immune evasive functions have been attributed to several ASFV gene products (Dixon et al., 2013). ASFV targets different components of the host defense system, including innate and intrinsic immune mechanisms like type I interferon (IFN) responses, inflammation, and apoptosis (Arias et al., 2017). The interferon (IFN) system is an early innate anti-virus defense mechanism of vertebrates, which is activated shortly after entry of a pathogen and long before adaptive immunity sets in. Secretion of IFNs by the infected cells leads to establishment of an anti-viral state in surrounding cells. Both type I and II ("immune interferon") IFNs are crucial in coordination of the innate and adaptive immune responses to an infection by a virus. Type III IFNs provide innate antiviral defenses at epithelial surfaces, which are the major entry portals for numerous viral pathogens. Viruses evade host defense mechanisms through two basic strategies, first.

through manipulation of the various components of the early, innate immune responses (IFN, cytokines, chemokines, apoptosis), and secondly through avoidance of recognition by the adaptive immune responses like cytotoxic T cells or neutralizing antibodies by continuous antigenic variation (Correia et al., 2013; Yanez et al., 1995). The ASFV A276R-, A528R-, and I329L proteins were found to reduce the interferon response through targeting different intracellular signaling intermediates (Correia et al., 2013). Successful evasion of IFN responses by ASFV is a prerequisite for the persistent infections which are observed in warthogs, and occasionally also in domestic pigs (Correia et al., 2013). Macrophages and monocytes play an important role in the immune response through phagocytosis, antigen presentation and secretion of cytokines. They are the main targets of ASFV infection in pigs (Carrillo et al., 1994; Sanchez et al., 2017). Zhang et al., 2006. elucidated differences in the amounts of pro-inflammatory cytokines, interleukin 1 beta and tumor necrosis factor alpha, secreted from macrophages infected with either the nonpathogenic ASFV isolate OUR T88/3, or the virulent Malawi LIL20/1. Interestingly, there was no virus-induced "shut-off" of host transcription, but an early transcriptional activation of many genes including those of pro-inflammatory cytokines IL-6, TNF-α, IFN-β and CC and CXC group chemokines. The increased expression of these cytokines and chemokines benefits the recruitment and proliferation of inflammatory cells, and might provide an extra pool of macrophages for infection. The outer envelope protein CD2v of most virulent ASFV isolates and its haemagglutinating activity are also considered to play a role in immune evasion either by mimicking the homologous host T-cell marker, or by masking the virions with host erythrocytes (Borca et al., 1998). On the other hand, warthogs which do not develop clinical signs when infected with ASFV, may block viral immune evasion more efficiently than domestic pigs. It has been speculated, although not proven, that three amino acid substitutions enhancing the activity of the RELA subunit of NF-κB might be responsible for the resistance of warthogs against disease (Palgrave et al., 2011). Non-lethal ASFV infections of warthogs and domestic pigs induce specific antibodies against several virion

proteins, in particular the phosphoprotein p30 (Alonso et al., 2018; Escribano et al., 2013). However, the relevance of these antibodies for virus neutralization is still controversially discussed.

1.9 Vaccine development – strategies and problems

Since the first introduction of ASF to Europe in the 1960s, it was attempted to prepare efficient vaccines against ASFV (Zakaryan & Revilla, 2016), which should induce both humoral and cell-mediated immune responses in pigs (Correia et al., 2013). Since this was not successful so far, the current prevention strategy depends on rapid diagnosis using enzyme linked immune sorbent assays (ELISA), PCR detection of virus DNA, or virus isolation in macrophage cultures (Arias et al., 2017). The disease is controlled by animal quarantine and slaughter (Vlasova et al., 2012), including culling of healthy animals in affected regions (Sanford et al., 2016). A number of reasons have been suggested to explain why the search for AFSV vaccines or antiviral drugs has not yet led to satisfying results (Argilaguet et al., 2012). The first approaches for vaccine development were based on inactivation of virulent ASFV, and classical in vitro attenuation of the virus e.g. by cell culture passage (Zakaryan & Revilla, 2016). More recent approaches included the use of recombinant peptides/proteins, antigen expression by viral vectors, and targeted attenuation of ASFV by genetic engineering (Arias et al., 2017; Zakaryan & Revilla, 2016). For initial vaccination attempts inactivated infected cell extracts, supernatants of infected pig peripheral blood leucocytes, purified and inactivated virions, infected glutaraldehyde-fixed or detergent-treated infected macrophages were used (Argilaguet et al., 2012; Arias et al., 2017). However, since cellular immunity seems to be essential for protection, inactivated ASFV preparations have always failed to confer protection, even in the presence of adjuvants (Blome et al., 2014). Antibody-mediated virus neutralization might be difficult to achieve since two forms of infectious particles exist, with and without an outer envelope and with different surface proteins (Arias et al., 2017). The functions of most ASFV proteins

involved in infection are incompletely understood, and cellular receptors are still unknown. This hinders targeted development of vaccines or antivirals which block viral entry. The identification of immunogenic proteins of ASFV that confer solid protective immune responses in domestic pigs is therefore necessary (Rock, 2017). For subunit vaccines involving the use of recombinant proteins, DNA or antigen delivery using viral vectors, the 167 proteins encoded by the ASFV genome make the selection of protective candidate antigens a difficult task (Arias et al., 2017; Rock, 2017). DNA immunization using the extracellular domain of ASFV hemagglutinin fused to antibody inducing ASFV proteins p54 and p30 exponentially improved both cellular and humoral responses, but no protection was conferred against lethal challenge with a virulent ASFV strain (Argilaguet et al., 2012). Viral vector-based vaccination approaches have been used to confer protective immunity to ASFV with varying results. Expression of several ASFV ORFs fused to ubiquitin conferred partial protection against challenge with a virulent strain via ASFV-specific T cells, but absence of antibody-mediated protection, indicating requirement of multiple ASFV antigens for protection (Arias et al., 2017). Further vaccination attempts included either naturally occurring low virulent ASFV isolates, cell culture passaged virulent strains, or engineered viruses (Arias et al., 2017; Rock, 2017). Live attenuated ASFV 9GL and UK gene deletion mutants were reported to confer protection against homologous challenge (O'Donnell et al., 2017). In general, pigs surviving infection with moderately virulent or attenuated ASFV variants develop long-term resistance against closely related, but not heterologous ASF viruses (Lacasta et al., 2015). Furthermore, attenuated viral strains raised some biosafety issues due to retention of virulence and occasional sub-clinical infections in pigs with some remaining chronically infected (Argilaguet et al., 2012). Noteworthy, results of gene deletions on ASFV attenuation and induction of protection may be dependent on the strain, and in some cases, the virulence of gene-deleted and parental viruses was indistinguishable, and some deletions affected the immunogenicity of the virus. Furthermore, some attenuated ASFV recombinants showed good protection against a related virulent strain, but themselves

induced low viremia, and undesirable side effects like arthritis and necrotic foci in the vaccinated pigs (Arias et al., 2017). Reis et al., 2016, have elucidated that the deletion of interferon modulators is a promising route for the development of rationally attenuated ASFV vaccine candidates. Thus, several single or multiple ASFV non-essential genes have been deleted and their impact on replication of the virus in cells and infection in pigs has provided valuable information required for the development of modified live vaccines (Dixon et al., 2019). Hakobyan et al., 2018, identified novel anti-ASFV rigid amphipathic fusion inhibitors with potential for future therapy, and Frouco et al., 2017, show that phenlbutyrate interferes with the replication of ASFV through disintegration of the hypoacetylation status induced by the virus and can potentially be used against ASFV. Several other antivirals could be used for early control of virus spread in new outbreak areas. These include resveratrol and oxyresveratol, microalgae, cholesterol lowering drugs or inhibitors of cholesterol transport, antitumoral lauryl-gallate, anticonvulsivant valproic acid, dynamin inhibitors, floroquinolones, serine protease inhibitors and specific peptides (Galindo & Alonso, 2017).

1.10 Pseudorabies virus as viral vector

Insertion of foreign sequences into other viral genomes has been used to achieve several purposes. For instance, sequences and genes were inserted into herpes simplex virus (HSV-1) DNA to identify putative *cis*-acting sites, to study heterologous complementation of mutated or deleted genes, or to use HSV-1 as a vector for foreign antigen expression (Roizman & Jenkins, 1985). Other DNA and RNA viruses like vaccinia virus, Newcastle disease virus, Fowl poxvirus and Pseudorabies virus (PrV) have been also used as vectors for foreign genes (Chen et al., 2011; Cruz et al., 2010; Hu et al., 2018; Roizman & Jenkins, 1985; Xu et al., 2015). Live attenuated PrV has been used as a vector to vaccinate against classical swine fever virus and porcine circovirus (Cruz et al., 2010). Recombinant Newcastle disease virus expressing the hemagglutinin (HA) of subtype H5 of avian influenza virus was considered a suitable bivalent

vaccine against both NDV and AIV, and usable as marker vaccine for the control of avian influenza (Veits et al., 2006). Methods to obtain transgene-expressing PrV recombinants using bacterial artificial chromosomes (BACs) containing the complete vector genome have been documented (Hubner et al., 2018b; Lerma et al., 2016). Some ASFV genes have previously been expressed in viral vectors. Replication-deficient human adenovirus and modified vaccinia virus Ankara have been used to express ASFV antigens (Lokhandwala et al., 2017). As will be continued in subsequent sections, our laboratory uses a PrV vaccine strain to express ASFV genes (Hubner et al., 2018b). PrV causes Aujeszky's disease (AD) in pigs, which can be efficiently controlled by vaccination using attenuated live vaccines. For vaccination against AD the first genetically engineered live virus vaccines were used, lacking genes that determine virulence. These genes encoded nonessential envelope proteins like glycoproteins E and I, or pUS9 that could be deleted from the genome without affecting viral replication in cell culture, or immunogenicity in pigs. This formed the basis for targeted genetic modification of PrV into a robust vector system for expression of foreign genes to make vaccines against several infectious swine diseases (Freuling et al., 2017).

1.11 Engineering of large DNA virus genomes

Due to the size of their genomes (100-300 kbp), and their high numbers of protein encoding genes, functional characterization of large DNA viruses like ASFV, poxviruses, herpesviruses and baculoviruses is still difficult. *In vitro* cleavage and ligation of DNA molecules of these sizes is usually not favorable because of the high frequency of conventional restriction endonuclease sites. Therefore, the traditional method for genetic manipulation of large DNA virus genomes utilizes homologous recombination with small engineered transfer plasmids in eukaryotic cell cultures. If, like in baculo- and herpesviruses, initiation of replication does not require virion proteins, virus recombinants can be generated simply by cotransfection of permissive cells with plasmid cloned DNA and genomic virus DNA, or with overlapping

plasmid or cosmid cloned DNA fragments spanning the complete virus genome (Roizman & Jenkins, 1985). However, if, like in poxviruses and ASFV, viral enzymes are essentially required to initiate viral gene expression and DNA replication, the cells have to be infected with related helper viruses, or with the targeted virus itself. To elucidate viral gene functions and to identify vaccine candidates, ASFV genomes have been mutagenized by homologous recombination in infected cells transfected with engineered plasmids to introduce deletions and/or reporter gene insertions for selection of recombinants (Afonso et al., 1998; Borca et al., 2017; Gallardo et al., 2018; Ramirez-Medina et al., 2019). Engineering of herpesviruses and poxviruses was significantly facilitated by BAC cloning and mutagenesis of their full-length genomes in *E. coli* (Domi & Moss, 2002; Messerle et al., 1997). Furthermore, methods have been described that enable more efficient cloning and manipulation of genomic DNA in bacteria like Gateway and bacteriophage-based homologous recombination systems, artificial restriction DNA cutters (ARCUT), and Quickchange mutagenesis (Rozwadowski et al., 2008; Yamamoto et al., 2005; Cotta-de-Almeida et al., 2003; Munteanu et al., 2012). However, in some cases propagation of viral DNA in bacteria is problematic since rearrangements or deletions of e.g. direct or inverted repeat sequences may occur, and transposable elements may be inserted (Merchlinsky & Moss, 1992; Rozwadowski et al., 2008; Mocarski & Roizman, 1980).

1.12 The CRISPR/Cas9 system

CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR associated nuclease) of Streptococcus pyogenes, and related systems have been initially identified as adaptive immune responses of bacteria to neutralize bacteriophages and other invading DNAs by RNA-guided site-specific cleavage (Jinek et al., 2012; Port & Bullock, 2016). After specific binding of a matching CRISPR RNA (crRNA) to an approx. 20-nucleotide sequence of the target DNA an interacting trans-activating crRNA (tracrRNA) activates the Cas9 nuclease which generates blunt DNA double strand breaks using its two catalytic domains (HNH and

RuvCI), at a site next to a protospacer acquisition motif (PAM) possessing the sequence (NGG) (Fig. 1-2).

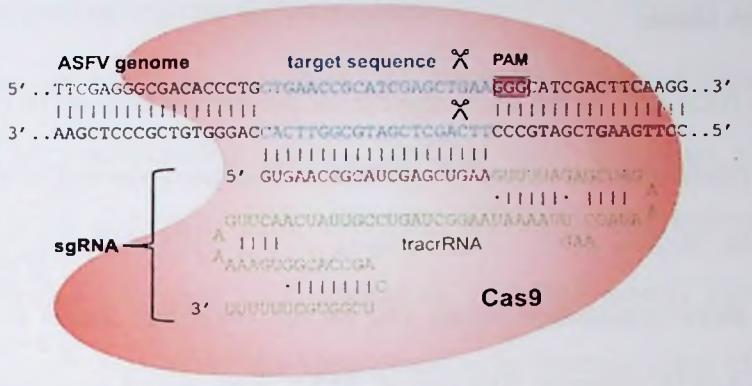


Figure 1-2: Modified CRISPR/Cas9 system consisting of a target-specific single guide RNA (sgRNA) and Cas9 nuclease. The essential protospacer adjacent motif (PAM: NGG) is also indicated.

The CRISPR/Cas9 system has been modified for efficient genome editing in eukaryotes (Cong et al., 2013; Pyzocha et al., 2014) by fusion of crRNA and tracrRNA genes to an artificial single guide RNA (sgRNA) gene that permits insertion of target specific sequences (Williams et al., 2016). Compared to previously used methods of eukaryotic genome editing CRISPR/Cas9 reduces cost and time required e.g. for generation of genetically modified mice that carry chromosomal deletions, point mutations, fusion tags, endogenous reporters and conditional alleles (Henao-Mejia et al., 2016). Modification, inactivation or substitution of Cas9 nuclease by other proteins enabled use of CRISPR/Cas9 also for epigenetic editing, gene regulation, chromatin engineering and imaging (Adli, 2018). The CRISPR/Cas9 system is also suitable for engineering of eukaryotic viruses. For instance, it has been used to mutagenize adenoviruses, HSV-1 and PrV (Bi et al., 2014), and to produce ASFV recombinants (Borca et al., 2018). CRISPR/Cas9 and CRISPR/Cas13 systems have been further used to establish resistance against DNA and RNA viruses in cell cultures, plants and animals either by mutation of host genes required for replication or by stable expression of the nuclease and antiviral sgRNAs (Bi et al., 2014; Mahas & Mahfouz, 2018). Whereas ASFV replication in cell culture could be substantially inhibited by CRISPR/Cas9 targeting of the viral p30 gene (Hübner et al., 2018a), partial deletion of the macrophage surface marker CD163 made CRISPR/Cas9-edited pigs

resistant against infection with porcine reproductive and respiratory syndrome virus (Burkard et al., 2017), but not against ASFV infection (Popescu et al., 2017).

1.13 Aims of the thesis

In Sub-Saharan Africa ASF is the most significant production disease in swine, harming pig farmers and pork producers due to loss of animals, animal products and by-products like meat, leather and gelatin (Bellini et al., 2016; Fasina et al., 2012; Stokstad, 2017). Even more devastating than direct production losses can be the economic losses resulting from trade restrictions after an ASF outbreak. The risk of ASFV introduction to and maintenance in new areas is mainly due to illegal and legal trade movement of live animals, and the importation or trade of animal feed, animal products and byproducts (Brown & Bevins, 2018; Gubbins et al., 2013). In Africa, many farmers own livestock as an insurance means during times of drought, and it has been shown that farming diversity stabilizes food production (Waha et al., 2018). Pigs are mainly kept for income generation, and as a demand-driven protein source (Mulumba-Mfumu et al., 2019). To date there are no licensed vaccines against ASFV (Argilaguet et al., 2012; Arias et al., 2017), which is still endemic in approx. 28 African countries, with a continued risk of transmission to Europe and other geographical areas where it has not been before (Arias et al., 2017; Stokstad, 2017). First-time outbreaks have recently occurred in new geographical areas like China, the biggest producer and consumer of pork, and have set an unprecedented disaster that is getting hard to control (Ge et al., 2018; Normile, 2018; Wang et al., 2018). In face of a business volume of global pig industry estimated at 150 billion dollar/year, control of ASF has to be improved urgently (Costard et al., 2009; Halasa et al., 2016). In the absence of an effective vaccine this can be only achieved by strict compliance to control measures (Jurado et al., 2018). Nevertheless, there is need for intensified research to find suitable vaccines, antiviral drugs or methods to improve ASF resistance of pigs (Arias et al., 2017; Stokstad, 2017; Zakaryan & Revilla, 2016). Owing to these facts, this PhD study

was designed to contribute to development of vaccine candidates and/or ASF resistant animals, improvement of diagnostic options and understanding of the complexity and diversity of ASFV. The experiments were set up with the following aims;

i) Generation of ASFV recombinants with defined deletions of possibly nonessential but virulence-determining genes (encoding viral thymidine kinase, dUTPase, ribonucleotide reductase, 9GL, pK145R, pA104R), and insertion of selectable reporter genes (encoding GFP, DsRed or truncated human CD4), supported by CRISPR/Cas9 targeting of the virus genes to be deleted. After characterization of their replication properties in cell culture the obtained ASFV mutants might be evaluated as potential attenuated live virus vaccines in pigs.

ii) Preparation of trans-complementing cells lines stably expressing presumably essential ASFV genes (e.g. p12), to permit isolation of corresponding gene deletion virus mutants which might then be used as safe "disabled in second cycle" (DISC) live vaccines.

iii) Improvement of reverse genetics by cotransfection of cells with genomic ASFV DNA and recombination plasmids, followed by booting up with phylogenetically distant ASF helper viruses which can be selectively inhibited by CRISPR/Cas9 using genotype-specific sgRNAs.

iv) Next generation sequencing (NGS) analyses of ASFV recombinants and parental strains.

v) Inhibition of ASFV replication in cell culture by stable expression of Cas9 and sgRNAs (Hübner et al., 2018a) against single or multiple essential virus genes (encoding p30, p12, and pE199L). Subsequent *in vitro* replication studies using reporter-gene labelled ASFV mutants might support selection of suitable transgenes for cloning of ASFV-resistant pigs.

vi) Expression of immunogenic ASFV proteins (p12, p54) in a PrV vaccine strain using CRISPR/Cas9 supported BAC mutagenesis (Hübner et al., 2018b), followed by *in vitro* characterization of the obtained PrV recombinants, which might be suitable as vectored vaccines against ASFV.

vii) Expression of ASFV gene products (p54, p12, p11.5, pK145R, p285L) as bacterial fusion proteins for preparation of monospecific rabbit antisera, which should allow identification through localization of their target proteins in infected cells and virions.

2 CHAPTER TWO: MATERIALS & METHODS

2.1 Materials

Name	*Company*
2.1.1 Equipment	
Acryl glass protection shields	Nalgene
Airflow controller AC3	Asecos waldner
AriaMx- Real-Time PCR system	Agilent Technologies
BAS screens (for radioactive probe detection)	FujiFilm
Fluorescent laser imaging scanner FLA 3000	FujiFilm
Biotrap Elutor (electroelution system)	Schleicher & Schuell, Whatmann
Centrifuges:	
Cooling centrifuge 5430R	Eppendorf
Micro centrifuges	Neolab
Multifuge 1S-R	Heraeus
Optima LE-80K ultracentrifuge	Beckman Coulter
Centrifuge 5810R	Eppendorf
Centrifuge 5403	Eppendorf
CO_2 incubators for cell culture	Sanyo
Contamination monitor LB124	Berthold Technologies
Cryotherm (Liquid Nitrogen tanks)	Biosafe
Electrophoresis PowerPac Basic	Bio-rad
Electrophoresis PowerPack P24	Biometra
Eraser (IP)	Raytest
Freezer (-20°C)	Liebherr
Freezer (-80°C)	New Brunswick Scientific
Gel dryer Model 583	Bio-rad
Genetic Analyzer 3130	Applied Biosystems
Icemaker	Manitowoc
Incubators (for bacteria)	Heraeus
Shaker Maxq 8000 (for bacterial cultures)	Thermo Scientific
Laminar Airflow Workstation class II	Thermo Scientific
Liquid Nitrogen Flask	Isotherm
MACS Multistand	Miltenyi Biotec GmbH

MACS µColumn	Miltenyi Biotec GmbH
µMACs separator	Miltenyi Biotec GmbH
Magnetic Stirrer M30E	Framo Gerätetechnik
Micro-electroelutor	Centrilutor
Micropulser	Bio-rad
Microscope Axio Vert A1 (Fluorescence)	Zeiss
Microscope Eclipse Ti-S (Fluorescence)	Nikon
Microscope Eclipse TS100	Nikon
Microwave	Privileg
Milli-Q water purification system	Millipore
Mini Sub™ electrophoresis cell	Bio-rad
Mini-Protean Tetra Cell	Bio-rad
Molecular Imager Gel Doc XR	Bio-rad
Multipette E3	Eppendorf
Mr. Frosty	Nalgene
Nanophotometer	Implen
Peqlab electrophoresis system	Peqlab
pH Meter (Microprocesor pH539)	Wtw
Pipettes (Multichannel)	Micronic systems
Pipettes	Eppendorf
Refrigerator	Liebherr
Rocker	Edmund Bühler
Rocker Duomax 1030	Heidolph
Rocking Shaker DRS-12	neoLabline
Safe cabinet	Berner Flowsafe
Safe cabinet	Thermo Electron Corp'n
Safety Bunsen burner + Butane gas	Wld-tec
Southern Blot Oven 7601	Gfl
Scale H-G Tuning-Fork	Vibra Shirko Denshi
Semi-Dry blotter unit V20-SDB	Biostep GmbH
Shaker Reax2000 (primers)	Heidolph
Sonifier (Digital) 450D	Branson
Suction Aspirator	Inotech, Knf Neuberger
Thermal cycler C100	Bio-rad

Thermal cycler	Biometra Tone
Thermomixer (1.5ml)	Eppendorf
Thermostat plus	Eppendorf
Tubes and micro-tube racks	Nalgene
Unijet II Refrigerated aspirator	Uniequip
UV light transilluminator	Biostep
UV Stratalinker 1800	Stratagene
Vacuum system BVC 21 NT	Vacuubrand
Versa Doc Imaging system 4000MP	Bio-rad
Vortex-Genie 2	Scientific industries
Waterbath	GFL
Weigh scales	Kern, Sartorius
Western blot MP 3AP (Power supply)	Major Science
Wide Mini SubCell	Bio-rad

2.1.2 Disposables

Cell culture flasks (25-, 75-, 162 cm^2)	Corning Inc., USA
Amersham Hybond-N^+	GE Healthcare
Cell culture plates (6-, 24-, 48-, 96-well)	Corning Inc., USA
Cell scrapers	Sarstedt
Combitips	Eppendorf
Cryoboxes	NeoLab
Cryotubes	Nunc
Desmanol industrial pure disinfection	Schülke
Disposable serological pipettes (sterile): 2, 5, 10, 25 ml	Costar
Centrifuge tubes: 15 ml (120 x 17 mm), 50 ml (114 x 28 mm)	Sarstedt
Filter unit	Millex
Filters (nitrocellulose)	Sartorius
Folded filter paper 125 mm	Mn
Glass plates	Biorad
Glassware	Simax/Duran
Hand gloves Eco Nitrile PF 250	ecoShield
Hand gloves latex free powder free	Medimex
Magnetic stirrers	

Microtube tough spots	Roth
Mikrozid AF/ sensitive liquid	Schülke
Nalgene bottle	Nalgene
Nitrocellulose blotting membrane	GE Healthcare
Parafilm	Bemis
PCR tubes	Biorad
Perform advanced alcohol EP disinfection	Schülke
Pipette filter tips for PCR	Nerbe plus
Pipette tips	Eppendorf
Plastic petri dishes and covers (sterile): 90 x 16 mm	Sarstedt
Plastic ware	Vitlab/Brand
Reaction tubes (0.5-, 1.5-, 2 ml)	Eppendorf, Sarstedt
Semi-micro disposable cuvettes	Brand
Single fold hand towel	Tork
Special autoclavable bags	nerbe plus
Stericup and Steritop vacuum driven disposable filtration system	Millipore
Syringes	Omnifix
Ultra centrifugation tubes	Beckman
Virkon S	DuPont

2.1.3 Software and websites

https://www.ncbi.nlm.nih.gov/	NCBI
https://www.ncbi.nlm.nih.gov/pubmed	NCBI
AIDA (Advanced Image Data Analyzer) version 5.0 SP1	Raytest
BASReader control for Fujifilm FLA 3000 scanner version 3.14	Raytest
Geneious software version 11.1.5	Biomatters
Image Lab 5.2.	Bio-Rad
Microsoft Office Professional Plus 2016	Microsoft
NIS Elements software version 4.20 (for fluorescence images)	Nikon
https://www.expasy.org	Swiss Institute of Bioinformatics
www.uniprot.org	UniProt Consortium
Quantity one 1-D analysis software version 4.6.9 (WB)	Bio-Rad

2.1.4 PCR kits

Go *Taq* PCR	Promega
KOD-Xtreme PCR	Novagen
SuperFi DNA Polymerase PCR	Invitrogen

2.1.5 Other Kits

BigDye terminator v1.1 cycle sequencing kit	Applied Biosystems
NucleoSeq columns	Macherey-Nagel
QIAamp DNA Mini kit	Qiagen
QIAfilter Plasmid MAXI kit	Qiagen
QIAfilter Plasmid MIDI kit	Qiagen
QuikChange II XL Site-Directed Mutagenesis Kit	Agilent
RediPrime II system	GE Healthcare
Zymoclean Gel DNA Recovery kit	Zymoresearch
MACS molecular µcolumns	Miltenyi Biotech GmbH

2.1.6 Enzymes for DNA cloning

Alkaline phosphatase (AP)	Roche
DNaseI	Roche
Klenow DNA Polymerase I	New England Biolabs
Phusion DNA polymerase	New England Biolabs
Restriction enzymes	New England Biolabs
RNaseA	Serva
T4 DNA Ligase	Roche
T4 Polynucleotide kinase	New England Biolabs
T5 Exonuclease	New England Biolabs

2.1.7 Bacteria

E. coli strain XL1-Blue MRF'	Agilent

2.1.8 Transfection systems for mammalian cells

FuGENE HD Transfection system	Promega
K2 Transfection system	Biontex
X-tremeGENE 9 DNA transfection system	Roche

X-tremeGENE HP DNA transfection system	Roche

2.1.9 Mammalian Cell lines

Rabbit Kidney 13 (RK13) cell line	Bio-bank, FLI
Wild boar lung (WSL-HP) cell line	Bio-bank, FLI
WSL-Bu25 cell line	Bio-bank, FLI

2.1.10 Plasmids

2.1.10.1 General cloning vector

pUC19 (Norrander et al., 1983)	New England Biolabs

2.1.10.2 Plasmid vector for reporter gene fusions

pEGFP-N1	Clontech

2.1.10.3 Mammalian expression vectors

pcDNA3, pcDNA3.1(+)	Invitrogen

2.1.10.4 Bacterial expression vectors

pGEX-4-T (-1, -2, -3)	GE Healthcare

2.1.10.5 Original plasmid vectors for CRISPR/Cas9 cloning

pX330-U6-Chimeric_BB-CBh-hSpCas9 (Cong et al., 2013)	Addgene
Multiplex CRISPR/Cas9 Assembly System Kit (Sakuma et al., 2014)	Addgene
pSB-U6-gRNA-eCas9-EGFP-Neo	kindly provided by B. Petersen (FLI)

2.1.11 Antibiotics

	Final Conc'n	*Company*
Ampicillin (50 mg/ml in H_2O)	100 µg/ml	Serva
Chloramphenicol (30 mg/ml in EtOH)	30 µg/ml	Serva
G418 Sulphate (50 mg/ml)	500 µg/ml	Corning
Kanamycin (50 mg/ml in H_2O)	50 µg/ml	Roth
Streptomycin (50 mg/ml in H_2O)	50 µg/ml	Sigma
Tetracyclin (10 mg/ml in 50% EtOH)	12.5 µg/ml	Roth
Penicillin/Streptomycin (10000U/ml/10000µg/ml)	1%	Biochrom GmBH
Amphotericin B (2.5 mg/ml in DMSO)	2.5 µg/ml	Sigma

2.1.12 Chemicals

2-Mercaptoethanol	Merck
2-Propanol	Roth
Acetic acid	Roth
Acrylamide	Roth
Adenosine 5'-triphospate (ATP)	Sigma
Agar (Bacterial grade)	MP Biomedicals
Agarose	Seakem/Biozym
Ammonium Persulphate (APS)	ThermoFisher
Bicinchoninic acid (BCA)	Pierce
Bisacrylamide	Roth
BlueJuice	Invitrogen
Boric acid	Roth
Bovine serum albumin, fraction V (BSA)	Serva
Bromodeoxuridine (BrdU)	Roche
Bromophenol blue	Sigma
$C_2H_3NaO_2$ - 3 H_2O	Roth
Casein hydrolysate	Oxoid
Chloroform	Roth
Coomassie brilliant blue	Roth
Cu_2SO_4	Roth
D(+)-Glucose	Roth
DABCO	Sigma
Dextrose	Sigma
Dimethyl formamide	Roth
Dithiothreitol (DTT)	Sigma, Promega
DMSO	Merck
DNA from fish sperm	Serva
DNA Ladder	Invitrogen
dNTPs	Roche
Ethylenediaminetetraacetic acid (EDTA)	Roth, Serva
Ethanol	Roth
Ethidium bromide	Roth
F-12 (Ham's)	gibco

Fetal Bovine Serum	Biochrom GmbH
Formaldehyde	Roth
Glucose	Roth
Glutamax II	gibco
Glycerol	Roth
Glycin	Roth
Hydrochloric acid (37%)	Roth
Iscove's Modified Dulbecco's medium (IMDM)	gibco
Isoamyl alcohol	Merck
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	MP Bio
KCl	Roth
KH_2PO4	Reanal
Magnesium chloride	Roth
MEM (Earles' salts)	gibco
MEM Eagle (Hanks' salts)	gibco
Methanol	Roth
Methylcellulose	Sigma
$MgCl_2$	Roth
$MgSO_4$	Roth
Na_2CO_3	Roth
$Na_2HPO_4.2H_2O$	AppliChem, Roth
NaCl	Roth
$NaHCO_3$	Roth
NaOH	Roth
n-Butanol	Veb Laborchemie Apolda
Non-essential amino acids	Gibco
Opti-MEM I	Gibco
Page Ruler	ThermoScientific
Paraformaldehyde	Roth
Polyethylene glycol (PEG)-8000 (or -4000)	Sigma
Phenol/chloroform/isoamyl alcohol	Roth
Pronase E	Serva
Propidium iodide	ThermoFisher
RNase A	Serva

Sarcosyl/ N-lauroylsarcosine	Sigma
Sodium Dodecyl Sulfate (SDS)	Roth
Sodium acetate trihydrate	Roth
Sodium pyruvate	Merck
Sodium tartrate	Merck
Sucrose	Roth
Supersignal West Pico Chemiluminescent Substrate	ThermoScientific
Tetramethylethylenediamine (TEMED)	Roth
Tri-Natrium citrate	Roth
Tris (Ultrapure)	Invitrogen
Triton X-100	Serva
Trypsin	Invitrogen
Tween 20	Applichem
Water – nuclease free	Roth
X-Gal	Invitrogen
Xylencyanol (FF)	Ferak
Yeast extract	MP Biomedicals

2.1.13 Buffers and solutions

2.1.13.1 For agarose gel electrophoresis

DNA marker

1 Kb plus DNA Ladder	50 µl
10X BlueJuice	50 µl
H_2O	400 µl

50X TAE buffer

Tris (Ultrapure)	242 g
Acetic acid	57.1 ml
0.5M EDTA (pH 8)	100 ml
H_2O	up to 1 L

0.7% Agarose gel

Agarose	2.1 g
1X TAE buffer	300 ml

- Melt for 7-8 minutes in microwave, cool to 56°C, then add

Ethidium bromide (10 mg/ml)	10 µl

- Pour gel, or store at 56°C

10X TBE buffer

Tris	107.8 g
Boric acid	55 g
0.5M EDTA (pH 8.0)	40 ml (9.3 g)
H_2O	up to 1 L

10X DNA sample buffer

0.25% Bromophenol blue	50 mg
0.25% Xylencyanol (FF)	50 mg
- Solve in 14 ml 0.5M EDTA (pH 8.0)	
30% Glycerol	6 ml

2.1.13.2 For Southern blot analyses

Denatured fish sperm DNA (10 mg/ml)

DNA from fish sperm	1 g
H_2O	100 ml

- Boil for 15 minutes, sonicate until completely dissolved, store at 4°C.

