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Review

African swine fever virus organelle rearrangements

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ABSTRACT

Like most viruses African swine fever virus (ASFV) subsumes the host cell apparatus in order to facilitate its replication. ASFV replication is a highly orchestrated process with a least four stages of transcription, immediate-early, early, intermediate and late. As the infective cycle progresses through these stages most if not all of the organelles that comprise a nucleated cell are modified, adapted or in some cases destroyed. The entry of the virus is receptor-mediated, but the precise mechanism of endocytosis is a matter of keen, current debate. Once ASFV has exited from the endosomal–lysosomal complex the virus life-cycle enters into an intimate relationship with the microtubular network. Genome replication is believed to be initiated within the nucleus and ASFV infection completely reorders the structure of this organelle. The majority of replication and assembly occurs in discrete, perinuclear regions of the cell called virus factories and finally progeny virions are transported to the plasma membrane along microtubules where they bud out or are propelled away along actin projections to infect new cells. The generation of ASFV replication sites induces profound reorganisation of the organelles that comprise the secretory pathway and may contribute to the induction of cellular stress responses that ASFV modulates. The level of organisation and complexity of virus factories are not dissimilar to those seen in cellular organelles. Like their cellular counterparts the formation of virus factories, as well as virus entry and exit, are dependent on the various components of the cytoskeleton. This review will summarise these rearrangements, the viral proteins involved and their functional consequences.

Contents

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1. Incoming African swine fever virions are delivered to perinuclear sites along microtubules

1.1. Virus entry

Like all intracellular pathogens the first part of the host cell that African swine fever virus (ASFV) encounters is the plasma membrane. *In vivo* viral replication can be detected in a number of different cell types in the soft tick host (Kleiboeker et al., 1998), however virus replication is principally restricted to cells of the mononuclear phagocytic system in the domestic pig and bushpig (Oura et al., 1998a). *In vitro* ASFV can enter a wide range of cell types from a number of different species including those within which its replication is then restricted (Alcamí et al., 1990; Carrascosa et al., 1999). ASFV can enter Vero cells and porcine macrophages by receptor-mediated endocytosis (Alcamí et al., 1989, 1990), but can be also taken up non-specifically by rabbit macrophages but the latter infection is not productive (Alcamí et al., 1990). Successful infection of porcine macrophages is linked to the expression of the CD163 scavenger receptor (Sánchez-Torres et al., 2003) and antibodies to CD163 inhibit virion binding to macrophages. In addition to these routes, virus enters midgut digestive cells in ticks bound to red blood cells (Kleiboeker et al., 1998, 1999), and loss of the CD2v gene responsible for hemadsorption to red blood cells significantly reduces viral replication in ticks (Rowlands et al., 2009). Dissemination to other cell types of the tick is not dependent on CD2v as virus lacking the gene replicates to similar levels as virus expressing CD2v if it is injected across the gut wall (Rowlands et al., 2009). ASFV may also be able to directly infect neighbouring cells through virus-tipped actin projections that form late during infection (see Section 5), although this mechanism could also serve to embed virions on the surface of adsorbed red blood cells. Lastly, ASFV induces apoptosis in infected cells (Gómez-Villamandos et al., 1995; Oura et al., 1998b; Ramiro-Ibáñez et al., 1996) and it is possible that intracellular virus may be taken up as part of apoptotic cell bodies by phagocytic cells.

1.1.1. Virus structure

The structure and morphogenesis of the ASF virion will be discussed thoroughly in another chapter of this special issue (Salas and Andrés, 2012), but a brief description is merited here to aid the discussion on entry. Virions are icosahedral structures approximately 200 nm in diameter, the centre of the virus contains the genomic DNA which is then surrounded by matrix proteins which are primarily derived by processing of two viral polypeptides, pp220 and p62. The matrix is built up on the internal face of viral membranes (see Section 4), while the capsid is assembled on the outer face of the viral membranes (Andrés et al., 1997). A further membrane, the external envelope, is obtained when the virus buds out through the plasma membrane, however the importance of this envelope is unclear as it is not required for infectivity (Andrés et al., 2001). The p12 protein has been identified as the viral attachment protein (Carrascosa et al., 1991) and immunogold electron microscopy has localised this protein to the external envelope (Carrascosa et al., 1993). However, immunofluorescence microscopy showed that p12 is incorporated into virions within the virus factory which occurs before virus exit (Angulo et al., 1993) implying that p12 is part of the intracellular virus and this has recently been confirmed by immunoelectron microscopy (Salas and Andrés, 2012).

1.1.2. Virus entry mechanisms

The mechanism of ASFV uptake *in vitro* has been the subject of two recent detailed studies which separately concluded that clathrin dependent endocytosis (Hernaez and Alonso, 2010) and macropinocytosis (Sánchez et al., 2012) were the primary route of virus entry. The authors of these two papers used different methodologies to determine what constituted a successful entry event and this may have contributed to the different conclusions from apparently similar experiments. Sánchez et al. defined entry as the presence of the major capsid protein p72 1 h post infection as detected by flow cytometry and reported the percentage of cells staining positive for p72. Using this assay they showed that 5-ethylisopropyl amiloride (EIPA), IPA-3 and cytochalasin D, which are drugs that inhibit Na^+/H^+, Pak1 phosphorylation and actin polymerisation respectively and have a downstream effect on macropinocytosis, inhibited virus entry. Sánchez et al. also showed that treatment with chlorpromazine, a drug that interferes with clathrin-mediated endocytosis, had no effect on ASFV entry, but did affect late protein synthesis. Sanchez et al. therefore concluded that macropinocytosis was the primary cell entry method for ASFV. Hernaez and Alonso used the expression of the early protein p30 6 h post infection as a definition of successful virus entry, detected this by flow cytometry and reported the percentage of cells staining positive for p30. Using this method Hernaez and Alonso reported that EIPA only had a moderate effect on ASFV entry and that the actin disrupters jasplakinolide and latrunculin A had none at all. Hernaez and Alonso also reported that chloropromazine significantly inhibited virus entry and so concluded that clathrin-mediated endocytosis was the primary mechanism for ASFV entry, consistent with early EM observations showing virus in coated pits (Alcamí et al., 1989, 1990; Valdeira and Geraldes, 1985). One of the criticisms by Sánchez et al. of the Hernaez and Alonso paper was the use of p30 expression as a read-out for virus entry, this is a reasonable criticism as expression of early genes is dependent on a number of distinct events post entry (see below). However, the method used by Sánchez et al. to determine virus entry did not define the threshold for a p73 positive cell (i.e., entry of one virions or ten virions) and it is unclear whether a cell infected with one virion would count the same as cell infected with one hundred. To complicate matters further a number of the experiments, such as those with chloropromazine, in the two papers directly contradict each other and other researchers using [^{3}H]labelled ASFV have shown that cytochalasin D had no effect on ASFV binding to, or up take within, Vero cells (Valdeira et al., 1998). Furthermore experiments in macrophages with wortmannin, another drug that can influence macropinocytosis, does not affect virus replication (Basta et al., 2010). Lastly it is worth considering that the different authors discussed above all used different virus preparation methods, Basta et al. used neat tissue culture supernatants, Sánchez et al. concentrated their virus by centrifugation, and Hernaez and Alonso used virus purified by sucrose sedimentation. Valdeira et al. used Percoll purification which has been shown to eliminate contamination with membranes and vesicles (Carrascosa et al., 1985).

