Vaccinia virus requires heat-shock proteins for genome replication and virion assembly

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Declaration

I, Harriet Mok, confirm that the work presented in this thesis is my own. Where information has been used and derived from other sources, I confirm that this has been indicated within the thesis and referenced accordingly.

Signed…………………………………………….………

Date………..……………

Abstract

The large double-stranded DNA virus, vaccinia virus (VACV), is closely related to variola virus, the causative agent of smallpox. While the poxvirus lifecycle has been studied for over 50 years, much is still unknown about the complex multi-step process of genome uncoating. Viral and cellular factors involved in poxvirus uncoating have been identified, including host cell proteasomes, ubiquitin, and heat-shock proteins (HSPs), which play a critical role in protein folding and the prevention of protein aggregation. Although HSPs have been linked to the lifecycle of many viruses including VACV, the role of these proteins in poxvirus infection has not been defined. Using various small compound inhibitors and RNAi, in combination with a battery of virus-specific assays, I show that heat-shock proteins are required for multiple stages of the VACV lifecycle. I demonstrate that Hsp90 is required for two stages of infection: release of the genome from the viral core and assembly of new virions. Following the finding that HSF1, the transcription factor for inducible HSPs, is required for infection, I also identify the Hsp105 as the inducible HSP required for post-replication formation of nascent virions. Given the clinical relevance of HSP inhibitors these results highlight HSPs as potential anti-poxviral drug targets. Collectively, through this work I define new cell factors required for poxvirus infection and demonstrate that subjugation of cellular HSPs during the poxvirus lifecycle is far more complex than previously appreciated.

Impact Statement

Viral diseases represent a major global threat that cost millions of lives each year. HIV, influenza, and the more recent outbreaks of Ebola and Zika virus, place a huge burden on global healthcare systems, and affect the economic stability of developing nations. This continual threat is combatted with research into the basic biology of viruses and their interactions with host cells that serves to aid the development of effective anti-virals and vaccines. In contrast, the existence of viruses is not all negative. At the same time they are of increasing importance in biotechnology, as viruses are more often used as vectors for vaccines, and gene and cancer therapies. This again emphasises the importance of understanding viruses and their complex lifecycles, in order to utilise them for our own benefit.

Smallpox, caused by the poxvirus variola, remains the deadliest virus in human history, having killed more than 500 million people. To date, it is the only human virus to have been successfully eradicated by the WHO. The smallpox vaccination programme has since been suspended, thus leaving the current population vulnerable. The threat of smallpox re-emergence through bioterrorism, and possible outbreaks of zoonotic poxviruses, such as monkeypox, confirms the need for continued research into this family of pathogens. Understanding the role of host proteins in poxvirus infection will enable greater preparedness for potential poxvirus outbreaks, through developments in vaccines and anti-viral treatments.

This study demonstrates how this ancient family of viruses use a highly conserved set of cellular proteins, molecular chaperones, for various stages of their lifecycle. Other viruses have also been shown to require these proteins, although the mechanisms of how they are needed have not been well defined. This study will not only enable a deeper understanding of the poxvirus

lifecycle and the interactions with host factors, but may also provide insight into how other viruses use this set of proteins. Molecular chaperones are of the upmost importance in maintaining a healthy cell, and are frequently associated with diseases such as cancer, where abnormal cells exploit their function to promote tumour proliferation and invasion. They have therefore been targeted for the development of inhibitory compounds. For this study, we utilised several inhibitors, which have been evaluated for cancer treatment in the clinic, and demonstrated their anti-viral activities. If these inhibitors, or their derivatives, were to gain approval for clinical use, then there could be potential for re-purposing them as broad-spectrum anti-viral agents.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

17AAG	Tanespimycin
7PC	7-protein complex
AD	Alzheimer's disease
AraC	Cytosine arabinoside
CEV	Cell-associated extracellular virion
CHX	Cycloheximide
CLEM	Correlative light and electron microscopy
CPXV	Cowpox virus
Cul3	Cullin 3
EdC	5-ethynyl-2'-deoxycytidine
EdU	5-ethynl-2'-deoxyuridine
EEV	Extracellular enveloped virion
EFC	Entry-fusion complex
EM	Electron microscopy
ER	Endoplasmic reticulum
EV	Enveloped virion
GA	Geldanamycin
GAG	Glycosaminoglycans
HD	Huntingdon's disease
HepB	Hepatitis B
HOP	Hsp70/hsp90 organising protein
HSE	Heat shock element
HSF	Heat shock factor
HSP	Heat shock protein
HSV	Herpes simplex virus
HU	Hydroxyurea
IFN	Interferon
IV	Immature virion
IVN	Immature virion with nucleoid

LB	Lateral body
MOCV	Molluscum contagiosum
MPXV	Monkeypox virus
MuV	Mumps virus
MV	Mature virion
ORF	Open reading frame
PD	Parkinson's disease
PS	Phosphatidylserine
PTM	Post-translational modification
Rbx1	RING-box protein 1
RNP	Ribonucleoprotein
RSV	Respiratory syncytial virus
SIM	Structured illumination microscopy
TPR	Tetratricopeptide repeat
UPS	Ubiquitin-proteasome system
VACV	Vaccinia virus
VARV	Variola virus
VITF	Vaccinia intermediate transcription factor
WT	Wild-type
WV	Wrapped virion

I Introduction

1 *Poxviruses and vaccinia virus*

1.1 Poxviruses: A general overview

Poxviruses represent some of the largest and most complex mammalian viruses that have co-evolved with hosts over thousands of years, from the insect poxviruses, *Entomopoxvirinae*, to those that can infect vertebrates, *Chordopoxvirinae* (Hughes et al., 2010), however the definitive origin of poxviruses still remains enigmatic. Whilst poxviruses are able to utilise their hosts in many ways, unlike other viruses, they do not exploit the cellular DNA replication machinery, and instead encode a set of polymerases, transcription factors, and other necessary proteins, to enable infection in cells independent of the nuclear machinery. This enables them to be unique in their replication cycle, which occurs exclusively in the cytoplasm of the cell. Poxviruses encode more than 260 open reading frames within their double stranded DNA genome (Cyrklaff et al., 2005, Moss, 2013), including many proteins that enable subversion of the host immune system and promote successful replication, as well as proteins that are homologs to host immune signalling molecules, gained through horizontal gene transfer (McFadden et al., 1995).

The poxvirus host range is extremely broad, and although the primary cell type infected is considered to be epithelia, due to their most common route of infection (airways and skin lesions), they are able to infect nearly every cell type (Hruby, 1990, McFadden, 2005, Vermeer et al., 2007). The most famous of the classified poxvirus families are the chordopoxviruses, of which there are eight genera that are closely related, and infect a wide range of hosts. Chordopoxviruses include the human pathogens molluscum contagiosum virus (MOCV) and variola virus (VARV), but also other zoonotic viruses such as monkeypoxvirus (MPXV), cowpoxvirus (CPXV), and vaccinia virus (VACV). Whilst VACV is known for its use as the model poxvirus, and can also be a

model for other dsDNA viruses such as iridoviruses and asfiviruses, CPXV is most notable for its use by Edward Jenner in establishing the initial idea for vaccination, in which he showed inoculation with this virus could protect against the much more severe smallpox infection. Smallpox, caused by VARV, presents itself with non-specific symptoms such as fever and fatigue, but is then followed by the development of highly contagious sores and lesions that spread across the whole body of an infected individual. These sores make smallpox highly contagious, through direct contact with the pox lesions or inhalation of droplets. Whilst some poxvirus infections remain restricted to the skin, systemic spread of VARV to lymphoid organs and hematopoietic cells, and pulmonary and neurological complications, is common, and deadly, with death in up to 50% of cases. It is known as one of the most deadly diseases in the history of mankind and potentially the most ancient, with around 300 million deaths attributed to this VARV infection in the 20th century alone (Geddes, 2006) and smallpox scars seen on the mummified remains of Ramses V (Strouhal, 1996).

Over 150 years after Jenner's original CPXV inoculations, in 1986 after many successful developments in vaccination and a WHO immunization campaign, smallpox was declared eradicated (Okwo-Bele and Cherian, 2011, Verardi et al., 2012). Although a huge advancement in terms of global vaccination campaigns, and preventing millions more smallpox related deaths globally, poxviruses still remain a threat, in the form of VARV being used as a bioterrorist agent in combination with very few people being protected by vaccination, and in the spread of the other aforementioned human poxviruses. MPXV, for example, is fatal in around 10% of cases, and although endemic to Africa, there have been recent outbreaks in the USA, and cases also detected in the UK in passengers returning from Nigeria (Nolen et al., 2016, Vaughan et al., 2018).

To combat this threat, until recently, the treatment of poxvirus infections relied on more general anti-virals, such as cidofovir, which was approved for treatment of cytomegalovirus, and has a broad spectrum of activity against other DNA viruses, including that of VACV, MXPV, and VARV. Problems with

specificity and bioavailability however have left the search for a more effective treatment open (De Clercq, 2002). In 2018, Tecovirimat, also known as TPOXX, became the first globally approved orthopoxvirus specific anti-viral, approved by the FDA for treatment of smallpox infections in adults and children (Hoy, 2018, Merchlinsky et al., 2019). Although a huge advancement in poxvirus therapeutics, this remains the only orthopoxvirus specific anti-viral, and its interactions with other drugs and vaccines still need to be studied. Continued investigation on poxvirus biology, therefore, is critical for the development of future treatment options, and the generation of additional vaccines should the threat of a pandemic arise.

Of the vaccines used for the WHO eradication of smallpox, one of the live strains utilised the prototypic poxvirus, VACV, as the infectious agent, as it shares high genetic similarity with VARV. In addition to its use in the smallpox vaccine, and as the model poxvirus in the lab, it also has become increasingly popular as a vector and for use in gene therapy (Smith and Moss, 1983, Hruby, 1990, Dai et al., 2014). So whilst it remains vital to study viruses and their infectious capabilities and the immune responses of hosts, it is becoming ever more important to also learn about their function as tools to probe cellular mechanisms and in medical technology.

1.2   Vaccinia virus morphology

The prototypic poxvirus, VACV, produces two infectious forms during its replication cycle; mature virions (MVs), and enveloped virions (EVs); EVs contain an MV surrounded by a second lipid bilayer (Fig1.1). VACV virions are large, brick-shaped particles, approximately 360 x 270 x 270 nm in size, and encode approximately 200 genes within the genome (Goebel et al., 1990, Johnson et al., 1993, Roos et al., 1996, Hollinshead et al., 1999, Griffiths et al., 2001, Sodeik and Krijnse-Locker, 2002, Cyrklaff et al., 2005, Lefkowitz et al., 2005).

The VACV particle was first visualised by electron microscopy (EM) (Nagler and Rake, 1948), with later studies then revealing a more detailed look at the

sub-structures that reside within the single lipid bilayer viral membrane; it contains a dumbbell shaped core, which packages the dsDNA genome, and two lateral bodies (LBs), which flank the core (Fig.1.1) (Easterbrook, 1966, Ichihashi et al., 1984, Goebel et al., 1990, Hollinshead et al., 1999, Pedersen et al., 2000, Cyrklaff et al., 2005, Condit et al., 2006). Various approaches have been used to identify proteins that reside in each structure; virus fractionation studies enabled separation of core and membrane proteins (Ichihashi et al., 1984, Jensen et al., 1996), immuno-EM has been used to identify and confirm the localisation of various proteins within the virus (Roos et al., 1996, Schmidt et al., 2013), and more recently mass spectrometry has been used to study the composition of the particle (Chung et al., 2006, Yoder et al., 2006). The overall consensus now is that the VACV particle contains over 80 different viral proteins, with at least 25 of these making up the complex virus membrane.

Whilst the viral core is required for protection and condensation of the large genome, the LBs are proteinaceous structures that are not well studied. Only recently have a few proteins and their functions been identified as residing in the LBs, thus leading to speculation over the role of these structures in the viral lifecycle. It has been suggested that they are responsible for immune evasion, due to their early role in the infection cycle and the pre-packaging of proteins that function as cytoplasmic immuno-modulators (Schmidt et al., 2013).
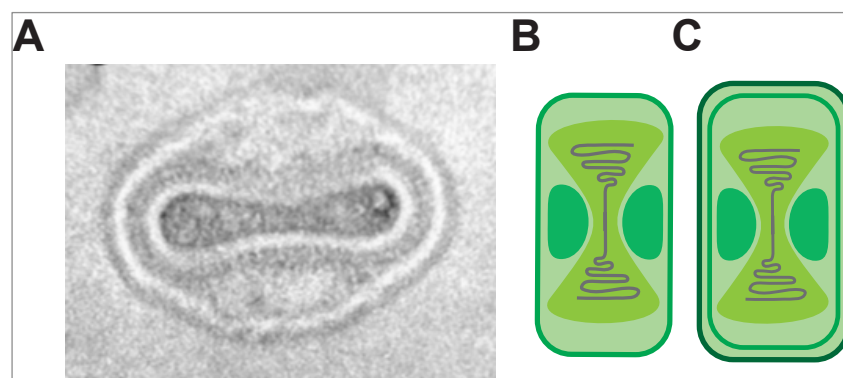
Figure 1. 1 Vaccinia virus structure

A) Electron micrograph of a VACV particle (Hollinshead et al. 1999). VACV produces two infectious particles, B) the mature virion (MV), and C) the enveloped virion (EV).

1.3 The vaccinia virus lifecycle

VACV is a promiscuous virus with a broad host range; it has no single cell-surface receptor that dictates its specificity and is therefore able to infect a variety of cell types (Chung et al., 1998, Hsiao et al., 1998, Lin et al., 2000). Although epithelia is considered to be the primary cell type due to it being the most common route of infection via the airways and skin (Hruby, 1990, McFadden, 2005, Vermeer et al., 2007). Whilst VACV is able to enter dendritic cells, infection has seen to be aborted and VACV is unable to induce maturation (Engelmayer et al., 1999, Drillien et al., 2000). For these reasons, cell lines are commonly used for studying VACV and establishing further knowledge on its replication cycle. Detailed studies of poxvirus infection of primary human cells have not been conducted, and therefore life cycles have been established in immortalised cell lines.

The complex lifecycle of VACV takes place in the cytoplasm of infected cells. It begins with virions entering the cell via macropinocytosis, using apoptotic mimicry to induce the macropinocytic response in cells, and allow for uptake of the virus (Fig.1.2) (Mercer and Helenius, 2008, Mercer and Helenius, 2010, Mercer et al., 2010). Macropinosomes then mature in the cytoplasm, thereby releasing the viral core into the cell cytosol (Armstrong et al., 1973, Carter et al., 2005, Rizopoulos et al., 2015). Following this, viral cores are activated and the first set of its viral genes, the early genes, which include all the viral proteins required for genome replication, are transcribed and delivered to the cytoplasm through pores on the surface of the viral cores. These vRNAs are then translated by host ribosomes, which can occur as early as 2 hours post infection (hpi) (Kates and McAuslan, 1967, Munyon et al., 1967). The viral genome is then uncoated and released entirely in to the cytoplasm where it becomes accessible to the viral DNA replication machinery (Mercer et al., 2012, Schmidt et al., 2013). DNA replication occurs in viral factories, which can be visualised in the cytoplasm from 4 hpi, followed by expression of intermediate genes, largely encoding late transcription factors, and late viral genes. The late genes encode for structural proteins that are then required for the assembly and formation of new virions, as well as early transcription

factors that are packed into the assembling virions for use in secondary infected cells (Moss, 2013). The proceeding virion assembly steps occur within electron-dense areas of the cytoplasm, termed replication sites, located near the nucleus in the vicinity of the MTOC (Tolonen et al., 2001). Viral membranes begin forming as viral crescents, which enclose a portion of the replication site, presumed to contain all of the factors required to generate an infectious virion, with the exception of the viral genome (Szajner et al., 2003, Szajner et al., 2004). Once formed, these immature virions (IVs) acquire viral DNA through a poorly characterized injection mechanism, at which stage proteolytic processing is required to transform IVs to MVs (Byrd et al., 2002, Ansarah-Sobrinho and Moss, 2004, Byrd and Hruby, 2005). MVs then either remain in the cell as single membrane virions until lysis at around 72 hpi, or can be further transformed by gaining two additional membranes to become wrapped virions (WVs) inside the cell (Condit et al., 2006, Sivan et al., 2016). These WVs can then be transported to the plasma membrane where they are able to fuse their outer-most membrane and be released as double-membrane bound extracellular enveloped virions (EEVs) or remain attached to the cell as cell-associated enveloped virions (CEVs). These particles are released prior to cell lysis, from around 12hpi, thereby generating multiple routes of further infection through the two types of particles.
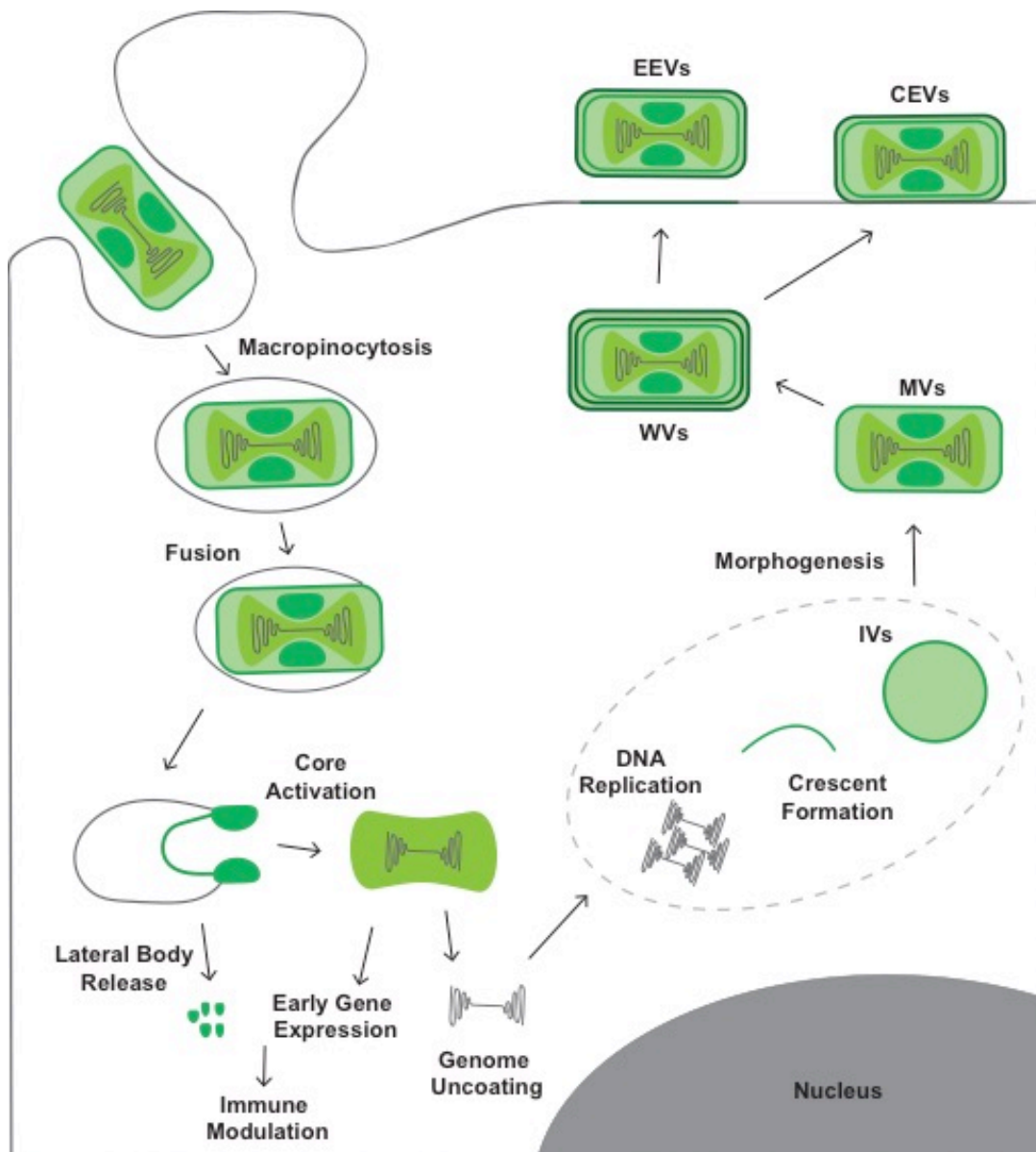
Figure 1. 2 The lifecycle of vaccinia virus

The lifecycle of VACV occurs in the cytoplasm, replicating its DNA in peri-nuclear sites, and forming immature virions (IVs) before generating mature, and enveloped virions (MVs and EVs). Infectious particles then go on to infect other cells through egress or cell lysis.

1.3.1   Virus entry and fusion

VACV entry into host cells is initiated by attachment of the MVs to factors on the cell surface, such as glycosaminoglycans (GAGs), extracellular laminin, heparin sulfate, and chondroitin sulfate (Chung et al., 1998, Hsiao et al., 1998, Lin et al., 2000, Moss, 2016). Although this is the primary mechanism of binding, there is also evidence for MVs binding to cells without GAGs, which

may account for the ability of VACV to infect a wide range of cell types (Foo et al., 2009).

Entry mechanisms of poxviruses have been controversial, and may be strain-dependent, but recent studies suggest that virus entry is pH dependent, with the main route being through macropinocytosis (Fig.1.3) (Townsley et al., 2006, Huang et al., 2008, Mercer and Helenius, 2008, Sandgren et al., 2010, Chang et al., 2019). This endocytic process, which involves a large amount of plasma membrane activity, is used by cells for the non-selective internalisation of fluid and membrane (Mercer and Helenius, 2009), and occurs during VACV infection when the virus induces a signalling network to trigger uptake by the target cell it is engaged with. Phosphatidylserine (PS) on the MV membrane is required for infection, as it activates receptors in this signalling cascade leading to actin rearrangement and engulfment of the bound virions (Mercer and Helenius, 2008). PS marks apoptotic bodies for endocytic uptake, and it is its exposure on the virus membrane that leads to events that are similar to apoptotic clearance, so apoptotic mimicry can be used by VACV to enter cells. Although macropinocytosis is the main route of entry for both MVs and EEVs, it appears that a minority can also use the route of plasma membrane fusion, where they are able to fuse their outer viral membrane with that of the cell (Carter et al., 2005, Schmidt et al., 2011).

Following virus uptake, the macropinosome undergoes maturation, with the resulting lowered pH activating the entry/fusion complex (EFC) that resides in the MV membrane (Townsley et al., 2006, Rizopoulos et al., 2015). This 11-protein complex, which is highly organised into functional domains (Gray et al., 2019), brings the viral and endosomal membranes together and mediates their fusion, resulting in release of the viral core into the cytosol. It is at this point, where the lateral bodies separate from the viral core, that the enzymatic and immunomodulatory LB proteins are released for perturbation of the cell, such as H1, which dephosphorylates host STAT1 to inhibit interferon (IFN) mediated antiviral responses. The lateral bodies are then degraded in a proteasome-dependent manner, whilst the core moves into the cytoplasm (Fig.1.2) (Schmidt et al., 2013).
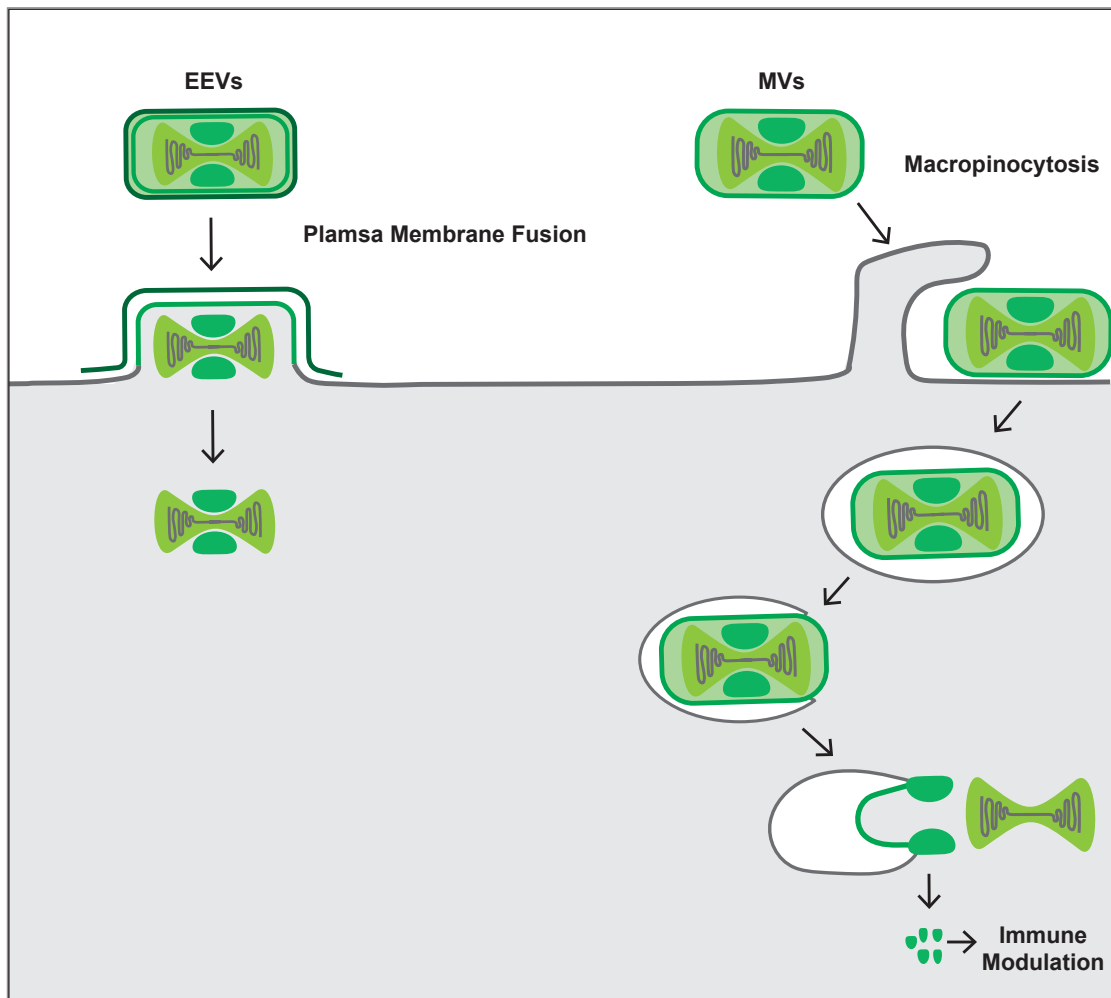
Figure 1. 3 Macropinocytosis is the main route of VACV entry

Entry of both MVs and EEVs occurs through macropinocytosis, whereby apoptotic mimicry triggers uptake of the virus to the cells. The alternative route of entry is through fusion of the viral membrane at the plasma membrane.

1.3.2 Viral genome uncoating and DNA replication

Genome packaging allows for the poxvirus dsDNA to be condensed and protected by the viral core during the extracellular phases of its lifecycle. Uncoating and viral core disassembly must therefore occur to allow the replication machinery access to the genome at the right time during its infection cycle. Poxviruses accomplish this in a unique two-step uncoating process.

Upon release into the cytosol, VACV cores undergo various morphological changes through the first stage of uncoating; core activation (Fig.1.4). As the core expands from its biconcave dumbbell to an oval shape, disulphide bonds in the core are reduced (Locker and Griffiths, 1999, Schmidt et al., 2013). From within this activated core, VACV has the ability to transcribe a first set of its viral genes, as the virus packages polymerases and transcription factors that are pre-bound to early promoters (Rohrmann et al., 1986, Broyles, 2003, Yang and Moss, 2009). Early mRNAs from approximately 80 genes are then exported into the cytosol where they are translated by host ribosomes (Chou et al., 2012). Amongst the early genes are proteins that are required for the proceeding steps; genome uncoating, DNA replication, intermediate gene transcription, and immuno-modulation. The second stage of uncoating requires the early protein, D5, and is known as the actual uncoating step where the genome is released from the viral core (Fig.1.4).

D5 is a multifunctional AAA+ ATPase, which has been identified as the viral factor needed for genome uncoating (Kilcher et al., 2014). Although the detailed mechanism of D5 mediated uncoating is still unknown, it has been shown that it gets recruited to subdomains of incoming cores and its ATPase activity is required (Kilcher et al., 2014). D5 is a highly conserved protein that is encoded by all poxviruses, and contains a primase and helicase domain in addition to its ATPase (Pedley and Cooper, 1987, Evans et al., 1995, Pedersen et al., 2000). Further to the requirement of D5, the genome-

uncoating step has also been shown to require the ubiquitin-proteasome system (UPS); proteasomal degradation of the core is needed, alongside ubiquitination events (Fig.1.4) (see section 3.1) (Satheshkumar et al., 2009, Mercer et al., 2012, Schmidt et al., 2013).

Once the core is uncoated and degraded, and the genome is successfully released, replication of the dsDNA occurs at about 2 hours post infection in viral factories. These infection specific cytoplasmic clearings are of uniform density, absent of most cellular organelles, and often surrounded by endoplasmic reticulum (ER) membrane (Tolonen et al., 2001). The virus encodes the replication machinery required for the replication process; including the DNA polymerase E9, the single-stranded DNA binding protein I3, and D5, the helicase primase, is again needed (Boyle et al., 2007, Moss, 2013, Hutin et al., 2016, Czarnecki and Traktman, 2017).
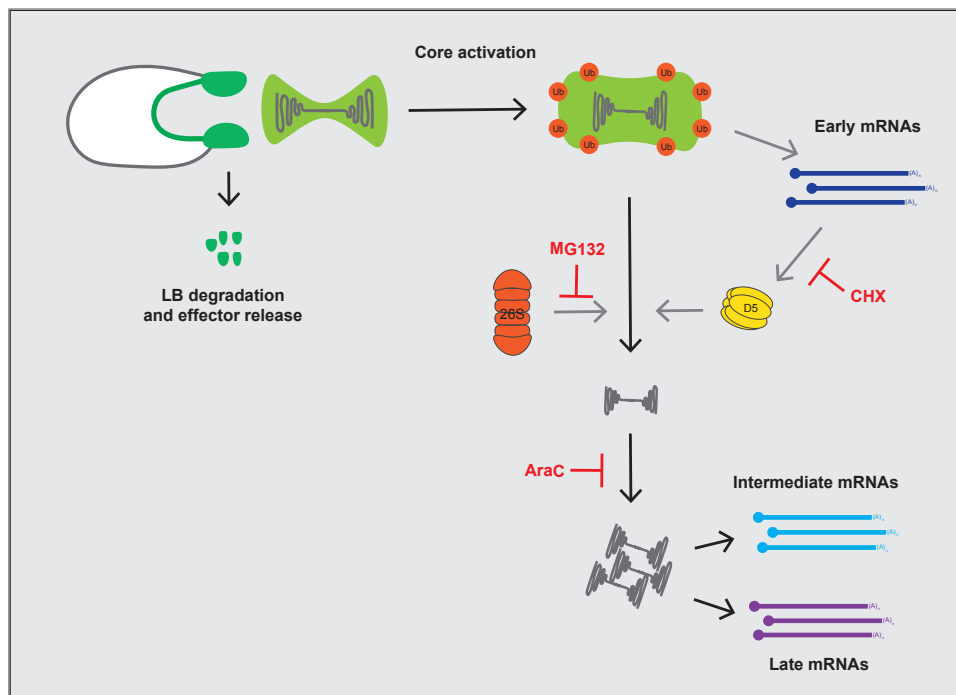
Figure 1. 4 VACV genome uncoating and replication

VACV genome release occurs in a two-step process. Upon fusion of the virus to the macropinosome membrane, the core is released into the cytosol where it is activated. The UPS, along with the viral factor, D5, is required for full genome release. DNA replication can then occur and intermediate and late genes be expressed.

The viral E9 DNA polymerase shares homology to eukaryotic polymerases, and synthesises VACV DNA from the 3' to 5' end, via the rolling hairpin strand displacement mechanism (Tattersall and Ward, 1976, Wang et al., 1989). Concatemers of two genomes are generated in DNA replication, through extensions of nucleotides towards the hairpin termini, and are later resolved into singular genomes by the resolvase, A22, encoded by the virus (Moyer and Graves, 1981, DeLange and McFadden, 1987, Garcia et al., 2000).

Although encoding its own machinery for the DNA replication process, it now appears that VACV also sequesters host proteins for viral replication; components of cellular DNA replication and repair machinery are seen to associate with the viral polymerase or viral genome, and the DNA damage response pathway of host cells is activated prior to genome release, again demonstrating the reliance of VACV on host factors (see section 3.1) (Postigo et al., 2017).

Successfully replicated genomes provide the template for the next two rounds of temporal gene expression, that of the intermediate and late genes; accounting for 93 open reading frames (ORFs) within the virus, which can then be transcribed in the cytoplasm.

Intermediate transcription occurs after DNA replication, and requires early gene products for synthesis of the intermediate mRNAs. Subunits of the vaccinia intermediate transcription factor (VITF), for example, are encoded within the early genes, and its assembly into a multimeric complex occurs at the intermediate promoter sequence (Rosales et al., 1994a, Rosales et al., 1994b, Sanz and Moss, 1999). Intermediate genes encode for late transcription factors, DNA binding and packaging proteins, and proteins that are associated with the viral core (Yang et al., 2010, Yang et al., 2011). Whereas late viral genes mainly encode for proteins that make up the newly formed virion membranes and sub-structures, and the enzymes required for this morphogenesis step. In turn, the transcription of the late viral genes, which makes up the final stage of the temporal gene cascade, is dependent upon intermediate gene products. Proteins such as A3, A10, and L4, the

major structural core proteins (Katz and Moss, 1970a, Resch et al., 2007, Jesus et al., 2015), F17, the abundant lateral body protein (Wickramasekera and Traktman, 2010, Schmidt et al., 2013), and components of the entry-fusion complex are all encoded late.

1.3.3 Morphogenesis, virion formation, and release

The process of MV formation is a highly complex process that can been visualised using EM; although the details of pathways and factors involved still remain unsolved (Dales and Siminovitch, 1961, Dales et al., 1978, Condit et al., 2006). After genome replication, assembly of IVs occurs in the cytoplasmic viral factories, where replicated viral DNA and late proteins accumulate (Fig.1.5). The first morphological form of VACV assembly is the single lipid bilayer crescent, which uses the viral protein, D13, as a scaffold to facilitate generation of a membrane layer associated with a honeycomb lattice (Heuser, 2005, Szajner et al., 2005). It has only recently been established that these viral crescents derive from endoplasmic reticulum membranes, and that they are stabilised by viral proteins at either end of the crescent, in addition to the D13 scaffolding (Bahar et al., 2011, Weisberg et al., 2017, Moss, 2018). The viral membrane crescents grow in length over time, whilst maintaining their curvature, until they close in on themselves to form spherical IVs, which package dense viral matter, termed viroplasm, which contains the constituents of the virion. This viroplasm is more electron-dense than the surrounding viral factory, and is also seen to localise to the crescents as they elongate, before they close to spheres, and also as large dense masses within the replication sites. At least seven VACV gene products are required for this process of membrane and viroplasm association and IV formation, and of these, six belong to the complex of proteins termed the seven-protein complex (7PC). Within the 7PC, the proteins A15, A30, D2, D3, G7, and J1, are found alongside the viral kinase F10. Mutations within this complex, result in defective wrapping of the viroplasm, with large areas still remaining in replication sites, and empty IVs forming (Cassetti et al., 1998, Chiu and Chang, 2002, Chiu et al., 2005). The kinase member, F10, is shown to associate to membranes and phosphorylate both membrane and core

proteins, playing a crucial role in virus assembly (Derrien et al., 1999, Szajner et al., 2004, Punjabi and Traktman, 2005). In addition, many of these proteins are found packaged in the viroplasm, and are shown to interact with viral cores, suggesting a role for this complex in the association of viroplasm with viral membranes (Szajner et al., 2001a, Szajner et al., 2001b). Further to members of the seven-protein complex, the core protein p4a, encoded by A10, is required for IV formation, and gets proteolytically processed into two polypeptides, which are packaged into virions, and a smaller peptide, who's fate is still undetermined (Wittek et al., 1984, VanSlyke et al., 1991a, Vanslyke et al., 1991b, Vanslyke and Hruby, 1994, Cudmore et al., 1996). When the gene product is absent in inducible mutants of A10, viral crescents appear normal in the replication sites, but IV formation is impaired; with no viral matter inside or containing unusual dense DNA material (Heljasvaara et al., 2001).

Following successful IV formation, these intermediates progress to a form of virus referred to as immature virions with nucleoid (IVN), where the viral DNA is injected and condenses into a nucleoid, and can be visualised in EM (Morgan, 1976, Condit et al., 2006). The initial mechanism of how newly synthesised viral genomes are trafficked to, and packaged within IVs, is not well understood, other than it occurs independently from morphogenesis. The viral core and lateral bodies are then able to form within the intact viral membrane of an IVN, and in the process the immature form matures into a MV, which are found outside, at the peripheries of the viral factory (Katz and Moss, 1970a, Katz and Moss, 1970b, Ansarah-Sobrinho and Moss, 2004). Although these separate forms of VACV virions can be visualised using EM, the processes of how each form progresses from one to the other is poorly understood.

This maturation step involves the formation of disulphide bonds in structural proteins of the membrane and core, and proteolytic cleavage carried out by the viral protease I7, as well as proteins from the previously mentioned 7PC (Locker and Griffiths, 1999, Senkevich et al., 2002, Byrd et al., 2002, Byrd and Hruby, 2005, Novy et al., 2018). The D13 membrane crescent scaffold is

removed, through proteolysis of the A17 membrane protein by the viral protease I7, and at least three viral core proteins get cleaved at the time of maturation, with their final cleavage products making up the structure of the inner and outer core (Katz and Moss, 1970a, Katz and Moss, 1970b, VanSlyke et al., 1991a, Vanslyke et al., 1991b, Vanslyke and Hruby, 1994, Ansarah-Sobrinho and Moss, 2004, Unger et al., 2013). The major core protein p4a, encoded by the A10L gene, is amongst the most abundant components of the VACV virion. As previously mentioned, it is synthesised as a late protein, and is one of the three core proteins to be post-translationally cleaved into smaller peptides (Heljasvaara et al., 2001); the cleavage products are associated with the core of the MV particle, specifically with the outer core structure (Vanslyke and Hruby, 1994). Additionally, this crucial activity of the I7 protease is dependent on the lateral body phosphoprotein, F17, and if absent, core precursors do not undergo cleavage and abnormal IVs result (Hiller and Weber, 1982, Kao and Bauer, 1987, Zhang and Moss, 1991, Schmidt et al., 2013).