20X SSC

3M NaCl	175.3 g
0.3M tri-Natrium citrate – 2 H_2O	88.2 g
H_2O	up to 500 ml

- Adjust to pH 7.0

20X SSPE

3.6M NaCl	105.0 g
0.2M $Na_2HPO_4.2H_2O$	17.7 g
20mM EDTA	3.72 g
H_2O	up to 1 L

Depurination buffer (0.25N HCl)

37% HCl	20.81 ml
H_2O	979.2 ml

Denaturation buffer

0.5N NaOH	40 g
0.9M NaCl	105.19 g
H_2O	up to 2 L

Neutralization buffer

0.9M NaCl	105.19 g
1M Tris	242.14 g
H_2O	1.8 L
- Adjust pH to 7.4 with 37% HCl,	add H_2O to 2 L

Hybridization buffer

H_2O	9 ml
4X SSPE	3 ml 20X SSPE
1% SDS	1.5 ml 10% SDS
500 µg/ml Fish sperm DNA	0.75 ml 10 mg/ml solution
0.5% Low fat milk	0.75 ml 10% suspension

2.1.13.3 For DNA preparation and cloning

TE buffer

10mM Tris	1.21 g
1mM EDTA	0.372 g
H_2O	1 L

- Adjust to pH 7.4 or 8.0, autoclave

TEN buffer

20mM Tris	1.21 g
1mM EDTA	0.186 g
150mM NaCl	4.35 g

H_2O	500 ml
- Adjust to pH 7.4, autoclave	
Sarcosyl buffer	
3% Sarcosyl/ N-lauroylsarcosine	15 g
75mM Tris	4.54 g
25mM EDTA	4.65 g
H_2O	500 ml
- adjust to pH 8.0	
5X Pronase buffer	
0.5M NaCl	29.22 g
0.5M Tris	60.57 g
H_2O	up to 1 L
- adjust to pH 7.2	
Pronase E solution (10 mg/ml)	
Pronase E	1 g
5X Pronase buffer	100 ml
- Incubate for 1h at 37°C, store at -20°C	
RNase A solution (10 mg/ml)	
RNase A	500 mg
10mM Tris	60.6 mg
15mM NaCl	43.8 mg
H_2O	50 ml
DNA-miniprep solution I	
20% D(+)-Glucose	10 ml
0.5M EDTA (pH 8.0)	4 ml
1M Tris HCl (pH 8.0)	5 ml
H_2O	181 ml
DNA-miniprep solution II	
5N NaOH	10 ml
10% SDS	25 ml
H_2O	215 ml
3 M Na-acetate	
$C_2H_3NaO_2$ - 3 H_2O	81.65 g
H_2O	up to 200 ml
- Adjust to pH 4.8 with acetic acid, autoclave	
5X Pre-assembly buffer	
0.5mM Tris-HCl pH 7.5	0.5 ml 1M solution
50mM $MgCl_2$	50 µl 1M solution
1mM dATP, dCTP, dGTP, dTTP	100 µl 10mM mix
50mM DTT	50 µl 1M DTT
25% PEG-8000	250 mg
H_2O	150 µl
- Store at -20°C	
2X Hot fusion buffer	
5X Pre-assembly buffer	160 µl
T5 Exonuclease (10 U/µl)	0.3 µl
Phusion DNA Polymerase (2U/µl)	10 µl
H_2O	230 µl
- Store 10 µl aliquots in PCR tubes at -20°C.	

2.1.13.4 For bacterial cultures

2X YT-medium

Casein hydrolysate	16 g
NaCl	5 g
Yeast extract	10 g
H_2O	up to 1 L

- Autoclave

LB-medium

Casein hydrolysate	10 g
NaCl	10 g
Yeast extract	5 g
H_2O	up to 1 L

- Autoclave

LB-medium low salt

Casein hydrolysate	5 g
NaCl	2.5 g
Yeast extract	2.5 g
H_2O	up to 500 ml

- Adjust to pH 7.5, autoclave

LB-agar

NaCl	5 g
Casein Hydrolysate	5 g
Yeast Extract	2.5 g
Agar (bacterial grade)	10 g
H_2O	up to 500 ml

- Autoclave, cool to 56°C, add antibiotics, pour plates, store at 4°C

2 % X-Gal solution

X –Gal	200 mg
Dimethyl formamide	10 ml

- Store 2 ml aliquots at -20°C.

SOA-Medium

2% Casein hydrolysate	20 g
0.5% Yeast extract	5 g
10 mM NaCl	580 mg
2.5 mM KCl	185 mg
H_2O	up to 1 L

- Autoclave

SOB-Medium:

SOA-Medium	98 ml
1mM $MgSO_4$	1 ml 1M solution
1mM $MgCl_2$	1 ml 1M solution

- Add autoclaved salt solutions!

SOC-Medium:

SOB	10 ml
2mM Glucose	100 µl 2 M solution

- Add autoclaved glucose solution!

TSS – Transformation medium

LB medium	85 ml
10% PEG 4000	10 g
5% DMSO	5 ml

$MgCl_2 - 6\ H_2O$	2.17 g

- Sterile filtration

TSS –Storage medium

LB medium	65 ml
10% PEG 4000	10 g
5% DMSO	5 ml
$MgCl_2 - 6\ H_2O$	2.17 g
20% Glycerol	20 g

- Sterile filtration

2.1.13.5 Cell culture media and reagents

ZB 28 (prepared by the cell culture laboratory of the FLI)

F-12 (Ham's)	5.32 g
IMDM	8.80 g
$NaHCO_3$	2.45 g
H_2O	up to 1 L

- Adjust to pH 7.2, sterile filtration
- Add 10% FBS, store at 4°C

ZB 28b

ZB28 with only 5% FBS

ZB5 (prepared by the cell culture laboratory of the FLI)

MEM Eagle (Hank's salts)	5.32 g
MEM (Earles' salts)	4.76 g
$NaHCO_3$	1.25 g
Non-essential amino acids (100x)	10 ml
Na-pyruvate	120 mg
H_2O	up to 1 L

- Adjust to pH 7.2, sterile filtration
- Add 10% FBS, store at 4°C

ZB 5b

- ZB5 with only 5% FBS

ATV and ATV-D (prepared by the cell culture laboratory of the FLI)

	ATV	ATV-D
NaCl	8.5 g	8.5 g
KCl	0.4 g	0.4 g
Dextrose	1.0 g	1.0 g
$NaHCO_3$	0.58 g	0.58 g
Trypsin	0.5 g	1.0 g
EDTA	0.2 g	0.2 g
H_2O	1 L	1 L

- Adjust to pH 7.2, sterile filtration

5% formalin for fixation

37% formaldehyde solution	50 ml
H_2O	up to 1 L

1X Methocel

Methylcellulose	8 g
MEM (w/o L-glutamin)	3.76 g
H_2O	390 ml

- Autoclave with stir bar, cool to RT

$NaHCO_3$	880 mg
PBS	6 ml

- Solubilize by heating, sterile filtration, add to autoclaved medium
- Glutamax II 4 ml
- Add to autoclaved medium and stir overnight at 4°C, store at 4°C

0.25X Methocel

1X Methocel	100 ml
ZB 5b	300 ml
Penicillin/Streptomycin (100x)	4 ml

10X PBS

NaCl	80 g
KH_2PO_4	2 g
KCl	2 g
$Na_2HPO_4.2H_2O$	11.5 g
H_2O	up to 1 L

- Adjust with NaOH to pH 7.4

2.1.13.6 For SDS-PAGE and Western blot

SDS sample buffer

Glycerol	10 ml
0.5M Tris HCl (pH 6.8)	12.5 ml
1% Bromphenol blue in H_2O	0.5 ml
20% SDS in H_2O	10 ml
2-Mercaptoethanol	5 ml
H_2O	12 ml

0.5M Tris HCl pH 6.8

Tris	30.28 g
H_2O	300 ml
- Adjust to pH 6.8 with HCl,	fill up to 500 ml H_2O

1.5M Tris HCl pH 8.8

Tris	90.82 g
H_2O	300 ml
- Adjust to pH 8.8 with HCl,	fill up to 500 ml with H_2O

30% Acrylamide solution

Acrylamide	29 g
N, N´-Methylenbisacrylamide	1 g
H_2O	up to 100 ml

- Filtration

Protein minigels

Separating gel	**7.5%**	**8%**	**10%**	**12%**	**13%**	**14%**	**15%**	**Stacking gel**	**4 %**
1.5M Tris-HCl (ml)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.5M Tris-HCl (ml)	1.25
30% Acrylamide (ml)	2.5	2.7	3.33	4.0	4.33	4.67	5.0		0.65
H_2O (ml)	4.95	4.7	4.1	3.6	3.2	2.9	2.6		3.15
10% APS (µl)	50	50	50	50	50	50	50		25
TEMED (µl)	10	10	10	10	10	10	10		5
10% SDS (µl)	100	100	100	100	100	100	100		50

Preparative protein gels

Separating gel (10 % acrylamide)

H_2O	40 ml
1.5M Tris-HCl (pH 8.8)	25 ml

30% acrylamide	33.5 ml
10% SDS	1 ml
- Degas for 15 min	
10% APS	340 µl
TEMED	60 µl
- Pour gels, overlay with 1 ml water-saturated n-butanol	
Stacking gel	
H_2O	12.5 ml
0.5M Tris-HCl (pH 6.8)	5 ml
30% acrylamide	2 ml
10% SDS	200 µl
- Degas for 15 min	
10% APS	200 µl
TEMED	10 µl
10X PAGE buffer	
250mM Tris	30.285 g
2M Glycin	150.14 g
H_2O	up to 1 L
1X PAGE buffer	
10X PAGE buffer	100 ml
10% SDS	10 ml
H_2O	890 ml
10X TBS buffer	
150mM NaCl	87 g
10mM Tris	12.1 g
H_2O	up to 1 L
- Adjust to pH 8.0	
TBS-T buffer	
10X TBS	100 ml
Tween 20	2.5 ml
H_2O	897.5 ml
Transfer buffer	
25mM Tris	3.03 g
200mM Glycin	14.4 g
0.01% SDS	100 mg
H_2O	800 ml
Methanol	200 ml

*2.1.13.7 **For protein purification***

BCA solutions

Solution A:	
25.8 mM Bicinchoninic acid	2.218 g
2% Na_2CO_3	5 g
0.16% Sodium tartrate	538 mg
0.95% $NaHCO_3$	2.375 g
H_2O	250 ml
- pH (5M NaOH)	11.25
Solution B:	
4% $CuSO_4$ in H_2O	
Working solution:	198 parts (v/v) solution A + 2 parts (v/v) solution B

Staining solution for analytic protein gels

0.2% Coomassie brilliant blue R250	1.25 g
H_2O	200 ml
10% Acetic acid	50 ml
50% Methanol	250 ml

Staining solution for preparative protein gels

0.2% Coomassie Brilliant blue R250	1.25 g
H_2O	400 ml
0.2% Acetic acid	1 ml
20% Methanol	100 ml

Fixing solution for analytic protein gels

10% Acetic acid	250 ml
40% Methanol	1 L
H_2O	1.25 L

2.1.14 Virus strains

ASFV Kenya 1033 genotype IX virus	provided by Richard Bishop, ILRI, Nairobi
ASFV-Armenia genotype II virus	provided by Sandra Blome
PrV vaccine strain Bartha	provided by Thomas C. Mettenleiter

2.1.15 Antibodies

2.1.15.1 Primary antibodies

Rabbit α-GST (IUL34)	provided by Walter Fuchs
Rabbit α-MalA104Rtick	provided Katrin Giesow
Rabbit α-GST-p72A	provided by Walter Fuchs
Mouse α-T Cell CD4 mAb (clone MT310)	Sigma
Mouse α-FLAG mAb (M2)	Sigma

2.1.15.2 Secondary antibodies

Horseradish peroxide (HRP) goat α-rabbit IgG	(Dil.: 1:20000) Jackson Immuno Research
Horseradish peroxide (HRP) goat α-mouse IgG	(Dil.: 1:20000) Jackson Immuno Research
AlexaFluor 488 goat α-rabbit IgG	(Dil.: 1:1000) Invitrogen
AlexaFluor 488 goat α-mouse IgG	(Dil.: 1:1000) Invitrogen

2.1.15.3 CD4 Microbeads

MACS CD4 microbeads	Miltenyi Biotech GmbH

2.1.16 Animals

Chinchilla bastard rabbits bred at FLI

2.2 Methods

2.2.1 Tissue culture

2.2.1.1 Propagation of mammalian cell lines

Wild boar lung (WSL-HP) cells were cultivated in MEM (ZB 28) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 2.5% CO_2 and water-saturated atmosphere. To split the cells, confluent T25/T75/T162 flasks were washed with ATV-D once and incubated with 1/3/6 ml ATV-D at 37°C for 10 min. Cells were homogenized with an equal volume of ZB 28 and diluted up to 1:12 in used or new flasks containing 5/15/30 ml medium. Neomycin-resistant cell clones from this and earlier (Hübner et al., 2018b) studies (e.g. WSL-gRp30, WSL-gRAKp30, WSL-gRE199L, WSL-gRAO61R, WSL-eCas9neoR, WSL-eCas4xASFVgR and WSL-4xASFVgR) were propagated in medium supplemented with 500 µg/ml G418. Rabbit kidney (RK13) cells used for propagation of PrV were cultivated and split similarly, but with ZB 5 instead of ZB 28 medium and ATV instead of ATV-D trypsin solution.

2.2.1.2 Preparation of cell samples for Western blotting

Samples for Western blot analysis were prepared by transferring 1 ml homogenized cell suspension into reaction tubes and centrifuging for 3 min at 15000 rpm. Pellets were washed with 1 ml PBS, centrifuged 3 min 15000 rpm, resuspended in 100 µl SDS sample buffer (SDS-SB) and stored at -20°C.

2.2.2 Transfection of mammalian cells for generation of transgene-expressing cell lines and virus recombinants

WSL and RK13 cells or their derivatives were used for transfection and/or combined transfection and infection experiments.

2.2.2.1 K2 Transfection System (Biontex)

The K2 system was used for transfection of WSL-HP derived cells with expression, CRISPR/Cas9 and recombination plasmids because of low cytotoxicity and higher efficiency compared to other reagents tested. The cells were grown overnight to subconfluent monolayers in 12-well plates, washed once with ZB 5b, and overlaid with 1 ml/well of ZB 5b containing 1% penicillin/streptomycin solution, followed by addition of 20 µl of K2 Multiplier to each well and incubation at 37°C for 2 hours. Two tubes with 60 µl Opti-MEM medium per well were prepared, and 1.5 µg plasmid DNA or 3.5 µl K2 Reagent were added, respectively, and mixed thoroughly. Then the reagent dilution was mixed gently with the DNA dilution and incubated at RT for 20 min. The solution was added dropwise to one well of Multiplier treated cells, followed by rocking at RT for 20 min. Then the plates were centrifuged for 1 hour at 2000 rpm 25°C and incubated at 37°C. After one day, the cells were washed once with ZB 28b, overlaid 1 ml ZB 28b + 1% penicillin/streptomycin, and incubation at 37°C was continued.

2.2.2.2 Transfection with X-tremeGeneHP Reagent (Roche)

X-tremeGENE HP was preferred for plasmid transfection of RK13 cells because of easier application and lower costs. Again the cells were grown overnight in 12-well plates, washed once with ZB 5b, and overlaid with 1 ml/well of ZB 5b containing 1% penicillin/streptomycin solution. Approximately 1.0 µg of plasmid DNA were diluted in 100 µl Opti-MEM and mixed by vortexing. Then 1.5 µl XtremeGENE HP were added slowly, mixed gently, and incubated for 20 min at RT. The solution was added dropwise to one cell culture well, followed by rocking for 20 min at RT, and centrifugation for 1 hour at 2000 rpm and 25°C. After overnight incubation at 37°C, cells were washed once with ZB 5b, overlaid with 1 ml fresh ZB 5b + 1% penicillin/streptomycin, and incubation was continued.

2.2.2.3 Transfection with FuGENE HD Reagent (Promega)

For co-transfection of cells with plasmids and genomic ASFV or PrV DNA the FuGENE HD reagent was used since it facilitates uptake of large DNA molecules. Cells grown in 12-well

plates were washed with ZB 5b, and overlaid with 1 ml fresh ZB 5b + 1% penicillin/streptomycin. Approximately 1 µg of virus DNA, and 250 ng of plasmid(s) were diluted in 50 µl Opti-MEM and mixed, before 4 µl FuGENE were added slowly, and mixed gently. After 20 min at RT the solution was added dropwise to the cells, followed by shaking for 20 min at RT, and centrifugation for 1 h at 2000 rpm and 25°C. After overnight incubation RK13 cells were washed and overlaid with fresh ZB 5b containing 100 U/ml penicillin and 100 µg/ml streptomycin, and incubation at 37°C was continued until pronounced CPE was observed.

2.2.2.4 ASFV infection after transfection

Since ASFV DNA, unlike herpesvirus DNA, is not infectious, native or transgene-expressing WSL-HP cells transfected with recombination plasmids, or co-transfected with plasmids and genomic DNA, had to be additionally infected with ASFV 5 hours after transfection at a multiplicity (MOI) of approximately 0.1. To this end, the medium was aspirated from transfected and control wells, 500 µl per well of virus dilutions in ZB 28b were added, and the 12-well plates were centrifuged for 1 hour at 2000 rpm and 25°C. Then the inoculum was aspirated, 1.5 ml ZB 28b with 1% penicillin/streptomycin per well were added and incubation was continued at 37°C until development of CPE.

2.2.2.5 Preparation of virus-infected cells for Western blot analysis

Confluent T25 flasks of WSL-HP cells for ASFV-Kenya and ASFV-Armenia viruses, or RK13 cells for PrV-Bartha derivates were infected at a multiplicity (MOI) of ≥ 1 in 1-2 ml medium. After 2 h at 37°C the inoculum was replaced by 5 ml ZB 28b or ZB 5b containing 1% penicillin/streptomycin, and incubation at 37°C was continued until pronounced CPE became visible. Then the cells were completely detached by vigorous shaking, the lysates were transferred to 15ml tubes and centrifuged for 5 min at 4000 rpm. The supernatant was aspirated and pellets were resuspended in 1 ml PBS, transferred to reaction tubes and centrifuged for 3 min at 15000 rpm. The supernatant was again aspirated and pellets were resuspended in 250 µl

SDS-SB and stored at -20°C. Prior to export from the BSL4 containment, ASFV-infected samples were incubated for 30 min at 95°C.

2.2.2.6 Generation of CRISPR/Cas9- and ASFV protein expressing WSL cell lines

Following plasmid transfection in 12well plates WSL-HP cells were incubated for 1-2 days at 37°C. Three 50 ml tubes were prepared containing 18 ml of fresh ZB 28, 4.5 ml used ZB 28 (from confluent WSL-HP cell cultures) and 225 µl G418 (500 mg/ml) each. The transfected well was washed with ATV-D, incubated with 300 µl ATV-D for 10 min at 37°C, homogenized with 700 µl of the medium from first 50 ml tube, and returned. After mixing, 2.5 ml were transferred into second tube, mixed and 2.5 ml were transferred in third tube. The cell dilutions were seeded into three 96well plates (200 µl/well), and incubated at 37°C. After approximately 14 days wells containing one single cell colony covering ≥ 30% of the area were washed with ATV-D, incubated with 75 µl ATV-D for 10 min at 37°C, homogenized with 150 µl medium and added to 1.5 ml ZB 28 + 500 µg/ml G418 in 12well plates and incubated at 37°C. Confluent wells were washed with ATV-D, incubated with 300 µl ATV-D for 10 min at 37°C, homogenized with 700 µl medium and 100 µl of the cells with 1.5 ml ZB 28 + 500 µg/ml G418 were further propagated in the 12well plates, whereas the rest was used for preparation of DNA (see 2.2.4) or Western blot (see 2.2.1.2) samples for transgene characterization. Positively tested wells were trypsinized as above and cells were added to 5 ml ZB 28 + 500 µg/ml G418 in T25 flasks and further propagated.

2.2.3 Cryopreservation of cells

For storage at -80°C the trypsinized cells of T25 flasks were homogenized with 10 ml ZB 28 and centrifuged in 15ml tubes for 2 min at 2000 rpm. The pellets were resuspended in 1 ml ZB 28 and 80 µl DMSO as cryoprotectant, and transferred to cryo-vials. The vials were initially stored at -80°C using a Mr. Frosty freezing container, which cools down the samples at a constant rate of -1°C/min. For long term storage the vials should be transferred to liquid

nitrogen. For reuse, the cells were quickly thawed at 37°C, added to 10 ml prewarmed ZB 28 in a T25 flask, and incubated overnight at 37°C. Then the medium was replaced by 5 ml fresh ZB 28 optionally containing 500 µg/ml G418.

2.2.4 Preparation of cells for DNA extraction

For DNA extraction from cells, 1 ml homogenized cells were transferred into reaction tubes and centrifuged 3 min at 15000 rpm. The pellets were washed with 1 ml TEN buffer, centrifuged 3 min 15000 rpm, resuspended in 250 µl TEN buffer, and stored at -20°C.

2.2.5 Virus and cell DNA preparations (Fuchs & Mettenleiter, 1996).

2.2.5.1 DNA extraction from cell lysates

To cell lysates resuspended in 250 µl TEN buffer (see above) subsequently 125 µl sarcosyl buffer, followed by incubation for 15 min at 65°C, 2.5 µl RNase A (10 mg/ml), followed by incubation for 30 min at 37°C, and 25 µl Pronase E (10mg/ml), followed by incubation for 2 h at 42°C, were added.

2.2.5.2 DNA preparation from virus-infected cells

After pronounced CPE developed ASFV- or PrV-infected T25 WSL-HP or RK13 cells were frozen at -80°C and thawed at 37°C. The content was transferred, centrifuged for 10 min at 4000 rpm and the virus supernatants were stored in aliquots at -80°C. The pellets were washed with 10 ml TEN buffer, centrifuged again for 10 min at 4000 rpm, and resuspended in 3 ml TEN buffer. For export from the BSL4 facility, ASFV-infected cell lysates were resuspended in 500 µl TEN instead and incubated with 15 µl 10% SDS for 30 min at 75°C to inactivate the virus reliably. Afterwards 2.5 ml TEN were added. Subsequently to all samples, 1.5 ml sarcosyl buffer, followed by incubation at 65°C for 15 min, 30 µl RNase A (10 mg/ml), followed by incubation at 37°C for 30 minutes, and 300 µl Pronase E (10 mg/ml), followed by 2 h or overnight incubation at 42°C, were added.

2.2.5.3 Virion DNA preparation

To prepare high quality ASF virion DNA for next generation sequencing (NGS) or transfection experiments (e.g. of ASFV-KenyaΔCD2vDsRed) from WSL-HP cells ≥ 2 infected T162 flasks were freeze-thawed (-80°C/37°C). Lysates were centrifuged in 50ml tubes for 15 min at 4000 rpm. The supernatant was put into SW32 tubes, ultra-centrifuged for 1 hr at 20,000 rpm and 4°C. After aspiration, the pellets were resuspended in 500 µl of TE (pH 7.4) buffer each, and transferred to reaction tubes. 10 µl 1 M $MgCl_2$ + 20 µl DNaseI (5 U/µl) + 10 µl RNase A (10 mg/ml) were added followed by incubation for 1 h at 37°C. Approximately 30 ml PBS were added to an SW32 tube, underlaid with 6 ml 30 % sucrose in PBS, and the enzyme treated virus suspensions were carefully added on top. Ultra-centrifugation for 2 hr at 20,000 rpm and 4°C followed and the supernatant was aspirated completely. The pellet was resuspended in 500 µl TEN buffer, transferred into a reaction tube, and 15 µl 10 % SDS were added followed by incubation for 30 min at 75°C. Afterwards the treatment was continued as described in the previous chapter (See 2.2.5.2).

2.2.6 Extraction and ethanol precipitation of DNA

Crude cellular or viral DNA solutions were extracted with 1 volume (300 µl/5 ml) phenol in 1.5 or 15ml tubes. The tubes were shaken vigorously for 30 s (not vortexed), incubated for 5/10 min on ice and centrifuged for 5/10 min at 15000/4000 rpm at 4°C. The upper aqueous phase was extracted in fresh tubes with 1 volume phenol-chloroform, shaken vigorously for 30 s, incubated on ice and centrifuged as before. Then, the upper phase was extracted in fresh tubes with 1 volume chloroform-isoamyl alcohol (IAA, 24:1), shaken vigorously, and centrifuged again. Finally, the upper aqueous phase was transferred to fresh tubes and 30/300 µl 3M Na-acetate and 0.75/12.5 ml ethanol were added, mixed and stored overnight at -20°C or for 1 hour at -80°C. The DNA was sedimented by centrifugation for 20/30 min at 15000/6000 rpm at 4°C. The pellets were washed with 500 µl/5 ml 70 % ethanol, and centrifuged again for 5/10 min at

15000/6000 rpm. The pellets were dried (at 56°C or under lamina flow), resuspended in 100/200 µl TE (pH 7.4), and the DNA concentrations were determined using a Nanophotometer (IMPLEN).

2.2.7 Generation of ASFV gene deletion mutants

To generate ASFV deletion mutants, standard cloning protocols were used for construction of recombination plasmids with substitutions of the cloned ASFV target genes by reporter gene cassettes. The cloning strategy mainly involved compatible restriction enzyme digestions of both the PCR amplified DNA insert and plasmid vector, followed by ligation, transformation of competent E. coli cells and selection of transformants by antibiotic resistance on agar plates.

2.2.7.1 PCR amplification and cloning of ASFV Kenya DNA from genome regions to be mutated

To obtain recombination plasmids, the target gene regions were amplified by PCR from genomic DNA of ASFV-Kenya IX 1033. Custom made amplification primer pairs for the thymidine kinase (K196R) and K145R, dUTPase (E165R), ribonucleotide reductase (F334L and F778R), p12 (O61R), 9GL (B119L), and pA104R gene regions (Table 3) were dissolved in DEPC-treated H_2O for molecular biology to obtain concentrations of 100 pmol/µl, and stored at -20°C. After PCR amplification using KOD-Xtreme Hot Start Polymerase according to the manufacturer's instructions (Table 2) the products were separated on agarose gels, the desired DNA bands were excised and extracted, followed by digestion at artificial restriction enzyme cleavage sites within the primer sequences (Table 3), a second gel purification step and ligation with a similarly digested plasmid vector, usually pUC19. The resulting plasmids pUC-AK9GLR, pUC-AKDUTR (Fig. 2-1), pUC-AKTKR, pUC-AKO61RR and pUC-AKRiReR were characterized by restriction analyses and sequencing with vector specific primers.

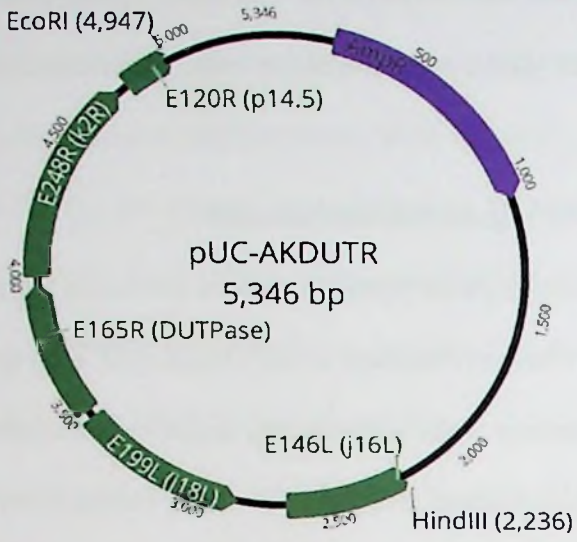


Figure 2-1: *Transfer plasmid pUC-AKDUTR showing inserted sequences containing the ASFV-Kenya dUTPase ORF (AK-E165R) and adjacent virus genes (green) in pUC19 with an Ampicillin resistance gene (purple). Restriction sites used for cloning are indicated.*

2.2.7.2 Generation of gene deletion and reporter gene plasmids

To generate gene deletion plasmids, the initially obtained clones of ASFV-Kenya genome fragments were digested with selected restriction enzymes to remove the open reading frames (ORFs) of interest, retaining > 1 kbp of viral sequences at both sides for subsequent homologous recombination. Instead of the deleted ASFV gene, a double reporter gene cassette containing the ORFs for enhanced green fluorescent protein (GFP) under control of the ASFV p72 promoter, and truncated human CD4 under control of the ASFV p30 promoter flanked by LoxP sites (Fig. 2-2) was isolated as a 2.5 kbp BamHI/HindIII fragment from plasmid pUC-LoxP-Pp72GFP-Pp30huCD4 (Hübner et al., 2019b) and inserted.

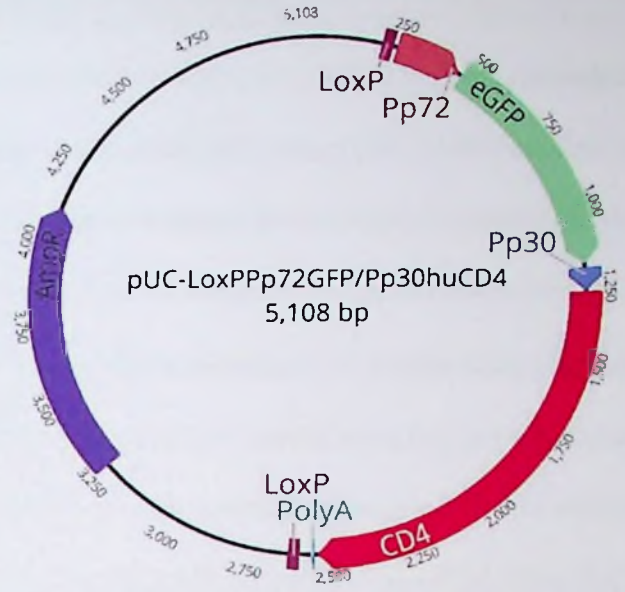


Figure 2-2: Double reporter gene plasmid containing the open reading frames for eGFP (light green) and huCD4 (red) flanked by ASFV-specific promoters (Pp72 and Pp30) and polyadenylation signals (PolyA), and loxP sites.

