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Review

African swine fever virus-cell interactions: From virus entry to cell survival

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ABSTRACT

Viruses have adapted to evolve complex and dynamic interactions with their host cell. The viral entry mechanism determines viral tropism and pathogenesis. The entry of African swine fever virus (ASFV) is dynamin-dependent and clathrin-mediated, but other pathways have been described such as macropinocytosis. During endocytosis, ASFV viral particles undergo disassembly in various compartments that the virus passes through en route to the site of replication. This disassembly relies on the acid pH of late endosomes and on microtubule cytoskeleton transport. ASFV interacts with several regulatory pathways to establish an optimal environment for replication. Examples of these pathways include small GTPases, actin-related signaling, and lipid signaling. Cellular cholesterol, the entire cholesterol biosynthesis pathway, and phosphoinositides are central molecular networks required for successful infection. Here we report new data on the conformation of the viral replication site or viral factory and the remodeling of the subcellular structures. We review the virus-induced regulation of ER stress, apoptosis and autophagy as key mechanisms of cell survival and determinants of infection outcome. Finally, future challenges for the development of new preventive strategies against this virus are proposed on the basis of current knowledge about ASFV-host interactions.

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1. Entry of African swine fever virus

The entry of a virus into host cells is not only the first step that initiates infection, but also a key determinant of viral tropism and pathogenesis. For an intracellular pathogen, the crucial issue is not merely the crossing of the cytoplasmic membrane since the entry pathway determines whether a productive infection takes place or not. There is also a substantial degree of complexity associated with the entry pathways of large DNA viruses. ASFV interaction with cellular receptor/s promotes subsequent entry steps involving the activation of signaling and endocytosis. However, early studies on ASFV entry in Vero cells and porcine macrophages characterized this event as a low pH- and temperature-dependent process consistent with saturable and specific receptor-mediated endocytosis (Alcami et al., 1989a,b; Alcami et al., 1990; Valdeira and Geraldes, 1985). An interesting observation was that the virus entered the macrophages of another species (rabbit), thus leading to an abortive infection when using a different mechanism mediated by non-saturable or non-specific receptors. These data are consistent with clathrin-mediated entry. In fact, early electron microscopy observations found ASFV particles frequently adsorbed to invaginations similar to clathrin-coated pits (Alcami et al., 1989a).

1.1. ASFV entry is dynamin-dependent and clathrin-mediated

Clathrin-mediated endocytosis is regulated by a network of proteins and lipids that are recruited in a dynamic temporal sequence. These molecules take part in membrane bending and elongation, and final fission of the endocytic vesicle (Fig. 1) (Merrifield et al., 2005; Taylor et al., 2011). The cell invaginates the plasma membrane, thus giving rise to a small intracellular vesicle composed by a clathrin coat with adaptor proteins, Epsin15 (Ede1), and dynamin. The latter recruits BAR domain proteins, which in turn recruit actin-related signaling molecules (Merrifield et al., 2002; Traub, 2009). Dynamin and actin nucleation at the base and the neck of the vesicle would propel the membrane inward and promote scission of the clathrin-coated pit (Taylor et al., 2012). Epidermal growth factor receptor (EGFR) and transferrin are characteristic proteins that are internalized through this endocytic pathway.

Biochemical and molecular analysis of ASFV entry, using the specific dynamin inhibitor dynasore, but also a dominant-negative mutant of dynamin-2, have revealed that viral endocytosis depends on dynamin GTPase, which participates in vesicle fission from the plasma membrane (Hernaez and Alonso, 2010). Clathrin-assembly inhibitors, such as chlorpromazine, and also knock-out of clathrin-adaptor Epsin15 by expression of a dominant-negative mutant, profoundly affect virus infectivity and subsequent virus production. This was shown using a highly adapted virus isolate (BV71V), a low passage one in Vero cells, and also in the WSL cell line, derived from wild boar lung cells (Hernaez and Alonso, 2010). Moreover, at very early post-infection times, virions colocalize with clathrin-heavy-chain antibodies on the cell surface. Jointly, these findings led to the conclusion that ASFV entry involves dynamin-dependent and clathrin-mediated endocytosis (Hernaez and Alonso, 2010). In addition, this entry mechanism requires cholesterol (Bernardes et al., 1998) as it is sensitive to membrane cholesterol depletion by cyclodextrin. Conversely, it is insensitive to nystatin, a drug that disorganizes cholesterol in lipid rafts without reducing cholesterol levels (Hernaez and Alonso, 2010). These data are not consistent with a caveolae-dependent pathway for entry, which is another dynamin-dependent endocytic route. Other information about the relevance of the cholesterol biosynthesis pathway for virus entry is discussed below.

Although it is tempting to exclude clathrin-mediated endocytosis because of the large size of ASFV particles (200 nm), there is increasing scientific evidence that the direct participation of actin in membrane dynamics during clathrin-mediated endocytosis promotes the efficient internalization of large viruses, such as vesicular stomatitis virus (70 × 200 nm) (Cureton et al., 2009, 2010), and even bacteria (Pizarro-Cerda et al., 2010; Veiga and Cossart, 2005) and fungi (Moreno-Ruiz et al., 2009). This may be the case of ASFV.

1.2. Entry by macropinocytosis. The role of actin

Recent studies on ASFV entry, using BA71V or E70 isolates either in Vero or IPAM cells, have demonstrated the activation of the small Rho-GTPase Rac1 immediately after infection (Quetglas et al., 2012; Sanchez et al., 2012). Rac1 has been implicated in the modulation of actin dynamics and in the stabilization of microtubules by acetylation. Disruption of actin cytoskeleton with cytochalasin D alters infectivity (Sanchez et al., 2012), in contrast with others reporting scarce effects on infectivity using jasplakinolide and latrunculin A (Hernaez and Alonso, 2010). Field emission scanning electron microscopy has revealed that actin is involved in

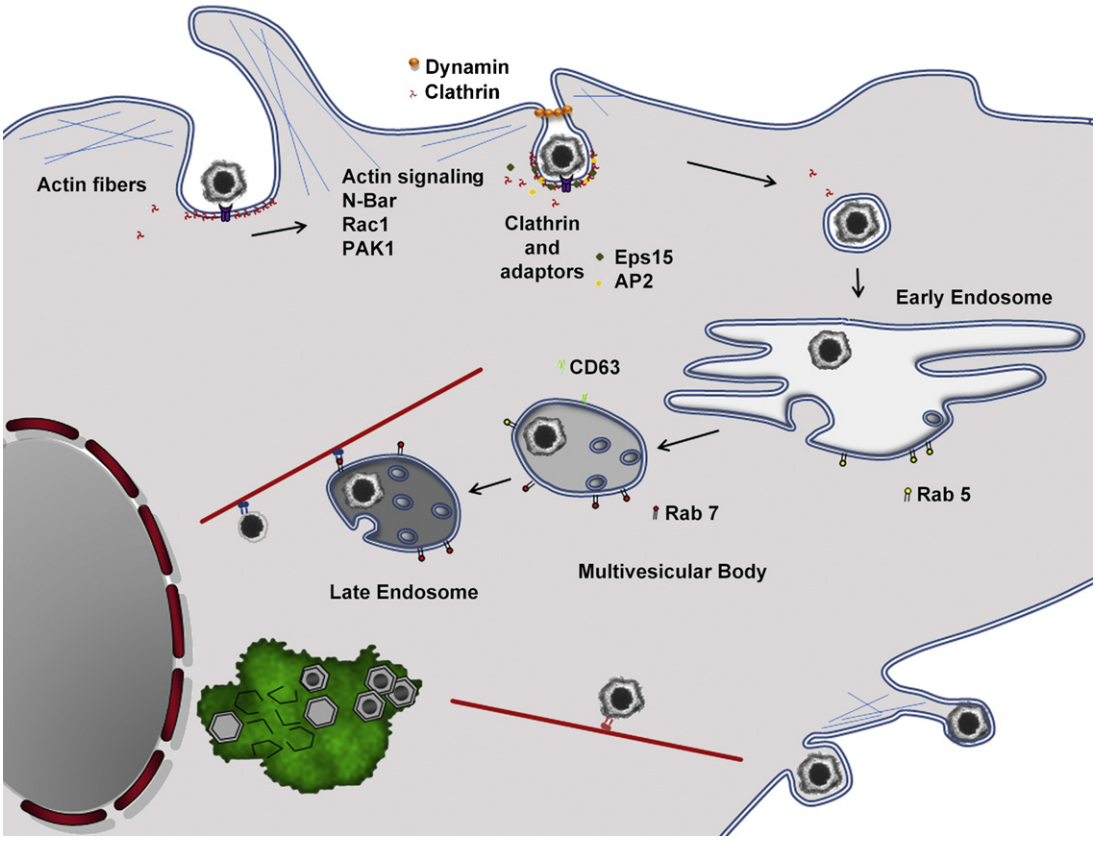

Fig. 1. Replication cycle of ASFV and first interactions with the host cell upon entry. The proposed model for virus entry would include dynamin and clathrin-mediated endocytosis and macropinocytosis. Clathrin-mediated endocytosis could be aided by actin reorganization and blebbing during macropinocytosis. Clathrin and adaptor molecules Eps15 and AP2 at clathrin-coated pits on the membrane promote actin nucleation (N-BAR) and recruit temporally successive modules of lipids and proteins necessary for the invagination and elongation of the vesicle. Finally, dynamin and actin induce constriction at the tail and neck produce final scission of the vesicle to the cytosol. In a few seconds, Rab5 and related molecules on the vesicle membrane give rise to the early endosome (EE). Further maturation of the early endosome requires acidification of the pH, phosphoinositide interconversions mediated by PI3K and PIKfyve, and finally Rab7 GTPase activation. Acidic late endosomal compartments produce virion desencapsidation, which will take place within the first 30–45 mpi.

the induction of the ruffles and blebs observed during the first hour post-infection (hpi). Sanchez et al. (2012) reported that ASFV infection is impaired by EIPA-amiloride, a potent inhibitor of the sodium/proton exchanger (Na^+/H^+), which has traditionally been used as a hallmark of macropinocytosis. On the basis of these data, they concluded that ASFV-induced macropinocytosis was a mechanism of virus entry, as previously described for vaccinia virus (VACV) (Mercer and Helenius, 2008). Nevertheless, macropinocytosis is recognized to be dynamin-independent. This conclusion contrasts with other reports (Cuesta-Geijo et al., 2012; Hernaez and Alonso, 2010).

