Investigating the interaction between Marek’s Disease Virus US3 kinase and the host cytoskeleton

By
Jonathan Brandt

Supervisors
Prof. Anne Straube
Prof. Andrew McInish

Thesis
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Finally I thank my family for their continued support and enormous amounts of encouragement they have given over the years.
Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree.

The work presented (including data generated and data analysis) was carried out by the author except in the cases outlined below:

• The synthesis of the BioID2-HA gene fragment and cloning into pKan-CMV-Kif1C_RIP2-GFP was carried out by Alex Zwetsloot.

• Mass spectrometry was carried out by Cleidi Zampronio of the Proteomics Research Technology Platform at the University of Warwick.

• Single molecule assays with microtubule motors and EB3 was done in collaboration with Alex Zwetsloot and Jack Chen.
Abstract

Marek’s Disease Virus (MDV, Gallid alphaherpesvirus-2) is an oncogenic alphaherpesvirus that affects poultry and results in severe economic losses. The US3 kinase is conserved amongst alphaherpesviruses and facilitates several steps in viral replication, including viral budding from the nucleus and rearrangement of the host cytoskeleton. US3 from MDV remolds the actin cytoskeleton in a kinase-independent manner, the mechanism for which remains to be understood. Here, using proximity-dependent biotinylation and proteomic mass spectrometry, the actin-crosslinking protein alpha-actinin was identified as a new interactor of MDV US3. Using purified proteins, we demonstrate that US3 directly binds alpha-actinin in a Ca\(^{2+}\)-dependent manner and blocks its actin-bundling activity in vitro. Ab initio protein structure prediction and molecular modelling suggest that the N-terminal 111 amino acids of US3 bind to the calponin-homology domains of alpha-actinin in their “closed” conformation, which is adopted in the presence of Ca\(^{2+}\) and incompatible with binding actin. Indeed, an ~7-fold increase in the affinity between MDV US3 and chicken alpha-actinin occurs in the presence of Ca\(^{2+}\) ions. Overexpression of alpha-actinin-4 counteracted the removal of stress fibres by kinase-dead, but not wild-type MDV US3. In addition to actinin, we also identified the microtubule motor dynein as an interactor of US3. Using fluorescent single-molecule motility assays, US3-GFP was observed being transported by both dynein and the kinesin KIF1C. This finding could have implications for the intracellular transport of the virus that needs to be further investigated. Together, these findings determine the mechanism for kinase-independent removal of stress fibres and identify new host interactors of MDV US3.
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<tr>
<th>Abbreviation</th>
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<td>ABD</td>
<td>Actin Binding Domain</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis Of Variance</td>
</tr>
<tr>
<td>APEX</td>
<td>Ascorbate Peroxidase 2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BHV</td>
<td>Bovine Alphaherpesvirus</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CASP</td>
<td>Critical Assessment of protein Structure Prediction</td>
</tr>
<tr>
<td>CH</td>
<td>Calponin-Homology</td>
</tr>
<tr>
<td>CHPK</td>
<td>Conserved Herpesvirus Protein Kinase</td>
</tr>
<tr>
<td>DDH</td>
<td>Dynein Dynactin Hook3</td>
</tr>
<tr>
<td>DDHU</td>
<td>Dynein Dynactin Hook3 US3</td>
</tr>
<tr>
<td>DHC</td>
<td>Dynein Heavy Chain</td>
</tr>
<tr>
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<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DU</td>
<td>Dynein US3</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>EHV</td>
<td>Equine Herpesvirus</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast Fourier Transform</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine Diphosphate</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>Haemagglutinin</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>HMM</td>
<td>Hidden Markov Model</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat Shock Protein</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
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<td>HVT</td>
<td>Herpesvirus of Turkey</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>----------------------------------</td>
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<tr>
<td>KH</td>
<td>KIF1C Hook3</td>
</tr>
<tr>
<td>KHU</td>
<td>KIF1C Hook3 US3</td>
</tr>
<tr>
<td>MD</td>
<td>Marek's Disease</td>
</tr>
<tr>
<td>MDV</td>
<td>Marek's Disease Virus</td>
</tr>
<tr>
<td>MST</td>
<td>Microscale Thermophoresis</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule Organisation Centre</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear Export Signal</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localisation Signal</td>
</tr>
<tr>
<td>NTA</td>
<td>Nitrilotriacetic Acid</td>
</tr>
<tr>
<td>PAM</td>
<td>Point Accepted Mutation</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PRV</td>
<td>Pseudorabies Virus</td>
</tr>
<tr>
<td>PSI-BLAST</td>
<td>Position-Specific Iterative Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>RMSD</td>
<td>Root Mean Square Deviation</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>TMR</td>
<td>Tetramethylrhodamine</td>
</tr>
<tr>
<td>UL</td>
<td>Unique-Long</td>
</tr>
<tr>
<td>US</td>
<td>Unique-Short</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VZV</td>
<td>Varicella Zoster Virus</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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### Amino acids abbreviations

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<th>Amino Acid</th>
<th>Abbreviation</th>
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<td>Alanine</td>
<td>A</td>
</tr>
<tr>
<td>Arginine</td>
<td>R</td>
</tr>
<tr>
<td>Asparagine</td>
<td>N</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>D</td>
</tr>
<tr>
<td>Cysteine</td>
<td>C</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Q</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>E</td>
</tr>
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<td>Glycine</td>
<td>G</td>
</tr>
<tr>
<td>Histidine</td>
<td>H</td>
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<tr>
<td>Isoleucine</td>
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<tr>
<td>Leucine</td>
<td>L</td>
</tr>
<tr>
<td>Lysine</td>
<td>K</td>
</tr>
<tr>
<td>Methionine</td>
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</tr>
<tr>
<td>Phenylalanine</td>
<td>F</td>
</tr>
<tr>
<td>Proline</td>
<td>P</td>
</tr>
<tr>
<td>Serine</td>
<td>S</td>
</tr>
<tr>
<td>Threonine</td>
<td>T</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>W</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Y</td>
</tr>
<tr>
<td>Valine</td>
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1 Introduction
Viruses are intracellular parasites that use host cellular machinery to reproduce. For viruses that replicate in the nucleus, the distance to where it exits through the plasma membrane or contacts another cell is vast. For human herpesviruses that are neurotropic, this may be up to 1 meter (Fletcher & Theriot, 2004). It is therefore unsurprising that while each virus has evolved unique strategies for entry, replication and egress, the majority if not all viruses hijack the host cytoskeleton in some way.

The cytoplasm is a crowded environment, and the free diffusion of large biological complexes that are greater than 500 kDa is highly restricted (Luby-Phelps, 1999). Large organelles, cargo-carrying vesicles and chromosomes require active mechanisms of directed transport. Generally, viruses use two alternative methods of intracellular transport. Viruses can either mimic cargo and use the host mechanisms for endocytic trafficking or directly interact with the host transport machinery. In addition, viruses often alter the inherent characteristics of their host cell to promote viral replication. Changes in cell morphology, mechanical structures, and the cell cycle are often consequences of viral infection. The cytoskeleton plays a significant role in all of these aspects.

1.1 The cytoskeleton
The cytoskeleton is the component of a cell that provides shape, motility and the physical pathways for intracellular transport of cargo (Figure 1.1). It was first proposed by Koltzoff (1903) that a network of very thin fibrils allowed a cell that mainly comprises of liquid to change and maintain shape. It was later suggested by Peters (1963) that a three-dimensional mosaic of proteins was responsible for coordinating the biochemical reactions within the cytoplasm. Electron microscopy finally revealed the presence of filamentous structures in the cytoplasm that were considered as possible functional elements of the cytoskeleton (Wohlfarth-Bottermann, 1962).
The cytoskeleton is comprised of three major components: microtubules (Figure 1.1B), actin microfilaments (Figure 1.1C) and intermediate filaments (Ishikawa et al., 1969; Manton & Clarke, 1952). The dynamics of the cytoskeleton allows a cell to modify its intracellular organisation and respond to its external environment. These dynamics are inherent from the ability of each component to polymerise and depolymerise in a controlled manner.

### 1.1.1 Actin

Actin was first identified as one of two major muscle components within actomyosin by Straub (1943) and is often regarded as the most abundant intracellular protein in the eukaryotic cell. There are six different isoforms of actin encoded by six different genes. Through peptide sequencing of purified actin from different muscles and organs, it was found that the α-skeletal, α-cardiac, α-smooth, and γ-smooth isoforms are found in their respective muscle types, while β-cytoplasmic and γ-cytoplasmic isoforms are expressed ubiquitously in all cells (Vandekerckhove & Weber, 1978). It is unknown what different purposes the cytoplasmic actin isoforms have. In non-motile cells, both isoforms are found in stress fibres. However, in motile cells, stress fibres are predominantly formed of β-cytoplasmic actin while cortical structures and cellular protrusions are formed primarily of γ-cytoplasmic actin (Dugina et al., 2009).

Actin microfilaments are polymers of G-actin subunits. G-actin contains ‘barbed’ and ‘pointed’ domain, separated by a cleft containing binding sites for adenosine triphosphate (ATP) or adenosine diphosphate (ADP) and a divalent cation (Ca$^{2+}$ or Mg$^{2+}$) (Kabsch et al., 1990). The barbed and pointed domains of the actin monomer are biochemically different, with the barbed domain having a greater binding affinity than the pointed domain. This biochemical difference results in a polar actin microfilament, with a barbed end that polymerises up to ten times faster than the pointed minus-end (Pollard, 1986). The continuous polymerisation of actin at the barbed end and depolymerisation at the pointed end can result in no overall change of filament length, but a phenomenon known as treadmilling. Treadmilling is driven by
ATP hydrolysis (Wegner, 1976) as one ATP is consumed for the turnover of each actin monomer.

Figure 1.1 The microtubule and actin cytoskeleton. A: Bovine pulmonary artery endothelial cells. DNA is highlighted in blue; actin and beta-tubulin are highlighted in red and green, respectively. Source: ImageJ demo image https://imagej.nih.gov/ij/images/. B: Schematic of microtubules and their dynamic instability that is driven by the hydrolysis of GTP bound to the beta-tubulin subunit. Growing microtubules maintain a GTP cap; its loss leads to depolymerisation. C: Schematic of F-actin polymerisation. ATP G-actin is incorporated into the barbed end of the growing filament. After a delay, ATP is hydrolysed into ADP, causing the loss of ADP G-actin at the pointed end of the filament.
When the concentration of actin, in any given solution, is higher than the critical concentration, G-actin polymerises in a nose-to-tail fashion to produce F-actin (Wegner, 1976) (Figure 1.1C). The actin nucleation rate is proportional to the cube of the actin concentration (Watanabe & Higashida, 2009). In vitro, the rate of polymerisation and elongation is proportional to the concentration of free actin (Nishida & Sakai, 1983). In order for the polymerisation and depolymerisation to occur within a controlled and purposeful manner, there is a complex mechanism of regulation. Triggered by several signalling pathways, typically through the Rho GTPase family, actin-binding proteins (ABPs) control all aspects of actin dynamics: nucleation, elongation, depolymerisation, capping, severing and cross-linking (Dos Remedios et al., 2003).

1.1.2 Non-muscle actin structures

While actin was initially characterised as being a significant muscle component, in non-muscle cells the actin cytoskeleton is best characterised for its role in cell motility, adhesion and cytokinesis. The cell achieves this by organising actin into dynamic and functional structures (Figure 1.2).

Cellular protrusions

During migration, the cell polarises to form a leading edge and a rear trailing edge. A dense actin network at the leading edge pushes the plasma membrane into the direction of travel forming flat, sheet-like protrusions known as lamellipodia that are 0.1 – 0.2 µm thin and 1 – 5 µm long (Innocenti, 2018; Mattila & Lappalainen, 2008). Within lamellipodia, F-actin is orientated in such a way that the growing ends face outwards towards the plasma membrane (Small et al., 1994). The branched nature of F-actin within lamellipodia is the result of daughter filaments nucleating from pre-existing filaments via the Arp2/3 complex that is downstream and consequently regulated by the Rac small GTPases (Ridley et al., 1992).

Behind the dynamic leading edge is a cross-linked actin gel known as the lamella. As the more branches and filaments are created, the density within
the lamellipodia increases and it protrudes forward (Zimmermann & Falcke, 2014). Capping and severing proteins are found abundantly close to the edge of lamellipodia. As the filaments of the dynamic leading edge increase, the rate of capping and severing also increases. When this reaches a certain point, the density of filaments decreases and the lamellipodium retracts (Zimmermann & Falcke, 2014).

Often found protruding from the lamellipodia network are finger-like protrusions known as filopodia. Filopodia are usually 0.1-0.3 µm in diameter and less than 10 µm in length (Mattila & Lappalainen, 2008). Filopodia contain parallel actin filaments that are cross-linked by fascin (Mellor, 2010). Filopodia grow by the addition of actin at their tips and shrink by the physical pulling of filaments by motors and subsequent depolymerisation or by cofilin-mediated disassembly at tip regions (Breitsprecher et al., 2011; Mallavarapu & Mitchison, 1999).

There are two proposed mechanisms for the initiation of filopodia, which are both only partially resolved (Yang & Svitkina, 2011). Firstly, the convergent elongation model proposes that a branched actin network is formed within lamellipodia by Arp2/3 (Svitkina et al., 2003). In this model, elongation factors maintain the elongation of some filaments. Parallel filaments are then cross-linked by fascin to form a protrusion containing an actin bundle. Secondly, another mechanism is proposed that relies on the Diaphanous-related formin, Dia2 (Pellegrin & Mellor, 2005). Here, filopodia are formed from the de novo nucleation of filaments.

Filopodia contain a diverse range of receptors for extracellular matrix molecules and signalling molecules (Mattila & Lappalainen, 2008; Mattes & Scholpp, 2018). Therefore, filopodia are often thought as protrusions that probe and allow the cell to sense the microenvironment, and play roles in cell migration, wound healing and chemotaxis.
**Actin cortex**

The actin cortex is a layer of short, highly cross-linked F-actin immediately underneath the plasma membrane (Bray & White, 1988). The actin cortex is connected to the plasma membrane by a variety of proteins such as the Ezrin/Radixin/Moesin (ERM) family, filamin A, ankyrin and spectrin (Tsukita & Yonemura, 1999; Stossel *et al.*, 2001; Bennett & Baines, 2001). The F-actin filaments within the cortex are held together by cross-linkers such as alpha-actinin (Tsukita & Yonemura, 1999; Mukhina *et al.*, 2007). In addition to cross-linking proteins, the cortex contains myosin II motors that act as cross-linkers themselves, but also generate forces and contribute to the stability of the plasma membrane (DeBiasio *et al.*, 1996; Stewart *et al.*, 2011).

Besides its role in maintaining cell shape, the actin cortex is also essential for cellular division. During mitosis, the actin cortex interacts with astral microtubules, via dynein, enabling spindle positioning (Théry *et al.*, 2005). In addition to this, the actin cortex is required for the progression of the cleavage furrow during cytokinesis (O'Connell *et al.*, 2001).

Lastly, the actin cortex is important for cellular migration. During migration, non-muscle myosin II generates higher cortical tension at the rear of the cell, causing cell body retraction (Paluch *et al.*, 2006). This contractility also gives rise to cortical flows that are involved in generating forces to move the cell forward (Bergert *et al.*, 2015). At the front of the cell, contractions of the actin cortex lead to the formation of blebs, which are leading-edge protrusions when cells move in physically confined spaces (Paluch & Raz, 2013).

**Stress fibres**

Actin stress fibres consist of bundled, anti-parallel F-actin filaments (Cramer *et al.*, 1997). The central cross-linking protein in these structures is alpha-actinin, although other cross-linking proteins such as filamin and epsin have been detected (Lazarides & Burridge, 1975; Chen *et al.*, 1999; Wang *et al.*, 1975).
Stress fibres can be broadly classified into four categories depending on their orientation and positioning within the cell. Dorsal stress fibres are distally anchored to focal adhesions, do not contain myosin II and are therefore non-contractile (Tojkander et al., 2011). Unlike dorsal stress fibres, transverse arcs are not anchored to focal adhesions and contain myosin II. The force generated by the contractile transverse arcs is transmitted to the surrounding area through the connections with dorsal stress fibres. Ventral stress fibres are anchored at either end to focal adhesions and are contractile due to the presence of myosin II (Mitchison & Cramer, 1996). Lastly, the perinuclear cap consists of stress fibres located above the nucleus and is thought to regulate nuclear shape during interphase and transmit force from the cellular environment to the nucleus (Khatau et al., 2009).

**Podosomes**

Podosomes are actin-rich adhesion structures commonly formed by cells that need to migrate through the extracellular matrix of basement membranes (Schachtner 2013). Podosomes consist of a core of dense, cross-linked actin filaments, alpha-actinin and nucleation factors such as the Arp2/3 complex, cortactin, and WASP (Kaverina et al., 2003; Tehrani et al., 2006; Linder et al., 1999). This actin core is surrounded by an adhesive ring, connected by radial actin filaments (Luxenburg et al., 2007). The adhesive ring consists of integrins vinculin and talin and anchors the podosome to the underlying matrix (Marchisio et al., 1988; Zambonin-Zallone et al., 1989). Podosomes can be found as scattered adhesion structures, but also as clusters or rings connected by a heterogeneous F-actin network (Destais et al., 2003).
Figure 1.2 Non-muscle actin structures. Clockwise: Filopodia are finger-like protrusions extending from lamellipodia at the leading edge of the cell. The cortex provides mechanical strength and contractile forces to the plasma membrane. Podosomes are highly localised cellular protrusions that interact with the extracellular matrix. Stress fibres can be contractile or non-contractile, are usually anchored at either end to focal adhesions and facilitate cellular contraction.
1.1.3 Microtubules and intracellular transport

Microtubules are hollow tube-like structures, with a diameter of approximately 25nm, comprising of heterodimeric alpha- and beta-tubulin (Ledbetter & Porter, 1963; Nogales et al., 1998). Microtubules are polar structures, having a fast growing plus-end and a slow growing minus-end. The plus-end is usually oriented towards the cell periphery, while the minus-end are nucleated and anchored at the microtubule organising centre (MTOC) in the cell centre via gamma-tubulin ring complexes (Howard & Hyman, 2003; Wiese & Zheng, 2000). Microtubule polarity is an inherent property due to the uniform orientation of alpha- and beta-tubulin in the microtubule lattice with beta-tubulin pointing and being exposed at the plus ends of microtubules.

The dynamic instability of microtubules is driven by GTP hydrolysis (Erickson & O'Brien, 1992) (Figure 1.1B). GTP is associated with beta-tubulin and GTP-tubulin added to the microtubule end (Mitchison, 1993). The incorporated GTP-tubulin creates a GTP cap at the growing end, behind which GTP is being hydrolysed to form a microtubule lattice of predominantly GDP-tubulin (Drechsel & Kirschner, 1994). The GTP cap allows relatively stable phases of microtubule assembly. If GTP-tubulin is not incorporated into the plus end at a sufficiently high rate, the stabilising cap is lost. When GDP-tubulin is exposed at the plus end, depolymerisation of the microtubule will be triggered.

The dynamic state of microtubules is therefore dependent on the local concentration of GTP-tubulin and the rate of its replenishment. The cell is, however, able to control the dynamics of microtubules through regulatory microtubule-associated proteins (MAPs) that either binds along the microtubule lattice or at the microtubule ends. Microtubule polymerases localise to tips of microtubule plus ends and accelerate the polymerisation of tubulin by recruiting tubulin dimers through their arrays of the conserved tumour overexpressed gene (TOG) domains (Akhmanova & Steinmetz, 2015; Widlund et al., 2011). On the other hand, kinesin-13 family depolymerases
and severing proteins promote disassembly of microtubules (Feng et al., 2017; Moores & Milligan, 2006).

Microtubules provide the tracks for numerous molecular motors that consume ATP to perform mechanical work. Dynein is a class of minus end-directed microtubule motors that move from the periphery to the centre of the cell (Schroer et al., 1989). Cytoplasmic dyneins transport various cargos, and when cortically localised, is crucial for proper positioning of the mitotic spindle (Reck-Peterson et al., 2018; Kotak et al., 2012). In addition to this, axonemal dynein provides the force required for cilia and flagella to beat (Vale & Toyoshima, 1988). In contrast to dyneins, most kinesins are plus-end directed microtubule motors (Vale et al., 1985; Endow & Waligora, 1998). Many kinesins primarily transport cargo, others crosslink and slide microtubules to organise the mitotic spindle or specialised as microtubule depolymerases (Hirokawa et al., 2009; Cross & McAlinsh, 2014).

1.2 The Alphaherpesvirus

Herpesviruses are a large family of double-stranded DNA viruses of approximately 150 to 200 nm in diameter, characterised by their icosahedral capsid morphology, which is surrounded by a tegument layer and an envelope (Mettenleiter, 2002) (Figure 1.3 and Figure 1.4). Within the capsid is a linear genome. The genomes of herpesviruses can be separated into six classifications depending on their structure (Roizman & Baines, 1991). The genome of Group A herpesviruses contains a single set of repeat sequences at both termini that are in the same orientation. Group B and C herpesviruses have multiple sets of repeat sequences at both termini that are in the same orientation. However, Group C herpesviruses contain additional tandem repeats of variable length internally. Group D herpesviruses have a unique region inserted between a single set of inverted repeats. Group E herpesvirus genomes consist of unique-long (UL) and unique-short (US) genes flanked by inverted repeats. Group F herpesviruses genomes do not contain any terminal tandem or inverted repeats.
The alpha subfamily of the herpesviruses is distinguished by a Group D/E type genome, a short replication cycle, and ability to establish latent infections. Alphaherpesviruses represent a diverse group of pathogens that pose a risk to the health of humans and animals.
Figure 1.3 Structure of an alphaherpesvirus. Alphaherpesviruses consist of an icosahedral capsid containing double-stranded DNA. The capsid is surrounded by a tegument layer and a host-derived envelope. Figure adapted from an illustration by Anne Straube that is based on the ViralZone online resource provided by the Swiss Institute of Bioinformatics.

Figure 1.4 Schematic of alphaherpesvirus proteins. UL- and US-designations indicate the location within the viral genome. Figure adapted from an illustration by Anne Straube that is based on the ViralZone online resource provided by the Swiss Institute of Bioinformatics.
1.2.1 Alphaherpesvirus replication

Viral entry

Alphaherpesviruses carry at least eleven glycoproteins on the exterior of the viral envelope. Glycoproteins gB and gC, which overlap in function, bind to chondroitin sulphate proteoglycans and heparin sulphate on the host cell surface to mediate initial attachment (Herold et al., 1991; Shieh et al., 1992). Initial attachment is independent of viral entry, and as such, the removal of gB or gC heparin-binding domains has no significant effect on viral entry (Laquerre et al., 1998).

Glycoproteins gB, gH and gL are essential for the entry of all herpesviruses. In the majority of alphaherpesviruses, these glycoproteins form a complex with gD that enables viral entry (Turner et al., 1998). Varicella-Zoster virus (Human alphaherpesvirus 3, VZV) is an exception to this, as it does not have a gD homolog within its genome and gB, gH, gL alone are sufficient for fusion with the plasma membrane (Cohen, 2010). Viral entry is dependent on cell type and can occur via direct fusion with the plasma membrane, or receptor-mediated endocytosis via an acidic- or non-acidic-endosome (Koyama & Uchida, 1987; Nicola et al., 2003; Milne et al., 2005).

Glycoprotein D uses various members of the nectin family as receptors (Campadelli - Fiume et al., 2000). Nectin-1 is used by Herpes Simplex Virus 1 (HSV-1), Herpes Simplex Virus 2 (HSV-2), Bovine alphaherpesvirus 1 (BHV-1) and Pseudorabies Virus (Suid alphaherpesvirus 1, PRV) (Geraghty et al., 1998; Cocchi et al., 1998b). Nectin-2 is used by PRV and some mutant strains of HSV-1 and HSV-2 (Warner et al., 1998; Cocchi et al., 1998a). The gD of PRV and BHV-1 interact with the poliovirus receptor (nectin-like-5, CD155) (Geraghty et al., 1998). In addition to nectins, HSV-1/2 gD can interact with two unrelated receptors to mediate entry. First, a member of the Tumour Necrosis Factor (TNF) receptor family, the herpesvirus entry mediator (HVEM) (Montgomery et al., 1996). Second, heparin sulphate that has been specifically modified by 3-O-sulfotransferases (Shukla et al., 1999).
For any alphaherpesvirus that tries to enter the cell, the actomyosin cortex provides an obstacle consisting of highly cross-linked F-actin immediately underneath the plasma membrane (Bray & White, 1988). The average pore size of the cortex has been measured using electron microscopy to be approximately 10 - 15 nm in diameter (Clausen et al., 2017). The alphaherpesvirus nucleocapsid has a diameter of 125 nm and is, therefore, unable to pass through after direct fusion with the plasma membrane without altering the morphology of the actin cortex (Steven & Spear, 1997). Nectins normally regulate the actin cytoskeleton through Ras/Rho GTPases, Rac1 and Cdc42. In cells overexpressing Nectin-1, upon HSV-1 attachment, a brief period of Cdc42 activation followed by an extended period of RhoA activation has been observed, which leads to the formation of large vesicles that mediate entry (Clement et al., 2006).

After the viral envelope fuses with the plasma membrane, the outer tegument layer of the alphaherpesvirus is lost. The inner tegument consisting of US3, UL36 and UL37 is retained and interacts primarily with dynein to enable the virus to be transported along microtubules towards the nucleus where viral replication occurs (Brzozowska et al., 2010; Zaichick et al., 2013; Pitts et al., 2014).