The resulting deletion plasmids pUC-AKΔ9GL-LoxGFPCD4, pUC-AKΔDUT-LoxGFPCD4 (Fig. 2-3), pUC-AKΔK145R-LoxGFPCD4, pUC-AKΔTK-LoxGFPCD4, pUC-AKΔRiRe-LoxGFPCD4, and pUC-AKΔO61R-LoxGFPCD4 were characterized by restriction analyses and sequencing using the reporter gene-specific primers EGFP-SNR and huCD4-OUT (Table 3).

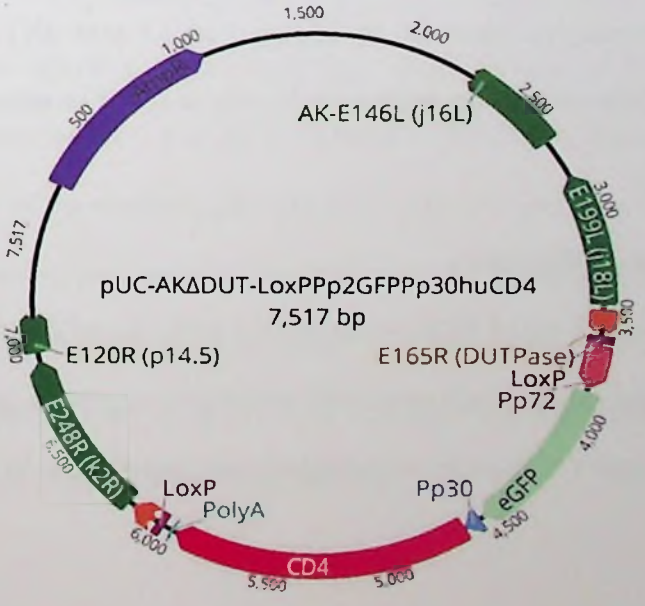


Figure 2-3: *Deletion plasmid pUC-AKΔDUT-LoxGFPCD4 showing deletion of the E165R (DUTpase) gene and insertion of the BamHI/HindIII fragment of pUC-LoxP-Pp72GFP-Pp30huCD4 (red and light green colors) flanked by two LoxP sites.*

2.2.7.3 Agarose gel analysis

To separate PCR amplicons and DNA fragments after restriction enzyme digestion, DNA loading buffer was added, and samples were loaded together with a size marker onto agarose gels (mostly 0.7% in TAE) containing 0.3 µg/ml ethidium bromide, and run at 90V for 1 hr.

2.2.7.4 Insert extraction, phosphorylation, and Klenow treatment

Correct size DNA products were excised from the agarose gel and either extracted with phenol or Zymoclean gel extraction kit. For phenol extraction, the excised gel piece was weighed, shredded and volume was brought to 300 µl with water. Then 300 µl TE buffer-saturated phenol was added, the sample was vortexed for 1 min, put into liquid nitrogen for 1 min, centrifuged for 20 min at 15000 rpm and room temperature. The upper phase was extracted with 300 µl chloroform-IAA, DNA was precipitated with ethanol as described above (see 2.2.6), and resuspended in 11 µl TE (pH 7.4). For control, 1 µl was loaded and run on an agarose gel.

To obtain phosphorylated PCR products, to approximately 1 µg of DNA in 15 µl water 2 µl 10X PNK-Buffer, 2 µl 10mM ATP, and 1 µl (10 U) T4 polynucleotide kinase (PNK) were added, and incubated for 1 h at 37°C. Single-stranded overhangs were removed after PNK treatment or restriction enzyme digestion by adding 1 µl 2.5 mM dNTPs and 0.5 µl (2.5 U) Klenow polymerase, followed by incubation for 30 min at RT. The enzymes were inactivated for 20 min at 70°C.

2.2.7.5 Vector and insert digestions

If possible, approximately 1 µg of PCR-product DNA and 2 µg of plasmid vector DNA were digested under recommended conditions with restriction enzymes generating compatible fragment overhangs. After 1 h aliquots were loaded onto agarose gels to control the success of digestion.

2.2.7.6 Dephosphorylation of vector DNA

If the vector DNA was completely digested, its 5'-ends were dephosphorylated by addition of 1X dephosphorylation buffer to a final volume of 270 µl, and 2 µl (2 U) alkaline phosphatase from calf intestine, followed by incubation for 1 hr at 37°C, addition of 30 µl 100 mM EGTA and incubation for 10 min at 75°C. After phenol extraction and ethanol precipitation (see 2.2.6) the DNA was resuspended in 11 µl of TE (pH 7.4) and 1 µl was run on an agarose gel in TAE to quantify the DNA amount required for ligation.

2.2.7.7 Ligations

Approximately 200 ng each of digested insert and vector DNA in a final water volume of 17 µl were ligated using 2 µl 10X ligase buffer and 1 µl T4 DNA ligase (1U/µl) by incubation for 15 min at 37°C, 1 h at 20°C and overnight at 15°C.

2.2.7.8 Hot Fusion cloning (Fu et al., 2014).

For cloning and mutagenesis of the ASFV A104R gene, a ligase-free protocol was used. First the A104R gene region of ASFV Kenya was amplified by PCR using primers AKA104RRHF-F and AKA104RRHF-R (Table 3) which provided 19 to 20 bp overlaps with the ends of *Sma*I-digested vector pUC19. Approximately 200 ng each of the purified PCR product and the linearized vector were mixed in 10 µl H_2O, and, after addition of 10 µl Hot Fusion mix, incubated in a thermocycler for 1 h at 50°C, ramped to 20°C (0.1 C/s) and then to 10°C, and stored at -20°C. DNA of the resulting plasmid pUC-AKA104RR (Fig. 2-4) was diluted to 10 ng/µl, and amplified using primers AKΔA104RHF-F and AKΔA104RHF-R (Table 3). The resulting product lacked A104R but contained the flanking regions, and was fused to the overlapping SmaI/HindIII insert of pUC-LoxP-Pp72GFP-Pp30huCD4 (Fig. 2-2) as above resulting in pUC-AKΔA104R-LoxPGFPhuCD4 (Fig. 2-5).

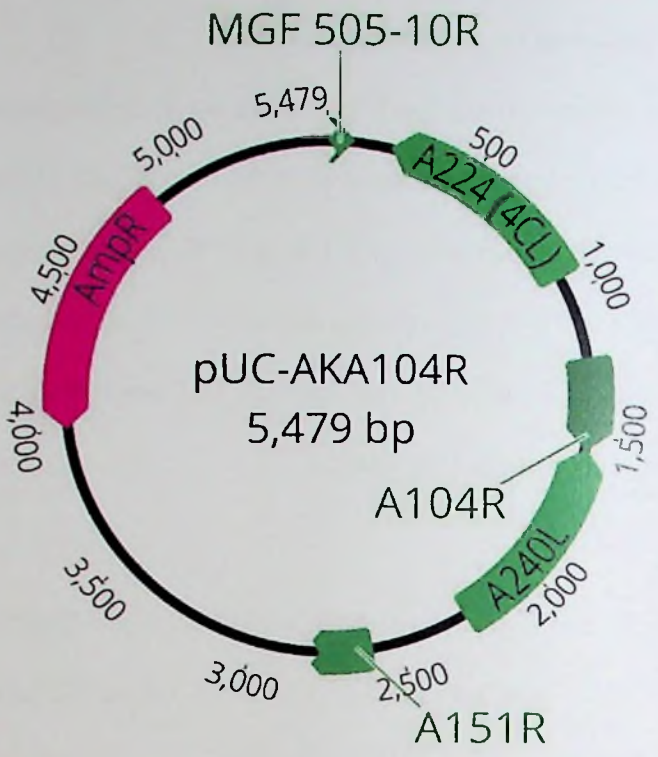


Figure 2-4: *Hot-fusion plasmid pUC-AKA104R showing inserted sequences containing the ASFV-Kenya A104R ORF and flanking virus genes (green) in pUC19 with an Ampicillin resistance gene (purple*

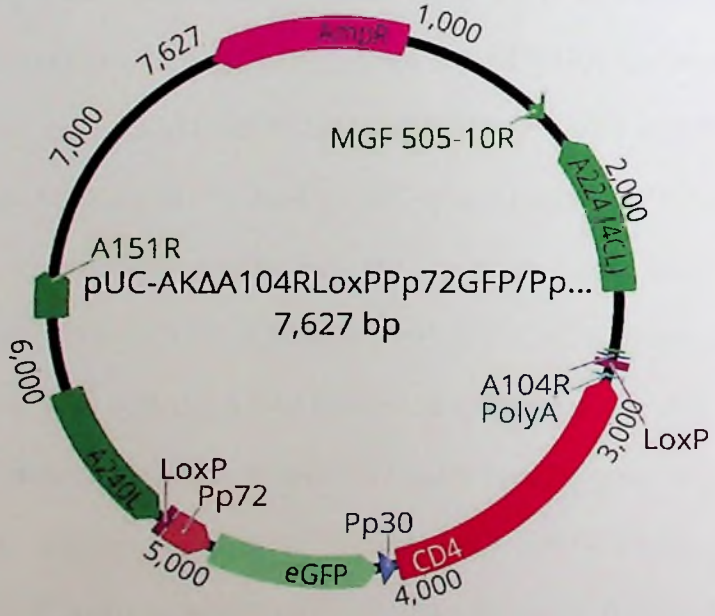


Figure 2-5*: Deletion plasmid pUC-AKΔA104R-LoxPGFPhuCD4 showing deletion of the A104R gene and insertion of the SmaI/HindIII fragment of pUC-LoxP-Pp72GFP-Pp30huCD4 (red and light green colors) flanked by two LoxP sites.*

2.2.7.9 Golden gate cloning of CRISPR/Cas9 plasmids

Golden Gate cloning is a method used to conveniently digest and then ligate multiple sequences with the same type IIS restriction enzymes (usually BsaI or BbsI) in a single reaction. These enzymes cut double-stranded DNA outside of their recognition site hence allowing the assembly of DNA fragments seamlessly. Using this type of cloning, 4 sgRNA expression plasmids were assembled, and a Multiplex CRISPR/Cas9 Assembly System kit (Sakuma et al., 2014) was used to generate the pX330eCasΔNLS-4xASFVgNeoR plasmid (Fig 3-16). Golden mutagenesis allows for cloning and assembly which enables single-reaction restriction-ligation procedures. First PCR fragments are generated that carry the terminal type IIs recognition sites which then introduce additional specific randomization sites outside of the binding primer sequence. These generated PCR products are further reassembled in a target expression vector, and the resulting genetic library is directly transformed into an E. coli expression strain. The structure of the generated PCR products (designed oligonucleotides) follows this order: type IIS recognition site, a specified four bp overhang, randomization site and template binding sequence (5' to 3' direction) (Pullmann et al., 2019).

2.2.7.10 Transformation of E. coli

To prepare competent E. coli XL1-blue MRF' cells, fresh overnight cultures were diluted 1:100 in LB medium, and shaken at 37°C to an optical density at 600 nm (OD600) of 0.6 to 0.8. The cells were cooled on ice for 30 min, centrifuged for 10 min at 3000 rpm and 4°C, and resuspended in 1/10 volume of TSS or TSS storage medium (for freezing of 200 µl aliquots at -80°C). 5 µl of ligations, Hot Fusion or Golden Gate reactions were added to 200 µl portions of competent bacteria, incubated on ice for 30 min, heated for 2 min to 42°C, cooled on ice for 2 min, and, optionally, shaken with 800 µl SOC for 1 h at 37°C. The cells were spread on LB-amp or LB-strep plates, optionally containing 0.005% X-gal and 50nM IPTG for blue-white selection of pUC19 and multiplex CRISPR/Cas9 clones.

2.2.7.11 Plasmid DNA mini-preparations

After overnight incubation of the plates, single (white) colonies were picked into 2 ml LB-amp or LB-strep and incubated overnight at 37°C in a shaker incubator at a speed of 200 rpm. To make DNA mini-preparations, the overnight cultures were transferred to 2ml reaction tubes, centrifuged 2 min at 15000 rpm and aspirated. The pellets were resuspended in 200 µl solution I, mixed with 200 µl Solution II, and subsequently 300 µl 3M Na-acetate were added and mixed gently. After 10 min on ice, the samples were centrifuged for 10 min at 15000 rpm. The supernatant was transferred to fresh 1.5ml tubes, mixed with 450 µl isopropanol, and centrifuged for 15 min at 15000 rpm. The pellets were washed with 300 µl 70% ethanol, and spun for 5 min at 15000 rpm. The ethanol was carefully aspirated not to lose the pellets, which were then dried for 5 min at 56°C, resuspended in 50 µl TE-RNase and incubated with shaking for 30 min at 37°C. The plasmids were tested for insertions by agarose gel electrophoresis using the empty vector as control. To confirm the insertions promising plasmids were digested with restriction enzymes (mostly the ones used for cloning), and loaded on agarose gels. Furthermore the inserts were checked by DNA sequencing with suitable primers. Correct clones were streaked onto LB-amp plates and incubated overnight at 37°C.

2.2.7.12 Plasmid DNA midi-preparations

Single colonies of the desired plasmid clones were picked into 2 ml LB-amp, shaken for 6 h at 37°C and used to inoculate 50 ml LB-amp in Erlenmeyer flasks. After overnight incubation at 37°C and 200 rpm, DNA preparation was done using the QIAfilter Plasmid MIDI kit (Qiagen) as recommended by the manufacturer. The DNA was resuspended in 200 µl TE (pH 7.4), and concentration was determined. After test digestions and sequence analyses, the plasmids were ready for e.g. transfection of mammalian cells.

2.2.7.13 DNA sequencing

For Sanger sequencing, approximately 250-500 ng DNA in 6 µl H_2O, 1 µl 5x sequencing buffer, 1 µl 5 µM primer and 2 µl terminator mix (BigDye Terminator v1.1 cycle sequencing kit) were mixed and tubes were placed in a thermal cycler. Cycle sequencing was performed under the following conditions:

Cycling sequencing conditions			
Step		**Temp**	**Time**
Initial Denaturation		96°C	1 min
25 cycles	Denaturation	96°C	15 sec
	Annealing	55°C	15 sec
	Extension	60°C	3 min
Hold		20°C	Infinitely

The sequencing reactions were purified using NucleoSeq spin columns (Macherey-Nagel) as described below. 10 µl of the discharge were added to 10 µl formamide and handed in for sequence analysis using a 3130 Genetic Analyzer (Applied Biosystems). The obtained output files were imported into Geneious® software package in version 11.1.5 (Biomatters) for further investigation.

2.2.7.14 DNA purification using spin columns

The NucleoSeq columns (Macherey-Nagel) were briefly centrifuged at 2800 rpm (750 x g), and after addition of 600 µl H_2O and mixing, they were incubated for 30 min at room temperature. The caps of the columns were removed, followed by centrifugation for 2 min at 2800 rpm. The columns were put into new reaction tubes and the probes (20-50 µl) dropped on top of the resin and centrifuged for 4 – 6 min at 2800 rpm. The discharge was kept.

2.2.8 Virus propagation and titration

2.2.8.1 MOI determination

The ratio of infecting viruses to host cells (MOI) was determined from estimated cell numbers:

Flask/Plate	Medium (ml/well)	Cell number
T25	5	3×10^6
T75	15	$1\text{-}3 \times 10^7$
T162	40	$2\text{-}5 \times 10^7$
6-well	2.5	8×10^5

12-well	1.5	3×10^5
24-well	1	1.8×10^5
48-well	0.5	8×10^4
96-well	0.2	6×10^3

2.2.8.2 Preparation of virus stocks

Cells were grown overnight in 25 to 162 cm^2 culture flasks to subconfluent monolayers and infected at a MOI of approximately 0.01 with ASFV (WSL-HP) or PrV (RK13) after substitution of the medium by ZB 28b or ZB 5b containing 1% penicillin/streptomycin, followed by incubation at 37°C until pronounced CPE developed (PrV: 2 to 4 d, ASFV: 5 to 10 d). Then the cells were lysed by freeze-thawing of the flasks at -80°C and 37°C, respectively. The lysates were transferred to sterile 15ml or 50ml tubes, and centrifuged for 10 min at 4000 rpm. The virus containing supernatants were stored in aliquots at -80°C. ASFV stocks could be also stored at 4°C for rather long periods without significant titer reduction. The cell pellets still contained considerable amounts of virus DNA, which could be prepared e.g. for characterization of virus recombinants (see chapter 3, section 3.2.2.1).

2.2.9 Plaque assays, virus purification and *in vitro* replication studies

2.2.9.1 Preparation and infection of cells

Plaque assays were performed for determination of virus titers, purification of virus recombinants, or quantification of virus spread. Approximately 2.5×10^6 WSL-HP, RK13 or recombinant cells were mixed with 24 ml ZB 5 or ZB 28, and 1 ml/well were added to a 24well plate. The cells were grown overnight at 37°C to confluent monolayers. Virus stocks or transfection progenies were serially diluted from 10^{-1} to maximally 10^{-8} in ZB 5b or ZB 28b. and 333 µl/well of ASFV were added to aspirated 24well plates with WSL-HP derivatives, which were subsequently centrifuged for 1 h at 2000 rpm and 25°C, and optionally incubated for an additional hour at 37°C. Since Pseudorabies virus enters its host cells more efficiently, aspirated plates with RK13 cells were inoculated with 250 µl/well of the virus dilutions and

incubated on a rocking platform for 1 h at RT. In both cases the inoculum was then aspirated, and 1 ml/well 0.25x methocel medium containing 1% penicillin/streptomycin was added, and cells were incubated at 37°C until virus plaques became clearly visible (PrV: 2 to 3 d, ASFV 5 to 8 d).

2.2.9.2 Fixation and staining of cells

To fix 24well well plates for immunofluorescence tests (see section 2.2.9.1), the methocel medium was aspirated, the cells were washed with PBS and fixed with 0.5 ml/well cold methanol/acetone for 30 min at -20°C. The plates were dried in the laminar flow cabinet and stored at RT. Alternatively the washed cells were fixed with 0.5 ml/well 3% paraformaldehyde in PBS for 30 min at 4°C. Optionally, the cells were additionally permeabilized by incubation with 0.5 ml/well of 0.5% Triton X-100 in PBS for 15 min at RT. Subsequently, cells were washed thrice with PBS for 5 min with rocking and stored at 4°C. For staining the cells were fixed with 5% formalin overnight (or at least for one hour) at RT. The plates were washed with water, and the bottom of all wells was covered with 1% crystal violet in 50% ethanol with rocking for 30 min at RT. Then the plates were washed again with water and dried for 30 min at 45°C.

2.2.9.3 Plaque purification of virus recombinants

To select virus recombinants obtained after transfer plasmid transfection and wild-type ASFV infection of permissive WSL cells, or cotransfection of RK13 derived cells with BAC DNA of pPrV-BaΔgGDG (Hübner et al., 2018b) and recombination plasmids (see section 2.2.7.2) the used 12well plates were checked for almost complete lysis and then frozen for at least one hour at -80°C. Plates were thawed at 37°C and lysates of transfected wells transferred into reaction tubes, and eventually again stored at -80°C. Serial dilutions of transfection progenies were analyzed by plaque assays as described above. For purification of virus recombinants single, usually fluorescent plaques were picked from the highest positive dilutions into 500 µl ZB 5 or

ZB 28, and frozen in reaction tubes at -80°C. Plaque purification was repeated until the virus population appeared homogeneous, and the final plaque isolates were used for infection of T25 flasks of the appropriate cell line.

2.2.9.4 Purification of virus recombinants by limiting dilutions

An alternative method for final purification of enriched ASFV recombinants was performed on 96well plates of WSL-HP or derived cell lines, which had been inoculated with approximately 2 x 10^6 cells in 20 ml ZB 28 (200 µl/well). After overnight growth at 37°C, the medium was replaced by 100 µl/well ZB 28b + 1% pen/strep. For virus dilution 96well plates with 150 µl/well ZB 28b + 1% pen/strep were prepared. 8 x 50 µl of an inhomogeneous plaque isolate were added into column 1 of one plate and mixed by pipetting up and down with an electronic 8channel pipet. Then the tips were changed, 50 µl/well were transferred from column 1 to column 2, mixed again, and stepwise 1:4 dilutions were continued until column 12. Finally, 100 µl/well of all virus dilutions were added to the prepared cell culture plates, which were incubated at 37°C until fluorescent cells and CPE became visible. Then single wells from the highest positive dilutions were harvested, and the virus was further propagated in T25 flasks of suitable cell lines.

2.2.9.5 Isolation of virus recombinants by Magnetic-Activated Cell Sorting (MACS)

To evaluate the suitability of MACS for purification or at least enrichment of CD4-expressing ASFV mutants, virus stocks of the reporter gene-labelled recombinants ASFV-KenyaΔK145RloxPGFPhuCD4 (generated in this thesis) and ASFV-ArmeniaΔp22loxPDsRed (kindly provided by Katrin Pannhorst), were mixed at a ratio of approx. 1 : 100 PFU. 1 ml of this mixture were incubated with 30 µl of magnetic bead-tagged anti CD4 antibodies (CD4 MicroBeads, human; Miltenyi Biotec) for 30 min at RT on a rocking platform. A sterile MACS µ column was placed in a µMACS separator (Miltenyi Biotec) and equilibrated with 1 ml ZB 5b + pen/strep. The virus-MACS bead mixture was added to the column in the magnet

separator. The discharge was reapplied to the column a second time, collected again, and stored at 4°C. Then the column in magnet was washed twice with 1.5 ml ZB 5b + 1% pen/strep, and the collected discharges were stored at 4°C. After removal of the column from the magnet bound virus was eluted with 2 x 1 ml ZB 5b + 1% pen/strep, and the discharges were stored at 4°C. Single and mixed virus supernatants, discharge, wash solutions and eluates were serially diluted from 10^0 to 10^{-7} and titrated on WSL-HP cells as described above (section 2.2.9.1).

2.2.9.6 Virus titer and plaque size determination

Autofluorescent virus plaques, or plaques visualized by indirect immunofluorescence (see section 2.2.9.1) or crystal violet staining (see section 2.2.9.2) were counted and titers determined using the formula: PFU/ml = number of plaques / volume of inoculum [ml] x dilution factor. For comparative analysis of virus spread, virus mutants were titrated in parallel on 24 well plates of the same cell batch, and diameters or areas of at least 20 plaques per virus were measured by fluorescence microscopy.

2.2.9.1 Indirect immunofluorescence tests

For better visualization of wild-type ASFV plaques without reporter genes, selected wells on 24well titration plates were washed thrice with 1X PBS for 5 minutes at RT. Blocking was done with 300 µl/well 10 % FBS in PBS with rocking for 1 hr at RT. As first antibody, the monospecific rabbit α-GST-p72 serum detecting the major capsid protein (Hübner et al., 2018a), was diluted 1:100 in 10 % FBS in PBS, and 200 µl were added per well and incubated with rocking for 1 hr at RT. Cells were washed thrice with PBS for five minutes at RT. The second antibody, AlexaFluor 488 α-rabbit IgG, was diluted 1:1000 in PBS, and 200 µl were added per well and incubated with rocking for 1 hr at RT. Cells were washed four times with PBS for 5 minutes at RT with rocking. Optionally, the samples were preserved, and nuclear chromatin was stained by overlaying with 10% PBS + 90% glycerol + 2.5% DABCO + 1 µg/ml propidium iodide. The plates were analyzed by fluorescence microscopy.

2.2.9.2 Growth kinetics of ASFV recombinants

WSL-HP or inhibitory CRISPR/Cas9 expressing derivatives of them were grown in 24well or 96well plates to subconfluent monolayers. Virus stocks of ASFV-Armenia (passage 21), ASFV Kenya, or of ASFV Kenya and Armenia mutants were diluted to approximately 3 x 10^4 PFU/ml. For each time and cell line medium was aspirated from 2 to 4 wells per mutant, and 333.3 µl (24well) or 75 µl (96well) of virus dilutions were added to each well (MOI approx. 0.03). After centrifugation for 1 hr at 2000 rpm 25°C, and incubation for 1 hr at 37°C the inoculum was aspirated, cells were washed once with ZB 28b, and 1 ml or 200 µl per well ZB 28b + 1% pen/strep added followed by incubation at 37°C. Immediately thereafter (0 hr), and after 24, 48, 72, 96, 120, and optionally 192 hours at 37°C, single plates were sealed and frozen at -80°C. To determine progeny virus titers the plates were thawed at 37°C, and serial virus dilutions of all samples were prepared with electric 8 or 12 channel pipets. To this end 96well plates with 180 µl ZB 5b/well were prepared, 20 µl of homogenized lysates were added to the first row, and mixed by pipetting up and down (about 5x). After change of tips 20 µl were transferred to second row, mixed by pipetting up and down, and serial 1:10 dilutions were repeated until 10^{-4} to 10^{-8}, depending on the expected titers. Medium was aspirated from subconfluent 48well plates with WSL-HP cells, and 150 µl per well of decreasing virus dilutions were added with the same tips, centrifuged for 1 hr at 2000 rpm 25°C, and incubated for 1 hr at 37°C. Then the inoculum was aspirated, and 500 µl per well 0.25 x methocel + 1% pen/strep were added, followed by incubation at 37°C. After 3-5 days, fluorescent plaques were counted directly, or cells were fixed as described above, and titers were determined after indirect immunofluorescence analyses (see sections 2.2.9.1).

2.2.10 Southern blot analyses

For subsequent blotting 1-2 µg genomic DNA of the investigated PrV or ASFV mutants were digested with *Bam*HI or *Eco*RI for ≥ 3 hrs at 37°C, loaded on large (15 x 20 ml) 0.7 % agarose gels in TAE together with a DNA ladder, run overnight at 30 V and photographed.

2.2.10.1 Blotting of agarose gels

To depurinate the DNA, gels were incubated in 0.25N HCl for 10 min at RT with rocking, and rinsed thrice with water. Then the gels were incubated for 2 x 15 min at RT in 0.5M NaOH/0.9M NaCl to denature the DNA, rinsed thrice with water, and neutralized in 0.9M NaCl/1M Tris-HCl (pH 7.4) for 2 x 30 min at RT. A nylon membrane and 3 filter papers (15x19 cm), plus 1 filter paper (15x38 cm) were cut. The membrane was moistened in deionized water, whilst 250 ml 12x SSC in a tub were prepared, and a glass plate was placed on it. The long filter paper was wetted and laid on the plate, with both ends dunking into the buffer reservoir. The gel was put on the filter paper, the membrane was placed on the gel, and air bubbles were squeezed out using a glass pipet. Two filter papers wetted in water followed by 1 dry filter paper were placed onto the membrane and air bubbles were removed again. Folded cellulose sheets were stacked on top (about 10 cm height), covered with a glass plate, and weighted with approximately 1 kg. The DNA was transferred to the membrane by capillary flow overnight at RT.

2.2.10.2 Blot hybridization

After transfer the membrane was UV cross-linked to immobilize the DNA, and rinsed in 2x SSC. The prehybridization solution was prepared (see 2.1.13.2) and boiled for 5 min at 100°C. The membrane was put into a wetted hybridization tube using a glass pipet, and incubated with 15 ml prehybridization buffer for 4 hr (or overnight) at 65°C. To prepare the radiolabeled probe approximately 100 ng of the desired plasmid or DNA was added to 200 µl H_2O, incubated for 5 min at 100°C and chilled on ice immediately. 45 µl of the diluted DNA and 5 µl α^{32}P-dCTP (code SCP-205, Hartmann analytic) were added to a RediPrime II tube (GE Healthcare), containing lyophilized deoxynucleotides, random oligonuclucleotide primers and DNA polymerase and incubated for 1 hr at 37°C. Unincorporated nucleotides were removed using NucleoSeq spin columns (Macherey-Nagel) as described above (see section 2.2.7.14). The discharge was mixed with 50 µl formamide, and radioactivity of the probe was measured with

a contamination monitor (LB124, Berthold). Prior to use the probe was incubated for 5 min at 100°C, chilled on ice, added to the prehybridization solution, and the membrane was further incubated overnight at 65°C.

2.2.10.3 Washing and exposure of Southern blots

Wash solutions were heated in a water bath to 65°C. The hybridization solution with the ^{32}P-labelled probe was decanted, and stored at 4°C. The membrane was briefly rinsed with 2X SSC / 0.5 % SDS, washed twice with 2X SSC / 0.5 % SDS for 30 min at 65 to 72°C (depending on the G/C-content of DNA probe), and 4 times with 0.1X SSC / 0.5% SDS for 20 min at the same temperature. Radioactivity of the wash solutions was measured (LB124, Berthold), and as long as exceeding background level (> 30 IPS), they were stored as contaminated liquid for at least one year. The membranes were briefly dried, packed into saran wrap, and exposed overnight to previously erased BAS phosphor screens (Fuji). Then the screens were analyzed in a FLA-3000 fluorescence imager (Fuji), and results were evaluated using the AIDA software package in version 5 (Raytest).

2.2.11 Codon optimization for expression of ASFV proteins in mammalian cells and viral vectors

To optimize transgene expression in mammalian cells (like WSL-HP), or viral vectors (like pPrV-BaΔgGD, Hübner et al., 2018b), codon usage has frequently to be adapted to that of the host species (Rosano & Ceccarelli, 2014). Therefore, the p12 (pO61R) and pA104R amino acid sequences of ASFV Kenya 1033 were back translated and adapted to codon usage of *Sus scrofa* as a reference (using Geneious software), and purchased as cloned synthetic genes (Fig. 2-4 containing synthetic gene sequences). These genes were recloned into pUC-BaKJCAGPLnew (Hübner et al., 2018b) to generate the virus mutants PrV-BaΔgG-AKO61Rporc and PrV-BaΔgG-AKA104Rporc according to the published protocols. From the recombination plasmid pUCBaKJCAG-AKO61Rporc, the synthetic p12 gene together with the preceding CAG

promoter was further recloned into the mammalian expression vector pcDNA3.1 (+) (Invitrogen) to obtain plasmid pCAGGS-AKO61RneoR which was used for stable transfection of WSL-HP cells (see section 2.2.2).

2.2.12 Expression of ASFV proteins in bacteria

The protein p12 (pO61R) of ASFV Kenya 1033 (genotype IX), as well as p54 (pE183L), p11.5 (pA137R), pK145R and p285L of ASFV-Armenia (genotype II) were expressed as GST fusion proteins in *E. coli* and used for preparation of monospecific rabbit antisera.

2.2.12.1 Cloning of expression plasmids

The synthetic ORF encoding p12 of ASFV Kenya was recloned as a *Bam*HI/*Not*I fragment into similarly digested expression vector pGEX-4T2. The ORF encoding p54 was amplified by PCR from genomic DNA of ASFV Armenia using primers AGp54-F and AGp54-R (table 3). After digestion with *Eco*RI and *Xba*I the PCR product could be inserted into the *Eco*RI/*Sal*I cleaved vector pGEX-4T3, since *Xba*I and *Sal*I generate compatible single-stranded overhangs. Correct plasmid clones obtained after transformation of *E. coli XL1-Blue MRF'* were identified by test digestion of DNA mini-preparations, and confirmed by subsequent sequencing using primer PGEX-5' (table 3). Further expression plasmids (see above) were cloned by colleagues.

2.2.12.2 Protein expression

To express the GST-AKp12 or GST-AGp54 fusion proteins, single plasmid clones were grown overnight in 3 ml LB-Amp at 37°C with shaking (200 rpm). The cultures were diluted 1:25 in 50 ml LB-Amp + 50mM glucose and shaken at 37°C until an $OD_{600} \geq 0.8$. 1 ml of the non-induced cultures was centrifuged for 2 min at 15000 rpm, and pellets were resuspended in OD_{600} x 100 µl SDS sample buffer and stored at -20°C. The remaining cultures were centrifuged in 50ml tubes for 15 min at 4000 rpm. The pellet was resuspended in 50 ml 2x YT medium with 1mM IPTG followed by incubation for 3 hr at 37°C in a bacterial shaker. The OD_{600} of the induced cultures was determined, and 1 ml of them was centrifuged for 2 min at 15000 rpm and

resuspended in OD_{600} x 100 µl SDS sample buffer and stored at -20°C. The remaining cultures were centrifuged in 50ml tubes for 15 min at 4000 rpm, drained and pellets were stored at -20°C.