1.2. Delivery to perinuclear sites requires microtubules

After entry virions locate to the endosomal–lysosomal complex from where they exit into the cytoplasm. How this is achieved is poorly understood, but is dependent on pH as treatment with lysosomotropic weak bases such as ammonium chloride or chloroquine prevent virion escape into the cytoplasm (Alcamí et al., 1989; Geraldes and Valdeira, 1985; Valdeira and Geraldes, 1985). Virions can be maintained in neutralised vacuoles for at least 72 h without any significant loss of infectivity, as wash out of chloroquine allows replication to progress normally (Geraldes and Valdeira, 1985; Valdeira and Geraldes, 1985). Exit from endosomes/lysosomes may also be dependent on proteolysis as treatment with phenylmethylsulphonyl fluoride produces a similar phenotype to that seen with lysosomotropic drug treatment (Valdeira et al., 1998). Electron micrographs hint at a fusion between one of the viral envelopes and the membrane of the cellular compartment (Valdeira et al., 1998) and this may be dependent on cholesterol as viral DNA fails

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to enter the cytoplasm when this lipid is depleted in target cells (Bernardes et al., 1998). If fusion does occur then it is likely that the external viral envelope is involved because virions can be detected within infected cells by staining for p72 and pE120R (Quetglas et al., 2012) or through p54-GFP signal (Hernaez et al., 2006) and all of these viral proteins are associated with the capsid and/or internal envelope of the ASF virion (Andrés et al., 2001; Cobbold et al., 1996; Rodríguez et al., 2004). p54 is still associated with the remainder of the virus particle after it has left the endosomal–lysosomal complex because it is directly interacts with the cytoplasmic, cellular protein dynein light chain (DYNL11/DLC8) (Hernáez et al., 2010) to deliver virions to perinuclear sites. If exit from endosomes and lysosomes requires fusion of the external envelope with cellular membranes then it is difficult to envisage how this can be accomplished by intracellular virus. Interestingly, in the VACV model intracellular mature virus can infect cells by direct fusion between the internal envelope and the plasma membrane (Carter et al., 2005). ASFV can enter cells directly by fusion under certain experimental conditions, however, this is not thought to be a natural route of infection (Valdeira and Geraldes, 1985).

Microtubules are crucial components of the cytoskeleton, providing structural support for the cell and the major long-range intracellular transport mechanism within the cytoplasm. Movement along microtubules can be driven by growth of the tubules themselves or through the use of motor proteins. Microtubules are organised at the centrosome, also called the microtubule organising centre (MTOC), and dynein motors are responsible for driving cargo towards the MTOC while kinesin motors drive transport to the periphery (see Section 5). Incoming ASFV virions are associated with microtubules (see Fig. 2A) (Quetglas et al., 2012), eventually localising to regions of the cell that are close to the nucleus and this movement can be followed in cells infected with recombinant ASFV encoding the p54 protein fused to GFP (Hernaez et al., 2006). Treatment with nocodazole, a drug that depolymerises microtubules, prevents correct localisation of fluorescently tagged ASFV at perinuclear sites showing dependence on an intact microtubule network (Hernaez et al., 2006). ASFV infection stabilises microtubules by acetylation shortly after infection and this may involve Rac1 because inhibitors of this Rho GTPase with drug treatments inhibits ASFV induced microtubule acetylation (Quetglas et al., 2012). Rac1 inhibition does not prevent viral entry, but does prevent correct localisation to perinuclear sites (Quetglas et al., 2012). Note that this conclusion is controversial as the same Rac1 inhibitor has been reported to prevent ASFV entry due to its effects on macropinocytosis (Sánchez et al., 2012). ASFV p54 protein is an essential structural protein that binds the light chain of dynein through a domain of thirteen amino acids near the C-terminus of the viral protein (Alonso et al., 2001). Cell-soluble peptides corresponding to the p54 dynein binding motif also inhibit incoming virions from correctly localising to perinuclear sites and subsequent steps in virus replication (Hernáez et al., 2010). This shows that the delivery of virions to specific parts of the cell is crucial for efficient replication and that the interaction between p54 and dynein light chain is an important driver of this process.

The conflicting interpretations of the mechanism of African swine fever virus entry (illustrated in Fig. 1) may simply reflect the fact that the virus can infect a wide variety of different cell types and that both intracellular and extracellular virions are infectious. It is not unreasonable to think that ASFV may utilise different mechanisms to enter different types of cells. An analysis directly measuring virus entry by counting virions (Quetglas et al., 2012) after infection with virus prepared using different purification methods with careful consideration as to the proportion of intracellular and extracellular virus may show that ASFV uses different cell-entry methods under differenct circumstances as seen for vaccinia virus (VACV) (Locker et al., 2000), all of which could be relevant to ASFV dissemination *in vivo*. The events that underpin the transition to early gene expression after delivery of virions to perinuclear sites has not been studied in any great detail, however it is known that this process is dependent on the product of the *E248R* gene (Rodríguez et al., 2009). After virus delivery ASFV replication requires the host cell nucleus and this is discussed in Section 2.