The majority of MVs accumulate in the cytoplasm of the cell during infection, and are eventually released when the cell lyses at around 72 hours post infection. These particles, released late in infection, are important for host-host spread. A sub-set of particles, however, acquire two additional membranes, from 12 hpi, to become WVs at wrapping sites in the cytoplasm, at which point they are transported to the plasma membrane along microtubules, where the outer envelope fuses with the cell membrane to release the double-membraned EV outside of the cell (Fig.1.5) (Smith et al., 2002, Roberts and Smith, 2008, Leite and Way, 2015). The two additional membranes of WVs have been shown to derive from the trans-Golgi network or early endosomes, with the final released EV consisting of the original MV particle surrounded by an additional membrane that has at least 6 additional unique proteins (Hiller and Weber, 1985, Tooze et al., 1993, Sivan et al., 2016). The wrapping process of MVs, and transport of wrapped particles to the plasma membrane has been shown to be dependent on several viral proteins, including A27, in addition to microtubules and their motor proteins (Dodding et al., 2009, Dodding et al., 2011, Bidgood and Mercer, 2015,

Carpentier et al., 2015). It has been proposed that the MV particles that go on to become EVs may already be destined to do so, due to a variation in their MV membrane composition and lack of the A25-A26 fusion suppressors, however this is still unclear (Howard et al., 2008).

Most EVs remain associated to the cell after fusion with the plasma membrane, as CEVs. It is signalling by viral proteins, particularly A36, which initiates the formation of actin tails at these sites that then enables movement of the bound virus away from the primary infected cell (Fig.1.5) (Frischknecht et al., 1999, Smith et al., 2002, Doceul et al., 2010). EVs can therefore mediate spread between neighbouring cells within an infected host with EV spread occurring prior to MV release, at about 8 hpi; the cell-free EVs enable spread between tissues (Payne, 1980, Smith et al., 2002).
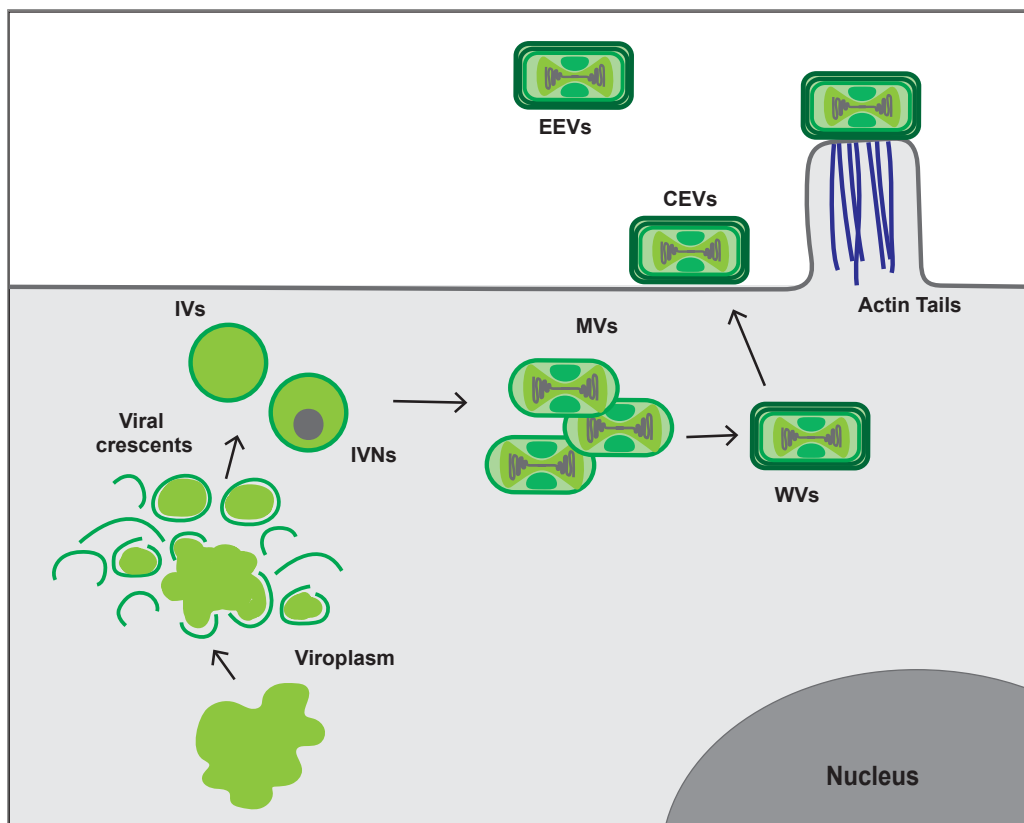
Figure 1. 5 Nascent virion formation

After viral DNA replication, viroplasm accumulates within replication sites, and localises to viral crescents, which begin to form viral membranes. Crescents wrap viroplasm and fuse to form IVs. Viral DNA is encapsidated and condenses to form IVNs, before proteolytic cleavage to form MVs. A subset of MVs are wrapped to form WVs, which become CEVs and EEVs through actin tail polymerisation to propel virions away from the cell membrane.

1.3.4   Summary

In order to form its two infectious particles, MVs and EVs, VACV undergoes a complex replication cycle in the cytoplasm of an infected cell. As a large virus that encodes many genes, vaccinia packages most of the proteins required for the various stages of replication. The activity of these viral proteins is critical to ensuring new virion production, and modulating cellular activity for the benefit of the virus. In addition to its own proteins, it is evident that VACV also requires action of cellular proteins.

2   Heat shock proteins

2.1   Overview of molecular chaperones

For proteins to carry out their function in a cell they need to be folded correctly. This correct folding of newly synthesised proteins requires the interaction of protein cofactors known as molecular chaperones. Molecular chaperones are diverse families of multi-domain proteins that are highly conserved amongst species, as they are essential for cellular proteostasis and survival during cellular stress. These proteins recognise and bind to polypeptide chains and folded intermediates of proteins to prevent their aggregation or mis-folding, which can be detrimental to the cell, and provide a route for protein degradation to regulate cellular pathways. They are able to promote folding and maturation of client proteins, as well as aid in the assembly of complexes and trafficking throughout the cell (Fig.1.6).

Heat-shock proteins (HSPs) are ATP-dependent molecular chaperones that take their name from their up-regulation during heat stress; although they can also be found constitutively expressed in cells (Taylor and Benjamin 2005). The heat shock response is the most conserved genetic system, and is found in all organisms in a multitude of stress conditions where proteins are often denatured; heat, toxin, and pathogen exposure for example can trigger the response (Morimoto, 1993, Wu, 1995).

HSP induction requires the activation of heat shock factor (HSF) (reviewed in (Wu, 1995, Morimoto, 1998, Gomez-Pastor et al., 2018)) via trimer formation, (Lis and Wu, 1993, Morimoto, 1993, Voellmy, 1994), which then binds to the heat shock promoter element (HSE) to trigger the induction of HSPs. HSF1 in vertebrates is analogous to the single yeast, and drosophila, HSF, and is the principle stress transcription factor (Sarge et al., 1991, Nakai et al., 1997). In normal cellular conditions, HSF1 exists in the cytosol or nucleus in an inactivated monomeric state. Progression to activation is a complex cycle that involves its repression and negative regulation by a cycle of constitutive

molecular chaperones (Zou et al., 1998), amongst other protein-protein interactions and post-translational modifications (PTMs) such as the phosphorylation of serine residues (Sarge et al., 1993, Kline and Morimoto, 1997, Zou et al., 1998).
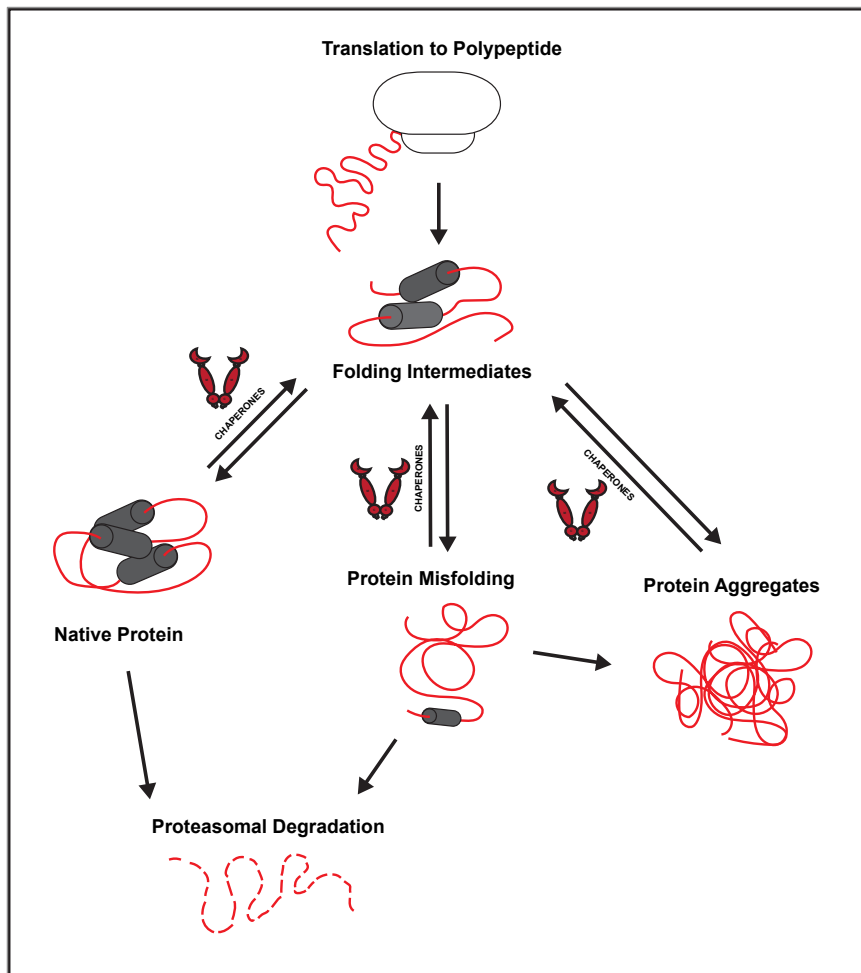
Figure 1. 6 Chaperones are crucial for cellular proteostasis

Incorrect folding of proteins or the accumulation of protein aggregates can be detriment to the cell. Molecular chaperones play a role in stabilising protein intermediates to help them fold or unfold correctly, or act as disaggregases.

As well as the conservation between organisms, HSPs are also found throughout the cell in different organelles and compartments, and have been classified into protein families according to their molecular size. These many families of HSPs play different roles within cells and have a wide range of substrate or client proteins that they act upon. Human Hsp60 (or GroEL in *Escherichia coli*), Hsp70 (DnaK), Hsp90 (HptG), and Hsp100 (ClpA and ClpB)

family members are all able to interact with aggregation-prone, non-native polypeptides, or proteins tagged for degradation, but have differing responsibilities within the cell. Hsp70 is able to direct its substrates for unfolding, refolding, disaggregation or degradation; Hsp90 acts at a later stage of folding and often with substrates important in cellular signalling, whereas Hsp60 acts at the earliest folding stages to coordinate the process. Hsp100 meanwhile, is known most for its unfolding capabilities. In addition to their involvement in protein folding and unfolding, HSPs also assist in stabilising proteins to help form complexes (extensively reviewed in (Lindquist and Craig, 1988, Saibil, 2013)).

Despite the fact that the HSP families have individual roles within the cell, they do not work alone and there is a large degree of interactivity and overlap between them to control their chaperone function. They rely heavily on protein-protein interactions for their function, to make up a complex network of regulation. Proteins that aid HSPs and designate them for a particular function or substrate are known as co-chaperones. Hsp70 for example, which is important in stabilising proteins for transport and translocation across organelle membranes (Sharma et al., 2010), commonly acts together with several co-chaperones to target it to a specific site within the cell (Laufen et al., 1999, Kampinga and Craig, 2010). Hsp40 acts as a co-chaperone to stimulate Hsp70 ATPase activity (Xu, 2018), and act as a substrate recruiter, whilst Hsp100 co-operates with Hsp70 for a protein disaggregation role (Shorter, 2011, Rampelt et al., 2012, Mattoo et al., 2013). Hsp90 and Hsp70 have also been shown to interact via the protein co-factor HOP (Edkins, 2015, Quintana-Gallardo et al., 2019). Moreover, Hsp90 has the largest set of identified co-chaperones that have been shown to be able to target it to specific client proteins or to a specific function; steroid receptor specific co-factors have been identified, as have those that are involved in targeting client proteins to degradation (see section 2.1.1).

Though some chaperones, like Hsp70 and Hsp90, have many interaction sites identified and rely on co-chaperones and other protein interactions to regulate their activity (reviewed in (Echtenkamp and Freeman, 2012,

Calderwood, 2013)), others such as the Hsp60 and Hsp100 families are mainly regulated through their stress-induction by HSF, as mentioned above. Thus, demonstrating that the heat shock response is a complicated, but well-regulated system, of incredible importance to the cell.

2.1.1   Heat shock protein 90

A wide range of proteins that are involved in fundamental cellular processes are dependent on the highly abundant molecular chaperone, Hsp90, for their activity. Hsp90 is not only one of the most abundant molecular chaperones, but also one of the most abundant cytosolic proteins. The Hsp90 family consists of many isoforms within human cells, which reside in different compartments and carry out different functions (reviewed in (Subbarao Sreedhar et al., 2004). Of the two cytoplasmic isoforms, Hsp90α (HSP90AA1) is stress inducible whereas Hsp90β (HSP90AB1) is expressed constitutively in cells. In addition, the Grp94 (HSP90B1) isoform is the endoplasmic reticulum (ER) Hsp90, and TRAP1 is mitochondrial (Rosser et al., 2004, Whitesell and Lindquist, 2005, Altieri et al., 2012, Jackson, 2013).

The domain of ATP binding and hydrolysis is conserved amongst these Hsp90 isoforms, and throughout those of bacterial species, as an ATP-coupled cycle is critical for its chaperone activity (Chen et al., 2005). Members of the Hsp90 family exist mostly in homo-dimers within mammalian cells, and consist of three domains. The N-terminal domain functions as the main ATP/ADP binding domain, but also contains conserved amino acid residues that form a lid, which opens and closes with the ATP cycle to cover the nucleotide binding pocket when Hsp90 is ATP-bound, and is vital to its chaperone function (Panaretou et al., 1998). The M-domain is also involved in the ATP cycle, as it contains residues for forming the ATPase site, although its main role is as an interaction site for client proteins. The 3rd C-domain meanwhile, is required for dimerisation of Hsp90 protomers, and for binding and docking of co-chaperones, which are able to regulate its activity (Prodromou et al., 1997a, Prodromou et al., 1997b). This ATP cycle of Hsp90

triggers a series of conformational changes and repositioning of the N- and M-domain from an open to a closed state, under an ATP 'molecular clamp' model (Fig.1.7) (Csermely et al., 1993, Sullivan et al., 1997). Within this Hsp90 ATPase coupled molecular clamp, binding of ATP promotes the association of the two N-terminal domains within the dimer, resulting in N-M-domain docking, to form a catalytically active 'closed' state. ATP hydrolysis, in turn, relaxes this dimer into an 'open' confirmation, where the reaction products can be exchanged for new ATP (Prodromou et al., 1997a, Prodromou et al., 1997b, Pearl and Prodromou, 2000). Although this ATPase activity, and the confirmation chaperone cycle is quite well understood, the nature of the client interaction and how the cycle drives substrate manipulation is not well defined (Pratt and Toft, 2003, Vaughan et al., 2006, Cox and Johnson, 2011)

Notably, nucleotide exchange is not the only factor influencing the conformational changes and substrate binding of Hsp90. As previously mentioned, chaperones rely heavily on their interactions with co-factors, and to date, more than 20 Hsp90 co-chaperones have been identified (Taipale et al., 2010, Li et al., 2012a). Hsp90 is frequently found in complexes with such proteins, which regulate its function by modulating the ATPase cycle or recruiting specific client proteins to its domains. One of the main classes of Hsp90 co-chaperones are those known as the tetratricopeptide repeat (TPR) containing co-chaperones. The C-domain of Hsp90 contains a conserved MEEVD motif that serves as the binding site for these TPR containing proteins, such as HOP, CHIP, and Cyp40 (Rohl et al., 2015). Many co-chaperones of this class function to regulate the ATPase activity of Hsp90 or provide a route for protein-protein interactions (Prodromou et al., 1999). HOP, or Hsp70/Hsp90 organising protein, has been implicated in a number of important cellular pathways and is both intra- and extracellularly functional. It functions as a bridge between multiple chaperones, recruiting Hsp70 to Hsp90 to create a handover complex for clients such as the most extensively studied set, the steroid receptors (Chen and Smith, 1998, Morishima et al., 2000, Kirschke et al., 2014). Protein substrates such as these commence folding from their nascent protein state using Hsp70 on the ribosome, after

which, they are recruited to Hsp90 by HOP for their final manipulation into a fully functional polypeptide (van der Spuy et al., 2001, Gray et al., 2008, Schmid et al., 2012). Furthermore, alongside CHIP, which has E3 ubiquitin ligase activity, HOP can aid in targeting Hsp90 substrate proteins for degradation (Muller et al., 2013, Chung et al., 2016), demonstrating that even individual co-chaperones have the ability to turn Hsp90 into a multi-functional cellular tool (Ballinger et al., 1999, Zhang et al., 2005, Stankiewicz et al., 2010, Quintana-Gallardo et al., 2019).
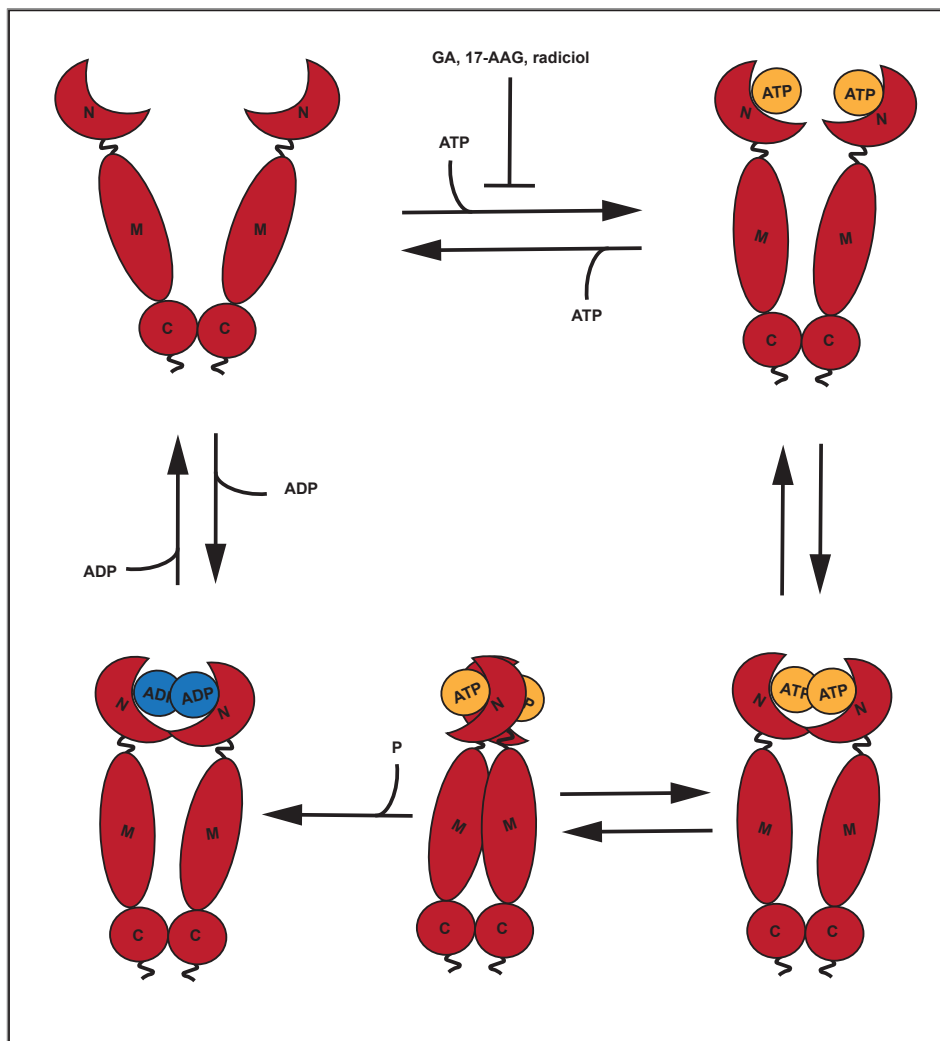
Figure 1. 7 The Hsp90 ATPase cycle induces conformational changes

The ATP cycle of Hsp90 is critical for its chaperone function. Conformational changes of its 3 domains are induced upon nucleotide binding. Inhibitors of Hsp90 can act on specific conformations, and prevent binding and exchange of nucleotides. Geldanamycin (GA), tanespimycin (17-AAG), and radiciol, inhibit the ATP-binding domain.

Alternatively, other non-TPR co-chaperones that do not interact with the C-domain, can still interact with Hsp90 through binding to exclusive confirmations of the chaperone during its cycle. This includes the proteins p23, Cdc37, Aha1, and P50, which are all able to modify the ATPase activity of Hsp90 in some way. Both p23 and Cdc37 function to prolong client interactions with Hsp90, by binding to the N-terminal domain and inhibiting ATPase activity. This stabilises the closed confirmation of the chaperone and therefore elongates its interaction time with a client protein to ensure it will leave the chaperone fully functional (Grammatikakis et al., 1999, Richter et al., 2004, McLaughlin et al., 2006, Gray et al., 2008). Aha1, meanwhile, is involved in client protein maturation through Hsp90 activation, and P50, conversely acts as an Hsp90 repressor. Aha1 is the most powerful activator of Hsp90 ATPase activity, and is able to bind the N- and M-domains to induce a partially closed confirmation and accelerate the ATPase cycle (Panaretou et al., 2002, Hessling et al., 2009, Retzlaff et al., 2010, Li et al., 2013), whereas P50 is able to recruit kinases to inhibit its ATPase activity. Such recruited proteins are able to further regulate Hsp90 activity through PTMs such as phosphorylation and acetylation, thereby adding another level of chaperone regulation (Li et al., 2012a). Hsp90 activity needs this tight level of control due to the amount of cellular processes it is involved in. It is known to participate in cell cycle control, cell survival, intracellular transport, and the cell stress response, amongst others (reviewed in (Caplan, 1999, Young et al., 2001, Li and Buchner, 2013)), and its clients include protein kinases, steroid hormone receptors, and transcription factors, thereby involving it not just in the folding or unfolding of a specific protein, but in a multitude of cellular processes and pathways (Wayne et al., 2011, Theodoraki and Caplan, 2012). Steroid receptors, as previously mentioned, make up some of the most extensively studied Hsp90 clients. Expressed in nearly every cell in the human body, the glucocorticoid receptor (GR), for example, is known to require Hsp90, Hsp70, and the co-chaperones, HOP, Hsp40, and p23, for its assembly; with Hsp90 also needed for its translocation to the nucleus (Sanchez et al., 1985, Mishra et al., 2016). Many proteins involved in the cell cycle have also been confirmed as main substrates of Hsp90 (Prince et al., 2005, Arlander et al., 2006, Zhang et al., 2015, Chen et al., 2016, Park et al., 2016).

Hsp90 client proteins are broad and varied, and as of yet, no singular specific factor that is an Hsp90 substrate determinant, has been identified. Some mechanisms, however, for how co-chaperones, such as Cdc37, influence substrate binding have been proposed, as have particular domains that Hsp90 detects in kinases (Citri et al., 2006, Taipale et al., 2012). Although both this and the mechanistic chaperone action of Hsp90 are not well understood, it is known that select client proteins are involved in the progression of some diseases. Investigation of how Hsp90 inhibition or activation affects both healthy and diseased cells, therefore, is highly sought after (discussed in section 2.3).

2.2   Heat shock proteins in viral infection

The cellular heat shock response and up-regulation of HSPs has been seen to occur during pathogen invasion, with viruses, parasites, and bacteria all able to trigger this. This may be a host reaction to the stress caused by the invasion and synthesis of foreign proteins, or may be a consequence of pathogens deliberately activating heat shock mechanisms within the cell. It could be that due to their role in facilitating protein folding, and trafficking, and assembly of complexes, pathogens have evolved alongside hosts to utilise these chaperones for successful replication. This could be especially true in the case of viruses, for which there are a large array of both DNA, and RNA viruses that induce the transcription of stress related genes (Peluso et al., 1978, Collins and Hightower, 1982, Kao and Nevins, 1983, Khandjian and Turler, 1983, Kennedy et al., 1985, Muller et al., 1989, Santomenna and Colberg-Poley, 1990, Phillips et al., 1991). Additionally, viruses package and transcribe many of their own complex proteins which need to be functional, and are known to rely on host cell machinery during their infection cycles. Some HSPs are now shown to be amongst these host factors that play a critical role in the replication of viruses, suggesting that whichever way the heat shock response to infection evolved, viruses have adapted in order to utilise this system.

The availability of specific chaperone inhibitors (discussed further in 2.3.3) has aided the investigation of both the positive and negative roles of HSPs in the control of viral replication and their initial interactions, however the details of their involvement in many viral lifecycles are still not well understood. Here I will discuss the current and basic understanding of chaperone involvement in viral lifecycles.

2.2.1   Viral structural proteins

Although the most likely role for HSPs in viral lifecycles is to stabilise structural proteins during their folding, unfolding, or trafficking, there are currently only a few instances where HSPs have been shown to interact with structural proteins in both RNA and DNA viruses. Viruses are complex protein structures that may face issues during infection, as cores and capsids require disassembly, proper folding, and correct assembly in cells in one single round of infection, whilst also avoiding detection and degradation as large protein aggregates. HSPs therefore may play a critical role in viral infection in aiding stabilisation and folding of these proteins.

Hsp90 is one such chaperone that has been implicated in an important viral core formation role; its inhibition impairs the replication of poliovirus, rhinovirus, and coxsackievirus in cell culture. During infection with picornaviruses it interacts with the viral capsid pre-cursor protein alongside the co-chaperone p23, and is required for its eventual cleavage into the viral capsid subunits (Geller et al., 2007). It is able to maintain the large capsid protein in a pre-processed form until cleavage is required for capsid assembly, at which point it can no longer interact with the viral protein. The reovirus attachment protein also undergoes a similar stabilisation by Hsp90. This attachment protein resides on the viral capsid as a heterotrimer to bind host receptors, and requires this chaperone for one stage of its folding. Here, Hsp90 is necessary for the post-translational folding of the C-terminus of the protein only, and does not interact with the fully assembled trimer, demonstrating the conformational specificity of the chaperone and ability for

viruses to use them at specific steps (Nagata et al., 1987, Mah et al., 1990, Gilmore et al., 1998).

2.2.2   Genome replication and viral polymerases

Although there appears to be some amount of interaction between HSPs and viral structural proteins, the most common stage of infection that chaperones have been implicated in is viral genome replication. Hsp90 in particular, is involved in the function of viral polymerases and other related enzymes in a wide range of viruses (Hu and Anselmo, 2000, Waxman et al., 2001, Burch and Weller, 2005, Connor et al., 2007, Geller et al., 2007, Naito et al., 2007, Chase et al., 2008, Smith et al., 2010, Geller et al., 2013).

The RNA polymerase of influenza A virus is one such enzyme, which requires Hsp90 activity for the replication of its genome. This highly abundant chaperone associates with two of the three subunits that make up the influenza A RNA polymerase, and additionally re-localises to the nucleus where it aids viral RNA synthesis (Momose et al., 2002, Naito et al., 2007). Treatment with Hsp90 inhibitors reduces the amount of successfully assembled polymerase complexes, and increases proteasomal degradation of their individual subunits (Chase et al., 2008). Inhibition of Hsp90 also results in degradation of the viral RNA polymerase of respiratory syncytial virus (RSV). Here, treatment with inhibitory Hsp90 compounds reduces virus production by over 50% and causes proteasomal degradation of the RNA polymerase (Geller et al., 2013). Proteasomal degradation is often investigated when determining an Hsp90 client, as when chaperone activity is inhibited it cannot stabilise and maintain the correct function of its substrate proteins, and instead the concurrent mis-folding or de-stabilisation results in proteasomal degradation of its client proteins. This degradation therefore suggests the influenza A polymerase subunits and those of RSV as clients of Hsp90. Another prime example for the stability of protein subunits requiring Hsp90 is the L protein of the paramyxovirus, mumps virus (MuV). In MuV replication, the viral RNA-dependent RNA polymerase consists of the L protein and cofactor P protein subunits and is needed for synthesis of its viral

RNA. L protein stability requires Hsp90, and inhibition of this chaperone results in its degradation and a reduction in viral RNA synthesis. It is important to note that L protein subunit stability can be attributed to the functional Hsp90 chaperone, as once in complex with the P protein cofactor, Hsp90 inhibition has no affect on RNA synthesis or L protein degradation (Katoh et al., 2017).

As previously mentioned, Hsp90 does not function alone, and co-chaperones and modifications are able to dictate its functionality and specificity, with viruses being no exception. Hepatitis B (HepB) virus requires the aforementioned co-chaperones Hsp70, Hsp40, HOP, and p23, alongside Hsp90 for its successful replication. During infection, this chaperone complex mediates the incorporation of RNA into nucelocapsids and its reverse transcription into DNA, with inhibition of Hsp90 reducing both of these steps (Hu and Anselmo, 2000, Hu et al., 2002). Further to this, Hsp60 is also implicated in a later stage of HepB infection, in the activation of its polymerase prior to incorporation of the enzyme into new viral cores (Park and Jung, 2001, Park et al., 2003). Further to its requirement for the folding and stability of many viral replication enzymes, the chaperone Hsp90 has also been demonstrated to be necessary for their correct localisation during infection. In the instance of herpes simplex virus (HSV), the ATPase activity of Hsp90 is required for the proper nuclear localisation of the viral DNA polymerase, with its inhibition resulting in distribution of the enzyme to the cytoplasm where it cannot function (Li et al., 2004, Burch and Weller, 2005).

As viral polymerases and HSP function are commonly linked, it has been hypothesised that there may be a conserved feature on these enzymes which the chaperones are able to interact with, or a common step in their folding or mechanistic function that the proteins are able to bind with, however this remains to be investigated.

2.2.3   Anti-viral roles of HSPs

Interestingly, in addition to their role in promoting viral replication, HSPs have also been shown to provide anti-viral activity in some instances. Hsp70

provides some examples of this. It associates with the viral ribonucleoprotein (RNP) complex of influenza A virus, through a direct interaction with two subunits, and translocates to the nucleus upon infection. In contrast to the role of Hsp90 in influenza A infection, and the perception that chaperones aid in stabilising proteins to benefit the virus, overexpression of Hsp70 results in reduced expression of viral proteins. Hsp70 expression in infected mice can also inhibit replication of the virus. Although this interaction has not been fully investigated, it has suggested that the interaction of Hsp70 with the RNP complex disrupts the binding of the polymerase to the viral RNA, and therefore inhibits transcription of its viral proteins (Li et al., 2011).

Chaperones such as Hsp70 may have a direct anti-viral role by acting on viral proteins and the transcription of viral genes, but they may also restrict viral infection through a role in the immune response (Li et al., 2002, Calderwood et al., 2007). This chaperone is known to support MHC I antigen expression and can be released extracellularly to promote the innate immune response in hosts (Wells et al., 1998, Basu et al., 2000, Vega et al., 2008). Hsp70 has been linked to interferon-β (IFNβ) induction in a mouse model of a measles virus brain infection, where it is released from infected neuronal cells and can interact with the pattern recognition receptors, TLR-2 and TLR-4, to induce IFN production (Vabulas et al., 2002a, Vabulas et al., 2002b). The resultant IFN proceeds to stimulate IFN stimulated genes; induce macrophage activation, antigen presentation, and T-cell activation, to aid clearance of the measles virus brain infection (Oglesbee et al., 2002, Kim et al., 2013b). The same is also seen in viral clearance of vesicular stomatitis virus in the brain (Kim et al., 2013a). This involvement of Hsp70 in innate immunity may support the idea that the up-regulation of HSPs during infection is part of the well-honed immune response in the host.

2.2.4 Summary

Viruses are highly complex pathogens with lifecycles that require a multitude of cellular factors to assist in replication; whether it is for direct assistance or shutting down and manipulating cellular pathways for its benefit. Thus, it is not

surprising that HSPs are required for the infection cycles of the viruses discussed here, as well as many others (Liu et al., 1998, Iordanskiy et al., 2004, Reyes-Del Valle et al., 2005, Young et al., 2008, Smith et al., 2010, Vozzolo et al., 2010, Anderson et al., 2014, Rathore et al., 2014, Taguwa et al., 2019). For the majority of viruses that utilise HSPs, it is still unclear whether the requirement for these chaperones is directly related to the folding and stabilisation of viral proteins by the chaperones, or due to effects arising from cellular chaperone targets and modified pathways in host cells. This may need to be further explored before chaperones can be considered a suitable target for anti-viral therapeutics, in case of off target affects.

2.3   Heat shock proteins as druggable targets in disease

The heat-shock response is a finely tuned, highly regulated system that is crucial to maintaining a healthy cell. In disease states with prolonged cellular stress, the system that maintains proteostasis can become overloaded and reach full capacity; as aggregates of mis-folded or non-functional proteins accumulate, and can exhaust and alter the activity of HSPs (Sherman and Goldberg, 2001, Winklhofer et al., 2008). Further to increased chaperone demand, amongst the wide array of HSP clients are many proteins that have been implicated in the development of various diseases, such as cancer and Parkinson's (Fig.1.8). I will herein present evidence for the involvement of these molecular chaperones in several diseases, and how this has formed the basis for HSP inhibitor development. I will not present evidence on HSP based anti-cancer vaccines, although many lines of research into this field exist and are reviewed in (Ciocca et al., 2012, Murshid et al., 2011).

2.3.1   Chaperones and cancer

Cancer is one of the leading causes of death worldwide, contributing to more than 1 in 4 deaths, and can develop from any cell type (Prager et al., 2018). The chaperoning function of HSPs is to maintain cellular proteostasis and therefore lead to cell survival. Cancer cells utilise this set of proteins for this

very function to ensure their progression and proliferation. They are often more dependent on HSPs for this reason, and thus levels of chaperones are commonly found up-regulated in tumour cells due to the accumulation of mutated, mis-folded, and over-expressed oncoproteins. Almost all classes of HSPs appear to be involved in cancer progression, although Hsp70 and Hsp90 are of the most importance (Fig.1.8). Elevated levels of HSPs are also usually associated with an increased resistance to cancer therapy and a poor outlook from diagnosis (Ciocca et al., 1993, Ciocca and Calderwood, 2005, Ciocca et al., 2013).

Hsp27 overexpression in cells can be found in breast cancer, endometrial cancer, and myeloid leukaemia, amongst others, where phosphorylation of the protein switches it from its usual chaperone form, to a pro-oncogenic HSP that is able to inhibit apoptosis and therefore promote cancer cell survival (Ciocca et al., 1993, Fuller et al., 1994, Conroy and Latchman, 1996, Wong et al., 1997, Thomas et al., 2005, Duval et al., 2006, Xu et al., 2006, Yu et al., 2010, Castro et al., 2012, Nagata et al., 2013, Roman et al., 2013, Yang et al., 2013, Wang et al., 2016). Similarly, the lesser-studied chaperones, Hsp40 and Hsp60, are found up-regulated in some types of cancer (Oka et al., 2001, Isomoto et al., 2003, Kanazawa et al., 2003, Castle et al., 2005, Mahoney et al., 2012). Like Hsp27, Hsp60 is able to protect cancer cells from apoptosis by preventing activation of the apoptotic pathway through formation of a Hsp60-p53 complex (Ghosh et al., 2008).

Of the better-studied chaperones, Hsp70 are a particular class that have been strongly implicated in cancer progression (Nylandsted et al., 2000a, Nylandsted et al., 2000b, Rohde et al., 2005, Nylandsted, 2009). Its overexpression positively correlates to increased cell proliferation and malignancy in a large number of cancers (Rerole et al., 2011, Murphy, 2013, Sherman and Gabai, 2015). Current evidence suggests that these abnormal cells become highly dependent on Hsp70 for its role in apoptosis, autophagy, and NF-κB signalling (Colvin et al., 2014), which in turn regulates metastasis and tumour progression (reviewed in (Murphy, 2013, Sherman and Gabai, 2015)). For example, Hsp70 is able to bind to the pro-apoptotic factor, BAX,

and block its translocation to the mitochondria, thereby inhibiting formation of the apoptosome and promoting survival of cancerous cells (Saleh et al., 2000, Gotoh et al., 2004, Stankiewicz et al., 2005). This is directly evidenced in breast cancer cells, where Hsp70 inhibition elicits apoptotic cell death (Wadhwa et al., 2002, Rohde et al., 2005, Powers et al., 2008).

Unsurprisingly, the most abundant chaperone, Hsp90, is found overexpressed in multiple cancer types, and associated with poor prognosis in lung, oesophageal, and bladder cancers, alongside melanoma and leukaemia (McCarthy et al., 2008, Zackova et al., 2013, Huang et al., 2014, Tian et al., 2014). Amongst the cellular isoforms, Hsp90α and Hsp90β are most significantly involved (Neckers and Ivy, 2003, Whitesell and Lindquist, 2005, Workman et al., 2007, Trepel et al., 2010). This family of chaperones have client proteins involved in oncogenic signal transduction and many oncogenic kinases, such as IKK, MEK, ERK, STAT4, and EGFR, which all rely on the activity of the chaperone for their maturation and functionality. Hsp90 has substrate proteins that contribute to all six 'hallmarks of cancer', which collectively describe the alterations in cell physiology that transform normal cells into those that are cancerous (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011). To list a few; p53, Akt, and survivin evade apoptosis, HER2 and EGFR function in the self-sufficiency of growth signalling, Cdk4 supports insensitivity to anti-growth signals, c-MET activation triggers tissue invasion and metastasis, VEGF and Akt are activators of sustained angiogenesis, and proteins such as hTert contribute towards limitless replicative potential (reviewed in (Workman and Powers, 2007). The extensive list of client proteins alone is evidence enough that cancerous cells utilise the multi-functional Hsp90 chaperone for their benefit, to not only evade apoptosis and cell death, but also promote the progression of cancer and tumour growth through multiple mechanisms.