2.2.13 Expression control

To check for presence and abundance of the desired proteins, small 12 % protein gels were prepared as described (see 2.1.13.6) for Coomassie staining and western blot analyses. 10 µl of non-induced and induced bacteria (from section 2.2.12.2), and, if already available, 500 ng of eluted fusion proteins (see section 2.2.13.4), as well as 250 ng to 1 µg BSA in SDS sample buffer were incubated for 5 min at 95°C, centrifuged for 3 min at 15000 rpm, and loaded onto the gels together with prestained molecular mass standards (PAGE-Ruler, Thermo-Fisher). Proteins were separated for 45 min at 200 V using a Mini-PROTEAN Tetra Cell (Bio-Rad).

2.2.13.1 Staining of analytic gels

Gels were stained with Coomassie brilliant blue solution for analytic gels for 30 min at RT with rocking, destained and fixed with 10% acetic acid / 40% methanol for 4 x 30 min at RT while rocking and rinsed with water for 2 x 10 min at RT. The gels were dried on filter paper for 1 h at 80°C under vacuum (Gel dryer Model 583, Bio-Rad).

2.2.13.2 Western blotting

Proteins were transferred to nitrocellulose membranes for 2 hr at 25 V using Semi-Dry blotters according to the manufacturer's instructions. Blots were blocked in TBS-T / 5% low-fat milk + 0.05% NaN_3 for at least 1 hr at RT with rocking. The 1st antibodies were diluted (usually 1:20000) in TBS-T / 0.5% milk and incubated with the blot for ≥ 1 hr (or overnight) at RT with gentle agitation. Blots were washed 3 x with TBS-T for 10 min at RT, and incubated with a species-specific, peroxidase-conjugated 2nd antibody diluted 1:20000 in TBS-T for 30 min at RT. Finally the blots were washed 5 x with TBS-T for 5 min at RT with rocking, and antibody

binding was detected by chemiluminescence reactions of Clarity Western ECL Substrate in a Versa Doc image analyzer using Quantity one 1-D software in version 4.6.9 (all from Bio-Rad).

2.2.13.3 Purification of the GST-fusion proteins

To isolate GST-fusion proteins, large preparative 10% protein gels were poured as described (2.1.13.6) using a PEQLAB system with ethanol-cleaned glass plates separated by 1.5 mm spacers and preparative combs inserted into the stacking gels. After polymerization the combs were removed, pockets washed with H_2O, gels inserted into chamber and filled with approximately 2 liters 1X SDS-PAGE buffer. The bacterial pellet of the induced 50 ml cultures was resuspended in 4 ml SDS sample buffer, sonified 3 x for 10 s at 70% amplitude and 1 ml per gel were incubated for 5 min at 95°C, centrifuged 3 min at 15000 rpm, and loaded into the large pocket, 30 µl non-induced bacteria or 10 µl PAGE-Ruler were loaded into the small control pockets. Proteins were separated overnight at 12.5 mA per gel (25 mA for two gels).

2.2.13.4 Staining and electrolution of fusion proteins

The elution buffer was prepared by adding 100 ml 10X PAGE-buffer and 100 ml methanol to 800 ml H_2O, filtrated through a 0.65 µm nitrocellulose filter in 1L NALGENE bottle, and, after addition of 10 ml 10 % SDS to the filtrated buffer, degassed for at least 2 hr using a Vacuubrand aspirator. Gels were stained with Coomassie brilliant blue solution for preparative gels for 30 min at RT, and destained in 30% methanol in H_2O for 4 x 45 min at RT with agitation. The bands of the induced GST-fusion proteins were excised, and the proteins were released using an Elutrap electroelution system (Schleicher & Schuell / Whatman). After assembly of the Elutrap system according to the manufacturer's instructions, the excised gel bands with the induced GST-fusion proteins were placed in the middle of the Elutrap device, which was then placed into a horizontal electrophoresis chamber. Protein molecules migrated from the gel slices into a trap area formed by BT1 and BT2 membranes after running overnight at 100V. To briefly detach all released protein molecules from the membranes, terminals were reversely inserted

very briefly at 200V for 30 seconds. The eluted fusion proteins were then aspirated into two well-labelled reaction tubes. The eluted fusion protein solutions were filled into the concentrator body of a Vivaspin 2 tube (PSE, 5 kDa) and filled up to 2 ml with sterile PBS. The tubes were inserted into an Eppendorf 5403 centrifuge and spun for 30 min at 6000 rpm at 4°C. The filtrate was removed and the concentrate was washed with 2 ml PBS in the concentrator body, and centrifuged again for 30 min at 6000 rpm at 4°C. The filtrate was again removed, and the tubes were vortexed briefly. Then the concentrator was inverted with a recovery cap, vortexed again briefly and centrifuged for 5 min at 3000 rpm at 4°C. The concentrate was kept. 200 µl PBS were added to the concentrator body, vortexed and inverted again, and centrifuged for 5 min at 3000 rpm. The two eluates were combined and stored at -20°C. The concentrator body was reinverted and stored at 4°C with 2 ml sterile water until reuse.

2.2.13.5 Determination of protein concentrations

To determine concentrations of the eluted fusion protein, a BCA working solution was prepared by mixing 10 ml solution A with 200 µl solution B (see 2.1.13.7) in a clean 50ml tube. 1 ml aliquots of working solution were transferred to reaction tubes and 0, 1, 2, 4, 8, and 16 µl BSA (1 µg/ml), or 2 µl of the eluted fusion proteins were added. The samples were mixed and incubated for 30 min at 60°C. Intensity of the staining reactions was determined by measuring the absorbance at 562 nm in a photometer (IMPLEN). The solution without protein was used as blank, and the BSA dilutions served as standards for calculation of the protein concentration of the eluates.

2.2.14 Preparation of rabbit antisera

For rabbit immunization 100 µg portions of the purified GST-fusion proteins were diluted to 500 µl with PBS, mixed with 500 µl complete Freund's adjuvant and subcutaneously injected into rabbits. In 4 week-intervals the immune response was boosted 3 to 6 times by repeated application of 100 µg of the same fusion proteins in PBS with incomplete Freund's adjuvant.

Blood samples taken before and 4 weeks after each immunization, and total blood collected at the end of the experiment were stored in 15 ml or 50 ml centrifuge tubes overnight at 4°C, stirred with inoculation loops, and centrifuged for 10 min at 4000 rpm, the sera were pipetted into fresh tubes and centrifugation was repeated if required. Then the sera were incubated for 30 min at 56°C for complement inactivation, and stored in 2 ml aliquots at -20°C.

3 CHAPTER THREE: RESULTS

3.1 Next generation sequencing (NGS) of the used ASFV strain

For most of the studies presented here, the virulent genotype IX field isolate ASFV Kenya 1033 was used. This virus had been adapted to efficiently replicate in a wild boar lung cell line (WSL) by several serial passages, and its genome sequence was determined (Keil et al., unpublished results). Furthermore, a reporter gene expressing CD2v deletion mutant of this virus was generated (Hübner et al. 2018a). Since ASFV-KenyaΔCD2vDsRed proved to be a useful test virus for different in vitro studies, and since it should be used as parental virus for multiple gene deletion mutants, its genome sequence was also determined by NGS.

Since DNA preparations from infected cells cultures usually contain only small proportions of ASFV DNA, virus particles of ASFV-KenyaΔCD2vDsRed were sedimented from infected culture supernatants, DNase-treated, and purified again through a 30% sucrose cushion prior to lysis and DNA preparation. The resulting DNA was digested with *Eco*RI and analyzed by gel electrophoresis which indicated a high purity, and a similar restriction pattern as predicted (Fig. 3-1).

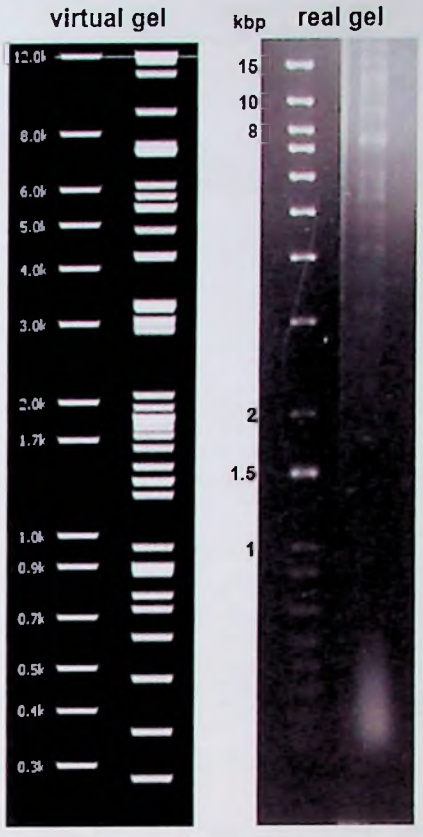


Figure 3-1: EcoRI-digested virion DNA of ASFV-KenyaΔCD2vDsRed separated on real (right panel), and on a virtual gel (left panel, generated with Geneious software). DNA size markers are indicated.

NGS analyses using Illumina MiSeq with v3 Kit in 2x 300 Bp PE mode were performed at the FLI (D. Höper et al.). Results revealed 1,357,748 ASFV specific reads (40.7%) out of 3,333,896 total reads which completely covered the virus genome. Identity of the assembled sequence was 99.6% to the previously determined one of ASFV Kenya, and 99.99% to the in silico assembled sequence of ASFV-KenyaΔCD2vDsRed. Besides the expected CD2v deletion (Fig. 3-2) there were only 8 single nucleotide substitutions, insertions or deletion with no or minor impact on the encoded ORFs (frameshifts in two nonessential MGF genes). Furthermore, some of the differences might have been due to errors in the original Kenya 1033 sequence.

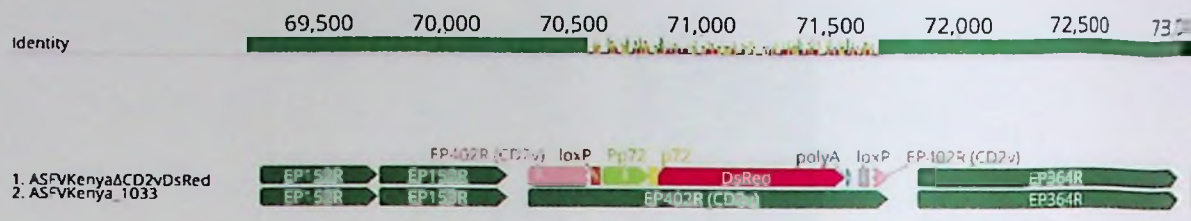


Figure 3-2: Excerpt from genome sequence alignment of ASFV-Kenya IX 1033 isolate and ASFV-KenyaΔCD2vDsRed confirming the deletion of the CD2v gene and substitution with a reporter gene for DsRed (red color) under control of the ASFV p72 promoter p72 (light green color).

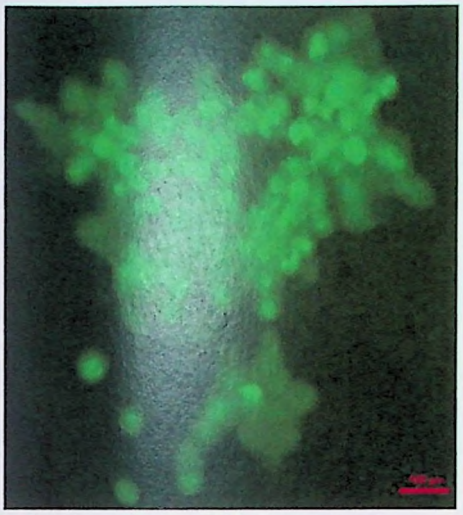
3.2 Deletion of non-essential ASFV genes

To develop experimental live vaccines against genotype IX ASFV in Eastern Africa it was attempted to delete several presumably non-essential genes (encoding viral thymidine kinase, dUTPase, ribonucleotide reductase, 9GL, pK145R, and pA104R) from ASFV Kenya 1033. The virus had been adapted to efficiently replicate in a wild boar lung cell line (WSL) by several serial passages. To facilitate gene deletion, selectable reporter genes (encoding GFP, DsRed or truncated human CD4) were inserted, and recombination was partly supported by CRISPR/Cas9 targeting of the virus genes to be deleted.

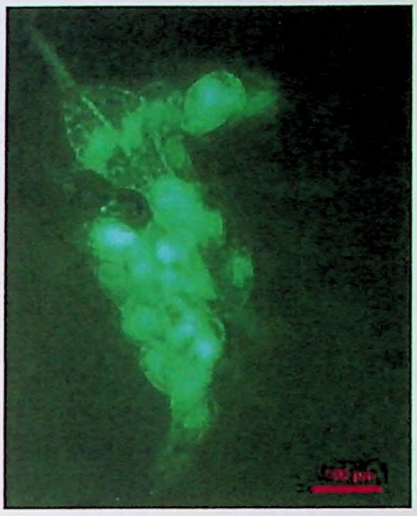
3.2.1 Substitution of ASFV genes with reporter genes

The viral genes were replaced with an expression cassette isolated from pUC-LoxP-Pp72GFP-Pp30huCD4 (see Fig. 2-2) containing LoxP sites, the GFP gene under ASFV p72 promoter control and the truncated human CD4 gene under control of the ASFV p30 promoter. For the mutant ASFV-KenyaΔA104R-LoxPGFPA104Rt, the CD4 ORF was replaced by an A104R-like ORF (A104Rt) present in certain soft tick genomes (Forth et al., unpublished data) to test its function in the viral context. A104Rt was also expressed under control of the strong ASFV p30 promoter. After transfection of WSL-HP cells with the resulting plasmids and infection with ASFV Kenya 1033, green fluorescent foci of infected cells were detected. GFP-expressing ASFV recombinants were purified by repeated plaque isolation on WSL-HP cells, through aspiration of green-fluorescent cell foci. Purification of the desired TK–deleted virus was facilitated by the use of a TK-negative cell line (WSL-Bu25), and selective medium containing

25 µg/ml bromodeoxyuridine (BrdU), which inhibited growth of TK-expressing ASFV ASFV (Keil et al., 2014). After 3-6 rounds of plaque purification, most GFP-expressing viruses appeared homogenous as shown in Fig 3-3.



ASFVKenyaΔTKLoxPGFPhuCD4



ASFV-Kenya-ΔK145R-LoxPGFPhuCD4



ASFV-KenyaΔA104R-LoxPGFPhuCD4

Figure 3-3: Fluorescence images of single plaques of ASFVKenyaΔTKLoxPGFPhuCD4, ASFV-Kenya-ΔK145R-LoxPGFPhuCD4 and ASFV-KenyaΔA104R-LoxPGFPhuCD4, on WSL-HP cells 5 days post infection.

Up to now, three genes could be successfully deleted from ASFV-Kenya during this study: the TK-gene K196R, the adjacent gene K145R, and A104R encoding a histone-like protein. Two further mutants, ASFV-Kenya-Δ9GL-LoxPGFPhuCD4 and ASFV-Kenya-ΔDUT-

LoxPGFPhuCD4 are currently plaque purified. Repeated attempts to delete genes encoding the two subunits of the viral ribonucleotide reductase (F334L and F778R) were not successful, indicating that these genes might be essential in ASFV.

3.2.2 Helper virus-mediated generation of ASFV recombinants

To improve reverse genetics of ASFV, it was tested whether the recently described CRISPR/Cas9 cell line WSL-gRp30 expressing a sgRNA which inhibits most ASFV strains but not ASFV-Kenya 1033 (Hübner et al., 2018a), can be used to facilitate generation of virus recombinants. Therefore, these cells were co-transfected with virion DNA of ASFV-KenyaΔCD2vDsRed and recombination plasmids for the nonessential virus genes described above (see section 3.2.1), as well as CRISPR/Cas9 plasmids containing sgRNAs targeting the genes to be deleted (Fig 3-15). Since ASFV DNA is not infectious and no heterologous or subviral helper system is available up to now, the cells were then infected with genotype II ASFV Armenia 2008 as helper virus. This virus is phylogenetically distant to ASFV Kenya (sequence identity 84.5%) and selectively inhibited on the used cell line, which might support booting up of the transfected, partly recombinant, virus DNA without unwanted additional recombinations with the helpervirus.

Using this approach a green and red double fluorescent, CD2v- and ΔK145R-deleted double mutant of ASFV-Kenya could be successfully isolated (Fig. 3-4), and a second double deletion mutant, ASFV-KenyaΔCD2vDsRed-Δ9GL-LoxPGFPhuCD4, is currently being plaque purified. To exclude recombination with the helper virus, NGS analyses of these virus mutants remain to be done.

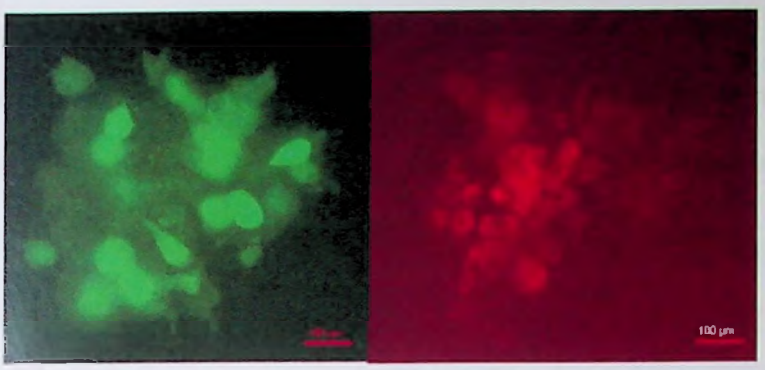


Figure 3-4: Fluorescence images of ASFV-KenyaΔCD2vDsRed-ΔK145R-LoxPGFPhuCD4 5 days after infection on WSL-gRp30 cells. GFP expression (left panel), and DsRed expression (right panel) of single virus plaques are shown.

3.2.2.1 PCR amplification and sequencing of the mutated ASFV genes

To confirm the desired mutations of the generated ASFV mutants, genomic DNA was prepared followed by PCR amplification of the affected genome regions using specific primers (Table 3). The studies revealed that all PCR products exhibited the calculated sizes (Fig. 3-5). Because of the reporter gene insertions, the products of the deletion mutants were always larger than those of the parental virus.

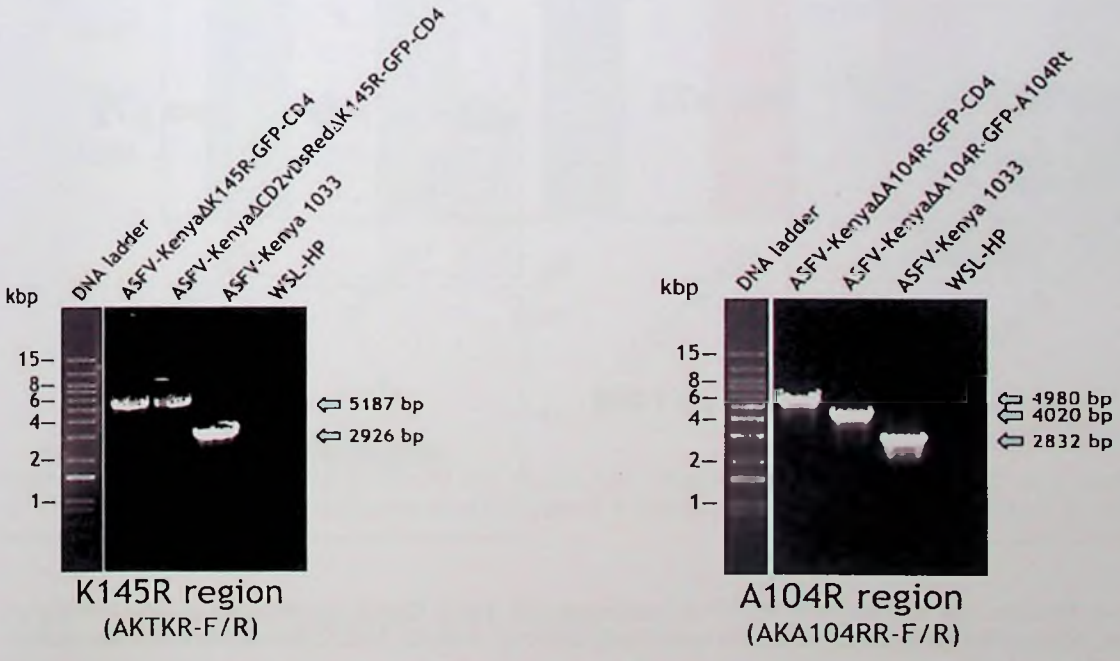


Figure 3-5: PCR products of the mutated gene regions of the indicated ASFV recombinants. In brackets are the primer pairs used for amplification, and expected product sizes are provided.

Furthermore, the PCR products were used for sequencing of the boundaries between the mutated ASFV genes and the reporter gene cassettes, and in all cases the obtained sequences were exactly as desired (not shown).

3.2.2.2 Western blot analysis of ASFV mutants

Lysates of WSL-HP cells infected with the mutant viruses were tested by Western blotting with monospecific rabbit antisera to ascertain protein expression profiles (Fig 3-6). The ASFV capsid protein (p72) was expressed in all infected cells, and served as loading control. In contrast, pK145R or pA104R were not detectable in cells infected with the respective deletion mutants. As expected, the viral and tick-encoded pA104R exhibit similar sizes (11.6 kDa), and are detected by the same antiserum (provided by K. Giesow & G.M. Keil).

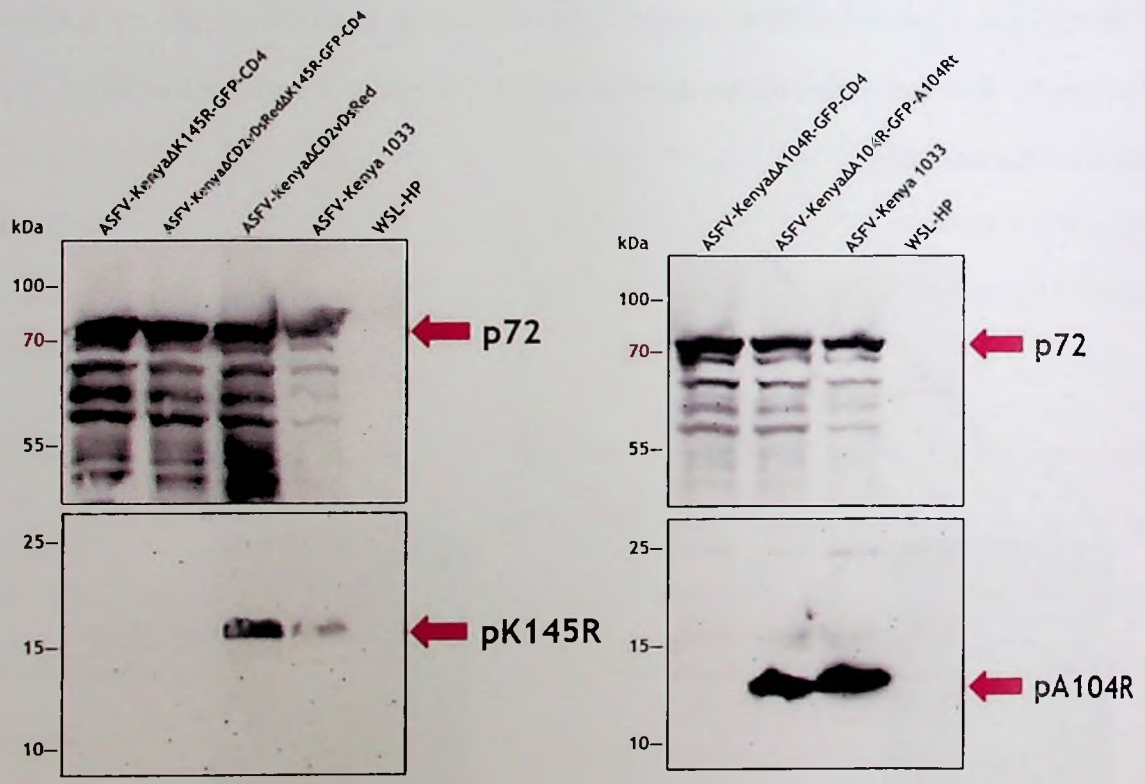


Figure 3-6: Western blot analysis of WSL-HP cells infected with ASFV Kenya recombinants or wild type Kenya virus at an MOI of 1 for 48 hr. Arrows indicate specifically detected proteins. Molecular masses of marker proteins are indicated at the left.

3.2.2.3 Cell-to-cell spread of ASFV Kenya recombinants

To investigate whether the introduced mutations affect spread of ASFV Kenya, WSL-HP cells infected at low MOI (< 0.001) with parental and mutant viruses were incubated under semisolid medium for 4 d at 37°C. The area of twenty plaques per virus were measured in a fluorescence microscope, either directly (GFP or DsRed expressing viruses), or after indirect fluorescence staining with a p72-specific antiserum (Fig 3-7). The studies showed that the deletion of K145R and/or CD2v did not affect virus spread of ASFV Kenya while deletion of A104R moderately impaired plaque formation. This defect was not corrected by substitutive expression of A104Rt from the tick genome.

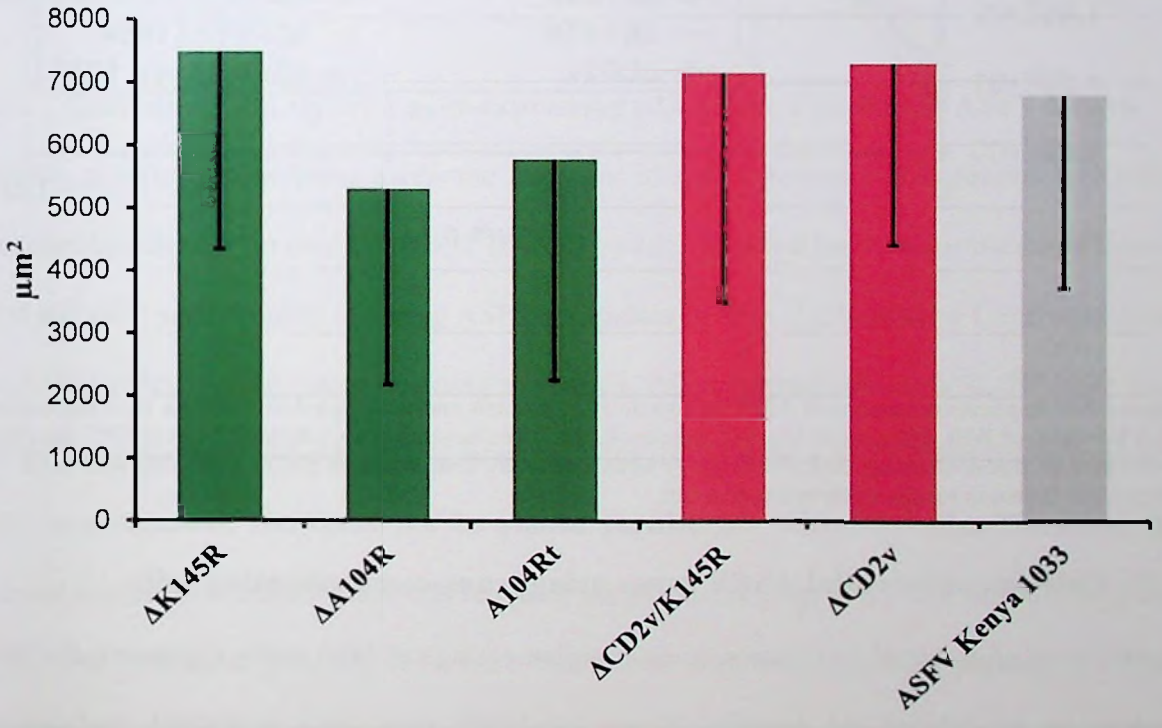


Figure 3-7: Comparison of plaque sizes of five mutant ASFV Kenya viruses and the parental strain Kenya 1033 on WSL-HP cells. 20 plaques were measured per virus at 4 days post infection. Negative standard deviations are indicated.

3.2.2.4 In-vitro growth kinetics of ASFV mutants

The four deletion mutants, ASFV Kenya-ΔK145R, -ΔA104R, -ΔA104Rt, -ΔCD2vK145R and -ΔCD2vDSRed and the parental strain ASFV Kenya 1033 were used to infect WSL-HP cells at

a MOI of 0.03. The cells were incubated for 0, 24, 48, 72, 96 and 120 h at 37°C, and then frozen at -80°C. Virus progenies were titrated on WSL-HP cells and 4 days post infection, plaques were counted and titers calculated. The results indicated no significant differences between replication of wild-type ASFV Kenya and the deletion mutants (Fig 3-8).

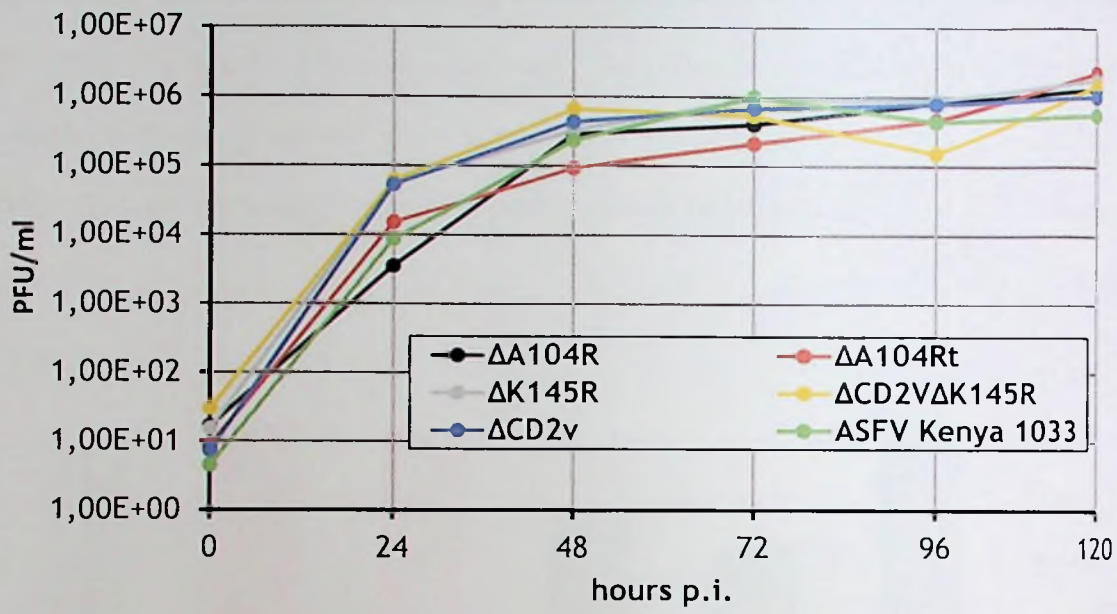


Figure 3-8: Replication kinetics of ASFV Kenya deletion mutants and wild-type ASFV Kenya 1033 determined after infection of WSL-HP cells at low multiplicity (0.03). After incubation for indicated times at 37°C the cells were lysed by freeze-thawing, and total progeny virus titers were determined by plaque assays on WSL cells. Shown are the mean results of three experiments.

3.3 Deletion of essential ASFV genes using trans-complementing cells

For deletion of essential viral genes, trans-complementing cell lines stably expressing the virus protein to be deleted are required. Once these cell lines were generated, isolation of corresponding gene deletion virus mutants could be performed as described above.

3.3.1 Preparation of p12-expressing cells lines

After transfection of WSL-HP cells with plasmid pCAGGS-AKO61RneoR, several stably neomycin resistant cell clones were obtained, and tested for p12 (pO61R) expression by Western blot analyses with a newly prepared monospecific rabbit antiserum (see section 2.2.14.

Fig. 3-9). Two cell clones unambiguously expressing the ASFV protein (WSL-CAGGS-O61R 11 and WSL-CAGGS-O61R 19) were used for generation of a p12 deleted ASFV recombinant.