Analysis of the mechanisms of entry in macrophages, the natural host cells of ASFV, is hindered by the fact that these cells have a heterogeneous surface marker profile and only restricted macrophage subpopulations are susceptible to this virus (McCullough et al., 1993, 1999; Sanchez-Torres et al., 2003). Moreover, permissive macrophage cell lines with the appropriate marker profile are not available (de Leon et al., 2012). Therefore, experimental approaches have frequently used the well-established laboratory model of ASFV BA71V isolate infection in Vero cells. In a wide analysis of various cell lines, it was shown that permissiveness for ASFV infection reaches different levels depending on the cell line analyzed and the restriction was found at the entry level or at later steps (Carrascosa et al., 1999).

Porcine CD163 scavenger receptor participates in the natural host cell infection (Sanchez-Torres et al., 2003). Expressed on most tissue macrophages but not on other myeloid cells, CD163 is one of the most reliable markers for cells of the monocyte macrophage lineage (Peréz et al., 2008). Nevertheless, some reports have shown how ASFV and other pathogens enter macrophages by fluid-phase uptake during macropinocytosis or by means of phagocytosis (Basta et al., 2010), which are receptor-independent.

Hence, the proposed routes of entry reported for the virus in the target cell include phagocytosis (Basta et al., 2010), macropinocytosis (Sanchez et al., 2012) and receptor-mediated endocytosis (Alcami et al., 1989a; Cuesta-Geijo et al., 2012; Hernaez and Alonso, 2010; Hernaez et al., 2012a). Nevertheless, these routes might not be equally effective to initiate infection. To evaluate infectivity, Sanchez et al. (2012) used p72 capsid protein expression at 1 hpi as measured by flow cytometry. This method does not discriminate viruses that entered the cytoplasm from those retained in membrane grooves. Virions that successfully entered the endocytic pathway and desencapsidated as a result of the acid pH of the late endosome are not detectable with p72 antibodies, as discussed below (Cuesta-Geijo et al., 2012). The dependence of infection on acid pH and endocytosis indicates that only desencapsidated virions will develop a productive infection. Thus, virions entering by pathways other than receptor-mediated endocytosis are not able to escape endosomes which is a crucial step for infection.

As occurs in poxviruses, ASFV mature intracellular virions (MVs) and extracellular virions (EVs) are infective (Andres et al., 2001). However, ASFV entry presents quite distinct features with respect to its mode of entry. VACV and Kaposi's sarcoma-associated herpesvirus use macropinocytosis and require this process for host cell entry and internalization (Mercer and Helenius, 2009; Raghu et al., 2009). Other viruses, such as species C Adenovirus (Ad) 2

and 5 and rubella virus, require macropinocytosis for entry but not for internalization. For Ad 2, macropinocytosis is required for the penetration of endosomal membranes after clathrin-mediated endocytosis (Meier et al., 2002).

VACV entry by macropinocytosis is followed by fusion of the viral membrane with the plasma membrane, which results in deposition of the viral core into the cytosol (Carter et al., 2005; Schmidt et al., 2012). Acid media treatment is sufficient to induce VACV membrane fusion (by removal of A25/A26 proteins); however, the need of endocytic passage is variable for MVs and EVs (Schmidt et al., 2011). Macropinosomes can undergo homo- and hetero-typic fusion and acidification but their relationship with endosomes and lysosomes remains elusive (Schmidt et al., 2012). Nevertheless, ASFV does not enter host cells by fusion at the plasma membrane, nor does it undergo acidic media-induced fusion, and it cannot circumvent the passage through acidic endosomes as shown by Cuesta-Geijo et al. (2012). Coincident with previous reports (Alcami et al., 1989a,b; Alcami et al., 1990; Valdeira and Geraldes, 1985), those authors concluded that both acid pH and endocytosis requirements are crucial for ASFV entry.

1.3. Open questions

Nevertheless, many questions regarding the ASFV entry mechanism remain unresolved. Could dynamin/clathrin-mediated endocytosis and macropinocytosis be alternative or even cooperative mechanisms of entry? If they are alternative, do they both lead to productive infection? Are both mechanisms consistent with saturable and specific receptor-mediated endocytosis? Could an alternative entry mechanism involve clathrin and some of the features described for macropinocytosis, such as actin-cytoskeleton and Rac1-dependent signaling? In this regard, it is conceivable that the activation of actin signaling elicited by macropinocytosis enhances clathrin-mediated endocytosis of the virus. A proposed model for the co-existence of both mechanisms is shown in Fig. 1.

Future research should clarify some of these questions, including the entry mechanism used in macrophages. However, after crossing the cell membrane, the next step for the virus involves the endocytic pathway.

2. ASFV at the endosomal pathway

2.1. Endocytosis maturation stages

Once the virus is internalized in primary endocytic vesicles, the intracellular pathways followed by incoming viruses are the same as those used by physiological cargoes. In a few seconds, various protein modules are recruited to clathrin-coated structures to enter the endocytic pathway (Taylor et al., 2011). Endosomal maturation requires the presence of some lipids, such as phosphoinositides, on the endosomal membrane for the specific incorporation of proteins involved in traffic and maturation termed Rab GTPases. Rab GTPases are regulators of the endocytic pathway, and each Rab protein incorporates to a specific compartment (Jordens et al., 2005). Shortly after the clathrin-coated vesicle pinches off the membrane, Rab5 effectors and Rab5 itself are recruited to the newly formed early endosome (EE) (Taylor et al., 2011). From this compartment, cargoes can be recycled to the membrane or progress and mature to late endosomes (LEs), which may fuse with lysosomes (LYs) for degradation. This pathway involves gradual acidification of the endosomal lumen, starting from the pH 6.5 of the EE, which, through invagination of small intraluminal vesicles (ILVs), becomes the multivesicular bodies (MVBs). These bodies then mature to Rab7-expressing LEs at pH between 6 and 5 (Huotari and Helenius, 2011). After fusion of LEs with LYs, which are characterized by Lamp1 expression, the pH drops to 5–4.5.

Many viruses have evolved to use the endocytic pathway for cell entry and transport (Mercer et al., 2010). For example, adenovirus (Ad) serotypes 3 and 7 have relatively long residence times in endosomes. The endosomal pathway was identified as the route used by Ad7, as virions were observed to colocalize with LE and LY marker proteins, including Rab7 and Lamp1, during viral entry and before viral egress from this compartment (Miyazawa et al., 2001). Despite trafficking through this pathway, Ad7 escapes degradation in these organelles. This virus traffics through low lysosomal pH, and the Ad fiber protein confers the Ad7 capsid the capacity to escape to the cytoplasm at low pH escape.

2.2. Virus entry is dependent on endosomal intraluminal acid pH

The dependence of ASFV infection on endosomal acid pH was reported several years ago (Valdeira and Geraldes, 1985) as infection was sensitive to a number of lysosomotropic agents (Alcami et al., 1989a). Fusion with the cell membrane artificially induced by lowering the pH of the medium was not followed by successful infection in cells treated with lysosomotropic drugs. This observation implied that this membrane fusion does not bypass the endocytic pathway for viral entry and that these virions are degraded in the cytoplasm (Valdeira et al., 1998). More recent studies showed that ASFV infectivity was severely decreased by drugs that block endosomal intraluminal acidification such as bafilomycin A1 (Baf) and ammonium chloride (Cuesta-Geijo et al., 2012). In fact, this blockage could not be reversed by exposure of the cells to an acidic medium. Similarly, an acidic medium cannot reverse dynasore-induced inhibition of endocytosis (Cuesta-Geijo et al., 2012). In conclusion, both endocytosis and intraluminal acidification of the endosome are required for successful ASFV infection. These results are summarized in Fig. 1.

2.3. ASFV desencapsidation occurs at late endosomes

The requirement for endosomal acidification was observed to be relevant before the first hour post-infection (30–45 mpi), but not thereafter. At this time, virions in the endosomes undergo desencapsidation, a necessary step for uncoating prior to egress to the cytosol to start replication.

ASF virions are *ca.* 200 nm in diameter and consist of a DNA-containing central nucleoid surrounded by core shell proteins derived by processing of viral polypeptides, pp220 and p62 (Salas and Andres, 2012). The ASF viral genome is protected by a protein shell termed capsid. The capsid has an icosahedral structure, which is composed of many subunits of structural protein p72. The capsid surrounds the inner envelope. The outer viral envelope is obtained by virus budding through the plasma membrane but is dispensable for infection (Andres et al., 2001).

Viral structure undergoes major conformational changes for an eventual release of genomic information, a stepwise process termed uncoating. It is crucial that uncoating does not prematurely expose the viral genome, since this would lead to degradation and/or failed transport to the replication site. Incoming virion capsids detected with antibodies against viral capsid proteins (p72 or pE120R) colocalize with early endosomes within the first minutes of infection (1–15 mpi) but not with other mature acidic compartments (Cuesta-Geijo et al., 2012). In fact, the inhibition of endosomal acidification with Baf impedes both acidification and viral desencapsidation, as shown by the detection of viral capsid protein staining in LEs expressing Rab7 exclusively under these conditions. Instead, viral core protein p150 colocalize with Rab7-positive LEs lacking viral capsid staining in control conditions (Cuesta-Geijo et al., 2012). Protein p150 is one of the products

obtained from the proteolytic cleavage of ASFV pp220 core shell protein (Salas et al., 2012; this issue). Moreover, recent electron microscopy studies showed that endocytic traffic through LEs is accompanied by changes in virion ultrastructure, these leading to the desencapsidation of genome-containing cores (Hernaez et al., 2012a).

All together, these data indicate that viral desencapsidation occurs in the acid pH of LE compartments between 30 mpi and 45 mpi. Moreover, this desencapsidation is a key step to ensure that the virion progresses through uncoating and egress in order to start replication. These data imply that ASFV belongs to the category of late-penetrating viruses (Brabec et al., 2006; Lozach et al., 2010; Mercer et al., 1996; Sieczkarski and Whittaker, 2003).