**Viral replication**

When the capsid has reached the outside of the nucleus, the dsDNA genome is inserted into the host nucleus via the capsid portal interacting with the nuclear pore complex in a process that requires importin-β (Ojala et al., 2000). Upon insertion into the nucleus, the transcription of immediate-early genes is induced by a combination of virion proteins and host transcription factors. Immediate-early Infected Cell Proteins (ICP) ICP0, ICP4, ICP22 and ICP27 act to limit the host immediate antiviral response and to up regulation the transcription of viral Early genes that are involved in viral DNA replication (Roizman, 1996; Silva et al., 2012).
Of the Early genes, seven proteins are required for viral DNA synthesis: an origin binding protein (UL9), a single-strand DNA binding protein (ICP8), a polymerase and a primase/helicase complex (UL5, UL8 and UL52). When the linear viral genome is inserted into the nucleus, it readily circularises through the fusion of its genomic termini, or in the case of alphaherpesviruses which lack genomic terminal repeats, direct end-to-end ligation (Garber et al., 1993; Strang & Stow, 2005). DNA synthesis begins at origin sequences. Depending on the virus, there may be several origin sequences; however, only one is required for DNA synthesis (Balliet et al., 2005).

Synthesis is initiated by the binding of ICP8 and UL9 to the origin sequences (Makhov et al., 2003). The binding of ICP8 and UL9 induces a formation of a DNA hairpin at the origin sequence (Olsson et al., 2009). The primase/helicase complex is then recruited to the destabilised viral origin (Chen et al., 2011). First, theta-type bidirectional replication occurs, where two DNA replication forks move forward independently around the circular DNA (Hammarsten et al., 1996). After this initial round, a concatemer copy of the viral genome is made using ‘rolling’ circular replication (Skaliter et al., 1996).

The replication of the viral genome activates the expression of Late genes that primarily encode proteins that are involved in the formation of the capsid and packaging of viral DNA. Capsid precursors are assembled by the interaction of UL19, UL6, UL26 and UL26.5 (Singer et al., 2005; Walters et al., 2003) UL6 forms the portal on the viral capsid for DNA entry (Newcomb et al., 2001). The alphaherpesvirus genome contains cleavage sites that allow individual copies of the genome within the concatemer to be separated for packaging into capsids (Varmuza & Smiley, 1985). Viral proteins UL15, UL28, and UL33 are thought to create an equivalent of a viral terminase (Beard et al., 2002). Together these viral proteins associate with stable capsids via UL6 and take the concatemer of the viral genome, cleave and subsequently package individual genomes into the capsids.

The role of actin in the alphaherpesvirus replication inside the nucleus remains poorly understood. G-actin has been reported to be essential for the nuclear expansion and remodelling of chromatin (Reynolds et al., 2004;
Simpson-Holley et al., 2005). This relaxation of the nucleus and chromatin is through to aid the movement of capsids to the nuclear membrane for egress.

The movement of capsids within the nucleus is active and dependent on ATP and temperature (Forest et al., 2005; Bosse et al., 2014). The extent to which actin or actin-associated proteins contribute to this movement remains yet to be determined. For HSV-1, this movement is shown to be sensitive to the actin depolymerisation drug latrunculin A, and inhibitor of myosin motors butanedione monoximine (Forest et al., 2005). It was also noted by Forest et al. (2005), that these filaments were resistant to depolymerisation by the drug cytochalasin D. Indeed, scanning electron microscopy has shown the presence of actin filaments within the nucleus of infected cells. Furthermore, it was found that these nuclear actin filaments are associated with viral capsids and myosin Va (Feierbach et al., 2006). Contradictory to this, Bosse et al. (2014) describes the transport to be actin-independent and argues that latrunculin A causes the formation of actin rods in a cell-dependent manner, and these rods can attach to and immobilise nucleocapsids. There are no alphaherpesvirus proteins that are homologs to actin nucleators, excluding the possibility of using actin tails similar to the vaccinia virus. However, it is not possible to exclude the association of capsids with nuclear myosin or kinesins.

**Viral egress**

Packaged capsids must egress from the nucleus by budding from the inner leaf, and fusing with the outer leaf of the nuclear membrane. This budding process requires the UL34 C-terminally anchored membrane protein and the UL31 nuclear phosphoprotein (Purves et al., 1992; Reynolds et al., 2001). The US3 serine-threonine kinase is known to regulate the process by phosphorylating UL34 (Purves et al., 1992).

Tegumentation of the viral capsid occurs within the cytoplasm, although the exact process remains mostly undefined. Tegument proteins UL13, US3, UL41, UL46 and UL47 can be deleted from the alphaherpesvirus genome with no effect on virion assembly (Roizman, 1996). However, at least for HSV-1, the absence of major tegument component UL48 interferes with the assembly
the tegument and integration of with membrane glycoproteins (Mossman et al., 2000). Alphaherpesvirus tegument proteins US9 and UL36 (VP1/2) are known to interact with motor proteins and are likely to be involved in transporting the viral capsid towards the cell periphery (Scherer et al., 2020; Zaichick et al., 2013).

The final alphaherpesvirus envelope is thought to be derived from the trans-Golgi network or endosomes (Granzow et al., 2001; Hollinshead et al., 2012). Tegument proteins interact with the tails of viral glycoproteins already incorporated in the membranes of the Golgi or endoplasmic reticulum. The exact location where final envelopment occurs remains unclear and may indeed be virus-specific. For alphaherpesviruses that release themselves into the extracellular environment, exocytosis must be triggered. Rab GTPases have found to be involved in alphaherpesvirus assembly and have been investigated using loss-of-function experiments. Rab1 is involved in the transport of the virus from the endoplasmic reticulum to the Golgi apparatus. Alphaherpesviruses are also known to use Rab5, Rab6, and Rab11 pathways that are involved in exocytosis in the early endosome, constitutive and recycling pathways, respectively (Hollinshead et al., 2012; Johns et al., 2014).

For alphaherpesviruses that are strictly cell-associated, such as Marek’s Disease Virus (Gallid alphaherpesvirus 2, MDV) and VZV, the cytoskeleton is significantly remodelled to form long cellular protrusions containing actin filaments and microtubules. These cellular protrusions enable direct cell-to-cell contact and allow the virus to be transported to an uninfected neighbouring cell.

For HSV-2, PRV, and MDV, the remodelling of the actin cytoskeleton during infection has been attributed to the alphaherpesvirus US3 serine-threonine kinase, and its interaction with Group A p21-activated kinases (PAKs) (Murata et al., 2000; Favoreel et al., 2005; Schumacher et al., 2005). Phosphorylation of PAK1 results in the formation of thin, branched actin-containing protrusions. Neither cells which have been infected with a US3-null virus or have their actin cytoskeleton stabilised through the drug
jasplakinolide are capable of forming such protrusions (Favoreel et al., 2005). In addition to this, US3 can remove actin stress fibres through the phosphorylation of PAK2.

The role of PAK1 and PAK2 has been explored in PRV infection (Van den Broeke et al., 2009b). In PAK1 knockout cells, viral spread was reduced when cells are grown sparsely; however, it was found to be dispensable in cell monolayers. This observation suggests that the PAK1 cellular protrusions are likely to be used to mediate direct contact and transmit the virus to distant cells. The viral spread was impaired in PAK2 knockout cells regardless of density. It is not known whether the impairment of viral spread is due to the inability to remove actin stress fibres or from the inability to phosphorylate another downstream component of the PAK2 pathway.

1.2.2 The alphaherpesvirus US3 kinase

The US3 serine/threonine protein kinase is found in all known alphaherpesviruses that have been identified to date, but is not conserved in beta- or gamma-herpesviruses (Jacob et al., 2011). The designation of US3 indicates its location within the third unique-short (US) region of the prototypical herpes simplex genome. The exception to this is the US3 orthologue of the Varicella-Zoster virus (VZV), where it is designated by the open reading frame number (ORF66). The presence of US3 within all alphaherpesviruses indicates that it is involved in the replicative fitness of the virus, but in some cases, has been seen to be not necessarily required for growth in cultured cells (Purves et al., 1987; Olsen et al., 2006; Takashima et al., 1999).

The most striking similarity between orthologues of the alphaherpesvirus US3 is the conserved kinase domain of ~290 amino acids, consisting of an ATP-binding site and an active catalytic site (Deruelle & Favoreel, 2011). The critical amino acids have been identified within these sites as being a lysine responsible for positioning the gamma phosphate of ATP, and an aspartic acid which deprotonates the acceptor serine or threonine (Knighton et al., 1991).
The identification of these amino acids has led to mutagenic analysis examining the differences in localisation and function between catalytic and not-catalytic variants of US3 (Schumacher et al., 2008; Ogg et al., 2004; Van den Broeke et al., 2009a).

_In vitro_ biochemical studies of pseudorabies virus (PRV) US3 have characterised a consensus minimal phosphorylation sequence of \((R)n\)-X-(S/T)-Y-Y, where \(n\geq 2\), X can be any amino acid or absent, S/T is the target serine/threonine, and Y is any non-acidic amino acid except proline (Leader et al., 1991). This minimal consensus sequence is similar to that of the cAMP-dependent protein kinase A (Benetti & Roizman, 2004).

1.2.3 Functions of US3

The viral genome is compact due to the limited size of the capsid, and because of this, the need for multifunctional genes arises. The US3 kinase is a prime example of this phenomenon (Figure 1.6). The flexible substrate sequence of US3 suggests that the kinase is indeed involved in many processes. It should be noted that when MDV is compared to the other US3 orthologues of major alphaherpesviruses, it has an average similarity of approximately 29% (Table 1.1). Because of this, the functions of US3 reviewed here may or may not be shared with MDV. A phylogenetic tree showing the distance between US3 kinases based on their amino acid sequence is shown in Figure 1.5, showing that MDV US3 highly dissimilar to other alphaherpesvirus orthologues.
Table 1.1 Percentage of amino acid identity between selected alphaherpesvirus US3 orthologues. Adapted from Deruelle and Favoreel (2011).

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<th>HSV-1</th>
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<th>VZV</th>
<th>PRV</th>
<th>BHV-1</th>
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<th>EHV-1</th>
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Figure 1.5 Phylogenetic tree of US3 kinases of selected alphaherpesviruses. The Jukes-Cantor model is used to determine genetic distance. Scale bar represents 0.08 substitutions per amino acid position.
Figure 1.6 Summary of alphaherpesvirus US3 functions. The alphaherpesvirus US3 kinase is a multifunctional protein involved in many aspects related to viral replication. In the nucleus, US3 is involved in preventing transcriptional repression by HDACs and is required for the development of virions emerging from the perinuclear space. US3 subverts the host immune response by inhibiting the production of IFN and maturation of MHC-1, as well as triggering the internalisation of gB present on the cell surface. US3 leads to cytoskeletal changes, including the disassembly of stress fibres and formation of cellular protrusions.
Gene expression

US3 has roles in modifying the transcriptional environment within the host nucleus in order to promote viral replication. The activity of histone deacetylases (HDACs) condenses chromatin and represses transcription (Grozinger & Schreiber, 2002). It has been observed that for HSV-1/2, PRV and VZV, US3 can, through what is likely to be an indirect mechanism, hyperphosphorylate and inhibit HDAC1 and HDAC2 (Walters et al., 2009; Walters et al., 2010). While interference with HDACs is common in alphaherpesviruses, there are notable differences in the role it may play in viral replication. Despite being an in vitro kinase substrate of PRV US3, it appears that PRV US3 is not essential for HDAC hyperphosphorylation (Walters et al., 2010). Similarly, when HDAC is inhibited in cells infected with US3-null virus, only increased viral growth was seen with PRV and VZV, but not HSV-1 (Walters et al., 2010).

In addition to the interference of HDAC1/2, there is increasing evidence to suggest that US3 can directly affect the expression of specific viral genes. Comparing the expression of viral genes from wild-type and a US3-null mutant PRV has highlighted that US3 may increase the expression of the UL29 major DNA-binding protein and UL39 ribonucleoside-diphosphate reductase, and decrease expression of UL42 DNA polymerase processivity factor (Skiba et al., 2010).

Nuclear egress of virions

While most DNA viruses exit the nucleus by rupturing the nuclear envelope or passing through nuclear pores, herpesviruses are unusual in the fact they have evolved a separate mechanism. Herpesvirus capsids bud from the inner nuclear membrane into the perinuclear space before fusing with the outer nuclear membrane and passing into the cytoplasm (Granzow et al., 2001). For HSV-1 and MDV, this has been shown to require the kinase activity of US3 (Reynolds et al., 2001; Schumacher et al., 2008).
Rearrangement of the actin cytoskeleton

Two actin cytoskeleton rearrangements occur during alphaherpesvirus infection: removal of actin stress fibres and the formation of actin- and microtubule-containing cellular protrusions. The cellular protrusions form direct cell-to-cell contacts, which for strictly cell-associated alphaherpesviruses such as VZV and MDV are used to transport virions and infect other cells.

For MDV, PRV and HSV-2, the actin remodelling has been attributed to the US3 kinase. The Rho family of GTPases is the primary regulator of the actin cytoskeleton. It is perhaps therefore not surprising that viruses, and in particular alphaherpesviruses, have evolved to interfere with this pathway in order to promote viral replication and spread. Through the phosphorylation of PAK1 and PAK2, the initiation of cellular protrusions and disassembly of actin stress fibres occurs (Van den Broeke et al., 2009b). Further to this, in PRV, US3 is seen to trigger the dephosphorylation and activation of the F-actin severing protein cofilin (Jacob et al., 2013).

MDV is currently the only known alphaherpesvirus that has been observed not to require its kinase activity to remove stress fibres (Schumacher et al., 2008). This observation suggests that for MDV, there is an alternative or additional mechanism to the primary Rho-GTPase mechanism.

Inhibition of apoptosis

Several US3 orthologues have shown to inhibit apoptosis induced by infection, UV damage and the Fas pathway (Jerome et al., 1999). Several mechanisms exist, but involve the Bcl-2 family of pro-apoptotic proteins. HSV-1 and PRV US3 phosphorylate and subsequently inactivate the Bcl-2 family member Bad (Ogg et al., 2004; Deruelle et al., 2007). In addition to this, Bid has been identified as a US3 substrate in vitro, and phosphorylation prevents the processing of Bid by granzyme B and blocks the cytochrome c apoptosis pathway (Cartier et al., 2003).
Subversion of host immune response

Interferons (IFN) are a class of cytokines that are produced during viral infection. These molecules trigger the production of antiviral effector proteins or act as signalling molecules for the immune response (Ivashkiv & Donlin, 2014). The alphaherpesvirus US3 kinase has been shown to interfere with IFN-alpha, -beta and –gamma through a variety of mechanisms. In the absence of US3, HSV-1 shows dramatically reduced growth in IFN-alpha treated cells (Piroozmand et al., 2004). Further to this, VZV reduces IFN-gamma signalling by reducing the phosphorylation of STAT1 (Schaap-Nutt et al., 2006). For HSV-1, US3 phosphorylates the alpha-subunit of the IFN-gamma receptor, inhibiting expression of IFN-gamma dependent genes (Liang & Roizman, 2008). In addition to this, HSV-1 US3 has been shown to impair the synthesis of IFN-beta by antagonising interferon regulatory factor 3 (Wang et al., 2013).

In order to prevent infected cells from being destroyed by cytotoxic T-lymphocytes, alphaherpesviruses have evolved a mechanism to prevent viral antigens from being presented at the cell surface by class 1 major histocompatibility complex (MHC 1). The inhibition of MHC 1 is typically achieved by preventing its cell surface expression. In the case of PRV and VZV, US3 is able, through an unknown mechanism, to retain the MHC 1:peptide complex at the Golgi apparatus (Deruelle et al., 2009; Abendroth et al., 2001).

Finally, there is increasing evidence that US3 modulates the expression of viral proteins at the cell surface in a way that reduces cellular and humoral immune responses. For HSV-1 and PRV, US3 is seen to phosphorylate the cytoplasmic domain of gB, causing its endocytosis and reducing its expression on the cell surface (Imai et al., 2010; Kato et al., 2009). Indeed, gB is a strong inducer of the immune response, with levels of cell surface gB correlating with cell lysis by natural killer immune cells (Kato et al., 2009).
1.3 Marek’s Disease Virus

Marek’s disease virus (MDV, Gallid herpesvirus-2) is an alphaherpesvirus under the genus *Mardivirus*. It is the causative agent of the Marek’s Disease (MD), an economically significant disease of common poultry that is characterised by T-cell lymphomas and peripheral nerve enlargement. It is estimated that MD results in US$ 1-2 bn of annual economic loss (Morrow & Fehler, 2004). MD was first characterised in 1907 by the Hungarian veterinarian Josef Marek, but only as polyneuritis with negligible mortality (Marek, 1907). It was only until 1968 when MDV was first isolated as the causative agent of MD (Churchill, 1968; Churchill & Biggs, 1967).

During the discovery of MD, histological examination highlighted the infiltration of the spinal cord and sciatic nerve by mononucleated cells. This observation can still be made in birds infected with modern strains of MDV (Bacon *et al.*, 2001; Gimeno *et al.*, 1999). Despite this, MD has evolved alongside the introduction of intensive poultry farming, into a highly infectious disease results in death if not vaccinated against (Gimeno, 2008). MD is characterised by T-cell lymphomagenesis, and the spread of transformed T-cells to skeletal muscle and visceral organs where tumours develop (Calnek, 2001; Burgess & Davison, 2002). In addition to this, MD also results in widespread damage and oedema to the brain and nervous system (Gimeno *et al.*, 1999).

Several serotypes of MDV exist, with significant differences in their overall genomic structure and replicative features. Serotype 1 is the most common wild-type virus and includes all oncogenic forms. Serotypes 2 and 3 are non-oncogenic and are isolated from chickens and turkeys, respectively (Gimeno 2008). Serotypes 2 and 3 are used in combination with attenuated strains of serotype 1 to produce polyvalent vaccines against MD (Sharma & Witter, 1983). While vaccination prevents the development of MD, it does not provide sterile immunity; MDV is still able to replicate and be shed from vaccinated birds (Read *et al.*, 2015). Because of this, an evolution towards
increasingly virulent strains occurs, which on several occasions has defeated vaccines available at that time (Witter, 1997; Nair, 2005).

Unlike many alphaherpesviruses, MDV is strictly cell-associated in cell culture (Nazerian et al., 1968). The replicative cycle of MDV strongly resembles that of VZV. Keratinised viral particles are shed from the feather follicle of infected birds and are inhaled by uninfected birds. The virus phagocytosed by macrophages and dendritic cells that reside in the lungs and airways, which transport the virus to the lymphoid organs, namely the bursa of Fabricius, spleen and thymus (Calnek, 2001). In the lymphoid organs, MDV undergoes its first cytolytic replication phase within B cells and later activated CD4+ T-cells (Calnek, 2001; Shek et al., 1983).

Infected CD4+ T-cells aside from being the target of viral transformation, are the reservoir for latent MDV genomes. During latency, viral genomes are maintained without the production of infectious virus (Grinde, 2013). Infected CD4+ T-cells also transport the virus to the feather-follicle epithelium, where infectious virus progeny is produced and shed into the environment (Johnson et al., 1975; Calnek, 2001). The sequence of events leading to the entry into latency or reactivation of the virus remains unclear as, with many other alphaherpesviruses (Efstathiou & Preston, 2005; Grinde, 2013). During lymphomagenesis, the population of transformed T-cells is identical to that where viral latency has been established. This observation suggests that the establishment of latent infection is required for transformation (Delecluse & Hammerschmidt, 1993; Delecluse et al., 1993). It is thought that only a small proportion transformed T-cells proliferate to form tumours (Calnek, 2001). The transformation has been attributed to the MDV basic leucine zipper protein Meq, which is highly similar to oncoproteins Jun and Fos (Jones et al., 1992). Meq interferes with cell cycle control by interacting with cyclin-dependent kinase 2 (CDK2), tumour protein p53, and retinoblastoma protein (Liu et al., 1999). The leucine zipper of Meq allows it dimerise, but also interact with Fos, c-Jun and JunB oncoproteins (Qian et al., 1996). Meq preferably interacts with c-Jun; from this, it is proposed that increased stability
of c-Jun allows it to activate heparin-binding epidermal-growth-factor-like growth factor (HB-EGF) and cathepsin-like protein JTAP-1. Both HB-EGF and JTAP-1 activation can independently lead to the transformation of chicken cells (Levy et al., 2003).

1.3.1 Marek’s Disease Virus US3

The MDV US3 gene encodes for a 402 amino acid protein containing a central kinase domain flanked by two predicted globular domains. As with other US3 kinases of the alphaherpesvirus family, its kinase activity has been explored in great detail. Previous work by Schumacher et al. (2005, 2008) removed catalytic activity through the amino acid substitution K220A. Similar to other alphaherpesvirus US3 homologs, removal of kinase activity resulted in reduced growth properties and smaller viral plaques compared to the wild type virus and also loss of anti-apoptotic effects (Schumacher et al., 2005). Electron microscopy also highlighted the accumulation of virions in the perinuclear space (Schumacher et al., 2005; Schumacher et al., 2008).

However, unlike other alphaherpesviruses, MDV US3 K220A was still able to alter the actin cytoskeleton of cells infected with a recombinant virus. Kinase-dead MDV US3 was able to remove actin stress fibres just as effectively as the wild-type MDV US3, although the formation of cellular projections was not reported (Schumacher et al., 2008). The kinase-independent mechanism for the remodelling of actin remains yet to be determined.

1.4 Outline of this work

As a result of the highly compact nature of the alphaherpesvirus genome, MDV US3 is a highly multifunctional viral protein. While the kinase region of the alphaherpesvirus US3 is conserved within the subfamily, MDV US3 only shares, on average, a 29 % sequence similarity between other alphaherpesvirus US3 homologs. While it has been shown for certain alphaherpesviruses that the remodelling of the actin cycle is kinase-dependent, this does not appear to be the case for MDV. In this regard,
identifying host interaction partners of MDV US3 will allow interrogating the mechanism of kinase-independent remodelling of the actin cytoskeleton.

Here we confirm the kinase-independent actin remodelling by US3 through mutagenesis and immunofluorescence microscopy (Chapter 3). We then screen for the potential interactors of MDV US3 using a proteomic discovery approach and identify the actin cross-linker alpha-actinin and the microtubule motor dynein as putative US3 interactors (Chapter 4). We use biochemical analysis to show the direct, calcium-dependent interaction between MDV US3 and alpha-actinin, demonstrate that kinase-dead MDV US3 can prevent alpha-actinin to cross-link F-actin and explore if the over-expression of alpha-actinin is capable of overcoming the US3-mediated stress fibre disassembly (Chapter 5). Finally, we follow up on the interaction between MDV US3 and dynein and show that US3 is co-transported by dynein along microtubules in vitro (Chapter 6).
2 Materials and Methods

2.1 Materials

2.1.1 Buffers

The composition of commonly used buffers is listed below.

**Phosphate-buffered saline (PBS)**
- 137 mM NaCl
- 2.7 mM KCl
- 10 mM Na$_2$HPO$_4$
- pH adjusted to 7.4 using HCl

**Tris-buffered saline (TBS)**
- 50 mM Tris-Cl pH 7.5
- 150 mM NaCl

**TBS-Tween (TBST)**
- 1x TBS
- 0.05 % v/v Tween 20

**Tris-EDTA (TE)**
- 10 mM Tris-Cl pH 7.5
- 1 mM EDTA

2.1.2 Size markers

**GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific SM1331)**
Contains 15 individual DNA fragments: 20000, 10000, 7000, 5000, 4000, 3000, 2000, 1500, 1000, 700, 500, 400, 300, 200, 75 bp

**Colour Prestained Protein Standard, Broad Range (NEB P7719)**
Contains 11 purified prestained proteins: 250, 180, 130, 95, 72 (Red), 55, 43, 34, 26 (Green), 17, 10 kDa.
Colour Prestained Protein Standard, Broad Range (NEB P7712)
Contains 12 purified prestained proteins: 245, 180, 135, 100, 80 (Red), 58, 46, 32, 25 (Green), 22, 17 kDa.