2. African swine fever virus DNA replication is initiated in the nucleus

Initial ultrastructural studies of ASFV infected cells defined cytoplasmic factories (Bresse Jr. and DeBoer, 1966; Moura Nunes et al., 1975) as the sites of viral replication. The development of methods to reliably enucleate cells allowed researchers to analyse the direct importance of the nucleus to viral replication. Like poxvirus replication, ASFV replication was shown to be dependent on the host cell nucleus (Hruby et al., 1979; Ortin and Vinuela, 1977), however, experiments since then have shown that part of ASFV DNA replication takes place within the nucleus whereas poxviruses only require nucleus-derived cellular factors. *In situ* hybridisation studies have detected viral DNA within the nucleus as well as within cytoplasmic factories (Ballester et al., 2010; Brookes et al., 1996; García-Beato et al., 1992; Rojo et al., 1999). The current hypothesis is that short sections of viral DNA are synthesised at early times post infection in the nucleus and then transported to cytoplasmic factories where these are used to prime synthesis of full length genomic DNA later in the replicative cycle. This is supported by the observations that radiolabelled DNA that has been synthesised in the nucleus can be chased into the cytoplasm (García-Beato et al., 1992), that viral DNA detected in the nucleus is smaller than that detected in the cytoplasm after equilibrium sedimentation (Rojo et al., 1999) and that the mature cross-linked viral DNA present in viral particles is derived from both nuclear and cytoplasmic DNA fragments (Ortin et al., 1979; Rojo et al., 1999). ASFV causes the breakdown of the nucleolus and the nuclear envelope early during infection and the redistribution of some nuclear membrane components to the cytoplasm (Ballester et al., 2011). Lamins A and C are phosphorylated and this is likely responsible for the disassembly of the nuclear lamina. Interestingly, lamin A/C is recruited to replication sites and the nucleoporin p62 is recruited to their periphery. Disruption of the nuclear sub-structure is concomitant with disruption of the localisation of SC35 splicing speckle marker and active RNA polymerase II. The disruption of nuclear structures may represent a mechanism by which ASFV alters patterns of cellular transcription and/or facilitates initiation of viral DNA replication (Ballester et al., 2011). Although ASFV is known to encode two DNA polymerases, little is known about the viral proteins that are involved in reorganising the host cell nucleus or that direct and control the synthesis of viral DNA. Recent experiments have shown that two viral structural proteins that are derived from pp220 polyprotein can be targeted to the nucleus (Eulálio et al., 2004). The viral matrix protein p37 co-localises with viral DNA in distinct foci in the nucleus at early time points in a manner that is independent of the CRM1-mediated nuclear-import pathway (Eulálio et al., 2004, 2006, 2007). Interestingly, p37 has been shown to shuttle between the nucleus and cytoplasm and its co-localisation with viral DNA in the nucleus at early time points suggests that it may be involved in the transport of viral DNA precursors to the factory (Eulálio et al., 2007).

3. The virus factory – a viral organelle

3.1. Virus factories resemble aggresomes

ASFV factories are similar to aggresomes (Heath et al., 2001) which are formed at the microtubule organising centre (MTOC)

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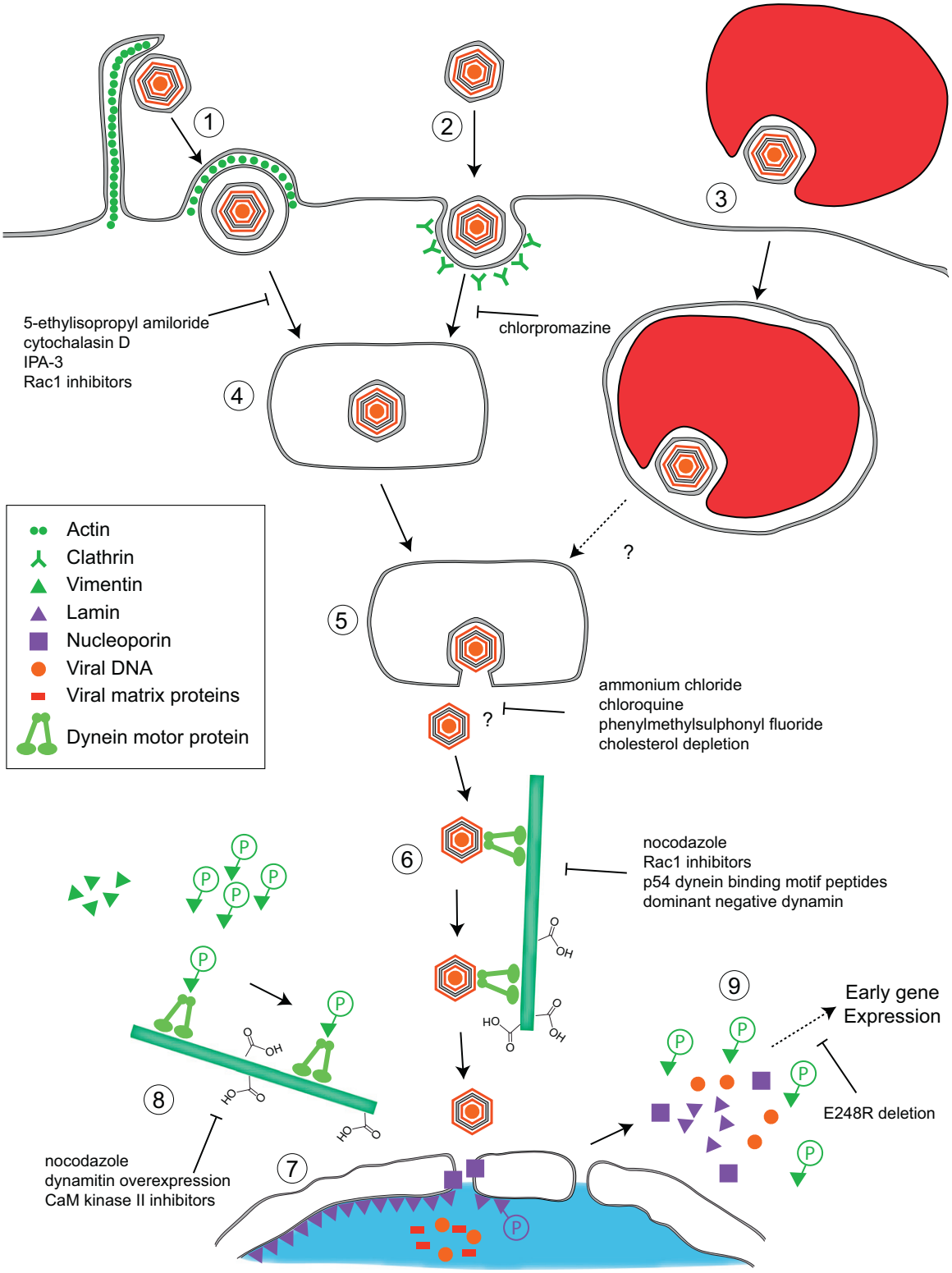