Furthermore, it has previously been discussed that Hsp90 does not function alone, and is highly regulated by modifications and interactions with co-factors. The same is true for its role in cancer development. Multiple co-chaperones have been implicated in tumour progression and formation of

cancers, with higher levels of the protein p23 associated with increased metastasis in breast cancer (Simpson et al., 2010), and its target genes found at higher levels in cells of mammary carcinoma and prostate cancer (Reebye et al., 2012). Additionally, expression of the kinase regulator, Cdc37, alongside c-myc in mice, also leads to prostate cancer (Stepanova et al., 2000), and is found at high levels in other types of malignancies such as lymphoma and leukaemia (Casas et al., 2003, Katayama et al., 2004, Thompson et al., 2005, Feo et al., 2006). Conversely, reducing Cdc37 levels through RNA interference decreases tumour growth (Gray et al., 2007, Smith et al., 2009).

Although it was originally hypothesised that HSF1 indirectly mediated cancer progression through its role in the induction of HSPs, recent evidence suggests that this transcription factor has its own role in cancer progression (Mendillo et al., 2012, Scherz-Shouval et al., 2014). HSF1 is a biomarker for mutation prone cancer cells, which show a higher level of HSF1 activity than normal, and increased translocation of the transcription factor to the nucleus (Solimini et al., 2007, Mendillo et al., 2012). These high expression levels also correlate to metastasis, and mice lacking HSF1 are protected from skin tumours (Wang et al., 2004, Dai et al., 2007). Notably, the transcriptional profile of HSF1 in tumour cells differs from that which is typical in thermal stress. The set of HSF1-regulated genes in cancer is instead much larger and broader that just the classical-heat shock genes, and although the mechanism of its regulation is still not well understood, it is evident that it has a more complex role in cancer progression that simply up-regulating HSPs (Mendillo et al., 2012).

This dependency of cancer cells on a multitude of heat-shock and heat-shock related proteins has made them particularly sensitive to chaperone inhibition, and therefore a suitable target for cancer therapy and the development of small compound inhibitors (Neckers, 2002, Trepel et al., 2010). The depletion of HSP activity in cancer cells from treatment with inhibitors would reduce the level of oncoproteins, and decrease survival of the abnormal cells through the activation of pathways that would lead to apoptosis for example. HSP

inhibitors have shown promising results in both pre-clinical and clinical treatments of such malignancies (discussed further in 2.3.3). Additionally, the use of HSPs as biomarkers in cancer screenings can be considered due to their up-regulation in most cancer types (Cho, 2007).

2.3.2   HSPs in mis-folding diseases

Further to the extensive role of HSPs in cancer, these molecular chaperones have also been associated with degenerative, age-related diseases. Aging, and its accompanying neurodegeneration, is often associated with an increase in proteotoxic stress, and thus an increased demand on the cells stress-response systems (Lee et al., 2000, Lu et al., 2004, Gleixner et al., 2014). Alzheimer's disease (AD), the most common neurodegenerative disease, is a progressive disorder characterized by the accumulation of amyloid-β and microtubule structure collapse, resulting from abnormal tau protein (Selkoe and Schenk, 2003, Paul and Mahanta, 2014). The second most common neurodegenerative disorder, Parkinson's disease (PD), presents with the death of dopaminergic neurons and the formation of α-synuclein protein aggregates, known as Lewy bodies. Alzheimer's and PD, alongside other neurodegenerative disorders such as Huntington's disease (HD), are 'protein folding' diseases; arising from the mis-folding and aggregation of specific proteins. Unlike their more detrimental role in cancer progression, HSPs appear to have a protective function in these disorders, where they attempt to combat toxic protein aggregation and the increase in cellular stress (Fig.1.8)

In disease models for both AD and PD, cells up-regulate various HSPs to try and reverse the negative effects of protein mis-folding (Sherman and Goldberg, 2001, Magrane et al., 2004, Dong et al., 2005, Muchowski and Wacker, 2005, Kumar et al., 2007, Nagel et al., 2008, Kalia et al., 2010). Up-regulated Hsp70 has been shown to enhance the re-folding of aberrant α-synuclein in PD, and is found localised to the toxic aggregates (Klucken et al., 2004, Luk et al., 2008), where it functions to aid their dispersion and degradation (Muchowski and Wacker, 2005, Leverenz et al., 2007). In AD,

where abnormal and mutated tau protein leads to fibrous accumulation within cells, Hsp70 members are also able to regulate degradation. Hsc70 is able to engage mutated tau proteins, and regulate their proteasomal degradation in conjunction with the E3-ligase co-chaperone, CHIP (Shimura et al., 2004, Elliott et al., 2007, Jinwal et al., 2010). Brain regions that express low levels of Hsp70 chaperones may therefore be more vulnerable to the detrimental affects of mutated tau accumulation.

Alongside Hsp70, Hsp90 works to protect from neurodegeneration by maintaining tau in a functional conformation, inhibiting its toxic aggregation, and facilitating clearance of amyloid-β as well as slowing its initial accumulation (Kakimura et al., 2002, Dou et al., 2003, Evans et al., 2006). Tau is a client protein for Hsp90 heterodimers, and when in an abnormal state can trigger the recruitment of CHIP co-chaperones. The Hsp90-CHIP complex thus induces the ubiquitination of tau, to target it for proteasome-dependent degradation (Dou et al., 2003, Dickey et al., 2007). In PD models, this complex is also of significance as it is able to modulate the stability of a protein kinase, which when mutated increases the risk of developing the neurodegenerative disorder (Hurtado-Lorenzo and Anand, 2008, Wang et al., 2008, Ding and Goldberg, 2009, Ko et al., 2009, Rudenko et al., 2012). Protein homeostasis in cells relies heavily on the UPS, and it is hypothesised that the increase in chaperone function during aging and neurodegenerative disorders is to combat the reduced activity of the proteasome that often comes with aging. In brain and liver cells, purified fractions of the 20S proteasomal subunit contain higher levels of Hsp90 in aged cells, suggesting there is an increased of this chaperone to the subunit to rescue cells from the decreased proteasomal activity (Dasuri et al., 2009). Although the majority of these roles are linked to the cytosolic isoforms of Hsp90, the mitochondrial isoform, TRAP1, may also be involved in protection from PD. Overexpression of TRAP1 is able to mitigate the toxicity induced by α-synuclein aggregation, and reverse any of the induced mitochondrial dysfunction (Butler et al., 2012). Hsp90 thus seems to have a two-pronged approach to neurodegenerative protection; it is able to re-fold and stabilise mis-folded client proteins, or

function alongside the UPS and the CHIP co-chaperone to target proteins for degradation, therefore preventing toxic aggregation.

Whilst the majority of research in to the intersection between HSPs and neurodegenerative diseases has been focused on Hsp70 and Hsp90, the other-lesser studied HSPs, such as Hsp27 and Hsp105, are still noted to play a role. In PD models, Hsp27 for example, has been shown to bind to α-synuclein and prevent its elongation into toxic aggregates (Outeiro et al., 2006, Cox et al., 2018). Moreover, there is evidence to suggest that Hsp105 is involved in protection from protein mis-folding diseases. The mutated anti-oxidant protein, SOD1, incites the degeneration of motor neurons, and is the most frequent cause of hereditary amyotrophic lateral sclerosis (ALS). Hsp105 is not only able to interact with mutant SOD1, but also suppress the formation of SOD1-containing aggregates (Yamashita et al., 2007). Additionally, in cystic fibrosis models, Hsp105 is able to regulate the folding of the important cystic fibrosis transmembrane receptor. The chaperone is able to rescue the folding of the mutated form, which is commonly found within the disease, and stabilise it to aid its cell surface presentation and functionality (Saxena et al., 2012).

Whilst evidence for the involvement of HSPs in only a few mis-folding diseases is presented here, both AD and PD are the two most common and important, with the pathogenesis of the diseases themselves still lacking in understanding. HSPs seem to provide a natural defence against aging and age-related neurodegeneration, and may therefore be a suitable target for therapy or prevention in these diseases. To increase the protective effects that can be seen, it could be important to boost the levels and activity of HSPs at particular times, to prevent the development or progression of these diseases, or to increase the impact of treatments that are already in place (reviewed in (Campanella et al., 2018)). Their increased activity in the progression of cancer, as already discussed (section 2.3.1), should be heavily weighted when considering this as a treatment option, however.
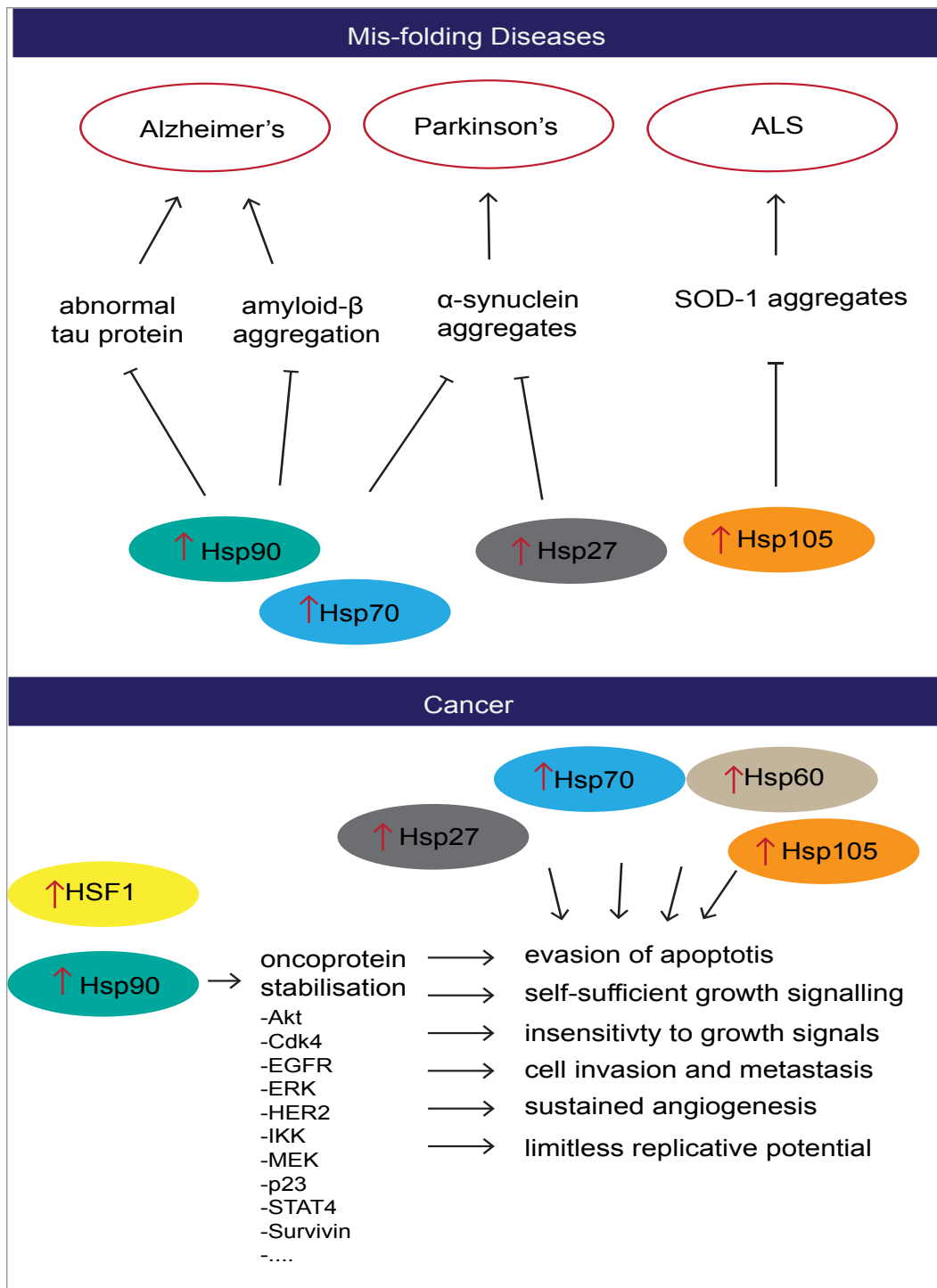
Figure 1. 8 The involvement of heat-shock proteins in disease

Various HSPs are found up-regulated in disease states. In aging related neurodegenerative and mis-folding diseases they offer a protective role by degrading toxic protein aggregates or helping mis-folding proteins re-fold. In many types of cancer they are found to help tumour growth and development through avoiding apoptotic cell death, and promoting cell growth. Hsp90 has many oncoproteins as clients which contribute to the 'six hallmarks of cancer'.

51

2.3.3 Targeting HSPs for small molecule inhibition

Understanding the complex role of HSPs in the aforementioned diseases has in turn been aided by the development of small molecule inhibitors. Hsp90 in particular, the most abundant and highly conserved chaperone, has been heavily targeted for drug development due to its vast role in cancer progression.

The naturally occurring compounds geldanamycin (GA) and radiciol (RD) were the first identified Hsp90 inhibitors, both of which target the ATP-binding pocket in the N-domain of the chaperone. GA was first revealed as an Hsp90 inhibitor in a study where its anti-cancer activity was demonstrated (Whitesell et al., 1994). This naturally occurring benzoquinone ansamycin antibiotic, from *Streptomyces hygroscopicus*, interacts with the N-terminal ATP-binding pocket of Hsp90 to prevent exchange of nucleotides, and therefore stops progression of the chaperone cycle and destabilises the interaction with client proteins (Fig.1.7) (Stebbins et al., 1997). Although able to induce anti-cancer effects, phase 1 clinical trials of GA were suspended due to high toxicity and poor solubility of the drug (Whitesell et al., 1994, Supko et al., 1995, Soga et al., 2003). More success for Hsp90 inhibition has been seen in derivatives of GA. Tanespimycin, also known as 17-AAG, and IPI-504, are more stable inhibitors based on the original compound, with better bioavailability than their predecessors and reduced liver toxicity (Banerji et al., 2005, Song et al., 2008, Hanson and Vesole, 2009). Although more successful in phase 1, 2, and 3 cancer trials than the original ansamycin class of inhibitors, their clinical evaluation still progressed no further (Modi et al., 2007, Kim et al., 2009, Modi et al., 2011). Nevertheless, the importance of such chaperones in cancer and disease has led to further development of many inhibitors using the same rationale of blocking the ATPase cycle (Patel et al., 2011). Of these, the most advanced is AUY922, or luminespib, of the resorcinol class of inhibitors, which shows stronger efficacy both in vivo and in the clinic (Shiotsu et al., 2000, Brough et al., 2008, Ueno et al., 2012).

Until recently, these inhibitors that targeted the N-terminal ATP binding pocket of the chaperone were the most utilised and promising (Whitesell et al., 1994, Neckers and Workman, 2012), but as many have proved ineffective in the clinic, the new rationale behind inhibitor development has been to target other domains of the chaperone (Fadden et al., 2010, Jhaveri et al., 2012, Lee et al., 2017a, Lee et al., 2017b, Lee et al., 2017c). A recently identified set of compounds have been found to target residues in the M-domain of Hsp90, and cause conformational changes in the chaperone that inhibit its interaction with clients, whilst still allowing ATP hydrolysis (Zhang et al., 2014). This is currently limited by the poor-specificity of these compounds but could provide a useful route for chaperone inhibition. The C-terminal domain can also be targeted as a site for inhibition by the courmarin class of antibiotics that include novobiocin, and coumermycin (Marcu et al., 2000, Matts et al., 2011). Although again limited by the specificity and efficacy of the compounds, one of the main benefits associated with C-domain inhibition is that there is a reduced feedback effect, which is often caused by N-terminal domain inhibitors. The feedback by ATP-binding inhibitors induces HSF-1 dependent transcription, and therefore increases the levels of Hsp70 and Hsp27, amongst other HSPs, which may in turn contribute further to cancer, and undo any positive effects of Hsp90 inhibition (Zuehlke et al., 2015). Amongst these various classes of Hsp90 inhibitors, the majority to enter the clinic have been of the ATP-binding class (Kim et al., 2009), although there are still no Hsp90 inhibitors that have received FDA approval. Treatment with such compounds has either proved too toxic, or not had sufficient anticancer effects to support further development in the clinic.

As well as the role of Hsp90 in tumour progression, the increased dependency of cancer cells on the chaperone Hsp70 also means it serves as an ideal target for therapy. A large amount of progress has been made in the development of Hsp70 inhibitors, although they have again been mostly unsuccessful in clinical trials. Similarly to Hsp90 inhibition there are various routes for inhibiting this abundant chaperone; PES targets its C-terminal domain and is able to disrupt interactions with clients such as p53, leading to apoptotic cell death (Gyrd-Hansen et al., 2004), whilst VER-155008 can target

the ATPase domain (Britten et al., 2000). Small molecule inhibitors have also been evaluated in cancer treatment for the lesser studied HSPs, Hsp27 and Hsp40. Both RP101, and quercetin have been shown to act as chemo-sensitizing agents that prevent the development of drug resistance in cancer cells (Hsu et al., 2011, McConnell and McAlpine, 2013), and whilst RP101 directly binds to Hsp27 to inhibit its function, quercetin targets HSF1-dependent HSPs (Yoshida et al., 1990, Hosokawa et al., 1992, Asaum et al., 2000, Elattar and Virji, 2000, Chen et al., 2012). Similarly, KNK437 is a pan-HSP inhibitor that has been shown to inhibit the expression of several HSPs, including Hsp40, Hsp105, and the inducible form of Hsp70 (Yokota et al., 2000). KNK437 is more effective and less toxic than quercetin, and differs in its mechanism of activity; whilst KNK437 is able to inhibit the binding of HSF1 to the HSE, either directly or through its initial activation into the trimeric form (Yokota et al., 2000, Ohnishi et al., 2004), quercetin does not affect its activation or binding, but instead directly decreases the levels of HSF1 available (Nagai et al., 1995). As the master regulator of the response to proteotoxic stress, and the enabler of adaption to stress in cancer cells, HSF1 may be a good solution to target for anticancer drugs.

The majority of the compounds discussed here are specific to particular chaperones, and as such, inhibit a wide array of cellular processes. An alternative mechanism for more successful targeting of these proteins in disease scenarios is to inhibit their mechanisms of regulation and control. Whilst general chaperone inhibition may lead to detrimental effects on the cell, due to the number of processes they are involved with maintaining, targeting an interaction with a specific co-chaperone, for example, may provide a better route to inhibiting a particular pathway or client protein. In cancer treatment in particular, where many Hsp90 client proteins are oncogenic, focusing on the chaperone complexes that maintain specific oncoproteins may help target tumours more effectively, and with less toxic side effects (Mimnaugh et al., 1996). Celastrol is a current example of an inhibitor that is not Hsp90 specific, but instead disrupts its interaction with the co-chaperone, Cdc37 (Zhang et al., 2008). Perturbation of this Hsp90-Cdc37 chaperone complex would then lead to destabilisation of kinase client proteins that are found to play a role in

cancer progression (Stepanova et al., 2000, Terasawa et al., 2006, Gray et al., 2007, Gray et al., 2008, Smith et al., 2009, Li et al., 2012b, Lu et al., 2014, Li et al., 2018). Furthermore, the PTMs of chaperones and their co-factors, provides an additional level of regulation that can be targeted by compounds. The anti-cancer activity of Hsp90 inhibitors can be enhanced with the combined used of kinase inhibitors, which prevent the regulatory phosphorylation of co-chaperones (Schwartz et al., 2015). Phosphorylation of both Hsp90 and Hsp70 has been shown to assist in the formation of the Hps90-Hsp70 chaperone complex, and phosphorylation of the Aha1 co-chaperone also promotes its interaction with Hsp90 (Dunn et al., 2015). This demonstrates that a further understanding of HSP co-chaperones and their PTMs is invaluable for the more specific targeting of chaperone-dependent cancers.

Although a large number of HSP inhibitors have been developed, and some evaluated in the clinic, there are still no chaperone inhibitors approved by the FDA. Strategies to enhance cancerous cell death are actively being sought; with one route of increasing their effectiveness to use them in combination or with other anti-cancer compounds. The Hsp70 inhibitors PES and VER155008 for example have been shown to be effective against certain cancers when administered in conjunction with the Hsp90 inhibitor 17-AAG (Massey et al., 2010, Braunstein et al., 2011, Kaiser et al., 2011), and 17-AAG in turn has shown promising activity against multiple myeloma when coupled with the proteasome inhibitor, bortezomib (Mimnaugh et al., 2006). Unsurprisingly, the development of HSP inhibitors for anti-cancer therapy has proved challenging. Due to their important role in cellular proteostasis it is probable that their use in the clinic will also have deleterious effects on healthy cells. Thus a deeper understanding of chaperones and their client proteins is vital for the development of successful HSP based anti-cancer treatments.

3 Vaccinia virus and cellular factors

Although vaccinia is a large virus that brings many of its own proteins necessary for replication, and is therefore less reliant on host systems than many smaller viruses, there are still many cellular proteins that are known to be required for its replication cycle. Various RNAi screens have uncovered host factors that VACV utilises for different stages of its lifecycle, some of which have already been briefly mentioned in detailing of the VACV replication cycle (section 1.3.3) (Moser et al., 2010, Mercer et al., 2012, Sivan et al., 2013).

From initial entry of the VACV particle, which requires the binding of cellular proteins and actin rearrangement for PS-mediated macropinocytosis (Chiu et al., 2007, Huang et al., 2008, Mercer and Helenius, 2008, Mercer and Helenius, 2010, Moser et al., 2010, Laliberte et al., 2011), to the surprising involvement of the host replication and repair machinery (Postigo et al., 2017), these cellular proteins range in function and are utilised throughout the lifecycle of the virus, although there is a greater amount of understanding of those involved in the earlier stages of infection. Notably, the large viral replication factories that are visible as clearings in the cytoplasm recruit a selection of cellular proteins and organelles that may also have some involvement. ER is needed for wrapping of the viral factory during DNA synthesis (Tolonen et al., 2001, Welsch et al., 2003, Oh and Broyles, 2005) and organelles such as mitochondria are also recruited, although their specific function during infection still remains to be understood (Tolonen et al., 2001). It has also previously been mentioned that at a later stage of VACV replication, organelles are also required for wrapping of MVs (section 1.3.3) (Hiller and Weber, 1985, Tooze et al., 1993, McNulty et al., 2010, Sivan et al., 2016).

Although various host factors necessary for the VACV lifecycle have been uncovered, cellular proteins that participate in the later stages of the lifecycle

remain largely undefined. Of particular interest to this thesis are the proteins involved in the problematic process of dis-assembly and re-assembly of virus whilst remaining protected from the cellular environment. Here, I will present details of the involvement of the UPS in viral core uncoating, which has been heavily implicated in this stage of the lifecycle (Satheshkumar et al., 2009, Teale et al., 2009, Mercer et al., 2012, Schmidt et al., 2013), and the lesser-understood participation of molecular chaperones (Jindal and Young, 1992, Sedger et al., 1996, Hung et al., 2002, Filone et al., 2014).

3.1   The ubiquitin-proteasome system

The UPS is the major degradation machinery of the cell and thus plays an important role in the regulation of many cellular processes, including cell cycle control, antigen presentation, DNA repair, and apoptosis (Grant et al., 1995, Loureiro and Ploegh, 2006, Koepp, 2014, Asaoka and Ikeda, 2015, Armstrong et al., 2016, Guo and Dixon, 2016). Degradation of proteins via this pathway involves conjugation of target proteins with ubiquitin molecules, followed by actual degradation by the proteasome. The process of ubiquitination is carried out by a set of sequentially activated enzymes: ubiquitin activation enzymes, conjugating enzymes, and ubiquitin ligases (Fig.1.9) (Lecker et al., 2006, Wang et al., 2007). Target proteins can be mono-ubiquitinated with a single ubiquitin moiety, or chains of ubiquitin can be conjugated together through sequential rounds of ubiquitination. This variation in mono- or poly-ubiquitination, as well as where the linkage within the chains of ubiquitin is found, determines its function (Petroski and Deshaies, 2005, Nathan et al., 2013a). The key component of the UPS is the multicatalytic machinery responsible for protein degradation, dependent on ubiquitin and ATP, the 26S proteasome (Voges et al., 1999, Zwickl et al., 1999, Schmidt et al., 2005, Wang et al., 2007, Zhang et al., 2007, Bedford et al., 2010, Wang et al., 2010, Xie, 2010, Kisselev et al., 2012, Nathan et al., 2013b). Composed of the 20S core particle, and two regulatory 19S subunits, its assembly, structure, and activity is determined by a combination of associating regulatory complexes, PTMs, and proteasome-interacting-proteins (Schmidt et al., 2005, Zhang et

al., 2007, Finley, 2009). Multiple stages of the VACV lifecycle have been identified that are dependent on the proteasome and ubiquitination (Satheshkumar et al., 2009, Teale et al., 2009, Mercer et al., 2012).
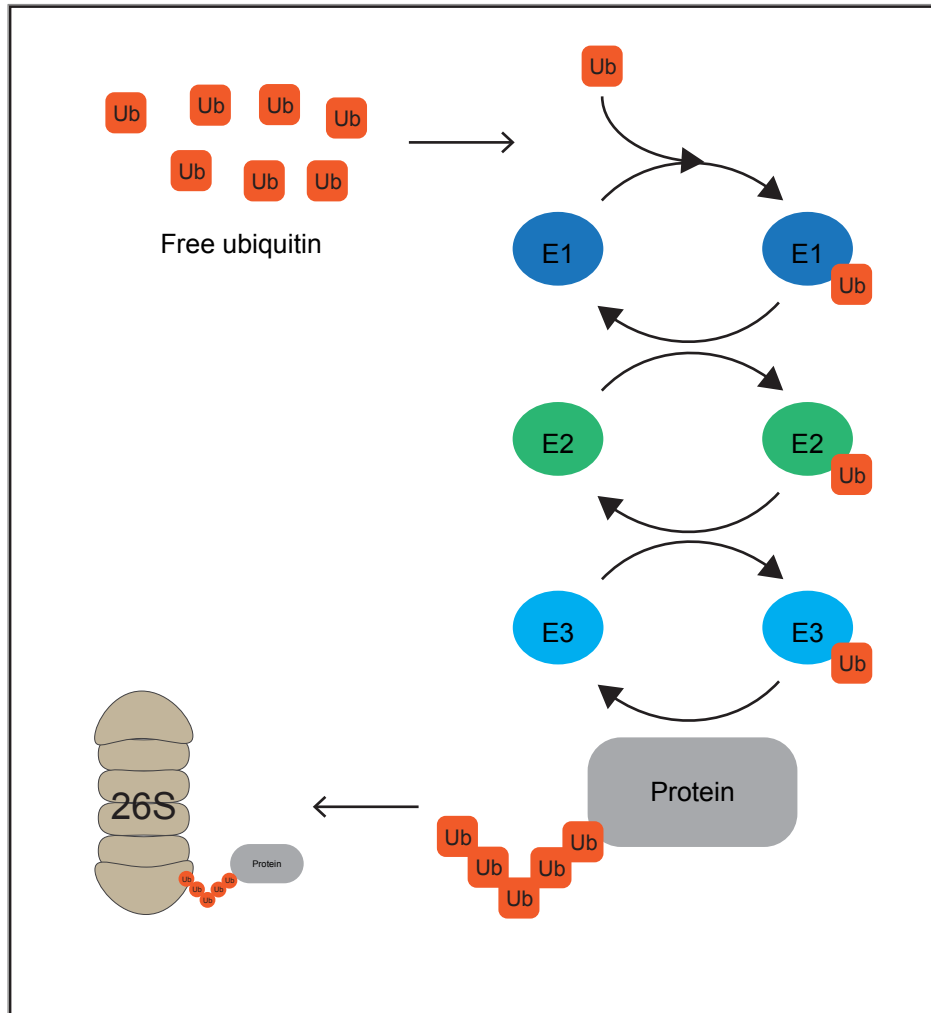
Figure 1. 9 Ubiquitination and proteasomal degradation

Conjugation of ubiquitin molecules to target proteins occurs through sequentially activated enzymes; E1 ubiquitin activators, E2 conjugating enzymes, and E3 ubiquitin-ligases. K48-polyubiquitination targets proteins for degradation by the 26S proteasome.

Ubiquitin is seen to accumulate in poxvirus replication sites, and viral core proteins are found to be K48-polyubiquitinated in the particle (Nerenberg et al., 2005, Chung et al., 2006). Inhibition of the ubiquitin-activating enzyme, E1, prevents the generation of new virus particles, suggesting that ubiquitination of viral core proteins is a vital step of virion assembly (Mercer et al., 2012). Although VACV encodes its own ubiquitin ligase, p28, this protein

is not required for virus production in tissue culture, and is therefore not responsible for this ubiquitination of viral core proteins (Nerenberg et al., 2005). K48-polyubiquitination targets proteins for degradation by the proteasome, and the proteasome inhibitors, MG132, MG115, lactacystin, and bortezomib, have been shown to inhibit poxvirus infection (Satheshkumar et al., 2009, Teale et al., 2009, Mercer et al., 2012). VACV infected cells treated with these inhibitors lack viral replication factories and do not express late viral genes, as when the proteasome is inhibited, viral cores are able to enter the cytoplasm but cannot undergo core uncoating and genome release (Mercer et al., 2012). At this stage of viral core uncoating, ubiquitination is not needed alongside proteasome activity, indicating that the previously ubiquitinated core proteins that are packaged in the virion, are sufficient enough for mediating proteasome-dependent genome uncoating (Fig.1.4, fig. 1.10).

Further to the role in the uncoating of the viral core, an additional step of the VACV lifecycle has been shown to require the UPS. Two components of the E3 ubiquitin-ligase complex, Cullin3 (cul3) and RING-box protein 1 (Rbx1), are required for initiation of DNA replication, after proteasome-dependent uncoating of the viral core (Mercer et al., 2012). Although VACV encodes multiple adaptor proteins of Cul3, these are not necessary for viral replication in tissue culture, indicating that they are not the adaptor proteins used by the ligase in DNA replication (Barry et al., 2010). Additionally, a second round of proteasome activity is also required for replication of viral DNA, independent of its role in release of the genome (Fig.1.10).

It is evident that the UPS plays an essential role in at least two steps of the VACV replication cycle; viral core proteins are ubiquitinated, ready for degradation by the proteasome upon release into the cell cytosol, which precedes a second step of proteasome activity and ubiquitin ligase action, necessary for the initiation of DNA replication (Satheshkumar et al., 2009, Teale et al., 2009, Mercer et al., 2012). Although the involvement of this system is somewhat understood, the lifecycle of VACV is complex and there are likely other proteins involved in these steps. It is still yet to be defined

which core proteins are ubiquitinated and degraded in the process of genome release, and how they are protected from proteasomal activity whilst being packaged into new viral particles. Further to this, viral cores released into the cytosol are not immediately degraded by the proteasome, suggesting the ubiquitinated core proteins need to be made more accessible as substrates, before uncoating can occur. The viral uncoating factor, D5, has been defined, but the link between these two uncoating stages is still yet to be understood (Fig.1.4) (Kilcher et al., 2014).
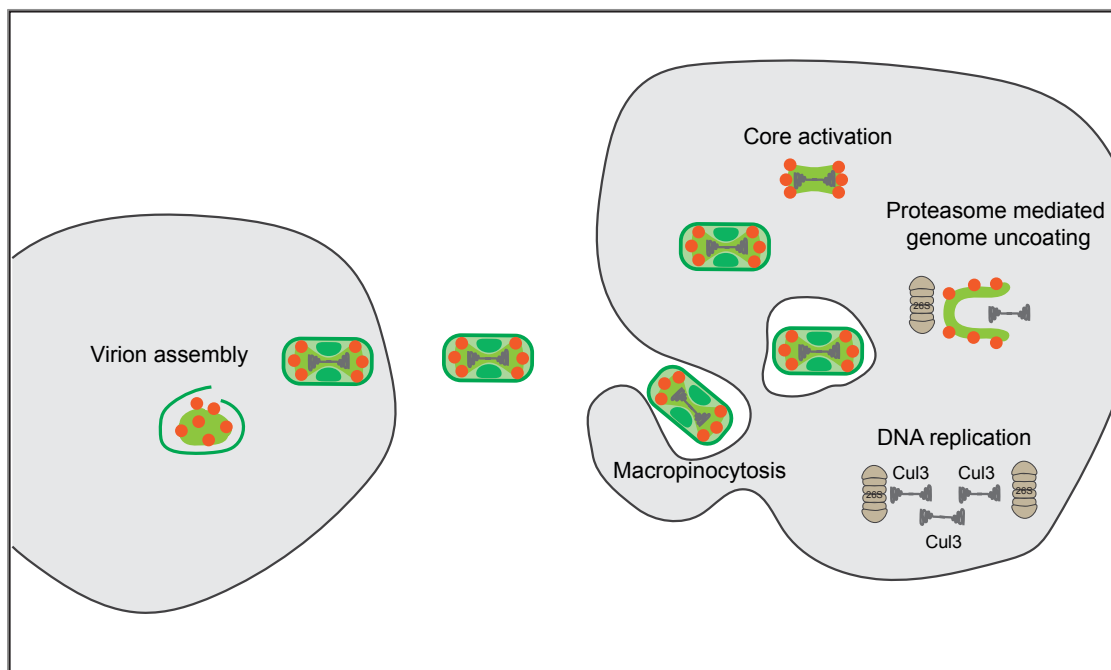
Figure 1. 10 Model of VACV core ubiquitination, uncoating, and replication

Viral cores are ubiquitinated in a K48-linked fashion in MV assembly. Upon infection, fusion of viral and cellular membranes releases ubiquitinated viral cores into the cytoplasm. Ubiquitinated cores become accessible and proteasome activity directs degradation of the cores, and release of the viral genome. A cullin3-based ubiquitin ligase, and second round of proteasome activity is required to initiate viral DNA replication.

3.2   Heat-shock proteins

Whilst both pro- and anti-viral roles of HSPs in other viral infections have been discussed, their function during viral replication is still not well understood (section 2.2). Their detailed mechanisms have not been described, and in VACV, the role of HSPs is both debatable and not well studied.

As with other viruses, some HSPs are found up-regulated upon infection with VACV (Sedger and Ruby, 1994, Kowalczyk et al., 2005), and viral factories have been seen to accumulate some of these chaperones (Hung et al., 2002). Whether these proteins are directly implicated in infection, however, is less understood. There have been discordant findings of the role of Hsp70 in VACV infection. The most significant difference in Hsp70 levels, in vaccinia infected human monocyte cells, has been seen at 12 hpi, where the chaperone also co-sediments with viral proteins in sucrose gradients, suggesting a direct interaction with the virus (Jindal and Young, 1992). Additionally, in mouse ovaries, where VACV can grow to high titers, members of the Hsp70 family, the inducible Hsp72 in particular, were the most abundant stress proteins found (Sedger and Ruby, 1994). Although the suggested interaction of Hsp70 with viral proteins and increase in expression, indicate that there might be a role for this chaperone in VACV replication, further evidence suggests otherwise. When Hsp72 is expressed in a recombinant VACV, there is no difference in virus growth kinetics or viral yields compared to that of the control virus, or infection of Hsp72-negative cell lines (Sedger et al., 1996). It would therefore appear that while VACV infection results in the induction of the inducible HSP, Hsp72, replication is independent of this protein, and instead may simply be up-regulated as part of the stress response to infection (Jindal and Young, 1992).

Although no apparent role for this particular chaperone, there is some initial evidence for the requirement of the highly conserved HSP, Hsp90, during poxvirus infection. Inhibition of this chaperone by GA reduces vaccinia 24-hour yield, and late viral gene expression (Hung et al., 2002). The virus appears to have delayed growth kinetics, and a block in DNA replication, when Hsp90 activity is inhibited, although the exact role of the chaperone in the lifecycle is still unknown. Interestingly, Hsp90 could be found interacting with the viral core protein, p4a, at a specific time-point through immuno-precipitation, potentially alluding to a mechanism of how the chaperone is utilised during replication (Hung et al., 2002).

Finally, the master regulator of the heat-shock response, HSF1, has also been implicated in the replication cycle of VACV. HSF1-regulated genes are at least two-fold enriched in infected cells, and during infection phosphorylated HSF1 is translocated to the nucleus, consistent with activation of this transcription factor. Depletion of HSF1, or its inhibition with either KNK437 or quercetin, reduces viral titers and thus blocks VACV infection in cultured cells (Filone et al., 2014).

It is not surprising that such important and highly conserved proteins may be utilised by poxviruses in their complex lifecycles. Current evidence suggests that Hsp90 and HSF1 are both necessary for VACV infection, but Hsp70 is not. Although there appears to be some interplay between HSPs and poxvirus infection, this still needs to be confirmed, and the exact mechanisms of their requirement, defined.

4 Aim of research project

Whilst there is a good level of understanding for many of the stages of the poxvirus lifecycle, detailed mechanisms of how the virus uncoats its genome, initiates DNA replication, and proceeds to make new virions still remain to be discovered. This thesis aims to clarify which HSPs are involved in the viral lifecycle, with a focus on the uncoating and assembly of VACV particles, and thus understand more about the cellular dependency of the virus. As previously discussed, HSPs have already been implicated in the lifecycles of other viruses, but an understanding of the specific details surrounding this involvement is lacking. Identifying their precise roles in VACV infection therefore would contribute to the wider knowledge of HSPs as pro-viral factors and provide further information for potential cellular targeting anti-viral development. As HSPs themselves are of high importance in cancer and the development of anti-cancer drugs, it may also provide more information on molecular chaperone biology and function.

I have therefore set out to further investigate the intersection of HSPs and VACV, which has been initially proposed (Hung et al., 2002, Filone et al., 2014). In addition to this previously existing evidence, a high-throughput RNAi screen of the human druggable genome was carried out in our lab to identify cellular factors involved in poxvirus lifecycles, and amongst the hits were the cytosolic isoforms of Hsp90, and the heat-shock regulator, HSF1 (data unpublished). As the host UPS is already known to be involved in multiple stages, I hypothesise that the involvement of HSPs is also likely to be complex, as the ties between molecular chaperones and proteasomal degradation themselves already involve multiple levels of regulation and various co-factors.

