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African swine fever virus replication events and cell nucleus: New insights and perspectives

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ABSTRACT

African swine fever (ASF) is currently matter for major concerns in global swine industry as it is highly contagious and causes acute fatal haemorrhagic fever in domestic pigs and wild boar. The absence of effective vaccines and treatments pushes ASF control to relay on strict sanitary and stamping out measures with costly socio-economic impacts. The current epidemic scenario of fast spreading throughout Asiatic countries impels further studies on prevention and combat strategies against ASF.

Herein we review knowledge on African Swine Fever Virus (ASFV) interactions with the host cell nucleus and on the functional properties of different viral DNA-replication related proteins. This entails, the confirmation of an intranuclear viral DNA replication phase, the characterization of cellular DNA damage responses (DDR), the subnuclear compartments disruption due to viral modulation, and the unravelling of the biological role of several viral proteins (A104R, I215 L, P1192R, QP509 L and Q706 L), so to contribute to underpin rational strategies for vaccine candidates development.

1. ASF – disease, epidemiological status and aetiology

African swine fever (ASF) is an infectious disease that can cause an acute hemorrhagic fever in domestic pigs, showing mortality rates up to 100% in domestic pigs and wild boars, also infecting bushpigs and *Argasidae* ticks of the genus *Ornithodoros* as vectors and infection reservoirs ('OIE Terrestrial Animal Health code', 2011).

To date, ASF is considered one of the most significant and devastating viral diseases of swine. Endemic in most of African Sub-Saharan countries were the wild suids (asymptomatic carriers) play an important role on disease maintenance. The first incursion outside the African continent was reported to happen in Portugal (1957), further spreading to neighbouring countries, and to South America, where it was eradicated, except on the island of Sardinia, where it is still endemic. In 2007, the disease was reported in Georgia, Armenia, Russia and Iran and then detected in Ukraine, Belarus, Baltic states and Poland having, more recently, been reported in the Czech Republic, Romania, Moldavia, Latvia and Hungary (Gulenkin et al., 2011; Sánchez-Vizcaíno et al., 2015; WAHID, 2018), mostly due to the movements of wild boars (More et al., 2018). From September 2018 to date, these wildlife movements have caused more than hundred occurrences in Belgium which still has managed to successfully implement sanitary measures, avoiding domestic swine outbreaks (Cwynar et al., 2019). Since August 3rd, 2018, ASF has been reported with multifocal outbreaks throughout China (the world's biggest pig producer), constituting a major concern for Chinese Veterinary Authorities (Ge et al., 2018) (FAO, 2018), causing the death of tens of thousands pigs until now and major production impact (Wang et al., 2018). Unfortunately, it also rapidly spread to neighbouring Asian countries as Cambodia, Korea, Laos and Vietnam (OIE, 2011), indicating a seemingly harder task to control the disease. Currently, the absence of effective vaccines and adequate treatment imposes strict control strategies for the containment of the disease insurgence based on stamping out measures and, trade bans of animals and pork products, leading to costly socio-economic losses in affected countries (Costard et al., 2013; Penrith and Vosloo, 2009).

The etiological agent of this suids disease, the African Swine Fever Virus (ASFV), is the sole member of the *Asfarviridae* family and *Asfivirus* genus and also, the only known arthropod-borne DNA virus, integrating the nucleocytoplasmic large DNA viruses (NCLDV) cluster (Dixon et al., 2005; King et al., 2011; Koonin and Yutin, 2010) enveloped virus, with

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an icosahedral capsid showing complex multi-layered structure protecting a linear double-stranded DNA (Breese and DeBoer, 1966; Carrascosa et al., 1984), and an inner viral core reviewed by Salas and Andrés (2013). Additionally, extracellular virions exhibit host membrane debris, acquired during the cellular egress hence contributing as evasion strategies (Andrés et al., 1998). ASFV shows a tropism for the mononuclear–phagocytic system cells (*e.g.* macrophages), infecting through clathrin- and dynamin- depended endocytosis and micropinocytosis (Cuesta-Geijo et al., 2012; Sánchez et al., 2012). The endosomal internalization may facilitate the access to host factors (Alonso et al., 2013a; Hernaez and Alonso, 2010), and an enhanced subversion of factors thus fostering the viral infection (Gómez del Moral et al., 1999), particularly through a tight regulation of cellular apoptosis (Revilla et al., 1997; Nogal et al., 2001; Granja et al., 2004; Hernáez et al., 2004; Hurtado et al., 2004). Following cell entry, ASFV uncoats within endogenous vesicles of acidic pH releasing the viral core (Hernaez and Alonso, 2010; Alonso et al., 2013a,b, Gómez del Moral et al., 1999). Recently, the ubiquitin-proteasomal pathway was also revealed as crucial for the final degradation of the viral cores, enabling the release of the viral DNA and the onset of viral DNA replication and transcription (Barrado-Gil et al., 2017).

2. ASFV infection and dynamics of its DNA replication

ASFV genome, a long linear double-stranded DNA genome flanked by inverted terminal repeats and closed by short hairpin loops has a guanine/cytosine content of approximately 39%, comprising several genes involved in nucleotide metabolism, transcription, replication, repair, immune evasion, and modulation of host cell apoptosis (Dixon, Chapman, Netherton & Upton, 2012). Its length ranges between 170 and 193 Kbp depending on the isolate (Chapman et al., 2008; Dixon et al., 2013; Yáñez et al., 1995), encoding between 150 and 167 open reading frames (ORFs), with several of them lacking known or predictable function (Chapman et al., 2008; Dixon et al., 2019; Yáñez et al., 1995). Closely spaced and/or superposed, ORFs display an upstream short A-T-rich sequence, which acts as promotor regions, recognized by the viral RNA polymerase complex and specific transcription factors of different temporal expression phases - early, intermediate and late phase (Dixon et al., 2013). Studies on point deletion showed that late viral promotors are located between −36 and +5 bp from the transcription initiation sites, also revealing that TATA box deletion severely disrupts viral transcription (García, Escudero, Eladio and Uela, 2000).