Fig. 1. Early events during ASFV infection: ASFV enters cells using macropinocytosis ① or clathrin coated pits ② . Virus can also enter certain cells attached to phagocytosed red blood cells ③ . Post-entry ASFV enters the endosomal–lysosomal system ④ from which it exits by fusing with the membrane and in doing so loses its external envelope ⑤ . Virions are directed to perinuclear regions by microtubules through the interaction between dynein motors and the structural protein p54 ⑥ . Viral matrix proteins and viral DNA enters the nucleus to initiate viral replication and co-incident with this is the phosphorylation and disassembly of nuclear lamins ⑦ . Vimentin is phosphorylated and recruited to perinuclear sites by dynein motor proteins ⑧ , movement of vimentin and virions require the acetylation of microtubules. Although the precise order is unclear, early viral gene expression begins, and the initial stage of factory formation begins including the recruitment of vimentin and nuclear proteins, and presumably the movement of viral DNA to replication sites ⑨ . Drug treatments and conditions that block different steps are indicated, please note that most of these are controversial, see text for details.

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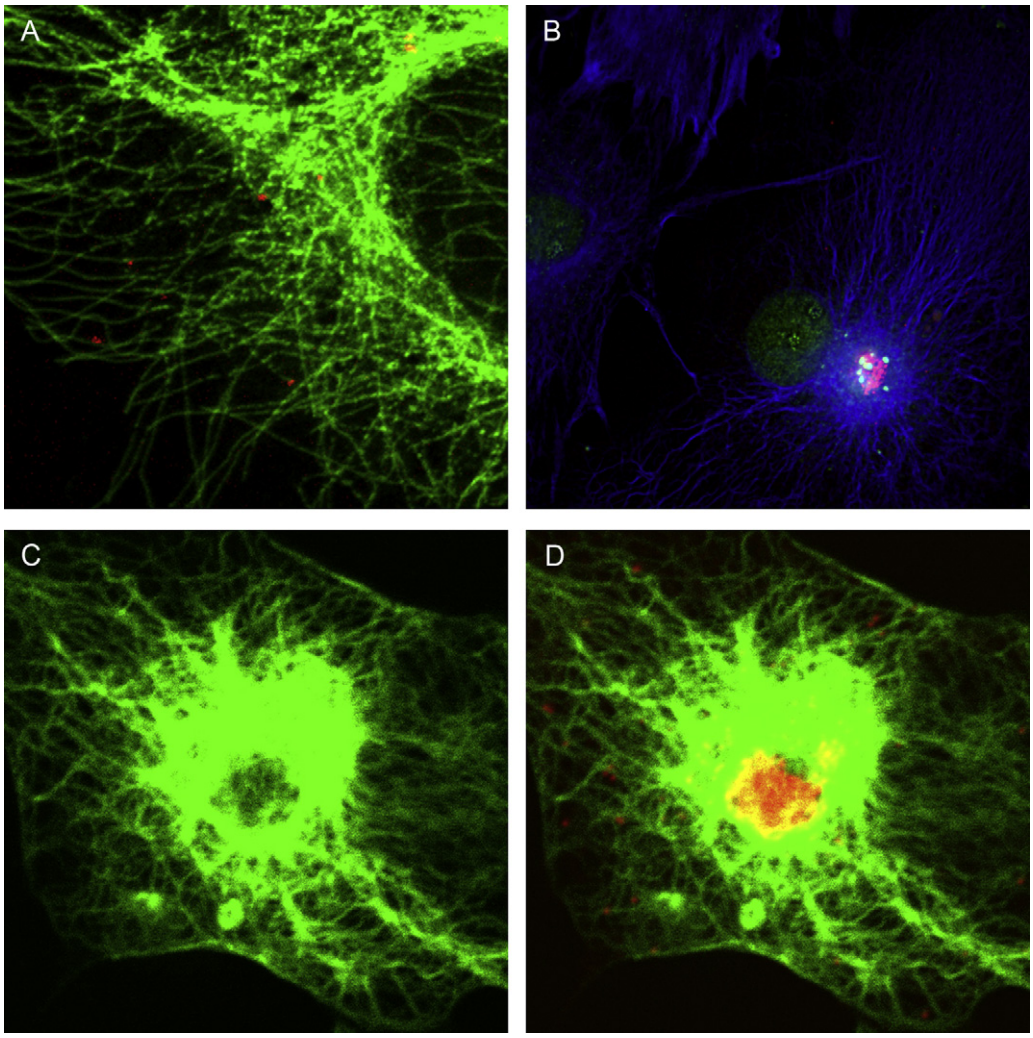

Fig. 2. ASFV and the cytoskeleton. (A) Vero cell 2 h post infection with Ba71v stained with antibodies to alpha-tubulin (green) and p72 (red), note the relationship between the virions and microtubules. (B) Porcine aortic endothelial cell 16 h post infection with Malawi Lil 20/1 stained with antibodies to vimentin (blue), p72 (red) and ubiquitin (green). (C) and (D) Vero cell 16 h post infection with Ba71v stained with vimentin (green) and p72 (red).

in response to the aggregation of proteins that form after misfolding (Johnston et al., 1998). These aggregates are highly toxic and are implicated in several neuropathological diseases such as Alzheimer's, Huntington's and the spongiform encephalopathies. Redirection of protein aggregates to aggresomes segregates them from the cell, reducing toxicity and facilitates their degradation by proteolysis by the proteasome or lysosomes after sequestration by autophagy (Garcia-Mata et al., 2002). Proteins are often ubiquitinylated prior to degradation and ubiquitinylated proteins can be detected in virus factories (Fig. 2B) some of which are viral in origin (Hingamp et al., 1995). The core particles of many viruses are similar in size (60–100 nm diameter) to protein aggregates and it is therefore conceivable that ASFV viral cores may be recognised as protein aggregates and are so delivered to the MTOC (Wileman, 2007).