Anti-viral effects of molecular chaperone inhibition have been previously demonstrated, but the specific stages of the poxvirus lifecycle where they are involved have not yet been defined. To achieve a more detailed

understanding of the role of this set of important proteins in VACV infection, small compound inhibitors in addition to RNA interference, alongside a toolbox of viral and cell biology assays were used to understand which molecular chaperones are needed for vaccinia infection, and the precise stages of the lifecycle at which they are utilised. In results chapters 1 and 2 I focus on the role for Hsp90 in viral genome release, and virion formation, respectively, before presenting evidence in chapter 3 for the role of an inducible HSP (Hsp105) in the formation of immature virions.

II Materials & Methods

1   General Materials and Methods

1.1   Cell culture

BSC40 cells and HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% Foetal Bovine Serum (FBS, Life Technologies) and 1% Penicillin-Streptomycin (Life Technologies) under standard conditions at 37 °C and 5% CO_2. Cell lines were maintained and passaged when confluent using Phosphate-buffered Saline (PBS) and Trypsin/EDTA (2.5 g Trypsin/litre, 0.2 g EDTA/litre). Cell counts were determined using Cellometer (Nexelcom).

1.2   Viruses

Recombinant viruses used in this study are part of the Mercer Lab collection, and were generated through homologous recombination, as previously described (Mercer and Helenius, 2008). Briefly, VACV infected BSC40s were transfected with linearized plasmid 4 hpi and harvested at 48 hpi. Plaques were selected by rounds of fluorescent purification and final plaques sequenced to confirm correct insertion of the construct. Recombinant strains bearing insertions in the thymidine kinase (tk) locus were generated using vectors based on the plasmid pJS4 (Chakrabarti et al., 1997). Other recombinants, including EGFP-A4 or mCherry-A4 encoded in the endogenous locus, were generated using vectors based on pBluescript II KS (ThermoFisher Scientific).

Virus Name	Description	Source/Reference
VACV WR	Wild-type Western Reserve strain	Jason Mercer
WR E EGFP	VACV WR encoding EGFP under the J2R early promoter in the tk locus	(Chakrabarti et al., 1997) (Stiefel et al., 2012)
WR I EGFP	VACV WR encoding EGFP under the G8R intermediate promoter in the tk locus	(Chakrabarti et al., 1997) (Stiefel et al., 2012) (Kilcher et al., 2014)
WR L EGFP	VACV WR encoding EGFP under the F17R late promoter in the tk locus	(Chakrabarti et al., 1997) (Stiefel et al., 2012) (Schmidt et al., 2013)
WR mCherry-A4	VACV WR encoding an endogenous C-terminal mCherry tagged A4 core protein	(Mercer and Helenius, 2008) (Schmidt et al., 2011) Previously referred to as mCherry-A5
WR mCherry-A4 EdU-DNA	EdU incorporated DNA virus based on WR mCherry-A4	(Kilcher et al., 2014) (Methods section 4.2.1)
WR EGFP-A4	VACV WR encoding an endogenous C-terminal EGFP tagged A4 core protein	(Mercer and Helenius, 2008) (Schmidt et al., 2011) Previously referred to as EGFP-A5
WR HA-D5	VACV WR encoding an endogenous HA-D5 fusion protein	(Kilcher et al., 2014)
WR HA-D5 EGFP-A4	VACV WR encoding an endogenous HA-D5 fusion protein, and endogenous EGFP tagged A4 core protein	(Kilcher et al., 2014)

Table 2. 1 A table of viruses used in this study

1.3 Reagents and antibodies

Cytosine arabinoside (AraC) and cycloheximide (CHX) were obtained from Sigma-Aldrich, and used at 10 µM and 50 µM respectively. KNK437 and hydroxyurea (HU) were used at 100 µM and 2.5 mM unless otherwise indicated, and also obtained from Sigma-Aldrich. AUY922 was purchased

from SelleckChem and used at 400 nM, and 17-AAG from Calbiochem, was used at 100 nM. These concentrations are correct unless others described in the text or figures. Tissue-culture grade DMSO was obtained from Sigma-Aldrich.

Anti-	Species	Source/Reference	Working Dilution
A14	Rabbit	Paula Traktman (Traktman et al., 2000)	1/500 WB (western blot)
Cullin3	Rabbit	Abcam	1/5000 WB
F17	Rabbit	Paula Traktman (Liu et al., 1995)	1/1000 WB
GRP94	Rabbit	Cell Signalling	1/1000 WB 1/100 IF (immunofluorescence)
HA	Mouse/Rabbit	BioLegend	1/1000 WB
HSP105	Rabbit	Abcam	1/500 WB
HSP40	Rabbit	Abcam	1/5000 WB
HSP72	Mouse	Enzo Life Sciences	1/500 WB
HSP90AA1	Mouse	Abcam	1/1000 WB 1/100 IF
HSP90AB1	Mouse	Abcam	1/500 WB 1/200 IF
I3	Rabbit	Paula Traktman (Rochester and Traktman, 1998)	1/1000 WB
p4a	Rabbit	Mariano Esteban (Demkowicz et al., 1992, Risco et al., 1999, Heljasvaara et al., 2001)	1/500 WB
α-tubulin	Mouse/Rabbit	Cell Signalling	1/1000 WB

Table 2. 2 A table of the antibodies used in this study

HRP linked secondary antibodies, anti-rabbit and anti-mouse, from Cell Signalling Technologies were used for western blot analysis at 1/1000 dilution as described below, and AlexaFlour coupled secondary antibodies were purchased from Thermo Fisher for fluorescence microscopy and used at a

1/1000 dilution. Hoechst 33258 (Life Technologies) was used for DNA staining at 1/10,000.

2 Virus Methods

2.1   Virus propagation and purification

Confluent 60 mm dishes of BSC40 cells were infected with the VACV WR at a multiplicity of infection (MOI, calculated from plaque forming units) of 1, and incubated under standard conditions for 2 days. Cells were harvested into 1 mM Tris pH 9.0 and lysed by freeze-thawing in liquid nitrogen, three times. The resulting cell extract was used to infect two 15 cm dishes of confluent BSC40 cells, which were then incubated as before for 2 days. For amplification of the virus, the cell extract from the two dishes, obtained using the same methods of repeated freeze-thaws, was used to infect 15 x 15 cm dishes of BSC40 cells, which were again incubated for 2 days. Infected cells were later harvested into PBS and pelleted by centrifugation at 300 x g for 5 min.

The cell pellet was resuspended into 10 mM Tris pH 9.0 and iced for 5 min. Cells were then disrupted with 25 strokes in a tight-fitting tissue homogenizer (Wheaton). The resulting cytosolic extract was centrifuged, 2,000 x g, 10 min, and the centrifugation was repeated on the supernatant, to remove cell debris from the extract. The cytosolic extract was then carefully layered on to 36% sucrose, 20m M Tris pH 9.0 in a SW32 ultracentrifuge tube (Beckman Coulter), and topped up to the final volume with 10mM Tris pH 9.0, before loading mineral oil on top to prevent virus aerosolisation. The virus was sedimented through the sucrose cushion in a SW 32 Ti Rotor (Beckman Coulter) for 80 min, 4 C, 43,000 x g, and resuspended into 1 mM Tris pH 9.0.

Sedimented virus was then further purified through sucrose band purification. A 25-40% sucrose gradient was prepared using the Gradient Master (Biocomp) set at 81.5°, 18 rpm, 3 min, in 10mM Tris pH 9.0 in SW41

ultracentrifuge tubes (Beckman Coulter). Sedimented virus was ultracentrifuged through the gradient, 12,000 *x g*, 50 min. A 21 G needle was then used to aspirate the band of purified virus through the tube, and pelleted out of the sucrose through centrifugation 43,000 *x g,* 40 min. Purified virus pellets were resuspended in 1 mM Tris pH 9.0 and stored at -80°C. Infectivity of purified virus stocks was determined through plaque assay (methods section 2.3).

2.2   Mature virion 24-hour yield

For 24 hr yields, 60 mm dishes of confluent HeLa cells were infected in DMEM at MOI 1 with WT VACV and incubated for 1 hr at 37°C with gentle shaking every 15 min. After incubation, infection media was aspirated and replaced with full DMEM, containing compounds of interest at concentrations previously indicated. Alternatively for yields of siRNA-transfected cells (methods section 8), cells were infected at 72 hr post siRNA transfection, as described above, and incubated in full medium.

After 24 hrs incubation, cells were scraped into media and spun at 300 x *g* for 5 min. Cell pellets were washed in PBS before re-pelleting. Final cell pellets were then resuspended in 100µl 1mM Tris pH 9.0 and freeze-thawed, in liquid nitrogen at a 37°C water bath, three times to obtain cell lysate with MVs.

2.3   Plaque assay

For determination of yields or virus titers from purified stocks, a plaque assay was performed. Of the resulting MV extraction or purified virus stock, 2 µl was diluted into 998 µl DMEM and 1 in 10 serial dilutions prepared. MV dilutions were used to infect each well of a 6-well plate, which contained confluent monolayers of BSC40 cells and incubated for 1 hr with gentle rocking every 15 min. Infection medium was aspirated after incubation and replaced with full supplementary media. After 48 hours, plates were aspirated of media and fixed with 0.1% crystal violet and 2% paraformaldehyde (PFA) and plaque-

forming units per millilitre (pfu/ml) were calculated by manually counting of plaques. Plaque areas were calculated using ImageJ measurements of scanned 6-well plates.

3 Drug Toxicity Assay

Cytotoxicity of small compound inhibitors was measured using the Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific). Staurosporine (Sigma Aldrich) was used as a comparative control for toxicity and serially diluted in supplemented DMEM in 10-fold dilutions, from 10 mM highest concentration down to 0.01 nM. 17-AAG and KNK437 were also 10-fold serially diluted in cell culture media, from 10 µM to 0.1 nM for 17-AAG and 1 mM to 1 nM for KNK437. Diluted compounds were added to HeLas that had been seeded in 96-well plates at 80% confluency and incubated at standard conditions for 24 hours. After incubation, the lysis buffer provided was added to a well of untreated cells and incubated for a further 30 minutes. Following incubation, 25 µl of medium was removed from each well and mixed with another 25 µl of the Pierce LDH reaction mix in a new 96-well plate. The cell medium and reaction mixture were incubated for 30 min RT in the dark, and 25 µl of stop solution was subsequently added. The absorbance was measured on VersaMax Microplate Reader (Molecular Devices) at 480 nm and 680 nm. All values were Min-Max normalised to the lowest and highest values on the plate. Lysis buffer samples provided the control for full cell toxicity and an untreated population of cells used as a negative control.

4 Flow Cytometry

4.1 Sample preparation

Confluent HeLa cells in 96-well plates were infected with either WR E EGFP, I EGFP, or L EGFP, at MOI of 10 in DMEM and left for 30 min at room temperature (RT) to bind. Unbound virus was aspirated and wells were fed

with full medium containing dilutions of the inhibitors, 17-AAG or KNK437. Plates were incubated at standard conditions and harvested at either 6 hpi or 12 hpi for early or intermediate and late EGFP viruses respectively. For harvesting, cells were aspirated of media and washed once with warm PBS before detaching with trypsin for 15 min at 37°C. After tyrpsinisation, blocking buffer (5% FBS in PBS) was added followed by equal amounts of 8% PFA PBS and left for 10 min for fixation at RT.

4.2 Flow cytometry acquisition and analysis

Fixed samples in 96-well plates were used directly for flow cytometry. Fluorescence in 488 nm channels was measured on a Guava easyCyte HT (Merck). 10,000 events (cells) were recorded per sample, and gated on the population of live cells from an uninfected HeLa cell sample control. The same control was used to create markers of 'non-fluorescence'. Cells with higher levels of fluorescence compared to those in uninfected cells were recorded as a fluorescent and therefore expressing GFP. The Guava Incyte Software (Merck) was used for analysis, and expression of GFP was measured as a percentage of an infected, untreated, negative control.

5 Microscopy Assays

5.1 Replication site formation

HeLa cells on 13 mm coverslips (VWR) were infected with WR EGFP-A4 at MOI 10 in DMEM, and bound for 30 min RT. Infection media was removed and replaced with full medium and treated with the indicated compounds. Cells were fixed with 4% PFA PBS at 8 hpi, permeabilised with 0.1% Triton X-100 (Sigma Aldrich) PBS 20 min, blocked with 5% bovine serum albumin (BSA, from Sigma Aldrich) PBS, 30 min, and stained with Hoechst 33258 for visualisation of viral replication sites for another 30 min. Samples were imaged using a 100x oil immersion objective (NA 1.45) on a VT-iSIM microscope (Visitech; Nikon Eclipse TI).

5.2 Genome release assay

5.2.1 EdU-DNA virus generation

WR mCherry-A4 EdU-DNA MVs were produced for the genome release assay. BSC40 cells were infected with WR mCherry-A4 as previously described for virus propagation and incubated in the presence of 1 µM of the nucleoside analogue, EdU. After homogenisation and centrifugation, post-nucleic cell extracts were incubated with 2 mg DNase I for 30 min at 37 °C to eliminate any non-viral, nucleic DNA contaminations. The virus was then purified as described.

5.2.2 Genome release assay

HeLa cells on coverslips were infected with WR mCherry-A4 EdU-DNA virus at a MOI of 10 and bound at RT for 30 min. Infection media was removed and cells were either fed with supplementary untreated, or treated DMEM with AraC, CHX, or 17-AAG. Samples were fixed at 4.5 hpi with 4% PFA in PBS and permeabilized with 0.1% Triton X-100 in PBS, 20 min, before blocking with 5% BSA PBS, 30 min. Click chemistry reactions were carried out to couple the EdU nucleoside to AlexaFluor 488 dye, according to the Click-iT EdU Imaging Kit protocol (Invitrogen) with minor alterations (described in (Mok and Yakimovich, 2019)), and stained with Hoechst for 30 min, for visualisation of viral replication sites. Samples were imaged on a Leica TCS SPE confocal microscope with a 63x oil objective. The spot-detection function of Imaris was used to quantify associated cores and released genomes. Green fluorescent genomes in Z-stacks were detected using the quality criterion (quality > 25; Imaris). Green spots with a mean red fluorescence > 55 were categorized as core associated genomes, whereas spots with background levels of red fluorescence were categorized as released genomes (at least 30 cells per condition and replicate).

5.3 EdC incorporation

For EdC incorporation assays, cells were infected as described for replication site formation assays and treated with indicated compounds alongside 10 mM final concentration of the nucleoside analogue 5-ethynyl-2'-deoxycytidine (EdC). At 4.5 hpi cells were fixed, permeabilized and blocked as before, and EdC was coupled to the AlexaFluor 594 dye according to the Click-iT EdU Imaging Kit protocol. After click chemistry reactions, nuclei and viral replication sites were stained with Hoechst for 30 min. Samples were imaged on the iSIM microscope as before.

5.4 Electron microscopy

5.4.1 Infection assays

HeLa cells were seeded onto 13 mm coverslips and infected at a MOI of 10 with WT VACV in infection media and left at RT for 30 min to bind. After binding infection medium was aspirated and cells were fed with full media and left either untreated or treated with the compound of interest. Alternatively, for RNAi cells, infection occurred 72 hr post siRNA-transfection and cells were fed with full cell media after infection. Cells were incubated for 8 or 12 hpi as indicated.

5.4.2 Sample preparation

After incubation, medium was aspirated and coverslips were fixed in EM-grade 2% PFA /2% gluteraldehyde (GA, both TAAB Laboratories Equipment), 0.1 M sodium cacodylate, secondarily fixed for 1 hr in 1% osmium tetraoxide/1.5% potassium ferricyanide at 4ºC and then treated with 1% tannic acid in 0.1 M sodium cacodylate for 45 min, RT. Samples were dehydrated in sequentially increasing concentrations of ethanol solution and embedded in Epon resin. Coverslips were inverted onto prepolymerised Epon stubs and polymerised by baking at 60ºC overnight. The 70 nm thin sections were cut with a Diatome 45º diamond knife using an ultramicrotome (UC7, Leica).

Sections were collected on 1x2 mm formvar-coated slot grids and stained with Reynolds lead citrate.

5.4.3   Serial sectioning

For serial sectioning EM, cells were infected and treated as previously described, and after incubation, aspirated before fixing in EM-grade 2% PFA/2% GA, 0.1 M sodium cacodylate, secondarily fixed for 1 hr in 1% osmium tetraoxide/1.5% potassium ferricyanide at 4ºC, and then treated with 1% Thiocarbohydrazide (TCH) (Fisher Scientific) for 20 min at RT. Samples were washed in dH_2O before a second osmium treatment in 2% osmium tetraoxide for 30 min at 4ºC, and again before an overnight incubation in 1% aqueous uranyl acetate at 4ºC. The following morning, coverslips were once more washed in dH_2O and incubated in lead aspartate for 30 min at 60ºC before final washes in dH_2O and dehydration and embedding as previously described.

All samples were imaged using a transmission electron microscope (Tecnai T12, FEI) equipped with a charge-coupled device camera (SIS Morada, Olympus). All electron microscopy sample preparation and imaging was kindly done by Ian White, LMCB deputy electron microscopy leader. Methods are adapted from Deerinck et al. (2010).

5.5   Hydroxyurea release

5.5.1   24-hour yields and immunofluorescence HU-release

For immunofluorescence experiments, HeLa cells on 13 mm coverslips were infected at a MOI of 15 with WR EGFP-A4 in DMEM for 30 min RT. Infection media was removed and replaced with full supplemented media, containing HU and incubated under standard conditions for 5 hours. At 5 hpi, samples were either left in the HU containing media, or washed in fresh DMEM 3x, before replacing with either untreated supplemented media, or treated with 17-AAG. Samples were incubated for a further 7 hr. All samples were fixed at

12 hpi with 4% PFA, permeabilised and blocked as before, and DNA stained with Hoechst before imaging with VT-iSIM.

For 24-hr yield, samples were infected as before with VACV WR, with HU in incubation media, and washed at 5 hpi for HU-release as described. After 24 hr incubation samples were harvested and tittered as previous.

5.5.2   HU-release for CLEM

For correlative light electron microscopy (CLEM), HeLa cells were seeded onto 14 mm gridded coverslips set in 35 mm dishes (MatTek), and infected with WR EGFP-A4 for fluorescence microscopy. Infected cells were treated and incubated with HU and washed in to 17-AAG as before. At 12 hpi, cells were fixed with EM grade 2% PFA/2% GA and washed in 5% BSA PBS 3 times. Samples were DNA stained with Hoechst for 30 min before washing in to PBS. Samples were first fluorescently imaged in PBS with 100x magnification on the VT-iSIM Nikon microscope, and EM sample preparation and imaging proceeded as previously described.

6   Western Blots

6.1   Infection timecourses

HeLa cells were seeded in 35 mm dishes for confluency at infection with VACV WR, or WR HA-D5, at a MOI of 10. Virus was bound for 30 min at RT, before aspirating and replacing with full medium. Cells were either left untreated or treated with the indicated compounds, and incubated at standard conditions. Samples were harvested at their respective timepoints, by removing media and scraping cells into PBS. Cells were pelleted and resuspended into loading buffer, containing DTT (Cell Signalling) and prepared for western blot (WB) analysis as described below (methods section 6.3).

6.2 Virus fractionations

Band purified VACV was diluted into fractionation buffer (10 mM Tris, 1.6% NP-40 (Sigma Aldrich), 50 mM DTT) and incubated at 37°C for 30 min, with mixing every 5 minutes. Samples were then centrifuged at 4°C, 20,000 x g, 1 hr. The top supernatant was collected as the first virus membrane fraction, and the remaining supernatant discarded. The remaining pellet was resuspended in 100 µl 10 mM Tris pH 9.0 and spun again 20,000 x g, 1 hr, 4°C. The top supernatant portion was collected as a membrane wash sample, and the remaining supernatant discarded. The final pellet was resuspended into 50 µl 10 mM Tris pH 9.0 as the core and lateral body sample. Each fraction was suspended into loading buffer, containing DTT, and prepared for WB analysis as described below (methods section 6.3). Fractions were blotted for either the viral core protein (p4a), membrane protein (A14), or the Hsp90 isoforms; α, β, or Grp94.

6.3 Western blot analysis

6.3.1 Sample preparation

Protein samples were sonicated for 15 min in a sonication water bath, incubated at 98 ºC for 10 min and spun at 20,000 x g, 1 min.

6.3.2 Western blot analysis

All protein samples were loaded into 4-12% Bis-Tris polyacrylamide gels and ran with MES SDS buffer (both Thermo Fisher Scientific) at 120 V, 1 hr 20 min. Transfers were carried out onto 0.2 µm nitrocellulose membrane using the semi-dry transfer system (both Biorad) at 15 V, with transfer buffer containing methanol (NuPAGE buffer, Thermo Fisher Scientific). Membranes were blocked with 5% milk TBS-T (Sigma Aldrich) for 1 hr before blotting. Primary antibodies were applied in 5% milk TBS-T for 1 hr, then was washed 3 x in TBS-T. Membranes were incubated with HRP-conjugated secondary antibodies for 1 hr, also in milk TBS-T, before washing 3 x into normal TBS-T.

Imaging of membranes was carried out with ImageQuant LAS 4000 Mini (GE Life Sciences) and Luminata Forte Western HRP Substrate (Merck) was used for detection. Western blot quantifications were done using ImageJ (Version 2.0.0) with α-tubulin used as a loading control where applicable.

7 Super-resolution of virions

High performance coverslips (18 mm, 1.5H, Zeiss) were washed with acetone, ethanol and MilliQ water. This was repeated three times before sonication in 1 M KOH for 20 min. Coverslips were then stored in 70 % ethanol til usage. Band-purified WR mCherry-A4 was diluted in 1 mM Tris pH 9.0 and bound to prepared coverslips for 45 min before fixing with 4 % PFA for 20 min at RT. Unbound virions were then removed by washing with PBS. For immuno-staining, bound virions were blocked and permeabilised with permeabilisation buffer (1 % Triton X-100, 1 % FBS, 5 % BSA PBS), for 30 min at RT on a low speed orbital shaker. Coverslips were then incubated with primary antibodies diluted in permeabilisation buffer, followed by fluorescent secondary antibodies, for 1 hour each at RT, with PBS washes in between staining. Coverslips were mounted in Vecta Shield (Vector Labs) before super-resolution structured illumination microscopy (SR-SIM) imaging. Imaging was performed using a Plan-Apochromat 63x/1.4 oil DIC M27 objective on an Elyra PS.1 microscopy (Zeiss). Images were acquired using 5 phase shifts and 3 grid rotations, with 647 nm (32 μm grating period), 561 nm (32 μm grating period), 488 nm (32 μm grating period), and 405 nm (32 μm grating period) lasers, and filter set at 3 (1850-553, Zeiss). Images were acquired using a sCMOS camera and processed using the ZEN software (2012, version 8.1.6.484, Zeiss). Multi-coloured TetraSpeck beads (Thermo Fisher Scientific) were used for channel alignment with the same acquisition settings.

8 RT-qPCR

Target	Primer Design
J2R	5′-TACGGAACGGGACTATGGAC-3′
	5′-GTTTGCCATACGCTCACAGA-3′
G8R	5′-AATGTAGACTCGACGGATGAGTTA-3′
	5′-TCGTCATTATCCATTACGATTCTAGTT-3′
F17R	5′-ATTCTCATTTTGCATCTGCTC-3′
	5′-AGCTACATTATCGCGATTAGC-3′
GAPDH	5′ AAGGTCGGAGTCAACGGATTTGGT-3′
	5′-ACAAAGTGGTCGTTGAGGGCAATG-3′

Table 2. 3 RTqPCR primer design

Designed by Yakimovich et al. (2017).

Confluent 6-well dishes of HeLa cells were infected with WT VACV at a MOI of 10. Cells were left for 30 min at RT for virus binding, before removing inoculum and replacing with full medium containing the DMSO control, AraC, CHX, or KNK437. Harvesting of cells at either 2, 5 or 7 hpi, and RNA extraction was done according the RNeasey Plus Mini kit (Qiagen) protocol, with homogenisation of the cell lysate carried out with a 21G needle. RNA was eluted using RNase-free water provided and RNA values read using the Nandrop 1000 Spectrophotometer (Thermo Fisher Scientific). First-strand cDNA synthesis was then carried out on the with the Oligo(dT)$_{12-18}$ Primer (Invitrogen), dNTPs (Thermo Fisher Scientific), and approximately 400ng RNA extract per sample, for 5 mins at 65ºC. RNaseOUT Recombinant Ribonuclease Inhibitor and the Superscript II 5 x First Strand Buffer (Invitrogen) were added alongside 0.1M DTT before running the thermocycler for 2 min at 42ºC. The Superscript II Reverse-Transcriptase was then added and samples run on the thermocycler for 50 min 42ºC followed by 15 min 70ºC. 96-well PCR plates and flat 8-cap strips (BioRad) were used for running of the qPCR samples. Amplification of cDNA of J2 (early) from 2-h samples, G8 (intermediate) from 5-h samples, F17 (late) from 7-h samples, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from all time points,

was performed using Mesa Blue qPCR MasterMix Plus for SYBR assay (Eurogentec) using primers designed previously (Yakimovich et al., 2017), specific for VACV J2R, G8R, F17R, and GAPDH (Table 2.3) on a CFX Connect (BioRad); 5 min at 95°C, 40 cycle of 15 s 95°C 1 min 60°C, then hold at 50°C. Viral mRNA threshold cycle (C_T) values were calculated and expression relative to GAPDH housekeeping gene determined.

9   siRNA silencing

9.1   siRNA-transfection

siRNA transfections were optimised 6-well dishes. Predesigned siRNAs as described in table 2.4, targeting Hsp40, Hsp72, and Hsp105 (FlexiTube from Qiagen) were diluted in RNase free water for use at a final concentration of 20 nM, and the transfection reagent, Lipofectamine RNAiMax (Thermo Fisher Scientific) was incubated in 1 ml of DMEM for 15 minutes, at an amount of 3 µl of reagent per well of a 6-well dish. siRNA dilutions and media containing Lipofectamine were then incubated together for a further 1 hr at RT. The AllStars Hs cell death control was used as a control for transfection efficiency, and AllStars negative as an off-target, scrambled siRNA control (both from Qiagen). siRNA-lipid complexes were then added simultaneous to seeding of HeLa cells to either 96-well glass-bottom plates (PerkinElmer) for microscopy-based high-throughput analysis or to 6-well dishes for all other siRNA experiments. Cells were then incubated at standard conditions for 72 hr before infection or harvesting.

To analyse knockdown efficiency, cells were harvested into WB loading buffer containing DTT 72-hours after siRNA-transfection. Western blot analysis was carried out as described above, and levels of targeted protein in siRNA-transfected cells were compared to to those of AllStars negative control (methods section 6.3). Replication site formation assays for both confocal and electron microscopy were carried out as described in methods sections 5.1

and 5.4 respectively, with infection taking place 72-hours after siRNA-transfection.

siRNA Target Gene	Target Sequence
Hsp40	5'-ATGAAGTGCGACAGCATTAAA-3'
Hsp72	5'-TCCTGTGTTTGCAATGTTGAA-3'
Hsp105	5'-AAGTGCGAGTCAACACCCATG-3'

Table 2. 4 Predesigned siRNA target sequences

9.2   High-throughput microscopy

For the original detection of late viral gene expression in inducible HSP KD conditions, siRNA-transfected cells in 96-well plates were infected with WR L EGFP at a MOI of 15 and incubated for 12 hours under standard conditions. CHX and AraC were also added to wells where no siRNAs had been transfected, as a controls for not late gene expression. Cells were then fixed with 4% PFA before washing in PBS 3 x. Hoechst diluted in 5% BSA PBS was then added to washed cells for 30 minutes before washing again with PBS. The Opera LX high-throughput microscope (PerkinElmer) was used for imaging with a 40x air objective and 405 nm and 488 nm lasers. CellProfiler was used to detect individual cells, based on Hoechst stained nuclei, and the intensity of GFP fluorescence as a read out of late expressing cells. siRNA-transfected cells that were able to express late viral genes were then calculated. Janos Kriston-Vizi kindly carried out the Opera based imaging, and Artur Yakimovich assisted with the analysis.

III Results

1 *Hsp90 is required for viral genome release and replication*

1.1 Introduction

Hsp90 is one of the most abundant, and highly conserved chaperones, with many client proteins that rely on its function. It is the chaperone most associated with viral infections, including that of VACV (Hung et al., 2002). VACV has a temporal gene expression profile and expresses its early genes from within the viral core shortly after entry into the cell cytoplasm, followed by the expression of intermediate and late genes after DNA replication. Previous work by Hung et al. (2002) showed that inhibition of Hsp90 prevented late gene expression and inhibited viral DNA replication, but had no impact on the initial entry of the virus to the cells. I set out to confirm the involvement of Hsp90 in the VACV lifecycle, and to determine at which stage it is required. Considering the requirement for ubiquitin and proteasome activity in VACV uncoating, I hypothesised that Hsp90 may also be required for viral core uncoating and genome release, as it functions in degradation, protein folding and stabilisation.

1.2 Hsp90 is needed for DNA replication and virion formation

1.2.1 *Hsp90 inhibition reduces viral yields and inhibits viral DNA replication*

Geldanamycin (GA) is a specific inhibitor of Hsp90 that blocks its chaperone activity by targeting the ATP-binding region on the N-terminal domain (Whitesell et al., 1994). GA was used by Hung et al. (2002) to inhibit Hsp90 during VACV infection. To confirm these previous findings, that inhibition of

Hsp90 activity blocked vaccinia infection, the less toxic and more stable derivative of GA, 17-AAG, was used throughout this thesis. An analysis of the toxicity of 17-AAG to HeLa cells was first carried out to ensure any anti-viral affects were true and not due to cell death caused by the drug. HeLa cells were treated with serial dilutions of the Hsp90 inhibitor and incubated for 24 hours, at which point the amount of released LDH was quantified using a colorimetric assay, as a read-out for cell death (Fig.3.1). Cells were also treated with dilutions of the microbial alkaloid Staurosporine, which induces cell death via apoptotic pathways, and could therefore be used for comparison. Whilst 10 µM of Staurosporine caused around 90% cell death, 100 nM of this compound resulted in less than 20%, and treatment with 10 nM caused no excess cell death and was similar to that of the untreated sample. Even at the highest concentration of 17-AAG tested, cell death did not exceed 50%. I therefore concluded that concentrations of 17-AAG lower than 1 µM were appropriate for use with HeLa cells as cell death did not much exceed that seen in untreated cell populations.
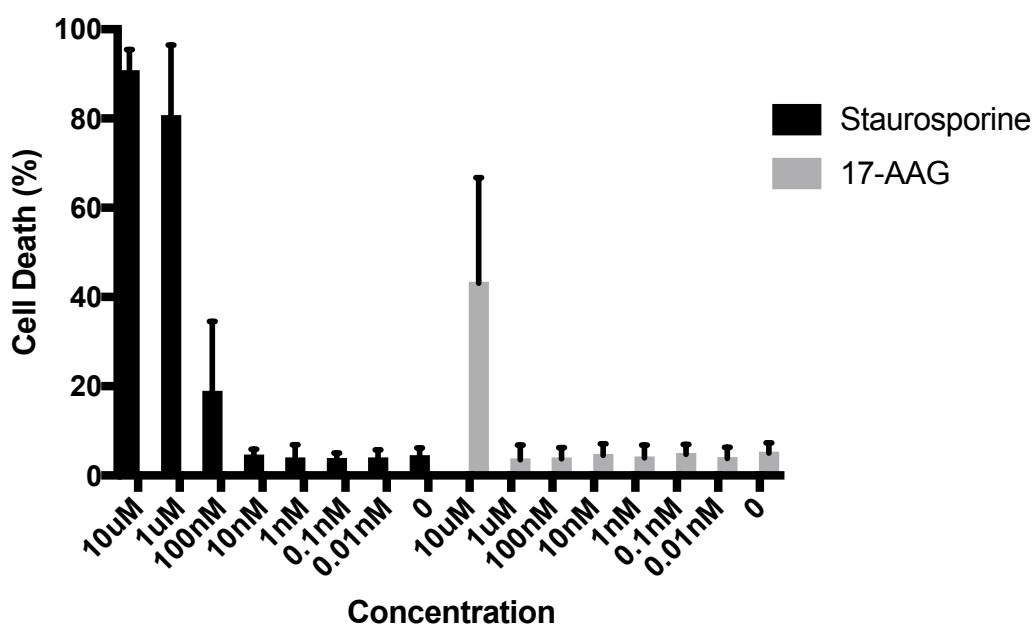
Figure 3. 1 Toxicity of 17-AAG on HeLa cells

Levels of released LDH were measured as a read-out for cell death of HeLa cells treated with dilutions of Staurosporine and 17-AAG. Cell death is shown as a percentage and was normalised to a cell lysis control. Bars shown mean from 3 repeats and error bars are SEM.

Once it had been established that the Hsp90 inhibitor was not highly toxic to cells, HeLas were infected in the presence or absence of the inhibitor, with wild-type (WT) VACV WR at an MOI of 1, and the MV yield was determined after 24 hours to assess the role of Hsp90 in VACV replication. Vaccinia formed far fewer plaques in the presence of the Hsp90 inhibitor and had a dramatically reduced MV yield compared to that of the control DMSO treated cells (Fig.3.2a). There was a reduction in plaque forming units of over 3-logs when Hsp90 was inhibited.

GA has additionally been shown to inhibit the expression of VACV late genes. To see if this could be recapitulated with 17-AAG treatment, and to begin to specify the point in the viral lifecycle where this chaperone is involved, the temporal gene expression of the virus was investigated. VACV has the capacity to encode a large amount of foreign DNA, and homologous recombination of its genome can occur naturally during virus replication (Smith and Moss, 1983, Moss, 1991). Both of these properties allow for the generation of recombinant poxviruses, which is a strategy commonly undertaken in the lab for insertion of fluorescent proteins for visual identification of the virus. To study different stages of the virus lifecycle when Hsp90 activity was inhibited, recombinant vaccinia viruses with EGFP under the control of the specific promoters, early, intermediate, and late, were used as a read out for the respective temporal gene expression of the virus (Kilcher et al., 2014, Yakimovich et al., 2017). Cells were infected with either early, intermediate, or late promoter driven GFP viruses at an MOI of 10 and their fluorescence measured using flow cytometry (Fig.3.2b). Increasing concentrations of 17-AAG had no impact on vaccinia early gene expression, as GFP remained stable in WR E EGFP infected cells. This demonstrates that stages of the lifecycle prior to early gene expression, such as virus entry, were not inhibited by addition of the Hsp90 inhibitor. On the other hand, GFP expression under the control of both the intermediate and late gene promoters, in cells infected with WR I EGFP or WR L EGFP, was perturbed by increasing concentrations of the compound; at the maximum concentration of 17-AAG, GFP expression was lower than 40% of the untreated, infected cells. This reduction in intermediate and late gene expression attests to

previous findings; that Hsp90 is needed for a productive lifecycle and is required for the expression of late viral genes.
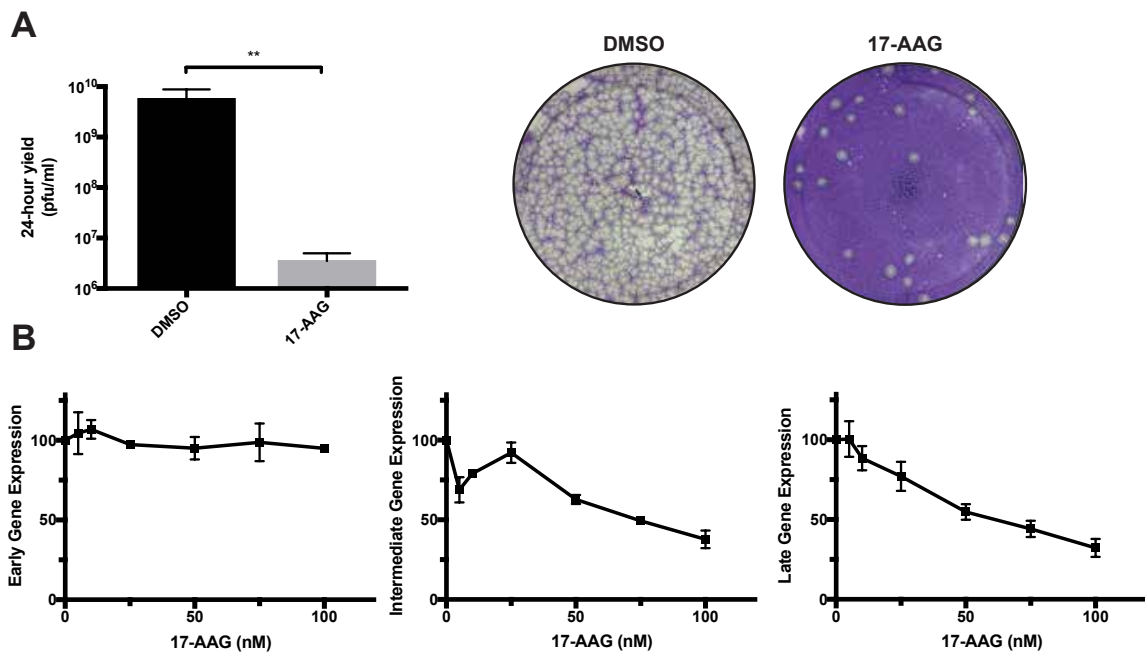
Figure 3. 2 Hsp90 inhibition reduces VACV yields and gene expression

A) MV 24-hr yield of DMSO control or 17-AAG (100 nM) treated HeLa cells and plaque formation. B) Flow cytometry expression assay of WR E EGFP, WR I EGFP, and WR L EGFP with increasing concentrations of 17-AAG. Data from 3 or more biological replicates, each data point shows mean ± SEM, **P < 0.01.