Even though ASFV genome fragments have been found in nuclear extracts (García-Beato et al., 1992a,b; Rojo et al., 1999), only its cytoplasmic DNA replication at later hours post-infection had been thoroughly explored. Significantly, early studies on enucleated cells proved to induce impaired ASFV-infection, strongly indicating that cellular nuclear factors would, most probably, be indispensable in the early stages of ASFV DNA synthesis (Dixon et al., 2013; García-Beato et al., 1992a,b; Netherton and Wileman, 2013; Ortin and Viñuela, 1977; Tabarés, 1987; Tabares and Sánchez Botija, 1979). Evidences also related to the possibility of ASFV full length genomes being formed in the cytoplasmic factories, possibly from nuclear synthesized fragments as replicative intermediate forms, like head-to-head or tail-to-tail genomic concatemer structures, along with proof of mature viral particles found to be composed both by nuclear and cytoplasmic fragments (Gonzalez et al., 1986; Caeiro et al., 1990; Brookes et al., 1996; Rojo et al., 1999). Altogether, these data were contrary to the initially accepted model of exclusive cytoplasmic DNA replication within perinuclear viral factories (vimentin cages). Recently, our lab proved that ASFV has an early intranuclear replication stage, similarly to other dsDNA virus (Sobhy, 2017). Additionally, it was shown that ASFV infection induced nuclear lamina disruption and recruitment of S phase-specific host translation factors (eIF4E, eIF4G and 4E-BP1) to its factories, enhancing its replication (Ballester et al., 2011), events suggesting an interplay of host cell nucleus and virus during viral infection.

A few years ago, our group detected “de novo” synthetized BrdU-labelled ASFV genomes inside the nucleus of Vero-infected cells, after prompting cells into an arrested G2/M phase (previous to infection), exposing that active viral DNA replication occurs within host cell nucleus, at an initial phase of infection (Simões et al., 2015a). Confirmatory studies were also performed with swine monocyte-derived macrophages (MDMs), infected with the virulent ASFV/Lisboa 60 isolate. Foremost, 4hpi, in both infected cell lines, viral “de novo” synthesised DNA had an exclusive intranuclear BrDU staining distribution. This comparative study also allowed to uncover morphological differences in what was considered to be virus replication foci as in Vero-infected cells a discrete pan-nuclear pattern of foci was observed, whilst in MDMs clusters of dense BrdU-staining nucleic acids were observed. These differences were accepted as being related with the biological characteristics between dividing Vero cells and non-dividing cells (*e.g.* MDMs) in regard to nucleotide pools, as previously debated by Dixon et al. (2008). These results, added to earlier published data, constitute compelling evidence of a two-phased replication of ASFV genome, with and early nuclear onset, followed by cytoplasmic DNA replication and packaging phase.

3. ASFV-host cell nucleus interplay

Cellular mechanisms which recognise viral genomes as foreign molecules soon after virus internalization still lack full characterization (Whittaker et al., 2000; Knipe, 2015), as the understanding of the struggle between the cell survival mechanisms and the virus subversion events to replicate. In order to shed light on ASFV-host cell interactions, we performed studies which revealed this virus infection to activate DNA Damage Response (DDR) pathways, changing the nuclear landscape and cellular epigenetic signatures, modifications ultimately essential for effective infections and progeny formation (Simões et al., 2013, Simões, M et al., 2015b). The obtained results were worthy of comparison with many other viruses that also show an intranuclear stage of replication (Schmid et al., 2014), and those exploiting subnuclear domains for successful infections (Chang et al., 2011; Salsman et al., 2008).

Many factors, internal and external, can induce DNA injuries to cells, which if not detected and corrected appropriately pose a threat to genomic integrity and cellular survival. Cellular DNA damage response (DDR) mechanisms refer to a myriad of surveillance and responsive factors that once activated maintain the integrity of its genome and cellular homeostasis, or induce programmed cell death (insults beyond repair) (Ciccia and Elledge, 2010; Freeman and Monteiro, 2010; Roos and Kaina, 2013). This broad selection of host signal-transduction cascades regulate cell cycle progression, DNA replication and repair (Lilley et al., 2010; Pancholi et al., 2017; Weitzman et al., 2004). Viral genomes sensed inside cellular nucleus trigger sensing-repair factors and pathways (reviewed by Syed and Tainer, 2018). As counter-offensive strategies, viruses hijack DDR factors into viral replication sites or exotic domains, to subvert, explore or degrade/downregulate the activated DDR components which could negatively interfere with the viral replicative success (Lilley and Weitzman, 2010; Luftig, 2014; Weitzman and Fradet-Turcotte, 2018). Curiously, some viruses are known to target and enhance apoptotic pathways activation for their own success (Li and Hayward, 2011), as does ASFV (Dixon et al., 2017). ASFV genome may be recognized as an aberrant dsDNA molecule by the host nucleus, thus activating DDR factors, especially double strand breaks (DSBs) repair machinery as other viruses and reviewed by Pancholi, Price and Weitzman (2017). The phosphorylation of both histone H2 variant H2AX, at serine 139 (γH2AX), and Replication Protein A 32 kDa subunit (pRPA32) are commonly regarded as DNA damage response markers. This phosphorylation (γH2AX) is regarded as a hallmark of DSBs signals, activated from stalled replication forks to DSBs (Nichols et al., 2009), whereas pRPA accumulates on single-strand DNA ends, created by replication stress or stalled replication forks