Protein aggregates are surrounded by a collapsed cage of the intermediate filament protein vimentin during aggresome formation. Virus factories are also surrounded by vimentin at the MTOC during ASFV infection (Fig. 2C and D). Vimentin is re-arranged at the centrosome into a star shaped structure that resembles the microtubule aster formed during mitosis (Stefanovic et al., 2005) and this process can be blocked by over expression of p50 dynamitin indicating involvement of the dynein motor protein. The vimentin aster provides the basis for the cage that surrounds the factory following the onset of virus DNA replication and synthesis of late structural proteins (Heath et al., 2001; Monaghan et al., 2003; Stefanovic et al., 2005). Rearrangement of vimentin by ASFV requires phosphorylation of vimentin at serine 82 by calcium calmodulin-dependent protein kinase II (Stefanovic et al., 2005) and this is required for late gene expression. A link between vimentin rearrangement and phosphorylation has also been reported for the iridoviruses that like ASFV are members of the nucleo-cytoplasmic large DNA viruses. Temperature sensitive mutants of frog virus 3 are unable to rearrange vimentin or progress to late gene expression and this is linked to the inability to phosphorylate the intermediate filament (Chen et al., 1986; Willis et al., 1979). Aggresomes recruit a variety of different cellular components to facilitate protein folding and/or degradation, including cellular chaperones, proteasomes and mitochondria. Cellular chaperones, such as hsp70 and mitochondria are also recruited to ASFV factories (Castelló et al., 2009; Heath et al., 2001; Rojo et al., 1998). The chaperones may facilitate folding of viral structural proteins, although ASFV encodes a chaperone that is responsible for folding the major capsid protein p72 (Cobbold et al., 2001). The similarity between factories and aggresomes raises the possibility that ASFV may use the aggresome pathway to facilitate assembly. Microtubules can concentrate viral and cellular proteins needed for replication at the MTOC and vimentin can provide a physical scaffold within the factory, or act as a cage to prevent movement of viral components into the

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cytoplasm. Alternatively, aggresomes may be part of an innate defence against virus infection able to recognise viruses as foreign and/or misfolded so that they can be stored in inclusions and removed by autophagy and delivery to lysosomes for degradation (Wileman, 2006, 2007) and presentation by major histocompatibility complexes (Nimmerjahn et al., 2003; Paludan et al., 2005; Schmid et al., 2007).

3.2. Organisation within virus factories

The major steps in ASFV assembly occur at a single site where all of the components that go on to form nascent virions; DNA, proteins and membranes accumulate (Brookes et al., 1996; Moura Nunes et al., 1975; Rouiller et al., 1998). The staining patterns of individual viral structural proteins within an infected cell typically have very strong signals co-incident with the virus factory and a less intense signal in the surrounding cytosol (Cobbold et al., 1996; Sanz et al., 1985). However, the mechanisms that govern the distribution of viral components within the host cell are only beginning to be understood. Recent work on ASFV and VACV has begun to define subdivisions within virus factories that are specialised for different parts of the replication process. DNA and RNA are spatially separated within the VACV factory (Katsafanas and Moss, 2007). The VACV transcription factor VITF-3 localises to factory subdomains that also contain poly(A) mRNA. Ribonucleoproteins such as G3BP and p137, eukaryotic initiation factors and ribosomal subunits are recruited to the RNA subdomains. This strongly suggests that these RNA positive domains are transcriptionally active and not accumulations of dsRNA. Taken together these results suggest that transcription and DNA synthesis are segregated within the replication site. In a similar manner, eukaryotic initiation factors are recruited to ASFV factories while ribosomal proteins, poly(A)RNAs and viral RNAs are recruited to the periphery of ASFV factories (Castelló et al., 2009). The recruitment of translation machinery is an early step in factory morphogenesis and the relationship between the localisation of the translation machinery and viral proteins within the factory appears to change over time suggesting that the organisation of the virus factory is a dynamic process. These results indicate that specialised domains form within the ASFV factory in an analogous manner to those formed in VACV replication sites. It will be interesting to relate the location of ribosomal proteins, RNAs and nucleoporin to the vimentin cage that surrounds the viral DNA and structural proteins to see if the vimentin cage represents the boundary between the ASFV replication site and the rest of the cell or a subdivision of the factory (see Fig. 3).

3.3. Virus factories utilise microtubules

Maintenance of the ASFV factory at the MTOC may require motor proteins as depolymerisation of microtubules results in the dispersal of ASFV factories (Alonso et al., 2001; Alves de Matos and Carvalho, 1993; Carvalho et al., 1988; Heath et al., 2001). As mentioned previously the ASFV structural protein p54 interacts directly with dynein motor protein (Alonso et al., 2001; Hernáez et al., 2010) and interestingly, repression of the *E183L* gene that encodes p54 disrupts ASFV assembly in factories (Rodríguez et al., 2004). In the absence of *E183L* expression other virus structural proteins are recruited to zippered membranes of ER that appear in the cytosol rather than in the factory (Rodríguez et al., 2004). One potential explanation of these results are that p54 binding to dynein motors is important for the correct localisation of membrane precursors to viral replication sites as well as its established role in delivering cores to the MTOC. However, treatment of cells with a tyrosine phosphatase inhibitor that inhibits dynein dependent transport does not affect virus replication when added 6 hpi which argues against this interpretation (Alonso et al., 2001). The Rho GTPase family may also govern the correct formation of the virus factory as incubating cells with *Clostridium difficile* toxin B induces an enlarged, aberrant replication site without directly affecting the synthesis of viral structural proteins (Quetglas et al., 2012).

4. Origins of membranes used for assembly of African swine fever virus

The inner envelopes of ASFV and VACV are comprised of a single lipid bilayer that is tightly associated with a protein layer (Heuser, 2005; Hawes et al., 2008). In the case of VACV the protein layer is comprised of a honeycomb lattice of the D13 scaffold protein (Szajner et al., 2005) and intriguingly the major capsid protein of ASFV, p72 shares a loose structural similarity to D13 (Benson et al., 2004). ASFV factories contain viral membranes upon which new virions are formed by the ordered assembly and recruitment of viral proteins. One of the key questions since ASFV replication sites have been imaged in the electron microscope is where do the viral membranes originate from? Some viral membranes found in factories may be connected to cellular organelles and viral membranes label with resident ER markers as well as viral proteins such as p17, p54 and pB318L in the electron microscope (Rouiller et al., 1998; Alejo et al., 1999; Andrés et al., 1998). These observations are consistent with biochemical experiments that show that p72 and p17 co-sediment on sucrose gradients with the luminal ER protein protein disulphide isomerase (PDI) as well as with enzymatic activity associated with the ER (Cobbold et al., 1996). In addition membranes close to virus particles label with antibodies against cellular and viral ER proteins (Rouiller et al., 1998; Andrés et al., 1998). Perhaps counter-intuitively, immunoflourescence analysis shows an apparent absence of resident ER proteins from virus factories relative to the rest of the cytoplasm (Andrés et al., 1998; Netherton et al., 2006). This led to the development of a model whereby a section of ER cisternae collapsed leading to the exclusion of most resident ER proteins, subsequently capsid and matrix proteins were recruited to opposite sides of this membrane and this would lead to the formation of the six-sided assembly intermediates and ultimately nascent virions (Andrés et al., 1998; Rouiller et al., 1998). This model explained the observations that ER proteins can be detected in viral membranes by electron microscopy or in purified virus by western blot, but appear to be absent at a larger scale when analysed by fluorescence microscopy. However, this model needs modification to fit with the more recent observation that the internal envelope is comprised of a single lipid bilayer. The question has become how do you derive a single lipid bilayer from an ER cisternae? One possibility is that viral membrane formation may be mechanistically related to lipid droplet formation (Heuser, 2005; Martin and Parton, 2006) or that viral infection ruptures existing cellular membranes which are then stablised by viral proteins (Chlanda et al., 2009, 2011).