As VACV intermediate and late gene expression require viral DNA replication. To determine if the reduction in GFP controlled by the intermediate and late gene promoters was due to a direct block of intermediate and late gene transcription and translation, or inhibition of DNA replication, viral replication site formation was investigated. The compounds cycloheximide (CHX) and cytosine arabinoside (AraC) were used as controls for no replication site formation. Both compounds inhibit VACV infection; CHX inhibits early genes and therefore prevents degradation of the core and genome uncoating, whereas AraC blocks viral DNA replication (Moss and Filler, 1970, Vos and Stunnenberg, 1988). Both of these compounds therefore prevent the formation of viral replication sites, and were used as controls for no replication site formation. Infection and formation of virus factories could be visualised using confocal microscopy. Cells were infected with the GFP-tagged virus, WR EGFP-A4 (green-fluorescent core), and as VACV replicates its dsDNA in

the cytoplasm, Hoechst staining was used for detection of viral replication sites (Fig.3.3). At 8 hpi, replication sites, visible at peri-nuclear regions in the cytoplasm, were able to form as normal in the infected sample with DMSO, and contained new GFP-tagged viral intermediates. Treatment with either CHX or AraC blocked formation of replication sites as expected. In cells treated with 17-AAG, replication sites could also not form, indicating that Hsp90 inhibition blocked viral DNA replication and the subsequent formation of new virions. Infected cells were classified as containing no replication sites, containing replication sites without cores, or containing replication sites with cores. The quantification of 17-AAG treated cells resembled that of the controls, CHX and AraC, with a significantly high number of Hsp90 inhibited cells containing no replication sites and no new viral cores (Fig.3.3).
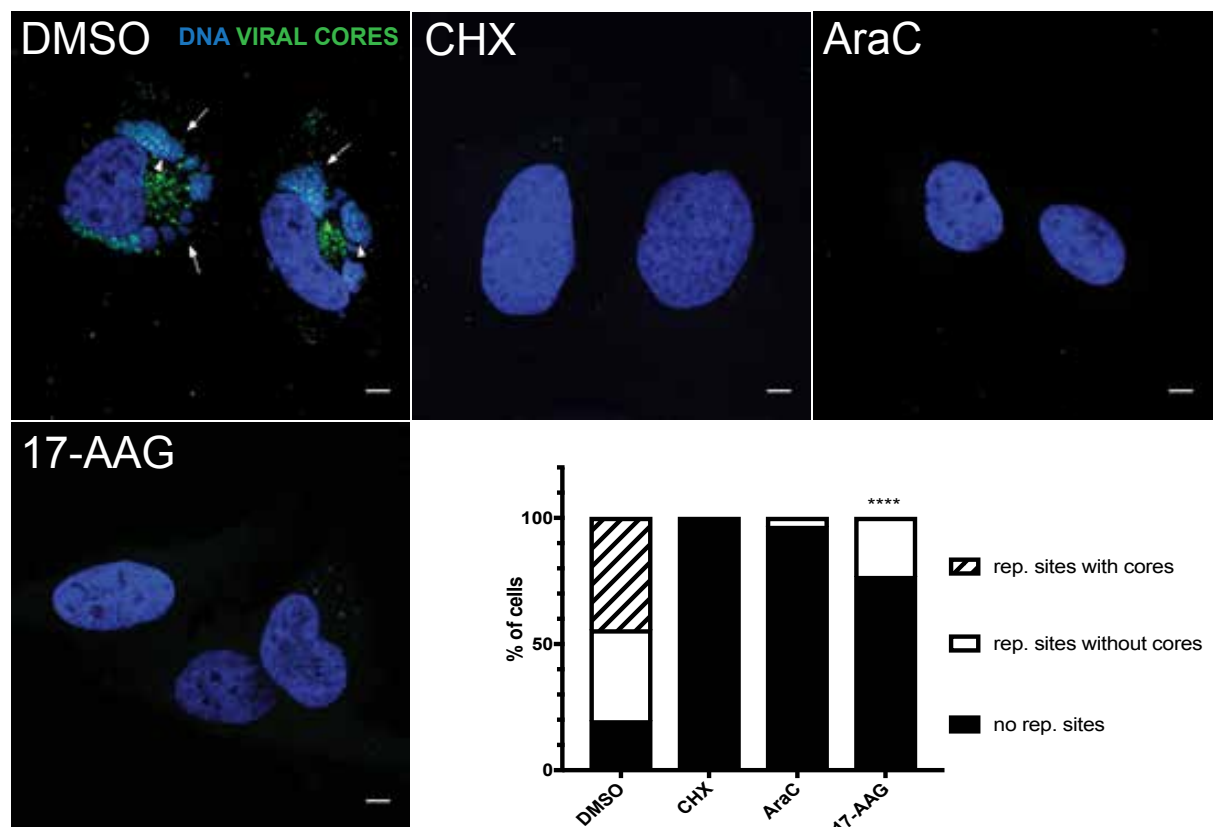
Figure 3. 3 Replication sites cannot form in the presence of 17-AAG

Confocal microscopy images of VACV infected HeLa cells at 8 hpi, in the presence of DMSO, CHX, AraC, or 17-AAG. Cells were infected with WR EGFP-A4 (green) and stained for DNA (blue). White arrows show viral replication sites and white arrowheads show newly forming virions. Scale bars = 5 μm. Images are representative of 3 biological repeats. Replication sites were quantified, and replication sites with cores were compared to the infected DMSO control. 35 cells were quantified from each replicate, ****$P < 0.0001$.

Collectively, this data corroborates what has previously been demonstrated by Hung et al. (2002), that Hsp90 inhibition blocks the formation of new vaccinia virions and reduces virus titers, and does so at a step prior to intermediate and late gene expression, by preventing replication of the viral DNA.

1.2.2   Viral genomes remain core-associated

As I have confirmed that Hsp90 inhibition blocks VACV DNA replication, but does not affect the expression of early genes, I was able to narrow down the stage of the lifecycle at which it is involved. As previously mentioned, I hypothesised that Hsp90 may be necessary for uncoating of the viral core due to its role as a molecular chaperone, in protein folding, un-folding and degradation, and its ATPase activity that is similar to that of the viral uncoating factor, D5 (Kilcher et al., 2014). To investigate if Hsp90 inhibition altered viral core uncoating and genome release, a microscopy-based uncoating assay was used that relies on the incorporation of 5-ethynl-2'-deoxyuridine (EdU) into the viral DNA (Kilcher et al., 2014, Mok and Yakimovich, 2019). WR mCherry-A4 (red-fluorescent core) was grown in the presence of EdU to produce the WR mCherry-A4 EdU-DNA virus. After infection and fixation at 4.5 hpi, EdU-containing viral genomes were labelled by coupling fluorescent azides to EdU, thus allowing for the simultaneous detection of viral cores and genomes and differentiation of the released and non-released viral DNA (Fig.3.4).

HeLa cells were infected with WR mCherry-A4 EdU-DNA in the presence of 17-AAG, and confocal microscopy images were acquired (Fig.3.5a). The controls CHX and AraC were again used, and viral genomes were classified as released or core-associated. Cells infected in the presence of DMSO contained released genomes, and replication sites with newly forming virions. CHX prevents early gene expression, and therefore blocks core degradation and genome uncoating, whereas AraC allows for the release of genomes but blocks DNA replication. The majority of green viral genomes co-localised with magenta cores in CHX treated cells, whilst they were mostly released when

AraC was present. In Hsp90 inhibited cells viral cores accumulated in the cytoplasm, and co-localised with the green fluorescent genomes. When quantified, the level of core-associated genomes in 17-AAG treated cells was similar to that of the CHX control, indicating that Hsp90 is required for uncoating of the viral core (Fig.3.5b).
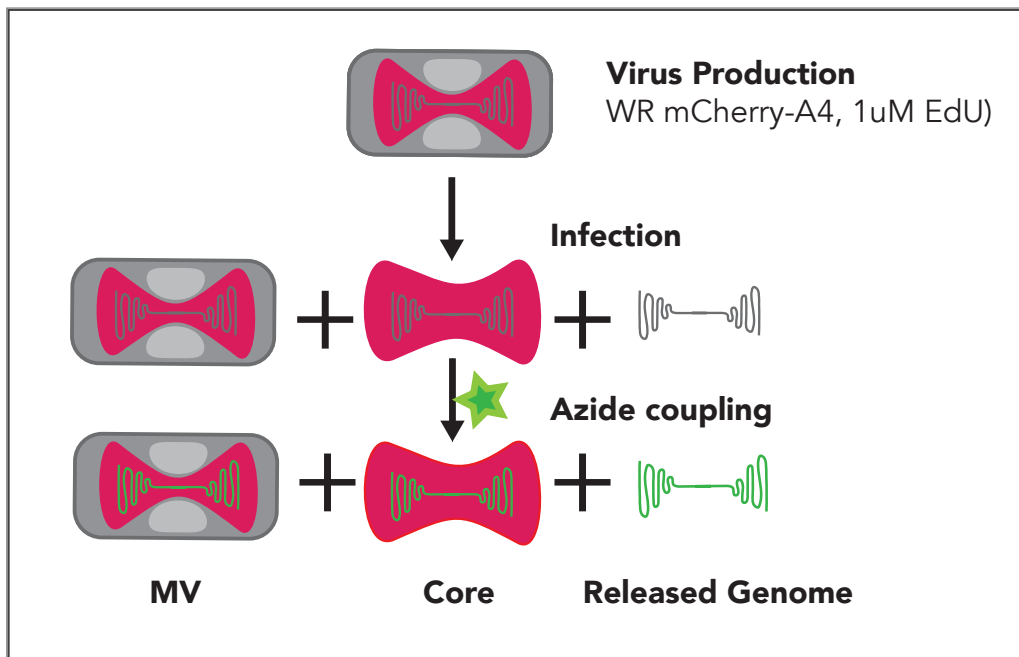
Figure 3. 4 Generation of an EdU-DNA virus for a core-uncoating assay

An EdU labelled virus was produced for a microscopy-based uncoating assay by infecting cells with WR mCherry-A4 in the presence of EdU. Coupling of the nucleoside analogue to a fluorescent azide enables distinction of released genomes to those that are non-released and still within the viral core.
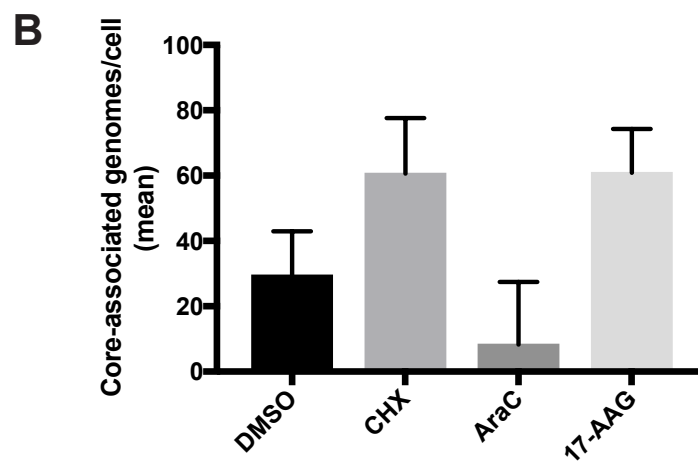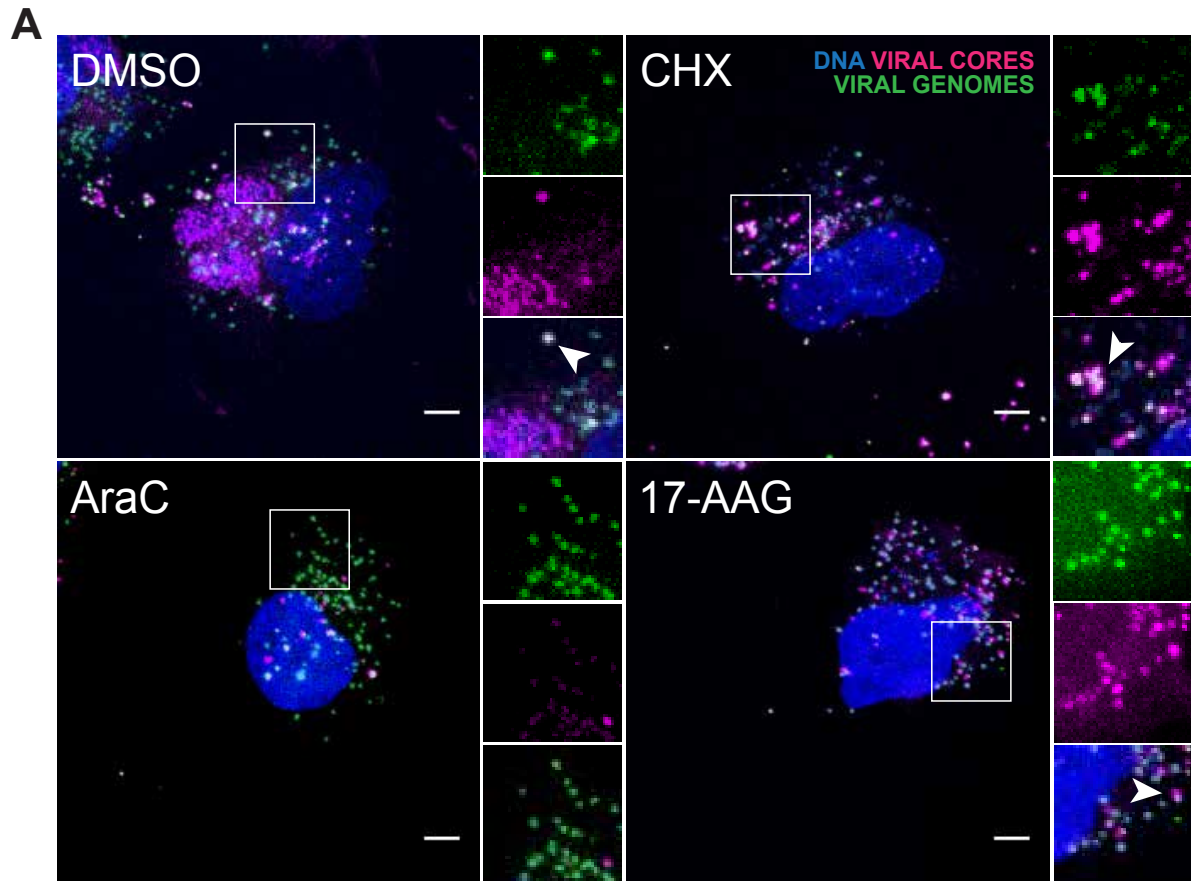
Figure 3. 5 VACV genomes localise with cores when Hsp90 is inhibited

Microscopy-based viral genome uncoating assay. A) Max projections of HeLa cells infected with WR mCherry-A4 EdU-DNA. Incoming viral DNA (green) and viral cores (magenta) are visible and nuclei were stained (blue). CHX and AraC were used as control inhibitors for core-associated genomes and released genomes respectively. Expanded selected regions are shown in side panels. White arrowheads show example core-associated genomes. Images are representative of 3 biological repeats. Scale bars = 5 μm B) Viral genomes were classified and quantified and the average number of core-associated genomes per cell is shown. At least 20 cells per repeat and condition quantified. Error bars are SEM.

1.3 Hsp90 inhibition results in an genome-release intermediate

1.3.1 Hsp90 inhibited cores are broken in the cytoplasm

I have so far demonstrated that Hsp90 is required by VACV for uncoating of the viral core and subsequent release of the genome. I sought to find out if the viral cores that accumulated in the cytoplasm of 17-AAG treated cells resembled those that are present when uncoating is inhibited through other means, such as the cores of the control drug, CHX. As although genomes were still core-associated in the microscopy-based uncoating assay, the patterns of co-localisation between green genomes and magenta cores appeared to differ. To do this I made use of electron microscopy to visualise viral intermediates in detail. Cells were infected with WT virus in the presence of CHX or 17-AAG and fixed at 4.5 hpi before imaging with transmission electron microscopy (Fig.3.6)

Viral cores that accumulate in the cytoplasm of CHX treated cells cannot release their genomes because of an inhibition of early gene expression, and lack of D5 expression. At 4.5 hpi these cores appear as large brick-shaped objects in the cytosol that still contain electron dense viral DNA (Fig.3.6a). Surprisingly, there were no viral cores that resembled these in Hsp90 inhibited cells. Instead the cores were stable in the cytoplasm and not degraded, but partially 'broken' open with patches of the core wall missing (Fig.3.6b). Notably, they also appeared to not contain the de-condensed viral genome, which is what had been suggested from the microscopy-based uncoating assay. Upon further investigation using serial sections of Hsp90 inhibited cells, these broken cores were still associated with the viral DNA, which appeared to be in the process of being released from the viral core. As has been previously described for early viral replication sites, these genomes were often found surrounded by ER membrane (Fig.3.6c). Although the appearance of viral DNA surrounded by ER resembles viral pre-replication site formation, this is a completely novel viral intermediate, whereby the viral genome is released from the broken core but remains core associated. These

results indicate that Hsp90 is involved in the complete release of the dsDNA from the viral core.
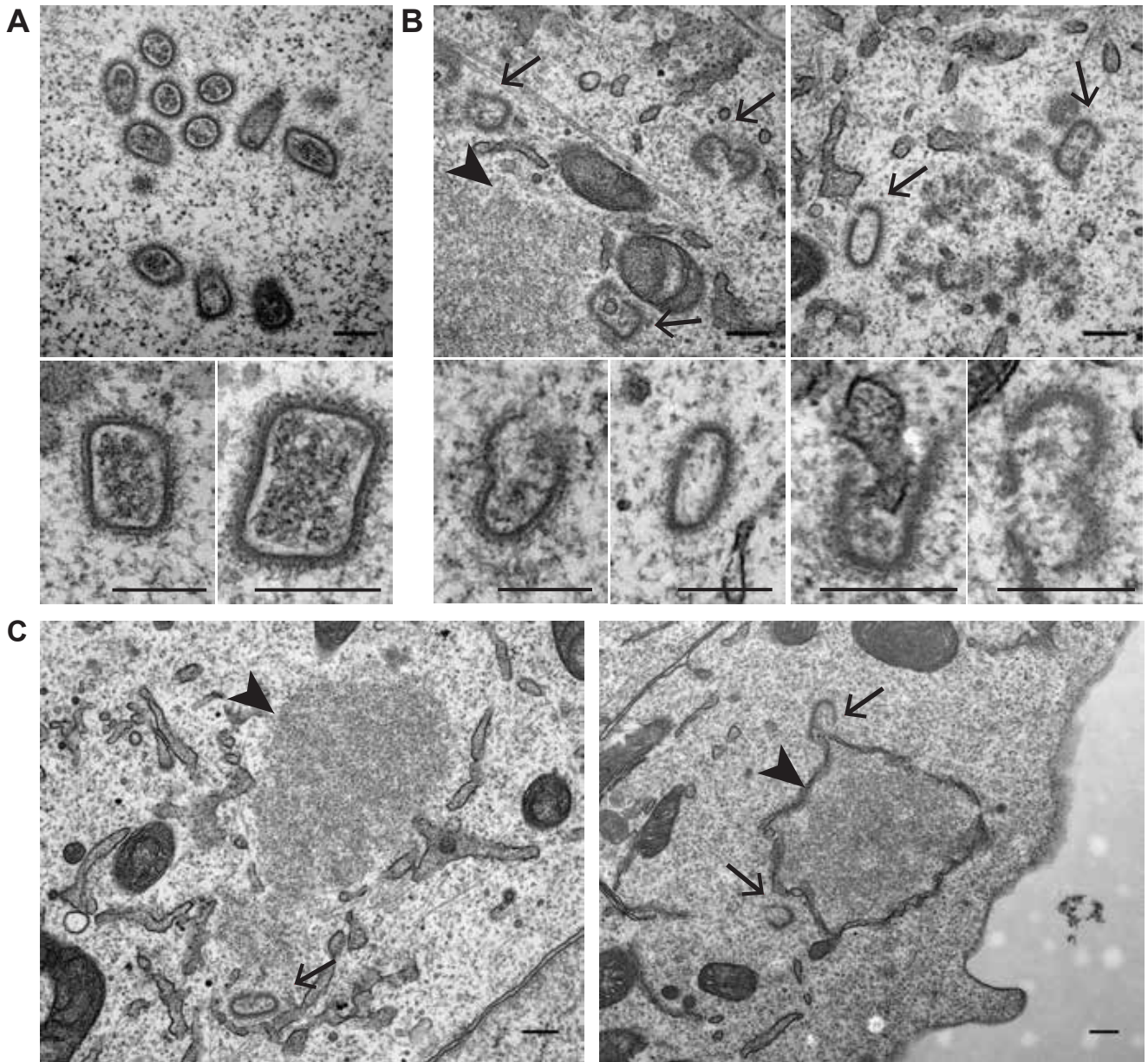
Figure 3. 6 A genome-release intermediate is present with Hsp90 inhibition

Electron micrograph of cytoplasmic VACV cores. A) CHX treated viral cores accumulate in the cytoplasm and contain electron dense viral DNA. B) Hsp90 inhibited cells accumulate viral cores in the cytoplasm that are broken open. C) Serial sectioning of infected Hsp90 inhibited cells show viral cores associate with viral DNA. Black arrowheads show cytoplasmic viral DNA, black arrows shown stabilised viral cores. Scale bars = 200nm.

1.3.2   Hsp90 works independently from the uncoating factor D5

The helicase primase D5 is the viral uncoating factor that is needed for the release of VACV genomes from viral cores, although the mechanism of how it facilitates uncoating is still unknown (Kilcher et al., 2014). As Hsp90 is a molecular chaperone that functions to fold and stabilise client proteins, I considered whether its role for genome release could be related to its requirement by a client protein. The viral uncoating factor D5 stood out as potential Hsp90 client as helicases have been found amongst the substrates of the molecular chaperone Hsp90 (Miao et al., 2018), and the microscopy-based uncoating assay of 17-AAG resembled that of D5 depletion, carried out previously by Kilcher et al. (2014).

To investigate whether the viral uncoating factor D5 is a client protein of Hsp90, the expression level of D5 protein in infected cells were quantified. As previously mentioned, an indicator of Hsp90 chaperone activity on a protein is degradation of the client by the proteasome when the chaperone is inhibited. To measure the levels of D5, cells were infected with a recombinant vaccinia virus in which D5 has been tagged with HA (Kilcher et al., 2014), and harvested over a timecourse of infection for analysis by western blot (Fig.3.7). Over the course of an 8-hour infection, the levels of D5 and pattern of its expression in infected cells treated with 17-AAG did not vary from that of WT infection indicating that the uncoating factor is not a client of Hsp90. Although this suggests Hsp90 activity is not needed for stable expression of D5, it has previously been seen that the chaperone is also required for the correct localisation of some enzymes, and its inhibition can result in their mis-localisation and therefore interfere with their function. Confocal microscopy was used to analyse the localisation of D5, which accumulates on incoming viral cores, and then in virus factories in the cytoplasm. Cells were infected in the presence or absence of 17-AAG with a green-fluorescent core virus that also had D5 tagged with HA and analysed at 5 hpi (Fig.3.8). Whilst D5 localised mostly to the replication sites in WT infected cells, as expected, its localisation in 17-AAG treated cells was surprising. In these cells, rather than appearing solely at the viral core where it is needed for core uncoating, D5

accumulated in nearby punctae, which would appear to be the partially released viral DNA visible in EM.
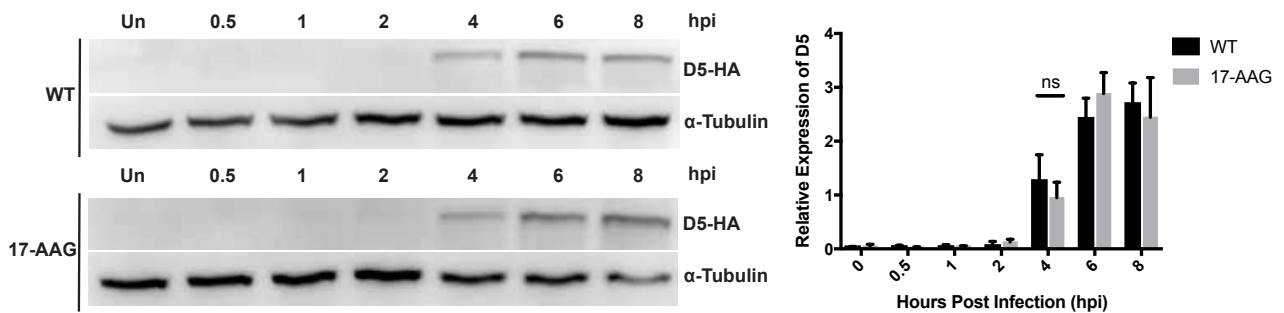
Figure 3. 7 D5 expression is not affected by Hsp90 inhibition

Western blot and quantification of D5 expression in cells infected with WR HA-D5, in the presence of absence of the Hsp90 inhibitor, 17-AAG. D5 was detected with anti-HA. Protein expression was quantified relative to the α-tubulin loading control. Blots are representative of 4 biological repeats.
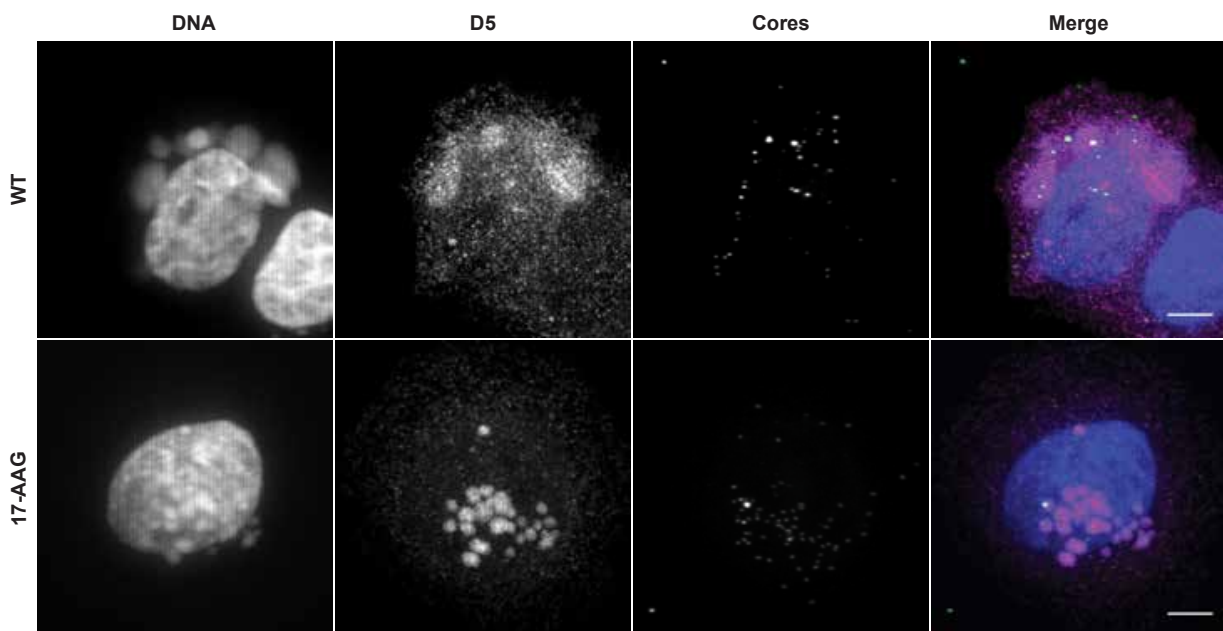
Figure 3. 8 D5 punctae localise to viral cores in Hsp90 inhibition

Confocal microscopy images of cells infected with WR EGFP-A4 HA-D5 at 5hpi in the absence or presence of 17-AAG. D5 was detected with anti-HA. Scale bars = 5µm.

1.3.3   Replication is initiated in the genome-release intermediate

In addition to its requirement for core uncoating and genome release, the enzymatic activity of D5 is also needed for the initiation of viral DNA

replication, where it is hypothesised to act at the replication fork (Boyle et al., 2007). Seeing that this protein localised next to the broken viral cores in Hsp90 inhibited cells, with the closely associated viral DNA, led us to consider that the partially released DNA may be primed for DNA replication and may require other proteins to necessitate the rest of the uncoating and replication step.

I first sought to investigate whether other proteins required for the initiation of DNA replication were client proteins of Hsp90, and therefore had their activity perturbed by Hsp90 inhibition. The levels of I3, the VACV single stranded DNA binding protein, and Cul3, the cellular Ub-ligase required for DNA replication, were measured over a timecourse of infection by western blot (Fig.3.9). The expression of the cellular protein Cul3 did not vary between WT infection and infection in the presence of 17-AAG (Fig.3.9a). Expression of the viral protein I3 across 8 hours of infection also showed no change when Hsp90 was inhibited (Fig.3.9b). This indicated that neither of these proteins require Hsp90 for their stable expression during infection.

As these three proteins, which are needed for viral DNA replication were unaffected by Hsp90 inhibition I hypothesised that the partially released viral DNA in 17-AAG treated cells may be replication competent and contain newly synthesised genomes. To investigate if viral DNA replication can occur in the genome-release intermediate of Hsp90 inhibited cells, cells were infected with the green-fluorescent core virus, WR EGFP-A4, and incubated with the nucleoside analogue 5-ethynyl-2'-deoxycytidine (EdC), which was coupled to a fluorescent azide after fixation. At 5 hpi, confocal microscopy was used to visualise the incorporation of EdC into newly synthesised DNA (Fig.3.10). In WT cells, EdC can be seen co-localising to viral replication sites, as new viral DNA is synthesised and the lifecycle progresses. The infected control cells, CHX and AraC, contain no incorporated EdC, other than that associated with the cellular nuclei. Interestingly, in the presence of 17-AAG, fluorescently labelled EdC can be seen localising near green viral cores, indicating that viral DNA is being synthesised in these genome-release intermediates. This demonstrates that Hsp90 inhibition does not prevent DNA replication, and the

areas of DNA that are seen associated to broken viral cores in EM, contain newly replicated viral DNA and is not solely from incoming genomes.
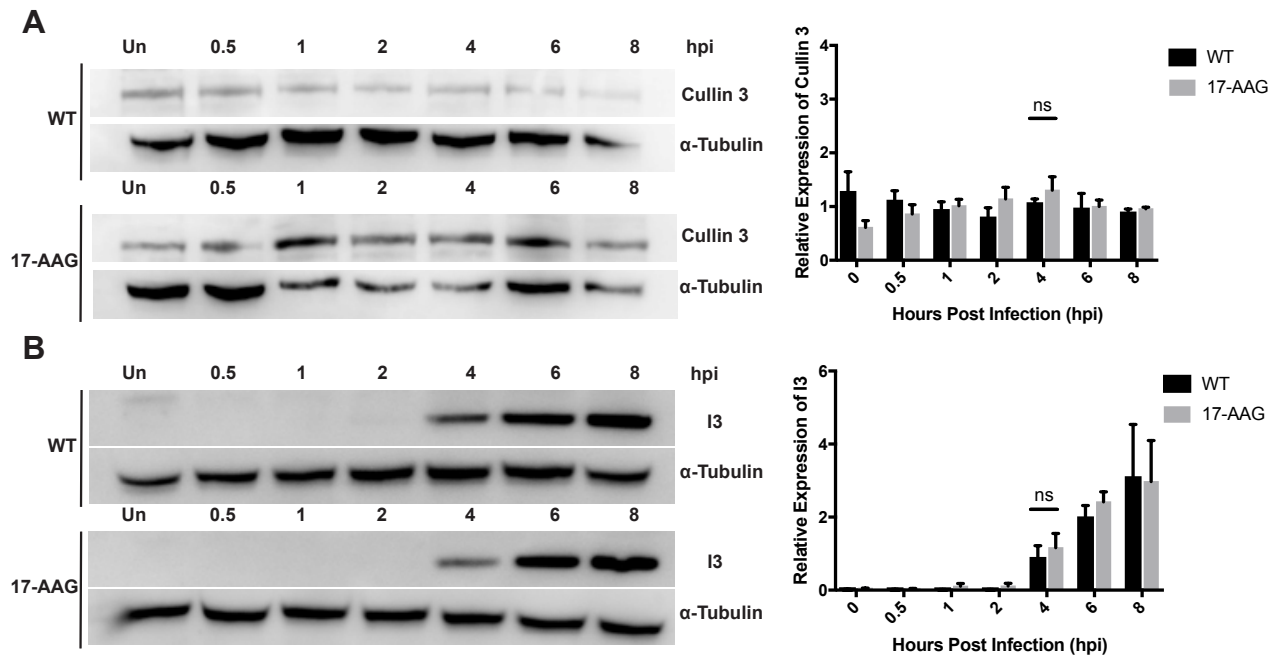
Figure 3. 9 Protein levels of Cul3 and the VACV protein I3 are unaffected by Hsp90 inhibition

Western blots and quantifications of protein expression in cells infected with WT VACV, in the presence of absence of the Hsp90 inhibitor, 17-AAG. A) Cullin 3 and B) I3 expression was detected with anti-cullin and anti-I3 respectively. Protein expression was quantified relative to the α-tubulin loading control. Blots are representative of 3 biological repeats.
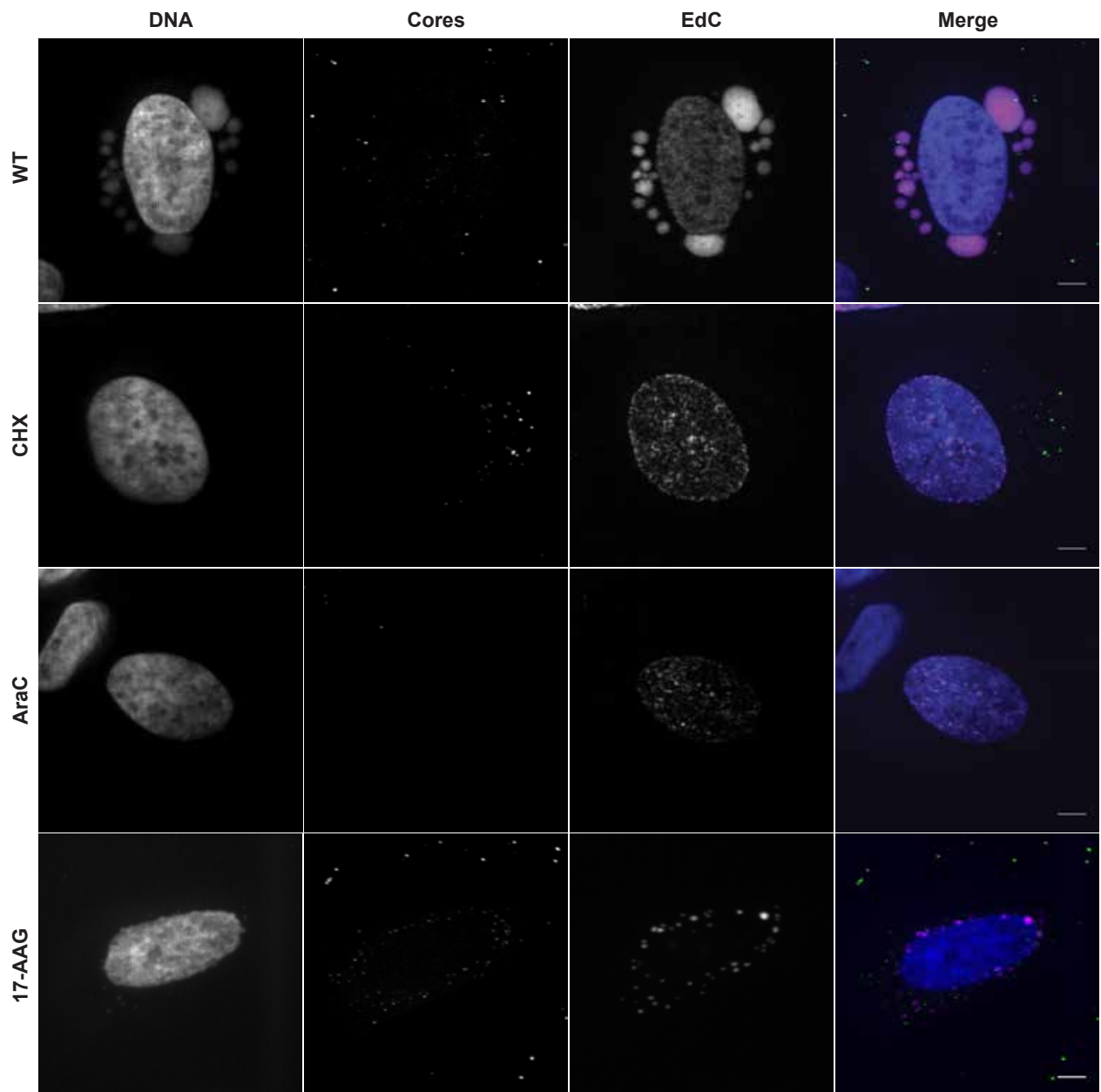
Figure 3. 10 EdC incorporates into newly synthesised viral genomes

Confocal microscopy of cells infected with WR EGFP-A4, in the presence of the nucleoside analogue EdC. DNA (blue,) viral cores (green), and EdC incorporated into newly synthesised DNA (magenta) is seen. Images representative of 2 biological repeats. Scale bars = 5 µm.

1.4   Summary

Although previous work indicated that the chaperone Hsp90 played a role during VACV infection, details of its involvement still remained elusive. Here I have confirmed that the virus requires Hsp90, as inhibition of the chaperone

with the inhibitor 17-AAG resulted in reduced viral yield and diminished late gene expression, and I have additionally built upon this by specifying the step of VACV infection for which it is required.

Within this chapter I have identified a role for Hsp90 in the core uncoating and genome-release stages of vaccinia infection, although it is different from one that requires the viral uncoating factor D5, or proteasomal activity (Fig.3.10).

As early gene expression was seen to occur independent of Hsp90 activity, but for viral DNA replication it was a requirement, the microscopy-based uncoating assay was used to demonstrate that Hsp90 is needed for release of VACV genomes from their cores. Initial data from this assay alone suggested that the viral DNA genome remains stuck inside the cores when Hsp90 is inhibited, and it was therefore predicted that these cores would appear similar to those seen in CHX treatment; large, activated cores accumulating in the cytoplasm, which contain de-condensed viral DNA. However, visualisation of Hsp90 inhibited cores by electron microscopy demonstrated that viral DNA had been partially released, and instead of the cores being degraded and DNA detaching for replication, they stayed closely associated. This showed a novel viral-core-genome intermediate, whereby viral cores are stable in the cytoplasm and released their genomes through breaks in the core wall. Through incorporation of the nucleoside analogue and fluorescent labelling through click chemistry, it was then shown that the partially released DNA could still replicate while associated with the broken cores, demonstrating that Hsp90 activity is not required for replication itself.