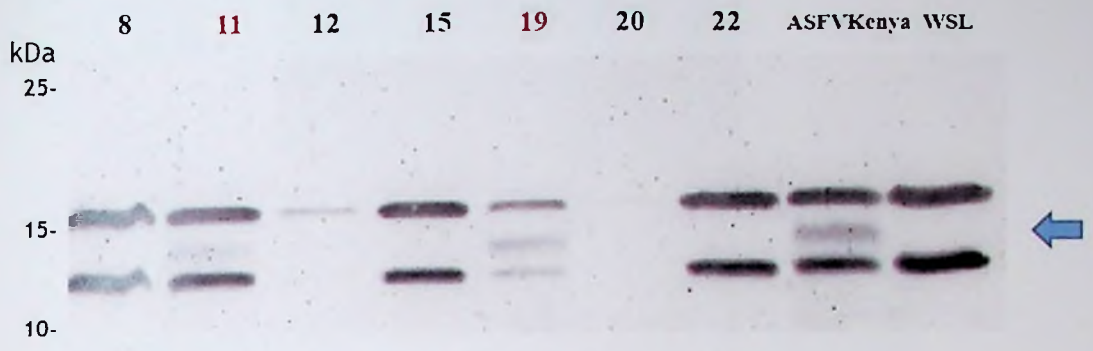


Figure 3-9: Western blot analysis of WSL-CAGGS-O61R cells, normal WSL-HP cells and ASFV Kenya-infected cells (MOI 1, 2 d.p.i.) with an anti-p12 rabbit antiserum. Two cell clones (red) showed expression of a 12-kDa protein similar to that found in infected cells (arrow). The serum also reacted nonspecifically with smaller and larger cell proteins.

3.3.2 Generation of a reporter gene-expressing p12 deletion mutant of ASFV Kenya

Initially, a plasmid containing a genome fragment of ASFV Kenya 1033 (genotype IX) was modified by substitution of the p12 ORF (O61R) by an expression cassette for truncated human CD4 and GFP under control of strong ASFV promoters (p30, p72) flanked by LoxP sites using the highly efficient "hot fusion" cloning technique. After transfection of WSL-HP cells with the resulting plasmid, and subsequent infection with ASFV Kenya 1033 green fluorescent cell foci were observed, but could not be plaque purified on WSL-HP cells. Therefore, the transfection/infection experiment was repeated on p12-expressing WSL cell lines. In addition to the O61R deletion plasmid, cells were transfected with an expression plasmid for Cas9 nuclease and a p12 gene-specific guide RNA to facilitate deletion by site-specific cleavage of the ASFV DNA followed by homology-directed repair (HDR). This was possible since WSL-CAGGSO61R cells expressed p12 from a synthetic gene exhibiting a DNA sequence different from that of the viral O61R gene. GFP-expressing ASFV-recombinants presumably representing the desired mutant ASFV-Kenya-ΔO61R-LoxPGFPhuCD4 were observed in plaque assays on WSL-CAGGSO61R cells (Fig. 3-10) and could be enriched by repeated

plaque purification. Ongoing experiments have to prove whether the virus mutant can be purified to homogeneity, and whether subsequent DNA analyses confirm the desired deletion.



Figure 3-10: *Fluorescence image of a single "plaque" of GFP-expressing ASFV-Kenya-ΔO61R-LoxPGFPhuCD4 on stably p12-expressing WSL-CAGGSO61R cell clone 19, recorded 4 dpi.*

3.4 Separation of ASFV recombinants expressing human CD4

3.4.1 Expression huCD4 in WSL cells

The expression of the truncated human CD4 gene used as selectable reporter protein was first tested after transfection of WSL-HP cells with a plasmid encoding the huCD4 under control of the human cytomegalovirus immediate-early promoter, or the empty vector. Indirect immunofluorescence analyses of non-permeabilized cells with a monoclonal antibody showed that the CD4 gene is abundantly expressed at the surface of the transfected cells (Fig. 3-11).

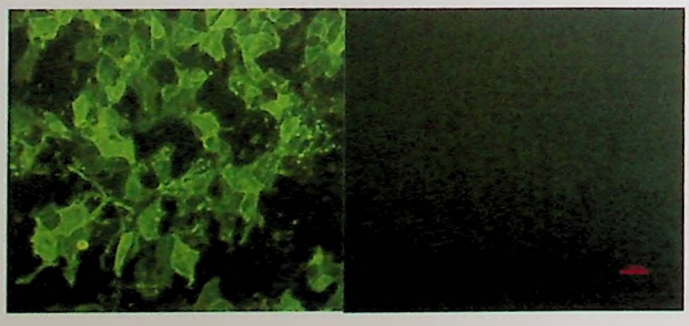


Figure 3-11: Indirect immunofluorescence analysis of WSL-HP cells two days after transfection with pcDNA-huCD4 (left panel) or pcDNA3 (right panel). Cells were fixed with 3% paraformaldehyde (PFA) and incubated with a CD4-specifc mAb (MT310) as first antibody, and AlexaFluor 488 goat α-mouse IgG as second antibody

In WSL-HP cells infected with the generated ASFV recombinants containing GFP and CD4 reporter genes (see above), surface expression of CD4 under control of the ASFV p30 promoter could be also demonstrated (Fig. 3-12).

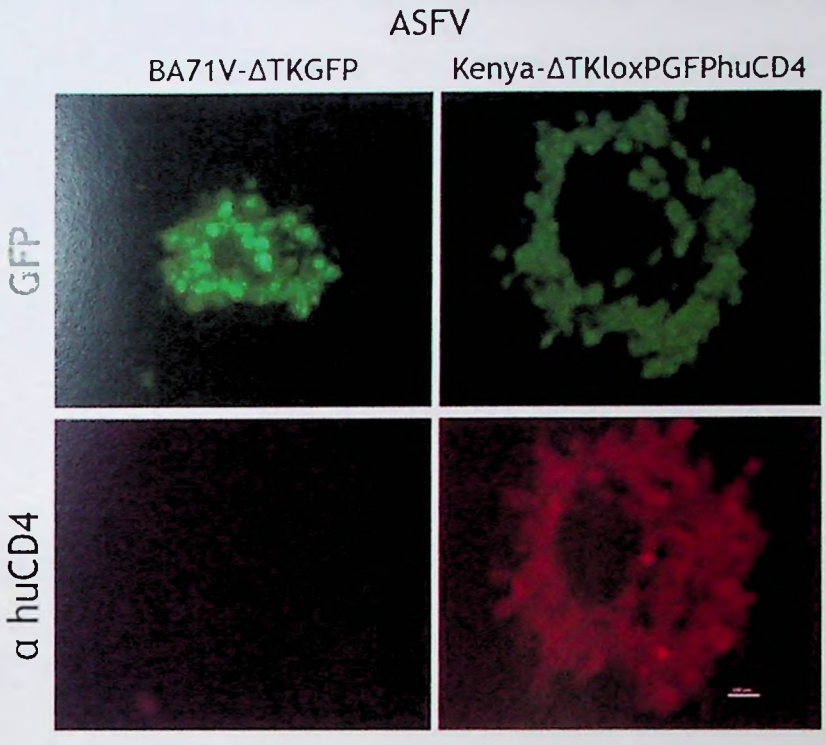


Figure 3-12: Indirect immunofluorescence analysis of WSL-HP cells fixed with 3% PFA 5 d after infected with ASFVBA71V-ΔTKGFP (left panels), or ASFVKenya-ΔTKloxPGFPhuCD4 (right panels) and incubated with a CD4-specifc mAb (MT310) as first antibody, and AlexaFluor 594 goat α-mouse IgG as second antibody. The upper panels show GFP autofluorescence (green), and the lower panels show CD4 specific immunofluorescence reactions (red) of the same plaques.

3.4.2 MACS sorting of virus particles of CD4-expressing ASFV recombinants

Since human CD4 was abundantly expressed at the surface of cells transfected with corresponding plasmids or infected with ASFV mutants, it appeared conceivable that the protein was also incorporated into the outer envelope of virus particles, which is derived from the plasma membrane. To test for this, WSL-HP cells were infected at a MOI of 0.1 with ASFV-Kenya-ΔK145R-LoxPGFPhuCD4, or ASFVArmeniaΔp22LoxPDsRed (kindly provided by K. Pannhorst, FLI), and supernatants were harvested and cleared by low-speed centrifugation 3 d p.i. without freeze-thawing to avoid release of infectious ASFV particles lacking an outer

envelope. Virus mutants were incubated with magnetic bead-tagged anti CD4 antibodies and separated on MACS columns (Miltenyi Biotech). The virus content of the original mixture, the flow through, wash solutions and eluates was analyzed by plaque assays on WSL-HP cells (Fig 3-13). The studies revealed that the portion of GFP and CD4 decreased from 28.9% in the original mixture to 0.6% in the flow-through, and 4% in the wash solutions. In the eluates, the portion of ASFV-Kenya-ΔK145R-LoxPGFPhuCD4 was 97.7%. However, whereas the control virus ASFVArmeniaΔp22LoxPDsRed was almost completely recovered, more than 90% of the CD4 expressing virus was lost during the experiment, presumably due to irreversible binding to the MACS column. Nevertheless the method should be suitable for enrichment or even purification of correspondingly labeled ASFV recombinants.

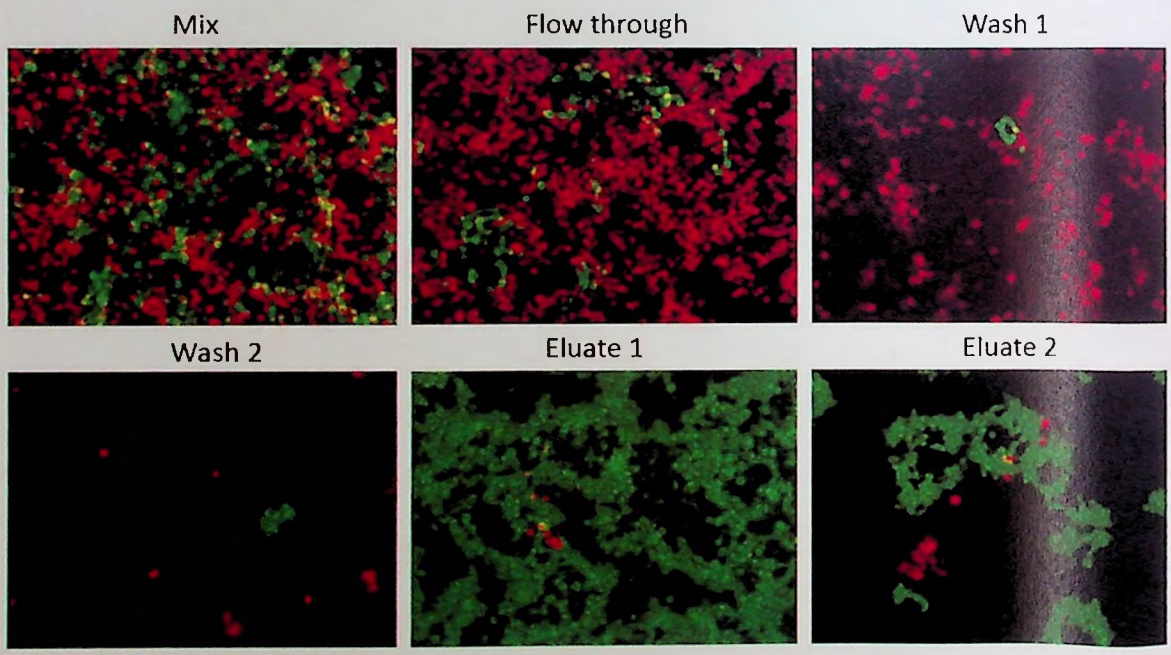


***Figure 3-13**: Autofluorescence images of titrations following MACS separation of two mixed ASFV mutants. ASFV-Kenya-ΔK145R-LoxPGFPhuCD4 (green), and ASFVArmeniaΔp22LoxPDsRed (red), on WSL-HP cells at 4 dpi.*

3.5 Inhibition of ASFV replication in cell culture by stable expression of Cas9 and sgRNAs against single or multiple essential virus genes

After successful CRISPR/Cas9-mediated inhibition of ASFV Armenia and other genotype II and I virus isolates in cell culture (Hübner et al., 2018a), a similar approach was desirable for

inhibition of genotype IX ASFV Kenya. To achieve this, essential ASFV genes encoding p30, p12, and pE199L were targeted using the triple expression vector pX330-ΔNLSneoR for neomycin resistance, single guide RNAs (sgRNAs), and a FLAG-tagged Cas9 nuclease without nuclear localization signals (ΔNLS) since ASFV replicates in the cytoplasm (Fig. 3-14).



Figure 3-14: CRISPR/Cas9 expression vector used for transfections. The gene-specific target sequences were inserted between two BbsI sites preceding the Cas9-activating tracrRNA gene. A neomycin resistance gene (neoR) permitted selection of stably transformed cell clones.

The 20 bp target sequences of the sgRNAs (Fig. 3-15) were inserted as hybrids of complementary synthetic oligonucleotides with matching single stranded overhangs into the *Bbs*I-digested vector. Besides the described gR-CP204LA targeting the p30 gene of ASFV Armenia (Hübner et al., 2018a), gR-CP204LK was designed to target the corresponding gene region of ASFV Kenya exhibiting 4 base substitutions. gR-E199L was also originally developed for ASFV Armenia but exhibited only one mismatch compared to ASFV Kenya. gR-O61R targeted a fully conserved sequence of the p12 gene of both viruses.

gR-CP204LA	GCAAGGGTATACTGAACATCAGG	(codons 71 to 78)
gR-CP204LK	GACAGGGATATACTGAACAGCAGG	(codons 71 to 78)
gR-O61R	GCACTTGATGGTTCAAGTGGTGG	(codons 2 to 8)
gR-E199L	GCATTTTTATTTCCATTACACGG	(codons 98 to 92)

Figure 3-15: *gRNA target sequences used in the study. Codon positions within the ASFV genes are indicated. The PAM sequences (green) were not included in expression plasmids. Base substitutions (red) between ASFV-isolates Armenia and Kenya are also shown.*

Besides plasmids for the individual sgRNAs, the plasmid pX330eCasΔNLS-4xASFVgRneoR was designed for co-expression of all four ASFV gene-specific sgRNAs (Fig. 3-16). This plasmid was generated using the Multiplex CRISPR/Cas9 Assembly System Kit (Sakuma et al., 2014), and assembled from four single sgRNA expression plasmids by Golden Gate cloning (see section 2.2.7.9). The resulting plasmid was modified by substitution of the Cas9 by the eCas9 gene exhibiting three substitutions of basic amino acids (lysine or arginine) to alanine to minimize off-target cleavage (Slaymaker et al., 2016). As above the NLS of eCas9 was removed, and a neomycin resistance gene was inserted to permit selection of stably transfected WSL-HP cell clones.

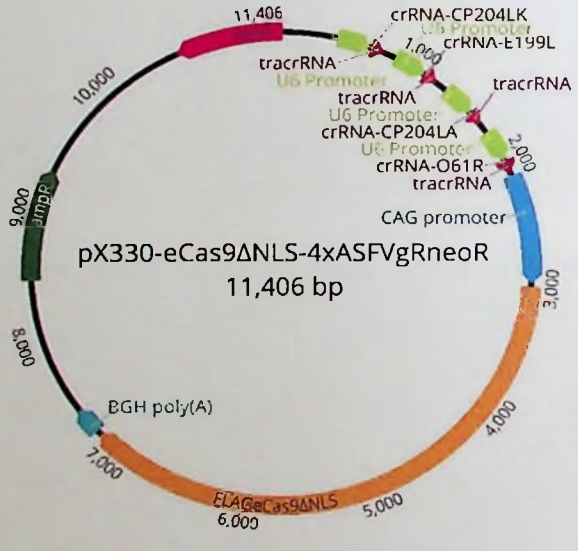


Figure 3-16: *Plasmid used for targeting 4 ASFV genes, the expressed Cas9 nuclease (FLAGeCas9ΔNLS) lacks nuclear localization signals, and exhibits point mutations which should reduce off target activity.*

3.5.1 Transfection and selection of resistant cell clones

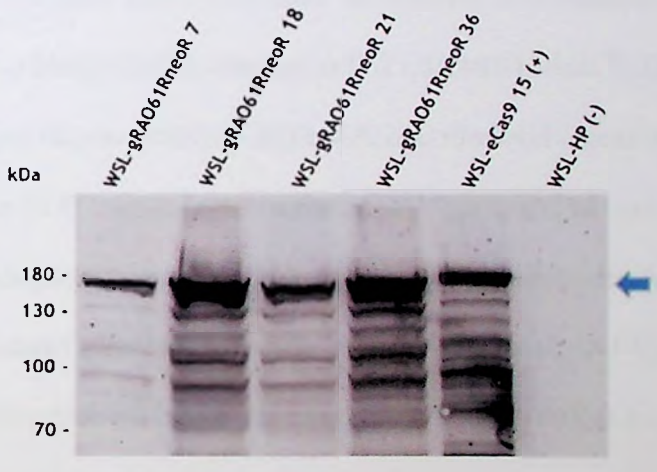
WSL-gRAO61RneoR, WSL-gRACP204LKneoR and WSL-4xASFVgRneoR cells were isolated from WSL-HP cells transfected with the corresponding CRISPR/Cas9 plasmids by limiting dilutions, and propagation of single cell colonies exhibiting resistance to 500 µg/ml

G418 sulfate. WSL-gRAE199LneoR cells have been prepared previously (kindly provided by A. Hübner, FLI).

3.5.2 Investigation of Cas9 expression

To analyze stable expression of Cas9 or eCas9 nuclease, cell samples were prepared and tested by Western blotting with a FLAG tag-specific monoclonal antibodies (Fig. 3-17). The studies revealed that most cell lines expressed various amounts of large proteins matching the calculated mass of tagged-Cas9ΔNLS (161 kDa). Abundance of the Cas9 expression of individual cell clones remained constant over ≥ 25 passages (not shown).

(A)



(B)

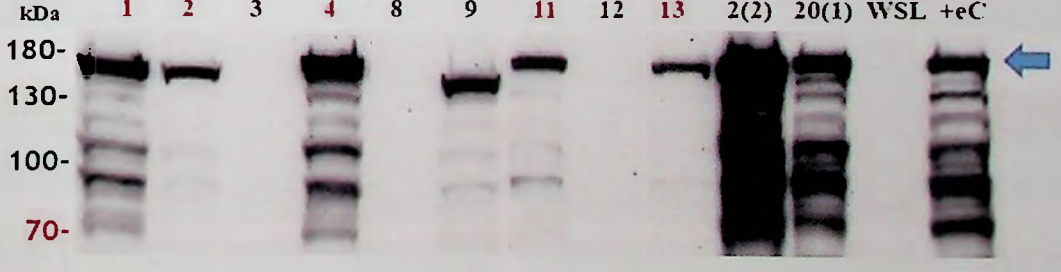


Figure 3-17: Western blot analysis of WSL-gRO61RneoR cell clones with a monoclonal antibody against the FLAG-tag of Cas9 nuclease. All shown cell clones express the full length 161 kDa protein (arrow). Molecular mass markers are indicated at the left. (A) is expression after the after 25 passages and (B) desirable WSL-X330eCasΔNLS4xASFVgRneoR cell clones are indicated in red color.

3.5.3 Sequencing of inserted sgRNA genes

Genomic DNA of Cas9-expressing cell clones was prepared, and a 468 bp fragment of the nuclease gene was amplified by PCR with primers Cas9-RNA-1 and Cas9-RNA-u (Table 3). Sequencing of the PCR products with these primers permitted differentiation of normal Cas9 and eCas9 genes, and confirmed presence of the desired variants (results not shown). The single sgRNA genes of WSL-gRACP204LKneoR and WSL-gRAO61RneoR cells were amplified with primers X330GRR-F2 and X330GRR-R2 (Table 3), and primer X330GRR-R2 was used for sequencing (Fig. 3-18). All tested Cas9-expressing cell clones also contained the desired sgRNA gene. The multiplex sgRNA gene cluster of WSL-eCasΔNLS4xASFVgRneoR cell clones was amplified using primers CRISPR-step2-F2 and CRISPR-step2-R (Table 3), and sequenced using the same two primers. In most tested cell lines the obtained PCR products exhibited the expected size of 1842 bp and contained all four sgRNA cassettes as desired (Fig. 3-19). From few clones, however smaller PCR products were amplified, and sequencing revealed loss of one or two sgRNA genes (not shown). This was presumably due to recombination between different copies of the almost identical genes forming a tandem repeat sequence (see Fig. 3-16, plasmid map). Cell clones abundantly expressing Cas9 nuclease and containing all desired sgRNA genes were stored and tested for susceptibility to ASFV infection.

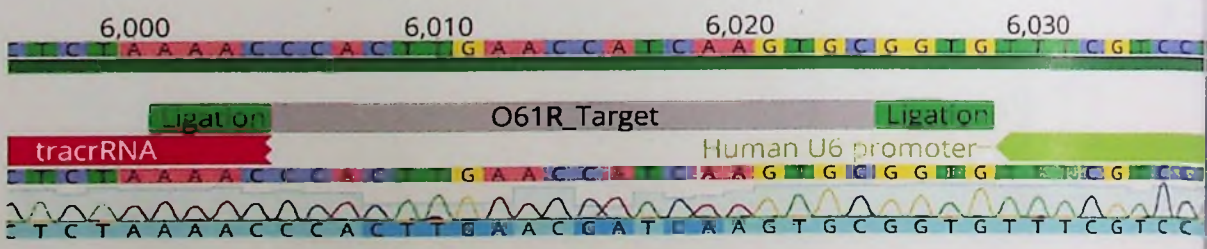


Figure 3-18: Confirmation of the ASFV O61R specific target sequence insertion at the 5'-end of the tracrRNA gene in a Cas9-positive WSL-gRAO61RneoR cell clone (No. 36). Bottom-up the determined sequence (including the chromatogram of base reads), the relevant part of the desired sequence, and the consensus sequence confirming 100% identity are shown.

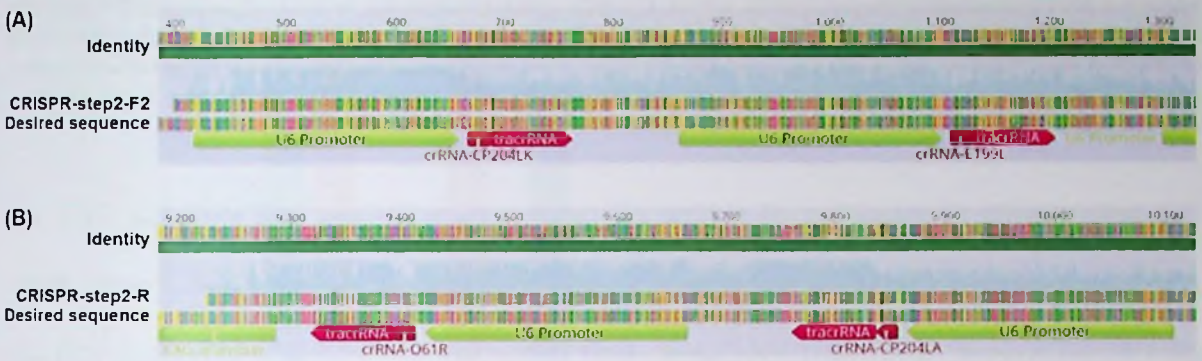


Figure 3-19: Sequence analysis of a WSL-eCasΔNLS4xASFVgRNeoR cell clone using CRISPR-step2-F2 primer (A) and CRISPR-step2-R (B) primers, confirmed correct insertion of all the four ASFV-specific target sequences (crRNA-CP204LK, -E199L, -CP204LA, and – O61R) at the 5'-ends of the tracrRNAs.

3.5.4 In vitro replication studies of wild-type and reporter-gene labeled ASFV mutants in positive cell clones

Cell clones exhibiting abundant Cas9 or eCas9 expression and correct gRNA sequence insertions were infected with WSL-HP adapted ASFV Armenia, ASFV Kenya 1033 or the reporter gene expressing mutants ASFV KenyaΔCD2vDsRed (Hübner et al., 2018a), and ASFVArmeniaΔ285L-LoxPGFPhuCD4 (Hübner et al., 2019). Initial experiments showed that spread of wild-type and CD2-deleted ASFV Kenya was almost abolished on a WSL-gRAO61RneoR cell line, and also severely impaired on WSL-eCas4xASFVgRneoR cells (Fig. 3-20).

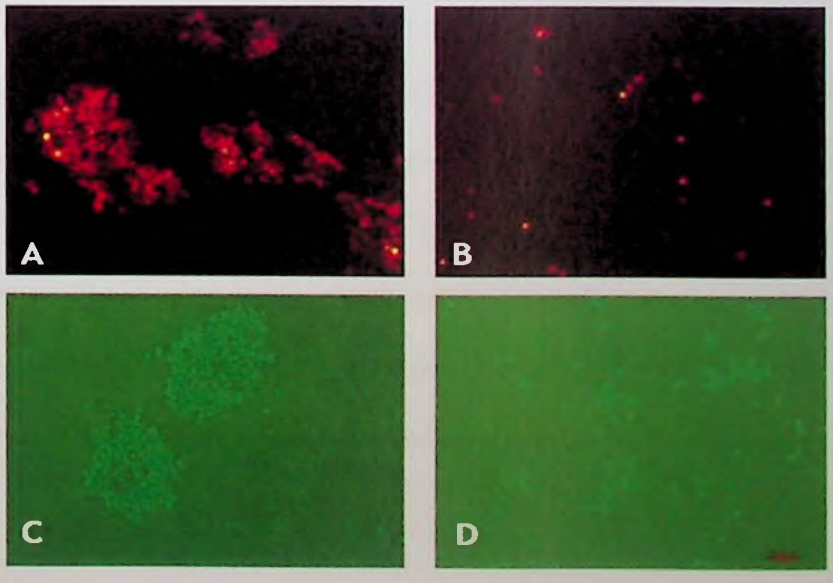


Figure 3-20: *Fluorescence images of WSL-HP (A, C), WSL-gRO61RneoR (B), or WSL-eCas4xgRASFVneoR (D) cells 5 days after infection with 10^3 PFU/well (A, C) or 10^5 PFU/well (B, D) of ASFV-KenyaΔCD2vDsRed (A, B) or ASFV-Kenya 1033 (C, D). Cells infected with the latter virus were visualized by indirect immunofluorescence reactions of a monospecific rabbit antiserum against the major capsid protein p72 of ASFV, and Alexa-Fluor 488-conjugated secondary antibodies.*

For comparative investigation of plating efficiencies of wild-type ASFV-Kenya 1033 and ASFV-Armenia the CRISPR/Cas9 cells WSL-gRO61RneoR, WSL-gRCP204LAneoR, WSL-gR-CP204LKneoR, WSL-gR-E199LneoR, WSL-eCas4xgRASFVneoR, and normal WSL-HP cells were grown to monolayers in 24well plates and infected with serial virus dilutions by subsequent centrifugation at 2000 rpm and incubation at 37°C for 1 hour each. After 6 days under semisolid medium virus plaques were visualized by indirect immunofluorescence assay using a monospecific rabbit antiserum against the major capsid protein p72, and apparent titers were calculated (Fig. 3-21). The studies revealed a complete inhibition of plaque formation but not infection, resulting in single fluorescent cells of ASFV Armenia on all CRISPR/Cas9 cell lines except WSL-gRCP204LKneoR, which expresses a non-matching sgRNA (see Fig. 3-15). In contrast, spread of ASFV Kenya was completely inhibited on WSL-gRO61RneoR cells, and to lower extents also on WSL-gRCP204LKneoR, WSL-gRE199LneoR, and WSL-eCas4xgRASFVneoR. In agreement with previous findings (Hübner et al., 2018a) replication of ASFV Kenya was not inhibited on WSL-gRCP204LAneoR cells.

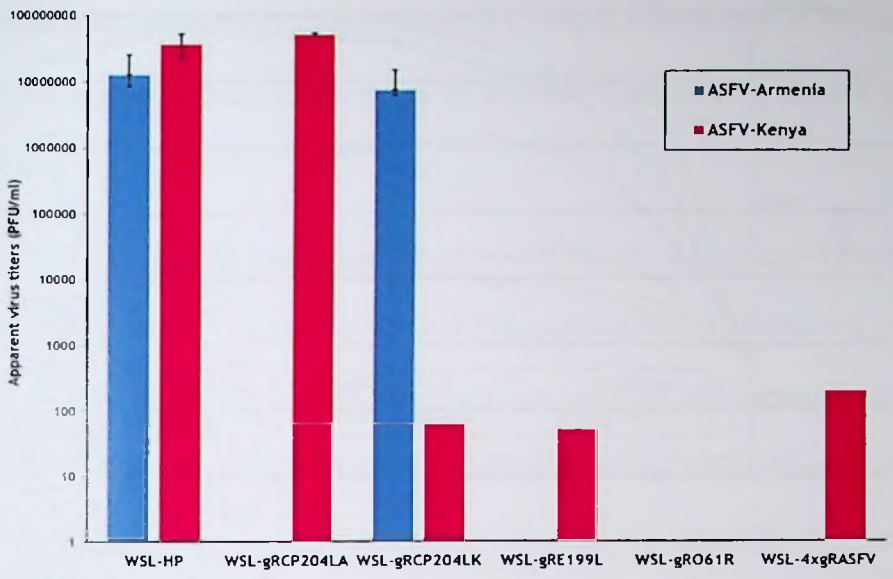
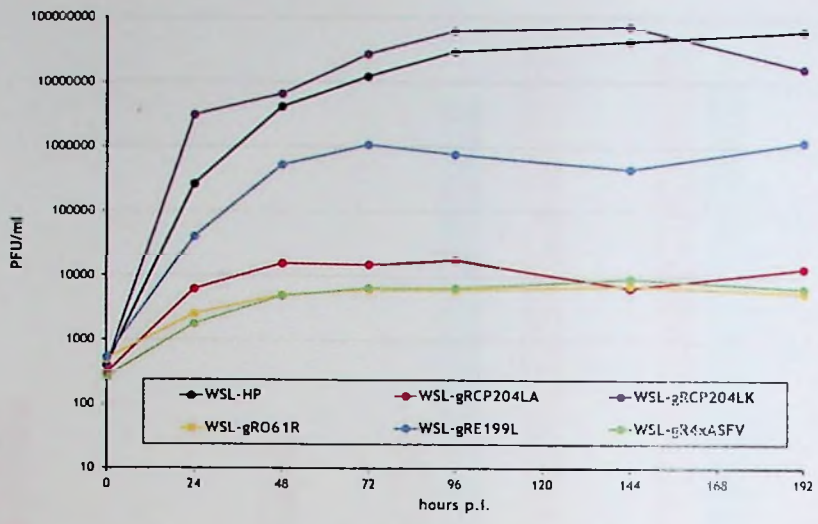


Figure 3-21: To determine plating efficiencies, WSL-HP cells and cells expressing gRCP204LA, gRCP204LK, gRE199L, gRO61R, or all four gRNAs (4xgRASFV) were infected in parallel with serial dilutions of ASFV-Armenia and ASFV-Kenya. After 6 days at 37°C apparent virus titers, and standard deviations were determined.