Zipper-like stacks of membrane can also be detected in ASFV factories and maybe similar to the complex concentric whorls detected in factories of an iridovirus of *Rana temporaria* (Cunningham et al., 1996). These zipper structures can be readily detected in cells that have been infected under non-permissive conditions with inducible recombinant viruses where specific structural proteins are under the control of the lac repressor and are seen in cells infected with wild type virus as well (Andrés et al., 1998; Rouiller et al., 1998). ASFV zippered membrane stacks are generated in the absence of p72, pB438L, CAP80/pB602L, or p54 (Andrés et al., 2002a, 2002b; Epifano et al., 2006a, 2006b; García-Escudero et al., 1998). Similar stacks of membranes derived from the ER can be seen in cells infected with VACV that are unable to make major core protein A10L (Heljasvaara et al., 2001; Rodriguez et al., 2006). There is a large body of evidence that shows that these stacks are associated with the ER membranes (Andrés et al., 1998; Cunningham et al.,

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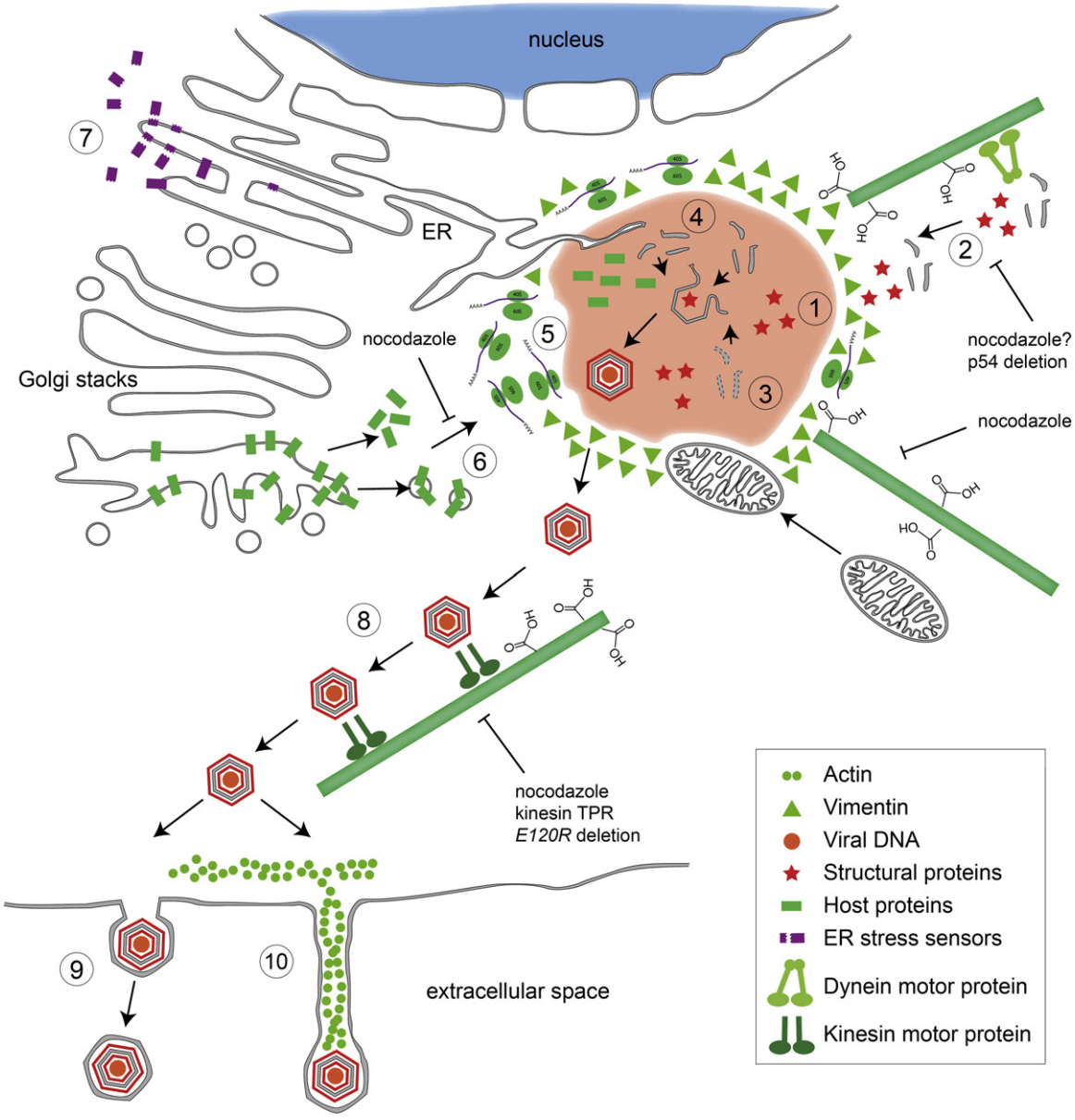

Fig. 3. Late events during ASFV infection: Viral replication occurs in perinuclear regions of the cell called virus factories ①. These contain viral DNA, viral membranes and viral structural proteins as well as certain host proteins (see text for details) and are surrounded by a vimentin cage and mitochondria. Localisation of virus factories to perinuclear sites requires microtubules which might be involved in delivering viral proteins or membranes ②. Viral membranes may also be derived by disruption and reformation of existing cellular membranes ③ or by collapse of organelles such as the ER that are in continuity with viral membranes ④. Ribosomes and viral RNA are also recruited to the virus factory, but in areas that are distinct from viral DNA ⑤. Components of the secretory pathway are redistributed by ASFV infection, particularly those of the *trans*-Golgi network ⑥, the net effect of this redistribution appears to be a slowing of protein trafficking to the cell surface and lysosomes. Disruption of the cellular homeostasis leads to the induction of cellular stress responses and the release of ATF6 and caspase-12 from the cytoplasmic face of the ER ⑦. Fully assembled virions are transported along microtubules to the plasma membrane by kinesin motor proteins ⑧ and are then exit the cell by budding ⑨ or travelling along actin projections ⑩. Drug treatments and conditions that block different steps are indicated.