This therefore leads us to suggest that whilst Hsp90 activity is not directly required for viral DNA replication, it is needed for full release of the genome and the dissociation of viral cores, which in turn allows for the formation of full replication sites and the subsequent production of new virions (Fig.3.11).
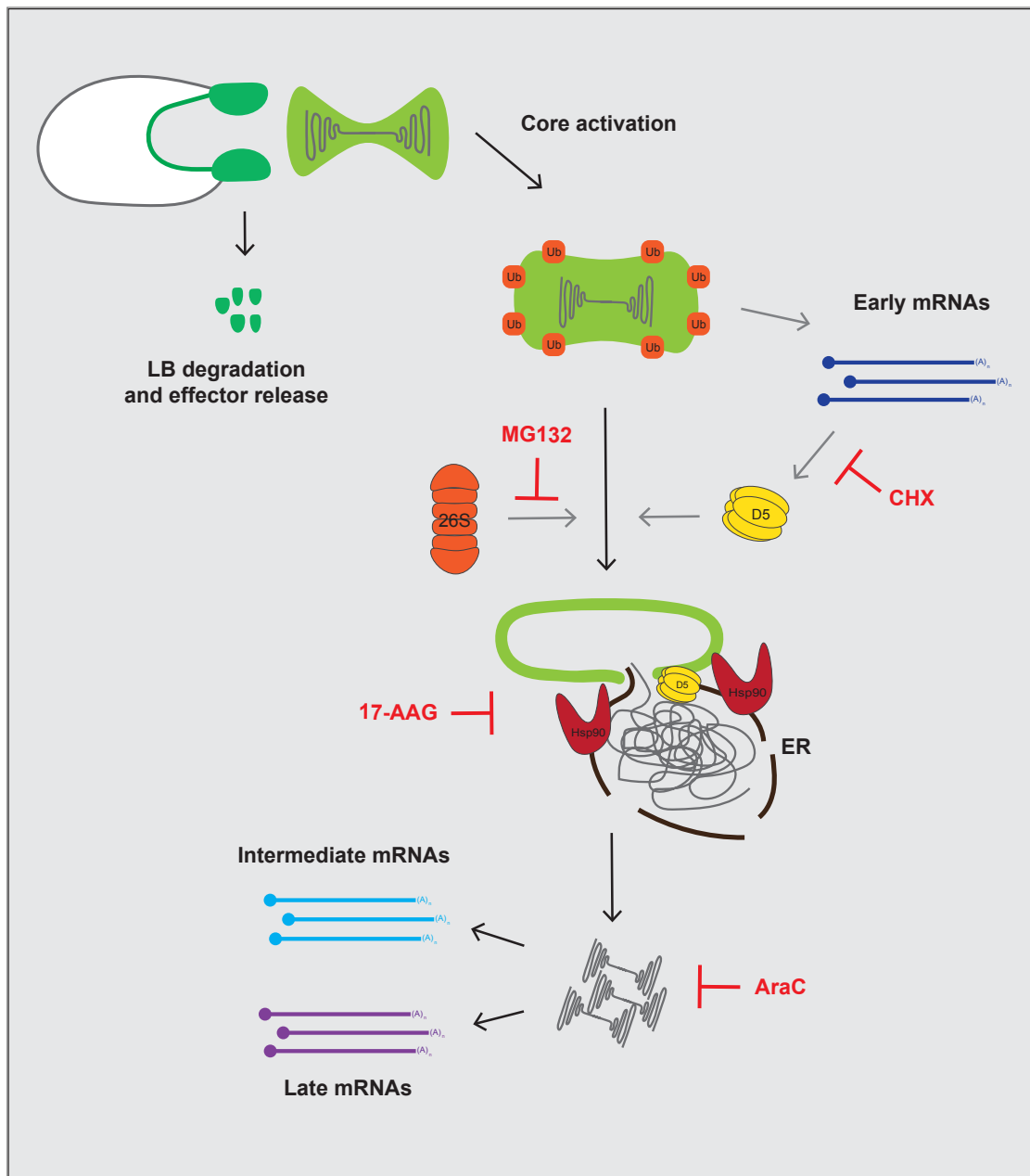
Figure 3. 11 Model of VACV genome release involving Hsp90

VACV genome release occurs in a multi-step process. After core activation the UPS and the viral ATPase D5 is required initial uncoating of the viral core. Hsp90 is needed for full release of the genome from cores in the cytoplasm, and formation of replication sites. Inhibition of the virus with 17-AAG produces stable uncoating intermediates where viral DNA remains associated to the core wall.

2 Hsp90 is required for the formation of new virions

2.1 Introduction

In chapter 1 I demonstrated that Hsp90 is required for the core uncoating and genome-release stages of the VACV lifecycle, and that this stage is different from the ones in which uncoating factors have already been identified. I have shown that Hsp90 is necessary for the dissociation of the genome from the viral core, and its full release into the cytoplasm, hereby uncovering an entirely new viral core uncoating intermediate. As the viral DNA still has the ability to replicate when only partially released into the cytoplasm this suggests that dissociation of the core is the limiting-step of VACV replication site formation in the presence of 17-AAG. I therefore sought to investigate whether Hsp90 is only required for this step, or if it is also needed for additional stages in the VACV lifecycle genome release.

2.2 Hsp90 is required for a second step of VACV infection

2.2.1 VACV also requires Hsp90 after core-uncoating

In order to investigate if release of the viral genome was the sole step in VACV infection where Hsp90 activity is needed, I needed to bypass genome release and then the effect of Hsp90 inhibition. To do this I used the compound hydroxyurea (HU), a reversible late stage inhibitor of VACV replication that allows for core uncoating but blocks viral DNA replication (Moss et al., 1969, Slabaugh et al., 1991). Cells were infected in the presence of HU to allow for the accumulation of released viral genomes in the cytoplasm. After 5 hours of infection in its presence, to allow sufficient time for viral cores to enter the cytoplasm of the cell and release their dsDNA, cells were thoroughly washed to reverse the DNA replication block. To investigate

the impact of 17-AAG treatment after core uncoating, the washed cells were then released into media containing no compound or media containing the Hsp90 inhibitor. Infection was continued for 19 hours and the 24-hour yield of cells infected with VACV WR was then determined (Fig.3.12). As a control for its inhibitory effects on VACV, cells were incubated for the full 24 hours in the presence of HU. In this instance plaque-forming units were drastically reduced as expected. Conversely, viruses that had been inhibited by HU for 5 hours, but then released into fresh media free of any compound, were able to recover and produce an MV yield comparable to that of WT infected cells, demonstrating the reversibility of the drug. When infected cells were released from HU treatment into 17-AAG, virus yields did not recover to WT levels. The 1-log reduction in MV production showed that Hsp90 inhibition perturbs a post-replicative step in the lifecycle, indicating that the chaperone is required for a second, later step in the VACV lifecycle.
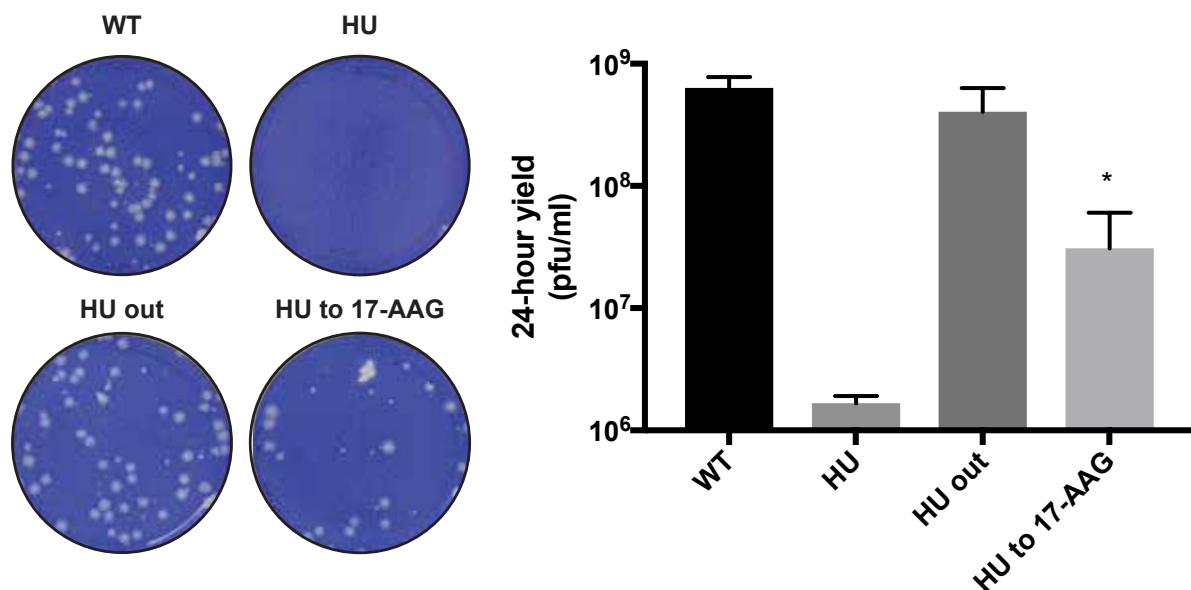
Figure 3. 12 Hsp90 is required at a second step in the vaccinia lifecycle

MV 24-hour yield of WT infected cells from hydroxyurea release assay. Cells were infected and either; left untreated (WT), incubated in the presence of HU for 24-hrs (HU), incubated with HU for 5-hrs and washed into media (HU out), or incubated with HU for 5-hrs and washed into 17-AAG (HU to 17-AAG). Data represents 3 biological repeats. Mean + SEM. *$P < 0.05$.

2.3   Hsp90 is necessary for the formation of immature virions

2.3.1   *Secondary inhibition of Hsp90 prevents virion formation*

In chapter 1, I showed that VACV still had the ability to replicate its genome in the absence of Hsp90 activity and suggested the association of the partially released genome to the viral cores limited its ability to progress. As this indicates that viral DNA replication can occur in the presence of 17-AAG, it reasoned that the second block may occur at the level of new virion formation.

To assess this possibility I needed to check for viral DNA replication and nascent virion formation. Cells were infected with WR EGFP-A4, and incubated as before with HU for 5 hours, followed by shifting into 17-AAG. Replication sites and newly formed virions were then visualised by confocal microscopy at 12 hpi (Fig.3.13). Cells infected in the presence of HU for the entire 12-hours did not form replication sites or produce new green fluorescent virions, as anticipated. When HU was removed from cells, and the effects of its inhibition reversed, VACV could recover and was able to replicate its dsDNA. Newly formed green viral particles could also be seen within the cytoplasmic replication sites. As predicted from earlier EdC incorporations assays with 17-AAG, in cells where HU was washed into the Hsp90 inhibitor DNA replication could occur, as replication sites are visible in infected cells. However, their appearance differs dramatically from that of both WT and HU released infected cells. First they lack green newly formed virions. Second, they contain very dense areas of DNA within the replication sites not seen in in the other conditions. Although these images provide limiting information regarding the nature of these dense areas, it is clear that viral DNA is accumulating in infected cells. This lack of new virions and presence of viral DNA in infected cells further suggested that the second stage of the lifecycle where Hsp90 is needed is in the formation of new virus particles.
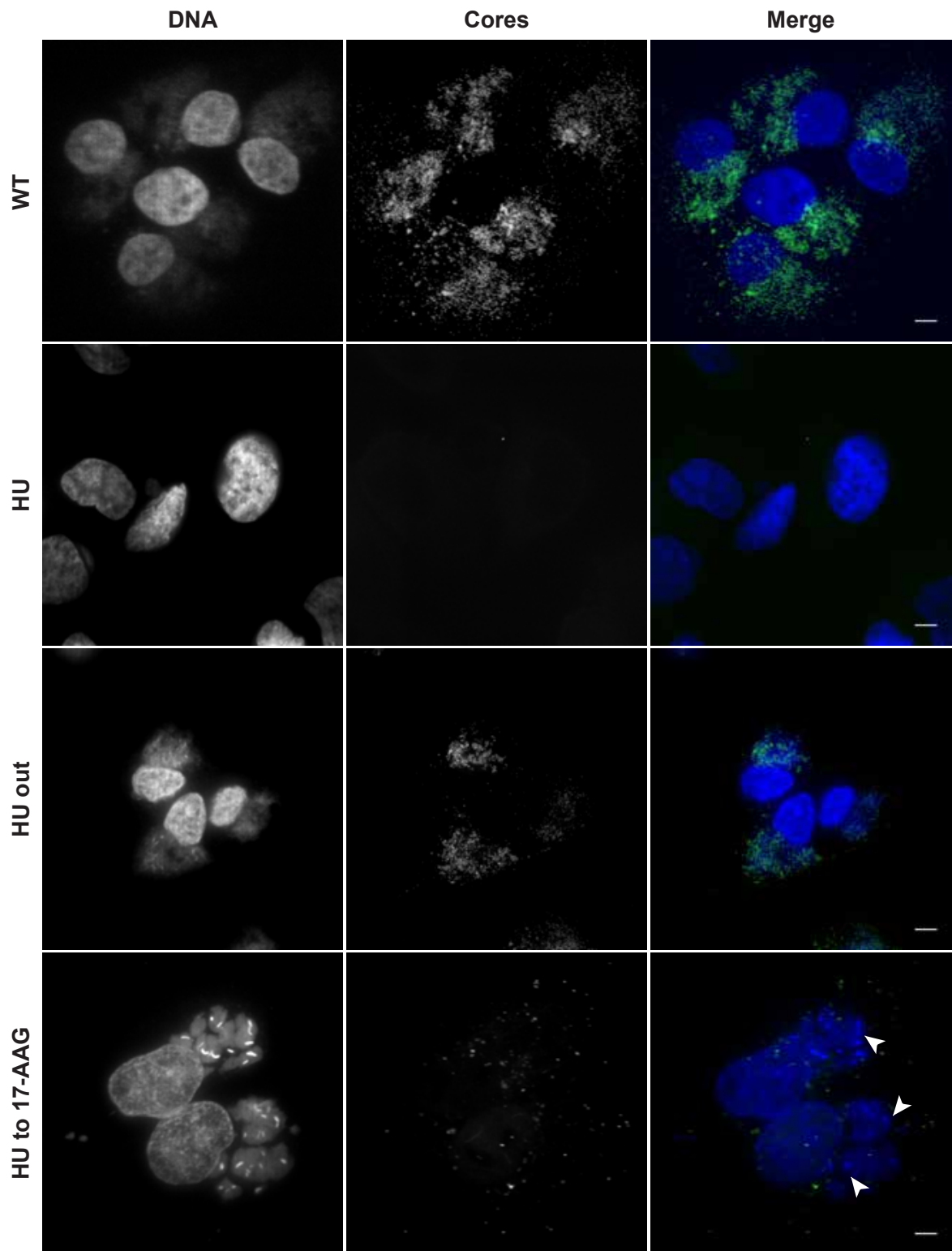
Figure 3. 13 Secondary Hsp90 inhibition prevents MV formation

Confocal microscopy of WR EGFP-A4 infected cells from hydroxyurea assay. Cells were infected and either; left untreated (WT), incubated in the presence of HU for 24-hrs (HU), incubated with HU for 5-hrs and released (HU out), or incubated with HU for 5-hrs and released into 17-AAG (HU to 17-AAG). Images are representative of 2 biological repeats. White arrowheads show areas of viral DNA condensation. Scale bar = 5 μm.

2.3.2   Viral DNA and proteins cannot associate in the presence of 17-AAG

Confocal microscopy of infected cells released from HU into 17-AAG demonstrated that the secondary role of Hsp90 in VACV infection is in the formation of new virions, as they are unable to form in the replication sites of these cells. In addition to the distinct lack of MVs, large DNA patterns occur in the viral replication sites when Hsp90 is inhibited after viral genome release. Although these striations clearly contain DNA, as they can be stained with Hoechst, it cannot yet be said whether the patterns are solely condensed areas of viral DNA, or clusters of viral intermediates that contain the genome but cannot progress through morphogenesis to form MVs. To further probe the secondary role of Hsp90 in MV formation, I returned to EM to investigate in more detail these DNA aggregates in the cytoplasm. Specifically, correlative light and electron microscopy (CLEM) was used to visualise cells at 12 hpi, infected with WR EGFP-A4 and released from HU into 17-AAG as previously described (Fig.3.14).

The areas of dense Hoechst staining in confocal microscopy images were correlated to electron micrographs of the cell and analysed. This confirmed them to be areas of electron-dense viral DNA, rather than viral intermediates containing genomes that are stuck in morphogenesis (Fig.3.14a). These areas of viral DNA condensation have a distinct morphology, and appear as dense rectangular areas that are associated with ER. Interestingly, they are similar to the previously termed 'DNA crystalloids', which have been identified in a few other instances of VACV perturbation, such as interference with DNA packaging and a rifampicin block. With Hsp90 inhibition however, they appear much larger and more consistent in their morphology (Grimley et al., 1970, Grubisha and Traktman, 2003). Although some areas of GFP fluorescence were seen in confocal microscopy, no viral intermediates were present in the correlated EM images. Membrane crescents, IVs, and IVNs were all absent. However, on closer inspection of the inhibited cells, the areas of GFP fluorescence, which did not have the same uniform appearance as normal fluorescent MVs, correlated to dense areas in electron microscopy that closely resemble the viroplasm seen in WT cells (Fig.3.14b). It would therefore

appear that whilst the GFP-A4 core protein can be translated, there is a defect in production of actual viral intermediates. Although it is evident that both viral DNA and viral proteins are produced and present in the cytoplasm of the cell, no viral crescents are formed, and the viral DNA and proteins do not localise and cannot associate to form IVs.

Although we can see DNA and evidence of viral proteins both in the cytoplasm of the cell, there are no viral crescents formed which would go on to form membranes, and the viral DNA and proteins do not localise and cannot associate to form IVs. This therefore indicates that Hsp90 plays an important role in the formation of IVs, and is critically involved with the packaging of viral proteins and viral membrane production.
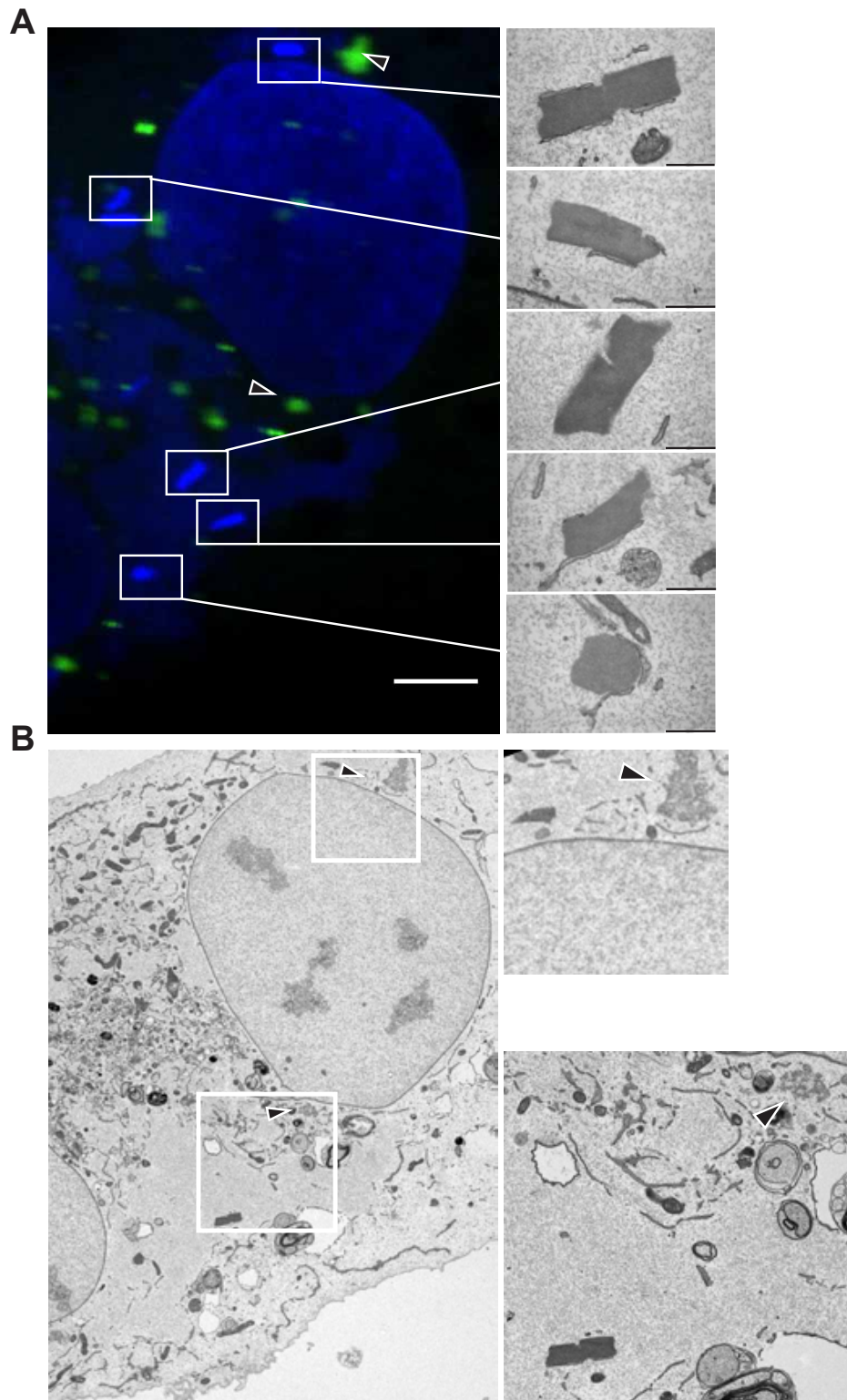
Figure 3. 14 DNA crystalloids are present in 17-AAG treated cells

Correlative light and electron microscopy of WR EGFP-A4 infected cells in the presence of HU released at 5-hrs into 17-AAG. A) Confocal microscopy image of an infected cells and electron micrographs of viral DNA condensation. B) Correlated electron micrograph of the same cell and expanded regions of interest show DNA crystalloids and areas of GFP fluorescence. Arrowheads show aggregates of viral proteins. White scale bar = 5µm, black scale bars = 500 nm.

2.4   Hsp90 is packaged for the next round of infection

2.4.1   The viral particle packages Hsp90 isoforms

As Hsp90 therefore appears to be involved in the formation of new virions, and perhaps in the association and packaging of viral proteins to DNA and membranes, I also wanted to investigate if the chaperone itself was packaged. VACV has been shown to package a selection of host proteins into its virion (Jensen et al., 1996, Webb et al., 1999, Chung et al., 2006), and although packaging of some chaperones by vaccinia has previously been suggested from analysis of the viral proteome, it is not been confirmed.

To identify if the virus packages Hsp90 I investigated the main cytosolic isoforms Hsp90α and β, alongside the ER isoform, Grp94. As VACV is a large virus, that can be visualised easily with confocal microscopy, I first utilised the super-resolution microscopy technique, structured illumination microscopy (SIM), to image individual virions. Band purified WR mCherry-A4 particles were permeabilised, and antibody detection used for investigating the presence of Hsp90 isoforms in the virus (Fig.3.15a). No Hsp90α was detected on viral cores, indicating this isoform is not packaged into the virus. However both Hsp90β and Grp94 were detected on viral particles by SIM, suggesting that the virus packages specific isoforms of the chaperone. To confirm the packaging of isoforms, and investigate which substructures these host proteins are packaged in, I carried out fractionation experiments. Purified WT virus was treated with detergent to produce fractions of viral membrane proteins, a wash fraction, and a fraction of core and LB proteins. Virion fractions were analysed by western blot, and compared to protein samples of whole purified virions and uninfected cell lysates (Fig.3.15b). The viral core protein, p4a, and the viral membrane protein, A14, were blotted for as a control for core and membrane fractions respectively. By SIM Hsp90α appeared not to be packaged by the viral particle, however by western blot analysis a small band was visible in the whole virus sample, and a small amount detected in the core and LB fraction. This indicates that VACV does package Hsp90α, but in very low amounts that were undetectable with the

previous immunofluorescence. Blotting for Hsp90β and Grp94 in whole virus samples confirmed the super-resolution data and showed they can both be found within the viral particle. Their specific location within the virions sub-structures differs however. A large band is seen for Hsp90β in the core and LB sample, and although a small amount is detected in the first membrane fraction, this is likely a small amount of the protein being removed by the detergent treatment, as conferred by the small amount of viral core protein also present in the membrane fraction. Conversely, Grp94 is detected at high amounts in the first membrane fraction, with only a minute amount appearing amongst core and LB proteins. SIM imaging and viral fractionations therefore demonstrate that Hsp90α and Hsp90β are both packaged by the virus into the core or lateral bodies, although the α isoform is packaged at much lower amounts, and Grp94, on the other hand, appears to be packaged into the viral membrane.
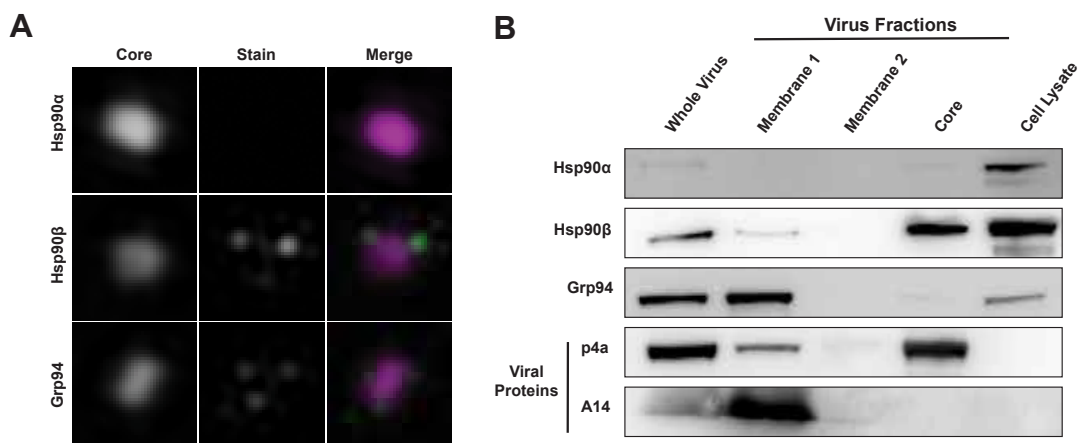
Figure 3. 15 Vaccinia packages Hsp90 isoforms

VACV packages Hsp90 isoforms demonstrated by A) structured illumination microscopy of individual virions. WR mCherry-A4 virions were permeabilised and probed for Hsp90 isoforms, and B) fractionation of virus particles analysed by western blot, and blotted for viral proteins and Hsp90 isoforms. Images representative of 3 biological repeats.

2.5   Summary

Within this thesis I had previously described the requirement of Hsp90 by VACV for the full release of its dsDNA genome from the viral core. I have now

demonstrated in this chapter that there is a further requirement for the chaperone later in the lifecycle, and that vaccinia packages Hsp90 isoforms within the virus sub-structures (Fig.3.16).

Inhibition of Hsp90 after successful viral genome release still reduced production of MVs over 24 hours, indicating a second block with 17-AAG treatment. I was able to further identify that this second role for Hsp90 in the vaccinia replication cycle is in the formation of immature virions. CLEM was paramount to this discovery and the identification of DNA crystalloids, which are associated with ER membranes, in cells with aberrant Hsp90 activity. Although DNA crystalloids have previously been described when viral morphogenesis is blocked by the depletion of either the vaccinia genome packaging protein I6 or the viral protease I7, the virus is still capable of producing viral intermediates and the crystalloids exist in addition to viral crescents and IVs in these cases (Ericsson et al., 1995, Grubisha and Traktman, 2003). Instead, the existence of the crystalloids produced from Hsp90 inhibition is most similar to the phenotype of a rifampin block of viral morphogenesis, where viral intermediates also do not form (Grimley et al., 1970). In addition to these areas of viral DNA condensation, CLEM also revealed that GFP-A4 was expressed in Hsp90 inhibited infected cells, but instead of remaining in the replication sites and co-localising to viral DNA it was found on the peripheries in large protein aggregates. I have also now confirmed that VACV packages multiple Hsp90 isoforms into its different substructures, and it could be considered that this variation in isoform location may be related to how the virus uses each protein differently in its lifecycle.

Collectively, this chapter demonstrates that Hsp90 is directly involved in the production of immature virions, and plays a critical role in the wrapping and encapsidation of viral proteins and DNA by crescent membranes. I have shown that the role for Hsp90 in VACV infection is far more complex than originally thought.
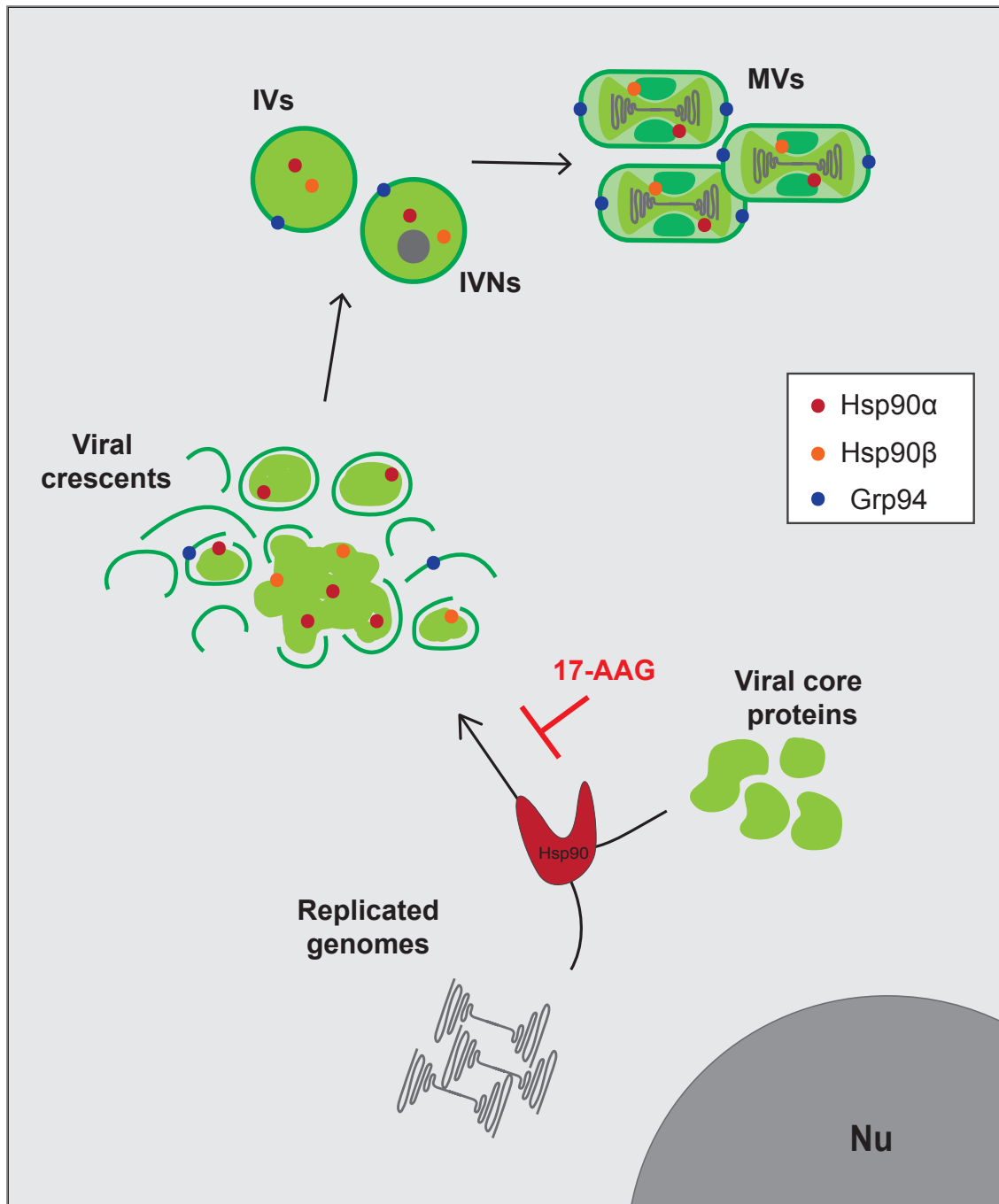
Figure 3. 16 Model of IV formation requiring Hsp90

Nascent genomes and viral proteins are present in cytoplasmic replication sites. Hsp90 is required for their encapsidation and wrapping by crescent membranes, and formation into IVs. The virus packages the chaperone isoforms Hsp90α, Hsp90β, and Grp94 (red circles) within the core/LBs and viral membrane of MVs.

3   An inducible HSP is required for virion formation

3.1   Introduction

We have now ascertained that the molecular chaperone Hsp90 is packaged by VACV and is required at multiple stages of its lifecycle. As HSPs are often found to work co-operatively, and additional members have been implicated in the replication cycles of other viruses, I decided to further investigate the role of host chaperones in the virus lifecycle. In addition to the previous study on Hsp90, a role for the transcription factor, HSF1, during VACV infection has been proposed (Filone et al., 2014). HSF1 is the master transcriptional regulator of the heat shock response, and its activation during cellular stress triggers transcription of a set of 'inducible' HSPs. Filone et al. (2014) found HSF1 to be activated during VACV infection, which increased transcription of its target genes to occur (Yang et al., 2010). Inhibition of the transcription factor was shown to prevent VACV and MPXV infection, as measured by virus titer, indicating that HSF1-regulated genes are involved in productive orthopoxvirus infection.

I therefore chose to investigate the role of HSF1 in VACV infection by taking a similar approach as the investigation into Hsp90. I sought to find out if the function of HSF1 in VACV is linked to the requirement for Hsp90, or if it plays an entirely separate role, and the activity of its transcriptional targets in the VACV lifecycle.

3.2   Inhibition of inducible HSPs prevents new virion formation

3.2.1   KNK437 treatment reduces late protein expression and MV formation

To investigate HSF1 activity relative to VACV infection, the compound KNK437 was used. This benzylidene lactam compound prevents binding of HSF1 to the HSE to inhibit HSF1 activity, and thus prevents the induction of

HSF1-regulated chaperones (Yokota et al., 2000, Ohnishi et al., 2004). As with 17-AAG, toxicity of this inhibitor was assessed in HeLa cells by carrying out an LDH assay to quantify cell death in the presence or absence of the compound (Fig.3.17). Dilutions of Staurosporine were again used for titrations of cell death, where the highest concentrations were almost 100% toxic to HeLa cells. At 1 mM, KNK437 resulted in over 50% cell death, whilst 100 µM caused only low levels that were similar to those found in untreated cell populations. I therefore concluded that KNK437 was appropriate for use with HeLa cells at concentrations of 100 µM or lower.
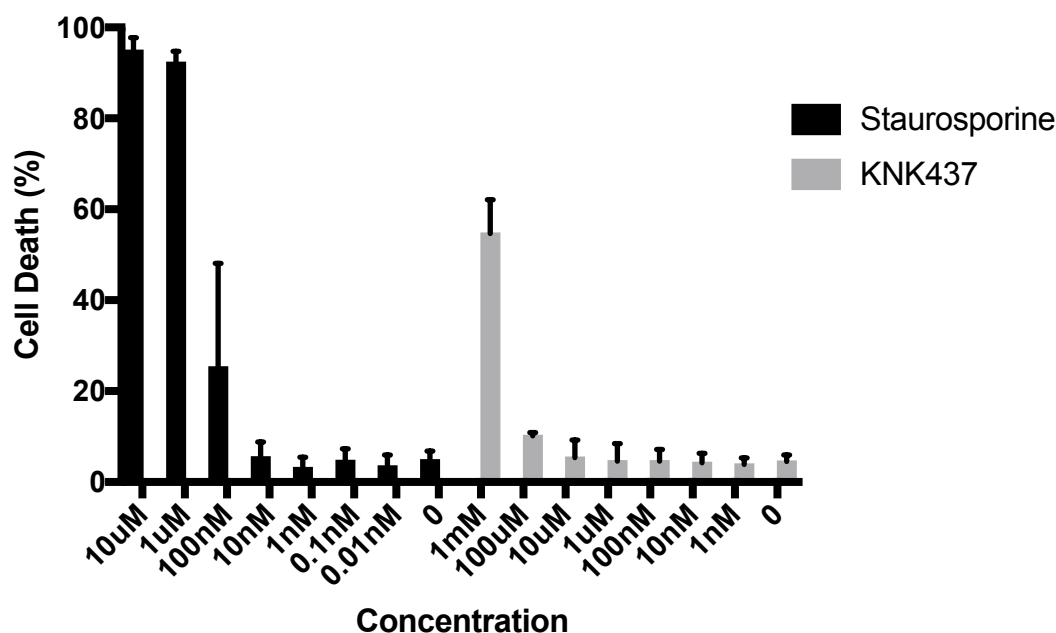
Figure 3. 17 Toxicity of KNK437 in HeLa cells

Levels of released LDH were measured as a read-out for cell death of HeLa cells treated with dilutions of Staurosporine and KNK437. Cell death is shown as a percentage and was normalised to a cell lysis control. Bars show mean from 3 repeats are error bars are SEM.