Moreover, Rab7 GTPase activity is crucial for ASFV infectivity, as shown with knock-out function dominant-negative mutants (Cuesta-Geijo et al., 2012). Similarly, the interconversion of phosphoinositides, which coordinate the assembly of effectors to allow endosomal maturation, is required for successful ASFV infection. The inhibition of enzymes that mediate this interconversion, such as phosphoinositide-3-kinase (PI3K), by wortmannin (Sanchez et al., 2012) and inhibitors of PIKfyve, an enzyme that mediates the conversion from phosphatidylinositol 3 phosphate (PtdIns3P) to phosphatidylinositol-3,5-bisphosphate (PtdIns(3,5)P_2) (Jefferies et al., 2008), profoundly impairs fusion endosome dynamics and consequently ASFV infection (Cuesta-Geijo et al., 2012). In conclusion, the early steps of ASFV infection are strongly dependent on endosomal pathway maturation. Future research should be conducted to identify the viral components involved in the further steps required to complete uncoating after desencapsidation and to determine the fate of other internal membranes and the precise mechanism of viral egress from the endosome before virus replication starts.

3. Microtubules during ASFV entry

Incoming ASFV virions reach the replication site in the perinuclear area, close to the microtubule organizing center (MTOC; Alonso et al., 2001). One of the steps required for endosomal maturation includes endosome progression toward the perinuclear area through microtubules (Huotari and Helenius, 2011). In fact microtubule depolymerizing agents, such as nocodazole, impair virus trafficking (Alonso et al., 2001; de Matos and Carvalho, 1993; Heath et al., 2001). Trafficking of ASFV relies on microtubules, and previous reports have shown that this virus requires functional microtubules for successful infection. Moreover, the activation of Rac1, a molecule that also triggers microtubule stabilization, is crucial during early infection (Quetglas et al., 2012).

3.1. ASFV p54 interaction with microtubule motor dynein

One of the major structural proteins of ASFV, p54, interacts directly with the 8-kDa light chain of the microtubule motor protein dynein (dynein light chain 1 or DLC1) (Alonso et al., 2001). Cytoplasmic dynein is a minus-end-directed microtubule motor protein that mediates a wide range of functions, including the transport of organelles, proteins and viruses to defined subcellular sites of action (Vallee et al., 2012). Binding of dynein to p54 is a high affinity chemical interaction that forms a stable-molecular-weight-complex *in vitro*. A putative p54 binding surface on DLC1 has been defined by nuclear magnetic resonance (NMR) spectroscopy (Hernaez et al., 2010). A short peptide sequence mimicking the viral protein DLC1-binding domain binds and competes for the binding of the viral protein. The relevance of the p54-dynein interaction in infected cells is highlighted by the observation that the use of this short sequence to compete with this interaction in infected Vero cells results in a marked decrease in virus infectivity, viral replication and finally virus production (Hernaez et al., 2010).

Interestingly, sera from pigs surviving infection presented antibodies against p54 DLC1-binding domain (DBD) and immune mice sera raised to this domain reduced virus infection plaques in neutralization assays (Escribano et al., 2012; this issue). These observations led to the conclusion that p54 DBD is implicated in antibody-mediated virus neutralization.

Moreover, other functions have been postulated for p54 at late stages of infection. This protein is important for virus morphogenesis, and it participates in the recruitment of viral membranes to assembly sites, as shown with the inducible mutant vE183Li (Rodriguez et al., 2004). This mutant triggers virus assembly arrest, and this phenotype is partially reversible when p54 expression is induced at 12 hpi, as would be expected for a p54 function exerted at late times after infection.

Interestingly, exposure of viral particles to an acidic medium can induce substantial changes that are relevant for transport linked to microtubules. Most Ad serotypes enter cells by clathrin-mediated endocytosis, and the pH inside the endosomes plays a crucial role by inducing conformational changes in a viral protein. Ad5 hexon protein exposed to an acid pH enhances dynein binding through intermediate and light-intermediate chains (Scherer and Vallee, 2011). These data provide physiological evidence of the relevance of Ad exposure to endosomal pH and dynein binding for efficient infection.

In contrast, ASFV p54 expressed in *E. coli* interacts with dynein at basic pH *in vitro*. ASFV p54 is located on the internal membrane of the virion and can be externally exposed between capsomers when the capsid is intact (Rodriguez et al., 2004). Nevertheless, further studies are required to clarify whether desencapsidation of the virus in acidic endosomes facilitates p54 interaction with dynein motor protein, thus driving desencapsidated virions to the MTOC to start virus replication. In fact, in several virus models, the low pH of endosomes is relevant for genome release by a number of mechanisms (Fuchs and Blaas, 2010; Zaitseva et al., 2010).

3.2. Open questions

Further structural studies are probably required in order to relate these results with the successive uncoating steps of the virion in order to establish at which step p54 may access to microtubules and motors. Future research should focus on the early steps of ASFV infection before replication takes place, as these phases are essential targets in the design of intervention strategies against the disease.

4. ASFV at the nucleus

Early ASFV transcription start using processing enzymes packaged in the virion core (Dixon et al., 2012). These enzymes required for DNA replication are expressed immediately following virus entry into the cytoplasm from partially uncoated core particles. ASFV site of viral replication is predominantly cytoplasmic in defined perinuclear factories as characterized by early ultrastructural studies (Breese and DeBoer, 1966). However, ASFV DNA replication presents an initial stage at the nucleus (Garcia-Beato et al., 1992; Tabares and Sanchez Botija, 1979). Like other viruses belonging to the nucleocytoplasmic large DNA virus superfamily such as poxviruses, ASFV requires intact nuclei for replication (Dixon et al., 2012; Ortin and Vinuela, 1977). Nevertheless, while poxviruses only require nucleus-derived cellular factors, ASFV DNA is detected in the nucleus and cytoplasmic replication sites by *in situ* hybridization and radioactive labeling (Ballester et al., 2010; Garcia-Beato et al., 1992; Rojo et al., 1999). Short viral DNA nuclear

fragments are synthesized in the proximity of the nuclear membrane and then, transported to the cytoplasmic replication factory (Garcia-Beato et al., 1992). ASFV DNA found in mature viral particles is derived from both nuclear and cytoplasmic fragments (Ortin et al., 1979; Rojo et al., 1999)

Moreover, viral proteins p37 and p14 can be targeted to the nucleus (Eulalio et al., 2004). These proteins are products of polyprotein pp220, a component of the ASFV core shell (Salas and Andres, 2012). ASFV p37 is transported to the nucleus and exported to the cytoplasm, independent of the CRM1-mediated nuclear import, and therefore, it may be involved in ASFV DNA nucleocytoplasmic transport (Eulalio et al., 2006, 2007). Recent studies reported that ASFV infection disrupts nuclear organization at an early stage of infection (Ballester et al., 2011). Increased lamin A/C phosphorylation is found at 4 hpi, followed by lamina network disassembly in the proximity of the replication site. Other nuclear elements that are redistributed include RNA polymerase II, the splicing speckle SC35 marker, and the B23 nucleolar marker. The impact of nuclear disorganization is reflected by the presence of lamin and other nuclear envelope markers in the cytoplasm at late infection stages (Ballester et al., 2011; Basta et al., 2010).

5. Viral factory formation

5.1. Aggresomes and HDAC6

ASFV specifically binds dynein and migrates toward MTOC to reach perinuclear viral replication sites and form structures known as viral factories (VFs) or the viral replication organelle. Similarities between aggresomes and ASFV VFs described several years ago (Heath et al., 2001) raised the possibility that ASFV uses the aggresome pathway to concentrate cellular and viral proteins, thus facilitating replication and assembly (Wileman, 2007). Cytoplasmic histone deacetylase 6 (HDAC6), through its simultaneous interaction with ubiquitinated proteins and dynein motors (Fig. 2F), is a key element that mediates the selective disposal of protein aggregates and cytotoxic misfolded proteins by sequestering activity in cellular "storage bins" called aggresomes (Boyault et al., 2007b; Rodriguez-Gonzalez et al., 2008). HDAC6 is a major cytoplasmic tubulin-deacetylase, a specific member of class II HDACs (Hubbert et al., 2002; Matsuyama et al., 2002; Zhang et al., 2003). HDAC6 binds to both mono- and poly-ubiquitinated proteins (Boyault et al., 2007a) and dynein proteins, thereby recruiting protein cargo to dynein motors in order to transport misfolded proteins on the microtubule cytoskeleton to aggresomes (Kawaguchi et al., 2003). Many cellular trafficking compartments are organized by microtubule motor proteins such as dynein, and they tend to cluster in the MTOC adjacent to the nucleus.

We report new results that suggest that HDAC6 is not involved in the formation of the ASFV VF. Inhibition of HDAC6 function was performed using the reversible inhibitor tubacin, which impedes the specific interaction of HDAC6 with dynein (Hideshima et al., 2005). Cells were pretreated for 3 h with tubacin at the indicated concentrations in growth medium at 37 °C, followed by cold synchronized infections with a m.o.i. of 1 pfu/cell the Ba71V or of the recombinant fluorescent virus B54GFP (Hernaez et al., 2006). The inhibitor was present throughout the experiment. At 24 hpi, cells were harvested by trypsinization for FACs analysis or lysed for Western blot. Infectivity rates obtained in tubacin-treated cells were normalized to the values in infected control cells. Concentrations of 1–2 μM tubacin (Ding et al., 2008) efficiently increased acetylated tubulin in Vero cells (over 4-fold starting 1 h after addition and reaching a peak at 16 h, as detected by Western blot (Fig. 2A) and confocal laser scanning microscopy (not shown). Nevertheless, at the same doses that increased microtubule acetylation, tubacin did not modify infected cell percentages, as shown by immunostaining for ASFV proteins p30 at 6 hpi and p72 at 16 hpi (Fig. 2B). Nor did this inhibitor alter the detection of infected cells with the recombinant virus B54GFP by flow cytometry (Fig. 2C). Moreover, tubacin did not change early or late viral protein expression (p30 or p72), as shown by Western blot (Fig. 2D). Similarly, viral production was not modified under tubacin-induced HDAC6 inhibition (not shown). Furthermore, confocal microscopy revealed that inhibition of HDAC6 did not alter the formation of VFs and their number, morphology and location were preserved under these conditions. Also, the characteristic vimentin cage was formed around the factory (Fig. 2E). Hence, although the morphology of the ASFV VF is similar to that of aggresomes, the mechanism of viral factory formation is apparently not related with the canonical aggresome pathway mediated by HDAC6 (Fig. 2F).