2.1.3 Bacterial strains

Table 2.1 Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosetta™ (DE3)pLysS</td>
<td>Novagen</td>
<td>str. B F- ompT gal dcm lon? hsdS_{B}(r_{B}^{-}m_{B}^{-}) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB^{+}]_{K^{+}}(λ^{S}) pLysSRARE[T7p20 ileX argU thrU tyrU glyT thrT argW metT leuW proL ori^{p15A}] (Cm^{R})</td>
</tr>
</tbody>
</table>
### 2.1.4 Plasmids

Table 2.2 Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEGFP-N1</td>
<td>Mammalian expression vector with c-terminal EGFP. Confers bacterial resistance to kanamycin.</td>
<td>Clontech</td>
</tr>
<tr>
<td>pET22b-mEB3-6His</td>
<td>A bacterial expression vector containing a c-terminal 6xHis tag. Confers bacterial resistance to ampicillin.</td>
<td>Anne Straube</td>
</tr>
<tr>
<td>pFLAG-MDV_US3</td>
<td>A mammalian expression vector containing FLAG-tagged wild-type MDV US3. Mammalian expression is driven by a CMV promoter. Confers bacterial resistance to ampicillin.</td>
<td>Klaus Osterieder</td>
</tr>
<tr>
<td>pKan-CMV-KIF1C-RIP2-GFP</td>
<td>A mammalian expression vector containing used to clone the HA-tagged BioID2 promiscuous biotin ligase. Mammalian expression is driven by a CMV promoter. Confers bacterial resistance to kanamycin.</td>
<td>Alex Zwetsloot</td>
</tr>
<tr>
<td>MDV RB1B UL35-GFP BAC</td>
<td>Bacterial artificial chromosome containing the genome of MDV strain RB1B that contains a GFP-tagged capsid protein. Confers bacterial resistance to chloramphenicol.</td>
<td>Venugopal Nair</td>
</tr>
</tbody>
</table>
pFLAG-MDV_US3(D218A, K220A)

To generate a kinase-dead mutant of US3, the amino acid substitutions D218A and K220A were introduced into pFLAG-MDV_US3 using site-directed mutagenesis by PCR. Mutagenic PCR is described in Section 2.2.4. The template was pFLAG-MDV_US3. The mutagenic primer was MDV US3 D218A K220A mut (AS543) and was used with the upstream primer MDV US3 926 Rev (AS542) and downstream primer MDV US3 259 Fwd (AS541). Both the mutagenic PCR product and pFLAG-MDV_US3 were digested using BsrGI before being ligated together. The fragment excised from pFLAG-MDV_US3 was replaced with the same fragment of the digested mutagenic PCR product.

pET22b-MDV_US3(D218A,K220A)-GFP

MDV US3 was cloned into the pET22b for bacterial expression and subsequent protein purification. MDV US3 was amplified from pEGFP-MDV_US3(D218A,K220A) using PCR with primers MDV US3 AseI Fwd (AS672) and GFP NotI Rev (AS673). pET22b-mEB3-6His was linearized and insert removed by digesting with AseI and NotI. The PCR product was digested with AseI and NotI before being ligated into the pET22b vector.

pEGFP-MDV_US3(D218A,K220A)-GFP

Kinase-dead MDV US3 was cloned into the pEGFP-N1 vector containing eGFP for expression and visualisation in mammalian and chicken cells. Using pFLAG-MDV_US3(D218A, K220A) as a template, kinase-dead US3 was amplified using primers MDV US3 NheI Fwd (AS632) and MDV US3 BamHI Rev (AS583). Both the pEGFP vector and PCR product were digested using NheI and BamHI before being ligated together.

pKan-MDV_US3-BioID2-HA

US3 was amplified using PCR from a bacterial artificial chromosome containing the genome of MDV strain RB1B with primers MDV US3 NheI Fwd (AS632) and MDV US3 BamHI Rev (AS583). The PCR product was digested with NheI and BamHI. BioID2-HA was artificially synthesised as a gene
fragment using the sequence of MCS-BioID2-HA from Kim et al. (Addgene #74224). The codon sequence was optimised for mammalian cell expression. The repeats and GC content was adjusted to reduce complexity for artificial synthesis. The gene fragment was digested with \textit{BamHI} and \textit{NotI} and ligated into pKan-CMV-Kif1C_RIP2-GFP (carried out by Alex Zwetsloot). The resulting plasmid was then digested with \textit{NheI} and \textit{BamHI} and ligated to the digested MDV US3 PCR fragment.

\textit{pKan-MDV\_US3(1-111)-BioID2-HA}

Using a bacterial artificial chromosome containing the genome of MDV strain RB1B as a template, the US3 region 1-111 was amplified with restriction sites introduced using primers \textit{NheI US3 Fwd (AS578)} and \textit{US3 P111 BamHI Rev (AS579)}. The PCR product and pKan-MDV\_US3-BioID2-HA were digested with \textit{BamHI} and \textit{NheI} before the PCR product was ligated into the vector backbone.

\textit{pKan-MDV\_US3(1-322)-BioID2-HA}

Using a bacterial artificial chromosome containing the genome of MDV strain RB1B as a template, the US3 region 1-322 was amplified with restriction sites introduced using primers \textit{NheI US3 Fwd (AS578)} and \textit{US3 P322 BamHI Rev (AS581)}. The PCR product and pKan-MDV\_US3-BioID2-HA were digested with \textit{BamHI} and \textit{NheI} before the PCR product was ligated into the vector backbone.

\textit{pKan-MDV\_US3(111-402)-BioID2-HA}

Using a bacterial artificial chromosome containing the genome of MDV strain RB1B as a template, the US3 region 111-402 was amplified with restriction sites introduced using primers \textit{NheI-ATG-US3 P111 Fwd (AS580)} and \textit{US3 BamHI Rev (AS583)}. The PCR product and pKan-MDV\_US3-BioID2-HA were digested with \textit{BamHI} and \textit{NheI} before the PCR product was ligated into the vector backbone.
pKan-MDV_US3(111-322)-BioID2-HA
Using a bacterial artificial chromosome containing the genome of MDV strain RB1B as a template, the US3 region 111-402 was amplified with restriction sites introduced using primers Nhel-ATG-US3 P111 Fwd (AS580) and US3 P322BamHi Rev (AS581). The PCR product and pKan-MDV_US3-BioID2-HA were digested with BamHI and Nhel before the PCR product was ligated into the vector backbone.

pKan-MDV_US3(322-402)-BioID2-HA
Using a bacterial artificial chromosome containing the genome of MDV strain RB1B as a template, the US3 region 322-402 was amplified with restriction sites introduced using primers Nhel-ATG-US3 P322 (AS582) and US3 BamHi Rev (AS583). The PCR product and pKan-MDV_US3-BioID2-HA were digested with BamHI and Nhel before the PCR product was ligated into the vector backbone.

2.1.5 Oligonucleotides
Table 2.3 Primers generated in this study

<table>
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<tr>
<th>#</th>
<th>Name</th>
<th>Description</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>AS541</td>
<td>MDV US3 259 Fwd</td>
<td>Amplify MDV US3 from nucleotide position 259</td>
<td>GAA ACG GTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ACG GAC ATT G</td>
</tr>
<tr>
<td>AS542</td>
<td>MDV US3 926 Rev</td>
<td>Amplify MDV US3 from nucleotide position 926</td>
<td>GAT CTC AGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TGA GAA CCT G</td>
</tr>
<tr>
<td>AS543</td>
<td>MDV US3 D218A K220A mut</td>
<td>Mutagenesis primer to introduce D218A and K220A substitutions</td>
<td>GTA TAA TAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ATC GTG CTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TAG CAA CTG</td>
</tr>
<tr>
<td>AS578</td>
<td>Nhel MDV_US3 Fwd</td>
<td>Introduce Nhel site at the start of US3</td>
<td>TTT AAT GCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AGC GAT GTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TTC GAC TCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GGA G</td>
</tr>
<tr>
<td>AS579</td>
<td>MDV_US3 P111</td>
<td>Amplify up to amino</td>
<td>CTT CAG GAT</td>
</tr>
<tr>
<td>AS580</td>
<td>BamHI Rev</td>
<td>acid position 111 of MDV US3 and introduce <em>BamHI</em> site at the end of the gene</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>----------</td>
<td>--------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nhel-ATG-MDV_US3 P111 Fwd</td>
<td>Amplify from amino acid position 111 of MDV US3, introduce start codon and <em>Nhel</em> site at the start of the gene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS580</td>
<td>CCG GCG GTA ACG ATG AAA CAA TG</td>
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<tr>
<td>AS581</td>
<td>Nhel-ATG-MDV_US3 P322 BamHI Rev</td>
<td>Amplify up to amino acid position 322 of MDV US3 and introduce <em>BamHI</em> site at the end of the gene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS581</td>
<td>GAA ATG GAT CCG GAT GGA CTT GCA GGC ATC</td>
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</tr>
<tr>
<td>AS582</td>
<td>Nhel-ATG-MDV_US3 P322</td>
<td>Amplify from amino acid position 322 of MDV US3, introduce start codon and <em>Nhel</em> site at the start of the gene</td>
<td></td>
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<tr>
<td></td>
<td>AS582</td>
<td>GCC TGG CTA GCA TGC CGT TGG AAT TTC CAC AGA AC</td>
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<tr>
<td>AS583</td>
<td>MDV_US3 BamHI Rev</td>
<td>Introduce <em>BamHI</em> site at the end of US3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS583</td>
<td>CCT TGG GAT CCA TAT GAG CGG CAG TTA TCG</td>
<td></td>
</tr>
<tr>
<td>AS632</td>
<td>MDV US3 Nhel Fwd</td>
<td>Introduce <em>Nhel</em> site at the start of US3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS632</td>
<td>TTT AAT GCT AGC ATG TCT TCG ACT CC</td>
<td></td>
</tr>
<tr>
<td>AS672</td>
<td>MDV US3 Asel Fwd</td>
<td>Introduce <em>AseI</em> site at the start of US3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AS672</td>
<td>AGG GTG ATT AAT GTC TTC GAC TCC GGA</td>
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</tbody>
</table>
Amplify GFP, introduce NotI site at the end of GFP

Table 2.4 Primary antibodies used in this study

<table>
<thead>
<tr>
<th>Target</th>
<th>Species</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-actinin</td>
<td>Mouse IgG</td>
<td>IF: 1:500</td>
<td>Santa Cruz sc-17829 Lot A1518</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WB: 1:1000</td>
<td></td>
</tr>
<tr>
<td>FLAG tag</td>
<td>Mouse IgG</td>
<td>IF: 1:200</td>
<td>Sigma F3165 Lot SL08344</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WB: 1:2000</td>
<td></td>
</tr>
<tr>
<td>GFP</td>
<td>Mouse IgG</td>
<td>WB: 1:2000</td>
<td>Sigma G6539</td>
</tr>
<tr>
<td>HA-tag</td>
<td>Rabbit IgG</td>
<td>IF: 1:800</td>
<td>CST #3724 Lot 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WB: 1:1000</td>
<td></td>
</tr>
<tr>
<td>Polyhistadine tag</td>
<td>Mouse IgG</td>
<td>WB: 1:3000</td>
<td>Sigma H1029 Lot</td>
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</tbody>
</table>

IF: Immunofluorescence
WB: Western Blot
Table 2.5 Secondary antibodies used in this study

<table>
<thead>
<tr>
<th>Target</th>
<th>Conjugate</th>
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<th>Dilution</th>
<th>Source</th>
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<tr>
<td>Mouse IgG</td>
<td>HRP</td>
<td>Goat</td>
<td>1:4000</td>
<td>Promega W402B</td>
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<td>Mouse IgG</td>
<td>Alexa 488</td>
<td>Donkey</td>
<td>1:500</td>
<td>Molecular Probes A-21202</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>Alexa 594</td>
<td>Donkey</td>
<td>1:1000</td>
<td>Molecular Probes A-21203</td>
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<tr>
<td>Mouse IgG</td>
<td>Alexa 647</td>
<td>Donkey</td>
<td>1:300</td>
<td>Molecular Probes A-31571</td>
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<td>Rabbit IgG</td>
<td>HRP</td>
<td>Goat</td>
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<tr>
<td>Rabbit IgG</td>
<td>Alexa 488</td>
<td>Donkey</td>
<td>1:500</td>
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<tr>
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<td>Alexa 594</td>
<td>Donkey</td>
<td>1:1000</td>
<td>Molecular Probes A-21207</td>
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<tr>
<td>Rabbit IgG</td>
<td>Alexa 647</td>
<td>Donkey</td>
<td>1:300</td>
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</tr>
</tbody>
</table>

2.1.7 Stains

Table 2.6 Fluorescent stains used in this study

<table>
<thead>
<tr>
<th>Stain</th>
<th>Target</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acti-stain 555 phalloidin</td>
<td>F-actin</td>
<td>1:1000</td>
<td>Cytoskeleton, Inc # PHDH1-A</td>
</tr>
<tr>
<td>Acti-stain 670 phalloidin</td>
<td>F-actin</td>
<td>1:500</td>
<td>Cytoskeleton, Inc # PHDN1-A</td>
</tr>
<tr>
<td>DAPI</td>
<td>DNA</td>
<td>1:10000 of 1 g/ml stock</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

52
### 2.1.8 Streptavidin conjugates

**Table 2.7** Streptavidin conjugates used in this study

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptavidin-HRP</td>
<td>1:10000</td>
<td>GE-Healthcare RPN1231</td>
</tr>
<tr>
<td>Streptavidin-FITC</td>
<td>1:10000</td>
<td>Sigma S3762</td>
</tr>
</tbody>
</table>

### 2.1.9 Software

**Table 2.8** Software used in this study

<table>
<thead>
<tr>
<th>Software</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ClusPro</td>
<td>Protein-protein docking</td>
<td>cluspro.bu.edu</td>
</tr>
<tr>
<td>FIJI</td>
<td>Image analysis software</td>
<td>fiji.sc</td>
</tr>
<tr>
<td>Jalview</td>
<td>Sequence alignment, visualisation of protein structure</td>
<td><a href="http://www.jalview.org">www.jalview.org</a></td>
</tr>
<tr>
<td>Prism</td>
<td>Statistical analysis</td>
<td><a href="http://www.graphpad.com">www.graphpad.com</a></td>
</tr>
<tr>
<td>Scaffold</td>
<td>Analysis of proteomic mass spectrometry data</td>
<td><a href="http://www.proteomesoftware.com">www.proteomesoftware.com</a></td>
</tr>
<tr>
<td>Serial Cloner</td>
<td>Virtual cloning, PCR and restriction digest</td>
<td>serialbasics.free.fr</td>
</tr>
<tr>
<td>SnapGene Viewer</td>
<td>View DNA sequences, PCR primer design</td>
<td><a href="http://www.snapgene.com">www.snapgene.com</a></td>
</tr>
<tr>
<td>QUARK</td>
<td>Ab initio structure assembly</td>
<td>zhanglab.ccmb.med.umich.edu/QUARK/</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 DNA gel electrophoresis

Agarose gel was prepared by dissolving 1% (w/v) agarose in TAE buffer, and boiling until all agarose had dissolved. The solution was allowed to cool to approximately 60-70 °C before 10 μl of SafeView nucleic acid stain (NBS Biologicals) was added per 100 ml of agarose gel. Gels were cast using the RunOne system (Embitec). Once the agarose had set, the combs were removed and the gel placed in the electrophoresis cell containing TAE buffer. Samples were combined with 6x DNA loading dye buffer to a final concentration 1x before being loaded into the gel. Electrophoresis took place at 100V for approximately 20 minutes. DNA was visualised using UV transillumination.

Tris-acetate-EDTA (TAE)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>40 mM</td>
<td>Tris base</td>
</tr>
<tr>
<td>2 mM</td>
<td>EDTA</td>
</tr>
<tr>
<td>20 mM</td>
<td>Acetic acid</td>
</tr>
</tbody>
</table>

DNA loading dye buffer (6x)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM</td>
<td>50x TAE</td>
</tr>
<tr>
<td>0.15 % w/v</td>
<td>Orange G</td>
</tr>
<tr>
<td>60 % v/v</td>
<td>Glycerol</td>
</tr>
</tbody>
</table>

2.2.2 Purification of DNA from agarose gel

After gel electrophoresis, DNA bands were excised from the gel, and their weight measured. Purification was done using the EZ-10 Spin Column DNA Gel Extraction Kit (BioBasic Inc). 400 μl of Binding Buffer II was added for every 100 mg of gel. The gel fragment was incubated in the binding buffer at 60 °C for 10 minutes until the agarose gel had completely dissolved. The binding mixture was loaded DNA binding spin column and centrifuged at 10,000 × g for 2 minutes. The column was washed twice by adding 750 μl of wash solution and centrifuging the column at 10,000 × g for 2 minutes. DNA
was then eluted by adding 30 μl of water to the column and centrifuging at 10,000 × g for 2 minutes.

### 2.2.3 Polymerase chain reaction (PCR)

Phusion High-Fidelity DNA Polymerase (New England Biolabs) was used in the amplification of DNA for molecular cloning. For a 50 μl single reaction, the following PCR mix was created:

- 5 μl 10× Buffer-HF
- 0.5 μl Phusion DNA polymerase
- 2.5 μl 10 μM Forward Primer
- 2.5 μl 10 μM Reverse Primer
- 1 μl 10 mM dNTPs

Variable Template

PCR reactions were run according to the following profile. The annealing temperature was adjusted depending on primer pairs. An extension time of 15 seconds per kb of the amplicon was used.

- Pre heat 98 °C
- Initial denaturation 98 °C 2 minutes

30 cycles of:
- Denaturation 98 °C 10 seconds
- Annealing 60 °C 30 seconds
- Extension 72 °C 30 seconds

- Final extension 72 °C 10 minutes
- Hold 4 °C
After each PCR reaction, the presence of the correct product was checked by DNA gel electrophoresis. The PCR product was purified before restriction digest or ligation into the plasmid vector.

**PCR Buffer (Phusion HF) (10x)**

- 300 mM Tris-HCl pH 10
- 100 mM KCl
- 100 mM NH₄OAc
- 20 mM MgSO₄
- 1 % v/v Triton X-100
- 1 mg/ml BSA

### 2.2.4 Mutagenesis PCR

The generation of mutations that resulted in amino acid substitutions was carried out using the three-step megaprimer method of site-directed mutagenesis (Picard *et al.*, 1994). This method uses a mutagenesis primer containing the desired mutation in addition to an upstream and downstream primer.

5 μl  10× Buffer-HF
0.5 μl  Phusion DNA polymerase
2.5 μl  10 μM Upstream Primer
2.5 μl  10 μM Mutagenesis Primer
1 μl  10 mM dNTPs
Variable Template

PCR reactions were run according to the following profile. The annealing temperature was adjusted depending on primer pairs. An extension time of 15 seconds per kb of the amplicon was used.

Pre heat  98 °C
Initial denaturation  98 °C  2 minutes
10 cycles of:
Denaturation 98 °C 10 seconds
Annealing 60 °C 30 seconds
Extension 72 °C 10 seconds

Hold 72 °C Add 0.5 μl 100 μM downstream primer.

10 cycles of:
Denaturation 98 °C 10 seconds
Annealing 60 °C 30 seconds
Extension 72 °C 20 seconds

Hold 72 °C Add 0.5 μl 100 μM upstream primer.

10 cycles of:
Denaturation 98 °C 10 seconds
Annealing 60 °C 30 seconds
Extension 72 °C 30 seconds

Final extension 72 °C 10 minutes
Hold 4 °C

2.2.5 Phosphorylation of DNA

T4 polynucleotide kinase (T4 PNK, New England Biolabs) was used to phosphorylate DNA.

The following reaction condition was used:

DNA variable (up to 500 pM)
T4 PNK Reaction Buffer (10X) 5 μl
ATP (10 mM) 5 μl
T4 PNK 1 μl
The reaction mixture was incubated at 37 °C for 30 minutes, then heat-inactivated by incubation at 65 °C for 20 minutes.

### 2.2.6 Dephosphorylation of DNA

Calf intestinal alkaline phosphatase (CIAP, Fermentas) was used to dephosphorylate the 5’ end of vector DNA to prevent self-circularisation.

The following reaction condition was used:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>variable</td>
</tr>
<tr>
<td>CIAP</td>
<td>1 μl</td>
</tr>
<tr>
<td>Dephosphorylation buffer (10X)</td>
<td>5 μl</td>
</tr>
<tr>
<td>Dilution buffer</td>
<td>up to a final volume of 50 μl</td>
</tr>
</tbody>
</table>

The mixture was incubated at 37 °C for 5 minutes (DNA with 5’ overhang) or 50 °C for 5 minutes (blunt-ended DNA). DNA was gel-purified, or the CIAP was heat-inactivated by the addition of 1mM EDTA and incubation at 65 °C for 15 minutes.
2.2.7 Determination of nucleic acid concentration

The DNA concentration was measured on a Nanodrop 2000 (ThermoFisher). The concentration of nucleic acid within a sample was determined by the Beer-Lambert equation:

\[ c = \frac{A \cdot \varepsilon}{b} \]

- \( c \) Nucleic acid concentration (ng/\( \mu \)l)
- \( A \) Absorbance (A.U.)
- \( \varepsilon \) Wavelength dependent extinction coefficient (ng-cm/\( \mu \)l)
- \( b \) path length (cm)

The following extinction coefficients were used:

- DNA (double-stranded) 50 ng-cm/\( \mu \)l
- DNA (single-stranded) 33 ng-cm/\( \mu \)l
- RNA 40 ng-cm/\( \mu \)l

The absorbance of light with wavelengths of 220 nm to 340 nm and path lengths of 1.0 mm to 0.05 mm was used. In addition to measuring the concentration, the purity of nucleic acid was assessed by using A260/A280 and A260/A230 ratios.
2.2.8 Ligation

Ligation of DNA fragments was carried out at room temperature for 1 hour. To 6 µl of insert DNA, 1 µl of vector plasmid DNA, 2 µl of 5x rapid ligation buffer and 1 µl of T4 DNA ligase (Thermo Scientific) were added.

Rapid ligation buffer (5x)

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 mM</td>
<td>Tris-HCl pH 7.6</td>
</tr>
<tr>
<td>50 mM</td>
<td>MgCl₂</td>
</tr>
<tr>
<td>5 mM</td>
<td>ATP</td>
</tr>
<tr>
<td>5 mM</td>
<td>DTT</td>
</tr>
<tr>
<td>25% w/v</td>
<td>Polyethylene glycol-8000</td>
</tr>
</tbody>
</table>

2.2.9 Small scale plasmid purification (Miniprep)

Plasmids were purified either using an EZ-10 Spin Column Plasmid DNA Miniprep kit (BioBasic) following the manufacturer’s instructions or by alkaline lysis. 1.5 ml of the overnight culture was harvested by centrifugation at 12000 x g for 2 minutes.

For plasmid DNA extraction by alkaline lysis, each cell pellet was resuspended in 100 µl of Solution 1. 200 µl of Solution 2 was added to the resuspension, gently mixed by inversion and incubated at room temperature for 5 minutes. To this, 150 µl of Solution 3 was added, gently mixed by inversion and incubated at room temperature for 5 minutes. The resulting mixture was centrifuged at 20000 x g for 5 minutes (Eppendorf miniSpin plus, F-45-12-11 rotor) and the supernatant was carefully separated and retained.

The DNA was precipitated by adding 1 ml of ethanol to the supernatant and incubating at room temperature for 5 minutes. The DNA was pelleted by centrifugation at 20000 x g for 10 minutes (Eppendorf miniSpin plus, F-45-12-11 rotor). Pellets were washed with 500 µl of 70 % ethanol and re-pelleted by centrifugation at 20000 x g for 3 minutes (Eppendorf miniSpin...
plus, F-45-12-11 rotor), then dried at room temperature for 5 minutes. Pellets were resuspended in 50 µl of TE containing RNase and incubated at 60 °C for 20 minutes.

For the purification of bacterial artificial chromosomes (BACs), the method is the same as extraction by alkaline lysis, with the following modifications:

• Bacterial culture increased to 10 ml
• Solution 1 volume increased to 100 µl
• Solution 2 volume increased to 200 µl
• Solution 3 volume increased to 300 µl

**Solution 1**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>50 mM</td>
</tr>
<tr>
<td>Tris-HCl pH 8.0</td>
<td>25 mM</td>
</tr>
<tr>
<td>EDTA pH 8.0</td>
<td>10 mM</td>
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</table>

**Solution 2**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
<td>0.2 M</td>
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<tr>
<td>SDS</td>
<td>1 % w/v</td>
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</table>

**Solution 3**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium acetate</td>
<td>3 M</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>11.5 % v/v</td>
</tr>
</tbody>
</table>

### 2.2.10 Large scale plasmid purification (Midiprep)

Plasmids were purified using GenElute HP Plasmid Midiprep Kit (Sigma NA0200) following the manufacturer's instruction. For plasmids, a 50 ml bacteria culture was used, while a 300 ml bacteria culture was used for BAC DNA.
2.2.11 Ethanol precipitation

In order to sequence plasmid DNA that was in TE-buffer, it was necessary to replace the TE-buffer with water. To the DNA, $\frac{1}{10}$ volume of 3 M pH 5.2 sodium acetate was added. To this mixture, 3 volumes of 95 % ethanol were added and incubated at room temperature for 15 minutes.

The DNA was centrifuged $> 14,000 \times g$ for 30 minutes at 4 °C (Eppendorf Centrifuge 5417R, FA45-30-11 rotor). The supernatant was removed, and the pellet was washed with 70 % ethanol before being centrifuged $> 14,000 \times g$ for 15 minutes at 4 °C (Eppendorf Centrifuge 5417R, FA45-30-11 rotor). The supernatant was discarded, and the DNA was resuspended in the desired volume of water.

2.2.12 DNA sequencing

DNA sequencing was carried out by GATC Biotech, Germany. A total volume of 10 μl was submitted. Samples contained 80-100 ng/μl of plasmid DNA or 20-80 ng/μl of PCR product in H₂O, with 5 μM of primer. Sequences were viewed in the SerialCloner software (SerialBasics).

2.2.13 Bacterial culture

Bacteria were grown in double yeast tryptone (DYT) or LB medium. The medium was supplemented with 100 μg/mL ampicillin or 50 μg/mL kanamycin, dependent on the resistance conferred by the plasmid. Cultures were inoculated by picking a single colony or stab from a frozen glycerol stock. For starter cultures for protein purification and large-scale plasmid preparation, 50 ml cultures were used. For small-scale plasmid preparation and screening, 4 ml cultures were used. Unless specified, liquid bacterial cultures were grown at 37 °C with shaking at 180 rpm.
Double yeast tryptone-extract (DYT)
16 g/L Bacto-tryptone
10 g/L Yeast extract
5.0 g/L NaCl

Luria-Bertani (LB) medium
10 g/L Bacto-tryptone
5 g/L Yeast extract
10 g/L NaCl

2.2.14 Competent cells

During the preparation of chemically competent Top10 *E. coli*, LB media was used without any antibiotic supplementation. Top10 *E. coli* was grown in 600 ml LB with 20 mM MgSO$_4$ from a starter culture to an OD600 of 0.48. Cells were then chilled on ice for 10 minutes before being harvest by centrifugation at 4000 x g for 5 minutes at 4 °C. All buffers, pipettes and tubes were precooled to 4 °C and all work were carried out in a cold room.