For comparative multistep replication studies of wild-type ASFV-Kenya 1033 and ASFV-Armenia, monolayers of the same cell lines were infected at a MOI of approximately 0.03. After removal of the inoculum and washing, the cells incubated for 0, 24, 48, 72, 96, 120, 144, 168 and 192 h at 37°C, and then frozen at -80°C. Progeny virus titers in the thawed lysates were determined by plaque assays on WSL-HP cells (Fig. 3-22). The studies revealed an approximately four log reduction of final titers of ASFV-Armenia on WSL-gRO61RneoR, WSL-eCas4xgRASFVneoR and WSL-gRCP204LAneoR cell clones, and a moderate growth inhibition on WSL-gRE199LneoR cells (Fig. 3-22A). As expected, replication of ASFV Armenia was similar on WSL-gRCP204LK and WSL-HP cells. Titers of ASFV Kenya were reduced by approximately five log on WSL-gRO61RneoR cells, and by four log on WSL-eCas4xgRASFVneoR and WSL-gRCP204LK (Fig. 3-22B). Productive replication of ASFV Kenya was only negligibly inhibited on WSL-gRE199LneoR cells, presumably due to only a single mismatch in the guide RNA target sequence (see above). On WSL-gRCP204LAneoR no growth inhibition of ASFV Kenya was observed compared to WSL-HP cells.

(A)



(B)

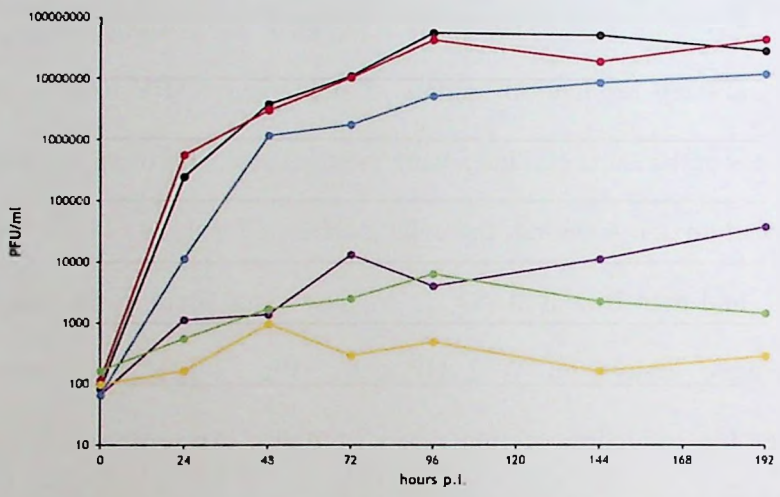


Figure 3-22: *Growth kinetics of (A) ASFV-Armenia, and (B) ASFV-Kenya on WSL-HP cells, and cells expressing gRCP204LA, gRCP204LK, gRE199L, gRO61R, or all four gRNAs (4xgRASFV) were determined after infection at low multiplicity (0.03). After incubation for indicated times at 37°C the cells were lysed by freeze-thawing, and total progeny virus titers were determined by plaque assays on WSL cells. Shown are the mean results of two experiments.*

Since growth inhibition of both ASFV Kenya and ASFV Armenia was more pronounced on WSL-gRO61RneoR than on WSL-eCas4xgRASFVneoR cells which expressed the same p12 gene-specific sgRNA together with three additional ones, it was speculated that this might be due to a lower activity of eCas9 compared to the wild-type Cas9 nuclease present in the first cell line. To test this, the mutated part of the gene in pX330-eCasΔNLS4xASFVgRneoR was replaced by the wild-type sequence. The obtained plasmid was used to generate stable

recombinant WSL-HP cell clones as described above, which were tested by Western blotting, PCR, and sequencing for abundant expression of ordinary Cas9 and the desired four sgRNA genes (not shown).

Several new WSL-Cas4xgRASFVneoR cell clones, old WSL-eCas4xgRASFVneoR and WSL-HP cells were infected with either ASFVArmeniaΔ285L-GFPhuCD4 or ASFV-KenyaΔCD2vDsRed. The studies indicated similarly pronounced, but incomplete inhibition of both viruses on both Cas9- and eCas9-expressing cells (Fig. 3-23). Thus, although more precise comparative in vitro replication studies remain to be done, other reasons like competitive effects might be responsible for the equal or even reduced inhibition of ASFV by four coexpressed sgRNAs compared to single ones.

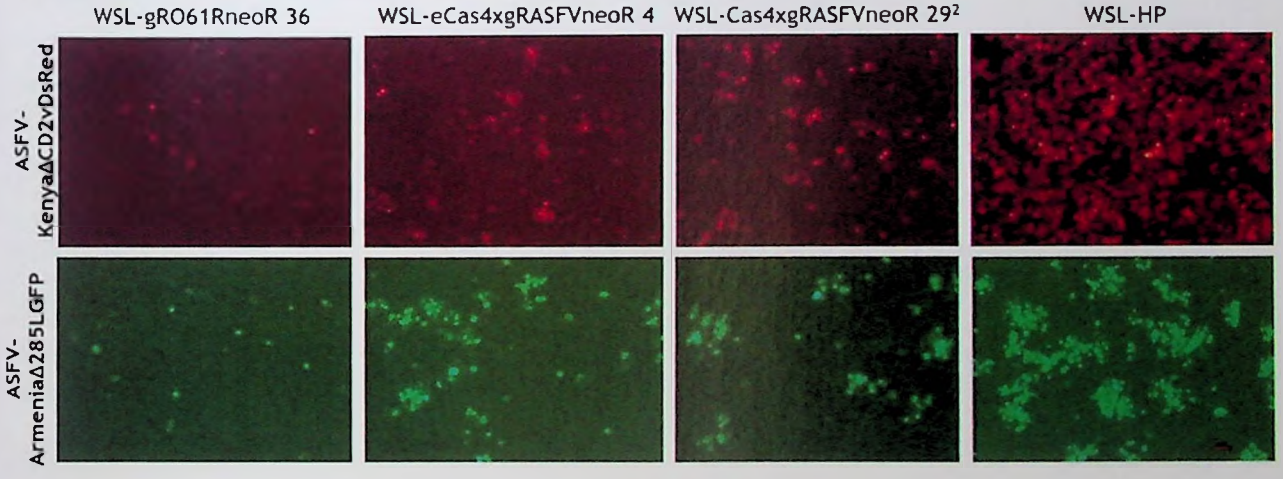


Figure 3-23: Autofluorescence images of WSL-gRO61RneoR, WSL-eCas4xgRASFVneoR, new WSL-Cas4xgASFVneoR and WSL-HP cells 5 days after low MOI infection with ASFV-KenyaΔCD2vDsRed or ASFVArmeniaΔ285L-GFPhuCD4.

3.5.5 Escape mutants

To prove the CRISPR/Cas9 specificity of virus inhibition on WSL-gRCP204LKneoR and WSL-gRAO61RneoR cells, they were infected with ASFV-KenyaΔCD2vDsRed, and serially passaged in T25 flasks. After 3 to 5 passages plaque forming and replication competent escape virus mutants occasionally occurred. Sequencing of the PCR-amplified p30 or p12 genes of these mutants showed nucleotide substitutions or deletions within the target sequences (Fig. 3-

24), which obviously inhibited sgRNA binding without seriously affecting function of the proteins. In the obtained CP204L escape mutant this was not surprising, since the single silent nucleotide substitution did not alter the amino acid sequence of p30 (Fig. 3-24A). In O61R however, the mutations in the two escape mutants led to an in-frame deletion of three or four amino acids from the N-terminal part of p12, respectively (Fig. 3-24B).

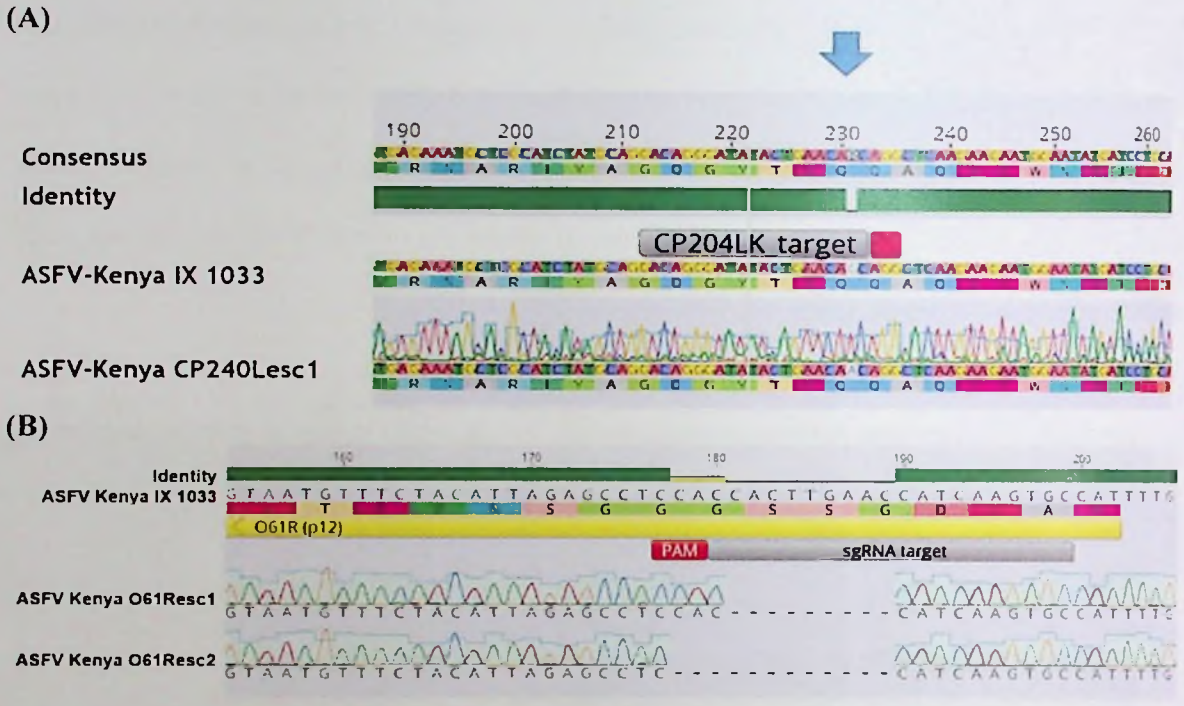


Figure 3-24: *Sequence characterization of replication competent escape mutants of ASFV-Kenya obtained on (A) WSL-gRCP204LKneoR or (B) WSL-gRO61RneoR cells. Amino acid sequences of p30 and p12, sgRNA target regions and PAM sites (red) are indicated.*

3.6 Expression of ASFV proteins in a PrV vaccine strain using CRISPR/Cas9 supported BAC mutagenesis

The PrV vaccine strain Bartha was shown to be a suitable vector for expression of ASFV genes, which could be easily inserted by CRISPR/Cas9 supported BAC mutagenesis (Hübner et al., 2018b). Therefore, we aimed to express additional ASFV proteins using a similar approach. For the present study the immunogenic ASFV proteins p12 (pO61R) and p54 (pE183L) were chosen.

3.6.1 Generation of PrV-BaΔgGAKO61Rporc and PrV-BaΔgGFLAGAKO61Rporc

To generate the desired virus mutants the plasmids pUC-BaKJCAGGS-AKO61Rporc and pUC-BaKJCAGGS-FLAGAKO61Rporc were cloned, which contained the codon-adapted ORF O61R from ASFV Kenya under control of the CAG promoter, and flanked by sequences from the PrV-Bartha glycoprotein G (gG) gene region to permit homologous recombination. The N-terminally FLAG-tagged O61R gene was included since at this time no p12-specific antiserum was available. The recombination plasmids were used for cotransfections of cells together with a bacterial artificial chromosome (BAC) containing the complete PrV-Bartha genome (Fig. 3-25A). To facilitate isolation of virus recombinants the infectious BAC pPrV-BaΔgGG (Klingbeil et al., 2014) containing the bacterial replicon and a reporter gene cassette encoding enhanced green fluorescent protein (EGFP) at the nonessential gG locus had been modified by deletion of the promoter and the N-terminal part of the essential gene for the receptor-binding glycoprotein gD which is located immediately downstream of the gG gene (Fig. 3-25A). Therefore, the resulting BAC pPrV-BaΔgGΔgD (Hübner et al., 2018b) was defective and had to be rescued by recombination with the plasmids providing an intact gD gene. Transfection experiments were performed in the recombinant rabbit kidney cell line RK13-Cas9EGFPgRneoR (Hübner et al., 2108b), which stably expresses Cas9 nuclease and an EGFP gene-specific sgRNA. Cleavage of the transfected BAC DNA, followed by homology directed repair by the recombination plasmids led to deletion of the EGFP gene and the bacterial vector, and substitutive insertion of the desired ASFV genes (Fig. 3-25B). This method permitted fast and highly efficient transgene substitution. Thus, PrV-BaΔgGAKO61Rporc and PrV-BaΔgGFLAGAKO61Rporc could be isolated after a single round of plaque purification on normal RK13 cells. In a similar manner PrV-Ba BaΔgGAE183Lporc expressing the p54 gene of ASFV Armenia was generated. Genomic DNA of all PrV recombinants was analyzed by PCR and sequencing for correct transgene insertions (results not shown).

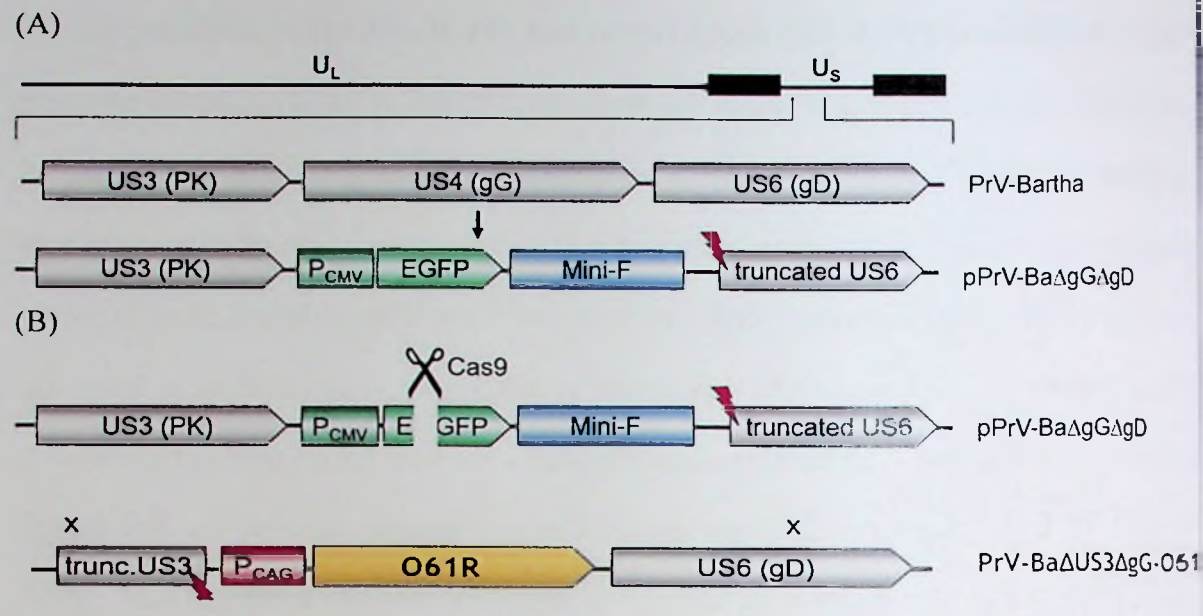


Figure 3-25: *(A) Genome map of PrV-Bartha showing long and short unique regions (UL, US), and an enlarged section showing the protein kinase (US3), gG (US4) and gD (US6) genes, and the eventual defective BAC pPrV-BaΔgGΔgD. (B) cotransfection of either pUC-BaKJCAGGS-AKO61Rporc and pUC-BaKJCAGGS-FLAGAKO61Rporc with pPrV-BaΔgGΔgD on RK13-Cas9EGFPgRneoR cell clones led to cleavage of the BAC DNA at the EGFP site (marked with scissors) and subsequently to insertion of the desired transgene like O61R and E183L by homologous recombination.*

3.6.2 Investigation of protein expression by Western blot analysis of infected cells

To investigate protein expression, RK13 cells were infected (MOI 5) with the obtained PrV recombinants PrV-BaΔgGAKO61Rporc and PrV-BaΔgGFLAGAKO61Rporc, or transfected with the O61R plasmids and harvested after approximately 24 h at 37°C. Western blot analyses of the lysates using a FLAG tag-specific mAb revealed expression of tagged p12 (Fig 3-26), which exhibited an apparent molecular mass of ca. 16 kDa. This was significantly larger than calculated (8.1 kDa), and presumably due to post-translational modifications. Detection of untagged p12 was not possible at this time, since a "commercial" p12 monoclonal antibody turned out to be specific for p11.5 (A137R) instead (results not shown). This finding prompted us to generate a polyclonal ASFV p12-specific rabbit antiserum (see section 3.7).

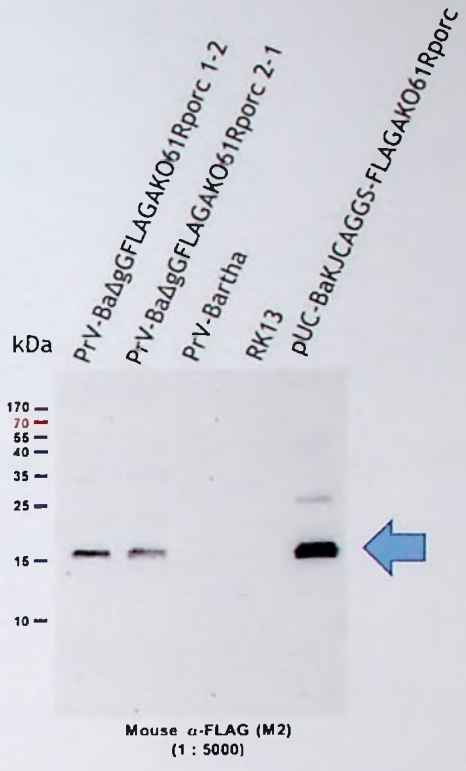


Figure 3-26: Western blot analysis of PrV recombinants or plasmid transfected cells transfected cells expressing FLAG-tagged O61R protein with an anti-FLAG mAb. The specifically detected 16 kDa protein (arrow) was not found in uninfected or PrV-Bartha-infected RK13 cells. Molecular masses of marker proteins are indicated.

3.6.3 Southern blot analysis of PrV recombinants

To further confirm that the obtained PrV-BaΔgGAKO61Rporc recombinants contain the desired mutations genomic DNA prepared from infected RK13 cells was digested with *Bam*HI, separated on a large (20 cm) agarose gel, transferred to nylon membranes and hybridized with the radioactively labelled recombination plasmid pUC-BaKJCAGGS-AKO61Rporc. The analyses revealed the expected fragment size alterations compared to the parental virus strain PrV-Bartha (Fig. 3-27).

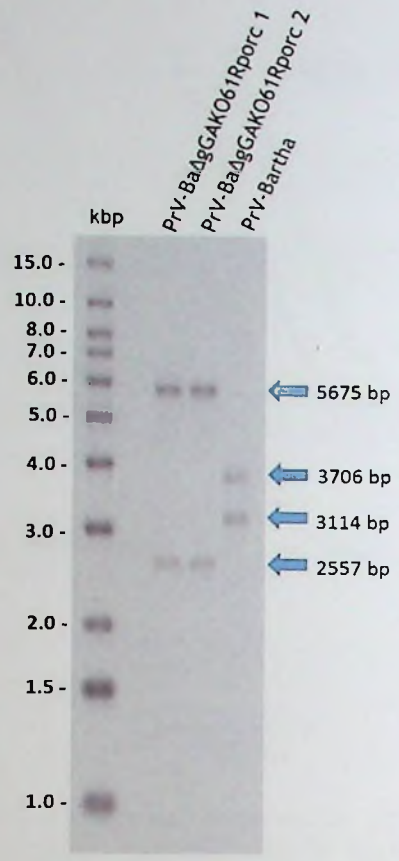


Figure 3-27: Southern blot analysis of BamHI-digested DNA of PrV-BaΔgGAKO61Rporc (1, 2), and PrV-Bartha with ^{32}P-labelled pUC-BaKJCAGGSAKO61Rporc. Sizes of DNA markers and calculated sizes of the expected fragments are indicated.

3.7 Bacterial expression of ASFV genes for preparation of monospecific rabbit antisera

Since many predicted ASFV gene products possess unknown functions, and have not yet been identified at the protein level, monospecific rabbit antisera were prepared to localize them in infected cells and virions, and to confirm their presence or absence in generated ASFV recombinants. In the present study, sera against p54 (pE183L), p12 (pO61R), p11.5 (pA137R), pK145R, and p285L were prepared, after expression of the corresponding ORFs as bacterial fusion proteins with glutathione S-transferase (GST).

The monospecific rabbit antiserum against the proposed attachment protein p12 (pO61R) had to be prepared, since a commercial "p12"-specific monoclonal antibody turned out to be

directed against p11.5 (pA137R). Analyses of the deduced O61R translation products of ASFV Kenya and Armenia using Geneious indicated that p12 is an integral membrane protein containing a central anchor domain (Fig. 3-28), with a calculated molecular mass of 6.8 kDa. The amino acid sequences of the two virus strains differ at 4 positions.

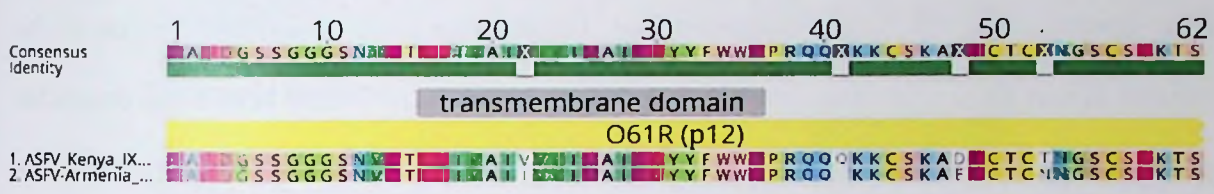


Figure 3-28: Amino acid sequence alignment of p12 of ASFV Kenya 1033 and ASFV Armenia. Using Geneious and Uniprot, the predicted transmembrane domain and orientation are indicated.

3.7.1 Expression of ASFVp12 and p54 in E. coli

The codon-adapted ASFV ORF O61R from strain Kenya (93.5% identity to Armenia) and the original ORF E183L from strain Armenia (79.1% identity to Kenya) were cloned into prokaryotic expression vectors pGEX-4T1 or -4T3, respectively. After transformation of *E. coli* and IPTG (Isopropyl β-D-1-thiogalactopyranoside) induction, fusion proteins with GST were expressed, and could be detected by SDS-PAGE and subsequent Coomassie staining (Fig. 3-29).

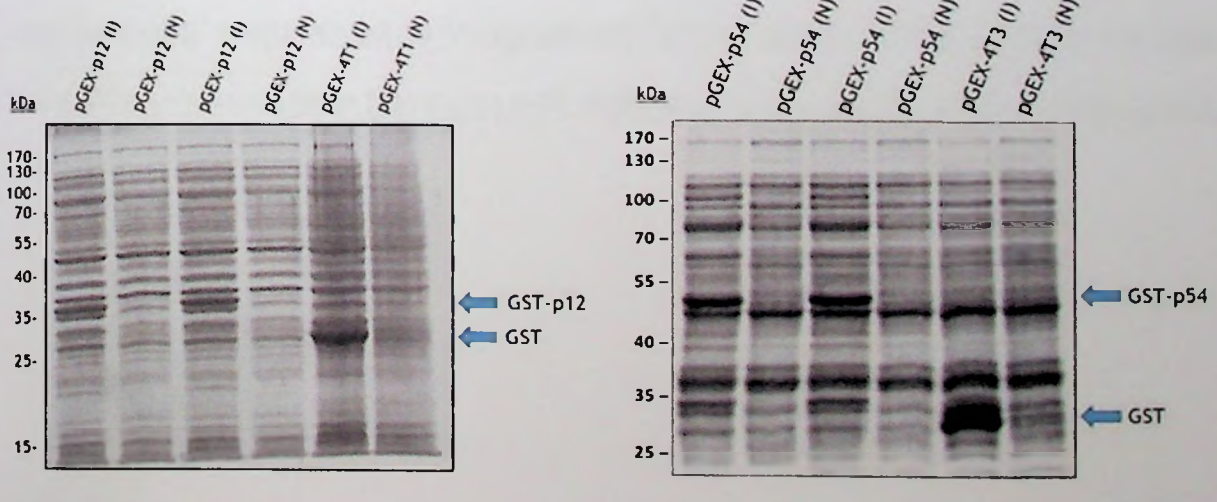


Figure 3-29: GST fusion protein expression in lysates of induced (I) and non-induced (N) bacteria transformed with pGEX-p12 (left panel) or pGEX-p54 (right panel). Cells transformed with the empty vectors served as controls. Stained SDS-10% polyacrylamide gels are shown, and molecular mass markers as well as induced proteins are indicated.

The expressed fusion proteins GST-p54 (414 amino acids, 46.7 kDa), and GST-p12 (289 amino acids, 33.16 kDa) exhibited approximately the calculated molecular masses, and were isolated from preparative polyacrylamide gels by electro-elution.

3.7.2 Preparation and testing of monospecific rabbit antisera against ASFV proteins

After determination of protein concentrations, rabbits were immunized with 100 µg of the isolated fusion proteins at least four times in 4-week intervals. However, to obtain a detectable p12-specific antibody response, seven immunizations were required. For Western blot analyses the newly prepared antisera were used at dilutions of 1:2,500 (anti-p12) to 1:20,000 (anti-p54).

The rabbit serum raised against GST-p12 exhibited strong reactions with homologous and heterologous GST-fusion proteins expressed in bacteria, and also unspecific reactions with 17 and 13 kDa proteins of mammalian cells (Fig 3-30). In contrast, the specific reactions with the authentic or PrV-BaΔgGAKO61Rporc-expressed p12 of ASFV-Kenya were rather weak. Again the apparent molecular mass of the protein (approx. 14 kDa) was higher than the calculated mass (6.8 kDa), indicating post-translational modifications. The serum showed no specific reaction with ASFV Armenia-infected cells, presumably because of the above mentioned amino acid sequence differences of p12 (see Fig. 3-28). Apparent absence of specific reactions with the FLAG-tagged p12 of PrV-BaΔgGFLAGAKO61Rporc might be also explained by a size shift to the strong unspecific 17 kDa band (Fig. 3-30).

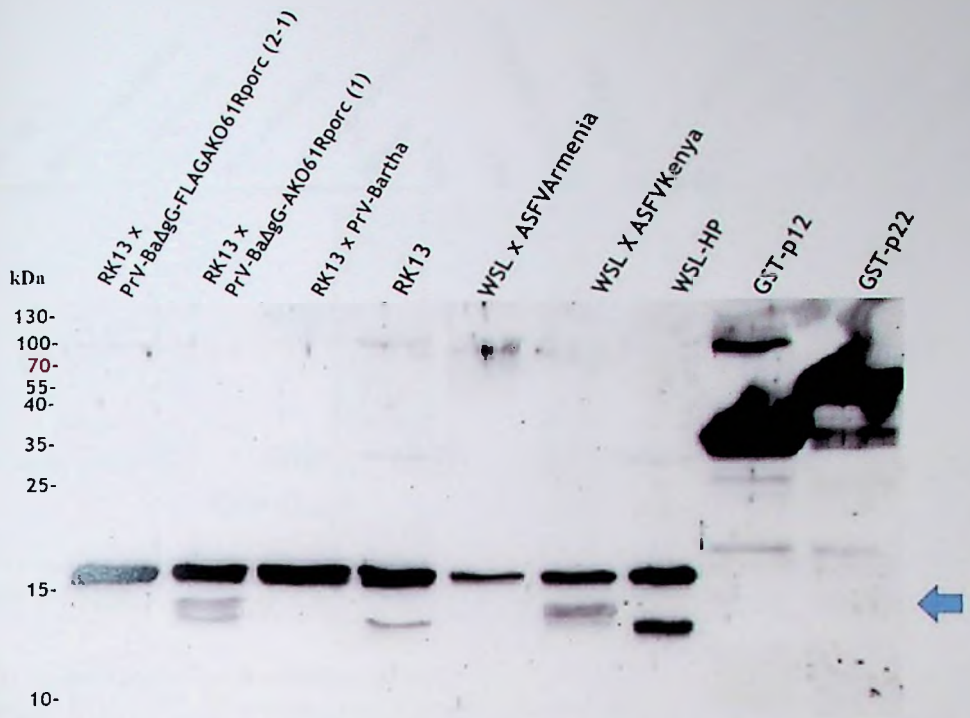


Figure 3-30: Western blot analysis of proteins from RK13 cells infected with PrV mutants, WSL cells infected with wild-type ASFV Kenya and ASFV Armenia, and induced bacterial cultures expressing GST-p12 or GST-p22 (provided by A. Hübner, FLI). After separation on a SDS-15 % polyacrylamide gel the proteins were blotted and incubated with the anti-p12 rabbit serum (dilution 1: 2500). Molecular masses (kDa) of marker proteins and the specifically detected p12 signal (arrow) are indicated.

Western blot analyzes of the obtained anti-p54 serum specifically detected approximate 27 and 57 kDa proteins in RK13 cells transfected with expression plasmids for native and codon-optimized (porc) p54, as well as in ASFV-infected WSL-HP cells (Fig. 3-31). Remarkably, the large form of p54 was always under-represented, but the dominant smaller protein was still larger than calculated from the amino acid sequence (20 kDa for the ASFV Armenia gene product).

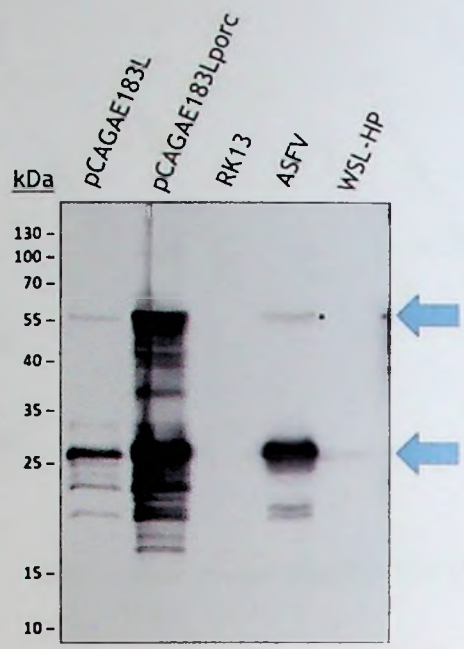


Figure 3-31: Western blot analysis showing expression of p54 in plasmid-transfected RK13 cells and ASFV infected WSL-HP cells. After separation on an SDS-10% polyacrylamide gel the proteins were blotted and incubated with anti-p54 serum (dilution 1: 20.000). Molecular mass markers (kDa) and specifically detected proteins (arrows) are indicated.

Monospecific rabbit antisera against the proteins p11.5, pK145R and p285L of ASFV Armenia were produced using similar approaches and tested in Western blot (Fig 3-32) and indirect immunofluorescence tests (Fig 3-33) for reactivity with WSL-HP cells infected with ASFV Armenia or ASFV Kenya. Whereas p11.5 and pK145R were similarly detected in both virus strains, the anti-p285L serum detected only the homologous protein of ASFV Armenia. This was somewhat surprising since ASFV-Kenya has been shown to possess a 285L ORF, exhibiting 86.2% identity of the deduced amino acid sequence. In immunofluorescence tests the anti-p285L showed no unambiguous reactions with either virus.