1996; Rodriguez et al., 2006; Rouiller et al., 1998). The precise role of the zippered stacks of ER in the assembly of ASFV and how they relate to the inner envelope of the virus is unclear. Two types of zippered stacks were originally described, those associated with the periphery of the virus factory and those present within the factory (Andrés et al., 1998). Although zippers within the factory can form icosahedral shapes reminiscent of assembly intermediates these are morphologically distinct from true virions and are aberrant symmetrical structures formed by unprocessed pp220 and pp62 polyproteins (Andrés et al., 2002b). It is not known if the peripheral zippers are precursors to those located with the factory or whether the peripheral zippers are formed in the cytosol and then move into the factory.

How ASFV recruits and modifies host cell membranes is not certain, however two potential mechanisms have been described. Firstly, ASFV encodes a family of secretory proteins with conserved cysteine motifs, several of which are retained within the early secretory pathway by KDEL recycling motifs. Over-expression of one of these proteins causes the dramatic redistribution of resident ER proteins (Netherton et al., 2004a). It is possible that these proteins are involved in diverting host-cell membranes to the factory and/or causing their collapse prior to assembly. Interestingly, the KDEL receptor that is responsible for recycling KDEL bearing proteins within the secretory pathway has been implicated in the recruitment of membranes to VACV replication sites (Zhang et al., 2009). An alternative route is indicated by the formation of

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collapsed ER cisternae in cells over-expressing the structural protein p54. In addition to the C-terminal dynein binding motif p54 has a transmembrane domain and very short N-terminal luminal domain that contains a single cysteine. Disulphide bonding between p54 proteins across the lumen of the ER draws two membranes into apposition and initiates the collapse of a cisternae (Windsor et al., 2012). Crucially the cytosolic C-terminal region of the protein can interact with other p54 molecules in both a parallel and anti-parallel manner. This enables p54 to facilitate the ordered collapse of large stretches of multiple ER cisternae although the direct importance of this in infected cells is unclear (Windsor et al., 2012). As mentioned in Section 3.3 p54 is required for the correct localisation of membranes to the virus factory, however it is difficult to correlate the structures formed in transfected cells to those seen in cells infected with ASFV. The combination of p54 expression in combination with other as yet unidentified viral factors may lead to the delivery of viral membranes to the factory. However, neither of these models provide for a satisfactory derivation of the single membrane found in the final ASFV particle. It is possible that recruitment and collapse of ER membranes precedes formation of the viral membranes that are incorporated into virions.

5. The role of the cytoskeleton in virus egress

5.1. Virions use microtubules to exit the virus factory

ASFV also utilises the cytoskeletal network to facilitate its egress from the virus factory to the plasma membrane and out of the infected cell. Early work showed that after microtubule depolymerisation with colchicine ASFV particles were retained at the edge of replication sites (Carvalho et al., 1988) and that ASFV bound to microtubules *in vitro* (de Matos and Carvalho, 1993). These results were consistent with later immunofluorescence analysis of ASFV infected cells that showed that newly assembled viruses in the cytoplasm were frequently aligned with microtubules (Jouvenet et al., 2004). Further experiments with microtubule-destabilising agents showed that intact microtubules were required for the transport of virions from factories to the plasma membrane (Jouvenet et al., 2004). The late structural protein pE120R is essential for virus transport from assembly sites to the plasma membrane (Andrés et al., 2001). Repression of *E120R* gene expression leads to a phenotype that is very similar to that of cells treated with nocodazole or colchicine. ASFV particles assemble normally but the nascent virions remain confined to virus factories (Andrés et al., 2001). Moreover, EM analyses of virus structure suggest that pE120R is part of the outer layer of the capsid and therefore should have access to the microtubules motors required to drive transport to the plasma membrane (Andrés et al., 2001).

The kinesin family of motor proteins govern movement along microtubules from the MTOC towards the plasma membrane (Vale, 2003). Conventional kinesin is a heterotetramer of two heavy chains and two light chains. The N-terminus of each heavy chain binds microtubules and contains a motor domain. The C-terminus of a kinesin light chain protein binds cargo via six tetratricopeptide repeat (TPR) motifs, while the N-termini of the light chains provide the link to the heavy chains (Vale, 2003). Immunolabelling of infected cells showed that kinesin light chain was recruited into ASFV factories, and to most viruses in the cytoplasm. GFP-TPR fusion proteins provide a useful tool for identifying kinesin cargo and due to their inability to interact with microtubules act as dominant negative inhibitors when over-expressed. Expression of GFP-TPR by a viral promoter showed that the probe bound 100% of viruses indicating that virions were recognised as cargoes by kinesin. High-level expression of the GFP-TPR prevented ASFV anterograde transport and led to virions being retained within the virus factory (Jouvenet et al., 2004). These results demonstrate that virus infection induces the recruitment of conventional kinesin to virus factories and uses the motor to move progeny virions from the assembly sites to the periphery. As GFP-TPR-KLC over-expression prevented almost all virus dispersal from replication sites, conventional kinesin is probably the principal anterograde directed motor used by ASFV to reach the plasma membrane. It is likely that kinesin recruitment to virus factories is driven by late gene expression as the GFP-TPR signal was distributed in the cytoplasm early during infection and only appeared when p72 was expressed. It is possible that an ASFV structural protein, or perhaps correct structural maturation is responsible for kinesin recruitment. This is the case for VACV where the intracellular enveloped virus envelope protein A36R recruits conventional kinesin via a direct interaction with TPR-KLC (Ward and Moss, 2004). It will be interesting to see if pE120R and the TPR domain of kinesin interact during infection.

5.2. ASFV induce filopodia like projections at the plasma membrane

ASFV infection induces long unbranched actin projections that originate from the plasma membrane (Jouvenet et al., 2006). ASFV particles can also be detected in alignment with unbranched cortical actin fibres underneath the plasma membrane. The actin filaments present in the actin-projections were long, unbranched and parallel and are similar to those detected in filopodia. Electron micrographs revealed that the viruses at the tip of the projections were beneath the plasma membrane and are therefore intracellular. Each filopodia-like extension appeared to be associated with a single virus that was present at the tip of each structure. Live cell microscopy of ASFV infected cells showed that virion-tipped projections can grow and shrink over time and also suggested that pre-existing virion-tipped projections aided the growth of subsequent ones (Jouvenet et al., 2006). These movements may represent surfing of extracellular ASFV along filopodia, a phenomenon that has been described for several other viruses as well (Sherer et al., 2007).