(Wilkinson and Weller, 2006). Several viruses are known to induce both γH2AX and RPA32 phosphorylation, determining the cascade-type of events, triggering other DDR mediators, as tumour suppressor p53 (also known as "guardian of the genome") and specific transduction kinase effectors of the Phosphoinositide 3-kinase (PI3K) family, *e.g.* Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia Mutated-Rad3 related (ATR). These effectors are modulated by the activity of downstream checkpoint kinases, ChK1 and ChK2, respectively, overall interfering with the cell cycle and modulating the virus replication (reviewed in Nikitin and Luftig, 2012). The main PI3-kinases involved in DSBs promote DNA lesions repair by one of two mechanism, Homologous Recombination (HR) or Non-Homologous End-Joining (NHEJ). ATM and ATR pathways are mainly responsible for high-fidelity HR processivity by which the restoring process uses a template sister chromatid maintaining genomic congruency. In contrast, DNA-dependent protein kinase catalytic subunit (DNA-PKcs) pathway is mainly responsible for NHEJ repair, which randomly fills the recognized genomic gaps, acting as a more error-prone mechanism (Lilley et al., 2007). Experiments performed in our laboratory revealed that from an early stage of infection (4hpi onwards), ASFV is responsible to induce a strong phosphorylation of H2AX and RPA32 (key sensors for DDR), as well as the phosphorylation of the mediator p53 and the activation of ATR and Chk1 factors. The importance of ATR pathway activation by ASFV infection was further confirmed with pharmacological trials using caffeine and wortmannin, each used at ATR-specific inhibitory concentrations, causing a disruption of ASFV protein synthesis. Additionally, the use of ATR-kd cells proved a dominant-negative effect also inducing decay of viral protein synthesis, and the reduction of viral yields (Simões et al., 2013). Virus-host DDR interplay is well characterized as in the case of HSV1, a NCDLV member that uses ATM-mediated DDR activation to promote viral production, results confirmed by the use of cells defective for ATM in which a reduced HSV1 replication was present. Also for Epstein Barr virus (member of the herpesvirus family) and for Adenovirus studies proved the widespread sensor signal activation and subvertion of DSBs repair mechanisms (Nikitin and Luftig, 2011; Li and Hayward, 2011), a strategy reported to induce cells into S phase hence providing loads of host factors needed for these viruses DNA synthesis (reviewed in McFadden and Luftig, 2013). Alongside, ATR activation has been described for other DNA viruses (Adeyemi et al., 2010; Weitzman et al., 2010; Edwards et al., 2017), and an array of evidences suggest that HR facilitates viral infectivity by enabling viral DNA recognition as genomic DSBs assisting viruses on easy access to host DNA replication and repair machinery, subverting host proteins and tempering host immune responses (Mboko et al., 2012; Nikitin and Luftig, 2012). ATR pathway activation has also been demonstrated to facilitate adenoviral genome concatemerization (Lakdawala et al., 2008), most likely used for its high fidelity mechanism, also supporting arguments for ASFV concatemers formation. The long before described presence of ASFV in the host cell nucleus and proof of its involvement in initial viral replication, encouraged further virus-host nucleus interaction studies, ultimately revealing a more sophisticated viral subversion of this prominent cellular compartment (Simões et al., 2015b).

The eukaryotic nucleus is a highly organized structure, composed by several subdomains where specific factors and biological processes are restrained to limited spaces, optimizing the nucleus performance and dynamics (reviewed in Dundr and Misteli, 2010; Dundr, 2011). Primarily, the nuclear architecture is based on a complex model, based on structural hierarchical levels designed from bare DNA molecules to chromatin arrangements and epigenetic modulation. Contrasting to chromatin occupied regions, distinguishable areas persist deprived of it (interchromatin compartments), even though filled with other different constituents such as protein aggregates of immune-related factors in 'storage-like' territories (Lallemand-Breitenbach and de Thé, 2010), domains of transcription and splicing factors (Lamond and Spector, 2003; Cioce and Lamond, 2005; Morris, 2008), and depots of DNA replication/repair machineries (Cremer and Cremer, 2010; Kosak and Groudine, 2004; Nagele et al., 1998; Zuleger et al., 2011).

Promyelocytic leukaemia nuclear bodies (PML-NBs), mainly associated to cell cycle control, apoptosis and immune responses (Everett and Chelbi-Alix, 2007), and both nuclear speckles and Cajal bodies, functionally related with RNA biogenesis, transcription and splicing events, are examples of this unique organization of the cellular nucleus. Studies on nuclear spatial organization have unravelled strategies of distinct viruses, which proved to have coevolved exploiting their host cell nucleus (reviewed in Lieberman, 2006; Toth, and Jung, 2013). Morphofunctional changes of subnuclear domains and/or modifications of the pre-determined epigenetic status of cellular chromatin are some of the viral-induced modification aiming at enhanced productive infections (reviewed in Everett, 2001; Ihalainen et al., 2009). Often, PML-NBs are reshaped by viruses for their activities in antiviral responses and stimulation of DNA damage sensing (reviewed in Lallemand-Breitenbach and de Thé, 2010), for which most viruses colocalize their replication centres next to these domains either to benefit from retained factors or to subjugate antiviral responses (reviewed in Everett, 2001; Möller and Schmitz, 2003; Tavalai et al., 2008; Sarkari et al., 2011). Along the ASFV infection, PML-NBs were shown to reduce in number and get oversized according to changes also reported in other DNA virus infections (reviewed in Jiang and Imperiale, 2012). By PML-knockdown experiments, ASFV infection proved to be PML-dependent, as this depletion of PML-related factors lead to irregularly shaped viral factories and to a significant decline in viral progeny. Concomitantly, microscopy studies revealed that ASFV disrupted PML-NBs are in close proximity to reorganized foci of phosphorylated-p53 (activation of the factor regarded as 'guardian of the genome') and accumulations of phosphorylated-ATR (Simões et al., 2015b). Also revealing that, the cellular transcription switch and/or downregulation of splicing events during ASFV infections resulted in nuclear speckles enlargement (captions on accumulations of SC-35 considered to be nuclear speckles dominant protein), although reduced in number, as equally reported for other viral infections (reviewed in Lamond and Spector, 2003). Significantly, Cajal bodies (CBs) which are commonly reshaped by viral infections apparently due to accumulations of small nuclear ribonucleoproteins and the inhibition of RNA processing (Chang et al., 2011; Gama-Carvalho et al., 2003; James et al., 2010; Kim et al., 2007; Salsman et al., 2008; Schneider et al., 2008), were also disrupted by ASFV infection. Indeed, CBs shifted into 'comma-shaped' structures (unlike their usual dot pattern), even displaying higher numbers, possibly to favour a host transcriptional switch previously described and reviewed in Sánchez et al. (2013).