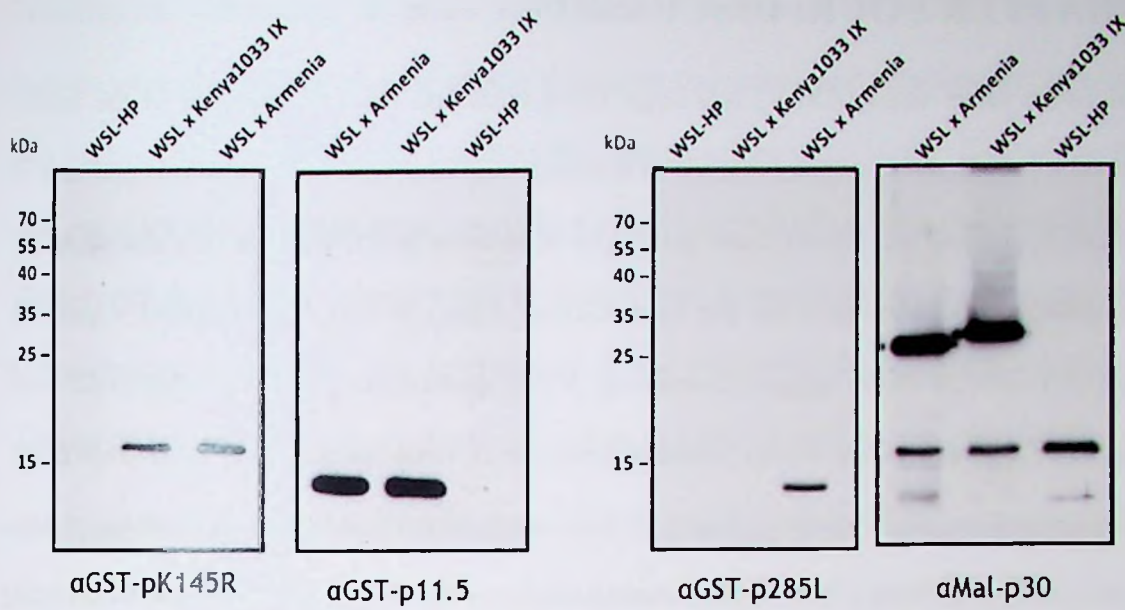


Figure 3-32: Western blot analysis of WSL-HP cells infected with ASFV Armenia (genotype II) or ASFV Kenya (genotype IX) using monospecific rabbit antisera raised against the indicated proteins of ASFV Armenia. The serum against p30 fused to maltose-binding protein (αMal-p30) was kindly provided by G.M. Keil (FLI).

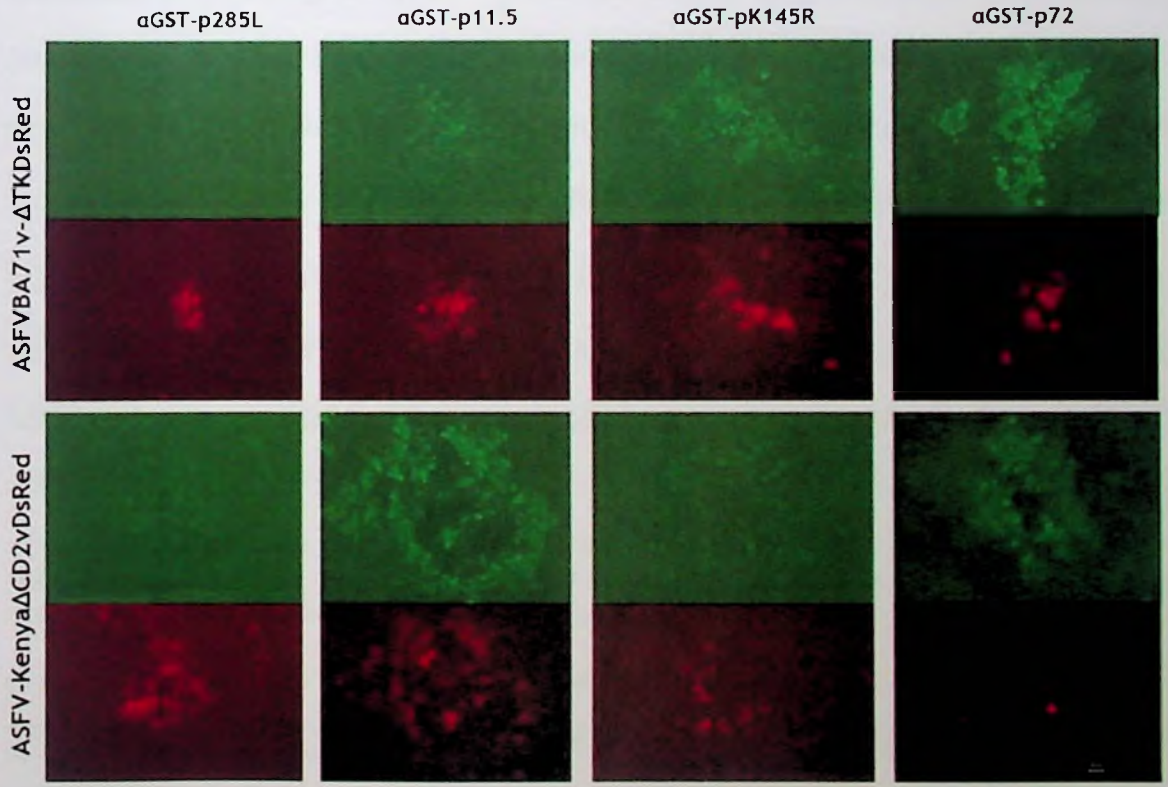


Figure 3-33: Indirect immunofluorescence reactions of monospecific rabbit antisera (diluted 1 : 100) on cells WSL-HP cells fixed 5 d after infection with ASFV-KenyaΔCD2vDsRed (lower panel), or ASFVBA71v-ΔTKDsRed (upper panel, kindly provided by G.M. Keil, FLI) expressing red fluorescent reporter proteins. Green fluorescence was obtained after incubation with AlexaFluor 488-conjugated anti-rabbit antibodies.

4 CHAPTER FOUR: DISCUSSION

African swine fever virus (ASFV) is a large DNA virus and the sole member of the family *Asfarviridae*, which causes a fatal disease of domestic pigs and wild boar. ASFV is endemic in 28 African countries, and 24 different genotypes have been described. For this study, ASFV control and diagnostic options for the East African region should be developed where the situation is complicated by several coexisting ASFV genotypes. Several of them had been introduced to Europe, and a highly virulent genotype II virus spread since 2007 over many Eurasian countries (Gaudreault & Richt, 2019). Most recently ASFV was introduced into Belgium, and spread from China to neighboring countries like Mongolia, Vietnam, Cambodia, Laos, the Philippines and the Democratic People's Republic of Korea. Up to now no ASF vaccines are available. Therefore, we aimed at developing control options for ASFV through the construction of putative attenuated live, single cycle or vectored vaccines by targeted gene deletions. Furthermore, intrinsic ASFV-resistance was engineered and tested in vitro, that may form the basis of ASFV-resistant transgenic pigs. Finally ASFV diagnostic options were improved through polyclonal antibody production.

Of the roughly 150 genes encoded by ASFV, it is predicted that a half to two-thirds play a role in virus survival and transmission in the hosts, but are not essential for replication (Dixon et al., 2004). We, therefore, utilized several ASFV genes to achieve our aims as described in the following sections.

Deletion of non-essential genes of ASFV for generation of live attenuated ASF vaccine candidates

In the present study gene deletion mutants were generated from a genotype IX wild-type virus isolate, designated as ASFV-Kenya 1033. In order to develop putative live or single cycle vaccine candidates, we generated gene deletion mutants lacking known non-essential genes (encoding thymidine kinase, dUTPase, CD2v, 9GL), potentially essential genes (p12, pA104R,

ribonucleotide reductase), or genes with unknown functions (pK145R). Recombination plasmids were prepared by cloning of ASFV genome fragments, and substitution of the desired open reading frames by reporter cassettes for fluorescent proteins (GFP, DsRed), or human CD4. After transfection with these plasmids, a permissive wild boar lung cell line (WSL) was infected with ASFV, and mutants raised by homologous recombination were selected utilizing the fluorescence of infected cells. Furthermore, it was evaluated whether surface expression of CD4 can be utilized for antibody-mediated sorting of infected cells or virus particles.

Although ASFV DNA is found in the host cell nucleus during the early phase of infection, viral DNA replication and mRNA transcription take place mainly or exclusively in the cytoplasm under the control of viral cis-acting elements and proteins (Netherton & Wileman, 2013). Therefore, it was crucial for the generation of ASFV recombinants to express the used reporter proteins under control of strong ASFV promotors. Our studies showed that the gene for green fluorescent protein (GFP) under control of the major capsid protein p72 promoter, and the gene for truncated human CD4 protein under control of the secreted phosphoprotein p30 promoter, were abundantly expressed, and, in case of CD4, incorporated into the plasma membrane as well as into the outer envelope of virions. This enabled direct detection and isolation of the desired infected cells in plaque assays or limiting dilution experiments by fluorescence microscopy and subsequent aspiration, or enrichment of recombinant virus particles by magnetic activated "cell" sorting (MACS) using tagged anti-CD4 antibodies. Since the expressed reporter genes might influence the virus phenotype *in-vivo*, and facilitate unwanted recombination with the host or other organisms in the environment, the transgene insertions of all newly generated ASFV mutants were flanked by LoxP sites to permit their rapid removal by the transiently expressed Cre-recombinase.

In earlier studies, several non-essential genes of ASFV have been deleted singly or in combination, and their impact on virus replication in cells and virulence in pigs has provided

valuable information for the development of potential modified live vaccines (Dixon et al., 2019; O'Donnell et al., 2015; Ramirez-Medina et al., 2019).

9GL-deletion mutants of ASFV have been regarded as potential and promising live-attenuated vaccines. The ASFV 9GL or B119L gene encodes the protein Sulfhydryl oxidase, an oxidoreductase, with Flavin-linked sulfhydryl oxidase activity that catalyzes disulfide bond formation in the host cell cytoplasm, and is involved in virion maturation (www.uniprot.org/uniprot/Q65163). 9GL was shown to encode a 14-kDa protein in infected macrophage cell cultures containing 119 amino acids, and is highly conserved both at nucleotide and amino acid level in different ASFV isolates (Lewis et al., 2000). 9GL influences maturation of virions, growth of virus in macrophage cell cultures and virulence of ASFV in swine (Lewis et al., 2000). O'Donnell et al., 2015, showed that even at low doses a recombinant ASFV-Δ9GL recombinant virus induced protection in swine against the highly virulent parental ASFV-Georgia strain. Although pigs developed ASF signs after a lethal challenge 21 days post vaccination, they survived. Protection was even more effective if the animals were challenged 28 days after vaccination. Previously engineered 9GL deletion mutants showed marked isolate-specific attenuation (O'Donnell et al., 2015). Δ9GL mutant viruses from pathogenic Malawi Lil-20/1 wild-type ASFV were also markedly attenuated in swine, and all vaccinated animals survived homologous wild type challenge infection (O'Donnell et al., 2015). Therefore, the introduced Δ9GL deletion of ASFV Kenya 1033 should also attenuate the virus, and might confer protection against infections with genotype IX wild-type ASFV which, however, needs to be tested experimentally.

The generated ASFV-KenyaΔDUT mutant lacks the gene E165R which encodes dUTPase (Deoxyuridine 5'-triphosphate nucleotidohydrolase) also known as dUTP pyrophosphatase. dUTPase is a hydrolase involved in nucleotide metabolism and may have a role in lowering the dUTP concentration in natural infections, which minimizes misincorporation of deoxyuridine

into the viral DNA and ensures the fidelity of genome replication (www.uniprot.org/uniprot/Q65199). The enzyme is expressed both at early and late times of ASFV infection and localizes in the cytoplasm of infected cells (Oliveros et al., 1999). Therefore, the DUT gene deletion may limit *in vivo* replication of ASFV Kenya, and attenuate the virus as described for other strains (Oliveros et al., 1999).

ASFV-KenyaΔA104R lacks the gene encoding a viral histone-like protein pA104R that is expressed late in the viral replication cycle. It is a DNA-binding protein with a proposed critical role in viral DNA replication, transcription and packaging. With a binding site spanning about 15 nucleotides, it binds to both ssDNA and dsDNA, and displays DNA-supercoiling activity in the presence of the viral DNA topoisomerase 2 (pP1192R) (www.uniprot.org/uniprot/P0C9E5; Frouco et al., 2017). It is highly conserved and was also found in ancient ASFV-like sequences found integrated in certain tick genomes (Forth et al., unpublished results). Mutants lacking pA104R have been suggested as candidates for development of ASFV DISC vaccines (Frouco et al., 2017; Freitas et al., 2019b). Gutierrez-Castaneda et al., 2008 have shown that remarkably high antibody responses were observed in infected animals against the DNA-binding protein pA104R, similar to those against structural proteins p30, p54 and p72. To investigate the role of pA104R in replication of ASFV Kenya 1033, two mutants, ASFV KenyaΔA104R and ASFV Kenya ΔA104R-A104Rt were prepared, with the latter containing inserted A104R like tick-origin sequences at the genome position of the original ASFV gene. Surprisingly, deletion of A104R did not significantly affect productive replication, and only moderately impaired plaque formation of ASFV Kenya on WSL-HP cells. This minor defect was not corrected by the substitution of pA104R with pA104Rt indicating that this protein is not functional. It remains to be clarified whether the discrepancies between the previously proposed essential function, and the dispensability of pA104R found by us, are virus strain or cell type-specific.

Nevertheless, the minor in vitro replication defect of ASFV KenyaΔA104R might be also evident *in vivo*, and make the virus suitable as an attenuated live vaccine candidate.

The K145R protein was identified as one of the 20 most abundantly expressed proteins in WSL, HEK293 and Vero cells (Kessler et al., 2018) although it is now known to be a true late protein (Hubner et al., 2019). In WSL cells infected with an ASFV strain OURT 88/3 recombinant (OURT 88/3-ΔTK-GFP), it was the most highly expressed ASFV protein and ranked no.2 in abundance of intracellular proteins (Kessler et al. 2018). In a previous study (Kollnberger et al., 2002), pK145R has been characterized as an immunogenic, antibody-inducing protein. pK145R has been further shown to be diffusely distributed in the cytoplasm of infected cells, but was not detectable in purified virus particles (Hubner et al., 2019). Whereas a K145R deletion mutant of genotype II ASFV Armenia exhibited slightly impaired replication in cell culture (Hübner et al., 2019), virus spread and growth kinetics of ASFV Kenya were not detectably affected by a similar deletion. The importance of pK145R for *in vivo* replication and virulence of ASFV in swine remains to be determined.

ASFV KenyaΔTK lacks the gene K196R which encodes thymidine kinase associated with virulence in many viruses, including ASFV (Sanford et al., 2016). It phosphorylates thymidine in an ATP-dependent reaction resulting in dTMP, which is a precursor nucleotide for DNA synthesis. The viral TK is dispensable in permanent cell lines but required for ASFV growth in swine macrophages, and is a virulence factor in vivo (www.uniprot.org/uniprot/P0C8I3; Hernandez et al., 1995). In other ASFV strains TK deletion mutants have been shown to be over-attenuated, and not suitable to confer protection against wild-type challenge (Sanford et al., 2016). It remains to be analyzed in animal trials whether this is also true for ASFV Kenya.

The gene deletion mutant ASFV KenyaΔCD2vDsRed used in this study has been described previously (Hübner et al., 2018a). CD2v is membrane protein of ASFV, which is encoded by ORF EP402R, and similar to the host CD2 protein expressed on NK and T cells. CD2v adsorbs

RBCs onto virus-infected cells and extracellular virus particles, and its expression aids dissemination of virus in pigs, and further impairs bystander lymphocyte function (Dixon et al., 2004). Virus replication in ticks is significantly reduced with the loss of the CD2v gene, although dissemination of virus in tick cells is not strictly dependent on CD2v (Netherton & Wileman, 2013; Rowlands et al., 2009; www.uniprot.org/uniprot/Q76VU8). The influence of CD2v on virulence largely depends on the genetic background of the virus, and in some cases deletion of the EP402R gene only led to a delay in the onset of clinical signs, but not to a reduction of lethality in pigs (Dixon et al., 2019). On the other hand, a study by Monteagudo et al., 2017, showed sufficient virus attenuation after the deletion of the EP402R gene from the ASFV BA71 virulent isolate, and induction of protection against challenge with virulent virus strains of different genotypes. Because of these ambiguous results, we decided to generate double deletion mutants of ASFV Kenya 1033 lacking CD2v and an additional proven or proposed virulence factor.

For these experiments we applied a new approach to improve reverse genetics of ASFV by cotransfection of WSL-gRp30 cells (Hübner et al., 2018a) with genomic DNA of ASFV KenyaΔCD2vDsRed and recombination plasmids containing the desired additional gene deletions and GFP reporter cassettes, followed by booting up with phylogenetically distant ASF helper virus genotype II ASFV Armenia, which was specifically targeted on this cell line by a transgenic CRISPR/Cas9 anti-p30 system (Hübner et al., 2018a). Furthermore, CRISPR/Cas9 plasmids targeting the ASFV Kenya genes to be deleted were included in the transfection/infection experiments to select for the desired recombinants.

To evaluate the risk of unwanted recombination of the donor virus DNA and the helper virus several DsRed-expressing plaque isolates of ASFV KenyaΔCD2vDsRed obtained after booting up from ASFV Armenia-infected WSL-gRp30 cells, were analyzed by PCR and sequencing of different genome regions. The studies almost exclusively revealed ASFV Kenya-derived

sequences (results not shown) demonstrating the stringency of the established selection system. Next generation sequencing (NGS) of one booted-up plaque isolate of ASFVKenyaΔCD2vDsRed proved an identity of 99.6% to the previously determined sequence of the parental virus ASFV Kenya 1033, and besides the desired substitution of the CD2v gene by DsRed only 8 single nucleotide polymorphisms were observed. There was no evidence for any recombination with the helper virus, which was presumably prevented by the low overall sequence identity between ASFV Kenya and ASFV Armenia of only 84.5%. Thus, our modified DNA transfection and ASFV helper virus infection system greatly increased the proportion of the desired recombinants in virus progenies. In the future it might be also used to boot up synthetic ASFV genomes cloned in bacteria or yeast to establish a reverse-genetics system based on a cloned infectious copy of the viral genome. NGS and determination of nucleotide identity between full-length ASFV genomes may be also used as a tool for tracing of geographical spread and biological evolution of ASFV (Mazur-Panasiuk et al., 2019;Gilliaux et al., 2019).

Two potential ASFV-Kenya vaccine candidates lacking CD2v and either K145R or 9GLwere constructed using the new method, and exhibited wild type-like growth in cell culture. As expected, none of our CD2v single or double mutants showed rosette formation in haemadsorption (HA) assays, whereas the parental strain ASFV Kenya 1033 did (results not shown).

Since both CD2v and 9GL have been identified as virulence factors in other ASFV strains, a corresponding double mutant may be even more attenuated in pigs than the single mutants. However, this has to be confirmed for the genotype IX virus used in this study, and the general role of pK145R in virulence also remains to be elucidated. Prior to in vivo experiments the reporter gene insertion of the CD2v mutants will be removed using Cas9 nuclease and a DsRed

gene-specific gRNA (available), followed by homology directed repair with a CD2v deletion plasmid.

Although live attenuated ASFV strains are promising vaccine candidates, they sometimes exhibit adverse effects like joint swellings and skin lesions, chronic infections or reversion to virulence (Gaudreault & Richt, 2019). Furthermore, unavailability of a stable cell line for high yield production of conventionally attenuated ASFV field isolates is one of the obstacles for ASFV vaccine production. However, our defined gene deletion mutants are based on cell culture-adapted ASFV and replicate well in WSL-HP cells, which is a promising cell line for production of live attenuated vaccine stocks.

Deletion of essential ASFV genes using trans-complementing cells for generation of DISC vaccine candidates

While this approach has been successfully used in many viruses, so far no ASFV deletion mutants in essential genes have been isolated which required trans-complementing cells stably expressing the essential viral protein to be provided in trans. A deletion plasmid for the presumably essential p12 gene (O61R) of ASFV Kenya 1033 was generated by substitution of the respective ORF by an expression cassette for truncated human CD4 and GFP under control of strong ASFV promoters flanked by LoxP sites. Like in the previously described experiments, WSL-HP cells were transfected with this plasmid, and infected with ASFV Kenya 1033. However, unlike in the previous experiments, foci of infected GFP-expressing cells were rarely detected in plaque assays of virus progenies, and the desired ASFVKenyaΔO61R deletion mutant could not be purified to homogeneity, strongly indicating that the proposed attachment protein p12 (Carrascosa et al., 1991; Netherton & Wileman, 2013) is indeed essential for productive ASFV replication.

Recent studies indicate that trans-complementing cell lines might be a potential avenue for generation of defective ASFV recombinants suitable as safe "disabled in second cycle" (DISC)

live vaccines (Freitas et al., 2019b). Remarkably, in these studies cells expressing the histone-like ASFV protein pA104R were prepared to isolate an A104R deletion mutant of genotype I strain BA71V. In our studies, however, A104R proved to be dispensable for *in vitro* replication, at least of genotype IX ASFV Kenya 1033 in WSL-HP cells, casting doubt on this claim.

Nevertheless, we used a similar approach to prepare trans-complementing WSL-HP cells lines that stably express the p12 gene (O61R) of ASFV under control of the strong CAG promotor. Western blot analyses confirmed that the transgene was present at constant amounts comparable to those observed after ASFV infection over ≥ 50 serial passages of WSL-CAGGS-O61R cells. Since the cell-expressed p12 was translated from a codon-adapted ORF, it did not interfere with CRISPR/Cas9 targeting of the viral O61R gene to enhance mutagenesis of ASFV. Using these methods GFP expressing virus mutants presumably representing ASFV-Kenya-ΔO61R-LoxPGFPhuCD4 (AKΔO61R) could be significantly enriched after several rounds of plaque purification on p12 expressing cells. If purification of AKΔO61R to homogeneity is possible it might become a promising DISC vaccine candidate. The virus particles obtained from trans-complementing cells should contain the attachment protein p12 cells enabling infection of host cells (i.e. macrophages) in vaccinated animals, followed by replication, production and release of progeny ASFV particles which are still able to induce host immune responses, but unable to infect other cells due to the lack of p12. This might be sufficient to confer protection at least against homologous wild-type ASFV challenge. Our study strongly indicates that ASFV p12 is an essential protein for replication of ASFV in cell culture, and, thus, may be a promising candidate for generation of an ASFV DISC vaccine.

Inhibition of ASFV replication in cell culture by stable expression of Cas9 and sgRNAs targeted towards single or multiple essential virus genes

No licensed vaccines are available to-date for ASF prevention. Thus, the generation of host organisms that are resistant to ASFV infection might be an alternative approach towards control

in endemic regions. In prokaryotes, adaptive immune systems were developed to inactivate invading phages or plasmids by RNA-guided site-specific DNA cleavage (Jinek et al., 2012; Port & Bullock, 2016). Such adaptive immune systems like the CRISPR (Clustered Regularly Interspaced Short palindromic repeats)/Cas9 system of Streptococcus pyogenes have been modified for efficient genome editing in eukaryotes (Cong et al., 2013; Pyzocha et al., 2014), allowing specific mutagenesis of viruses affecting eukaryotes, or for generation of virus resistant host organisms. Cas9 nuclease generates blunt DNA double strand breaks using its two catalytic domains (HNH and RuvCI), at a site next to a PAM sequence (NGG), and matching an approximately 20-nucleotide sequence determined by a CRISPR RNA (crRNA) (Williams et al., 2016). Modified artificial single guide RNAs (sgRNA) include a fused RNA sequence consisting of an arbitrary crRNA and the trans-activating crRNA (tracrRNA) that binds and stabilizes the Cas9 nuclease.

For *in vitro* evaluation of potential antiviral targets, we generated ASFV-resistant WSL cell clones that stably express Cas9 nuclease, and single or multiple sgRNAs against genes encoding presumably essential ASFV proteins. Three ASFV genes encoding p30, p12, and pE199L were selected. In ASFV Kenya p12 is a 62 amino acid membrane protein encoded by O61R, which is presumably involved in the attachment of the virus to the host cell, and predominantly located in the outer virus envelope. The phosphoprotein p30 encoded by the CP204L gene contains 201 amino acids. It is located in the cytoplasm of infected cells, secreted, and also incorporated into the viral particle. It is involved in virus entry, and represents one of the most antigenic proteins of ASFV (Prados et al., 1993). The open reading frame E199L encodes the cysteine-rich integral membrane protein j18L containing 199 amino acids, which is expressed late in ASFV infection and localized mainly in viral factories (Sun et al., 1996; www.uniprot.org/uniprot/Q65242). Its precise function is still unknown.

In an earlier study, a sgRNA against the p30 gene CP204L was shown to inhibit plaque formation and productive replication of ASFV almost completely (Hübner et al., 2018a). However, several African virus isolates were not inhibited because of altered target sequences. We have now developed a sgRNA targeting a modified CP204L sequence, which inhibited the African, but not recent Eurasian ASFV isolates.

To inhibit all ASFV isolates, a sgRNA targeting the highly conserved codons 2 to 8 of the p12 gene O61R, was stably co-expressed together with Cas9 nuclease in WSL-HP cells after transfection with a triple expression plasmid, additionally conferring neomycin-resistance. Two approaches were successfully used to generate the desired WSL-gRO61RneoR cell clones. The first involved limiting dilution of the transfected cells, followed by testing of single neomycin-resistant cell clones for Cas9 expression (by Western blotting), and sgRNA gene insertion (by PCR and sequencing) and finally for susceptibility to ASFV infection. In the second approach the transfected cells were propagated in the presence of neomycin (G418) and immediately infected with ASFV, followed by isolation of surviving cell clones, and testing them for Cas9 expression and sgRNA gene insertion. Success of the less laborious second approach demonstrated that the inhibition of ASFV replication by targeting of O61R was very fast and efficient to allow clearance of the infection prior to cell lysis. This might indicate a yet unknown function of p12 beyond virus attachment, e.g. in virus maturation. Even though the most inhibitory WSL-gRO61RneoR cell lines were passaged over 40 times, their growth and expression level of Cas9 nuclease remained constant.

Susceptibility of cell clones to infection was mainly tested with reporter gene-expressing virus mutants, like ASFV Kenya ΔCD2vDsRed, to facilitate evaluation. Upon infection the virus was obviously able to enter WSL-gRO61RneoR cells as well as WSL-HP control cells, and viral gene expression was initiated, since red fluorescence became visible. Remarkably, DsRed expression was under control of the late p72 capsid protein promoter, which is not activated

prior to viral DNA replication (Hübner et al., 2018a; Rodriguez & Salas, 2013). However, unlike on WSL-HP cells plaque formation of ASFV on WSL-gRO61RneoR cells was completely abrogated. In growth kinetic studies almost no increase of virus titers was observed from WSL-gRO61RneoR cells, and maximal titers were reduced by approximately 5 log10 compared to WSL-HP cells. The specificity of this effect could be confirmed by sequencing of PCR amplified DNA from the few replication competent escape virus mutants which could be isolated from resistant WSL-gRO61RneoR cells. These mutants exhibited in-frame deletions of 2 or 3 codons from the gRNA target site in the 5' part of O61R.

To reduce the frequency of such escape mutants, four different sgRNAs were stably co-expressed in WSL cells after transfection with a multiplex CRISPR/Cas9 vector (Sakuma et al., 2014). The obtained WSL-4xgRASFVneoR cell clones contained two strain specific CP204L sgRNAs, an E199L sgRNA matching e.g. genotype II ASFV Armenia perfectly, but exhibiting one mismatch compared to ASFV Kenya, and the ubiquitously active O61R-specific sgRNA. The obtained cell clones were tested for susceptibility to genotype IX ASFV Kenya and genotype II ASFV Armenia-derived virus mutants. Surprisingly, inhibition in these cells appeared slightly less efficient than in several of the single gRNA-expressing cells. It was speculated, that this was due to the use of a modified Cas9 nuclease (eCas9) which had been shown to exhibit reduced off-target effects (Slaymaker et al., 2016; Science. 2016; 351:84–8), but also lower specific activity in several cases (Kim et al., 2017). Therefore, the possibly attenuated nuclease eCas9 was replaced with the original Cas9, and new WSL-4xgRASFVneoR cell lines were prepared. However, virus inhibition assays showed no significant differences between eCas9 and Cas9 cell clones. Other explanations for the still incomplete virus inhibition on the quadruple gRNA-expressing cells might be the occasionally observed loss of single sgRNA genes from the tandem repeat-like array by recombination, as well as a general limitation of the achievable number of Cas9 cleavage events.

Comparison of growth properties of ASFV-Kenya and ASFV-Armenia on CRISPR/Cas9 cells WSL-gRO61RneoR, WSL-gRCP204LAneoR, WSL-gR-CP204LKneoR, WSL-gR-E199LneoR, WSL-eCas4xgRASFVneoR and on control WSL-HP cells showed that the two sgRNAs against CP204L worked strictly strain-specific, whereas the more conserved E199L- and O61R-specific ones proved to be efficacious against both ASFV genotypes. However, the effect of the E199L-specific gRNA on productive virus replication was only moderate, and less pronounced against ASFV Kenya, which was presumably due to a single mismatch to the target sequence of this virus type.

Based on our results, several of the prepared sgRNA and Cas9 expression plasmids have been already used for generation of transgenic pigs at the FLI Institute of Farm Animal Genetics, in Neustadt-Mariensee, Germany, which are currently investigated for transgene expression and susceptibility of their macrophages to ASFV infection. Virus-resistant transgenic pigs have been successfully produced before. For example, gene-edited pigs lacking a single domain of the macrophage surface protein CD163 proved to be resistant against infection with porcine reproductive and respiratory syndrome virus (PRRSV), but not against ASFV infection (Popescu et al., 2017; Burkard et al., 2017).

Expression of immunogenic ASFV proteins in a PrV vaccine strain using CRISPR/Cas9 supported BAC mutagenesis

The use of vectored or bivalent vaccines is a suitable and effective means for prevention of infectious diseases in farm animals (Klingbeil et al., 2014; Chen et al., 2011; Cruz et al., 2010; Hu et al., 2018; Roizman & Jenkins, 1985; Xu et al., 2015). Therefore, we developed vaccine candidates against ASFV using a previously described bacterial artificial chromosome (BAC) clone of the genome of pseudorabies virus (PrV) vaccine strain Bartha (Hübner et al, 2018b), which provided a robust vector system for efficient expression of foreign genes.

In this study, we selected two genes of ASFV-Kenya 1033, O61R coding for p12, and pE183L coding for p54, for expression in the PrV vector, since they have been described as immunogenic proteins in other ASFV strains (Netherton & Wileman, 2013; Alonso et al., 2001; Carrascosa et al., 1991). To facilitate substitutive insertion of the transgenes, rabbit kidney (RK13) cells stably expressing Cas9 nuclease and a GFP gene-specific guide RNA were used to induce site-specific cleavage of the BAC DNA that contained a GFP-expressing cassette. When these Cas9-expressing cells were co-transfected with the PrV BAC and recombination plasmids containing expression cassettes for p12 or p54 transgenes flanked by DNA sequences of the PrV genome, a majority of the virus progeny exhibited the desired ASFV gene insertions, and a complete removal of the reporter and antibiotic resistance genes and other BAC vector sequences. To further optimize transgene expression under control of the strong CAG promoter (Niwa et al., 1991), the codon usage of synthetic p12 and p54 genes was adapted to that of chromosomal porcine genes. Like PrV-genes (Klupp et al., 2004), the synthetic genes showed a substantially higher third-position GC content than authentic ASFV genes. The synthetic genes were also designed to avoid cryptic mRNA splice sites, since DNA replication, transcription and capsid assembly of herpesviruses including PrV occur in the nucleus of the host cell (Pomeranz et al., 2005), whereas ASFV replication takes place in viral factories in the cytoplasm where no RNA splicing occurs (Dixon et al., 2013).

The PrV mutants obtained in this study (PrV-BaΔgGAKO61Rporc, PrV-BaΔgGE183lporc) expressed the ASFV proteins abundantly, and grew to high titers (> 10^8 PFU/ml) in RK13 cells.