To first confirm that its inhibition results in diminished infectivity, cells were infected with VACV WR at an MOI of 1 and incubated with the inhibitor. MVs were harvested after 24 hours and virus titers deduced (Fig.3.18a). Inhibition of HSF1 by KNK437 resulted in reduced plaque formation, and a reduction in MV yield by 2-logs. This confirms that HSF1-regulated proteins are required for the replication cycle of VACV. To determine whether KNK437 produced an early or late block during infection, I again investigated the temporal gene

expression of VACV. As with our investigation into Hsp90, cells were infected at an MOI of 10 with recombinant viruses that express GFP under either an early, intermediate, or late viral promoter. Infected cells were incubated in the presence or absence of KNK437, and fluorescence measured by flow cytometry as a read out for their respective gene expressions (Fig.3.18b).
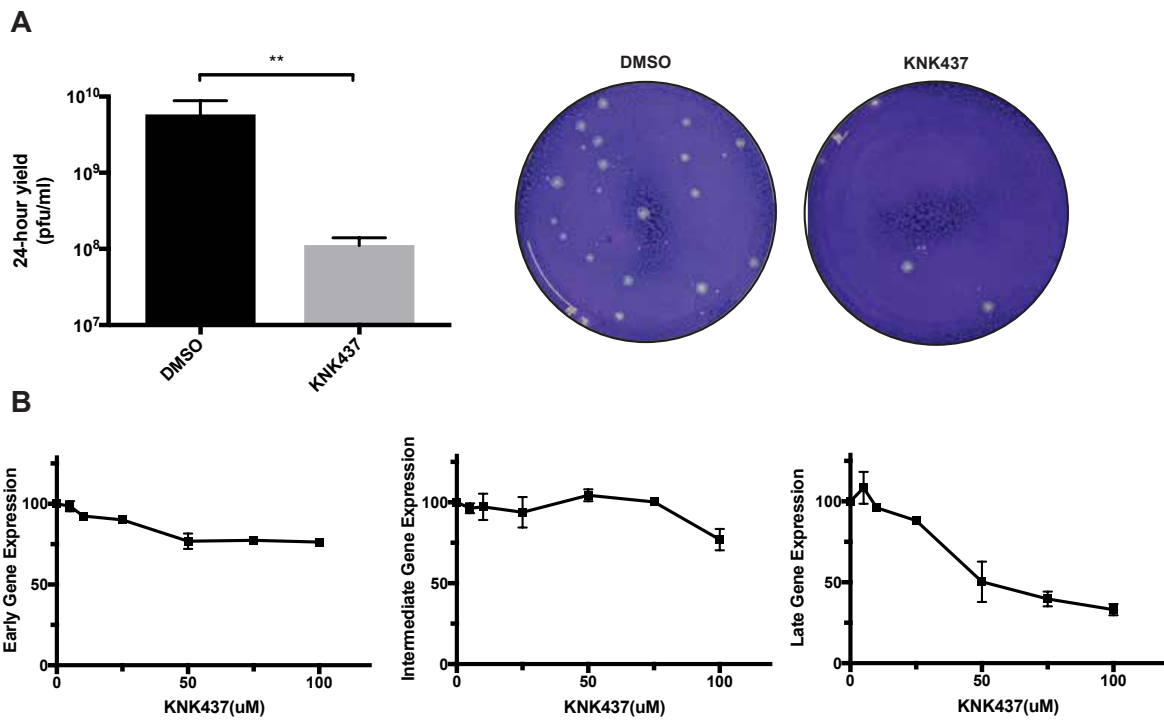
Figure 3. 18 Treatment with KNK437 reduces VACV yields and late protein expression

A) MV 24-hr yield of DMSO control or KNK437 treated HeLa cells and plaque formation. B) Flow cytometry expression assay of WR E EGFP, WR I EGFP, and WR L EGFP with increasing concentrations of KNK437. Data from 3 or more biological replicates, each data point shows mean ± SEM, **P < 0.01.

Expression of GFP under the early gene promoter was unaffected by an increasing amount of KNK437, indicating that the virus is able to enter the cytoplasm of cells and express its early genes. Interestingly, intermediate gene expression remained relatively stable, with only a slight decrease at the highest concentrations of the compound, but late gene expression was dramatically affected. GFP fluorescence under the late gene promoter decreased substantially with increasing drug concentrations, with 100 µM KNK437 resulting in less than 30% gene expression. Together this indicates

that inducible HSPs are involved in a post entry step, as viral cores can express their early genes, and intermediate genes that are only expressed after DNA replication were largely unaffected. Right off, this indicated that HSF1, and therefore inducible HSPs, are involved in a different stage of the VACV lifecycle to that of Hsp90.

To investigate the later steps of the virus lifecycle, and to confirm that viral DNA replication could occur, I used the GFP core virus, WR EGFP-A4, alongside Hoechst staining to image viral replication sites and newly forming virions. Cells infected at a MOI of 10 with the green-fluorescent core virus, were imaged at 4.5 hpi by confocal microscopy (Fig.3.19). Large viral replication sites containing newly forming virions can be seen in the cytoplasm of infected cells when incubated with DMSO. The drugs CHX and AraC were again used as controls for no replication site formation. In the presence of KNK437 we can see that replication sites can form within infected cells. However the lifecycle is still impaired, as these sites appear smaller than those in WT cells and no newly forming green-fluorescent virions are present. Compared to WT infected cells, where the population is distributed between cells that contain replication sites with cores, and cells that contain replication sites without cores, approximately 80% of KNK437 treated cells contain replication sites that have no cores. The result is consistent with what was seen by flow cytometry, that whilst VACV DNA replication can occur, and intermediate genes are able to be transcribed, when HSF1-activity is inhibited we get a block in late proteins and therefore an inhibition of virion formation.
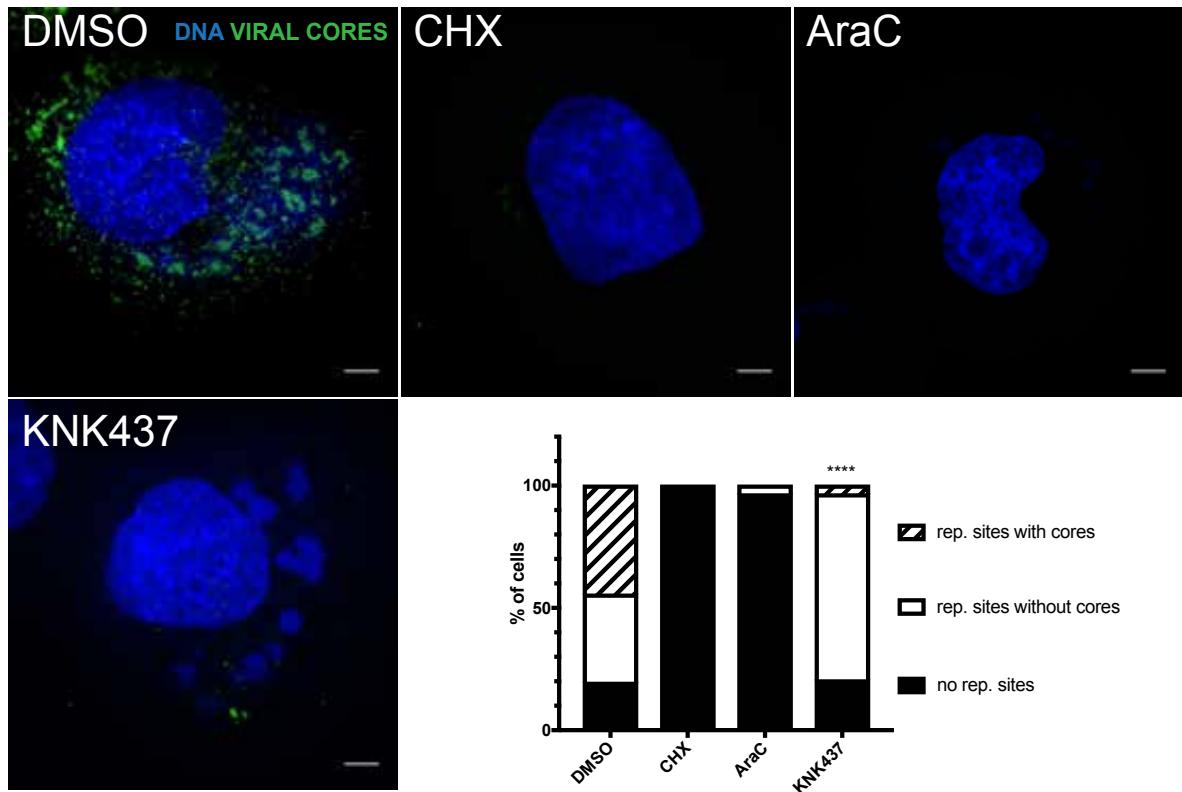
Figure 3. 19 KNK437 treatment alters replication site formation

Confocal microscopy images of VACV infected HeLa cells, in the presence of DMSO, CHX, AraC, or KNK437. Cells were infected with WR EGFP-A4 (green) and stained for DNA (blue). Scale bars = 5 µm. Images are representative of 3 biological repeats. Replication sites were quantified, and replication sites with cores were compared to the infected DMSO control. 35 cells were quantified from each replicate, ****P < 0.0001.

3.2.2   KNK437 treatment reduces accumulation of late viral proteins

I have now shown that inhibition of HSF1-regulated genes impacts the levels of late viral proteins and newly formed virions in VACV infected cells. This suggests that the inducible HSPs inhibited by KNK437 are involved in late-protein expression stages of the viral lifecycle, as these proteins encode for new viral structural components and the enzymes needed for morphogenesis and virion formation. The current data gives no indication however of whether these chaperones are involved in this step at the protein level, i.e. translation or folding of late gene products, or prior to this in the actual transcription of late viral mRNAs.

To determine how the inhibition of HSF1-activity by KNK437 reduced new virion formation, I investigated the direct impact of the inhibitor on late viral proteins (Fig.3.20). To determine the affect of KNK437 on VACV late gene mRNA levels I infected cells at a MOI of 10 in the presence or absence of inhibitors, and carried out RT-qPCR. Primers for the early, J2, intermediate, G8, and late, F17, viral proteins were used as standards to investigate the mRNA levels in infected cells relative to that of WT infected cells harvested at 2, 5, and 7 hpi respectively (Fig.3.20a). The control drug CHX allows for early gene expression but inhibits early protein synthesis and subsequent stages of VACV replication. We can see early viral mRNAs are still present in CHX treated samples but those of the intermediate and late genes are reduced. The control drug AraC, which inhibits VACV DNA replication, shows a reduction in both intermediate and late viral mRNAs as expected. When we inhibit inducible HSPs with KNK437, early and intermediate mRNAs are both transcribed, consistent with the flow cytometry and confocal microscopy data. When measuring the levels of late viral mRNAs, we see that F17 RNA is still present in cells infected in the presence of this drug. In KNK437 inhibited samples late mRNA levels are in fact higher than those of WT infected cells. It is possible that both intermediate late viral mRNAs accumulate in these infected cells due to a lack of feedback inhibition, which may usually occur in the presence of late viral proteins that shut off early and intermediate transcription.

The RT-qPCR analysis demonstrates that inducible HSPs are not involved in the inhibition of late viral proteins at a transcription level, and must therefore be involved at the protein level. To investigate this I blotted for the same late viral protein F17, in VACV infected cells in the presence or absence of KNK437 over a 24-hour timecourse (Fig.3.20b). In cells infected in the absence of KNK437 newly synthesised F17 is seen after 4 hpi upon DNA replication and late gene expression, with increasing expression over time. In HSF1-inhibited cells the protein levels of newly expressed F17 were drastically reduced across all timepoints compared to that of WT, with very little F17 present at 12- and 24-hour timepoints. This shows that inducible HSPs are required for the expression of the late viral protein, F17.
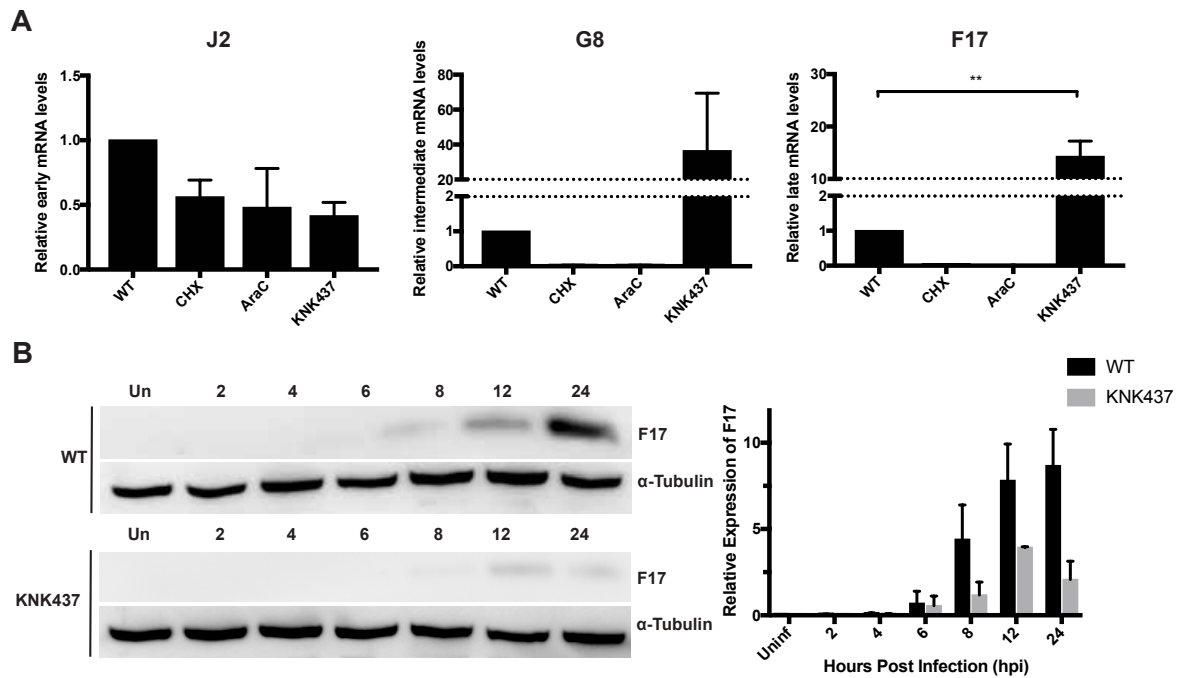
Figure 3. 20 KNK437 inhibits F17 at the protein level

A) Early, intermediate, and late viral mRNA levels were measured in VACV WR infected cells in the indicated conditions, by RTqPCR with primers against the viral genes J2, G8, and F17 as a readout for their respective mRNA transcription. B) Levels of the late expressed viral protein F17 were measured over a 24-hour timecourse by western blot in the absence or presence of the inducible HSP inhibitor, KNK437. Protein expression was quantified and calculated relative to the loading control. Data is representative of at least 2 biological repeats. ** P < 0.01

3.2.3   *KNK437 treatment impairs the formation of viral intermediates*

The late viral protein F17 (previously known as F18) is one of the most abundant structural proteins of VACV, with 27,000 copies per MV. It is a LB protein that has been shown to interact with itself and other viral proteins, and to localise throughout IVs (Sarov and Joklik, 1972, Kao and Bauer, 1987, Pedersen et al., 2000, Mercer and Traktman, 2005, Chung et al., 2006). In its absence, VACV exhibits morphogenesis defects and forms aberrant MVs that are non-infectious, contain the majority of viral proteins and DNA, but lack definable MV sub-structures (Wickramasekera and Traktman, 2010). Although I have demonstrated that inhibition of VACV with KNK437 results in a decrease of F17 protein levels, MVs could not be seen by immunofluorescence microscopy. If aberrant assembled MVs were present I

115

would have expected to see these as green-fluorescent virions. This suggested that VACV inhibition by KNK437 might block an early step of morphogenesis. To investigate the formation of viral intermediates in the presence or absence of the inhibitor, cells were infected with VACV WR at a MOI of 10 and visualised by EM at 8 and 12 hpi (Fig.3.21).

At 8 hpi, WT infected cells contain viral intermediates within the replication sites; membrane crescents closing around viral matter, IVs, IVNs, and some MVs are all present on the periphery of the replication site. ER, which surrounds the viral replication site at earlier timepoints, can also be seen starting to disperse as the infection cycle progresses (Fig.3.21a). In KNK437 treated cells replication sites are still visible in the cell cytoplasm, however no viral intermediates are detected. Instead these viral factories, which are absent of identifiable VACV structures, contain some small areas of higher electron density that could be accumulated viral matter that is unable to be packaged into virions (Fig.3.21a). Strikingly the ER is still tightly associated with these replication sites and is not distributed as much throughout the factory. To determine if HSF1-inhibition resulted in a delay in the formation of viral intermediates, replication sites were also imaged at a later timepoint of 12 hpi (Fig.3.21b). In cells with no inhibition, we can again see high amounts of viral intermediates produced in the replication sites, and the amount of IVNs and MVs is higher than at 8 hpi, as expected. When infected in the presence of KNK437 however replication sites at 12 hpi still remain empty. A very small amount of viral membrane intermediates can sometimes be seen, however these do not package viral matter and do not form any type of virion within the cytoplasm. When quantified we can see that WT infected cells are able to produce high amounts of IVs and MVs, and that KNK437 significantly reduces the amounts of virions formed at both 8 and 12 hpi (Fig.3.21c).

Perturbation of the VACV structural protein F17 results in non-infectious MVs that lack proper sub-structures. Although I showed that KNK437 treatment reduces the levels of F17, I have now demonstrated that this is not the sole reason for decreased viral yield, as we do not see the same aberrant MV phenotype (Wickramasekera and Traktman, 2010). Instead this suggests that

116

inducible HSPs are required for late proteins that are involved in the initial formation of viral membranes and IVs.

A

B

C
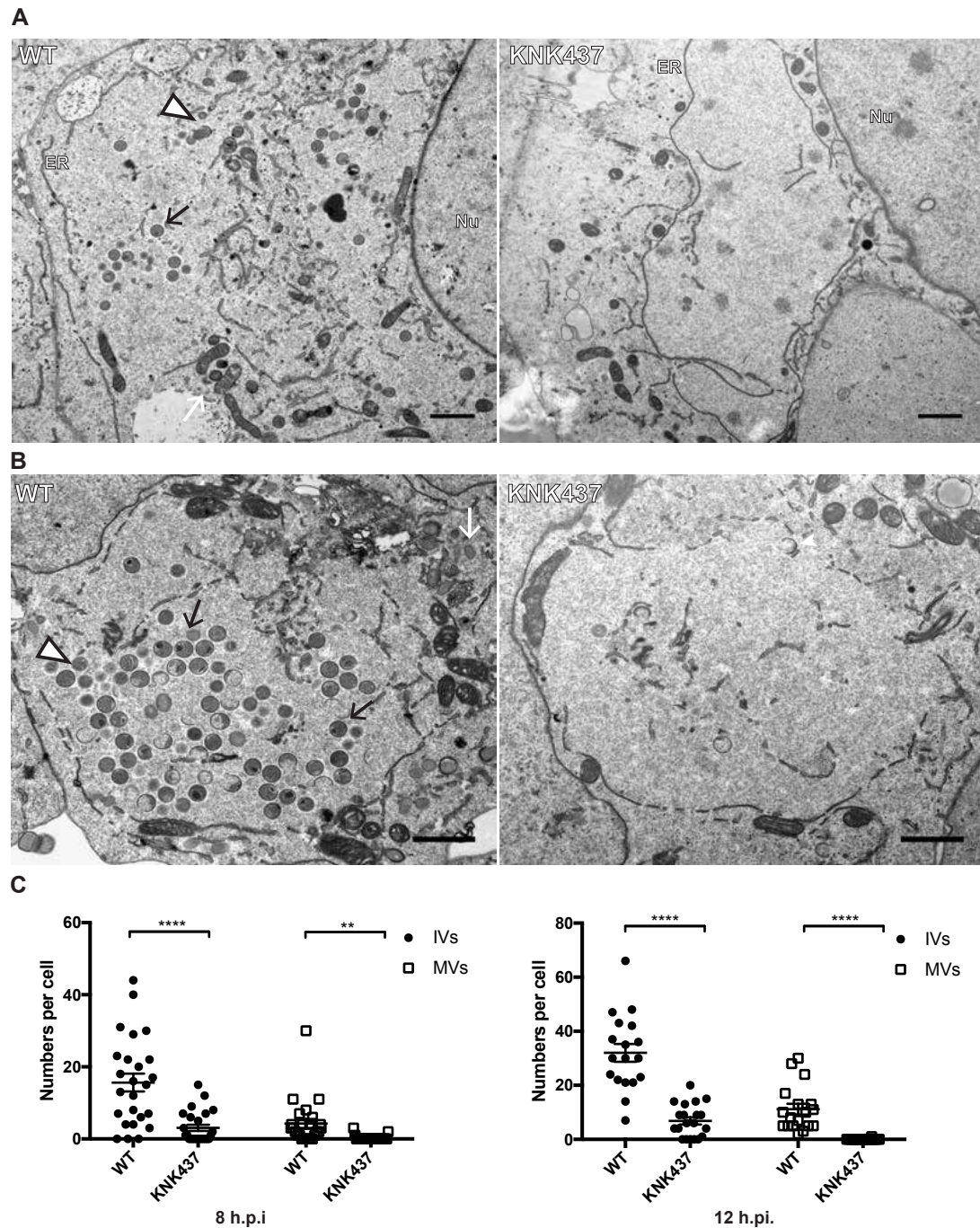
Figure 3. 21 Formation of viral intermediates is inhibited by KNK437

Electron micrograph of viral replication sites in WT and KNK437 treated cells. Cells were infected with VACV WR in the absence or presence of KNK437 and compared at both A) 8 hpi and B) 12 hpi. C) Numbers of IVs and MVs present in replication sites per cell. White and black arrowheads show IVs, and black arrows show IVNs. Images and quantifications represent 2 biological replicates. Mean + SEM is indicated on graph. **P < 0.01, ****P < 0.0001.

3.3 The inducible HSP, Hsp105, is required for virion formation

3.3.1 Perturbation of Hsp105 reduces the formation of new virions

Using the HSF1 inhibitor KNK437 I have now demonstrated that inducible HSPs are required for the formation of VACV IVs. In its presence, VACV late proteins are destabilised and viral crescent membranes and IVs cannot form. KNK437 inhibits HSF1 activity by preventing binding to the HSE and therefore inhibits transcription of the inducible set of HSPs. Amongst these HSF1-regulated genes are Hsp27, Hsp40, Hsp72, and Hsp105 (Yokota et al., 2000, Taba et al., 2011). Hsp27 is a small HSP that has been shown to have roles in protein disaggregation, and Hsp40 primarily functions to activate Hsp70 chaperones by stimulating their ATPase activity (Kampinga et al., 1994, Qiu et al., 2006). As previously mentioned, Hsp72 is the stress inducible member of the Hsp70 family, and although highly up-regulated in VACV infection, it has been suggested that it is not necessary for its replication cycle (Sedger et al., 1996, Park et al., 2001, Senf et al., 2008). Hsp105 is one of the lesser-studied molecular chaperones that belongs to the Hsp105/110 family; a sub-family of the Hsp70 chaperones (Ishihara et al., 1999, Yasuda et al., 1999). Whilst most of its biological functions remain to be discovered, it has been shown to play a role in the refolding and maturation of proteins, and in protecting cells from toxic protein aggregation (Hatayama et al., 1998, Yamashita et al., 2007, Yamagishi et al., 2010, Yamagishi et al., 2011). Although I have demonstrated the involvement of inducible HSPs in VACV infection using KNK437, the exact chaperones involved in IV formation still remain to be elucidated. I sought to investigate whether there was one single target of the drug required by the virus for virion formation, or if a combination of inducible HSPs were needed. As KNK437 destabilised late viral proteins, I chose to explore the role of Hsp105 in VACV infection, due to its function in cellular protein maturation.

To do this, I made use of RNA interference to target Hsp105 for knockdown. Lysates from HeLa cells transfected 72-hours earlier with either the scrambled negative control siRNA, or that which targeted Hsp105 were analysed by western blot to confirm efficient knockdown of the gene (Fig.3.22a). Once

confirmed, 24-hour yields were then carried out using siRNA-transfected cells that were infected with VACV WR at a MOI of 1 (Fig.3.22b). We see that the MV yield is reduced by approximately 1-log when Hsp105 is knocked down from cells, indicating that this HSP is required for infection. Although not as drastic a reduction as that seen with KNK437 treatment, virus produced from cells absent of Hsp105 also makes smaller plaques, as measured by plaque area (Fig.3.22c and d). This shows that knocking down the inducible HSP Hsp105 impacts VACV infectivity.
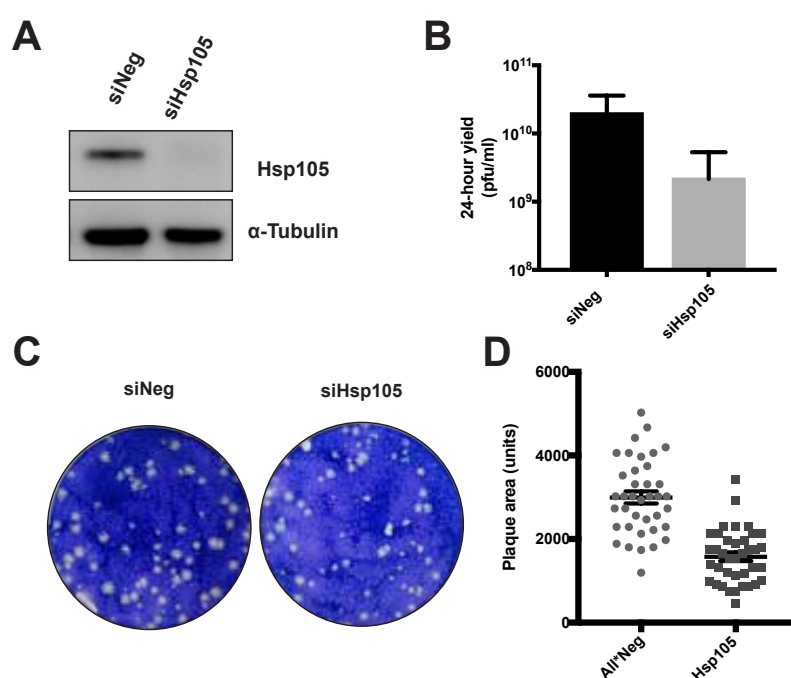
Figure 3. 22 Hsp105 knockdown reduces VACV MV formation

HeLa cells were transfection with scrambled siRNA (siNeg) or siRNA targeting Hsp105. A) Knockdown efficiency of Hsp105 was confirmed by western blot analysis. B) 24-hr yield of siRNA-transfected HeLa cells plaque assayed onto BSC40s. C and D) Plaque assay and plaque areas of siRNA-transfected HeLa cells. Data representative of 2 or more biological replicates, ****$P < 0.0001$.

To further study the later steps of the VACV lifecycle, and to determine whether knockdown of Hsp105 replicated the phenotype of KNK437 treatment, I again used the GFP core virus, WR EGFP-A4, alongside Hoechst staining to image viral replication sites and newly forming virions. In the presence of the HSF1 inhibitor KNK437 VACV can undergo viral DNA

replication but cannot form viral intermediates. Cells which had been siRNA-transfected 72-hours previous to deplete Hsp105, were infected at a MOI of 10 with the green-fluorescent core virus, and imaged at 8 hpi by confocal microscopy (Fig.3.23a). In the negative control siRNA-transfected cells VACV was able to undergo DNA replication, as seen by large cytoplasmic replication sites, and form new green-fluorescent virions. In Hsp105 knockdown cells we can see that although viral replication sites can form, they are absent of newly formed virions. Whilst all cells transfected with the scrambled siRNA had replication sites that contained viral cores, over 75% of Hsp105 knockdown cells contained replication sites without viral cores (Fig.3.23b).
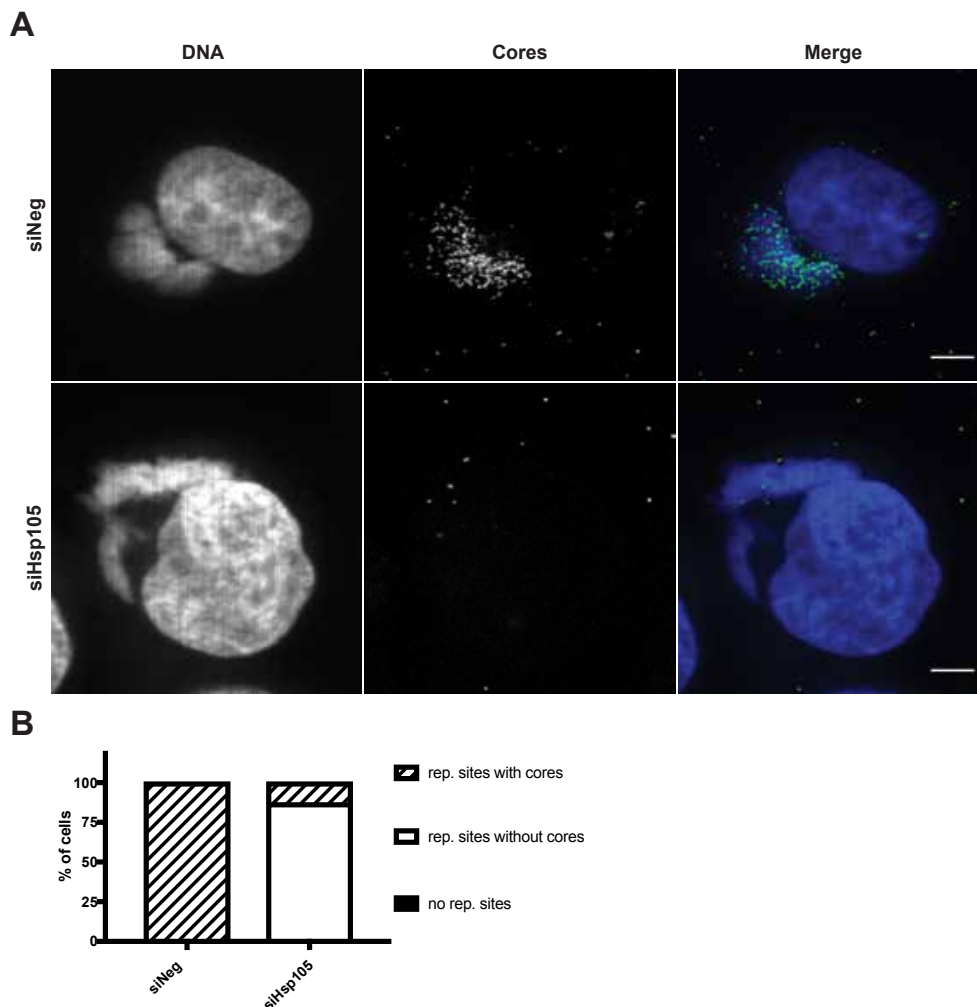
Figure 3. 23 Hsp105 is required for a post-DNA-replication step

Confocal microscopy of WR EGFP-A4 infected cells at 8 hpi, that had been transfected with either the control negative siRNA or siRNA against Hsp105 72-hours prior to infection. A) Virions (green) and cellular and viral DNA (blue) are visible. B) Cells were classified as before as containing no replication sites, replication sites with cores or replication sites without, and quantified. Images representative of 2 biological repeats. Scale bars = 5 µm.

Thus far I have shown that Hsp105 knockdown produces the same phenotype as HSF1 inhibition with KNK437, where VACV can undergo viral DNA replication but new virions cannot form and late gene expression is impaired. To further ascertain if Hsp105 is the inducible HSP required for the stabilisation of late viral proteins and new virion formation, siRNA knockdown cells were infected with VACV WR at a MOI of 10 and imaged by EM after 12 hpi to investigate the formation of viral intermediates (Fig.3.24). In control siRNA-transfected cells the cytoplasmic replication sites contain various viral intermediates; IVs, IVNs, particles undergoing morphogenesis to become MVs, and many MVs are also present. Whilst in Hsp105 knockdown cells there are very few, if any, viral intermediates. Viral crescents, IVs, IVNs, and MVs are absent from the infected cells and replication factories, and instead we see the same areas of high electron density that occur during KNK437 treatment, that may be aggregated viral matter. This indicates that Hsp105 is required for formation of VACV intermediates during the production of new virions.
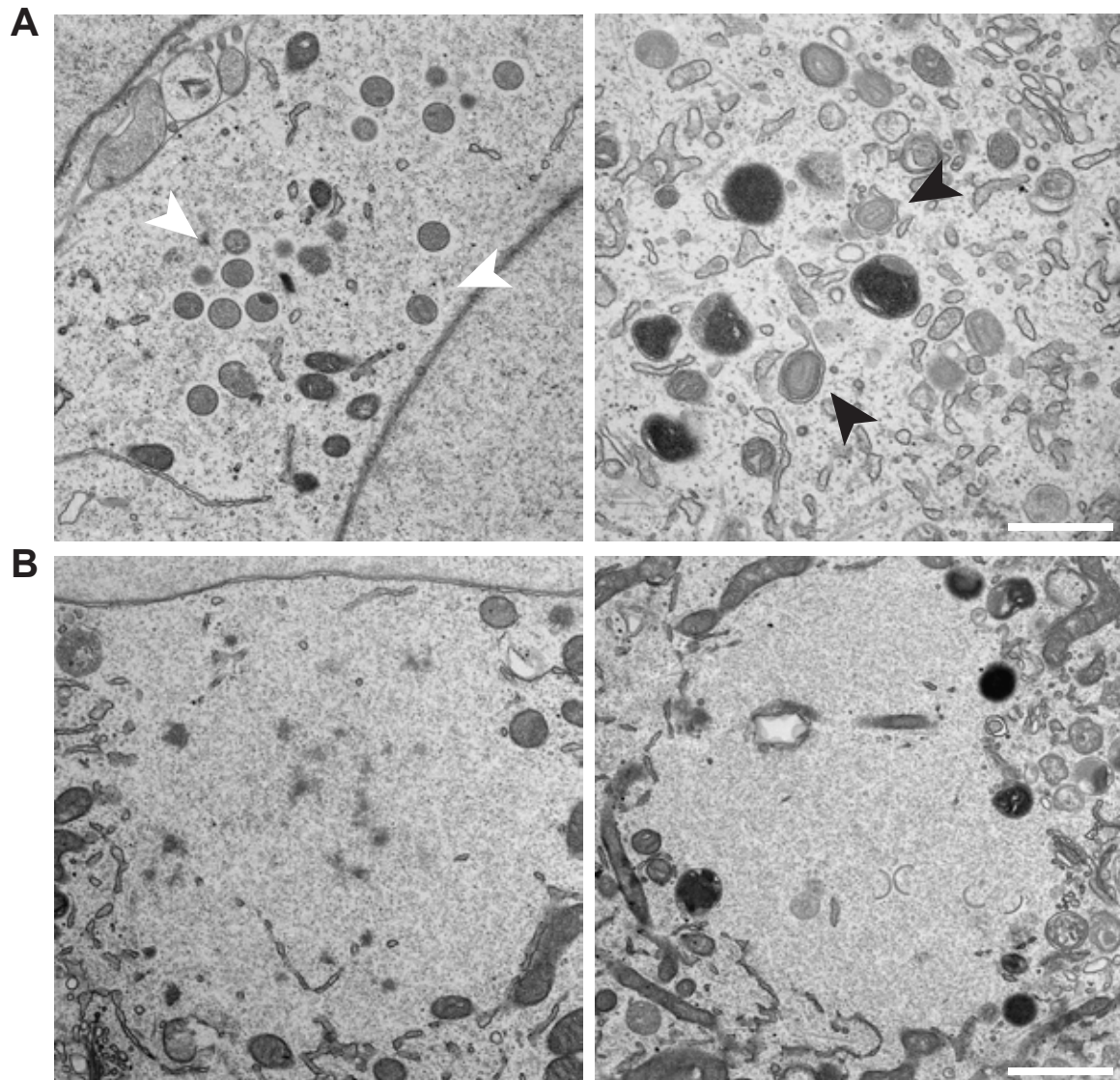
Figure 3. 24 Depletion of Hsp105 inhibits the formation of IVs

Electron micrograph of VACV infected siRNA-transfected cells at 12 hpi. A) IVs (white arrowheads) and MVs (black arrowheads) form in negative control RNAi cells. B) No viral intermediates or MVs are present in replication sites of Hsp105 KD cells. Scale bars = 1 μm.

3.3.2   Investigating the role of other inducible HSPs

As Yang et al. (2010) and Filone et al. (2014) had previously shown that Hsp105 was amongst the HSPs up-regulated during VACV infection, I questioned whether its up-regulation coincided with the formation of new virions, as the virus may wish to sequester more of this chaperone to aid in the formation of new viral particles. Cells were infected at a MOI of 10 with VACV WR and harvested at selected timepoints. Western blot analysis was then used to detect changes in protein levels over an 8-hour infection

timecourse (Fig.3.25). Levels of Hsp105 in cells increased steadily upon VACV infection and peaked at 8 hpi, around the time where IV formation and morphogenesis occurs (Fig.3.25a). This increase of over 3-fold compared to uninfected cells suggests that VACV is able to up-regulate the chaperone to fulfil its requirement for the chaperone in virion formation.