Potentially, epigenetic signatures enable the activity state shift of wrapped DNA like when acting as landing platforms for chromatin remodelling factors. The complexity of possible combinations of distinct epigenetic marks and its respective impact on cellular functions led to the known "histone code", stressing the biological importance of this epigenetic level juxtaposed to the genetic code, as previously reviewed (Jenuwein and Allis, 2001). Post-translational histone modifications still lack full understanding but its spatially reorganization within the intranuclear higher order is recognized, resulting in both functional and organizational changes of subnuclear domains and biological processes, (Bártová et al., 2008; Black et al., 2012; Kouzarides, 2007; Meaburn and Misteli, 2007; Zinner et al., 2006). Understandably, host chromatin status represents an additional challenge for viral infections, one to overcome in order to succeed. Several studies showed that control over histone methylation/acetylation status and distribution of HP1 isoforms, allow viruses to modify the chromatin texture, promoting a permissive nuclear environment and subverting host gene expression, as (Knipe et al., 2013; Lieberman, 2006; Weitzman et al., 2010). Eukaryotic cells functionality is related to the balance between euchromatin, an open-packed form of DNA, transcriptionally active and easily accessed for replication, and heterochromatin, which is constituted by genomic regions that are compacted and silenced

(Fillingham and Greenblatt, 2008; Soria et al., 2012). Not so long ago, studies revealed that heterochromatin establishment is dependent on specific histone H3 methylation marks enrichment, aided by additional heterochromatin protein 1 isoforms (HP1α and HP1β), which overall facilitate a repressive chromatin environment and an effective DNA damage response (Bartkova et al., 2011; Eskeland et al., 2007; Luijsterburg et al., 2009; Zeng et al., 2010). Diverse viruses are known to promote changes on the methylation patterns of histone H3, regulating the accessibility of cellular replication/transcription machineries to distinct domains and chromatin territories (Bartkova et al., 2011; Eskeland et al., 2007; Luijsterburg et al., 2009; Vogel and Kristie, 2013; Zeng et al., 2010). Viruses also interfere with histone deacetylases (HDACs) activities which are related to heterochromatin formation and spreading, in order to improve viral replication (Lukashchuk and Everett, 2010; Tang and Maul, 2003).

Studies performed in our lab, on epigenetic interference during ASFV infection, revealed a redistribution of heterochromatin-related marks (H3K9me3, H3K27me3, HP1α, HP1β and HDAC2), whilst the distribution of euchromatin marks (H3Kme1, H3K27me1, H3K36me3 and HP1γ) remained unchanged. These ASFV-induced epigenetic modifications reflect an heterochromatinization of the host nucleus, which most probably occurs to silence specific host genes encoding proteins perceived as detrimental for the virus processes (*e.g.* apoptotic and IFN responses). These virus-induced rearrangements coincide with HP1α and HP1β redistributions along ASFV infection, putatively explaining the nuclear lamina breakdown previously reported by Ballester et al. (2011), concurring to the known involvement of HP1 isoforms with the nuclear envelope assembly, also reviewed in (Ballester et al., 2011). These evidences combined with the clarification of an ASFV intranuclear DNA replication phase, help support the argument of nuclear lamina disintegration induced by structural changes related with ASFV replication compartments and/or by viral nuclear entry/egress, as reported in other viral infections (reviewed in Ballester et al., 2011); (Snoussi and Kann, 2014).

At the same time, we reported HDAC1, HDAC2 and HDAC3 being recruited to ASFV cytoplasmic factories, with special emphasis for HDAC2 displacement, for its main association to chromatin plasticity (Snoussi and Kann, 2014).These HDAC modulations not only change the host epigenetic state but mostly act in favour of ASFV replication events, as described for other viral infections reviewed in (Herbein and Wendling, 2010).Interestingly, HDAC6 role in ASFV cytoplasmic factories formation is still to be proven (Muñoz-Moreno et al., 2015), but findings on class I HDACs raise the possibility to use HDAC I inhibitors to disrupt ASFV infection, as demonstrated for other viruses (Adhya et al., 2013; Siddiquey et al., 2014).

Altogether, ASFV disruption of the subnuclear domains and the chromatin texture, inducing nuclear architecture changes, are orchestrated to enhance a repressive nuclear environment. This nuclear landscape is further sustained by combined subnuclear domains and heterochromatic regions close localization, underlining the possible ASFV-induced host transcriptional switch, as also described for Herpesvirus (reviewed in Conn and Schang, 2013; Toth et al., 2013). Supporting studies revealed the spatial proximity of the altered subnuclear domains, heterochromatic regions and DDR-related factors in ASFV-infected cells, most probably as part of ASFV strategy to induce nuclear heterochromatinization to silence adverse genes and to increase interchromatin territories, granting extra-space for viral intranuclear replication.