5.2. Time-lapse imaging of viral factory formation

VFs comprise a robust collection of newly synthesized viral proteins and viral DNA and are located at the perinuclear area corresponding to the MTOC. The formation of these factories remains intriguing. When the first recombinant ASFV expressing GFP fused to viral protein p54 (B54GFP) was used as a tool for live-imaging of the viral infection, the GFP fusion protein was observed to accumulate in a few discrete spots at the perinuclear area about 8 hpi (Hernaez et al., 2006). These multiple VFs or early factories are motile around the nucleus and coalesce in a single location coincident with the MTOC at subsequent time points. We have generated other fluorescent recombinant viruses, these expressing p12-GFP (B12GFP) under p72 promoter control, and p54-mCherry fluorescent protein (B54ChFP) under p54 promoter control, following a similar procedure to that described in Hernaez et al. (2006). These viral fusion proteins exhibited VF localization.

5.3. Morphometric analysis of the ASFV replication organelle

We report on the use of these recombinant fluorescent ASFVs to study the location, morphology and size of VFs in Vero cells and in WSL, a cell line of wild swine origin (Fig. 3). No significant differences in VF size were observed in cells infected with the different recombinant viruses in either cell line ($N_{Vero} = 140$, $N_{WSL} = 50$; ns, $p > 0.05$; Fig. 3A). Moreover, these recombinant viruses showed almost complete superposition in their distribution at the VF (Fig. 3B). VFs showed intense fluorescence as a result of the high amount of proteins accumulated in these replication and assembly areas (Fig. 3B and 4A–C). However, viral DNA, detected by TOPRO3 staining did not show a complete superposition in VFs. These observations suggest an organization with segregated functions for DNA replication and viral protein synthesis in VFs (Fig. 4C).

VFs are well-defined structures with a major axis size (X) *ca.* 5 μm at 16 hpi in Vero cells and *ca.* 6 μm in WSL cells (Fig. 4E). The only differences between these two cell lines were found in the size of VFs. The major axis of the factories was significantly larger in WSL than in Vero cells ($^{***}p < 0.001$ and $^{**}p < 0.01$; Fig. 4E).

At this time point, 34 and 39% of Vero and WSL infected cells, respectively, presented a marked cytopathic effect. Also, in 25% of infected Vero and in 12% of WSL cells, we found multiple VFs as several independent organelle-shaped fluorescent spots prior to coalescence (Fig. 4B and C). These infected cells bearing multiple VFs did not show a marked cytopathic effect in 83% of infected Vero or in 87.5% of WSL cells.

We also addressed organelle organization in Vero cells infected with recombinant viruses B12GFP and B54GFP at 16 hpi. VFs were characteristically devoid of organelle markers. ER staining in infected cells was disperse in the cytoplasm, and sometimes maintained an empty halo around the factory (Fig. 5). Consistent with

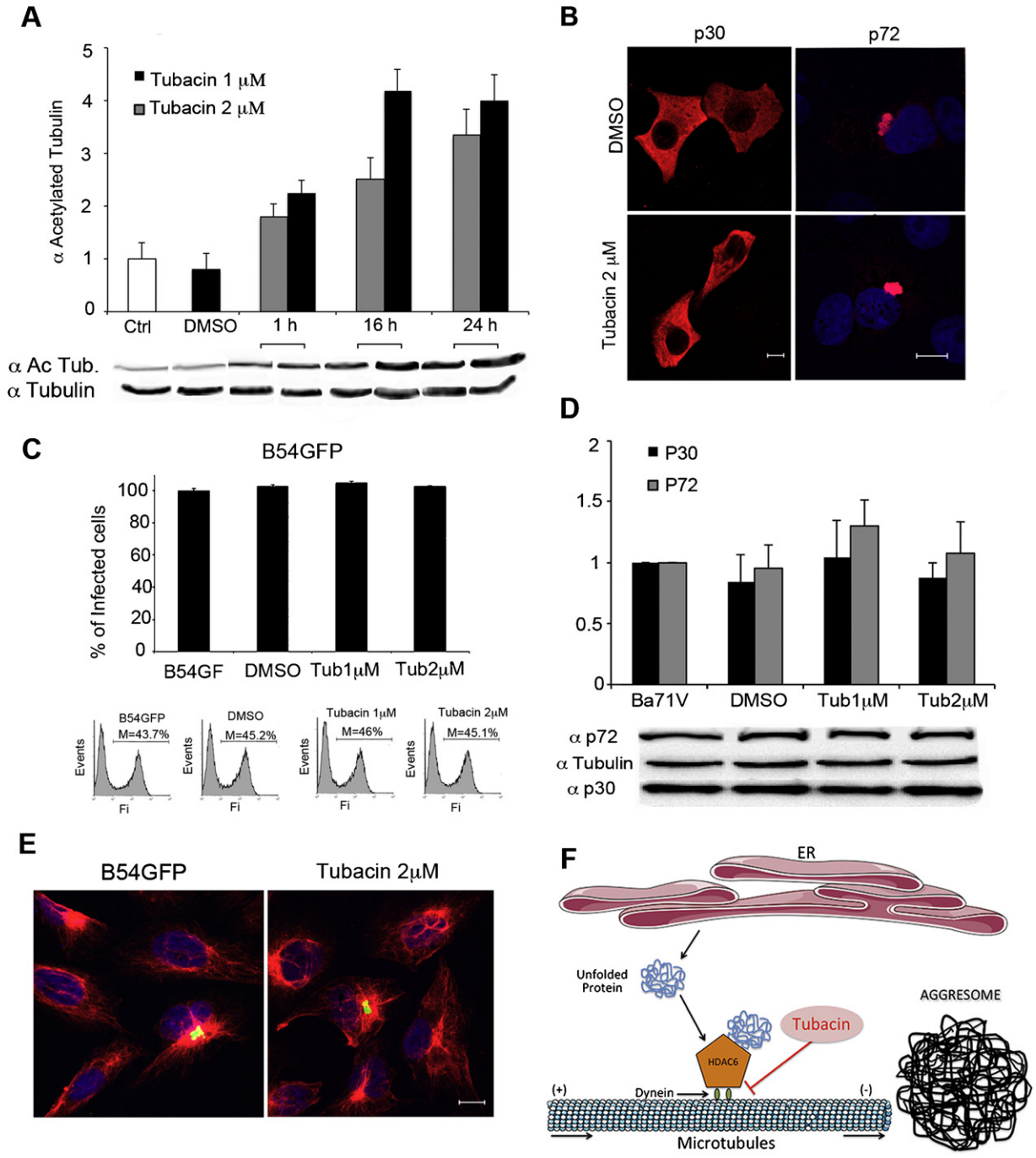

Fig. 2. HDAC6 participation in the viral factory formation. (A) Acetylated tubulin levels in Vero cells treated with HDAC6 inhibitor tubacin, as shown by Western blot. (B) Infectivity was analyzed by immunofluorescence using antibodies against ASFV proteins p30 and p72 to evaluate infected cell numbers. Representative micrographs of p30 (6 hpi) or p72 (16 hpi) in Vero cells infected with BA71V ASFV isolate (m.o.i. of 1 pfu/cell) and treated with tubacin. (C) Flow cytometry analysis of infectivity in Vero cells infected with recombinant B54GFP (m.o.i. of 5 pfu/cell) and treated with tubacin. (D) Western blot analysis of p30 and p72 viral protein expression of tubacin-treated infected cells or controls. (E) Vimentin-cage formation around the viral factories. Vimentin staining of Vero cells treated with tubacin and infected with B54GFP. Bar 10 μm. F. Role of HDAC6 in the canonical pathway of aggresome formation.

previous reports (Rojo et al., 1998), mitochondria were organized around the VFs and the Golgi complex disassembled following microtubules (Netherton et al., 2006), until the signal almost disappeared (Fig. 5). One of the consequences of trans-Golgi network dispersal is that the delivery of membrane protein to the plasma membrane is slowed down.

With respect to cytoskeleton organization, intermediate filaments stained with anti-vimentin antibody proliferated in the cytoplasm forming a robust vimentin cage around the factories (Fig. 5) (Stefanovic et al., 2005). Acetylated tubulin filaments were reduced, and actin cytoskeleton was progressively disassembled, as shown by the faint staining of the few remaining polymerized actin filaments (Fig. 5). Disorganization of cytoskeleton after 24 hpi could affect viral transport to the membrane itself. In fact, extracellular virus production was considerably lower when compared to the intracellular fraction in BA71V Vero infected cells at 24 hpi. This observation could be a consequence of less efficient virus exocytosis.

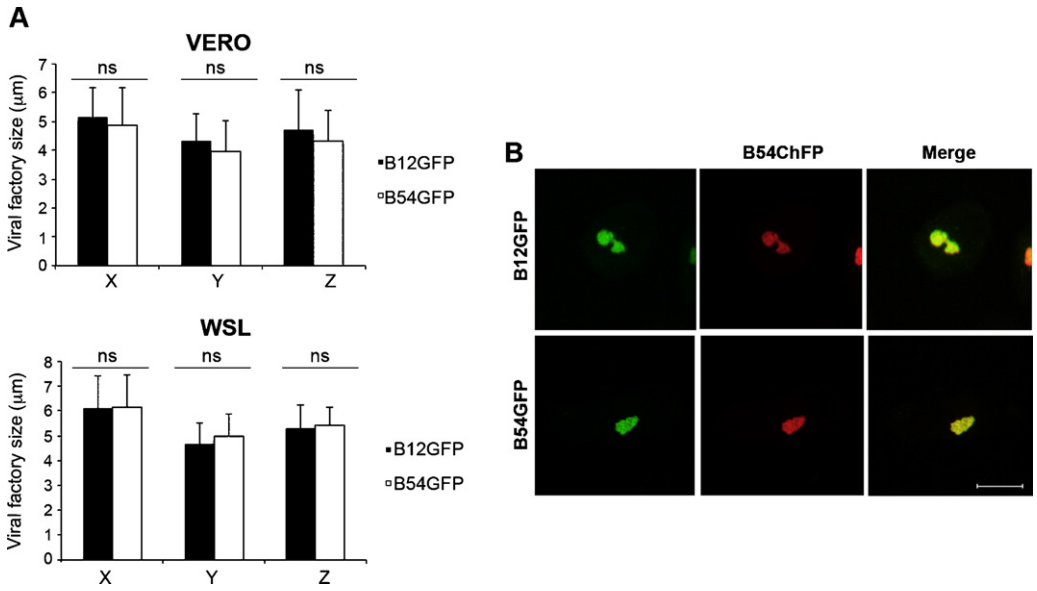

Fig. 3. Recombinant ASFV expressing fluorescent proteins. (A) Comparison of the size of the virus factories in Vero and WSL cells infected with recombinant viruses B12GFP and B54GFP (N_{Vero} = 140, N_{WSL} = 50; ns $p > 0.05$). (B) Representative confocal micrographs of Vero cells infected with the recombinant viruses in (A) and B54ChFP. The merged images show almost complete superposition of these fusion proteins at the viral factories. Bar 10 μm.