Cells were resuspended in Solution 1. 10 ml of buffer per centrifuge bottle was used. Resuspended cells were pelleted by centrifugation at 1500 x g for 10 minutes at 4 °C. Each pellet was resuspended in 4 ml of Solution 2 and incubated on ice for 15 minutes. The bacterial suspension was divided into 200 μl aliquots and flash-frozen in liquid nitrogen before being stored at -80 °C.

Solution 1
300 mM Potassium acetate
1 M RbCl$_2$
100 mM CaCl$_2$
1M MnCl$_2$
15 % v/v Glycerol
adjusted to pH 5.8.
**Solution 2**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M</td>
<td>MOPS</td>
</tr>
<tr>
<td>100 mM</td>
<td>CaCl</td>
</tr>
<tr>
<td>1 M</td>
<td>RbCl₂</td>
</tr>
<tr>
<td>15 % v/v</td>
<td>Glycerol</td>
</tr>
</tbody>
</table>

adjusted to pH 5.8.

### 2.2.15 Transformation

*E. coli* strains TOP10 (Thermo Fisher Scientific) was used for plasmid propagation, while Rosetta (Novagen) was used for protein production. 100 μl of chemically competent *E. coli* were incubated on ice with 1 μl of plasmid DNA or 10 μl of ligation mixture for 20 minutes. The competent *E. coli* were then heat shocked at 42 °C for 45 seconds. After allowing to cool for several minutes on ice, 1 ml of SOC media was added to cells which were then allowed to recover at 37 °C for 30 minutes with shaking. Cells were harvested by centrifugation at 2000 x g for 5 minutes. After removing 1 ml of SOC media, cells were resuspended in the remaining media and plated on LB agar plates with the appropriate antibiotics. Plates were incubated at 37 °C overnight.

**LB agar**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x</td>
<td>LB</td>
</tr>
<tr>
<td>15 g/L</td>
<td>agar</td>
</tr>
</tbody>
</table>

Antibiotics were used at the final concentrations:

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Antibiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 μg/ml</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>50 μg/ml</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>25 μg/ml</td>
<td>Chloramphenicol</td>
</tr>
</tbody>
</table>
To make agar plates, LB and agarose were combined and autoclaved to at least 120 °C under 20 psi for 30 minutes. After the LB agar was cooled to 60 °C, antibiotic was added to the concentrations mentioned above. Under sterile conditions, approximately 10 ml of molten LB agar was poured into each 60 mm x 15 mm dish.

### 2.2.16 Glycerol stocks

Equal amounts of 100 % glycerol and overnight bacterial culture were combined. The resulting mixture was flash-frozen using liquid nitrogen before being stored at -80 °C.

### 2.2.17 Cell lines and routine passage

**A7r5**

Rat vascular smooth muscle cells were a kind gift from Dr Irina Kaverina (Vanderbilt University, Nashville, USA). Cells were grown at 37 °C and 5 % CO₂ in Dulbecco’s Modified Eagles Medium with 1000 mg/L glucose, L-glutamine and sodium bicarbonate (Sigma D6046) supplemented with 10 % FBS (Sigma F7524) and 10 ml/L penicillin-streptomycin solution (10,000 units penicillin and 10 mg/ml streptomycin in 0.9 % NaCl, Sigma P0781). For routine passage, A7r5 cells were grown to 90 % confluence, washed twice with PBS, and incubated in 0.05 % w/v Trypsin 0.02 w/v EDTA solution (Sigma) until cells were detached. Cells were split in a 1:5 ratio in the pre-warmed growth medium.

**DF1**

DF1 chicken fibroblasts were a kind gift from Prof Venugopal Nair (Pirbright Institute, UK). Cells were grown at 38.5 °C and 5 % CO₂ in Dulbecco’s Modified Eagles Medium with 4500 mg/L glucose, L-glutamine, sodium pyruvate and sodium bicarbonate (Sigma D6429), supplemented with 10 % FBS (Sigma F7524) and 10 ml/L penicillin-streptomycin solution (10,000 units penicillin and 10 mg streptomycin per ml in 0.9 % NaCl, Sigma P0781). For routine passage, DF1 cells were grown to 90 % confluence, washed twice with PBS, and incubated in 0.05 % w/v Trypsin 0.02 w/v EDTA solution (Sigma)
until cells were detached. Cells were split in a 1:10 ratio in the pre-warmed growth medium.

**RPE-1**

Retinal pigment epithelium cells (Clonetech). Cells were grown at 37 °C and 5 % CO₂ in F12 Ham’s Dulbecco’s Modified Eagles Medium with 3150 mg/L glucose, 55 mg/L sodium pyruvate and sodium bicarbonate and 15 mM HEPES (Sigma D6421), supplemented with 10 % FBS (Sigma F7524), 2 mM L-glutamine (Sigma 59202C) and 10 ml/L penicillin-streptomycin solution (10,000 units penicillin and 10 mg streptomycin per ml in 0.9 % NaCl, Sigma P0781). For routine passage, RPE-1 cells were grown to 70 % confluence, washed twice with PBS, and incubated in 0.05 % w/v Trypsin 0.02 w/v EDTA solution (Sigma) until cells were detached. Cells were split in a 1:10 ratio in the pre-warmed growth medium.

**RPE-1 ACTN4-mNeonGreen**

Stable RPE-1 cell line expressing mNeonGreen-tagged ACTN4 (generated by Ainur Kakpenova). Cells were grown at 37 °C and 5 % CO₂ in F12 Ham’s Dulbecco’s Modified Eagles Medium with 3150 mg/L glucose, 55 mg/L sodium pyruvate and sodium bicarbonate and 15 mM HEPES (Sigma D6421), supplemented with 10 % FBS (Sigma F7524), 2 mM L-glutamine (Sigma 59202C) and 10 ml/L penicillin-streptomycin solution (10,000 units penicillin and 10 mg streptomycin per ml in 0.9 % NaCl, Sigma P0781). For routine passage, RPE-1 cells were grown to 70 % confluence, washed twice with PBS, and incubated in 0.05 % w/v Trypsin 0.02 w/v EDTA solution (Sigma) until cells were detached. Cells were split in a 1:10 ratio in the pre-warmed growth medium.

**2.2.18 Cryopreservation**

In order to maintain stocks of cells, cells were frozen for long-term storage. Cells were grown to 90 % confluence in T75 culture flasks. Cells were trypsinised with 1 ml of trypsin-EDTA and pelleted by centrifugation at 200 x g
for 5 minutes. After media and trypsin were removed, cells were resuspended in FBS (Sigma D6429) containing 10 % DMSO (Sigma D2438). For each T75 flask, two cryovials were used containing 1 ml cell suspension each. Cells were frozen at -80°C in a specialised cryogenic freezing container with isopropanol (Nalgene). After 24 hours at -80 °C, frozen cells were transferred into liquid nitrogen storage. To thaw cells, cryovials were briefly warmed in a 37 °C water bath. Cells were resuspended in pre-warmed media and transferred into T25 flasks. Media was renewed once cells had adhered to remove the DMSO.

2.2.19 FuGENE transfection

FuGENE 6 Transfection Reagent (Promega) was used for the transient plasmid transfection of A7r5 and DF1 cells. Cells were seeded in a 6-well plate 24 hours before transfection. Before transfection, the media was renewed. For each well of the plate, 4.5 μl of the transfection reagent was diluted in 100 μl of Opti-MEM serum-free media (Gibco), mixed well by vortexing and incubated at room temperature for 5 minutes. To the diluted transfection reagent, 1 μg of plasmid DNA was added, thoroughly vortexed and incubated for 45 minutes at room temperature before being added drop-wise to cells.

2.2.20 Polyethyleneimine (PEI) transfection

PEI was used for the transient plasmid transfection of DF1 cells. Cells were grown to a confluence of 80-90 % in 15 cm dishes. Before transfection, the media was renewed. For each dish, 22.5 μg of plasmid DNA was diluted in 1.5 ml of Opti-MEM serum-free media. 67.5 μg of PEI was added to the transfection mixture, which was vortexed thoroughly before being incubated at room temperature for 15 minutes. After incubation, the transfection mixture was added drop-wise to cells.
2.2.21 Immunofluorescence

Cells intended for immunofluorescence microscopy were seeded on 16mm circular glass coverslips within 12-well plates. Coverslips were cleaned by acid washing with 20% hydrochloric acid at 60 °C overnight. Coverslips were rinsed with 500 ml distilled water before being autoclaved between filter paper.

Fixation
Media was removed from cells before incubating with 4 % paraformaldehyde in cytoskeletal buffer for 15 minutes at room temperature. After briefly rinsing coverslips with PBS, cells were permeabilised by incubating with 0.1 % Triton-X 100 in PBS for 1 minute at room temperature. Cells were washed in PBST for 30 minutes twice, before being blocked with 5% BSA in TBST for 30 minutes.

Cytoskeletal buffer
10 mM MES pH 6.1
138 mM KCl
3 mM MgCl₂
2 mM EGTA
10 % w/v Sucrose

Staining
Antibodies were diluted to the appropriate concentration in PBST. Coverslips were inverted onto 25 μl drops of diluted primary antibody on parafilm in a humidified chamber. Coverslips were incubated with the primary antibody overnight at 4 °C. Coverslips were then washed in PBST for 5 minutes 3 times. After washing, coverslips were inverted onto 25 μl drops of diluted secondary antibody on parafilm in a humidified chamber and incubated in the dark for 1 hour at room temperature. Coverslips were then washed in PBST for 5 minutes 3 times. After washing a 25 ul drop of DAPI was added to each coverslip and incubated at room temperature for 1 minute. Cells were then washed in PBST for 5 minutes 3 times before being mounted onto slides.
with Vectashield anti-fade medium (Vector Laboratories). Slides were sealed with nail varnish and stored at 4 °C in the dark.

2.2.22 SDS-PAGE

For the separation and analysis of proteins, 10% gels were used. Gels were cast and ran using the mini-PROTEAN system (Bio-Rad). Gels were produced from Acrylamide/Bis-acrylamide in the compositions listed below. The resulting solution was poured between the assembled gel plates to a depth of approximately 1 cm below the comb. Isopropanol was used to level the separation gel, to remove any bubbles from the surface and to prevent the gel from drying out while polymerising. After polymerisation, isopropanol was removed by blotting before the stacking gel was layered on top of the separation gel. Samples were prepared by adding 6x loading dye to a final concentration of 1x and boiling at 98 °C for 5 minutes. Gels were run at constant voltage of 180 V and a variable current until loading dye ran out of the gel.

**Protein gel running buffer (PGRB)**

- 25 mM Tris-HCl
- 192 mM Glycine
- 0.1 % w/v SDS

**Protein sample buffer (Laemmli) (6x)**

- 375 mM Tris-HCl
- 9 % w/v SDS
- 50 % v/v Glycerol
- 127 mM Beta-mercaptoethanol
- 0.03 % w/v Bromophenol blue

**Separation gel (10 %)**

- 4.0 ml H₂O
- 2.5 ml Separation buffer (1.5 M Tris-HCl pH 8.8, 0.4 % SDS)
- 3.3 ml Acrylamide/bis-acrylamide (30 % solution)
- 200 μl Ammonium persulfate (APS) (10 % w/v solution)
20 μl Tetramethylethylenediamine (TEMED)

**Stacking gel (4 %)**

2.7 ml H$_2$O
1.1 ml Stacking buffer (0.5 M Tris-HCl pH 6.8, 0.4 % SDS)
0.6 ml Acrylamide/bis-acrylamide (30 % solution)
80 μl Ammonium persulfate (APS) (10 % w/v solution)
8 μl Tetramethylethylenediamine (TEMED)

### 2.2.23 Western blotting

**Electrotransfer**

After separation by SDS-PAGE, protein is transferred onto nitrocellulose membranes with a 0.45 μm pore size (Amersham Protran 0.45 NC, GE Healthcare) using the Trans-Blot wet blotting system (Bio-Rad). Protein gels were placed on top of 3 mm Whatman paper (GE Healthcare) before a pre-soaked membrane was applied onto the gel. A sheet of Whatman paper was put on top of the membrane. Upon adding each layer, the air was removed to ensure good contact between the gel and the membrane. The resulting sandwich was placed in the electrotransfer cassette between two fibre pads, which was inserted in the electrotransfer tank containing Towbin transfer buffer. To prevent overheating, an ice pack was inserted into the electrotransfer tank. Electrotransfer took place for 1 hour with a constant current of 445 mA.

**Detection**

After electrotransfer, membranes were blocked to prevent any unspecific binding by the primary antibodies by incubating with 5 % BSA (w/v) in TBST for 1 hour at room temperature. Membranes were briefly washed with TBST before being incubated with primary antibodies overnight at 4 °C. Antibodies were diluted to their correct concentration in 0.5 % BSA in TBST. After the primary antibody solution was removed, membranes were washed three times with TBST. Depending on the primary antibody used, anti-rabbit or anti-mouse HRP conjugate secondary antibodies diluted 1:400 in 0.5 % BSA in TBST was
added to membranes and incubated at room temperature for one hour. The secondary antibody solution was removed, and the membranes were washed three times with TBST.

HRP antibodies were visualised using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Equal volumes of stable peroxide solution and luminol/enhancer solution were combined before being applied to the membrane. Western blots were visualised using the G:Box Chemi-XRQ imaging system (Syngene), with automatic selection of exposure time.

In cases where the same membrane was probed multiple times, it was necessary to strip the membrane. Membranes were incubated in stripping buffer at 60 °C for 30 minutes. Membranes were then washed in TBST for 5 minutes, three times.

**Stripping buffer**

| 3 % v/v | Glacial acetic acid |
| 500 mM | NaCl |

**Transfer (Towbin) buffer**

| 25 mM | Tris-HCl |
| 192 mM | Glycine |
| 20 % v/v | Methanol |

adjusted to pH 8.6
2.2.24 Proximity-dependent biotin identification

Biotinylation

DF1 cells were seeded in triplicate 15 cm dishes and were allowed to grow until 80-90 % confluence. Cells were transfected with BioID constructs using PEI. 24 hours after transfection, media was renewed. 48 hours after transfection, biotin was added to media with a final concentration of 50 µM. Cells were incubated in biotin-containing media overnight before being harvested.

Pull-down

Cells grown in triplicate 15 cm dishes were trypsinised with 2 ml of trypsin-EDTA per dish, before being resuspended and pooled in 10 ml of media. Media was removed by centrifugation at 400 x g for 5 minutes (Eppendorf Centrifuge 5804R, A-4-44 rotor). Cells were washed by resuspending in 10 ml of PBS, before being centrifuged again at 400 x g for 5 minutes (Eppendorf Centrifuge 5804R, A-4-44 rotor). Cells were lysed in 750 μl of RIPA buffer containing 1x cOmplete™ protease inhibitor cocktail (Roche) and 1 mM PMSF. Cell lysis was achieved by repeatedly forcing lysate through a 19-gauge syringe needle containing two 90° bends. The lysate was clarified by centrifugation at 20,000 x g for 30 minutes at 4 °C (Eppendorf Centrifuge 5417R, FA45-30-11 rotor). The clarified cell lysate was incubated with 200 μl of streptavidin magnetic beads (New England Biolabs) equilibrated in RIPA buffer for 1.5 hours at 4 °C. After binding, magnetic beads were washed by collecting beads in a magnetic concentrator, removing the buffer and resuspending in PBS. The magnetic beads were washed a total of six times, with 2 ml of PBS at each wash step. The buffer was removed from beads that were then frozen for storage.

Radioimmunoprecipitation (RIPA) Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>150 mM</td>
</tr>
<tr>
<td>Nonidet P-40</td>
<td>1 % v/v</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>0.5 % w/v</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1 % w/v</td>
</tr>
</tbody>
</table>
Streptavidin magnetic beads were added to 45 µl of 50 mM ammonium bicarbonate pH 8.5. The resulting mixture was incubated at 70°C with 10 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and 40 mM chloroacetamide (CAA) for 5 minutes. Protein digestion was achieved using 1 µg of trypsin per 100 µg protein, at 37 °C overnight. The pH of the mixture was brought down to pH 3-4 using 1 % trifluoroacetic acid (TFA). Salt and magnetic beads were removed from the sample using 0.22 µm Costar Spin-X centrifuge tube filter (Sigma CLS8169).

Mass spectrometry was carried out by Cleidi Zampronio of the Proteomics Research Technology Platform at the University of Warwick. Peptides were separated using reverse-phase HPLC with a 50 cm C18 column on an UltiMate 3000 RSLCnano System (Thermo Scientific). A gradient of acetonitrile was used as the mobile phase. HPLC was carried out for 90 minutes.

Protein identification
Data collected from the instrument was used to search a curated proteome of Gallus gallus(Uniprot: UP000000539), the MaxQuant database of common contaminants and the sequences of the bait proteins. Data were processed and visualised using Scaffold software (Proteome Software). Peptide identifications were considered valid if they could be established with a greater than 95% probability when analysed with the Scaffold Local False Discovery Rate algorithm. Protein identifications were considered valid if they could be established with greater than 99% probability and contained at least two valid peptide identifications. Protein probabilities were assigned using the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be distinguished on mass spectrometer data alone were grouped to satisfy parsimony principles.
2.2.25 Ni-NTA purification

Cell harvest and lysis

BL21 Rosetta E. Coli was grown in LB broth supplemented with 10 mM MgCl2, 25 µg/ml chloramphenicol and 100 µg/ml ampicillin. Cells were grown to an OD of 0.6 before being induced to produce the protein of interest by the addition of 1 mM IPTG. Cells were harvested by centrifugation at 10,000 x g for 15 minutes. Culture media was removed, and cells were resuspended in Ni-NTA Lysis buffer. Cells were sonicated on ice for 1 minute, three times at 50% amplitude, with a profile of 10 seconds on and 10 seconds off. Cell lysates were clarified by centrifugation at 48,000 x g for 40 minutes at 4°C.

Ni-NTA Lysis buffer

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM</td>
<td>Tris-HCl pH 7.4</td>
</tr>
<tr>
<td>300 mM</td>
<td>NaCl</td>
</tr>
<tr>
<td>10 mM</td>
<td>Imidazole</td>
</tr>
<tr>
<td>10 % v/v</td>
<td>Glycerol</td>
</tr>
<tr>
<td>25 mM</td>
<td>Sucrose</td>
</tr>
<tr>
<td>0.1 % v/v</td>
<td>Triton X-100</td>
</tr>
<tr>
<td>1 mM</td>
<td>DTT</td>
</tr>
<tr>
<td>1 mM</td>
<td>PMSF</td>
</tr>
<tr>
<td>1x</td>
<td>cOmplete™ protease inhibitor cocktail (Roche)</td>
</tr>
</tbody>
</table>

Ni-NTA binding

Before the binding, Ni-NTA agarose beads were equilibrated by placing 1 ml of bead slurry into a disposable 5 ml polypropylene gravity flow column and flowing through 5 ml of Ni-NTA Lysis buffer. 5 mM ATP, 20 mM MgCl2 and 100 mM KCl were added to the lysate. Equilibrated beads were then resuspended in the lysate and incubated in an end-over-end rotator 4°C for 1 hour.

Wash and elution

After binding, beads were loaded onto the column. The column was washed with Ni-NTA wash buffer until A280 absorbance reached baseline. An additional wash step was carried out with wash buffer supplemented with
mM ATP, 20 mM MgCl2 and 100 mM KCl. The column was washed until the A280 absorbance reached baseline. After washing the protein was eluted with Ni-NTA elution buffer with fraction volumes equating to half the column volume.

**Ni-NTA Wash buffer**

- 50 mM Tris-HCl pH 7.4
- 300 mM NaCl
- 50 mM Imidazole
- 10 % v/v Glycerol
- 25 mM Sucrose
- 1 mM DTT

**Ni-NTA Elution buffer**

- 50 mM Tris-HCl pH 7.4
- 300 mM NaCl
- 250 mM Imidazole
- 10 % v/v Glycerol
- 25 mM Sucrose
- 1 mM DTT

### 2.2.26 Size exclusion chromatography

Size exclusion chromatography was carried out using a Superdex 200 HiLoad 16/60 FPLC column (GE Healthcare). Before size exclusion chromatography, the column was equilibrated with two column volumes of Size exclusion buffer at a flow rate of 0.5 ml/min. 2 ml of protein was injected and fractions of 500 µl were collected. Glycerol to a final concentration of 10% (v/v) was added to fractions containing US3-GFP, which were subsequently aliquoted and snap-frozen for long-term storage in liquid nitrogen.

**Size exclusion buffer**

- 50 mM Tris-HCl pH 7.4
- 300 mM NaCl
<table>
<thead>
<tr>
<th>Concentration</th>
<th>Substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 % v/v</td>
<td>Glycerol</td>
</tr>
<tr>
<td>25 mM</td>
<td>Sucrose</td>
</tr>
<tr>
<td>1 mM</td>
<td>DTT</td>
</tr>
</tbody>
</table>
2.2.27  Protein concentration by UV absorbance

Protein concentration was measured on a Nanodrop 2000 (ThermoFisher). The concentration of protein within a sample was determined by the Beer-Lambert equation:

\[ c = \frac{A}{(\varepsilon \times l)} \]

- \( c \)  Nucleic acid concentration (M)
- \( A \)  Absorbance (A.U.)
- \( \varepsilon \)  Molar attenuation coefficient (M\(^{-1}\) cm\(^{-1}\))
- \( l \)  path length (cm)

The following molar attenuation coefficients were used, which represents the weighted sum of the coefficients of tryptophan (W), tyrosine (Y) and cysteine (C) that absorb light at the 280 nm wavelength:

\[ \varepsilon = (nW \times 5500) + (nY \times 1490) + (nC \times 125) \]

- \( n \)  number of residues in primary sequence

The absorbance of light with a wavelength of 280 nm and path lengths of 1.0 mm to 0.05 mm.

2.2.28  Quantification of GFP fluorescence

GFP fluorescence (in RFU) was quantified using Nanodrop 3300 (ThermoFisher). The sample was excited with 470 nm light, and the emission spectra were measured from 500 nm to 750 nm. The peak at 509 nm was used to determine GFP fluorescence.
2.2.29 Stress fibre removal assay

Cells were seeded onto 16 mm coverslips and transfected using FuGENE (as described in Section 2.2.19) with kinase-active or kinase-dead MDV US3 24 hours later. Cells were fixed using paraformaldehyde and stained for F-Actin, DNA and if required, FLAG-tagged protein 24h after transfection as described in Section 2.2.21.

Fixed cells were imaged on a Deltavision Elite (GE Healthcare) Wide-field microscope. Images were captured using a CoolSNAP HQ interline CCD camera (Photometrics) under the control of the SoftWorks (GE Healthcare). Z-stacks of individual cells were acquired using a 40x objective (NA 1.30) for A7r5 cells and a 100x objective (NA 1.40) for RPE-1, DF1 and CEF cells, with a Z-spacing of 0.2μm. Samples were illuminated with 405 nm, 488 nm, 561 nm, and if necessary, 640 nm light.

Using FIJI, Z-stacks were projected based on their maximum intensity, and the presence of actin stress fibres was assessed. Cells were classified to have stress fibres removed if there was a complete absence, or at least a 90 % reduction compared to untransfected controls.

2.2.30 Microscale thermophoresis

Microscale thermophoresis (MST) was carried out on a Monolith NT.115 device (Nanotemper Technologies). Capillaries were filled with 10 µl of the protein mix, and MST was performed using 100% excitation with Nano Blue and medium MST laser power. Traces were analysed, and the Kd model was fitted using the MO.Affinity Analysis software (Nanotemper Technologies).
2.2.31  Actin bundling assay

Preparation of alpha-actinin

Lyophilised pan-alpha-actinin from chicken gizzard (2B Scientific) was resuspended in molecular biology grade water before being aliquoted and snap-frozen in liquid nitrogen. Alpha-actinin was resuspended to a concentration 1 mg/ml in a buffer containing 10 mM Tris-acetate pH 7.6, 0.1 mM EDTA, 2 mM DTT and 20 mM NaCl.

Preparation of F-actin

F-actin was prepared by resuspending a single 5 µl aliquot to a final concentration of 1 mg/ml with general actin buffer. The resuspended actin was left on ice for 30 minutes before 1/10th total volume of actin polymerisation buffer (500 mM KCl, 20 mM MgCl₂, 10 mM ATP) was added and left to incubate at room temperature for 1 hour.

Actin bundling assay

The total reaction for each tube in the assay was 30 μl. Separate tubes contained F-actin with buffer, F-actin with alpha-actinin, F-actin with BSA, F-actin and US3-GFP, F-actin with US3-GFP and alpha-actinin. Tubes were centrifuged at 14,000 x g for 1 hour at room temperature (Eppendorf miniSpin plus, F-45-12-11 rotor). The supernatant was carefully separated from the pellet, and each fraction was visualised using SDS-PAGE.