ASFV proteins have been already expressed in several virus-based vectors and tested for protection using experimental animal models. For example, replication-deficient human adenovirus has been used to express ASFV antigens (Lokhandwala et al., 2017), and baculovirus-expressed p30, p54, p72 and p22 have been shown to induce neutralizing antibodies which, however, were not sufficient for protection (Neilan et al., 2004). Similarly,

our group previously tested ASFV p30, p22, j18L and CD2v expressed in the PrV vector which were not sufficient to confer protection against ASFV lethal challenge (Hübner et al., 2018b). In their review, Gaudreault & Richt, 2019 highlight similar findings, showing insufficient success and inconsistence between different vector-based vaccine strategies. However, vaccination of pigs with certain pools of ASFV antigens expressed in adenovirus and modified vaccinia virus Ankara (MVA) was shown to reduce viremia after ASFV challenge significantly, indicating that protection may be achieved by improved protocols (Netherton et al., 2019).

Unlike inactivated whole virus or subunit vaccines, live virus vectored vaccines can be used to elicit not only humoral, but also cell-mediated immune responses. Another advantage of vectored vaccines is their potential applicability in differentiating infected from vaccinated animals (DIVA), since antibodies against ASFV immunogens not encoded by the vector can be used as markers for field virus infection. The vector chosen for our studies, PrV, causes Aujesky's disease (AD) in pigs, which is still a serious problem in many countries, but has been efficiently controlled by vaccination in Europe and North America (Freuling et al., 2017). Therefore, this vector offers the advantage that PrV-based ASFV vaccines could confer protection against two economically important swine diseases by one immunization without requiring any adjuvants. Furthermore, the PrV-based vector constructs can be used to investigate the immunogenic properties of individual ASFV proteins in the context of a non-lethal viral infection. This is particularly interesting since several ASFV antigens, like p30, are suspected to induce immune-mediated enhancement of ASFV infection (Gaudreault & Richt, 2019).

Expression of ASFV gene products as bacterial fusion proteins for preparation of monospecific rabbit antisera

The production of monoclonal antibodies (mAb) against ASFV has been used to identify dominant immunogenic antigens, for example the capsid protein p72 (Federspiel et al., 1991).

Furthermore, in vitro produced monoclonal or monospecific antibodies, together with virus neutralization tests can contribute to clarification of the role of antigen-specific humoral immune responses in protection against ASFV (Escribano et al., 2013). Last but not least, monospecific antisera can be used for detection and precise localization of barely characterized viral proteins in infected cells and virions, providing useful indications on their biological functions (Hübner et al., 2019). Our laboratories have established efficient protocols to obtain potent monospecific antisera by immunization of rabbits with bacterial fusion proteins containing the antigen of interest fused to glutathione S-transferase (GST) (Fuchs et al., 2002), and already produced monospecific corresponding antisera against ASFV p11.5, p22, p30, p72, j18L, p285L, pK145R, and pB602L (Hübner et al., 2018a, b, 2019). Using a similar approach sera against two additional ASFV membrane proteins, pO61R (p12) of ASFV Kenya and pE183L (p54) ASFV Armenia were prepared. p12 is presumably involved in attachment of the virus to the host cell by binding to a yet unknown cellular receptor. (Carrascosa et al., 1991; Netherton & Wileman, 2013). p54 binds the light chain of dynein, indicating a role in intracellular transport, and is important in viral morphogenesis by recruiting and transforming of host endoplasmic reticulum membranes into precursors of the inner viral envelope (Netherton & Wileman, 2013; Alonso et al., 2001; www.uniprot.org/uniprot/Q65194).

Both rabbit antisera produced in this study were shown to detect their viral target proteins in Western blot analyses of WSL-HP and RK13 cells transfected with expression plasmids, or infected with PrV vector constructs or ASFV. However, the specific reaction of the p12 antiserum was only weak and restricted to the homologous protein of ASFV Kenya exhibiting an apparent mass of approximately 14 kDa. p12 of ASFV-Armenia was not detected, which was presumably due to three amino acid substitutions and 1 amino acid deletion in the, according to predictions, highly antigenic C-terminal part of the protein (Fig. 3-28). Furthermore, the serum exhibited pronounced unspecific reactions with two cellular proteins.

Thus, an alternative approach may be required (e.g. anti-peptide sera) to provide improved tools for precise localization of p12 in infected cells and virions by immunofluorescence and immunoelectron microscopy. This is of particular interest, since we found out that a commercially distributed and highly specific monoclonal anti-"p12" antibody detects the A137R protein (p11.5) of ASFV instead, and, therefore, some of the previously published results on localization and function of the O61R protein might have to be verified.

The serum raised against ASFV Armenia p54 detected apparent 27 and 57 kDa proteins in RK13 cells infected with a p54-expressing PrV Bartha mutant as well as in WSL-HP cells infected with homologous and heterologous ASFV strains. A more detailed characterization of this serum is still in progress.

Recently prepared rabbit antisera against the abundantly expressed but poorly characterized proteins p11.5, pK145R and p285L (Kessler et al., 2018; Hübner et al., 2019) of ASFV Armenia (genotype II) were also tested for cross-reactivity with the ASFV Kenya (genotype IX) homologues. Whereas the α-pK145R and α-p11.5 sera detected the proteins of both ASFV genotypes equally well, the α-285L serum showed no reaction with ASFV Kenya-infected cells. It remains to be clarified whether this was due to the low amino acid sequence identity between the two predicted proteins (86.2%), or to a lack of expression of the ORF in ASFV Kenya. The latter possibility is conceivable, since 285L is nonessential in ASFV Armenia (Hübner et al., 2019), and completely absent from several published ASFV genomes.

Monoclonal antibodies against individual ASFV proteins like p30, and p72 are useful tools for serological detection of infections e.g. by sandwich or competitive enzyme linked immunosorbent assays (ELISA) (Vidal et al., 1997), or virus detection in blood samples by lateral flow tests (Sastre et al., 2016). However, in view of the high genetic and antigenic variability of the numerous ASFV types circulating in eastern and southern Africa the restricted specificity of monoclonal antibodies might be insufficient for detection of all infections. In

initial studies Pan et al., 1988 determined marked epitopic differences between several virus isolates using 20 monoclonal antibodies. Thus, monospecific but polyclonal antisera detecting multiple epitopes of their target proteins might provide an alternative for reliable diagnostics. Some of the antisera prepared or tested in this study (e.g. those against p11.5, p54 or pK145R) might be also usable as diagnostic tools.

SUMMARY

To enable control of African swine fever (ASF) in Eastern and Southern Africa, prototype live vaccine candidates were generated by targeted gene deletions from a Kenyan genotype IX ASF virus (ASFV). It was attempted to delete known nonessential genes involved in virulence (encoding TK, dUTPase, CD2v, 9GL), possibly essential genes (p12, pA104R, ribonucleotide reductase), and genes with widely unknown functions (pK145R). Isolation of the desired virus recombinants by plaque assays or limiting dilutions on a wild boar lung cell line (WSL-HP) was facilitated by substitutive reporter gene insertions encoding fluorescent proteins (GFP, DsRed), or the human membrane protein CD4. The latter protein permitted enrichment of recombinant virus particles by magnetic activated cell sorting (MACS). The isolated ASFV recombinants were characterized by PCR and sequencing of the mutated genome parts, and replication kinetics and virus spread in cell culture were investigated. Deletion of TK, CD2v, or pK145R had no detectable effect on in vitro growth of ASFV Kenya. Interestingly, virus mutants lacking the DNA binding protein pA104R which has been considered to be essential for DNA replication, also exhibited almost wild type-like growth properties.

In contrast, ASFV mutants lacking ribonucleotide reductase or p12 could not be purified to homogeneity on WSL-HP cells, indicating these proteins are essential for virus replication in cell culture. Therefore, trans-complementing cells lines stably expressing ASFV p12 have been prepared which can now be used for mutant virus purification. If this approach is successful the

resulting defective mutant ASFV Kenya-Δp12 might be suitable as a safe "disabled in second cycle" (DISC) live vaccine in swine.

In a novel approach to improve reverse genetics of ASFV the CRISPR/Cas9 cell line WSL-gRp30 (Hübner et al., 2018a) was co-transfected with genomic DNA of ASFV-KenyaΔCD2vDsRed, sgRNA plasmids targeting K145R or 9GL, and GFP-expressing recombination plasmids for homology-directed repair. For booting up of the noninfectious virus genome the cells were infected with phylogenetically distant helper virus (genotype II ASFV Armenia, 84% identity) which was selectively inhibited on the used cell line. The desired double-fluorescent double-deletion mutants could be isolated after few plaque purification steps on selective WSL-gRp30 cells. Next generation sequence (NGS) analyses of reconstituted ASFV Kenya genomes showed that no unwanted recombination with the helper virus occurred, indicating that the method might be also suitable for booting of synthetic ASFV genomes cloned and mutagenized in *E. coli* or yeast.

The modified CRISPR/Cas9 system of *S. pyogenes* might be also usable for generation of ASFV resistant pigs. To evaluate this alternative control measure WSL cell clones stably expressing Cas9 nuclease and single or multiple sgRNAs against essential ASFV proteins were prepared and tested for their susceptibility to infection. Strain specific sgRNAs targeting the p30 gene of ASFV Kenya or Armenia selectively inhibited the respective viruses, and a p12 gene-specific sgRNA abrogated replication of both genotypes almost completely. Interestingly, coexpression of four ASFV-specific sgRNAs did not enhance virus inhibition, but might help to reduce the frequency of escape mutants which were occasionally isolated from the single sgRNA-expressing cells, and exhibited silent base substitutions or in-frame deletions within the target genes. First attempts to express the *in vitro* tested CRISPR/Cas9 constructs in transgenic pigs are in progress.

CRISPR/Cas9 supported rescue of a defective BAC clone of pseudorabies virus (PrV) vaccine strain Bartha (Hübner et al., 2018b) was used to develop putative vectored vaccines against ASFV. In the present study expression cassettes for the codon-optimized p12 and p54 genes of ASFV were successfully inserted into the PrV genome. The insertions did not significantly affect PrV recombination in cell culture, and the transgenes were expressed at similar levels as in ASFV-infected cells. It has to be tested whether coinfection with vector constructs for these and other immunogenic ASFV proteins is able to protect pigs against a lethal challenge.

For characterization of the generated ASFV mutants and PrV vector constructs, monospecific antisera against several ASFV gene products (p11.5, p12, p54, pK145R, p285L) were prepared by immunization of rabbits with bacterial GST fusion proteins. The anti-p12 serum showed only weak and strain-specific reactions with the ASFV Kenya protein, but was nevertheless useful for identification of p12-expressing PrV recombinants and WSL cell lines. All other sera showed satisfying reactions in Western blot and mostly immunofluorescence analyses, and allowed i.a. precise localization of the pK145R and p285L proteins in ASFV-infected cells and virions (Hübner et al., 2019).

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Table 1: Known and putative functions of proteins encoded by the ASFV genome. Adapted from (Alonso et al., 2018).

Gene function	Gene name	Predicted protein mass (kDa)
Nucleotide metabolism, transcription, replication and repair		
Thymidylate kinase	*A240L*	27.8
Thymidine kinase	*K196R*	22.4
Deoxyuridine triphosphatase*	*E165R*	18.3
Ribonucleotide reductase (small subunit)	*F334L*	39.8
Ribonucleotide reductase (large subunit)	*F778R*	87.5
DNA polymerase α-like	*G1211R*	139.8
DNA topoisomerase type II*	*P1192R*	135.5
Proliferating cell nuclear antigen (PCNA)-like	*E301R*	35.3
DNA polymerase X-like*	*O174L*	20.3
DNA ligase*	*NP419L*	48.2
AP endonuclease class II*	*E296R*	33.5
RNA polymerase subunit 2	*EP1242L*	139.9
RNA polymerase subunit 6	*C147L*	16.7
RNA polymerase subunit 1	*NP1450L*	163.7
RNA polymerase subunit 3	*H359L*	41.3
RNA polymerase subunit 5	*D205R*	23.7
RNA polymerase subunit 7	*D339L*	39
RNA polymerase subunit 10	*CP80R*	9.0
Transcription factor IIB-like	*C315R*	37.0
Helicase superfamily II	*A859L*	27.8
Helicase superfamily II	*F1055L*	123.9
Helicase superfamily II	*B962L*	109.6
Helicase superfamily II	*D1133L*	129.3
Helicase superfamily II	*Q706L*	80.4
Helicase superfamily II	*QP509L*	58.1
Transcription factor SII	*I243L*	28.6
Guanylyl transferase*	*NP868R*	29.9
PolyA polymerase large subunit	*C475L*	54.8
FTSJ-like methyl transferase domain**	*EP424R*	49.3
ERCC4 nuclease domain**	*EP364R*	40.9
Lambda-like exonuclease	*D345L*	39.4
VV A2L-like transcription factor**	*B385R*	45.3
VV A7L-like transcription factor**	*G1340L*	155.0
VV VLTF2-like late transcription factor**	*B175L*	20.3
FCS-like finger DNA primase	*C962R*	111.3
Other enzymes		
Prenyltransferase*	*B318L*	35.9
Serine protein kinase*	*R298L*	35.1
Ubiquitin conjugating enzyme*	*I215L*	24.7
Decapping enzyme (g5R)*	*D250R*	29.9
Host cell interactions		

IAP apoptosis inhibitor*	*A224L*	26.6
Bcl-2 apoptosis inhibitor*	*A179L*	21.1
Inhibitor of host gene transcription*	*A238L*	28.2
C-type lectin-like*	*EP153R*	18.0
Similar to HSV ICP34.5 neurovirulence factor**	*DP71L*	8.5
Nif S-like	*QP383R*	42.5
ERV 1-like. Involved in redox metabolism*,**	*B119L*	14.4
Phosphoprotein binds to ribonucleoprotein-K	*CP204L*	30.0
UK virulence factor	*DP96R*	10.7
Structural proteins and proteins involved in morphogenesis		
p22	*KP177R*	20.2
Histone-like	*A104R*	11.5
p11.5	*A137R*	21.1
p10	*K78R*	8.4
pA151R. Contains CXXC motif similar to that in thioredoxins. Binds to E248R protein. Not incorporated into virions. Possible component of redox pathway.	*A151R*	17.5
p72 major capsid protein. Involved in virus entry	*B646L*	73.2
p49. Required for formation of vertices in icosahedral capsid	*B438L*	49.3
Chaperone. Involved in folding of capsid. Not incorporated into virions	*B602L*	45.3
SUMO-1-like protease. Involved in polyprotein cleavage	*S273R*	31.6
pp220 polyprotein precursor of p150, p37, p14 and p34. Required for packaging of nucleoprotein core	*CP2475L*	281.5
p32 (p30) phosphoprotein. Involved in virus entry	*CP204L*	23.6
pp62 (pp60) polyprotein precursor of p35 and p15	*CP530R*	60.5
p12 attachment protein	*O61R*	6.7
p17. Required for progression of precursor membranes to icosahedral intermediates	*D117L*	13.1
j5R. Transmembrane domain	*H108R*	12.5
p54 (j13L). Binds to DLC1 chain (8 kd) of dynein, involved in virus entry. Required for recruitment of envelope precursors to the factory	*E183L*	19.9
j18L. Transmembrane domain	*E199L*	22.0
p14.5. DNA-binding. Required for movement of virions to plasma membrane	*E120R*	13.6
E248R (k2R). Possible component of redox pathway required disulfide bond formation. Structural protein	*E248R*	27.5
XP124L. Multigene family 110 member. Contains KDEL ER retrieval sequence and transmembrane domain	*MGF 110-4L (XP124L)*	14.2
EP402R. Similar to host CD2 protein. Required for binding red blood cells to infected cells and extracellular virus particles. Glycoprotein inserted into external virus envelope*	*EP402R*	45.3
Multigene family members		
Multigene family 360	*MGF 360-1L (KP360L)*	41.7
	MGF 360-2L (KP362L)	42.6
	MGF 360-3L (L356L)	41.7
	MGF 360-4L (LIS382)	44.9
	MGF 360-5L (UP60L)	7.0
	MGF 360-6L (LIS375)	43.9
	MGF 360-7L (LIS375a)	44.1
	MGF 360-8L (J319L)	31.3
	MGF 360-9L (A125L)	14.5
	MGF 360-10L	41.6
	MGF 360-11L	41.6
	MGF 360-12L	41.1

	MGF 360-13L	41.0
	MGF 360-14L	41.3
	MGF 360-15R (A276R)	31.6
	MGF 360-16R (DP311R)	35.6
	MGF 360-17R (DP63R)	8.4
	MGF 360-18R (DP148R)	17.2
	MGF 360-19R (DP363R)	42.4
	MGF 360-20R (DP42R)	4.9
	MGF 360-21R	42.0
	MGF 360-22R	41.7
Multigene family 110	*MGF 110-1L (L270L)*	32.4
	MGF 110-2L (U104L)	12.2
	MGF 110-3L (LIS124-1)	14.3
	MGF 110-4L (XP124L)	14.2
	MGF 110-5L (V82L)	9.4
	MGF 110-6L (Y118L)	13.9
	MGF 110-7L (LIS137)	15.9
	MGF 110-8L (LIS124-2)	14.9
	MGF 110-9L (LIS290)	34.8
	MGF 110-10L (190-2)	22.7
	MGF 110-11L (LIS119-1)	32.5
	MGF 110-12L (LIS 119-2)	12.5
	MGF 110-13L (LIS 117)	18.3
	MGF 110-14L (LIS121-2)	14.7
Multigene family 300	*MGF 300-1L (J268L)*	31.3
	MGF 300-2R (J154R)	17.6
	MGF 300-3L (J104L)	12.5
	MGF 300-4L (J182L)	21.7
Multigene family 505	*MGF 505-1R*	62.6
	MGF 505-2R (A489R)	57.7
	MGF 505-3R (A280R)	32.5
	MGF 505-4R (A505R)	59.2
	MGF 505-5R (A498R)	58.7
	MGF 505-6R (A518R)	61.8
	MGF 505-7R (A528R)	61.7
	MGF 505-8R	61.7
	MGF 505-9R (A506R)	59.4
	MGF 505-10R (A542R)	59.4
	MGF 505-11L (DP542L)	63.1
Multigene family 100	*MGF 100-1R*	15.3
	MGF 100-2L (DP141L)	16.8
	MGF 100-3L (DP146L)	17.2

• The asterisk shows ASFV-encoded proteins whose function have been demonstrated experimentally.

Table 2: PCRs and their cycling conditions

KOD-Xtreme™ PCR (Novagen)					
Standard reaction conditions	(50 µl rxn)	**Cycling conditions (Three-step)**			
Water, nuclease free, PCR grade	X µl	**Step**		**Temp**	**Time**
2X KOD Xtreme™ PCR Buffer	25 µl	Polymerase activation		94°C	2 min
2 mM dNTPs	10 µl	20-35 PCR cycles	Denature	98°C	10 sec
10 pmol/µL Primer F	1.5 µl		Anneal	T_m minus 5°C	30 sec
10 pmol/µL Primer R	1.5 µl		Extend	68°C for 1 min/kb	3 min
KOD Xtreme™ Hot Start DNA Polymerase (1.0 U/µl)	1.0 µl	Final extension		68°C for 1 min/kb	10 min
				4°C	Hold
Genomic DNA	200 ng				
SuperFi DNA Polymerase PCR (Invitrogen by Thermo Fisher Scientific)					
Standard reaction conditions	(25 µL rxn)	**Cycling conditions**			
Water, nuclease free	X µl	**Step**		**Temp**	**Time**
5X SuperFi™ Buffer (includes 7.5 mM $MgCl_2$)	5 µl	Initial denaturation		98°C	30 sec
10 mM dNTP mix	0.5 µl	25-35 PCR cycles	Denature	98°C	5-10 sec
10 µM forward primer	1.25 µl		Anneal		-
10 µM reverse primer	1.25 µl		Extend	72°C	15-30 sec/kb
Template DNA(5-50 ng gDNA or 1 pg-10 ng plasmid DNA)	Varies (use T_m calculator)	Final extension		72°C	5 min
				4°C	Hold
5X SuperFi™ GC Enhancer (optional – recommended for targets with >65% GC sequences)	5 µl				
Platinum™ SuperFi™ DNA Polymerase (2 U / µL)	0.25 µl				
GoTaq® PCR (PROMEGA)					
Component	(50 µl rxn)	**Cycling conditions (Three-step)**			
Nuclease-free water	X µl	**Step**		**Temp**	**Time**
$MgCl_2$, 25mM solution	2.0-8.0 µl				
5X Colorless GoTaq® Flexi Buffer	10 µl	Initial Denaturation		95°C	2 min
PCR Nucleotide Mix, 10mM each	1 µl	25-35 PCR cycles	Denature	95°C	0.5-1 min
Upstream primer	50 pmol		Anneal	42-65°C	0.5-1 min
Downstream primer	50 pmol		Extend	72°C for 1 min/kb	3 min
GoTaq® DNA Polymerase (5 u/µl)	0.25 µl	Final extension		72°C	5 min
				4°C	Hold
template DNA	Variable (<0.5µg/50µl)				
Pfx PCR (Invitrogen)					
Component	(50 µl rxn)	**Cycling conditions (Three-step)**			
Autoclaved, distilled water	X µl	Step		Temp	Time
10X *Pfx* Amplification buffer	5.0 µl				
10 mM dNTP mixture	1.5 µl	Initial Denaturation		94°C	2-5 min
50 mM $MgSO_4$	1 µl	25-35 PCR cycles	Denature	94°C	15 sec
Primer mix (10 µM each)	1.5 µl		Anneal	55°C	30 sec
Template DNA(10 pg to 200 ng)	≥1 µl		Extend	68°C for 1 min/kb	
Platinum™ *Pfx* DNA Polymerase	0.4 µl	Hold		4°C	

Table 3. Primers for amplification, sequencing or cloning of ORFs of ASFV genes or other DNA probes

Primer	Primer Sequence	Modification
ASFVKenyaTKR-F	ATCTAAGGGCATTCCGATTTAC	
ASFVKenyaTKR-R	TTTATTATTTAATAGGTCGCAACC	
ASFVKenyaE199L-F	TTC**AGCT**TCTTCGGTGATAGGTTGG	*Alu*I site
ASFVKenyaE199L-R	AAGTCTTGCTCATGGCTTACGTTAC	
ASFV-KenyaCP204L-F	GCAAAACTGTTTCATGTTAGAGGGC	
ASFV-KenyaCP204L-R	GCAAAACTGTTTCATGTTAGAGGGC	
AKRiReR-F	CAC**GCATGC**GATGCCTTTGTCTCAATAG	*Sph*I site
AKRiReR-R	CAC**GAATTC**TAAGTGCGGCTTTAAATGC	*EcoR*I site
ASFVKenyaO61RR-F	AAATGACTACAATCTTTCACGC	
ASFVKenyaO61RR-R	CCCAAAGTCTCATTAACTACGG	
AKO61RR-R3	CAC**GCATGC**CAAAGTCTCATTAACTACGG	*Sph*I site
AKO61RR-R2	CAC**GGATCC**TCCACCACTTGAACCATC	*Bam*HI
AKA104RRHF-F	GAATTCGAGCTCGGTACCCAACCAAGGAAGAAATTAGCG	
AKA104RRHF-R	GTCGACTCTAGAGGATCCCCAATGTTGTTGGATGTTGCTC	
PAK9GLR-F	CAC**AAGCTT**GTACTTCAGCGTTTCAATGG	*Hind*III site
PAK9GLR-R	CAC**GGTACC**ATATTGTTACCTTGGTGCCC	*Kpn*I site
PAKDUTR-F	CAC**AAGCTT**GTAATCCGGTCAGAGTTG	*Hind*III site
PAKDUTR-R	CAC**GAATTC**TAGCGAAGATGATGTGGTAG	*Kpn*I site
AKT-PS	GGTTGCCCACTAGTATGC	
X330GRR-F2	ATGCTTACCGTAACTTGAAA	
X330GRR-R2	ATTTGTCTGCAGAATTGGCG	
Cas9-RNA-1	CTGTCTGCACCTCGGTCTTT	
Cas9-RNA-u	ACAAGCTGATCCGGGAAGTG	
CRISPR-step2-F2	GGACTTTCCACACCCTAACTGACAC	
CRISPR-step2-R	CGGGCCATTTACCGTAAGTTATGTAACG	
AGp54-F	CACGAATTCCACCATGGATTCTGAATTTTTTCAACCGG	
AGp54-R	CACTCTAGATTACAAGGAGTTTTCTAGGTCTTTATGCG	
pGEX-5'	GGTCTGGCAAGCCACGTTTGGTG	
EGFP-SNR	CGGACACGCTGAACTTGTGG	
huCD4OUT	GGAGAACAAGGAGGCAAAGG	

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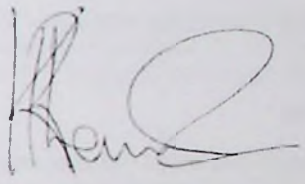
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Declaration of originality (Eigenständigkeitserklärung)

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.



Unterschrift des Promovenden

Curriculum vitae

tkabuuka@naro.go.ug or dr.kabuukat@gmail.com

Academic qualifications

- 2016 – 2019; PhD student at Friedrich-Loeffler-Institut, Germany
- 2012 - 2014; Master of Science in Veterinary Science, University of Pretoria, South Africa
- 2002 - 2007; Bachelor of Veterinary Medicine, Makerere University Kampala, Uganda

Continuous Professional Development

- 2019 – Good Scientific Practice, Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit, Insel Riems, Greifswald
- 2018; SciencePLUS Workshop – Academic Writing and Publishing, Golin Wissenschafts Management, Hamburg, Germany
- 2008 - Uganda Veterinary Association (UVA) Member

Work experience

2009 to date - Research Officer – Animal Virology, National Agricultural Research Organization (NARO) Uganda

Publications

1. Identification and characterization of the 285L and K145R proteins of African swine fever virus. Hubner, A., Kessler, C., Pannhorst, K., Forth, J. H., **Kabuuka, T.**, Karger, A., . . . Fuchs, W. (2019). J Gen Virol. doi:10.1099/jgv.0.001306

2. Efficient transgene insertion in a pseudorabies virus vector by CRISPR/Cas9 and marker rescue-enforced recombination. Hubner, A., Keil, G. M., **Kabuuka, T.**, Mettenleiter, T. C., & Fuchs, W. (2018) J Virol Methods, 262, 38-47. doi:10.1016/j.jviromet.2018.09.009

3. Drivers and risk factors for circulating African swine fever virus in Uganda, 2012–2013. **Kabuuka, T.**, Kasaija, P.D., Mulindwa, H., Shittu, A., Bastos, A.D.S., Fasina, F.O. Research in Veterinary Science 97 (2014) 218 – 225.

4. Seroprevalence and Risk Factors for Exposure of Free-Range Poultry to Avian Influenza Viruses in Important Bird Areas in Uganda, 2015. H. Kirunda, AD F. Kabi, A N. Muwereza, **T. Kabuuka**, A S. D. Kerfua, A P. D. Kasaija, A D. K. Byarugaba, B and F. Wabwire-Mangen C. Avian Diseases 59 :64–70.

5. Knowledge and perceptions of smallholder dairy farmers of cattle disease burdens in selected agro-ecological zones of Uganda. H. Kirunda, F. Kabi, N. Muwereza, **T. Kabuuka**, J.W. Magona and G. Lukwago. Uganda Journal of Agricultural Sciences, 2012, 13 (2): 107-116.

Conferences

1. Generation of defined gene deletion mutants of African swine fever virus as live vaccine candidates. **Tonny Kabuuka**, Katrin Pannhorst, Alexandra Hübner, Jolene Carlson, Günther M. Keil, Thomas C. Mettenleiter, and Walter Fuchs. EPIZONE meeting, August 2019, Berlin, Germany.

2. CRISPR/Cas 9-mediated inhibition of African swine fever virus replication in cell culture. **Tonny Kabuuka**, Alexandra Hübner, Björn Petersen, Günther M. Keil, Katrin Pannhorst, Jolene Carlson, Thomas C. Mettenleiter and Walter Fuchs. 29th Annual Meeting of the Society for Virology, Mar 20 – 23, 2019 in Düsseldorf, Germany.

3. CRISPR/Cas 9-mediated inhibition of African swine fever virus replication in cell culture. **Tonny Kabuuka**, Alexandra Hübner, Björn Petersen, Günther M. Keil, Thomas C. Mettenleiter and Walter Fuchs. Junior Scientists Symposium, 24 -26 September 2018, Greifswald, Germany.

4. CRISPR/Cas9-mediated inhibition of pseudorabies virus and African swine fever virus replication in cell culture. Alexandra Hübner, Bjorn Petersen, Gunther M. Keil, **Tonny Kabuuka**, Thomas C. Mettenleiter and Walter Fuchs. EPIZONE meeting, September 2017, France.

5. Is African swine fever changing in Uganda: A molecular insight. **T. Kabuuka**, P.D. Kasaija, H. Mulindwa, K.D. Atuhaire, J. van Heerden, L. Heath, F.O. Fasina, A.D.S Bastos. Uganda Veterinary Association Annual Symposium, Imperial Royale Hotel, 3-4 Dec 2015.

6. Multi-locus typing of African swine fever viruses from domestic pigs in Uganda using PACT. **Kabuuka T.**, Fasina, F.O., Bastos, A.D.S., Van Heerden, J., Heath, L. Global African swine fever Research Alliance Scientific Workshop, 10-14 Nov., 2014, South Africa.

7. Risk factors for African swine fever viruses in Uganda, 2012-2013. **T. Kabuuka**, P.D. Kasaija, H. Mulindwa, A. Shittu, A.D.S.Bastos, F.O. Fasina. Global African swine fever Research Alliance Scientific Workshop, 10-14 Nov. 2014, South Africa.

8. Risk factors and sero-prevalence of recent foot-and-mouth disease outbreaks in Uganda 2013-2014. Kerfua S.D, Kasaija P.D, Muwereza N, Azabo R, Ssekitto C, Sande R, Emudong P, Alingu R, Ongyera S, Walubengo J, Kirunda H, **Kabuuka T.** Uganda Veterinary Association Annual Symposium, 2014.

9. Exposure and potential risk factors for outbreaks and spread of influenza viruses in smallholder poultry production systems in Uganda. H. Kirunda, F. Kabi, N. Muwereza, **T. Kabuuka**, S. D. Kerfua, P. D. Kasaija, D.K. Byarugaba, F. Wabwire-Mangen. NARO Conference. Imperial Botanical Beach Hotel, Entebbe, Uganda, 1-3 Oct 2012.

10. Knowledge and perceptions of smallholder dairy farmers of cattle disease burdens in selected agro-ecological zones of Uganda. H. Kirunda, F. Kabi, N. Muwereza, **T. Kabuuka**, J. W. Magona, G. Lukwago. NARO Conference. Imperial Botanical Beach Hotel, Entebbe, Uganda, 1st – 3rd Oct 2012.

Awards

1) 2016 - Friedrich Loeffler Institute, PhD scholarship (AVSOA), Germany
2) 2014 - Academic Honorary Colors from the Student Representative Council (SRC) University of Pretoria, South Africa
3) 2012 - University of Pretoria Masters Bursary, South Africa
4) 2011 - Agricultural Technology & Agribusiness Advisory Services (ATAAS) scholarship from World Bank, Uganda

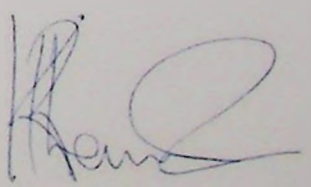
Referees

1) Dr. Walter Fuchs

Institut für molekulare Virologie und Zellbiologie, Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit, Federal Research Institute for Animal Health, Südufer 10, 17493 Greifswald - Insel Riems, Tel: +49 38351 7 1531/1258 | Fax: +49 38351 7 1526, http://www.fli.de/

2) Prof. Oludayo Fasina

Country Team Leader, Emergency Centre for Transboundary Animal Diseases - Food and Agriculture Organization of the United Nations (ECTAD-FAO), United Nations Office in Nairobi (UNON), UN Avenue, Gigiri, Kenya. Telephone: +254 (20) 762 5998; Cell phone: +27849581925; +254729840367, folorunso.fasina@fao.org



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