Nonetheless, the full role of the host cell nucleus in ASFV infection success is still unclear, and in recent years, our studies have contributed to improve knowledge on ASFV-cell interactions aiming to uncover new strategies on antiviral therapies (*e.g.* PML, HP1 and HDAC activity modulation).

4. ASFV replication events – innovative insights on the role of five major viral enzymes

4.1. ASFV-A104R – a viral histone-like protein

ASFV encodes for the A104R ORF, a putative histone-like protein (pA104R), described as the only histone-like protein encoded by a eukaryotic virus. This viral protein shares a sequence identity of 25–30% with two distinct families of bacterial histone-like proteins (HU and IHF) (Borca et al., 1996; Neilan et al., 1993). In addition, pA104R sequence comprehends a histone-like proteins signature, from residues 57 to 76, including 8 aminoacid residues responsible for DNA interaction (highly conserved between bacterial histone-like proteins). In prokaryotes, these nucleoid-associated proteins are involved in DNA supercoiling also playing an important role in DNA mechanisms including: replication, DNA repair, recombination and transcription (Hashimoto et al., 2003; Koli et al., 2011; Oberto et al., 2009). Some of our studies, revealed that purified recombinant pA104R binds in an ATP-independent manner, with higher affinity to dsDNA instead of ssDNA, suggesting that this protein is probably more apt to fold full-length ASFV genomes other than intermediate single-stranded genomes. Therein, demonstrations were made regarding pA104R binding site size, as being of around 14 to 16 nt and a minimal binding length of 11 to 20 nt (Frouco et al., 2017a,b,c), similarly to other viral DNA-binding proteins description (Loregian et al., 2007; Rochester and Traktman, 1998). To ensure an efficient DNA-binding activity of pA104R, the residue Arg69 has been identified as crucial, probably due to its positive charge, as its replacement by alanine (a nonpolar aminoacid), lead to a pA104R activity reduction. And, in accordance to its replacement by lysine (also positively charged amino acid) which lead to no change of pA104R DNA-binding properties (Frouco et al., 2017a,b,c).Likewise, when residue Pro74 that had previously been predicted as critical for DNA-binding (Borca et al., 1996; Neilan et al., 2004), was substituted by a lysine residue, the formation of complexes between pA104R and DNA was unchanged (Frouco et al., 2017a,b,c), even contrasting to previous reports on bacterial histone-like proteins (Luscombe, 2001; Luscombe and Thornton, 2002). Plus, in vitro assays proved a stable pA104R DNA-binding activity at a wide range of temperatures (4 °C to 37 °C) and pH values (4 to 11), possibly to support ASFV replication both in Argasidae vectors and in suids (Frouco et al., 2017a).pA104R was further characterized and proved its ability to supercoil DNA in the presence of ASFV topoisomerase II (P1192R). Advances on this enzyme revealed its critical role for ASFV replication (Coelho et al., 2016; Freitas et al., 2016). This enhance mechanism is very well described in bacterial histone-like proteins (Bensaid et al., 1996; Ghosh et al., 2014), and for some viral proteins involved in genome packaging (Bogner et al., 1998; Thoma et al., 2006), providing room for the hypothesis of pA104R participating in ASFV genome packaging.

This phenomena of viral packaging is still not fully understood, new evidences have brought to light the putative role of pA104R in promoting a stable, organized and compact nucleoid. Its participation is further supported by the observation of pA104R localized over the central nucleoid structure (Borca et al., 1996; Salas and Andrés, 2013). Also, the A104R gene transcription has been described as occurring mainly during the late phase of infection, showing a maximum accumulation peak at 16 hpi, although, in substantial lower levels when compared with two other ASFV structural genes, CP204 L and B646 L, suggesting that pA104R is more likely to be involved in fundamental viral processes, namely DNA replication and transcription, instead of viral capsid formation. These results are validated by pA104R expression from 12 hpi onward, and by the use of a potent transcription inhibitor (Arabinose, AraC) which completely halt pA104R expression. Concomitantly, the fact that this viral protein is recruited into viral factories reinforces the notion of its participation in ASFV genome packaging (Frouco et al., 2017a,b,c), like, its observed nuclear localization strongly suggests its contribution in viral DNA replication and/or

in heterochromatization of the host cell genome. As earlier described, the heterochromatization process may facilitate viral infection while leading to the silencing of particular host genes part of an effective immune response (Simões et al., 2015b). Further evidences were obtained by A104R downregulation using siRNA as its decline of 27% at 16 hpi, negatively impacted the production of new infectious particles. This pA104R downregulation induced a 82% reduction in viral yields, along with lower viral genome copy number (−78.3%) and a 47.6% repression of late viral transcription (B646),yet not interfering with the transcription of CP204 L, an early viral gene (Frouco et al., 2017a). All these finding add up to other known examples of viruses which alter the epigenetic status of their host cells chromatin in order to control cellular gene expression for its own benefit (de Souza et al., 2010; Knipe et al., 2013).