Alpha-actinin storage buffer

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM</td>
<td>Tris-acetate pH 7.6</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>EDTA</td>
</tr>
<tr>
<td>2 mM</td>
<td>DTT</td>
</tr>
<tr>
<td>20 mM</td>
<td>NaCl</td>
</tr>
</tbody>
</table>

General actin buffer

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM</td>
<td>Tris-HCl pH 8.0</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>CaCl₂</td>
</tr>
</tbody>
</table>
**Actin polymerisation buffer**

50 mM KCl  
2 mM MgCl\(_2\)  
1 mM ATP

### 2.2.32 Polymerisation of microtubules

Microtubules were assembled from 8 µl of 3.4 mg/ml unlabelled pig brain tubulin (kindly provided by Dr Manas Chakraborty), 0.5 µl biotin-tubulin (Cytoskeleton Inc. #T333P). The mixture was incubated on ice for 5 minutes before an equal amount of polymerisation buffer (2x BRB80 buffer supplemented with 20% v/v DMSO and 2 mM MgGTP) was added. Microtubules were assembled by incubating at 37 °C for 1 hour. The sample was diluted in 100 µl of MRB80 containing 50 µM taxol before use.

**Microtubule seeds**

Seeds for experiments with dynamic microtubules were assembled from pig brain tubulin, biotin-tubulin (Cytoskeleton Inc. #T333P) and HiLyte647-tubulin (Cytoskeleton Inc. #TL670M) (at a molar ratio of 25:1:2 in MRB80 supplemented with 1mM Guanosine-5′-[(α, β)-methylene] triphosphate (GMP-CPP). The mixture was incubated on ice for 30 minutes before seeds were assembled by incubating at 37 °C for 30 minutes. After assembly, seeds were diluted 1:20 with MRB80 containing 20 µM taxol and stored at room temperature.

**MRB80**

80 mM K-PIPES pH 6.8  
1 mM EGTA  
4 mM MgCl\(_2\)

**2x BRB80**

180 mM K-PIPES pH 6.8  
2 mM EGTA  
2 mM MgCl\(_2\)
2.2.33 Chamber preparation

0.17 mm thick 22x22mm coverslips (Scientific Laboratory Supplies) were acid-washed and plasma-cleaned before use. Coverslips were attached to slides (Menzel Gläser Superfrost Plus, Thermo Scientific) using double-sided tape (Scotch 3M 137D-GB). The resulting flow chamber was coated with 0.2 mg/ml PLL(20)-g[3.5]-PEG(2)/PEG(3.4)-Biotin (50%) (SuSoS AG) for 5 minutes. Microtubules or microtubule seeds were attached to this surface with 0.625 mg/ml streptavidin (Sigma S4762). Finally, the surface was blocked with 1 mg/ml κ-casein. Between each layer, the chamber was washed 3 times with MRB80 (for experiments involving EBs) or TAB (for experiments involving motor proteins). US3, and test proteins were incubated in the appropriate buffer for one hour before being added into the imaging chamber. Once the sample was added to the chamber, the chamber was sealed with paraffin wax.

### TIRF Assay buffer (TAB)

- 25 mM HEPES pH 7.2
- 25 mM KCl
- 5 mM MgSO₄
- 1 mM EGTA

2.2.34 Single molecule motility assay

The single-molecule motility assay was carried out on an Olympus TIRF system using a 100x NA 1.49 objective. The sample was placed in an environmental chamber and a stage-top-incubator (Okolab, Ottaviano, Italy), and illuminated with 488 nm, 561 nm, and 640 nm laser lines. Images were captured using an ImageEM emCCD camera (Hamamatsu Photonics) controlled by the Xcellence software (Olympus). Unless otherwise stated, images were acquired every 600 ms for 150 s at an exposure of 50 ms (561 nm and 640 nm) or 150 ms (488 nm). For analysis, kymographs were drawn and analysed using FIJI.
2.2.35  **Statistical analysis and figure preparation**

The significance of results, unless otherwise stated, was tested using Student’s T-test using Graphpad Prism. Results were considered significant when $p<0.05$. Graphs were made using Graphpad Prism. Figures were assembled in Adobe Illustrator. Microscope images were processed using FIJI. Immunofluorescence images were taken as Z-stacks, which were then flattened using a maximum intensity projection. When colocalisation is examined, different channels were displayed together as a false colour merge, otherwise images are displayed with an inverted lookup table. For motility assays, kymographs were drawn using the Multi Kymograph Tool with a line width of 1 and displayed using an inverted lookup table.
3 Kinase-independent remodelling of the actin cytoskeleton by MDV US3

3.1 Introduction

MDV US3 belongs to a class of enzymes known as serine/threonine protein kinases within the transferase family. These enzymes use the γ-phosphate of ATP or GTP to generate phosphate monoesters, with alcohol groups on serine and threonine side chains as phosphate acceptors (Edelman et al., 1987; Hanks & Hunter, 1995). Protein phosphorylation is known as a prominent means of signal transduction, where an intricate network of phosphorylation and dephosphorylation reactions controls effectors of cellular functions. Virus-encoded serine/threonine kinases are unique to large DNA viruses such as herpesviruses, poxviruses and baculoviruses (Jacob et al., 2011). All herpesviruses encode a conserved herpesvirus protein kinase (CHPK) that was initially discovered in VZV (Chee et al., 1989; De Wind et al., 1992). Only the alphaherpesvirus subfamily encodes a second kinase, US3 (Jacob et al., 2011).

Virally encoded kinases show minimal homology to known cellular kinases, apart from the conserved core catalytic domain. Within the catalytic domain are 12 smaller subdomains (I to XII). Subdomains I to IV represent the N-terminal lobe of the kinase domain that is involved in orientating the nucleotide, whereas subdomains VI to XI bind to the peptide substrate and initiate phosphotransfer. Domain V, located in the cleft and spanning the two lobes, is the site of catalysis (Hanks & Hunter, 1995). Within the catalytic site is a lysine critical for ATP binding and a conserved catalytic aspartate which deprotonates the acceptor serine or threonine (Figure 3.1) (Hanks & Hunter, 1995).

The amino acid residues involved in catalysis have been targeted by mutagenesis to generate kinase-dead US3 for expression within cells, but also alphaherpesvirus mutants for further characterisation of function. Using this approach, PRV, HSV-2, BHV-1 have been shown to require the kinase activity
of US3 to affect actin organisation (Brzozowska et al., 2010; Finnen et al., 2010; Murata et al., 2000; Van den Broeke et al., 2009a). In contrast, MDV US3 K220A has been observed to remove actin stress fibres through an unknown kinase-independent mechanism (Schumacher et al., 2008). To ensure that this observation was not a result of any residual kinase activity, the catalytic aspartate will be substituted, and the actin cytoskeleton will be assessed using immunofluorescence microscopy.

Figure 3.1 Conserved catalytic active site of the alphaherpesvirus US3. Clustal multiple alignment of selected alphaherpesvirus US3 kinases. Similarity is indicated by the following symbols * = positions that have fully conserved residues, : = strong conservation (PAM 250 matrix score > 0.5), . = weak conservation (PAM 250 matrix score < 0.5). Conserved catalytic aspartic acid and lysine responsible for the positioning of the ATP γ-phosphate are highlighted.
3.2 Results

In order to remove any residual kinase activity of US3, the substitutions D218A and K220A were introduced using mutagenic PCR. The presence of mutations was verified using DNA sequencing (Figure 3.2). Sequencing shows that both aspartic acid and lysine have been substituted with alanine residues. The mutated MDV US3 was cloned into a mammalian expression vector resulting in a C-terminal fusion with a 4xFLAG tag.

To test whether kinase-dead MDV US3 was capable of remodelling the actin cytoskeleton, A7r5 rat aortic smooth muscle cells (Figure 3.3) and RPE-1 human retinal pigment epithelium cells (Figure 3.4) were transfected with wild-type and kinase-dead MDV US3. Cells were fixed and stress fibres were assessed after 24 hours in line with previous experiments by Schumacher et al. (2008). Both wild-type and kinase-dead MDV US3 were found to remove actin stress fibres to a similar extent. For A7r5 cells, from three independent experiments, 40 ± 14% (n = 14) of cells transfected with wild-type and 55 ± 6% (n = 26) cells transfected with kinase-dead MDV US3 showed a reduction or removal of stress fibres.

In RPE-1 cells, from three independent experiments, 43 ± 12% (n = 30) of cells transfected with wild-type and 36 ± 7% (n = 23) of cells transfected with kinase-dead MDV US3 showed a reduction or removal of stress fibres. In 45 ± 5% (n = 33) of RPE-1 cells transfected with wild-type MDV US3, the formation of thin, branched cellular protrusions was observed. This did not occur with the kinase-dead construct. Protrusions had an average length of 97 ± 45 µm (n = 33). The presence of cellular protrusions did not correlate to whether stress fibres were present in the cell.
Figure 3.2 Amino acid substitutions introduced into MDV US3. A: Sequencing chromatogram showing the catalytic site of MDV US3. An asterisk indicates a mutation. B: DNA alignment and translation of US3 WT and US3 D218A K220A. Mutations change the critical aspartic acid and lysine to alanine residues, as indicated by the asterisk and boxes.
Figure 3.3 Kinase dead MDV US3 removes actin stress fibres in A7r5 cells. A: Representative images of A7r5 cells were transfected with either wild-type or kinase-dead MDV US3 and then fixed 24 hours later. US3 was visualised with a mouse anti-FLAG antibody while F-actin was visualised using fluorescent phalloidin. Scale bar represents 10 µm. B: Quantification of cells with no or reduced stress fibres. Data is from three independent experiments.
Figure 3.4 Kinase dead MDV US3 removes actin stress fibres in RPE-1 cells. A: Representative images of RPE-1 cells were transfected with either wild-type or kinase-dead MDV US3 and then fixed 24 hours later. US3 was visualised with a mouse anti-FLAG antibody while F-actin was visualised using fluorescent phalloidin. Arrow indicates a thin cellular protrusion characteristic of cells transfected with wild-type, but not kinase-dead MDV US3. Scale bar represents 10 µm. B: Quantification of cells with no or reduced stress fibres. Data is from three independent experiments.
3.3 Discussion

In line with previous reports, significant remodelling of the actin cytoskeleton can be observed when wild-type MDV US3 is overexpressed in cells. Here we used smooth muscle cells that are usually completely filled with actin stress fibres, highlighting the efficient removal of these upon US3 expression.

By introducing the substitutions D218A and K220A, the kinase activity of MDV US3 was removed. Transfection of rat A7r5 and human RPE-1 cells shows that unlike other alphaherpesvirus US3 orthologues, kinase activity is not required for the removal of stress fibres. Furthermore, the removal of actin stress fibres is conserved cross-species, which is expected given that the actin cytoskeleton and its regulatory mechanisms are highly conserved amongst higher eukaryotes. Cells transfected with kinase-dead MDV US3 do not form long cellular protrusions, actin punctae or show an increase in membrane ruffling. This is expected, as without catalytic activity, kinase-dead MDV US3 is unable to directly activate Cdc42/Rac1 signalling pathways. However, one cannot exclude that by altering actin dynamics, feedback mechanisms would eventually activate such pathways. Indeed, it is likely that such an event occurs, given that stress fibre reassembled within approximately 24 hours for both wild-type and kinase-dead MDV US3 (Schumacher et al., 2008).

For wild-type MDV US3, the formation of long cellular protrusions and removal of actin stress fibres is consistent with interference with Rho GTPase signalling, through the Cdc42/Rac1 signalling pathways (Murata et al., 2000). Similarly, the loss of stress fibres and formation of cellular protrusions is consistent with the activation of Group A P21-activated kinases (PAKs), which are downstream effectors of Cdc42/Rac1. Indeed, the alphaherpesvirus US3, at least for PRV, has been shown to directly activate PAK1/2 (Van den Broeke et al., 2009b).
The observation that kinase-dead US3 does not generate cellular protrusions would suggest that the kinase-independent mechanism of actin remodelling is not through the interference with Rho or Rac regulatory elements. Of the small GTPases that act as central regulators of the actin cytoskeleton, Rho plays a major role in the assembly of actin stress fibres through Rho-associated protein kinase (ROCK) (Ridley & Hall, 1992; Narumiya et al., 2009). Rho facilitates the formation of long parallel actin filaments by activating mDia1 (Otomo et al., 2005; Higashida et al., 2004). ROCK inhibits cofilin disassembly of actin filaments by activating LIM domain kinase 1 (LIMK1) (Ohashi et al., 2000; Yang et al., 1998). If kinase-dead MDV US3 inhibited the function of Rho and ROCK, the formation of stress fibres would be inhibited, and existing stress fibres will be disassembled by cofilin. However, the inhibition of Rho would lead to the release of Rac inhibition and promote the formation of cellular protrusions (Edelstein-Keshet, 2016).

The formation of actin stress fibres relies on several actin filament nucleators and crosslinking proteins, of which inhibition will result in the loss or reduction of stress fibres. Arp2/3 and mDia1/2 nucleated actin filaments are involved in the formation of stress fibres (Hotulainen & Lappalainen, 2006; Higashida et al., 2004). However, depletion of mDia1/2 reduces the number, but does not result in the complete loss of stress fibres (Hotulainen & Lappalainen, 2006). The actin filaments within stress fibres are crosslinked by alpha-actinin and in the case of contractile stress fibres, non-muscle myosin II (Lazarides & Burridge, 1975; Mitchison & Cramer, 1996). It is possible that MDV US3 can interact with and inhibit any of these stress fibres components. Therefore, in order to understand the kinase-independent mechanism of actin remodelling, the potential interactome of MDV US3 will be determined (Chapter 4).
4 Proximity dependent biotin identification of candidate MDV US3 host interactors

4.1 Introduction

The classic affinity-based approach to screening novel protein-protein interactions is well established, but not without limitations. For any affinity purification to be successful, the protein-protein complex must be maintained through the use of mild lysis and purification conditions. It is because of this that insoluble proteins, such as membrane proteins, are purified and identified with great difficulty. In addition to this, proteins that have weakly bound or create transient complexes are easily lost during purification (Smits & Vermeulen, 2016; Berggård et al., 2007). These issues can be resolved by using an approach that does not require direct interaction, but instead labels proteins that are in proximity to the bait protein such as ascorbate peroxidase for proximity-ligation (APEX) and proximity-dependent biotinylation (BioID) (Rhee et al., 2013; Roux et al., 2013).

Here, we investigate the interaction between the MDV US3 serine-threonine kinase and host cellular proteins using proximity-dependent biotinylation. This method from Kim et al. (2016), BioID2, is the second iteration of the original BioID system developed by Roux et al. (2013). The BioID approach is summarised in Figure 4.1. The BioID system uses a mutated BirA biotin ligase from *E. coli* that is fused to the bait protein. This R118G mutation causes BirA to release activated biotin prematurely into its immediate vicinity, subsequently biotinylating primary amines of any protein which is within a ~10 nm radius of the bait protein. Biotinylated proteins are purified using streptavidin affinity chromatography for downstream analysis and identification.

The BirA R118G promiscuous biotin ligase from *E. coli* is slightly larger than green fluorescent protein at 321 amino acids long. The size of BirA can, on occasions, lead to the improper sub-cellular localisation of the bait protein.
To address this issue, BioID2 uses a significantly smaller biotin ligase from *Aquifex aeolicus* (233 amino acids). Compared to its predecessor, BioID2 has been shown to improve the subcellular location of fusion proteins and requires less biotin (Kim *et al.*, 2016).

Compared to other methods such as yeast two-hybrid, BioID does not suffer from problems that involve and are caused by post-translational modification or incorrect folding, as the promiscuous biotin ligase is fused to a bait protein that is subsequently expressed in cellular conditions. However, these cellular conditions and the correct protein structure are not required for the streptavidin-based enrichment and purification of interactors, allowing for purification in harsh denaturing conditions if required. Together, these aspects of BioID allow for enhanced detection of novel interactions that may be weak and transient as well as being insoluble.

**Figure 4.1 Summary of the BioID approach.** In the method developed by Roux *et al.* (2013), a bait protein is created by fusing a promiscuous biotin ligase to the protein of interest and is expressed in cells. When free biotin is added to the cell culture media, proteins in close proximity to the bait are biotinylated. Biotinylated proteins can be captured using streptavidin-affinity purification and can be subsequently identified using mass spectrometry.
The determination of substrates for the alphaherpesvirus US3 kinase has been typically achieved by analysing the amino acid sequence of proteins and searching for consensus phosphorylation sites (Leader et al., 1991; Kato et al., 2005). However, in the case of lamin A/C which HSV-1 US3 phosphorylates in vitro, the phosphorylation of the substrate does not correspond to the defined consensus sequence (Mou et al., 2007). Therefore, the alphaherpesvirus US3 may interact with and phosphorylate a much larger pool of proteins than initially thought.

This consensus sequence approach, while identifying substrates for phosphorylation, does not necessarily identify non-catalytic interactions. Because of this limitation, the BioID approach was chosen for its capability to identify possible interaction partners regardless of their affinity and regardless if the interaction is catalytic or not. By generating an MDV US3-BioID2 fusion, the potential interactome of MDV US3 can be identified. To generate the largest pool of candidate interactors, wild-type US3 was used. By identifying candidate interactors that are involved in the regulation and formation of the actin cytoskeleton, possible mechanisms for the kinase-independent remodelling of the actin cytoskeleton will be explored further.

4.2 Results

Bait proteins maintain transcriptional and biotinylation activity

Bait proteins were generated by fusing HA-tagged BioID2 to the C-terminus of US3 serine-threonine kinase of MDV strain RB1B (Figure 4.2A). A flexible linker region (amino acid position 402 to 472) was introduced to maximise the potential area surrounding the bait protein where biotinylation can occur.

mCherry-BioID2-HA was used as the negative control. To confirm those fusion proteins were expressed correctly, DF1 cells were transiently transfected with bait proteins and analysed using immunofluorescence and western blotting. The presence of HA-tagged protein of the expected size in
cell lysate confirms that the bait proteins are indeed expressed in DF1 cells (Figure 4.2B).

To assess whether the activity of the promiscuous biotin ligase fused to the bait proteins maintained activity, DF1 cells transfected with US3-BioID2-HA and mCherry-BioID2-HA were grown overnight in culture media containing 50 μM of free biotin, and analysed using western blotting. Biotinylated proteins were detected using streptavidin-HRP. The analysis shows an abundance of biotinylated proteins in cell lysate, indicating that BioID2 constructs did maintain their ability to biotinylate proteins in the proximity of the fusion protein. However, it should be noted that LC-MS/MS analysis can identify proteins that would not be detectable visually, and the abundance of a specific biotinylated protein on the western blot does not necessarily correspond to its biological significance.
Figure 4.2 BioID2 constructs are expressed and retain biotinylation activity. A: Bait proteins were constructed by fusing mCherry or MDV US3 with a HA-tagged promiscuous biotin ligase (BioID2). B: Western blots probing for HA-tagged fusion proteins (US3-BioID2-HA 77 kDa; mCherry-BioID2-HA 58 kDa, indicated by arrows) and biotinylated proteins in cell lysate. Bait proteins were detected using anti-HA antibodies, while biotinylated proteins were detected using streptavidin-HRP.
Figure 4.3 Localisation of MDV US3-BioID2 and biotinylated proteins in DF1 fibroblasts. DF1 cells were transfected with MDV US3-BioID2 using PEI. After fixation, MDV US3 was visualised using anti-HA antibodies. Biotinylated proteins were visualised using streptavidin-FITC. F-actin was visualised using fluorescent phalloidin. Scale bar represents 10 µm.
To further confirm the activity of the promiscuous biotin ligase and to assess the targeting of bait proteins and the location of biotinylated interactors, cells were fixed and visualised by immunofluorescence microscopy (Figure 4.3). DF1 and A7r5 cells were transiently transfected with US3-BioID2-HA and grown overnight in culture media containing 50 μM of free biotin. Cells were probed for HA-tagged protein biotinylated proteins. The F-actin cytoskeleton was visualised with fluorescently labelled phalloidin. Fluorescence microscopy shows that US3 is primarily cytoplasmic, localising with F-actin structure and membrane ruffles. As expected, biotinylated proteins share a similar localisation to the US3 bait.

**BioID2 screen highlights candidate interactors for MDV US3**

To identify candidate interaction partners, samples were generated for LC-MS/MS analysis from cells transiently transfected with either MDV_US3-BioID2-HA or mCherry-BioID2-HA. Three 15 cm dishes were used for each condition. Prior to LC-MS/MS, the presence of biotinylated proteins was confirmed by western blotting. To the data generated by LC-MS/MS, a peptide identity threshold of 95 % was applied to proteins where at least two peptides were identified. A total of 1439 proteins were identified. Proteins that had less than log2-fold enrichment compared to the mCherry-BioID2-HA control or were not statistically significant as determined by the Fisher’s-exact test from triplicate results were excluded (Figure 4.4 and Figure 4.5).
Figure 4.4 BioID2 screen of MDV US3 reveals candidate interactors
Volcano plot shows the significance and relative log fold change of interactors compared to proteins identified in the mCherry control sample. The horizontal and vertical dashed lines represent cut-off p-value of 0.05 and cut-off enrichment of 2 fold. P-values were calculated using Fisher’s exact test from three independent experiments. For proteins that are within these cut-offs, the gene symbols are displayed.
Figure 4.5 Proteins identified from BioID2 screen of MDV US3 which are absent in control. Volcano plot shows the significance and average normalised total spectra of interactors that were absent in the mCherry control sample. The horizontal dashed lines represent cut-off p-value of 0.05. P-values were calculated using Fisher's exact test from three independent experiments. For proteins that are within this cut-off, the gene symbols are displayed.
Table 4.1 Proteins identified by BioID2 screen classified by function. Proteins were classified based on cellular function after applying a 95% peptide identity threshold. Proteins that are determined not significant (P>0.05) using Fisher’s exact test from triplicate results or not at least 2-fold enriched compared to the mCherry control are excluded.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
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<tbody>
<tr>
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<td>Importin-7</td>
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<td>Transportin-1</td>
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<td>Sideroflexin-3</td>
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<td>TSPO</td>
<td>Translocator protein</td>
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<td>Alpha-actinin-4</td>
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<td>Cyclin-dependant kinase 2</td>
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<td>Mitogen-activated protein kinase 2</td>
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<tr>
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<td>RANBP2-like and GRIP domain-containing protein 5/6</td>
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<td>USP9X</td>
<td>Probable ubiquitin carboxyl-terminal hydrolase FAF-X</td>
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<tr>
<td>A0A155PII9</td>
<td>Uncharacterised chicken protein</td>
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Examining alpha-actinin as a candidate interaction partner of MDV US3

Of particular interest was the actin-binding protein alpha-actinin-4, due to its presence in actin stress fibres. To validate this result from the BioID2 screen, the presence of alpha-actinin was detected in BioID2 samples after streptavidin affinity purification using western blot (Figure 4.6). Western blots were normalised by the total biotinylated protein present in the sample. Alpha-actinin was found to be present in the US3-BioID2-HA sample but not in the mCherry-BioID2-HA control. With the antibody that was used, the antigenic epitope is found conserved in all isotypes. It was not possible to determine which particular member of the alpha-actinin family of proteins was present.

Figure 4.6 Alpha-actinin is enriched in US3 BioID2 sample but not mCherry control after streptavidin affinity purification. Western blot of US3-BioID2 and mCherry-BioID2 samples showing the presence of alpha-actinin (approx. 100 kDa). The same membrane is then stripped and probed for total biotinylated protein with a streptavidin-HRP conjugate.
Truncated MDV US3 BioID2 constructs and their biotinylated interaction candidates have different localisations

To further clarify the possible functions of different regions within MDV US3, BioID2 constructs containing various combinations of the kinase domain and the flanking N- or C-terminus (Figure 4.7) were produced. These constructs were transiently transfected into A7r5 cells, and grown overnight in culture media containing 50 μM of free biotin before fixation. The presence of US3 and biotinylated proteins, the F-actin cytoskeleton, and the nucleus were visualised using immunofluorescence microscopy (Figure 4.8 and Figure 4.9).

**Figure 4.7 BioID2 MDV US3 truncations.** Truncations of MDV US3 were produced and fused to the HA-tagged BioID2 promiscuous biotin ligase. Truncations were formed of individual regions, or in combination with the protein kinase domain (PκC), the N-terminus (1-111) or C-terminus (322-402).
Full-length US3 is seen to be primarily cytoplasmic with weak localisation to actin stress fibres, actin hotspots and membrane ruffles. A similar localisation can be seen with the biotinylated proteins. The N-terminus of US3 (1-111) retains a similar localisation to the full-length protein. US3 1-111 is seen to be localised towards stress fibres and F-actin structures within membrane ruffles. Similar to the full-length protein, the localisation between US3 1-111 is shared, except for the nucleus, where US3 1-111 is excluded, but biotinylated protein is enriched. The C-terminus of US3 (322-402) and its biotinylated interactors do not show any specific localisation and are cytoplasmic.