Although I have demonstrated Hsp105 is required for VACV replication, the previous studies also showed other inducible HSPs to be up-regulated during VACV infection. I therefore wanted to investigate whether these other HF1-regulated chaperones may also be involved in this stage of viral replication, and contribute to the KNK437 phenotype along with Hsp105. Western blot analysis was again carried out to compare protein expression levels to those of Hsp105, which I have shown to be needed for VACV virion formation. Levels of Hsp40 were highly variable across the infection timecourse (Fig.3.25b). There were no consistent trends over the 8-hour infection period, and therefore no pattern of down- or up-regulation during VACV infection could be deduced. Concurring with previous literature, levels of Hsp72 are highly elevated upon infection, over a 3-fold increase compared to uninfected cells (Fig.3.25c). However, there is no further increase at the later timepoints of 6 and 8 hpi where virion formation would occur and the increase in Hsp105 is seen, and the levels of Hsp72 then remain at a relatively consistent level throughout the remaining timepoints. This increase in Hsp72 protein levels may therefore be a stress response by the cell to infection, as it occurs soon after initial virus entry. These protein expression levels show that Hsp105 is the only inducible HSP to be specifically up-regulated at certain timepoints of VACV infection, however this does not mean that these proteins are not required by the virus. To investigate whether the other inducible HSPs were also needed by VACV in addition to Hsp105, they were also targeted for siRNA knockdown. HeLa cells that had been siRNA-transfected 72 hours earlier were infected with the late expressing GFP recombinant virus, WR L EGFP, at a MOI of 10, Hoechst stained, and imaged after 12 hours using a high-throughput confocal microscope. A control scrambled siRNA was used, and AraC as a control for no late viral gene expression. Cells that had a GFP fluorescence intensity above that of an uninfected population of cells were

classified as expressing late viral genes, and normalised against the negative siRNA control (Fig.3.26). As expected due to its involvement in virion formation that I have now demonstrated, knockdown of Hsp105 resulted in less than 50% late gene expression. Hsp72 knockdown had minimal impact on late gene expression, and as previously discussed VACV infection can occur independently of the Hsp70 family of chaperones, so this was anticipated (Sedger et al., 1996). Interestingly, targeting Hsp40 with siRNA resulted in a large decrease in late viral gene expression. This data suggests that in addition to Hsp105, the inducible HSP Hsp40 may also be involved in late steps of VACV replication.
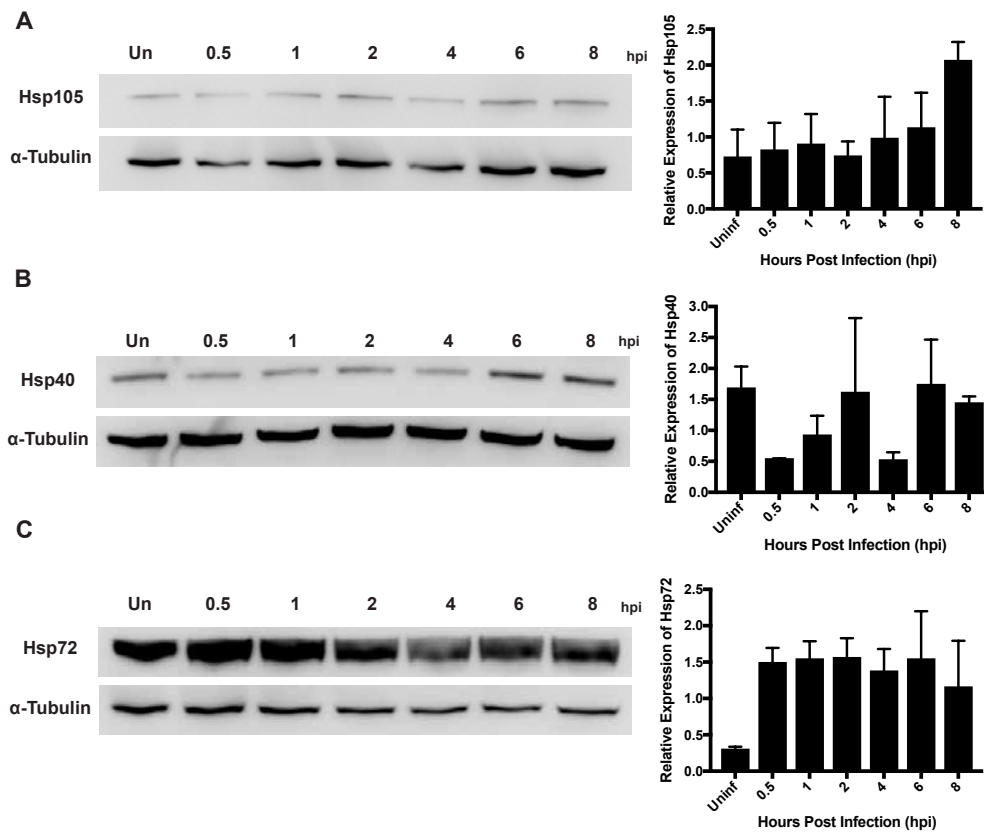
Figure 3. 25 Expression of inducible HSPs is altered in VACV infection

Western blot analysis of protein levels of the inducible HSPs A) Hsp105, B) Hsp40 and C) Hsp72 in WT infected cells across an 8-hour timecourse. Expression levels were quantified from a least 2 biological repeats and calculated relative to the expression of the α-tubulin loading control.
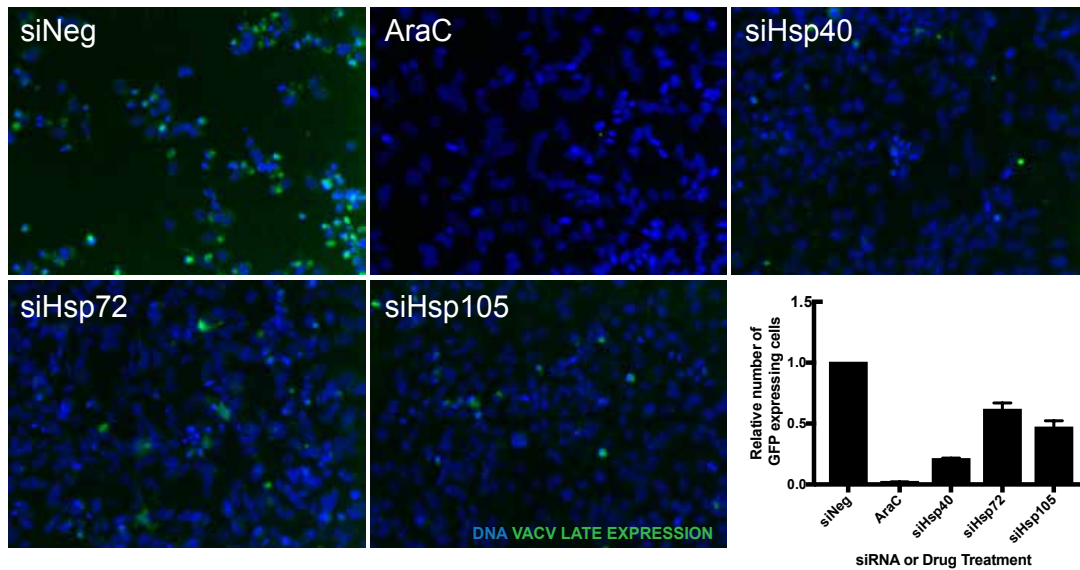
Figure 3. 26 RNAi of inducible HSPs reduces late viral genes

High-throughput microscopy of VACV late gene expression in cells transfected with siRNAs targeting inducible HSPs. Late gene expression was determined through GFP fluorescence from the late GFP expressing recombinant virus, and normalised to the negative scrambled siRNA control. AraC was used as a control compound for no late viral gene expression. Bars show mean of 2 repeats and SEM.

3.4   Summary

The master regulator of the cellular stress response, HSF1, had previously been implicated in VACV replication by Filone et al. (2014), although the exact details of its involvement were not explored. Within this chapter I have described the stage at which HSF1 activity is needed for VACV replication and determined that it is different from the stages in which Hsp90 is required. I showed that inhibition of the transcription factor, and therefore its regulated genes, destabilised late viral proteins thereby preventing the formation of intermediate viral structures during new virion formation. I also demonstrated that although the VACV structural protein F17 is affected by HSF1 inhibition, it is not this effect that leads to perturbation of virion formation.

Using the well-studied benzylidene lactam KNK437 was paramount to this discovery, however as treatment with this inhibitor then results in the inhibition of inducible HSPs regulated by HSF1 I then sought to investigate if there was a specific inducible chaperone that is involved in this stage of infection. I demonstrated that siRNA knockdown of the inducible HSP, Hsp105, resulted in reduced late viral gene expression and prevented formation of viral

intermediates, therefore concluding that this HSP is required by VACV for this late step (Fig.3.27). Although little is known about the functions of Hsp105, roles in protein maturation and refolding have been proposed, and it could be that this is needed for stabilisation of late viral proteins which are in turn required for the formation of IVs. Additionally, as RNAi of Hsp40 also appeared to impact late viral gene expression its involvement in VACV replication should be further investigated.
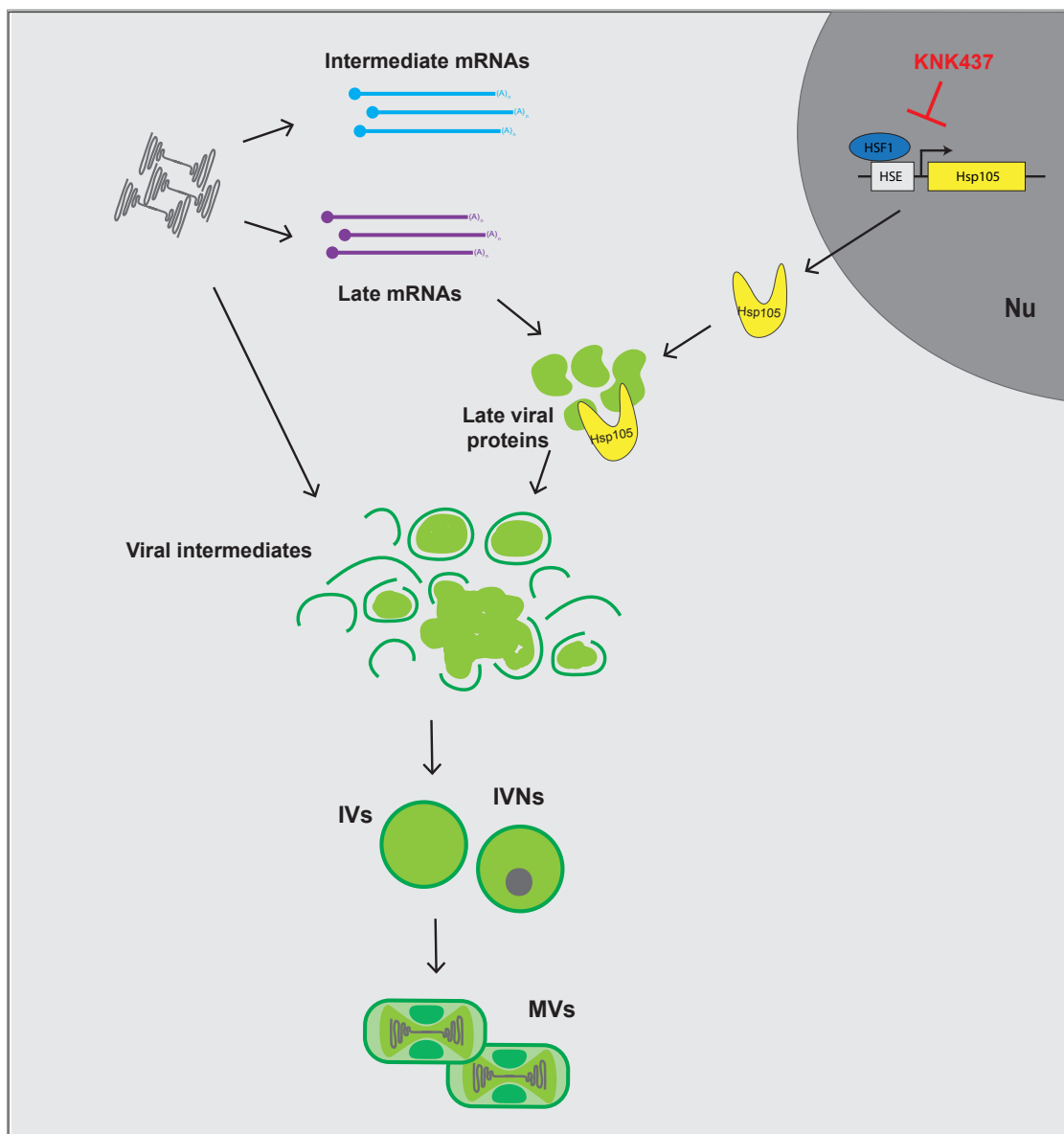
Figure 3. 27 Model of Hsp105 involvement in VACV virion formation

Hsp105 is required for the formation of VACV viral intermediates. KNK437 inhibits HSF1-activity by preventing binding of the transcription factor to the HSE, and therefore prevents the transcription of Hsp105. KNK437 inhibition destabilises late viral proteins and prevents the formation of VACV virions.

IV Discussion

1 Conclusions

Whilst there is a good level of understanding for many of the stages of the poxvirus lifecycle, detailed mechanisms of how the virus uncoats its genome, initiates DNA replication, and proceeds to make new virions still remain to be discovered. Although the model poxvirus, VACV, is a large virus that brings many of its own proteins necessary for replication, and is therefore less reliant on host systems than many smaller viruses, there are still many cellular proteins that are known to be required for these complex stages of its replication cycle, and likely many more to be discovered. Various RNAi screens have previously uncovered such host factors, such as host cell proteasomes and ubiquitin, amongst others, and HSPs have additionally been loosely tied to VACV infection in a handful of studies. These highly conserved molecular chaperones have already been implicated in the lifecycles of a wide range of other viruses, although an understanding of the specific details surrounding their involvement is lacking in most instances. I therefore set out to determine their precise roles in VACV infection, to contribute to the wider knowledge of HSPs as pro-viral factors and provide further information for potential cellular targeting anti-viral development. Throughout this thesis, I have presented evidence for the involvement of multiple HSPs in the VACV replication cycle. I have demonstrated that the highly conserved chaperone Hsp90 is required for two separate stages of the viral lifecycle and the inducible HSP, Hsp105, is also needed (Fig.4.1).

Viruses pose a problem in the complexity of their uncoating and reassembly within the same cell, termed virus metastability (Kilcher and Mercer, 2015). Although the VACV protein D5 has previously been shown to be involved in viral genome uncoating for which its ATPase domain is required, the

mechanism of how this viral factor facilitates release of the genome is still unknown (Kilcher et al., 2014). Additionally VACV core proteins are ubiquitinated during MV formation, so that upon entry into the cell cytoplasm the viral core is presumably targeted for proteasomal degradation (Satheshkumar et al., 2009, Teale et al., 2009, Mercer et al., 2012). The VACV genome release stage is described as a two-step uncoating process, although we are now able to say there are multiple steps and intermediates involved. Until now the actual genome release stages have not been well defined and have only been visualised in rare instances (Pedersen et al., 2000, Mallardo et al., 2002). Here I have been able to stabilise these genome release intermediates and show that full genome release is dependent on Hsp90 activity, thus filling in gaps in the complex genome-uncoating step of VACV. That this late uncoating intermediate was never visualised indicates that the D5 and UPS steps of uncoating occur prior to this stage.

Within this thesis I have used a combination of assays and imaging techniques, especially EM, to visualise new viral intermediates. Further to the discovery of an Hsp90-dependent uncoating intermediate, I have also alluded to mechanisms by which the virus undergoes reassembly, and demonstrated that Hsp90 inhibition results in DNA crystalloids that need to associate with viral matter for IV formation. Additionally I found Hsp105 to be involved in the formation of IVs through its requirement for late viral proteins at a post-transcription level. I have therefore not only built on the knowledge of chaperone involvement in virus lifecycles, but also explored and detailed new stages at which VACV requires host proteins. Although I have enabled a better understanding of the intersection of poxviruses and HSPs, my work has also shown that their utilisation by VACV is incredibly complex. Thus, I have provided new routes of investigation for further study of molecular chaperones in poxvirus infection, in order to determine the exact biological mechanisms and protein interactions that occur between HSPs and VACV, and also to determine if other chaperones are involved. Here I will present some concluding thoughts regarding the key findings of my work, and the future outlook surrounding questions that still remain.
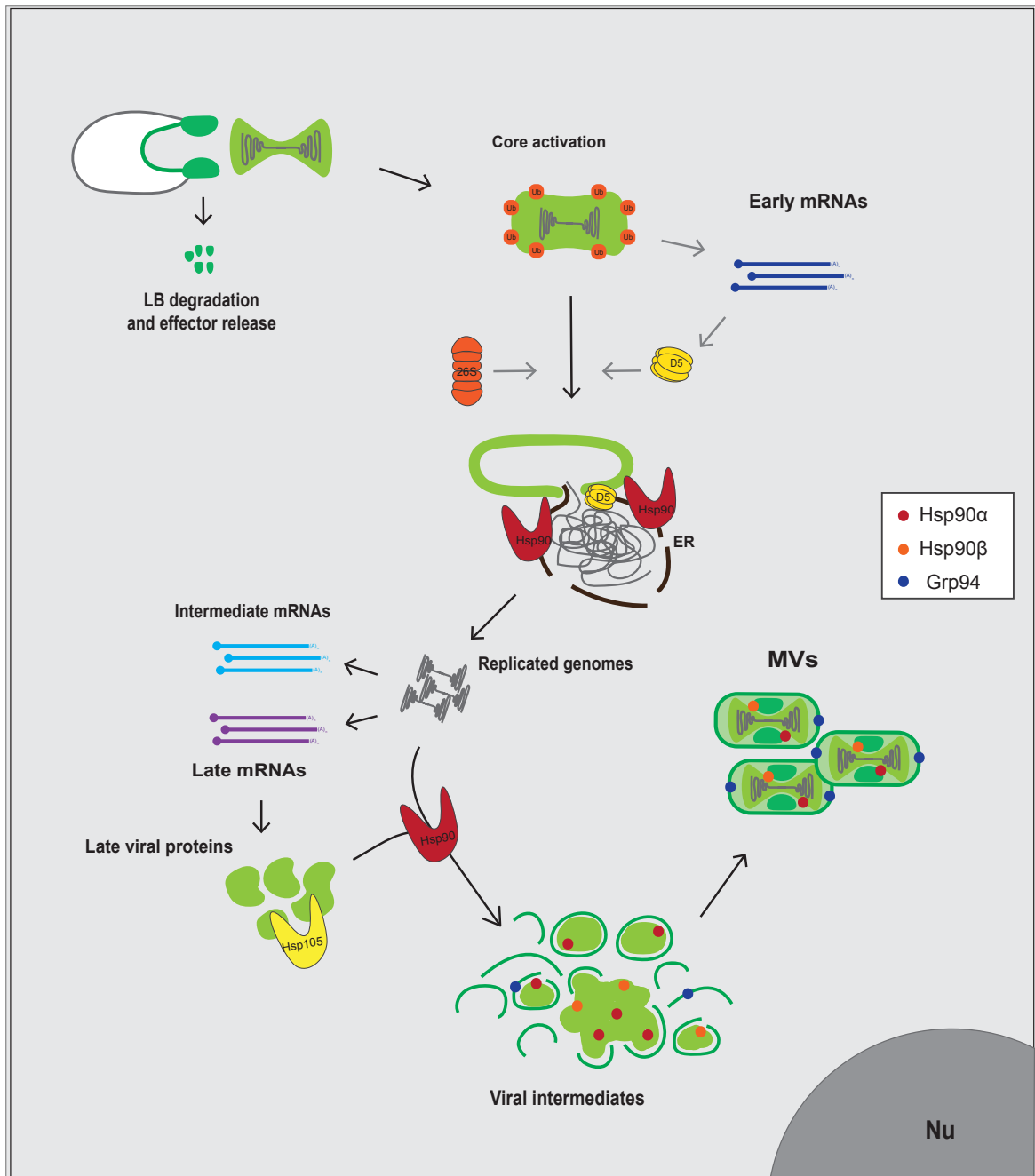
Figure 4. 1 A Model of HSP involvement in VACV replication cycle

The VACV uncoating and reassembly process involves multiple HSPs. Hsp90 is required for full release of the viral genome from the viral cores in the cytoplasm. After DNA replication and intermediate gene expression, late viral proteins require the activity of the inducible HSP, Hsp105, for their stabilisation in order to form viral intermediates. A second round of Hsp90 chaperone action is then needed for the association of viral DNA and viral proteins to form IVs. VACV then packages multiple Hsp90 isoforms into MVs within its core/LBs and viral membrane.

1.1   VACV requires Hsp90 for full genome release

Previous work by Hung et al. (2002) indicated that the highly abundant and well-studied chaperone Hsp90 played a role in VACV infection, however details of its involvement were left undefined. In results chapter 1 of this thesis I first confirmed that inhibition of Hsp90 was indeed a detriment to the virus, resulting in a reduction of MV yield and decreased intermediate and late gene expression. I then further specified that Hsp90 was required for a step that preceded viral DNA replication and demonstrated that genomes remained associated to VACV cores in the cytoplasm when the chaperone was inhibited. This suggested that Hsp90 might be involved in uncoating of the viral cores and genome release, in a manner similar to that seen when the viral uncoating factor, D5, is perturbed. Visualisation of Hsp90 inhibited viral cores by EM however lead to the discovery of a genome release intermediate whereby viral DNA was partially released from broken viral cores and ER membranes were closely associated. This visualisation of a new VACV uncoating intermediate, which had not been stabilised in the cytoplasm before, suggests that Hsp90 is required for full release of the genome to allow for DNA replication to occur in the cytoplasm. Using click chemistry to fluorescently label viral genomes I also showed that this partially released DNA was still replication competent while associated with the viral cores, thus demonstrating that Hsp90 activity is not required for replication itself.

That Hsp90 is not required directly for VACV DNA replication but instead is involved in release of the genome, proposes a new role for Hsp90 in viral life cycles. As previously discussed, Hsp90 is the chaperone most commonly associated with other viruses, and although many of the roles have not had their mechanisms well defined, the majority of the intersection lies at the steps surrounding genome replication. Whilst Influenza A and RSV both utilise Hsp90 for stabilisation of their RNA polymerases, VACV appears to not have involvement in DNA replication itself, but in steps leading up to it that are necessary for its occurrence. This is the first instance where a HSP has been

shown to be involved in uncoating of a viral core or capsid, and release of viral genomes. Even within the cellular environment this is a new role for Hsp90 as previous to this, Hsp70 has been the chaperone most related to 'uncoating', due to its role in the uncoating of clathrin-coated vesicles (Rothnie et al., 2011, Ungewickell, 1985).

Although I have shown involvement of Hsp90 in VACV genome uncoating, the exact biological interactions for which Hsp90 is required are unknown. There are several hypotheses of how Hsp90 is involved in this step. One likely route whereby Hsp90 facilitates release of the viral genome is through interaction with a viral core protein, or proteins, to enable expulsion or detachment of the viral genome from within. Hung et al. (2002) showed that Hsp90 interacts with the viral core protein p4a, encoded by the A10 gene; it could be that this interaction is involved in the release and unpacking of DNA from within the viral core. P4a is a major core component that gets proteolytically cleaved during virus maturation. It plays a role in organisation of the viral core and in DNA encapsidation. In its absence, VACV forms abnormal empty IV-like virions, and dense aggregates in infected cells (Heljasvaara et al., 2001). As it is needed for productive virion assembly and organisation of the viral core, it could be that its unfolding or degradation is needed for complete disassembly of virions that then triggers DNA release (Fig.4.2). Likewise, there could be other viral core proteins that interact with this chaperone to enable full genome release and dissociation of the viral core. To address this hypothesis, the proposed interaction between Hsp90 and p4a should be further investigated during a timecourse of infection. Although p4a remains a likely candidate for Hsp90 driven genome release or core breakdown, as it is the most abundant structural core protein, with over 30 core proteins and multiple viral morphogenetic intermediates, it maybe necessary to investigate the Hsp90-VACV interactome during this uncoating step.

In the same respect, Hsp90 may not function by interacting with a viral protein, but rather it is required for folding, translocation, and functionality of other host proteins that are involved in this step (Fig.4.2). I began investigating this hypothesis by looking at the stability of several viral and

cellular proteins that are already known to have involvement in genome uncoating and pre-replication, as Hsp90 client proteins are often degraded when the chaperone is inhibited. Both D5 and Cul3 were investigated due to their roles in viral infection but also as previous studies have suggested that helicases like D5, and Cul3 are substrates of Hsp90 (Manjarrez et al., 2014, Miao et al., 2018). Although my current data suggested these specific proteins were not clients of the chaperone there may be other cellular factors that are directly involved with VACV in these two stages, and thus the virus may indirectly require Hsp90. As above, an analysis of Hsp90 interactions during infection should be carried out. IPs could be carried out in the presence and absence of the virus to investigate other client proteins that may be required during viral infection to investigate other client proteins that may be required. Although it should be noted that client-chaperone interactions can be fast and transient, and therefore classical immuno-precipitation approaches may not give the best indication of whether a protein is a chaperone substrate or not.

Asides from these hypotheses that involve the function of some other proteins belonging to either the virus or the host, it could be that Hsp90 involvement in core uncoating is linked to the role of the ER in VACV infection. The ER is a continuous membrane system that undergoes frequent dynamic changes in cells (Pendin et al., 2011, Chen et al., 2013). Previous studies have shown a link between VACV and ER, as it is often found near intracellular cores in the cytoplasm and the process of DNA replication occurs within an ER-enclosed paddock (Pedersen et al., 2000, Tolonen et al., 2001, Mallardo et al., 2002). In my Hsp90-dependent viral uncoating intermediate, partially released DNA that has the capacity to replicate was seen by EM, to be surrounded by ER in the cytoplasm. The ER appears to extrude directly from breaks within the viral core walls. We know that ER associates with WT replication sites, but dissipates to allow for formation of new virions and viral intermediates. Perhaps, during VACV genome release, ER wrapping of the genome is the cellular response to DNA in the cytoplasm. If this were the case, ER wrapping would serve to limit VACV replication and Hsp90 would be required to remove it. Interestingly, a recent study showed that Hsp90 has the ability to mediate membrane deformation, and thus it may be through a similar mechanism that

Hsp90 is involved with dissociation and manipulation of these genome-filled ER enclosures from the viral cores (Fig.4.2) (Lauwers et al., 2018). This hypothesis considers that the ER acts as a restriction factor at this point of infection, and Hsp90 is required to remove this restriction.

Although these hypotheses of how Hsp90 facilitates full VACV genome release differ, one question that still remains for whichever of these is true is which Hsp90 is active in this role? As previously mentioned Hsp90 has multiple isoforms within the cell, and I have shown within results chapter 3 of my thesis that VACV packages various amounts of these isoforms within its MVs. Of particular interest to the role of Hsp90 in genome release is the packaging of Hsp90β within the viral core or LBs. It is reasonable to suggest that VACV deliberately packages Hsp90 isoforms within specific substructures to assist with its replication cycle. Hsp90β may be packaged in the core for the following round of infection, to assist in uncoating of the core and release of the genome. This may also have some involvement with the aforementioned interaction with the p4a core protein. The same could also be true for the less abundant, inducible isoform, Hsp90α, although this appeared to be packaged in very small amounts, if at all. To address this theory, and investigate whether it is host Hsp90 in newly infected cells that is involved in genome release or if the packaged isoforms play a role in this step of infection it would be wise to produce vaccinia virions that do not package Hsp90 isoforms or to pre-treat MVs with the Hsp90 inhibitors prior to infection. To produce virions totally absent of Hsp90 isoforms, infection and propagation of VACV MVs could be carried out in cells absent of Hsp90β, although it should be noted that if Hsp90β is involved in the latter step of IV formation then this may not be possible. However, were this to occur, it would still provide some indication of which isoforms of Hsp90 are involved in these two steps of VACV infection.

1.2   Hsp90 is required for the formation of VACV IVs

In addition to its role in genome release, I showed Hsp90 to be required for a second step in the VACV lifecycle that occurs after DNA replication and

affects the formation of new virions. The second phenotype of Hsp90 inhibition produces viral DNA crystalloids that do not localise to aggregates of viral proteins in the cytoplasm. Not only is there a block in IV formation when Hsp90 is inhibited, viral membrane intermediates and crescents are also not seen. This phenotype appears most similar to that which is produced with a rifampicin block. Inhibition with rifampicin blocks the stage of membrane crescent and intermediate formation, rather than morphogenesis, as when membrane crescents are already present, morphogenesis can proceed even when rifampicin is later introduced (Moss et al., 1969, Grimley et al., 1970, Nagaya et al., 1970). This suggests that DNA crystalloids form when viral membranes cannot, as the genomes cannot be encapsidated. I also demonstrated that Grp94, the Hsp90 ER isoform, is packaged into the VACV membrane. This chaperone has functions in processing and transport of secreted proteins (Rosser et al., 2004, Ghosh et al., 2016). Its presence in MV membranes provides compelling evidence to support work showing that VACV membranes are derived from the ER (Weisberg et al., 2017).

Previous studies demonstrated that the VACV membrane proteins A14 and A17, as well as the viral membrane associated proteins (VMAPs) L2 and A30.5, colacalise to the ER (Weisberg et al., 2017). Conversely, ER markers that are found on ER membranes are also found close to viral crescents in the cytoplasm. The current model of VACV membrane formation suggests that A17 remodels the ER, where the scaffold protein D13 is then able to bind to stabilise the curvature. VMAPs are then thought to carry out scission of ER membranes, along with cellular proteins, to form the viral membrane crescents. This recruitment of D13 to viral membranes by A17 is inhibited by rifampicin, which binds to D13 preventing A17 binding and tethering (Moss et al., 1969, Nagaya et al., 1970). Although I have demonstrated that the ER Hsp90 Grp94 is packaged in VACV MVs, it remains to be seen whether this Hsp90 isoform is coincidentally packaged because of its ER localisation, or whether its packaging relates to a role in viral membrane formation. It may be that this ER isoform of Hsp90 is needed for membrane formation through either maturation of the viral proteins A14 and A17, or potentially in direct derivation of the ER (Fig.4.2) (Unger et al., 2013, Weisberg et al., 2017). To

further investigate the role of this specific isoform in membrane formation it would be pertinent to carry out Grp94 specific knockdowns or inhibitions to see if the DNA crystalloid phenotype can be replicated. When the viral proteins D13 and A17 are expressed in cells alone, crescent-like ER membranes can form in the cytoplasm. This experiment could be repeated in the presence of absence of Grp94 to determine if this isoform is directly involved in derivation of the viral membrane from the ER.

1.3   Hsp105, the inducible HSP, is required for formation of VACV virions

Filone et al. (2014) previously alluded to the involvement of inducible HSPs in the replication cycle of poxviruses. I used the HSF1 inhibitor, KNK437 to investigate the role of these chaperones in the VACV replication cycle. I demonstrated using RNA interference that Hsp105 is the inducible HSP that is involved in the formation of IVs. The reduction in IV formation when inducible HSPs are inhibited is likely due to the decreased amount of the late viral protein F17, which occurs when KNK437 is present. Although I was able to determine that the decrease in F17 occurs at a protein rather than mRNA level, what still remains unknown is how Hsp105 is involved with this F17; whether it regulates actual translation, folding of the viral protein, or for stabilisation in the cytoplasm. Additionally, this reduction in viral protein was demonstrated with the highly abundant structural protein F17, but it would be important to determine if all late viral proteins require the chaperone, as this could suggest a common principle in VACV late protein folding.

The biological functions of Hsp105 have not been as extensively studied as some of the other HSP members, and therefore the suggested mechanisms of action in VACV IV formation remain elusive. There are also no other viruses currently known to require Hsp105, and therefore there is a lack of direction for drawing hypotheses for its activity in poxvirus infection. Under normal conditions, at a molecular level, this inducible chaperone can act as a nucleotide exchange factor for other chaperones as well as working to aid

maturation of proteins, or as a disaggregase (Kampinga and Craig, 2010, Shorter, 2011, Saxena et al., 2012, Ravindran et al., 2015). A better understanding of its chaperone activity, and the cofactors and PTMs that regulate it, would enable a greater appreciation of how this protein may function in VACV virion formation and its relationship to the late viral proteins. Additionally, it would be interesting to see if Hsp105 is packaged within the sub-structures of VACV MVs, as I have demonstrated with Hsp90 isoforms.

Further to the involvement of Hsp105, Hsp40 also provides interesting scope for further analysis of its role within poxvirus infection, as its knockdown appears to impact VACV late gene expression (Fig.4.2). Whether this is as important during infection as the other chaperones that VACV requires is yet to be seen but investigating whether Hsp40 knockdown can also replicate the KNK437 phenotype of reduced viral intermediate formation would be the next step for better understanding the inducible HSPs.

1.4   Overall themes and limitations

As discussed in the introduction to this thesis, the heat-shock stress response is a complicated system that involves multiple levels of regulation through many mechanisms. Co-chaperones and post-translational modifications are involved in regulating substrate specificity and binding, and chaperone ATPase activity. Hsp90 especially, has a multitude of co-chaperones and proteins that are involved in client protein binding and acceleration of ATP hydrolysis. Investigating co-chaperone involvement could prove important for deciphering the activity of Hsp90 and Hsp105 in VACV infection; Hsp90 more so due to its abundance of regulatory proteins.

Additionally, Hsp90 has multiple isoforms, which I have previously mentioned, and these may have differing roles in VACV replication. Hsp90α and Hsp90β are both packaged within the virion, although Hsp90β we can say more confidently. Packaging of these proteins into the core or LBs of the virus could prove crucial to their role in infection of the next cell. I previously discussed

the requirement for ubiquitination of viral core proteins in cells, ready for the next round of infection, and this could be a similar mechanism of preparation that is employed by Hsp90 when it is packaged within the virus. For example, it could be the packaged isoforms of Hsp90 are needed for uncoating of the viral core and release of the genome from within the virion in the next round of infection. To investigate this hypothesis, it would therefore be interesting to attempt to produce virus in the absence of Hsp90 and to then infect cells with MVs that are lacking the Hsp90 isoforms. This could be achieved by producing virus in knockout or knockdown cells, or through MV preparations that are pre-incubated with a stable Hsp90 inhibitor.

Hsp90 is by far the most common chaperone found to be involved in the replication of other viruses, although it is more often involved in viral polymerase function and stabilisation of replication machinery and enzymes. It appears that in VACV this is not the purpose of Hsp90, but rather a new method of action in viral infection. The majority of studies on Hsp90 and viruses however, do not detail the isoforms of Hsp90 which are involved, and it would be interesting to know if there is an isoform of the chaperone that is more commonly taken advantage of.

Finally, to conclude a thorough discussion of this thesis, the limitations of the data presented here should be considered. For my investigation into VACV and HSPs I have used only the HeLa cells, which are adherent epithelial cells derived a cervical cancer patient. As heavily discussed in my introduction, HSP activity in cancer cells is perturbed and HSPs are most often found upregulated in tumours. Although HeLa cells are commonly used for studying poxvirology and are also used for investigating HSPs, replicating the phenotypes across other cell lines would allow us to consider how differing expression levels of the chaperones alter VACV infection. Furthermore, small-compound inhibitors like those used within this thesis are able to effectively inhibit sub-sets of chaperones but for greater specificity and confirmation of specific protein involvement, individual knockdown experiments could be carried out.
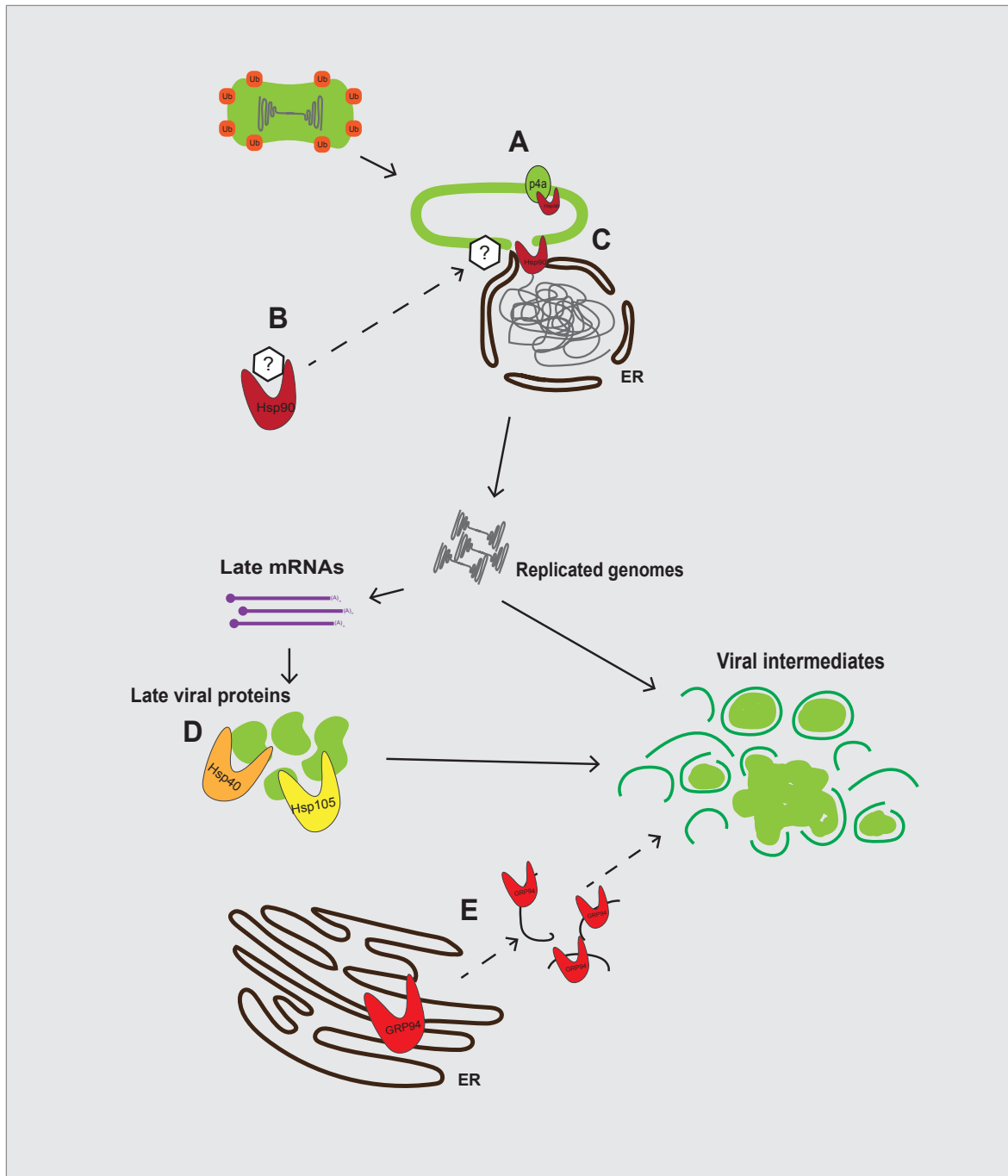
Figure 4. 2 Hypothesis of HSP mechanisms in the VACV lifecycle

A. Hsp90 may facilitate genome release through its interaction with a viral core protein, such as p4a. B. Another cellular protein that is a client of Hsp90 may be involved in uncoating and genome release. C. Hsp90 may be needed for dissociation of the viral core from the ER-enclosure. D. Late viral proteins may need Hsp40 in addition to Hsp105 for their stabilisation, translation or folding. E. Grp94 is the ER Hsp90 isoform and is packaged into viral membranes, it may be needed for deriving membranes from the ER.

2 Final Statement

Molecular chaperones are of the upmost importance in cells and are involved in a multitude of essential processes. Their involvement in diseases, specifically in cancer development, has given weight to the study of their functions within cells. They have previously been shown to be required by other viruses for processes ranging from viral polymerase function to capsid protein formation. They are often observed up-regulated during viral replication, however details surrounding their involvement are often lacking. Here I have detailed the stages where VACV utilises HSPs during its replication, which may provide insight into how other viruses also utilise these chaperones. Additionally, as these cellular chaperones are heavily targeted for drug development these studies may also provide a route for broad-spectrum anti-viral discovery through compound repurposing.

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157

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