How viral mechanisms act to modulate, interfere and reprogram cellular epigenetic landscape is still far from being fully understood although specific interactions with Histone Deacetylases (HDACs) and Histone Acetyltransferases (HATs) have been explored. These viral interferences seem to disrupt the host gene-expression program always favouring the viruses' infection thus avoiding cellular processes which could imperil the viral success. HDACs are known to catalyse the removal of the acetyl group from lysines, re-establishing histones' positive charge, hence inducing genome heterochromatinization and gene silencing (Dekker and Haisma, 2009). In mammals, eighteen HDACs have been identified and organized in four distinct classes (Bolden et al., 2006). Interestingly, both Class I and II can be pharmacologically modulated by inhibitors (HDACi). These inhibitors have also being used as promising antiviral agents since they activate latent HIV, Epstein-Barr Virus and Human Cytomegalovirus, and in doing so induce the depletion of virus persistent reservoirs and quiescent infections (Archin et al., 2012; Ghosh et al., 2012; Huber et al., 2011; Michaelis et al., 2005; Radkov et al., 1999); also limiting enveloped viruses replication by interfering with the stability of its particles (Vazquez-Calvo et al., 2011). Regarding the above data and acknowledging ASFV interference with the host chromatin epigenetic status, by promoting heterochromatinization and recruiting HDAC1, HDAC2 and HDAC3 to viral factories (Simões et al., 2015b), several studies were performed in order to explore the antiviral activity of four HDAC inhibitors: Trichostatin A (TSA), Vorinostat (SAHA), Valproic Acid (VPA) and Sodium phenylbutyrate (NaPB). The obtained results showed a full impairment of ASFV replication when using NaPB, whereas VPA lead to a significant reduction of viral progeny at 48 h post-infection (−73.9%, $p = 0.046$). Also, the two pan-HDAC inhibitors, TSA and SAHA, were tested promoting reductions of 82.2% ($p = 0.043$) and 73.9%, ($p = 0.043$), respectively. In addition, NaPB was also found to display protective effect in a dose-dependent manner, interfering with late viral expressions and reversing the ASFV-induced histone H3 lysine 9 and 14 (H3K9K14) hypoacetylation status. This particular hypoacetylation is known to induce an open chromatin state, possibly allowing the expression of host genes deleterious to infection progression. Again, a synergic antiviral effect was identified when NaPB was combined with Enrofloxacin (Frouco et al., 2017b). This fluoroquinolone is a specific ASFV-topoisomerase II poison which had already been described as an anti-ASFV drug (Mottola et al., 2013; Freitas et al., 2016).

4.2. ASFV-I215 L – a viral E2 ubiquitin-conjugating enzyme

In order to succeed, ASFV must override the anti-viral cellular intrinsic defences hence originating a productive infection. It is fairly established that diverse viruses modulate the ubiquitin-proteasome system of host cells, through several mechanisms, as encoding ubiquitin-related enzymes (Isaacson and Ploegh, 2009; Randow and Lehner, 2009). Studies presented in the 1990s´, showed that ASFV encoded a E2 ubiquitin conjugating enzyme, ORF I215 L (Hingamp et al., 1995, 1992), indicating that cellular ubiquitination pathway might be subverted during ASFV infection, similarly to other reported events for other viruses (González-Santamaría et al., 2011; Gustin et al., 2011). More recently, studies performed in our lab showed that this putative viral E2 enzyme acted as an ubiquitin conjugating enzyme, possible to mono and di-ubiquitinate. Its enzymatic ability to remain catalytically active under a wide range of pH values (4 to 9) was also revealed (Freitas et al., 2018), which could present critical effects during cell entry processes known to occur under a low-pH-dependent endosomal pathway (Alonso et al., 2013b; Cuesta-Geijo et al., 2012; Hernáez et al., 2016).Concomitantly, its enzymatic activity could facilitate *Argasidae* infection as the midgut epithelial cells of *Ornithodoros spp*., present pH levels much lower than 7 (Sojka et al., 2013). This catalytic plasticity was also proved under a broad range of temperatures (from 4 °C to 42 °C), as this could be of importance for the virus lifecycle continuity, remaining active during the high fever episodes (found in infected animals) but also inside the vector which usually sustain large temperature oscillations exposures (Lvov et al., 2015). Additionally, mono-, di-ubiquitinated and poly-ubiquitinated I215L-species were identified in detergent-soluble protein fractions extracted from ASFV-infected cells, suggesting that pI215 L will participate in a plethora of mechanisms, as the ability to generate diverse substrate-ubiquitin structures remains essential to target different host/viral proteins (Freitas et al., 2018). Indeed, it is described that monoubiquitination of several nuclear proteins may induce DNA repair modulation and variable cellular gene expression (Passmore and Barford, 2004; Sadowski and Sarcevic, 2010), whereas the poly-ubiquitination of a target protein occurs via K48 of ubiquitin ultimately leading to protein degradation through the 26S proteasome pathway (Feenstra et al., 2015; Matsuo et al., 2011), or even by endocytosis if poly-ubiquitination occurs via K63 residue of ubiquitin (Piper et al., 2014). Foremost, is the distinct pool of di-ubiquitinated forms detected in detergent-insoluble extracts, possibly caused by the pI215 L binding affinity to host proteins that contain an ARID DNA-binding domain (Bulimo et al., 2000; Hingamp et al., 1992).