5.4. Host factors in viral factory formation

VF formation is governed by several cellular determinants. For example, depolymerization of microtubules results in the dispersal of VFs (de Matos and Carvalho, 1993; Heath et al., 2001). Findings that Rho GTPase inhibitors impair virus morphogenesis, thus resulting in abnormally large VFs (Quetglas et al., 2012), indicate that Rho GTPases have an essential role in the formation of these factories. Transmission electron microscopy (TEM) revealed the accumulation of envelope precursors and immature virions at these enlarged VFs and fewer ribosomes. Also, in cells treated with a Rho GTPase inhibitor, instead of normal virion budding by filopodia, we observed the accumulation of immature virions at the plasma membrane and the absence of filopodia. Actin filopodia formation was described by Jouvenet et al. (2006). Rho-GTPase signaling inhibition may impede cortical actin regulation, thus explaining the absence of filopodia. However, we cannot exclude that mature ASFV particles are required for filopodia formation, as reported for VACV-induced actin tails (Smith et al., 2002).

In fact, host protein lipid modifications, such as the prenylation of small GTPases, are crucial for infection outcome (Quetglas et al., 2012). These post-translational modifications are required for the normal function of small GTPases belonging to the Ras superfamily. Isoprenoids are prenyl donors synthesized as intermediates of the cholesterol biosynthesis pathway. ASFV infection requires the integrity of the entire cholesterol biosynthesis pathway. Statins are potent drug inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the enzyme that catabolizes the conversion of HMG-CoA to mevalonate. Statins are widely used as cholesterol-lowering drugs in humans and can be used as antivirals. Statin treatment (Lovastatin) decreased ASFV progeny and infectivity in Vero cells. This effect is fully reversed by the addition of early precursor mevalonate. Isoprenoids generated in the cholesterol biosynthesis pathway, geranygeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP), are prenyl donors for protein posttranslational modifications. Farnesylation or geranylgeranylation of cellular and viral proteins are required at several infection steps. Intact pools of GGPP and FPP are required for viral replication (Quetglas et al., 2012). Rac1 is a geranylgeranylated protein that is important during early stages of infection (Quetglas et al., 2012), and its relevance has been discussed above.

ASFV encodes a transprenyltransferase (ORF *B318L*), which is an essential and late gene (Alejo et al., 1997). FPP and GGPP are formed in the reaction catalyzed by the viral enzyme. This enzyme has the unique characteristic that it is associated with precursor viral membranes derived from the ER at the viral assembly sites (Alejo et al., 1999; Andres et al., 1997). GGPP synthesized by B318L product serves as a substrate for protein prenylation, required during virus replication and morphogenesis.

6. ER stress and unfolded protein response

6.1. Overexpression of chaperones and ER stress caspase 12 activation

As obligate intracellular pathogens, viruses have evolved to exploit cellular responses to support viral replication. Viral infection leads to the modification of numerous signaling pathways including antagonizing or activating of specific cellular targets at distinct stages of the replication cycle. Several of these pathways belong to antiviral defense mechanisms such as cellular stress and/or host antiviral innate immune response.

By means of two-dimensional electrophoresis and matrix-assisted laser desorption/ionization peptide mass fingerprinting (MALDI PMF), a wide proteomic analysis of the cellular proteins that modify their expression upon ASFV infection led to identification of the overexpression of several chaperones, such as heat shock proteins 70, 27 and prohibitin, especially after 10–24 hpi (Alfonso et al., 2004). The high level of viral protein production at the ER saturates the protein folding capacity of chaperones. This saturation disturbs ER homeostasis, thereby inducing the so called Unfolded Protein Response (UPR).

ER stress after ASFV infection is reflected by the activation of caspase 12, which follows similar temporal dynamics to mitochondrial caspase 9 and effector caspase 3 activation. Also chaperones, calnexin and calreticulin, but not ERp57 or BiP, are over expressed after infection (Galindo et al., 2012).

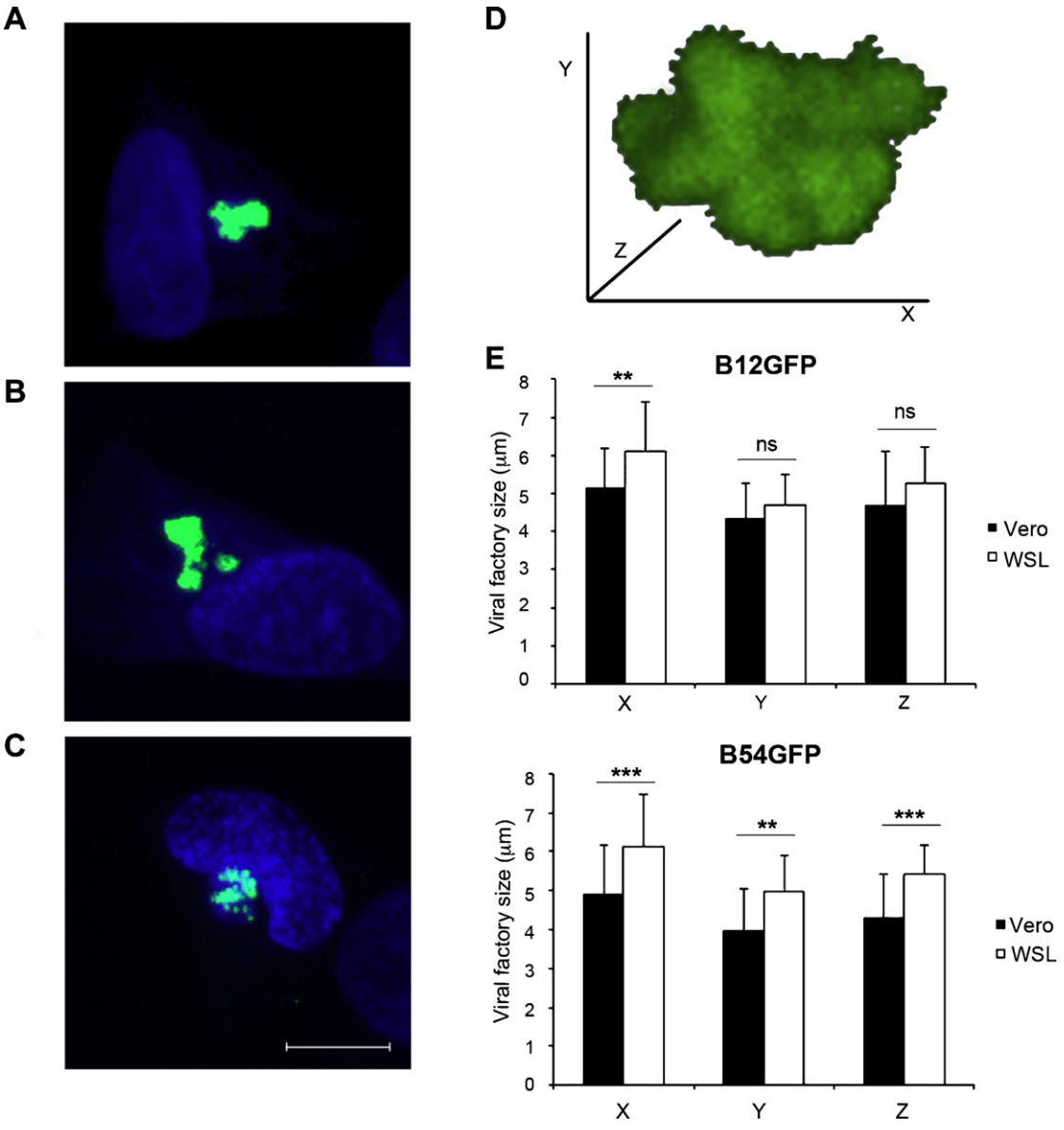

Fig. 4. Morphology of the ASFV viral factory. (A)–(C) are representative confocal micrographs of viral factories; as a typical single compact fluorescent spot (A) or as multiple viral factories (B) and (C). Bar 10 μm. (D) Three-axis dimensions of the viral factories in Vero and WSL cells infected with recombinant viruses B12GFP and B54GFP at 16 hpi were analyzed. Image acquisition of 13 *Z*-stacks per viral factory from $N_{Vero} = 140$ and $N_{WSL} = 50$ cells infected with these recombinant viruses was performed by confocal laser scanning microscope (Leica) and tridimensional reconstruction, and image analysis was done with the Leica Application Suite Advanced software. E. Graphics show means and standard deviations of *X*, *Y* and *Z* axis in μm. Major axis (*X*) was significantly larger in WSL cells when compared to Vero cells $^{***}p < 0.001$ or $^{**}p < 0.01$.

6.2. UPR pathways control and ATF6 translocation

Three ER transmembrane proteins function as UPR sensors, namely protein kinase-like ER resident kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activated transcription factor 6 (ATF6). In their steady state these proteins are associated with the chaperone BiP/Grp78, which prevents their aggregation and further activation. Under misfolded protein accumulation, BiP is released, thereby leading to the UPR (Fig. 6). UPR pathways transcriptionally activate a number of genes involved in protein degradation (Fig. 6). Nevertheless, according to previous data, several of these genes lack apparent activation (Galindo et al., 2012; Netherton et al., 2004).

ATF6 is activated and translocated from the ER to the nucleus and VFs (Galindo et al., 2012). Activation of the ATF6 branch and its transcriptional activation of chaperone-encoding genes might benefit the virus by assisting the folding of accumulated proteins and preventing protein aggregation (Fig. 6). It is relevant to mention here that VACV infection induces the sequester of crucial translation initiation factors within VFs in order to increase the efficiency of virus transcription and translation "on site". This is yet another mechanism by which viral gene expression is promoted (Katsafanas and Moss, 2007).