6. ASFV disrupts organelle function and modulates cellular stress

6.1. ASFV redistributes the secretory pathway

As has been discussed above ASFV replication affects the distribution of the ER, the protein synthesis apparatus, mitochondria as well as the three main components of the cytoskeleton, microtubules, intermediate filaments and microfilaments. All of these organelles and cytoskeletal elements contribute to a greater or less extent to the correct localisation and function of later parts of the secretory pathway. Examination of the localisation of a number of different markers of the Golgi apparatus shows redistribution and partial fragmentation of this organelle in cells infected with ASFV (Andrés et al., 1998; Netherton et al., 2006). The effect on the *trans*-Golgi network (TGN) however is more profound. Staining for TGN46, a type I membrane protein that cycles between the TGN and the plasma membrane, showed that this protein was lost in cells infected with ASFV (McCrossan et al., 2001). The clathrin adaptor protein AP-1 and the Golgin p230 are both involved in protein trafficking to the plasma membrane (Brémond et al., 2009; Edeling et al., 2006; Lieu et al., 2008; Ohno et al., 1995) and these two proteins were redistributed to vesicles surrounding the virus factory. TGN46 and p230 signals co-localise in uninfected cells, but separate into distinct localisations in infected cells in a manner that was dependent on intact microtubules (Netherton et al.,

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2006). Interestingly, disruption of conventional kinesin results in the clustering of the intermediate filament protein vimentin, and mitochondria, near the centrosome (Gyoeva and Gelfand, 1991; Stenoien and Brady, 1997; Tanaka et al., 1998); and also alters the integrity of the Golgi apparatus (Allan et al., 2002). Therefore it is possible that recruitment of kinesin into virus factories may contribute to many of the observed organelle phenotypes in infected cells. However, kinesin light chain is not recruited to virus factories until late in infection (Jouvenet et al., 2004) and the disruption of the TGN begins around 10 hpi and is not dependent on late gene expression (McCrossan et al., 2001). Although the precise mechanism by which ASFV reorganises the secretory pathway and the TGN in particular is unclear, the redistribution appears to have functional consequences. Two model proteins, cathepsin D and the vesicular stomatitis virus glycoprotein (VSV-G), have been studied and the transport of both of these markers through the secretory pathway is retarded in ASFV infected cells (McCrossan et al., 2001; Netherton et al., 2006). ASFV appears to upregulate MHC-I protein levels during infection, but this upregulation is not matched by a concomitant increase in levels of MHC-I at the cell surface (Netherton et al., 2006). Slowing of bulk secretion by ASFV infection may contribute to the observed reduction in the cell surface expression of MHC-I and possibly other important immunoregulatory molecules as well.

6.2. ASFV induces and controls cellular stress responses

The effect of ASFV on cellular organelles may have resulted in the virus needing to evolve mechanisms to fine tune the various stress response pathways that detect imbalances in cellular homeostasis. The critical importance of the ER to ASFV replication coupled with the effect on secretion led us and others (Galindo et al., 2012; Netherton et al., 2004b) to test whether virus infection induced the unfolded protein response (UPR). ER stress leads to the phosphorylation of PKR-like ER kinase (PERK) and inositol response element 1 protein (ire1p) as well as release of activating transcription factor 6 (ATF6) and caspase-12 from the cytosolic face of the ER. These signalling events can lead to a decrease in protein translation, lipogenesis, the upregulation of ER chaperones and ultimately apoptosis through the action of caspase-12 and the transcription factor C/EBP homologous protein (CHOP). ASFV infection led to the induction of the ER chaperones calnexin and calreticulin, but not ERp57, PDI (Galindo et al., 2012) or crucially GRP78 (Galindo et al., 2012; Netherton et al., 2004b) which is normally strongly upregulated by ER stress. However ASFV infection did lead to the activation of caspase-12 and cleavage of ATF6 suggesting that virus infection does induce ER stress (Galindo et al., 2012). ASFV can inhibit induction of CHOP in response to treatment with a variety of different stress inducers (Netherton et al., 2004b). The DP71L gene encoded by ASFV is a homologue of the herpes virus ICP34.5 protein and can inhibit CHOP induction *in vitro* by dephosphorylating eIF2α (Rivera et al., 2007). Intriguingly, however deletion of DP71L shows that this gene is not required for ASFV to inhibit CHOP expression (Zhang et al., 2010) raising the possibility that ASFV may encode novel regulators of ER stress. It is perhaps not surprising that ASFV closely modulates the ER stress response as the calcium gradient across the ER is critical for virus assembly (Cobbold et al., 2000). ASFV may also modulate other stress response pathways as infection also induces the expression of cpn60, mortalin (also known as mthsp70 or p74) and hsp70 (Netherton et al., 2006; Rojo et al., 1998). As mentioned previously hsp70 is recruited to virus factories and therefore ASFV may induce expression of this gene in order to facilitate the folding of viral structural proteins. Mitochondria are actively recruited to the periphery of virus factories in a manner that is also dependent on microtubules and careful morphological analysis showed that recruited mitochondria are actively respiring, suggesting they are producing ATP that is required for viral replication and assembly (Cobbold et al., 2000; Rojo et al., 1998). Alternatively mitochondrial recruitment may be part of an antiviral response involving mitochondrial driven apoptosis and ASFV encodes both a Bcl-2 and an IAP homologue that could attenuate this. Mortalin and hsp60 are regulated by the mitochondrial unfolded protein response (mtUPR) and their upregulation in ASFV infected cells in the absence of mitochondria biogenesis (Rojo et al., 1998) suggests induction of mtUPR. However, the available evidence suggests that the mtUPR is regulated by CHOP (Aldridge et al., 2007; Zhao et al., 2002) and as outlined above this transcription factor is actively inhibited during ASFV infection. The induction of mitochondrial stress genes in the absence of an important regulator of their expression suggests that ASFV may modulate the mtUPR as well as the conventional UPR. Unpicking the mechanisms by which ASFV selectively controls these pathways may reveal novel features of the two UPRs and suggests ASFV represents a useful model for study of cellular stress responses. However, as discussed in Section 2 ASFV infection induces profound changes in the nucleus and it is likely that these changes will have downstream effects on pathways that would normally lead to the transcription of host genes.

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