ASFV-infected Vero cells revealed that I215 L viral gene is transcribed from early infection times, showing two transcription peaks (at 2 and 16 hpi, respectively), indicating the possible involvement of pI215 L in distinct phases of the virus lifecycle, like viral transcription, genome replication and viral egress (Freitas et al., 2018), as similarly reported for several other viruses (Fukuyo et al., 2011). In addition, the detection of pI215 L from 4 to 20 hpi and the immunolocalization studies revealed that ASFV-pI215 L is recruited to viral factories, supporting the hypothesis of its participation in viral RNA transcription and/or DNA replication. Results also revealed that ASFV-pI215 L presents a diffuse distribution throughout the cytoplasm which may be related to the role in ubiquitination of viral proteins and/or host proteins engaged in nuclear functions (*e.g.* antiviral responses, DDR factors). Finally, siRNA experiments clarified the pI215 L involvement in the late viral transcription as its gene downregulation lead to the reduction of B646 L transcript copies, while mRNA levels of the early viral gene CP204 L remained unchanged, always attending to mock-transfected Vero cells mRNA profiles. Also in I215L-depleted cells, ASFV genomes were formed in a diminished number, between 63 to 68% and a reduced viral progeny (up to -94%) was confirmed. In sum, these results strongly suggest that the ubiquitin pathway mediates ASFV genome replication, viral late transcription and progeny production (Freitas et al., 2018), corroborating previous studies that pointed out the importance of the ubiquitin-proteasome system during this virus infection (Barrado-Gil et al., 2017).

4.3. ASFV-P1192R – a viral Topoisomerase type II

ASFV is the only known virus, infecting mammals, which encodes its own topoisomerase II - ORF P1192R (García-Beato et al., 1992a,b). Topoisomerases type II are found in all kingdoms of life and have been proved to regulate DNA topology during replication, transcription, chromosome condensation-decondensation and meiotic segregation, by catalysing transient double-stranded breaks in one helix DNA, pass a

second DNA helix, further closing the gap (Champoux, 2001; de Souza et al., 2010; Forterre et al., 2007). Curiously, phylogenetic studies revealed that viral topoisomerase II does not cluster with cellular topoisomerases (Gadelle et al., 2003), being most related with the bacterial DNA gyrases and topoisomerase IV, sharing homology of about 25% (Liu, 1994), hence raising the possibility of a common ancestor and the viability of using specific anti-bacterial topoisomerase/gyrase drugs (*e.g.* fluoroquinolones) to target this protein. Studies performed in our group showed that these anti-topoisomerase bacterial inhibitors induce a viral genome fragmentation and a delay of the viral protein synthesis (Mottola et al., 2013). Later, using Saccharomyces cerevisiae temperature-sensitive trials, the functionality of P1192R to relax supercoiled DNA through complementation was demonstrated, as with in vitro decatenation assays, further on confirmed by mutating its predicted catalytic residue (Coelho et al., 2015). Other studies relating optimal pH, temperature, salt and divalent ionic concentrations, and ATP conditions allowed the determination of optimal ones for the pP1192R activity, which were found to be similar to other type II topoisomerases (Coelho et al., 2016). ASFV-infected cells exposed to Enrofloxacin during the late phase of infection (from 15 to 16 hpi) showed induced-fragmentation of viral genomes, whereas no viral genomes could be detected whenever Enrofloxacin was added from 2 to 16 hpi, confirming previous reports (Freitas et al., 2016). These results suggest a similar mechanism of action of this enzyme when comparing ASFV and prokaryotes. In bacteria, topoisomerases play an important role in DNA replication as this class of enzymes is accountable for relieving torsional stress ahead of the replication fork, and in particular, the topoisomerase IV shows an effective decatenating activity essential for de novo separation of genomes. Further characterization revealed that ASFV P1192R gene is actively transcribed as early as 2 hpi, reaching a maximum peak of accumulation around 16 hpi (Freitas et al., 2016), colocalizing with the viral cytoplasmic factories at intermediate and late phases of infection, when viral DNA synthesis, transcription and translation are most active (Coelho et al., 2015). Following these assays, siRNA knockdown experiments revealed that ASFV Topoisomerase II plays a critical role in viral DNA replication and gene expression, with transfected cells revealing a decreased number of viral transcripts coupled with a reduced cytopathic effect whenever compared to ASFV-infected cells (control group). Besides, a significant decrease in the number of both infected cells and viral factories per cell along with a reduction of the virus yields was also confirmed (Freitas et al., 2016).

4.4. ASFV- QP509 L and Q706LSuperfamily 2 RNA helicases

ASFV gene temporal expression is characterized by a tight regulation of transcription initiation followed by a fast rate of mRNA degradation. In general, the early phase is established before the onset of DNA replication, and a late phase, after DNA replication has begun (Rodríguez and Salas, 2013). Early transcription or pre-replicative starts almost immediately after virus infection and continues until the initiation of DNA replication. The expression of a subset of immediately early genes is also recognized as initially being repressed and dependent on protein synthesis. Similarly to what is described for Poxvirus, after DNA replication, the transcription is divided in two stages, the intermediate transcripts which are synthesized immediately after DNA replication and of the later genes occurring after the intermediate ones, process which continues until the end of infection (Broyles, 2003). In terms of gene expression control, the regulatory factors for each phase are expressed in the subsequent stage with the exception of the early factors that result from a late phase of infection expression and are packaged into the viral particles (Salas and Andrés, 2013).

Following the onset of DNA replication in the cytoplasm, at about 6 hpi, a shift in the viral expression pattern occurs (Salas et al., 1986). ASFV genome encodes about 20 genes considered to be involved in the transcription and modification of its transcripts. Similar to Vaccinia virus, ASFV transcriptional machinery provides a high degree of independence from its host and an accurate temporal control of its own genes expression. Although little experimental data is available regarding the role of these proteins in transcription, the degree of homology between ASFV and other members of the NCLDV group can support predictions on many of the functions of these proteins (Koonin and Yutin, 2010). Among these genes and accounting ASFV, it encodes six members of helicase superfamily (A859 L, F105 L, B92 L, D1133 L, Q706 L, QP509 L), three of which are predicted to participate in transcription based on their similarity with Vaccinia virus, as being essential for transcript elongation, termination and release. Recently, several analyses were performed in CIISA-ASF Unit to better characterize the biological role of some of these viral enzymes, in particularly the QP509 L and the Q706 L enzymes. Phylogenetic analyses revealed that ASFV RNA helicases are highly conserved among virulent and non-virulent isolates, with ASFV-QP509 L and ASFV-Q706 L helicases belonging to distinct monophyletic lines. Although, a high homology was observed, it was possible to identify a geographic/genotype cluster segregation (personal communication) very similar to what was previously reported for ASFV-B646 L (Bastos et al., 2003; Boshoff et al., 2007; Lubisi et al., 2005).