Furthermore, Bap31 is not activated by the fragmentation of p20 in ASFV-infected cells. This observation indicates the absence of pro-apoptotic signaling between the ER and mitochondria. Interestingly, a serine protease inhibitor that impairs ATF6 activation abolishes both virus infectivity and virus production. This compound inhibits ASFV-induced activation of caspase 12, 3 and 9 but not staurosporine-induced caspase 3 activation. These findings reveal that this effect was highly specific for the virus infection. Conversely, inhibition of caspase 12 activation is not relevant for virus infection (Galindo et al., 2012).

Selective regulation of the UPR has been described for other double-stranded-DNA viruses, such as the cytomegalovirus (CMV)

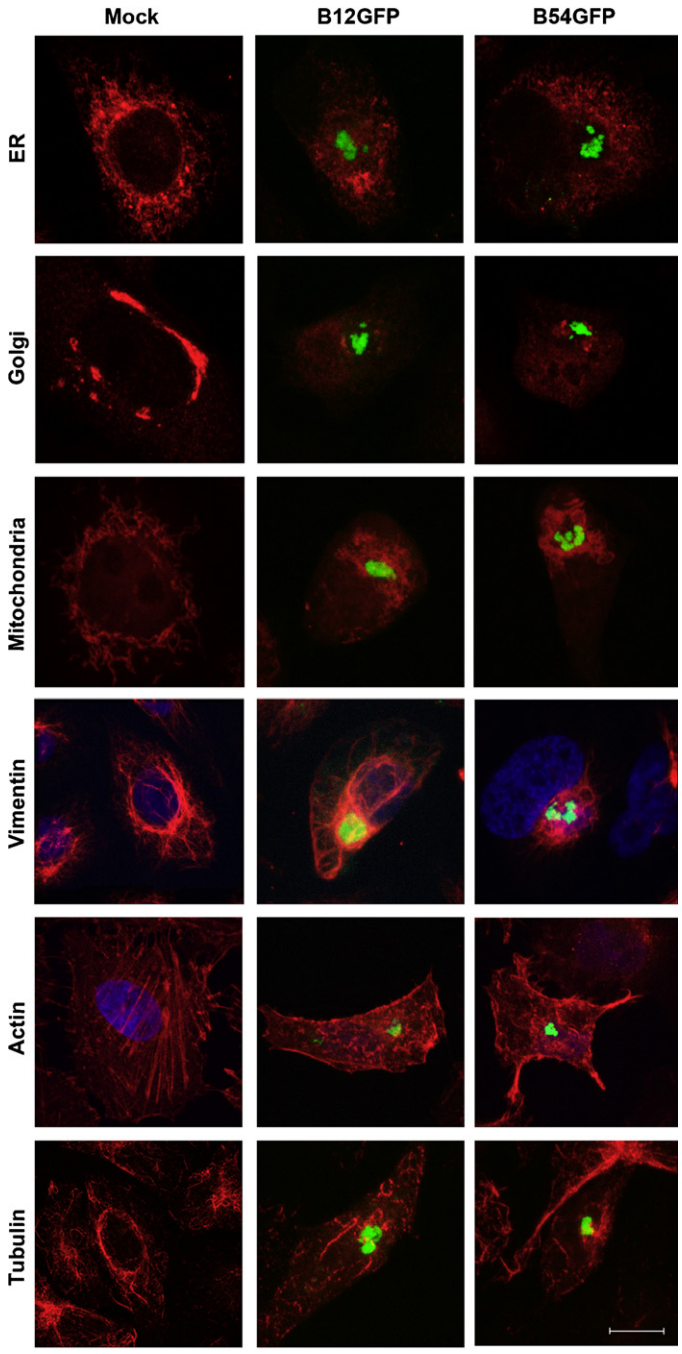

Fig. 5. Viral factories, cellular organelles and cytoskeleton. Intracellular structures in Vero cells infected with ASFV recombinant viruses B12GFP and B54GFP (m.o.i. of 1 pfu/cell) at 16 hpi and stained for ER (α-PDI), Golgi (α-TGN46), mitochondria (Mitotracker CMXRos), α-Vimentin-Cy3, AF594 phalloidin for actin and α-acetylated tubulin AF594. No labeling for any organelles inside the viral factories was observed. At this time point, ER staining was dispersed, Golgi apparatus had virtually disappeared, and mitochondria were organized around the viral factory. Vimentin filaments proliferated and accumulated in the viral factory forming a vimentin cage, along with actin cytoskeleton fiber disassembly. Acetylated tubulin lost its organization and accumulated around the viral factories. Bar 10 μm.

(Isler et al., 2005) and herpes simplex virus 1 (HSV-1) (Cheng et al., 2005). In fact, ASFV protein DP71L is involved in ATF4 downregulation and CHOP inhibition (Zhang et al., 2010). Future research should identify other possible viral protein candidates to mediate such regulation.

7. ASFV and apoptosis

7.1. Membrane blebbing and virus dissemination

Among the diverse ASFV-cell interactions, the manipulation of cell death and survival pathways is a key factor by which the cell lifetime is lengthened in order to ensure completion of viral replication. ASFV induces apoptosis in the target cell at relatively late times after infection (24–48 hpi) (Ramiro-Ibanez et al., 1996). The dynamics of caspase expression shows a late profile, starting with ER stress caspase 12 and mitochondrial upstream caspase 9 activation at 16 hpi, followed by executor caspase 3 activation at 48 hpi (Galindo et al., 2012). The activation of executor caspases causes proteolysis of DNA repair enzymes, DNA replication factors and cytoskeleton regulators, and cleavage of lamina, thus leading to final DNA fragmentation and chromatin condensation. Also, at the cytoplasm, cleavage of gelsolin, fodrin, and actin causes cytoplasmic vacuolization. Shortly after, the apoptotic cell starts to lose contacts with neighboring cells (Hernaez et al., 2006). At the end of an apoptotic process of any origin, caspase activation of Rock-I GTPase and myosin-actin contractile force generation produce the characteristic cytoplasmic membrane blebbing. Finally, cell shrinkage occurs, accompanied by the formation of membrane vesicles filled with fragmented nucleus, referred to as apoptotic bodies. The apoptotic bodies and vesicles derived from ASFV-infected cells are filled with viral particles and this has been postulated to be an efficient system for virus spread (Hernaez et al., 2006). In fact, Rock-I implication in membrane blebbing in ASFV-infected cells was demonstrated by using Rock-I and myosin-II ATPase inhibitors (Galindo et al., 2012). Moreover, we found that the inhibition of membrane blebbing reduces extracellular virus production (Galindo et al., 2012). Blebbing suppression at late infection, either using a Rock-I inhibitor (Y-27632) or a myosin-II ATPase inhibitor (Blebbistatin), reduces the extracellular virus fraction but does not modify total virus production. In comparison with common virus exocytosis, the process of membrane blebbing is crucial for efficient virus spread, especially at late post-infection times, when the microtubule and actin cytoskeleton are severely impaired.

7.2. ASFV induction of apoptosis

Cell death regulation by ASFV (Fig. 7) is a complex equilibrium between induction and inhibition signals (Reviewed in Hernaez et al., 2004). Although the execution of apoptosis in the target cell is a relatively late event, the signal triggering this process has been reported to occur early after virus interaction with the host cell. The apoptosis initiation signal occurs after virus binding but prior to ASFV early protein synthesis and virus replication (Carrascosa et al., 2002). Some viruses induce apoptosis solely by interaction with the cell membrane (Brojatsch et al., 1996). However, this was not found to be the case for ASFV, as UV-inactivated virus failed to induce caspase expression or apoptosis. ASFV uncoating is required to trigger apoptosis and is inhibited with lysosomotropic drugs that impair endosomal acidification (Carrascosa et al., 2002). VACV also induces apoptosis at a post-binding step associated with cell entry (Ramsey-Ewing and Moss, 1998). It has been proposed that the interaction of p54 with microtubule motor protein DLC1 during early virus transport competes for pro-apoptotic Bim binding to DLC1. This would free Bim in the cytosol to exert its apoptotic function at the mitochondrial membrane (Hernaez et al., 2004).

7.3. Apoptosis inhibitor ASFV genes

Despite this early trigger, ASFV-infected macrophages undergo apoptosis at late stages of infection, thus indicating that other virus genes negatively regulate apoptosis (Ramiro-Ibanez et al., 1996).