When the N-terminus and C-terminus are removed, the protein kinase region of US3 (111-322) is localised to the mitochondria of the cell as indicated by the mitochondrial marker TOM20 (Figure 4.10). The addition of the N-terminus (US3 1-322) reduces the strength of this localisation; however, the addition of the C-terminus region (US3 111-402) does not.
Figure 4.8 Full-length and truncated of MDV US3 and their biotinylated interactors show different localisations. A7r5 cells were transfected with different MDV US3 truncations fused to a promiscuous biotin ligase. 24 hours post-transfection, cells were allowed to grow in biotin enriched media overnight. After fixation, cells were stained for HA-tagged protein, biotinylated protein and F-actin. Scale bar represents 10 µm.
Figure 4.9 Truncated versions of MDV US3 and their biotinylated interactors show different localisations. A7r5 cells were transfected with different MDV US3 truncations fused to a promiscuous biotin ligase. 24 hours post-transfection, cells were allowed to grow in biotin enriched media overnight. After fixation, cells were stained for HA-tagged protein, biotinylated protein and F-actin. Scale bar represents 10 µm.
Figure 4.10 US3 111-322 shows mitochondrial localisation. A7r5 cells were transfected with MDV US3 1-111 fused to a promiscuous biotin ligase. 24 hours post-transfection, cells were fixed and stained for HA-tagged US3 (blue), mitochondrial marker TOM20 (red) and F-actin (green). Scale bar represents 10 µm.
4.3 Discussion

BioID2 screen identifies proteins involved in the actin cytoskeleton

The actin cross-linking protein Alpha-actinin 4 (ACTN4) has been identified in the mass spectrometry analysis for candidate interaction partners of MDV US3. Alpha-actinin is a ~100 kDa protein that cross-links F-actin filaments. Cytoplasmic isoforms (ACTN1 and ACTN4) are found on actin stress fibres, the actomyosin cortex and podosomes (Mukhina et al., 2007; Stickel & Wang, 1987; Cervero et al., 2012). The remaining isoforms, which were not found in the BioID2 screen (ACTN2 and ACTN3) are expressed in muscle cells and form the sarcomere (Beggs et al., 1992). It is interesting to note that ACTN1 and ACTN4 have different localisations within the cell. Work by Quick (2010) showed that ACTN1 was located at all types of stress fibres, whilst ACTN4 was located at the cell cortex, the perinuclear space and distal edge of stress fibres.

The BioID2 screen has also identified the F-actin binding protein Neurabin-2-like (NUR2L). The human orthologue of NUR2L, Neurabin-II (NUR2) is characterised as being highly expressed in neuronal cells and regulates dendritic spine morphology (Nakanishi et al., 1997).

BioID2 screen identifies nuclear transport proteins

As previously discussed, the alphaherpesvirus US3 kinase is multifunctional, having roles within and outside of the nucleus. The molecular weight of MDV US3 alone is 44 kDa, and the MDV US3-BioID2 fusion is 77 kDa. These sizes are larger than what is considered permissible for diffusion into the nucleus (40 kDa) (Freitas & Cunha, 2009). Because of this, it is likely that active nuclear import and export is required. It is therefore within expectations that nuclear transport proteins would be identified in the BioID2 screen for potential interactors. Four nuclear transport proteins have been identified: Importin 7, Transportin 1, Exportin 7 and Exportin T. These proteins are responsible for mediating, via the hydrolysis of RanGTP, the movement of macromolecules through the nuclear pore complex (Jäkel & Gürlich, 1998).
Studies on HSV-2 have highlighted nuclear-shuttling properties of the alphaherpesvirus US3. Application of the Exportin-1 (CRM1) specific inhibitor leptomycin B to transfected cells stops the nuclear export and results in the nuclear accumulation of HSV-2 US3 (Finnen et al., 2011) The export of proteins by Exportin-1 typically involves leucine-rich nuclear export signals (Wen et al., 1995). Indeed, HSV-1 US3 does contain two predicted leucine-rich nuclear export signals in the region of amino acids 242-259. Further analysis shows that this does not appear to be conserved in MDV, PRV or VZV (Finnen et al., 2011). It remains yet to be known whether the nuclear export of MDV US3 is inhibited by leptomycin B. Exportin-7 (RanBP16), compared to Exportin-1, is considered to have the second most diverse range of cargos. However, unlike Exportin-1, export proteins which share a common leucine-rich motif, cargos of Exportin-7 do not appear to have any functional or structural similarity or a common motif (Mingot et al., 2004). It does appear, however, that basic residues are important for cargo recognition. It is not unreasonable to speculate that MDV has alternative mechanisms for nuclear export. The absence of an identifiable NLS or NES within MDV US3 suggests that either it is non-canonical, or that it interacts with and is subsequently co-transported with substrates that do contain an NLS or NES.

**BioID2 screen identifies proteins involved in the HSP90 system**

Alphaherpesviruses are known to subvert the host immune system through their interaction with the Heat Shock Protein (HSP) 90 system. HSPs are chaperones that aid the proper folding, stabilisation and maturation through repetitive binding and unbinding in an ATP-dependent manner. The BioID2 screen identified HSP90-alpha class B (HSP90AB1) and HSP90-beta (HSP90B1). HSP90AB1 has a wide range of client proteins, and US3-BioID itself may require HSP90AB1 for proper folding. HSP90AB1, relative to others identified in the BioID screen, has a relatively low significance and enrichment compared to the mCherry control, supporting this.
BioID2 screen highlights proteins involved in dynein transport

The BioID2 screen highlights the heavy chain subunit of the dynein complex (DYNC1H1). Dynein is a minus-end directed microtubule motor and is known to transport incoming alphaherpesvirus visions to the nucleus, where replication occurs (Dohner et al., 2002). Dynein is a multi-subunit motor complex consisting of the heavy, intermediate, light intermediate and light chains (King et al., 2002). No other chains apart from the cytoplasmic heavy chain have been identified. In addition to this, no cargo adaptor proteins or dynein cofactors have been identified in the screen. This can be explained as the dynein complex is larger than the 10 nm range of biotinylation (Reck-Peterson et al., 2006).

Whilst absence of these proteins does not necessarily exclude such interactions; it may suggest that MDV US3 has a specific affinity for DNYC1H1, or that interactions with other subunits cannot be distinguished from those of the cytoplasmic mCherry-BioID control. Because of this potential interaction, the significance of dynein in alphaherpesvirus infection, and association of US3 and microtubules, this will be explored further in Chapter 6.

BioID2 screen highlights non-receptor kinases

Two non-receptor kinases have been identified in the screen for potential interactors, namely Mitogen-Activated Protein Kinase (MAP2K) and Cyclin-Dependent Kinase 2 (CDK2). As MAP2K and CDK2 regulate a wide variety of cellular processes, including cell cycle progression and apoptosis (Gil-Gómez et al., 1998; Wilkinson & Millar, 2000). Both these kinases will impact viral replication; it is, therefore, unsurprising that exploitation of these pathways is a common theme in large DNA viruses including alphaherpesviruses (Bonjardim, 2017; Fan et al., 2018).

MAP2K is an upstream regulator of CDK2, via ERK. CDK2 activity increases from mid- to late-G1 phase, where it targets functions that are required to initiate and sustain DNA replication (Bertoli et al., 2013). It is notable that in infected herpesvirus cells, MAP2K phosphorylation increases; however, the exact mechanism for this remains yet to be determined.
(Filippakis et al., 2010; Rahaus et al., 2004). The regulation of MAPKs and CDKs is through the phosphorylation of serine and threonine residues, respectively (Cargnello & Roux, 2011; Morgan, 1995). It is generally accepted that herpesviruses have evolved a mechanism to force quiescent cells to enter an S-phase-like status to create an environment favourable to replication, and MDV US3 as a serine-threonine protein kinase could potentially play a role in this (Colao et al., 2017).

**Truncations of MDV US3 BiolD2 constructs and biotinylated proteins show different localisations**

The full-length US3 protein appears to have localisation that is highly correlative with the F-actin cytoskeleton of the transfected cell. Truncating this protein to the first 111 amino acids does not appear to change this, suggesting that this region of MDV US3 is primary determinator of this localisation. This localisation and the localisation of biotinylated proteins would also suggest that region 1-111 alone can interact with components of the F-actin cytoskeleton.

It is interesting that for US3 1-111, there is a strong enrichment of biotinylated proteins in the nucleus, while US3 1-111 itself is excluded. This may indicate a mechanism that enriches the biotinylated candidate interactor in the nucleus or may indicate a nuclear export mechanism acting on US3 1-111. However, no nuclear export signal could be predicted in this region.

It is striking that the loss of N-terminus (1-111) or C-terminus (322-402) regions flanking the MDV US3 kinase domain can lead to colocalisation with the mitochondrial marker TOM20. Amphipathic helices can lead to the mitochondrial targeting of proteins (Lemire et al., 1989). It is plausible that truncating MDV US3 could expose such helices that would normally be buried. Of the four helices within MDV US3 111-322, none have been predicted to be amphipathic. Localisation to mitochondria is expected, given that there is strong evidence that MDV US3 and its orthologues within HSV-1 and PRV can protect the cell from virus-induced apoptosis through the kinase interaction of US3 with the Bcl-2 family proteins present on the external surface of the
mitochondrial membrane (Deruelle et al., 2007; Ogg et al., 2004).

The addition of the C-terminus to region 111-322 appears to cause a reduction of mitochondrial localisation. The behaviour shown here can be explained in two ways. Firstly, region 322-402 could mask the region within 111-322 responsible for mitochondrial localisation, resulting in reduced binding affinity. Secondly, it is plausible that 322-402 contains other localisation signals or interacting domains that compete with mitochondrial localisation of US3; however, given that the C-terminus region alone does not appear to have any specific localisation, this seems unlikely.

Limitations of the BioID2 approach

It is not surprising that this proteomic approach has not highlighted known interactors of MDV US3, particularly those that are located in the nucleus or within organelles. Even though the BioID2 promiscuous biotin ligase is substantially smaller than previous versions, it is possible that it could affect localisation or function of MDV US3. For example, it has been demonstrated that when proteins are extended beyond 60-70 kDa, they can be excluded by the nuclear pore complex (Soullam & Worman, 1995). Indeed, the addition of the BioID2 promiscuous ligase increases the size of full-length MDV US3 from 45 to 77 kDa. It is therefore plausible that the addition of BioID2 prevented the MDV US3 used for the BioID2 screen from entering the nucleus.

The control of a BioID2 screen is usually an unrelated protein with the same cellular compartment. In this investigation, mCherry was used. Indeed, US3 and mCherry share the same cellular compartment. However, it is unlikely that US3 and mCherry are both expressed in cells equally. If mCherry is expressed more readily than US3, the sensitivity of the BioID2 screen is reduced. A further variation is introduced by transient transfection. This could be overcome by stable transfection or viral transduction.

A common limitation of tandem mass spectrometry is that there are a limited number of ions that can undergo fragmentation (ms1) and secondary mass spectrometry in a given amount of time. Secondary mass spectrometry
is required to identify the sequence of a peptide. Typically, ions are prioritised based on their intensity in the ms1 spectrum. When the sample is complex or where there is a contaminant producing large and long-lasting ms1 peaks, an increasing number of ms1 peaks will be missed for secondary mass spectrometry. Typically, the prioritization of precursors is by a data-dependent strategy that is responsive to the signals generated from within a given sample. More recently, an alternative dataset-dependent strategy of precursor prioritization has been developed to increase selectivity and coverage (Broeckling et al., 2018). This method, however, requires a dataset of at least 20 MS/MS runs of each sample.

Using the BioID approach, a list of candidate interactors of MDV US3 has been generated. We have identified the actin cross-linker alpha-actinin and also the microtubule motor protein dynein. The BioID screen itself is not sufficient to validate an interaction or to draw conclusions on the function of US3. For alpha-actinin, the finding was further validated and showed that it is highly enriched in the MDV US3 sample compared to the mCherry control. Given the role that alpha-actinin has in the actin cytoskeleton, it will be further investigated to determine the nature of this interaction, and whether the product of such protein can explain the kinase-independent remodelling of the actin cytoskeleton. The presence of dynein heavy chain in the BioID2 screen is also interesting, given that dynein transports alphaherpesviruses to the nucleus where replication occurs during early stages of infection. Because of this, the interaction between dynein and US3 will also be explored.
5 Characterising the interaction between MDV US3 and alpha-actinin

5.1 Introduction

In Chapter 4, using a proximity-dependent biotinylation screen for interactors (BioID), alpha-actinin was highlighted as a potential interaction partner of MDV US3. To confirm and characterise the interaction between MDV US3 and alpha-actinin, further biochemical analysis was required. In addition to exploring the binding of the proteins, we also aimed to determine whether there is any interference with the normal function of alpha-actinin. For this reason, kinase-dead MDV US3 with amino acid substitutions D218A K220A was generated using site-directed mutagenesis and cloned into a bacterial expression vector containing a GFP tag. This fusion protein was expressed and purified from bacterial lysate.

Molecular structure and regulation of alpha-actinin

Alpha-actinin (Figure 5.1) is an F-actin crosslinking protein and part of the spectrin superfamily. It forms antiparallel homodimers with a subunit molecular weight of 94-103 kDa. From electron microscopy, it is visualised as a rod-shaped molecule that is approximately 30-40 nm long and 3-4 nm wide (Podlubnaya et al., 1975; Imamura et al., 1988).

Four major alpha-actinin isoforms have been isolated from different tissues, with the primary difference being their sensitivity to calcium. Non-muscle isoforms (ACTN1 and ACTN4) are calcium sensitive and are found in various crosslinked actin structures such as stress fibres, the actomyosin cortex and podosomes (Mukhina et al., 2007; Stickel & Wang, 1987; Cervero et al., 2012). Muscle isoforms (ACTN2 and ACTN3) are calcium insensitive and are found in the sarcomere (Beggs et al., 1992).

In the calcium-sensitive isoforms, calcium negatively regulates the ability of alpha-actinin to crosslink F-actin (Witke et al., 1993). The calmodulin-like (CaM) domain contains two EF-hand motifs, which in the presence of calcium, causes a conformation change within the CaM domain that associates with the
linker region between the two calponin homology (CH) domains in the second alpha-actinin subunit. This association causes the CH domains to rotate and move apart, resulting in a conformation which is unable to bind to F-actin (Tang et al., 2001; Liu et al., 2004). Non-calcium sensitive muscle isoforms of alpha-actinin contain non-functional EF-hands. The loss of function is due to the substitution of calcium-binding residue (L804Q), and the second binding loop having three amino-acid substitutions (S763N, T765L and G767D) (Arimura et al., 1988; Prebil et al., 2016). Muscle isoforms are autoinhibited by their CaM domains. To bind to actin, muscle isoforms rely on the release of inhibition that occurs when the CaM domain interacts with the Z-repeats of titin present in the sarcomeric Z-disk (Grison et al., 2017). Because of the stability of the Z-disk complex, muscle isoforms are permanently bound to F-actin when in the presence of titin.

**Interaction partners of alpha-actinin**

Asides from its role in crosslinking F-actin filaments, alpha-actinin can interact with numerous proteins via its actin binding domain (ABD), rod or CaM domains (Table 5.1). The rod domain consisting of spectrin repeats serves as a scaffold for other proteins, and such interactions should not alter the ability of alpha-actinin to crosslink F-actin. These proteins can be components that are involved with the actin cytoskeleton, such as integrins and zyxin, relying on their interaction with the rod domain of alpha-actinin for targeting or as a mechanochemical linkage (Roca-Cusachs et al., 2013; Crawford et al., 1992). In addition to this, the rod domain of alpha-actinin acts as a connection between the actin cytoskeleton and cell signalling by acting as a scaffold for kinases and other signalling molecules (Vallenius et al., 2000; Kigawa et al., 2004).

Interactions with the ABD and CaM domains of alpha-actinin are more functionally varied and in the case of calcium sensitive isoforms can be calcium dependent (Kim et al., 2002; Walikonis et al., 2001). Interactions with these domains are often used to regulate the activity of alpha-actinin. The ABD and CaM domains are phosphorylated by focal adhesion kinase and calmodulin dependent kinase II, respectively (Izaguirre et al., 2001; Dhavan et
Specifically for alpha-actinin-4, the ABD and CaM domains interact with steroid hormone receptors and transcriptional regulators (Khurana et al., 2012; Chakraborty et al., 2006).

Figure 5.1 The structure of alpha-actinin. A: Primary structure of an alpha-actinin subunit showing actin-binding domain (ABD) consisting of tandem calponin homology (CH1 and CH2) domains, the rod domain composed of four spectrin repeats (SR1 to SR4) and calmodulin-like domain (CaM) containing two calcium-sensitive EF-hands (EF1 and EF2). B: Cryo-electron microscopy reconstruction of chicken gizzard alpha-actinin dimer (PDB: 1SJJ). ABD and CaM domains are shown in blue and red respectively. Calcium binding sites within EF-hands are indicated by green spheres. C: Model of alpha-actinin bound to F-actin, from Liu et al. (2004). CaM domain (yellow) is flexible, allowing CH1 (purple) and CH2 (red) domains to be close together unhindered in a conformation that can bind to F-actin.
Table 5.1 Selected interaction partners that have been mapped to specific domains of alpha-actinin

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<tr>
<td>Estrogen Receptor α</td>
<td>(Khurana et al., 2012)</td>
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<tr>
<td>Cystic fibrosis transmembrane conductance regulator E3KARP (Ca²⁺ dependent)</td>
<td>(Kim et al., 2002)</td>
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<tr>
<td>Affixin</td>
<td>(Yamaji et al., 2004)</td>
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<tr>
<td>C-Reactive Protein 1</td>
<td>(Pomiès et al., 1997)</td>
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<tr>
<td>Focal Adhesion Kinase</td>
<td>(Izaguirre et al., 2001)</td>
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<td><strong>Rod domain</strong></td>
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<td>Humanin</td>
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<td>(Roca-Cusachs et al., 2013)</td>
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<td>(Vallenius et al., 2000)</td>
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<td>Zyxin</td>
<td>(Crawford et al., 1992)</td>
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<td><strong>Calmodulin-like domain</strong></td>
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<tr>
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<td>(Gonzalez et al., 2001)</td>
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<td>Calmodulin-dependent kinase II (Ca²⁺ dependent)</td>
<td>(Walikonis et al., 2001)</td>
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<td>(Dhavan et al., 2002)</td>
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<td>Densin</td>
<td>(Walikonis et al., 2001)</td>
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<tr>
<td>Histone deacetylase 7</td>
<td>(Chakraborty et al., 2006)</td>
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<tr>
<td>Membrane-associated guanylate kinase, WW and PDZ domain-containing protein 1 MAGI-1</td>
<td>(Patrie et al., 2002)</td>
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Ab initio protein structure prediction

Protein structure models are often generated following X-ray crystallography or cryo-EM reconstruction. While the number of protein sequences continues to increase, the number of solved protein structures is lagging. As of March 2020, there are ~162,000 PDB structures corresponding to ~28 % of curated protein sequences on UniProt. When solved protein structures are not available, or in the case of theoretical proteins that do not exist, 3D structural models can be generated computationally. If enough experimentally solved structures exist, it is possible to use template-based modelling. Here, based on the predicted secondary structure of the protein, similar structures are found in the structural library and used as a template to generate a 3D model that is further refined (Huang et al., 2014).

When no homology templates exist, *ab initio* protein structure prediction aims to predict the secondary and tertiary structure from the primary sequence of the protein. Generally, this method relies on a computationally intensive simulation of an atomic model where the positions of each atom are calculated using Newton’s laws of motions. Therefore, there is a trade-off between the duration that can be simulated, and subsequent accuracy of the model and the complexity of the protein. Because of this trade-off, *ab initio* structure prediction is of lower accuracy than experimental methods and is generally limited to small proteins.

To predict the structure a fragment of MDV US3, this study uses the QUARK programme (Figure 5.2) developed by Xu and Zhang (2012). The accuracy of publicly available structure prediction methods is quantitatively compared using the yearly Critical Assessment of Techniques for Protein Structure Prediction (CASP) benchmarking experiments. QUARK was chosen as it was the highest-ranked server for free modelling in CASP9 and CASP10 experiments. QUARK takes the amino acid input sequence and generates a multiple sequence alignment based on results from PSI-BLAST of a database of non-redundant sequences. These multiple alignments are used to train separate neural networks that subsequently predict beta turns, real torsion angles and solvent accessibility. The sequence profile from the multiple
alignments, the predicted torsion angles and predicted solvent accessibility are used to generate structural models of 1-20aa segments of the input sequence.

The segments of various lengths are randomly used to construct the initial atomic models for up to 40 replica-exchange Monte Carlo simulations that determine the 10 structures with the lowest energy. A total of 5000 models from the last 150 cycles of the replica-exchange Monte Carlo simulations of each of the 10 structures are randomly selected and clustered based on their RMSD using the revised SPICKER program (Zhang & Skolnick, 2004). The centres of these clusters are used as representative models which are then refined by adding missing atoms to reduced models and improving the quality of backbone and side chain atomic structures (Xu & Zhang, 2011).

**Protein docking**

In order to mechanistically understand a protein-protein interaction, the combined structures of the proteins must be determined at an atomic level, often through X-ray crystallography. Computational docking methods have been developed to use when this is not possible. Similar to protein structure prediction, both template-based and direct methods exist. Direct methods use thermodynamics to find the structure of the complex that has the lowest Gibbs free energy (Du et al., 2016). On the other hand, template methods use the similarity between sequence and interaction divergence to model interactions by homology (Sinha et al., 2010). However, because the coverage of templates is limited, the application of template methods is restrictive.

To predict and understand the nature of an interaction between MDV US3 and alpha-actinin, their 3D structures are docked using the ClusPro 2.0 server developed by Kozakov et al. (2013) (Figure 5.3). ClusPro is a direct docking method that uses Fast Fourier Transform to calculate the energy function of possible complexes. Both protein structures are modelled; however, the largest structure is fixed in space, and the smaller structure is rotated 5 degrees of Euler angles 70,000 times in all directions to calculate the energy function. This allows for an exhaustive evaluation of energy function for an
extremely large number of orientation and possible structures. From this, the lowest 1000 energy structures are then clustered based on pairwise RMSD measurements. The centres of the 30 largest clusters are then further refined to minimise their energy function by Monte Carlo simulation to give the 10 lowest energy structures ranked by their RMSD.
Figure 5.2 Workflow of the QUARK *ab initio* structure prediction. Adapted from Xu and Zhang (2012). QUARK analyses an input sequence to generate 3D segments. These segments are then used to construct a structure of the protein, which is then further refined.
Figure 5.3 Workflow of ClusPro 2.0 protein docking server. Adapted from Kozakov et al. (2017). ClusPro uses FFT to calculate the energy function of a receptor and its ligand in different orientations. These energy structures are subsequently ranked and clustered based on their similarity to each other. The centre structures of these clusters are then refined to reduce their energy function further to give the final models.
Microscale thermophoresis

Microscale thermophoresis (MST) is a technique that examines the directed movement of intrinsically fluorescent or fluorescently labelled biomolecules within a temperature gradient (Wienken et al., 2010). The movement of a biomolecule in a temperature gradient is dependent on a variety of factors: mass, size, charge and hydration shell. These factors can be altered when a biomolecule interacts with a ligand or another biomolecule. MST was chosen for its advantages over alternative methods as it requires small amounts of sample, does not require immobilisation of proteins, and can be carried out using crude cell lysates if needed.

During MST (Figure 5.4), biomolecules start evenly distributed within a small glass capillary. An infrared laser heats a point within the capillary, creating a temperature gradient. Biomolecules move along the temperature gradient, away from the hotspot, due to what is known as the Soret effect (Duhr & Braun, 2006). During this process, the fluorescence is continually measured.

By measuring the fluorescence before, during and after sample heating, several parameters can be determined. During approximately the first five seconds of sample heating, the measured fluorescence decreases due to the change in temperature (known as the T-jump) (Jerabek-Willemsen et al., 2014). Changes here are due to the altered local chemical environment of the fluorophore. These changes can be often brought about by a ligand binding near the fluorophore. After this point onwards, for approximately 20 seconds, measured fluorescence decreases due to thermophoresis of biomolecules within the temperature gradient. Once sample heating is stopped and the temperature gradient eliminated, biomolecules diffuse back to a homologous distribution. The rate of this diffusion gives information on the size and mass of the biomolecule complex.
Figure 5.4 A typical MST set-up and MST trace. During MST, a point in the sample capillary is heated using an infrared (IR) laser, and the fluorescence of the sample is measured at the same time. A: When there is no heating, molecules are in the initial state and there is no fluorescence change. B: When heating is applied, a temperature gradient is generated and molecules move away, resulting in a measured fluorescence decrease. C: During heating, molecules reach a steady state and there is no fluorescence change. D: When IR heating has stopped, molecules diffuse back, resulting in an increase of fluorescence.
5.2 Results

Actin bundling assay

Kinase-dead MDV US3-GFP6xHis was cloned into a bacterial expression vector containing a lac promoter. MDV US3-GFP was purified from bacterial lysate using Ni-NTA affinity purification and size exclusion chromatography (Figure 5.5). To determine whether MDV US3 interfered with the capability of alpha-actinin to crosslink F-actin, 3.3 µM of purified alpha-actinin from chicken gizzard was incubated with purified 4.7 µM of MDV US3-GFP and 7 µM of F-actin.

Individual F-actin filaments were separated from F-actin bundles using centrifugation (Figure 5.6). Despite the purified actin from rabbit skeletal muscle being >90 % pure, contamination by a 100 kDa protein was observed. Alpha-actinin is a common contaminant of actin preparations (Gordon et al., 2018). This contamination is likely to cause the small proportion of bundled F-actin present in the pellet when no crosslinking protein was added. F-actin in the absence of alpha-actinin or the presence of MDV US3-GFP alone was predominantly in the supernatant, indicating that F-actin was in individual filaments rather than in bundles. As expected, when F-actin was incubated with alpha-actinin, the majority of F-actin was crosslinked into bundles and sedimented in the pellet. When F-actin was incubated with alpha-actinin and MDV US3-GFP, a large reduction in F-actin bundling was observed, as seen in the higher proportion of F-actin remaining in the supernatant.
Figure 5.5  Purified kinase-dead MDV US3-GFP. A: Schematic of GFP-tagged MDV US3 (expected size: 73 KDa) B: Coomassie-stained protein gel of purified protein from size exclusion chromatography.