In addition, ASFV-QP509 L shares the same monophyletic group with Vaccinia A18R and Ranavirus ORF55 RNA helicases, whereas ASFV-Q706 L clusters with Vaccinia virus D6/D11 L and Marseillevirus marseillevirus MAR_ORF241 RNA helicases, corroborating previous studies (Baylis et al., 1993; Duffy et al., 2008; Roberts et al., 1993; Rodríguez and Salas, 2013; Yáñez et al., 1993).and supporting the idea that NCLDV members share a common ancestor (Yutin and Koonin, 2012). Interestingly, QP509 L and Q706 L share a sequence overlap however the orthologous genes in Vaccinia virus are found to be separated approximately 20,000 bp, suggesting a different evolutionary route for ASFV. Indeed, recent studies reported that ASFV presents a higher evolutionary rate than other DNA virus (Alkhamis et al., 2018; Duffy et al., 2008; Grenfell et al., 2004), probably due to its complex inter-species transmission (wild boars, ticks and domestic pigs), showing a diversity peak over the last 200 years (Alkhamis et al., 2018). Considering the transcript expression dynamics, both RNA helicases are actively transcribed from 2 hpi with maximum mRNA levels between 8 and 12 hpi, suggesting that both enzymes are mainly required during the intermediate and late stages of the infection cycle, while DNA replication and transcription events occur. In fact, pQP509 L is recruited from 12 hpi onwards to the viral factories and the host nucleus, whereas at the same period of infection pQ706 L could only be detected at viral factories, indicating different roles during the viral replication cycle. Despite an early intranuclear phase that has been proposed for ASFV (R. García-Beato et al., 1992a,b; Simões et al., 2015a), the presence of pQP509 L in this cellular compartment, at later times of infection, can be related to some viral events other than transcription and/or DNA replication as, for example, modulation of antiviral responses like those described for different viral RNA helicases, including: the NS3 RNA helicase of Hepatitis C virus (HCV) which is involved in the unwinding of the double-stranded RNA intermediates (Dumont et al., 2006) and viral assembly (Ma et al., 2008), and for the D6/D11 RNA helicase of Vaccinia virus that unwinds RNA-RNA, RNA-DNA and RNA-protein intermediates (Jankowsky et al., 2000). In a very similar way, down-regulation of QP509 L and Q706 L by siRNA leads to a reduction of viral late transcripts (ASFV-B646 L), although the expression of an early viral gene (ASFV-CP204 L) was not affected. Depleted cells also presented reduction in the number of viral genomes coupled with a decreased viral yield, indicating that both ASFV RNA helicases have essential and non-redundant functions, with their function not being rescued by cellular RNA helicases (personal communication).

In summary, last years, several studies provided enhanced knowledge on ASFV replication strategies, this virus subversion and control actions over host mechanisms and cellular factors essential for its own replicative success. Altogether, these studies also unravelled the

epigenetic modulation of the host nucleus and identified potential targets forwarding a rational development of treatments and vaccines against ASF. Alongside, several antiviral strategies were also provided, particularly the possibility to use: DDR-related enzymes specific inhibitors (caffeine and wortmannin), HDAC inhibitors (NaPB, SAHA), anti-ASFV-topoisomerase II poisons (fluoroquinolones) and anti-ASFV-topoisomerase II inhibitors (isoflavones) (Arabyan et al., 2018; Hakobyan et al., 2019). In addition, infectivity tests were performed on knockdown cells for specific host factors and/or on viruses generating" loss of function" mutations. The innovative insights herein disclosed open avenues to tackle and impair ASFV infection with the use of known Epigenetic therapies (commonly used both in oncology and human viral infections), and currently called as Epi-drugs. As for example, an increased expression of DNMTs, as reported for HBV, facilitates the methylation of the viral genome, impeding its replication in vitro, thus corroborating the host strategy of DNA methylation of viral genomes to enhance host-defence mechanism (Hong et al., 2017). Similarly to what is also described for many other viruses, ASFV infection promotes specific methylations/acetylations on host chromatin, probably to subvert host cell defence mechanisms thus enabling and boosting its replication success (Hartline et al., 2018; Kuss-Duerkop et al., 2018). So, inhibitors targeting histone acetyltransferases (HATs) and deacetylases (HDACs), lysine (K) methyltransferases (KMTs) and demethylases (KDMs), kinases and phosphatases, ubiquitin-ligases and deubiquitinases, and also DNMT inhibitors may have strong antiviral effects, as stated for other viral infections (Hartline et al., 2018), opening pharmacological possibilities against ASFV to be further explored. Nonetheless, on the development of vaccines against DNA viruses maintains its focus, with DISC viral particles as a promising strategy for safe delivery of specific antigens, prompting strong immunity responses and an effective way of disabling the fast paced spread of this dreadful swine disease. Indeed, our functional studies on the biological role of ASFV replication-related enzymes (*e.g.* A104R histone like, I215 L ubiquitin E2 conjugating enzyme, P1192R type II topoisomerase and the RNA helicases QP509 L and Q706 L) can be a good strategy to use for DISC particles designing, since each viral enzyme shares a conserved sequence (above 99%) among ASFV isolates (Freitas et al., 2019.and Coelho et al., 2015).

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