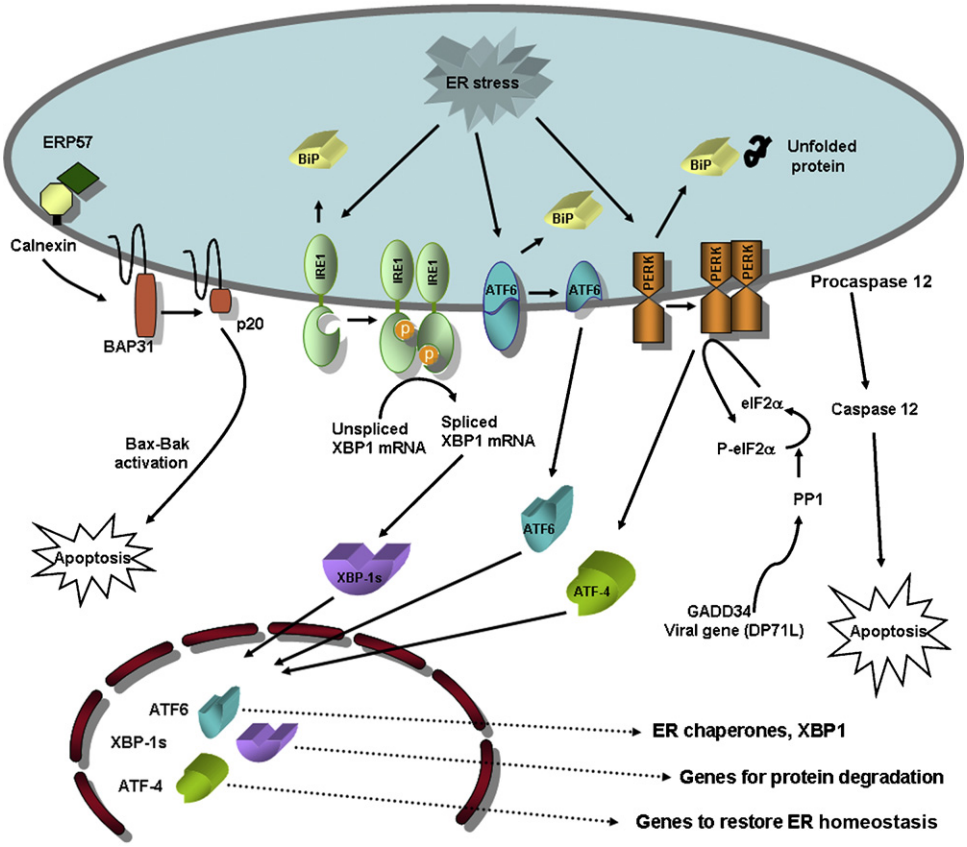

Fig. 6. ER stress and unfolded protein response pathways. When misfolded-proteins accumulate at the ER, sensor GRP78/BiP dissociates from the three endoplasmic reticulum stress receptors. Activated PERK blocks general protein synthesis by phosphorylating eukaryotic initiation factor 2 (eIF2a) and enables translation of ATF4, a transcription factor. ATF4 translocates to the nucleus and induces the transcription of genes required to restore ER homeostasis. ATF6 is activated by proteolysis and regulates the expression of ER chaperones and XBP1, another transcription factor. The spliced form of XBP1 protein, carried out by IRE1, controls the transcription of genes involved in protein degradation. Calnexin acts as a scaffold for the cleavage of the ER transmembrane protein Bap31 and thus for the generation of the pro-apoptotic p20 under ER stress. The Bap31 p20 fragment directs pro-apoptotic crosstalk between the ER and mitochondria. Caspase 12 is also cleaved to an active form in response to ER stress. ASFV-induced expression of caspase 12 and ATF6 is translocated to the nucleus and the viral factories while other UPR pathways can be tightly controlled by the virus.

To prevent premature cell death and ensure virus replication, ASFV, like other large DNA viruses, encodes for several apoptosis inhibitor genes (Fig. 7). The viral Bcl2 homolog (vBcl2) *A179L/5HL* is a conserved, essential gene encoding a 19-kDa protein named p21 (Neilan et al., 1993; Revilla et al., 1997). *A179L* protects cells from apoptosis, even when expressed in heterologous systems such as VACV or baculovirus (Brun et al., 1996, 1998). This vBcl2 contains the highly conserved domains of cellular Bcl2 (cBcl2)-related proteins, BH1, BH2 and BH3, but lacks the Bcl2 transmembrane domain (Afonso et al., 1996; Brun et al., 1996). *A179L* BH1 domain is conserved and functionally similar to cBcl2, including the relevant Gly-85 (Gly-145 in cBcl2), whose single mutation to Ala abrogates its capacity to protect cells from apoptosis (Revilla et al., 1997). vBcl2 *A179L* is expressed both at early and late times after infection, thus supporting the notion that this protein plays a crucial role in cell survival at various steps of the ASFV life cycle.

A179L product inhibits the action of several pro-apoptotic BH3-only proteins, known to be rapid inducers of apoptosis, such as activated Bid, BimL, BimS, BimEL, Bad, Bmf, Bik, Puma, and DP5 (Galindo et al., 2008). It also interacts at the mitochondrial membrane, A179L action is exerted on key pro-apoptotic Bcl2 family members, such as Bax and Bak (Fig. 7). Interestingly, A179L interacts only with active forms of Bid, not with the non-cleaved full-length Bid protein. Thus, A179L is a highly selective inhibitor.

Also, the late ASFV gene homolog to IAP proteins inhibits caspase 3 (Nogal et al., 2001) and activates NFkB (Rodriguez et al., 2002). Lectin-like E153R protein, which acts in the p53 pathway, was the first ASFV protein described with anti-apoptotic activity (Hurtado et al., 2004; Neilan et al., 1999). A238L is an early-late multifunctional protein that inhibits nuclear factors involved in immune responses NFkB (Powell et al., 1996), and the nuclear factor of activated T cells NFAT (Miskin et al., 1998). A238L inhibits NFkB interaction with the p65 subunit of NFkB (Revilla et al., 1998) by inhibiting CBP/p300 co-activators (Granja et al., 2006). A238L binds to calcineurin, thus impairing its phosphatase activity, which regulates NFAT (Abrams et al., 2008; Miskin et al., 2000). And NFAT modulates COX-2/PGE2 pro-inflammatory responses (Granja et al., 2004). The complex functions of this gene have been reviewed by Revilla et al. (this issue).

Moreover, ASFV encodes a homolog of the neurovirulence factor ICP34.5 of HSV-1 and the cellular gene *GADD34*. This homolog is the *DP71L* (*23NL/MyD 88*) gene (Zsak et al., 1996). The cytoprotective effect of *DP71L* is exerted by binding the catalytic subunit of protein phosphatase 1 (PP1). This binding causes the dephosphorylation of eukaryotic translation initiation factor 2α (eIF2α), thereby preventing the inhibition of protein synthesis produced by ER stress and the UPR (Rivera et al., 2007).

The prevention of the protein synthesis inhibition caused by eIF2α phosphorylation is an important virus-host interaction that ensures viral protein synthesis and cell survival in several virus models. HSV-1 ICP34.5 (He et al., 1997), papilloma virus (Kazemi et al., 2004), and coronavirus (Cruz et al., 2011) follow a similar strategy to that used by ASFV to overcome protein synthesis inhibition during its adaptation to the host. Moreover, a number of viruses have evolved mechanisms to inhibit viral nucleic acid sensing by interferon-inducible protein kinase (PKR) and activation of eIF2α,

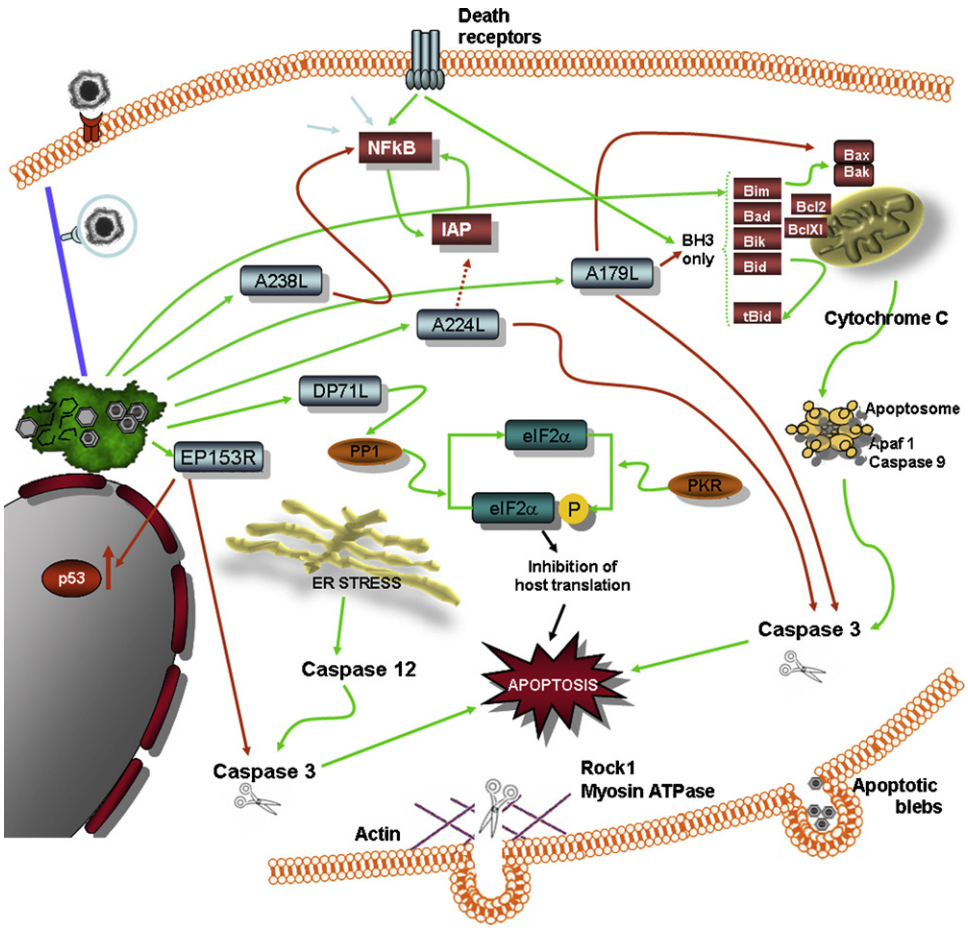

Fig. 7. Apoptosis pathways in ASFV infection. Major pathways of apoptosis activation (green) or inhibition (red) are summarized in this diagram. ASFV induces the activation of mitochondrial caspase 9, caspase 12 and executor caspase 3. The virus encodes several apoptosis inhibitor genes, namely *A238L, A224L, EP153R, DP71L* and *A179L*. Some of their functions are summarized. The function of these genes is required to prevent premature cell death, an event that would impair viral replication. Finally, late execution of apoptosis produces nuclear fragmentation, cytoplasmic vacuolization and membrane blebbing, giving rise to apoptotic bodies as cytoplasmic remnants surrounded by plasma membrane and filled with virus. These bodies are efficient vehicles for virus dissemination.

the latter promoting cell death (Domingo-Gil et al., 2011; Ramelot et al., 2002). The prevention of PKR-mediated translational arrest is shared by VACV (Sharp et al., 1997), HSV-1 protein Us11 (Poppers et al., 2000), and hepatitis C virus (He et al., 1997), among others.

Interestingly, deletion of DP71L from a virulent ASFV (isolate E70) reduces the virulence of the virus in pigs (Zsak et al., 1996); however, this effect was not reproducible for the highly pathogenic Malawi isolate. Moreover, deletion of this gene does not modify eIF2α phosphorylation. This observation thus suggests the presence of alternative mechanisms to prevent eIF2α phosphorylation (Zhang et al., 2010), as described for other DNA viruses (*e.g.* HSV-1). Also, DP71L inhibits the early induction of ATF4 and its downstream target CHOP (Zhang et al., 2010), a transcription factor that is commonly up-regulated as a result of the UPR, but not in ASFV infection (Galindo et al., 2012; Netherton et al., 2004).