Figure 5.6 Actin bundling assay. Coomassie-stained gel of supernatant containing individual F-actin filaments (S) and pellet containing bundled F-actin filaments (P). Purified MDV US3-GFP, chicken gizzard alpha-actinin and F-actin were pre-incubated for 30 minutes before bundles were separated from individual filaments by centrifugation.
Ab initio protein structure prediction of MDV US3 1-111

From biotinylation experiments using truncated MDV US3, the region 1-111 was identified as being responsible for the localisation of US3 to the actin cytoskeleton. Because of this observation, the amino acid sequence of MDV US3 1-111 was submitted to the QUARK webserver to generate a 3D protein model. The prediction generated 5 models that corresponded to the models with the lowest RMSD from clustering. The top-ranked model is shown in Figure 5.7. The model is characterised to have a primarily coiled secondary structure, with five extended strands at positions 9-11, 30-32, 55-56, 88-90 and 98-104. Two alpha-helices are predicted at positions 59-61 and 95-97. The unstructured region has a primarily negatively charged surface (Figure 5.8).
Figure 5.7 Predicted structure of MDV US3 1-111. The first 111 amino acids of MDV US3 were submitted to the QUARK prediction server. Models were ranked based on their RMSD from clustering. The top-ranked model is shown. The chain is coloured blue at the N-terminus and red at the C-terminus.

Figure 5.8 Electrostatic surface of the MDV US3 1-111 predicted structure. Positively charged surfaces are indicated in blue, whilst negatively charged surfaces are in red.
Docking of MDV US3 1-111 to chicken alpha-actinin

In order to understand the nature of the interaction between alpha-actinin and MDV US3, the predicted structure of US3 1-111 was docked to the structure of chicken alpha-actinin (PDB: 1SJJ) using the Cluspro 2.0 webserver. The protein structure of chicken alpha-actinin has both the calcium-bound and non-calcium-bound configurations of the calmodulin-like and actin-binding domains. A total of 10 models were generated that corresponded to the models with the lowest RMSD from clustering (Figure 5.9 and Figure 5.10). In all 10 models, MDV US3 1-111 was seen to dock between the two CH domains of alpha-actinin in the calcium-bound conformation. From this, it was predicted that the interaction between MDV US3 and alpha-actinin was calcium-dependent.

Examining the electrostatic surface of chicken alpha-actinin highlights differences between the actin-binding domains in their calcium-bound and no calcium states (Figure 5.11). The calcium-induced conformational change exposes a highly negative surface between the two CH domains. When MDV US3 1-111 is docked to chicken alpha-actinin, this negatively charged surface interfaces with the positive surface of US3 1-111 (Figure 5.12). Using the highest-ranked model, amino acids residues of US3 in the binding interface in close proximity to oppositely charged residues of alpha-actinin were identified for future investigation (Figure 5.13).

Alignment of MDV US3 1-111 to other alphaherpesvirus orthologues

In order to determine any similarity between MDV US3 1-111 and other alphaherpesviruses that have been studied, the sequence of MDV US3 and its orthologues was aligned using Clustal Omega (Figure 5.14). With the exception of vaccine strains CVI988 and HVT US3, no significant similarity was found between MDV US3 1-111 and other US3 orthologues. The amino acid residues identified for mutagenesis were found to be conserved between MDV and HVT.
Figure 5.9 Top 5 predicted docking configurations of MDV US3 1-111 and chicken alpha-actinin. The predicted structure of MDV US3 1-111 (red/green) was docked to the structure of chicken alpha-actinin (blue) (PDB: 1SJJ) using the Cluspro server. Structures were ranked based on their RMSD after clustering.
Figure 5.10 Predicted docking configurations of MDV US3 1-111 and chicken alpha-actinin ranked 6 to 10. The predicted structure of MDV US3 1-111 (red/green) was docked to the structure of chicken alpha-actinin (blue) (PDB: 1SJJ) using the ClusPro server. Structures were ranked based on their RMSD after clustering.
Figure 5.11 Domains and electrostatic surfaces of chicken alpha-actinin in a calcium-bound state and no calcium state. PDB: 1SJJ. CH: Calponin homology domain. CaM: Calmodulin-like domain. On electrostatic surfaces, red, white, and blue denote negative, neutral and positive charge respectively.
Figure 5.12 Domains and electrostatic surfaces of chicken alpha-actinin docked to US3 1-111. Highest ranked model. US3 is docked between CH1 and CH2 of chicken alpha-actinin (PDB:1SJJ) when actin binding domain is in the calcium bound, non-actin binding conformation. CH: Calponin homology domain. On electrostatic surfaces, red, white, and blue denote negative, neutral and positive charge respectively.
Figure 5.13 Amino acid residues of MDV US3 1-111 that have been identified for future mutagenic study. Red and blue denote amino acid side chains on MDV US3 and chicken alpha-actinin, respectively. Amino acids were identified based on their close proximity to amino acids on alpha-actinin that have opposite charge or aromatic side chains.
Figure 5.14 Clustal omega alignment of MDV US3 1-126 and other major alphaherpesviruses. Multiple alignments were carried out using EBI Clustal Omega that uses seeded guide trees and HMM profile-profile to generate alignments. Similarity is indicated by the following symbols * = positions which have fully conserved residues, : = strong conservation (PAM 250 matrix score > 0.5), . = weak conservation (PAM 250 matrix score < 0.5). Amino acid residues identified for mutagenesis and their aligned counterparts in other orthologues have been highlighted.
Microscale thermophoresis

In order to confirm the predicted calcium-dependent binding of MDV US3 to chicken alpha-actinin, a protein binding affinity curve was generated using microscale thermophoresis. For each MST sample, the concentration of US3-GFP was kept constant at 270 nM whilst increasing amounts of chicken alpha-actinin was added.

To determine the binding constant from MST, the normalised initial fluorescence was compared to the normalised fluorescence of samples 0.89 to 1.27s into the period of thermophoresis. From this, the fraction bound was determined using the following equation:

\[ F_{\text{norm}} = (1 - FB) F_{\text{norm, unbound}} + (FB) F_{\text{norm, bound}} \]

FB: fraction bound

\( F_{\text{norm, unbound}} \): normalised fluorescence of the unbound state

\( F_{\text{norm, bound}} \): normalised fluorescence of the bound state

To the calculated fraction bound values for the given concentrations of alpha-actinin, a Kd model was fitted (Figure 5.15). From this, the Kd for the interaction between US3 and alpha-actinin in the presence of 0.1 mM calcium or no calcium was calculated to be 1.29±0.54 µM and 9.45±3.67 µM, respectively.

Before the heating phase of MST was carried out, the initial fluorescence of US3-GFP was determined (Figure 5.16). Despite the concentration of US3-GFP being kept constant, the fluorescence of US3-GFP was observed to increase with the concentration of alpha-actinin. The change of fluorescence was found to be greater in the presence of 0.1 mM calcium.
Figure 5.15 Binding affinity curve of MDV US3-GFP in the presence of alpha-actinin. Concentration of US3-GFP was kept constant at 27 µM. Increasing concentrations of alpha-actinin was added to samples. Each data point represents the average of two repeat MST measurements. A Kd model was fitted to the data points. The shaded area represents the 95% confidence intervals of their respective Kd models.
Figure 5.16 Fluorescence of MDV US3-GFP increases with concentration of alpha-actinin. Initial fluorescence of MDV US3-GFP was measured before the heating phase of MST was carried out. Concentration of US3-GFP was kept constant at 270 nM. Increasing concentrations of alpha-actinin was added to samples. Each data point represents the average of two repeat MST measurements. Baseline of the raw fluorescence measurements was normalised. A Kd model was fitted to the data points.
Determining the specificity of calcium enhanced binding

To determine whether the fluorescence and binding change observed during MST experiments was specific to calcium and alpha-actinin, the fluorescence of US3 was measured in buffers containing 1 mM Ca\(^{2+}\) or 1 mM Mg\(^{2+}\) in the presence of alpha-actinin or BSA (Figure 5.17). Three repeated measurements were made in three independent experiments. Fluorescence baseline was normalised by dividing the fluorescence signal by the fluorescence of MDV US3 in buffer containing neither Ca\(^{2+}\) nor Mg\(^{2+}\). Mean relative fluorescence and their standard deviations are summarised in Table 5.1. After confirming normality of the data using the D'Agostino & Pearson omnibus normality test, statistical significance at the 95 % confidence limit was determined using a two-tailed paired t-test.

In all cases, the presence of Mg\(^{2+}\) does not change the fluorescence of US3 compared to the buffer (US3 and BSA P=0.2607; US3 and ACTN P=0.2986). A statistically significant increase in MDV US3 fluorescence was seen in the presence of alpha-actinin in 1 mM Ca\(^{2+}\) (P=0.0011) compared to 1 mM Mg\(^{2+}\). No significant increase was seen in US3 fluorescence in the presence of BSA in 1 mM Ca\(^{2+}\) compared to 1 mM Mg\(^{2+}\) (P=0.3818). This would suggest that the fluorescence change is specific to alpha-actinin in the presence of calcium.

Table 5.2 Normalised mean fluorescence of MDV US3-GFP in the presence of calcium and magnesium. Mean is calculated from 3 repeated measurements from 3 independent experiments. Baseline of each experiment was normalised to fluorescence of MDV US3-GFP in buffer containing no cations. Standard deviation is also shown.

<table>
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<tr>
<th></th>
<th>1 mM Ca(^{2+})</th>
<th>1 mM Mg(^{2+})</th>
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<tbody>
<tr>
<td>MDV US3 + ACTN</td>
<td>1.31 ± 0.25</td>
<td>0.94 ± 0.15</td>
</tr>
<tr>
<td>MDV US3 + BSA</td>
<td>0.87 ± 0.14</td>
<td>0.93 ± 0.18</td>
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Figure 5.17 MDV US3-GFP fluorescence in the presence of alpha-actinin in the presence of alpha-actinin or BSA with 1 mM calcium or magnesium. For each experiment, a master mix of MDV US3-GFP with alpha-actinin or BSA was made and then added to buffer containing 1 mM Mg\(^{2+}\) or 1 mM Ca\(^{2+}\). Data is from 3 repeated measurements from 3 independent experiments. Baseline of each experiment was normalised to fluorescence of MDV US3-GFP in buffer containing no cations. After confirming normality, statistical significance at the 95 % confidence interval was determined using a two-tailed paired T-test. ** P ≤ 0.01, ns P > 0.05.
Overexpression of alpha-actinin-4 in the presence of MDV US3

Both wild-type and kinase-dead MDV US3 was transfected and expressed in RPE-1 and RPE-1 cells stably expressing alpha-actinin-4-mNeonGreen to determine whether overexpression of alpha-actinin-4 could counteract the US3-induced disassembly of stress fibres (Figure 5.18). Cells were fixed 24 hours after transfection. 96 ± 4% of cells over-expressing alpha-actinin-mNeonGreen and kinase-dead US3 displayed intact stress fibres. This was considerably higher than the 64 ± 7% of RPE-1 cells expressing kinase-dead MDV US3 alone, indicating a rescue effect. No rescue effect was seen in cells expressing wild-type US3, where 57 ± 12% and 56 ± 2% of cells had intact stress fibres in cells overexpressing alpha-actinin-mNeongreen and in control cells, respectively.

The localisation of mNeonGreen-tagged alpha-actinin-4 was also examined. In untransfected cells, alpha-actinin-4 was seen to localise with crosslinked F-actin structures such as stress fibres Figure 5.19. Surprisingly, both wild-type and kinase-dead US3 were able to translocate alpha-actinin-4 to the cell nucleus. In cells transfected with wild-type US3, 30 ± 11% of cells showed an enrichment of alpha-actinin-4 within the nucleus. Translocation correlated with the loss of actin stress fibres, but did not necessarily correspond to colocalisation with US3. For cells transfected with kinase-dead US3, 14 ± 12% showed nuclear enrichment of alpha-actinin. Unlike cells transfected with wild-type US3, the translocation of alpha-actinin occurred while cells had intact stress fibres and in all instances resulted in colocalisation with US3 in the nucleus.
Figure 5.18 Overexpression of alpha-actinin-4 in RPE cells transfected with wild-type and kinase-dead MDV US3. RPE-1 cells and stable transfected RPE-1 cells expressing mNeonGreen tagged alpha-actinin-4 were transfected with either wild-type or kinase-dead MDV US3. Cells were fixed 24 hours after transfection. Results are from three independent experiments.
Figure 5.19 Translocation of alpha-actinin-4 in the presence of MDV US3. Stable transfected RPE-1 expressing alpha-actinin-4 mNeonGreen, and transfected with either wild-type or kinase-dead MDV US3. In cells expressing wild-type MDV US3, alpha-actinin-4 is enriched in the nucleus. In untransfected cells or cells that are expressing kinase-dead MDV US3, alpha-actinin is excluded from the nucleus. Scale bar represents 10 µm.
5.3 Discussion

**MDV US3 1-111 is predicted to interact with alpha-actinin**

As no structural models exist of MDV US3, one was created using *ab initio* structural prediction (Figure 5.7). Due to computational limitations, it was not possible to predict the whole structure of MDV US3. In Chapter 4, the localisation of MDV US3 truncations has highlighted the F-actin localisation of MDV US3 1-111. Because of this localisation, this region was chosen to be modelled and docked with the structure of chicken gizzard alpha-actinin (Figure 5.9 and Figure 5.10).

It is quite striking that the 10 highest ranked docking models show the region of MDV US3 1-111 interacting with the calcium-bound conformation of the alpha-actinin ABD. Closer examination of the electrostatic surfaces shows the highly negative charged area of MDV US3 1-111 (Figure 5.8) interfacing with highly positively charged area separating the two CH domains of alpha-actinin (Figure 5.11). Given the substantial differences in electrostatic charge, it is not surprising if this is the most energetically favourable conformation.

This area of positive charge is not exposed when the CH domains are close together in the non-calcium-bound state. The presence of the positively charged surface is counterintuitive of what is expected, given that F-actin has a net negative charge at physiological pH. It would appear that the separation of the two CH domains is sufficient to prevent actin binding. When the ABD is specifically in a conformation that does not accept F-actin, it still remains a region that can bind to other proteins in a calcium dependent manner (Kim *et al.*, 2002).

Several predictions can be made from the docked model. As the interaction surface only appears in the calcium-bound state of alpha-actinin, the interaction is likely to be calcium-dependent. The interaction of MDV US3 with alpha-actinin is through its N-terminus, and would not require the kinase domain or kinase activity. Additionally, this interaction surface is within the
ABD; it is quite possible that binding of MDV US3 to this would prevent the ABD from returning to its non-calcium-bound state.

It remains yet to be determined if other US3 orthologues share a similar negatively charged surface as seen within MDV US3 1-111. Similarity exists between this region and that of HVT US3. Further to this, the residues identified for mutagenesis in MDV US3 were found to be conserved in HVT US3. If these residues were indeed involved in the interaction with alpha-actinin, the conservation might indicate that HVT may also interact with alpha-actinin, and be capable of kinase-independent removal of actin stress fibres.

**Alpha-actinin relieves GFP quenching by MDV US3**

When the concentration of MDV US3-GFP is kept constant, increasing concentrations of alpha-actinin causes an increase in GFP fluorescence. The change in fluorescence cannot be replicated by the addition of BSA, suggesting the fluorescence change is due to a specific interaction with alpha-actinin.

The fluorophores of GFP and its derivatives are buried deep within beta-barrels; therefore, EGFP is not susceptible to quenching molecules (Yang et al., 1996). The fluorophore, however, is required to be in a deprotonated state and is consequently pH-sensitive (Shinoda et al., 2018). It is because of this sensitivity, that the pH is controlled in binding experiments. A possible explanation for the quenching of EGFP is alterations in its structure caused by an interaction with MDV US3. Proper folding of the beta-barrel of GFP and its derivatives are important for fluorescence. All 11 beta-strands must be assembled appropriately and precisely aligned (Nicholls & Hardy, 2013). Therefore, if unbound MDV US3 interacts with the EGFP tag in such a way that hindered proper alignment of the beta-strands, the structural environment for fluorescence would be sub-optimal and quenching would occur. Indeed, the fusion of proteins to GFP has been observed in several cases to quench or silence fluorescence and can indicate protein folding (Waldo et al., 1999; Nicholls et al., 2011). It is likely that the interaction of
alpha-actinin results in a conformation of MDV US3 that does not interact with the EGFP tag.

**Calcium increases the affinity of alpha-actinin to MDV US3**

Alpha-actinin purified from chicken gizzard was initially thought to consist predominantly of calcium insensitive muscle isoforms. Despite this, it can bind to calcium in a 1:4 molar ratio, which is expected if the 4 EF-hands in the alpha-actinin homodimer were functional (Wenegieme et al., 1994). Further to this, structural cryo-EM studies with chicken gizzard alpha-actinin have highlighted differences actin-binding domain conformation, which was linked to the changing conformation of the calmodulin-like domain (Liu et al., 2004). Together, these observations would suggest that alpha-actinin purified from chicken gizzard is calcium-regulated.

Both Ca$^{2+}$ and Mg$^{2+}$ are divalent cations that are closed shell and form ionic bonds. However, due to its larger size, only Ca$^{2+}$ can interact with the EF-hands within alpha-actinin (Grabarek, 2011). The ligand induced fluorescence change in MDV US3-GFP only occurs in the presence of calcium and alpha-actinin. This excludes the possibility that fluorescence change is due to cations acting on MDV US3-GFP. The observation would suggest that the interaction with MDV US3 is dependent on the conformational change in alpha-actinin brought about by the presence of calcium.

Despite the measured ~7 fold increase in affinity between MDV US3 and chicken gizzard alpha-actinin in the presence of calcium, this is lower than alpha-actinin to F-actin (Kd = 0.6 µM) (Wachsstock et al., 1993). If MDV US3 and actin shared the same binding site, alpha-actinin would preferentially bind to actin. Given that MDV US3 inhibits alpha-actinin function in the presence of F-actin, these observations together support that MDV US3 interacts with the ABD when it is in a non-actin binding state caused by the presence of calcium.

**MDV US3 interferes with the crosslinking of F-actin by alpha-actinin**

The actin-bundling assay highlights the capability of MDV US3 of inhibiting the crosslinking of F-actin. In all conditions, MDV US3 is present in the
supernatant only. This observation indicates that the inhibition of bundling is by direct interaction with alpha-actinin and excludes the possibility of MDV US3 preventing bundling by directly competing against alpha-actinin for actin.

From the bundling assay alone, it is not possible to determine the mechanism of inhibition. The ability of alpha-actinin to act as a crosslinker is dependent on its ability to bind to actin and for monomers to form antiparallel dimers. However, together with the calcium dependent interaction of MDV US3 and alpha-actinin, the inhibition of actin bundling would support the docked model of MDV US3 1-111 interacting with the alpha-actinin ABD.

**Overexpression of alpha-actinin-4 prevents stress fibre disassembly by kinase-dead MDV US3.**

Overexpression of alpha-actinin-4 was able to overcome the disassembly of stress fibres by kinase-dead, but not wild-type MDV US3; suggesting that there might be two distinct mechanisms of action. The alphaherpesvirus US3 is known, through its interaction with PAK1/2, to activate coflin, which severs F-actin and increases the monomer off rate (Jacob *et al.*, 2013; Bamburg, 1999). It would be expected, and in line with current observations, that overexpression of an F-actin crosslinker would have minimal effect if the majority of F-actin has been depolymerised. It is expected that kinase-dead MDV US3, from its inability to phosphorylate substrates, would not share this mechanism. The rescue effect from overexpressing alpha-actinin-4 would suggest that the removal of stress fibres is due to the inhibition of F-actin bundling.

**MDV US3 causes the nuclear translocation of alpha-actinin-4**

Alpha-actinin-4, asides from its role in the cytoskeleton, has been identified to be present in the cell nucleus and nuclear translocation can be triggered by the drug-induced depolymerisation of F-actin (Honda *et al.*, 1998). It would be expected, therefore, that loss of F-actin caused by the activation of coflin by US3 through PAK1/2 would lead to a loss of stress fibres and the translocation of alpha-actinin-4.
The nuclear enrichment of alpha-actinin-4, in cells transfected with kinase-dead MDV US3 with intact stress fibres suggest the existence of an alternative mechanism of nuclear translocation. The nuclear translocation of alpha-actinin-4 is an inherent feature of its spectrin repeats, and is prevented by its association with F-actin (Kumeta et al., 2010). Consistent with the findings of the actin bundling assay, a direct interaction and inhibition of alpha-actinin to bind and crosslink F-actin by MDV US3 would result in nuclear translocation.
6 Interaction of MDV US3 with microtubule motors

6.1 Introduction

The complete alphaherpesvirus capsid has an overall size of approximately 200 MDa (Heming et al., 2017). Crowding of the cytoplasm due to cytoskeletal filaments, organelles and the high concentration of protein restricts passive diffusion to molecules smaller than 500 KDa (Luby-Phelps, 1999), while larger objects require active transport. Therefore, active transport is critical for the replication of alphaherpesviruses. The microtubule cytoskeleton provides the physical pathways for the transport of alphaherpesviruses to and from the nucleus where viral replication occurs.

The dynein motor protein has been implicated in the retrograde transport of viral capsids from the cell periphery towards the microtubule organisation centre near the nucleus (Brzozowska et al., 2010; Zaichick et al., 2013; Pitts et al., 2014). However, the precise mechanisms that recruit dynein to viral capsids and activate retrograde transport remain unclear. The BioID2 screen (Chapter 4) highlighted two proteins involved in retrograde cargo transport, namely cytoplasmic dynein heavy chain (DYNC1H1) and coatamer subunit gamma 1 (COPG1). While cytoplasmic dynein 1 is the major motor protein that moves cargo towards the minus end of microtubules in animal cells, COPG1 is a subunit of the COPI complex, best known for surrounding vesicles budding from the Golgi apparatus destined for the endoplasmic reticulum (Aridor et al., 1995). However, it is emerging that that COPI is also involved in the transport of anterograde cargo (Orci et al., 2000; Yang et al., 2011).

Upon entry into the host cell, the majority of viral tegument proteins are released into the cytoplasm. However, US3 and other inner tegument proteins are thought to remain attached to the viral capsid during entry. For example, PRV US3 has been seen to co-transport with incoming PRV capsids using live- and electron-microscopy (Granzow et al., 2005). Further to this, when BHV-1 US3 is expressed within cells, it is found to be localised to microtubules (Olsen et al., 2006; Brzozowska et al., 2010).
Both enveloped and unenveloped capsids have been observed to undergo directed transport on microtubules, suggesting that the capsid proteins or viral tegument is sufficient for transport (Antinone & Smith, 2006; Antinone et al., 2010; Wisner et al., 2011) Together with the observed localisation of BHV-1 US3 along microtubules, the proteomic data described in Chapter 4, suggest a role for the alphaherpesvirus US3 in the microtubule-based transport of the capsid. For this reason, this interaction was tested using single-molecule motility assays with purified kinase-dead MDV US3-GFP and purified motor proteins.

Dynein

Cytoplasmic dynein is a large multimeric complex which is approximately 1.4 MDa in size (Zhang et al., 2017). Dynein consists of two light-intermediate-, two intermediate- and two heavy-chains complexed with three light chain dimers: LC8, RB, and Tctex1 (Figure 6.1A) (Paschal et al., 1987; Vallee et al., 1988; King et al., 1996a; Nikulina et al., 2004; King et al., 1996b). The motor domain of the multimeric protein complex is within the heavy chain and consists of an AAA+ ATPase domain from which a 15 nm long stalk extends with the microtubule-binding domain at its end (Samsó et al., 1998; Gee et al., 1997; Koonce, 1997). Dynein motility involves nucleotide-dependent conformational changes in the hexameric AAA ring that are propagated through the stalk to the motor domain and a powerstroke of the linker that swings across the face of the AAA ring (Burgess et al., 2003; Cho & Vale, 2012). The N-terminal tail is responsible for the dimerisation of the two heavy chains but also contains binding sites for the light-intermediate and light chains (Urnavicius et al., 2015). The different subunits of the dynein complex can interact with a wide range of cargo-binding adaptors, scaffolding and regulatory proteins (Fu & Holzbaur, 2014). Dynein is autoinhibited in the so-called phi conformation where the AAA rings are stacked, the linker is trapped and the microtubule binding stalk points in opposite directions (Torisawa et al., 2014; Zhang et al., 2017). In order to be activated for cargo transport, dynein first forms a ternary complex with dynactin and a cargo adapter such as BicD2, BicDR1 or Hook3 (Splinter et al., 2012; Schlager et al., 2014a; Olenick et al., 2016; McKenney et al., 2014).
Kinesins

Kinesins are a superfamily of predominantly plus-end-directed microtubule motors. There are approximately 15 subfamilies, based on phylogenetic differences, consisting of a total of 45 individual kinesin genes that are expressed in mouse and humans (Hirokawa et al., 2009). As with dynein, kinesins convert ATP into mechanical work (Schnitzer & Block, 1997). However, the motor domain can be located in the N-terminus, C-terminus or the middle of the protein (Vale & Fletterick, 1997). These different positions of the motor domains lead to several distinct functions. Kinesins that have an N-terminal motor domain are usually responsible for plus-end-directed transport (Hirokawa et al., 2009; Howard et al., 1989), while kinesins with a C-terminal motor domain are responsible for minus-end-directed transport (Hirokawa et al., 2009). Kinesins that have the motor domain located centrally are unable to walk, but instead have been associated with the depolymerisation of microtubules (Desai et al., 1999; Ogawa et al., 2004).