Other functions undertaken by the HSV-1-homologous gene, such as the inhibition of autophagy by means of Beclin-1 inhibition (Orvedahl et al., 2007); do not occur in ASFV DP71L, as described below.

7.4. ASFV regulation of cell survival

In general, the controversial effects of viruses on cell homeostasis are well illustrated in the host systems with which ASFV interacts. This virus encodes for several apoptosis inhibitor genes but finally induces the death of the infected cell. Also, most UPR genes are not activated upon infection; however, ASFV induces ER stress, casapase 12 activation and the UPR. Similarly, ASFV inhibits pro-inflammatory gene transcription; however, this infection induces the secretion of many cytokines both *in vitro* and *in vivo* that underlie the pathogenesis of this virus (Zhang et al., 2010). All together, these observations highlight that several cell responses to virus sensing are strongly counteracted by viruses.

8. ASFV and autophagy

Macroautophagy has the capacity to remove a wide variety of intracellular components, ranging from protein aggregates to whole organelles such as mitochondria, by sequestration and degradation (Mizushima et al., 2008). Cytoplasmic targets are captured within double membrane structures called autophagosomes, which subsequently fuse with lysosomes where the engulfed target is degraded or eliminated. The physiological functions of autophagy include the provision of a source of energy and amino acids by self-digestion in response to cellular stress or nutritional deprivation (starvation). Autophagy integrates with other cell stress responses upon nutrient deprivation, and the presence of reactive oxygen species, DNA damage, protein aggregates and intracellular pathogens (Fig. 8). Autophagy prevents cell death or senescence caused by the accumulation of damaged organelles and large macromolecular aggregates. Interestingly, autophagy may

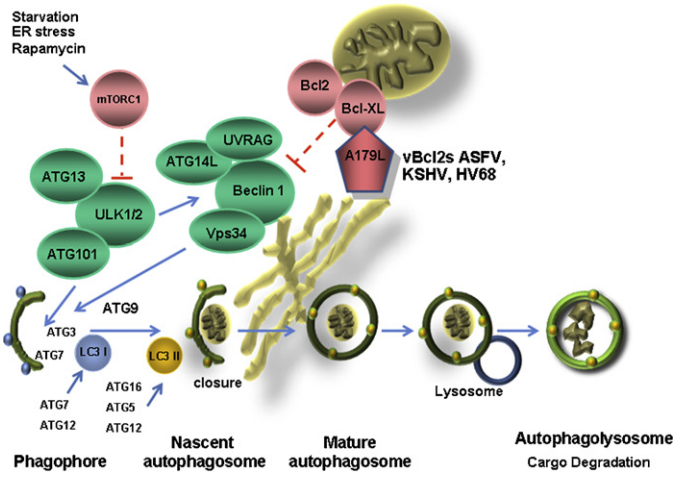

Fig. 8. Autophagic pathways and ASFV A179L Bcl2 homolog regulation. Autophagy is induced by starvation, ER stress, pathogen-associated molecular patterns, redox stress and mitochondrial damage. ULK1 and ULK2 play a key role in autophagy induction, acting downstream of mTORC1. Upon mTORC1 inhibition, for example by starvation, mTORC1 dissociates from the ULK complex, thus leading to its catalytic activation. ULK1 can phosphorylate Ambra1. Beclin1 is a multiprotein complex formed by the allosteric activation of the class III PI3K Vps34 to generate PI3P, which recruits FYVE proteins to mediate the initial stages of the isolation membrane nucleation and autophagosome formation. Anti-apoptotic Bcl2 family members are important regulators of autophagy that interact with Beclin1. Similarly, ASFV A179L homolog inhibits autophagy through interaction of its BH3-binding domain with the BH3 domain of Beclin1. The mammalian LC3 (ortholog of yeast Atg8) is translocated to the initiation membrane of the autophagosome and conjugated with lipids by means of different Atg proteins. This conjugation leads to the conversion of the soluble form of LC3 (LC3-I) to the lipidated LC3-II form. LC3-II is associated with the autophagic vesicle and its biochemical and microscopic detection is used to measure cellular autophagy.

constitute a cellular defense mechanism for virion degradation and it participates in innate immunity.

8.1. Regulation of autophagosome formation

Autophagy begins with the formation of an isolation membrane or phagophore (Fig. 8) and involves several molecules called authophagy proteins (atg). The Atg1/ULK (unc-51-like kinase) complex is downstream of the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) and it plays a key role in autophagy induction (Fig. 8). Upon mTORC1 inhibition, as by starvation, mTORC1 dissociates from the ULK complex, thus causing its dephosphorylation (Mizushima, 2010). Other key molecular complexes in this pathway include Atg6/Beclin1, class III phosphatidylinositol 3-kinase (PI3K), Atg9, and ubiquitin-like proteins Atg12 and Atg8/LC3 conjugation systems.

8.2. DNA viruses control of autophagy

Several DNA viruses keep autophagy under control, probably to prevent the degradation of replicating or newly assembled virions by lysosomal fusion. HSV-1 ICP34.5 targets Beclin1 autophagy protein and inhibits autophagy-dependent virion degradation (Alexander et al., 2007; Orvedahl et al., 2007). Viral Bcl2 homologs encoded by Kaposi's sarcoma herpesvirus (KSHV; (Pattingre et al., 2005) and murine γ-herpesvirus 68 (HV68); (Ku et al., 2008) also inhibit autophagy by a mechanism involving direct interaction with Beclin1. Therefore, there are at least two potential candidates by which to achieve Beclin1 regulation in ASFV, namely the viral homolog to HSV1 ICP34.5 DP71L, and the vBcl2 A179L.

We have shown that A179L interacts directly with Beclin1 while DP71L does not and that the A179L BH3 domain is required for binding (Hernaez et al., 2012b). Transient expression of A179L in HeLa cells inhibits starvation-induced autophagosome formation. Transient expression assays with A179L-GFP showed colocalization with both mitochondria and ER. This subcellular distribution makes it conceivable that A179L plays a dual role, on the one hand interacting with pro-apoptotic BH3-only proteins (Bim, aBid, Bad, Bmf, Bik, Puma, *etc.*) and Bax and Bak at the mitochondrial membrane, and on the other hand, with Beclin1 at the ER. In fact, cellular Bcl2 inhibits apoptosis at the mitochondrial membrane and also suppresses autophagy by interacting with Beclin1 at the ER. The UPR, the major ER stress pathway, is a potent stimulus of autophagy (Buchberger et al., 2010), hence this dual function of Bcl2 points to a close relationship between the two cascades.

In contrast, most RNA viruses have been reported to induce autophagy in infected cells, and in several cases autophagy may enhance viral replication (Reviewed in (Dreux and Chisari, 2010). A number of viruses replicate in multi-membrane vesicles that closely resemble autophagosomes (de Haan and Reggiori, 2008). Given the nature and location of these structures, autophagosomes may serve as sites of viral replication during some infections. Also, membranes associated with viral replication sites are often derived from the ER, which is a potential source for the autophagosomal membrane (Mijaljica et al., 2006). Nevertheless, VACV infection, which uses double-membrane vesicles, is not impaired in autophagy-deficient mice (Zhang et al., 2006). In other viral models, controversial results suggest that the impact of inhibition autophagy on viral infection varies depending on the cell type or the stage of the viral life cycle considered.

We found that ASFV does not induce autophagy in infected cells. ASFV infection did not induce LC3 activation or autophagosome formation in Vero cells infected with the ASFV BA71V isolate (Hernaez et al., 2012b). However, ASFV infection is strongly inhibited by lysosomotropic drugs because of its endosomal-dependent entry mechanism. This is a limitation when studying autophagic flux during infection in the presence of bafilomycin or protease inhibitors. Interestingly, induction of autophagy by starvation and rapamycin prior to ASFV infection reduces viral infectivity. This decrease could be due to the consumption of yet unknown factor/s from the core autophagic pathway required at an early stage of ASFV infection. This notion, together with the interconnection between autophagy regulation and its crosslinks with cell stress and apoptosis in ASFV infection, awaits further investigation.

9. Virus-cell interaction-based analysis of potential therapeutic intervention targets

9.1. Potential applications of antivirals

This chapter has reviewed some key ASFV interactions with the host cell that are crucial for the virus to start and complete productive infection. Several of these molecular systems are viewed as potential targets to consider in a rational vaccine design–something that continues to be an unmet need. Also, some of these systems are sensitive to antivirals. A possible application of antivirals would be to prolong survival in experimental infections with virulent ASFV isolates in order to gain further insight into the pathogenesis of this disease. Longer survival may change the acute course of the disease and eventually allow the swine host to generate an immune response against the virus. In addition, the combination antivirals with experimental vaccination protocols could be useful for the analysis of immune response required for effective protection against the disease. These antiviral/vaccine protocols should be further developed to refine the targets to be selected and to clarify the major obstacles that hinder achievement of a protective immune response against the virus.

9.2. "Druggable" targets at the virus-cell interface

Cholesterol-lowering drugs called statins effectively inhibit ASFV infection *in vitro* (Quetglas et al., 2012). These drugs are of generalized use in humans and their safety is widely proven. Valproic acid, which is used for treatment of neurological disorders, was found to have a potent antiviral effect against a number of enveloped viruses, including ASFV (Vazquez-Calvo et al., 2011) Also, resveratrol and other phytoalexins produced by plants effectively inhibit virus replication (Galindo et al., 2011). Together with extracts from marine microalgae (Fabregas et al., 1999), these plant compounds are antivirals derived from natural sources and they can be administered to animals as a dietary supplement. Other inhibitors that are used in oncological therapy in humans are effective antivirals against ASFV at different infection stages. Examples include serine protease inhibitors (Galindo et al., 2012), PI3K and/or PIKfyve inhibitors (Cuesta-Geijo et al., 2012) and microtubule-depolymerizing drugs (Basta et al., 2010).

Using our knowledge of ASFV-cell interactions, together with insights gained from NMR structure-based design, researchers face the challenge of further developing antiviral treatments and preventive strategies. Antiviral compounds targeting virus-host interactions are already under development. One example is an antiviral peptide that impairs infectivity and viral replication in cultured cells by competing with p54 binding to its cellular target dynein (Hernaez et al., 2010). Like the above-mentioned antivirals targeting cellular mechanisms, this peptide could be used to shed light on unknown cellular mechanisms targeted by ASFV infection and on the induction of protection.

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