Kinesin-1 a conventional plus-end directed motor, which is of relevance to the anterograde transport of viruses as depicted in Figure 6.1B (Dodding & Way, 2011). Kinesins contain a motor domain, which directly interacts with microtubules. The stalk, which is predominantly coiled-coil, allows for the dimerisation of the motor, but in some cases has a flexible region that allows the tail to fold back onto the head for autoinhibition when no cargo is present (Hackney & Stock, 2000). The tail of the kinesin is responsible for cargo-binding such as endosomes or vesicles, either directly or through adaptor proteins (Akhmanova & Hammer III, 2010).
Figure 6.1 Structure of cytoplasmic dynein and kinesin-1. A: Structure of cytoplasmic dynein. Dynein consists of light, light-intermediate and heavy chains. The motor domain contains a ring of AAA+ domains, from which a stalk extends from containing the microtubule-binding domains. B: Structure of the conventional kinesin-1. The tail and light chains facilitate the cargo binding, which through the stalk and linker is connected to the motor domain that binds to microtubules. The coiled-coiled nature of the kinesin stalk allows for the dimerisation of monomers. Adapted from Carter (2013).
6.2 Results

An in vitro motility assay was used to determine whether there was any interaction between kinase-dead MDV US3-GFP and dynein and kinesin motor proteins and whether MDV US3 would be co-transported by them. These experiments were performed in collaboration with Alexander Zwetsloot and Dr Jack Chen. In all conditions, test proteins were pre-incubated with 3.6 µM MDV US3-GFP for 1 hour at room temperature before adding to the imaging chamber.

First, the interaction between dynein alone (Figure 6.2A), and dynein complexed with dynactin and Hook3 activators (DDH) (Figure 6.2B) was explored. MDV-US3 was incubated with 100 nM dynein alone or with the addition of 100 nM dynactin and 1 µM Hook3. Hook3, in addition to its interaction with dynein, also binds to and activates kinesin-3 member KIF1C (Siddiqui et al., 2019). To test whether the interaction with MDV US3 was unspecific, another motor protein, KIF1C complexed with the activator Hook3 (KH) was used (Figure 6.2C). MDV US3 was incubated with 100 nM KIF1C with 1 µM Hook3. MDV US3 was observed to co-transport with dynein alone, DDH and KH. No instances where MDV US3-GFP colocalised with Hook3 alone were observed.

The microtubule-associated protein EB3 was investigated for its suitability as a negative control. MDV US3-GFP was incubated with 13.9 µM EB3. EB3 was seen tracking the polymerising ends of microtubules as expected (Roth et al., 2019) (Figure 6.2D). MDV US3 did not localise with EB3, or track polymerising microtubule ends.

To identify whether there was any difference in the efficiency of co-transport of US3-GFP with the different microtubule motors, a binding rate that represents the proportion of US3-GFP molecules localising with a motor was calculated for each of the conditions (Table 6.1). The highest binding rate of 50 % was observed with DDH, while dynein alone had a lower rate of 31 %. The lowest binding rate of 9 % was seen with KIF1C complexed Hook3. After
confirming no significant difference of standard deviations using an F-test ($p = 0.0765$), a one-way ANOVA determined that there was a significant difference between groups ($F(2,7) = 8.686$, $p = 0.0127$). A post hoc Tukey multiple comparison found a significant difference between DDHU and KHU ($p = 0.0127$, adjusted for multiple comparisons).

![Table 6.1 Binding rates of MDV US3-GFP and microtubule motors. Percentages indicate the proportion of MDV US3 colocalising with dynein alone (DU), dynein complexed with dynactin and Hook3 (DDHU) or in the case of unlabelled KIF1C, Hook3 (KHU).](table.png)
Figure 6.2 Microtubule transport of MDV US3. Representative kymographs of MDV US3-GFP with microtubule motors and EB3. A: dynein heavy chain (DHC) tagged with tetramethylrhodamine (TMR). B: DHC-TMR complexed with unlabelled dynactin and AlexaFluor 647 labelled Hook3 (Hook3-647). C: Unlabelled KIF1C complexed with Hook3-647. D: EB3-mCherry. Plus and minus signs indicate direction of microtubule based on the direction of motor or in the case of EB3, the rapidly polymerising end of the microtubule.
6.3 Discussion

MDV US3, for the first time, is seen to interact with microtubule motors directly. Both the anterograde and retrograde transport of US3 has been observed in interaction with dynein and KIF1C, respectively. The location of US3 at the inner tegument allows it to interact with motors when the virus is transported to the nucleus and during egress before complete envelopment. It is unlikely that US3 acts as the sole tether between the virus and these motors, given that MDV US3 deletion mutants are capable of reaching the nucleus for replication (Schumacher et al., 2005). The interactions seen here, however, may very well alter the recruitment or activation of motors, which is yet to be explored.

KIF1C is an 1103 amino acid long plus-end-directed kinesin which is primarily involved in the transport of vesicles from the Golgi apparatus to the endoplasmic reticulum and contributes to the Rab6A vesicle motility to the cell surface (Dorner et al., 1998; Lee et al., 2015). Rab6A is a key regulator of the plasma membrane-directed secretory pathway and is indeed present on alphaherpesvirus secretory vesicles (Hogue et al., 2014). It is likely, therefore, that KIF1C may contribute to the plus-end directed transport of alphaherpesviruses. Indeed, the kinesin-3 family, which KIF1C is a member of, contributes to the fast axonal transport of alphaherpesviruses within neurons (Kramer et al., 2012).

The direct interaction between MDV US3 and KIF1C is particularly interesting, as US3 is located at the inner tegument and is not expected to be exposed on the exterior of a vesicle. For alphaherpesviruses, the interaction with kinesin-3 family members has been attributed to the viral envelope proteins US9 and UL56 (Kramer et al., 2012; Daniel et al., 2016). However, both of these are dispensable for anterograde transport, suggesting that an alternative mechanism exists (Daniel et al., 2015; Daniel et al., 2016). For MDV, secretory vesicles or enveloped capsids do not dominate viral egress, with electron microscopy revealing that approximately 75% of capsids outside the nucleus are without envelopes, but it is unclear if these are associated with microtubules via motors (Tut, 2018). In addition to this, electron microscopy
studies of HSV-1 have shown the presence of capsids wrapped in membranes, with the capsid partially exposed (Buch et al., 2017). Together, these observations give rise to the possibility, at least for MDV, egress of virions can occur without the envelope, through the direct interaction of tegument proteins, such as US3, with motors.

In multiple cell types, KIF1C cargoes have been observed to move in a bi-directional manner, as KIF1C and dynein can exist in the same complex with the Hook3 scaffold protein (Siddiqui et al., 2019; Kendrick et al., 2019; Schlager et al., 2010). Further study is required to determine if the difference in affinity displayed by MDV US3 would alter in any way the bi-directional movement of KIF1C-dynein complexes and whether US3 shows preferential binding to other motors. It remains to be determined what similarity exists, if any, between KIF1C and dynein or other motors that allows for the shared interaction with MDV US3. KIF1C, dynein heavy chain and EB3 are all regulated by the phosphorylation of serine or threonine residues (Dorner et al., 1999; Runnegar et al., 1999; Ferreira et al., 2013; Dillman & Pfister, 1994; Komarova et al., 2012). No identified phosphoserine or phosphothreonine residues match the US3 consensus sequence as identified by Leader et al. (1991). It should be noted that the alphaherpesvirus US3 has been observed to phosphorylate residues that do not match the consensus (Mou et al., 2007). Despite this, the substrate specificity of the alphaherpesvirus US3 overlaps that of protein kinase A (PKA) (Benetti & Roizman, 2004). Indeed, PKA is known to interact with and modulate the motility of dynein and kinesin family motors (Reilein et al., 1998; Lee & Hollenbeck, 1995; Sato-Yoshitake et al., 1992) It remains yet to be determined if PKA interacts with EB3 or not.

The calculated binding rates highlight a significant difference in the binding of MDV US3 between DDHU and KIF1C. While an increase was seen with the addition of Hook3 to dynein, it was not statistically significant. The preference of US3 to interact with dynein rather than KIF1C could suggest that US3, in isolation, predominantly plays a role in minus-end directed transport.
Alternatively, it may suggest that the interaction with MDV US3 could alter the landing rate of motors on to microtubules. However, due to the low depth penetration (~100 nm), TIRF microscopy does not examine any protein interactions that occur in-solution that is not immediately adjacent to the coverslip. The co-localisation observed during TIRF microscopy warrants further biochemical characterisation by MST of the interaction between GFP-tagged MDV US3 with microtubule motors and their associated proteins.

7 General discussion and future directions

Herpesviruses remain a significant challenge to the health of humans and domestic animals. MDV is a significant economic burden, to which the current vaccination strategy is likely to be unsustainable. With poultry being the most affordable, and least environmentally impacting animal product available, its demand is forecasted to increase continually. The biosecurity of this food source, is, therefore, becoming increasingly important. Advancing our understanding of MDV by identifying and characterising virus-host interactions will enable us to develop new alternative interventions against MDV. The multifunctional US3 kinase is found in the genome of all members of the alphaherpesvirus family. The screen for the potential interaction partners and the biochemical assays carried out in this study highlight the increasingly apparent variation of substrates and function.

Schumacher et al. (2008) suggested that unlike other members of the alphaherpesvirus family, MDV US3 could remove actin stress fibres in a kinase-independent manner. We confirm this finding and propose a possible mechanism of this function. Using a BioID2 screen, and subsequent biochemical assays, we confirm and highlight the previously unknown interaction between MDV US3 and the F-actin crosslinker alpha-actinin. Further to this, we demonstrate that enzymatically inactive US3 can interfere with F-actin bundling. Unrelated to the actin cytoskeleton but still of particular interest, this study has shown the direct interaction between MDV US3, dynein
and KIF1C. The main findings of this study and their future directions of research are discussed below.

**Candidate interaction partners of MDV US3**

To identify potential interaction partners that may not necessarily be enzymatic substrates of MDV US3, we employed a BioID2 screen in the biologically relevant DF1 chicken fibroblast cell lines. While alpha-actinin and dynein were chosen for further study, there are still a significant number of candidates that warrant further exploration and may provide greater insight into the function of MDV US3.

The BioID2 screen has identified Transportin 1 (Importin β2). Through immunofluorescence microscopy and co-immunoprecipitation, Transportin 1 has been found to be involved in the nuclear import of viral nuclear egress protein UL31, a known interactor of US3 (Cai et al., 2016; Mou et al., 2009). UL31, when complexed with UL34, accumulates at the inner nuclear membrane. It is thought that these complexes interact with nucleocapsids and result in budding events. Phosphorylation of UL31 by US3 regulates the localisation of the UL31/UL41 complex. UL31 is known to have two NLS in the N-terminus of the protein. The second N-terminus NLS is preceded by serine residues which have been identified as a US3 phosphorylation site (Mou et al., 2009). The function of US3 phosphorylation of this NLS has not been investigated. Generally, it is understood that the phosphorylation of an NLS can either inhibit or activate nuclear transport. If phosphorylation of the UL31 NLS is indeed required for nuclear localisation and correct function of the UL31/UL34 complex, it could explain the accumulation of nucleocapsids in the perinuclear space as observed by Schumacher et al. (2008) in CEFs infected with MDV expressing only enzymatically inactive US3.

Another protein of interest that was identified in the BioID2 screen is HSP90B1, an essential chaperone involved in both the innate immune system, where it is involved in the maturation of Toll-like receptors (TLRs) (Liu et al., 2010). TLRs recognise molecular signatures of pathogenic microorganisms, including viruses, and when activated, a subset of TLRs
trigger the production of antiviral cytokines (Akira & Takeda, 2004). It is already known that HSV-1 US3 is able, through an unknown mechanism, to inhibit Toll-like receptor 2 from maturing and reaching the cell surface at or before ubiquitination by TRAF6 (Sen et al., 2013). Indeed, the involvement of HSP90B1 located in the endoplasmic reticulum is before TRAF6 ubiquitination which occurs at the mitochondria and plasma membrane (Koch et al., 1986; Hu & Sun, 2016). It is possible, therefore, that US3 may interfere with proper folding and maturation of TLRs within the Golgi apparatus, preventing their expression on the cell surface and any subsequent immune response.

It is notable that several known substrates of the alphaherpesvirus US3 were not identified in the BioID2 screen. A recent search of the literature has found that no substrates have yet been confirmed as specific interactors for MDV US3. The current understanding of MDV US3 is based on functional studies, with parallels drawn to other US3 orthologues. While several interaction partners of US3 orthologues have been confirmed, these have not been identified in the MDV US3 BioID2 screen. For PRV US3, direct interactions have been found with Group A p21-activated kinases PAK1 and PAK2 (Van den Broeke et al., 2009b). A study with HSV-1 US3 highlighted the direct interaction with Lamin A/C (Mou et al., 2007). Both of these interactions have been confirmed using in vitro kinase assays with purified proteins.

The BioID approach is regarded as being particularly useful for identifying kinase substrates and components of signalling networks due to its ability to identified highly transient interactions (Varnaitė & MacNeill, 2016; Zhou et al., 2015). Despite this, the BioID and BioID2 approaches suffer from slow kinetics, requiring >16h of labelling to produce sufficient quantities of biotinylated proteins for analysis (Branon et al., 2018; Kim et al., 2016). This creates a challenge in identifying proteins with short half-lives. PAK1, in HEK293-T cells, has a half-life of approximately 6 hours, which is then reduced to 1.5 hours upon activation or autophosphorylation (Weisz Hubsman et al., 2007). It is possible, therefore, that proteins with short half-lives such as PAK1 may not be detected. Such limitations could be overcome by using a promiscuous biotin ligase with a much higher kinetic activity such as TurboID.
BiolD2 was chosen for its small size, which is thought to improve the proper subcellular targeting. However, asides from nuclear import receptors, no proteins within the nucleus such as lamin A/C have been identified. When FLAG- and BiolD2- tagged wild-type MDV US3 are expressed in A7r5 cells, the cytoplasmic localisation and localisation to F-actin was visually similar. It was, however, not determined if the addition of the BiolD2 tag may have altered the nuclear-shuttling properties of MDV US3. Even so, the highest similarity by amino acid sequence between MDV US3 and the US3 orthologue of other major alphaherpesviruses is 31 % (HSV1) (Deruelle & Favoreel, 2011). Because of this, one cannot exclude the possibility that the difference seen in identified substrates partly due to the virus-specific variation of US3 and its interaction partners.

**Interaction between MDV and alpha-actinin**

As with most virus-host interactions, it is likely that the interaction with alpha-actinin results in enhanced replication. Previous work by Schumacher et al. (2008) characterised MDV mutants with kinase-dead US3 as having reduced viral growth. However, the presence of actin stress fibres within cells infected with kinase-dead US3 mutants has not been examined. It is quite possible that the kinase-independent remodelling of the actin cytoskeleton is a result of the overexpression of MDV US3. Further study using MDV mutants is required to determine whether the expression of kinase-dead MDV US3 at levels found during infection would inhibit alpha-actinin at a whole-cell level or only in the vicinity of a virion. Both of these outcomes would have significant implications in MDV infection.

Further study is required to determine whether the interaction of kinase-dead MDV US3 and alpha-actinin has a similar effect to that seen in uninfected cells where alpha-actinin has been depleted. In cells where alpha-actinin-1 or alpha-actinin-4 have been knocked down, cells often show reduced motility, but a higher number of cellular protrusions (Shao et al., 2010; Stanley et al., 2008). Specifically for T-cells, alpha-actinin is required for
adhesion to intercellular adhesion molecule-1 (ICAM1) (Stanley et al., 2008). MDV is a strictly cell-associated virus and is transferred from one cell to another by direct contact from protrusions. It stands to reason that the inhibition of alpha-actinin may be a mechanism to promote the formation of cellular protrusions for viral spread. It is unknown whether the lack of migration or in the case of T-cells, the additional lack of adhesion would be counterproductive for viral spread. Indeed, in the current model of MDV infection, migration of latently infected T-cells is crucial for the transport of the virus to the feather follicle epithelium (Calnek, 2001).

The interaction between MDV US3 and alpha-actinin may have effects in the immediate environment of a virion. Of particular interest is during initial infection where the cortical actin barrier, containing alpha-actinin, must be overcome. While the MDV genome contains the alphaherpesvirus glycoprotein D, it is not expressed in cell culture; and when it is expressed, it is non-functional (Tan et al., 2001; Anderson et al., 1998; Zelnik et al., 1999). The alphaherpesvirus glycoprotein D, during viral entry, interacts with nectins to induce actin cytoskeletal change through the modulation of Ras and Rho GTPases (Clement et al., 2006). Further to this, the interaction between gD and nectins triggers a local release of Ca\[^{2+}\] at the plasma membrane (Cheshenko et al., 2007). However, it is when there is a global release of intracellular Ca\[^{2+}\] brought about by the full complement of viral glycoproteins that viral entry occurs (Cheshenko et al., 2007; Cheshenko et al., 2013). If the release of calcium is inhibited, the fusion of the viral envelope occurs but, virions remain trapped at the plasma membrane due to the cortical actin barrier (Cheshenko et al., 2007).

The strictly cell-associated nature of MDV raises the question of whether the traditional alphaherpesvirus entry glycoproteins and their cell surface receptors are required, and if there is an alternative mechanism to change the morphology of the cortical actin barrier. Although MDV US3 is not critical for viral entry, it may, however, play a role in the relaxation of the cortical actin barrier to promote viral access to the cytoplasm. It has been shown that calcium increases the affinity between MDV US3 and alpha-actinin, but the
interaction can also occur in its absence. This observation would suggest that the ABD of alpha-actinin transiently enters the non-actin-binding conformation that interacts with MDV US3, and the presence of calcium through its action with the calmodulin-like domain favours this. It is, therefore, possible, that the direct interaction keeps the ABD in the non-actin-binding state and mimic the function of an increase in local Ca\(^{2+}\) concentration as seen during the interaction of gD and nectin.

Through \textit{in silico} experiments, MDV US3 is predicted to interact with the actin-binding domain of alpha-actinin whilst it is in a non-actin binding state brought about the presence of calcium. While several protein interactions with alpha-actinin have been mapped to the actin-binding domain, little is known whether they are promoted by the presence of calcium. Despite this, the actin-binding domain of alpha-actinin-4 is known to interact in a calcium-dependent manner with the second extended PDZ domain of the cystic fibrosis transmembrane conductance regulator E3KARP (Kim \textit{et al.}, 2002). It remains yet to be determined if MDV US3 has any similarity in the binding mechanism.

From the docked model, a list of MDV US3 amino acids that are candidates for future mutagenic study has been generated. Indeed, while these may help characterise the interaction, further structural studies are required to define an accurate structure of the US3 alpha-actinin complex. This would validate the current structural model and amino acids predicted to be involved binding. An accurate structure of the complex would enable research into inhibitors or genetically engineered interventions against this virus-host interaction. Multiple alignment of the candidate residues identified in this study appears to be conserved in MDV vaccine strains, but also HVT. It would be interesting to determine whether HVT US3 could also remove stress fibres in a kinase-independent way.

Tandem CH domains are found in a wide range of actin-binding proteins, particularly those within the spectrin superfamily. From this, the question arises whether MDV US3 could interact with such proteins in a similar
mechanism to that with alpha-actinin. Within the spectrin superfamily, similar to the non-muscle isoforms of alpha-actinin, the α-subunit of the non-erythroid spectrin heterodimer contains a calcium-sensitive calmodulin-like domain that is adjacent to the tandem CH-domains of the opposite β-subunit (Brown et al., 2015; Travé et al., 1995b; Travé et al., 1995a). The tandem CH-domain of chicken alpha-actinin-4 shares 67% amino acid similarity with the tandem CH-domain of chicken β-spectrin. Further examination highlights that the amino acids of chicken alpha-actinin-4 which contribute to the positively charged surfaces that interface with MDV US3 1-111 (R168, K169, R197, R273) are found to be conserved or substituted with similar negatively charged residues within chicken β-spectrin. As spectrin crosslinks actin underlying the plasma membrane, it may indeed have implications in viral entry and exit. Because of this, the possible inhibition of spectrin-mediated F-actin crosslinking by MDV US3 would warrant future investigation.

**Translocation of alpha-actinin during expression of MDV US3**

In untransfected cells, the nuclear translocation of alpha-actinin-4 can be replicated by the inhibition of phosphoinositide 3-kinase or the drug-mediated depolymerisation of F-actin (Honda et al., 1998). The nuclear translocation of alpha-actinin-4 has been functionally mapped to the rod domain that contains spectrin repeats (Kumeta et al., 2010). Together, these observations would suggest that the nuclear translocation of alpha-actinin-4 is inhibited by the interaction with the actin cytoskeleton.

The expression of wild-type or kinase-dead MDV US3 in RPE cells leads to the nuclear accumulation of mNeonGreen-tagged alpha-actinin-4. Further to this, the expression of kinase-dead MDV US3 resulted in the nuclear translocation of alpha-actinin-4 while the actin stress fibres were present in the cell. This gives rise to two distinct mechanisms for nuclear translocation and the removal of actin stress fibres. First, a conserved mechanism where US3 triggers the cellular loss of F-actin through the dephosphorylation and activation of the F-actin severing protein cofilin (Jacob et al., 2013). Second, a kinase-independent inhibition of the interaction between alpha-actinin and F-actin through direct interference by MDV US3.
Within the nucleus, alpha-actinin-4 has been reported to up-regulate genes involved in cell proliferation, survival, motility and the epithelial-mesenchymal transition (Khurana et al., 2011; Honda et al., 1998). Several studies have described the interaction between alpha-actinin-4 and the RelA/p65 subunit of NF-κB, which resulted in NF-κB-mediated transcription (Aksenova et al., 2013; Babakov et al., 2008). The persistent activation of NF-κB has been observed during alphaherpesvirus infection, but the mechanism of such activation is not fully understood (Patel et al., 1998; Romero et al., 2020). In HSV-1 infection, NF-κB activation is bi-phasic (Amici et al., 2006). An initial, rapid phase of activation (0-2 hours post-infection) is thought to be triggered upon viral entry through the interaction of the virus with a cell surface receptor of the tumour necrosis factor receptor (TNFR) superfamily. In later stages (3-4 hours post-infection), persistent NF-κB activation occurs. Given that several alphaherpesvirus genes contain NF-κB promoters, it is hypothesised that this is a mechanism to activate transcription of some viral intermediate-early genes (Amici et al., 2006).

The exact role of the alphaherpesvirus US3 in NF-κB modulation remains unclear. For HSV-1, US3 is reported to hyperphosphorylate RelA/p65 to prevent it from translocating to the nucleus and activating NF-κB (Wang et al., 2014). On the other hand, for PRV, expression of US3 results in NF-κB activation through the phosphorylation and release of the NF-κB inhibitor I-κBα (Chang et al., 2013). Disruption of the actin cytoskeleton by cytochalasin D also results in the phosphorylation of I-κBα and translocation of activated RelA/p65 to the nucleus (Kustermans et al., 2005). This observation, together with the findings here, would suggest that the phosphorylation and release of I-κBα reported by Chang et al. may not necessarily be the result of a direct interaction of US3, but could very well be a result from the loss of stress F-actin through the US3-mediated phosphorylation of PAK1/2 and activation of cofilin. While the difference in reported NF-κB activation by HSV-1 and PRV US3 appears to be contradictory, it is not known how much of this difference is due to virus-specific substrates of US3, expression levels of US3 or the stage of viral infection.
Alpha-actinin-4 has been shown to regulate the activity of HDAC7, a class II HDAC (Chakraborty et al., 2006). Under normal conditions, histone deacetylases repress transcriptional activity by binding to and inhibiting several transcription factors. Specifically, HDAC7 inhibits the transcriptional activation activity by myocyte enhancer factor 2 (MEF2). It has been demonstrated that alpha-actinin-4 and HDAC7 share binding sites on MEF2. Consequently, alpha-actinin-4 can antagonise the repressive action of HDAC7 on MEF2. MEF2 is involved in the regulation of cell survival, apoptosis and morphogenesis in a wide range of cell types (Potthoff & Olson, 2007). It is becoming increasingly evident that alphaherpesviruses interfere with the regular transcriptional activity of the cell, by interacting with the acetylation pathway or transcriptional co-activators. Several direct interactions between transcriptional regulators and viral proteins have been already identified, such as ICP0 with HDAC5 and US3 with HDAC2 (Lomonte et al., 2004; Walters et al., 2010). Despite this, very little is known if any indirect interactions with US3 exist.

The finding that alpha-actinin-4 translocates to the nucleus in cells that show a loss of stress fibres, together with our current knowledge of the role that alpha-actinin-4 plays as a transcriptional co-activator suggests a previously undescribed link between the remodelling of the actin cytoskeleton and transcriptional regulation in alphaherpesvirus infection. This mechanism is hypothesised to be conserved amongst alphaherpesviruses alongside the kinase-dependent ability of US3 to remodel the actin cytoskeleton. While this requires further investigation, these interactions combined are thought to contribute towards the inhibition of apoptosis and to maintaining an environment within the nucleus that favours viral transcription and replication. It remains yet to be determined if monomeric actin itself or other actin-binding proteins have potentially similar roles in the nucleus when cells exhibit a loss of stress fibres or F-actin.

**MDV US3 and microtubule motors**

Upon alphaherpesvirus entry, the viral capsid releases the majority of the envelope and tegument proteins into the host cytoplasm. An exception to this
are the inner tegument proteins US3, UL37 and VP1/2. Together these form the retrograde transport complex of the virus and are thought to interact with dynein and kinesin motors (Smith, 2012). While the deletion of US3 appears to be dispensable for the retrograde transport of the virus, it does not preclude the role of US3 from promoting or regulating microtubule-based transport (Smith, 2012; Schumacher et al., 2005). We have shown using purified proteins that US3 is capable of being co-transported along microtubules through the direct interaction with dynein and the kinesin-3 family motor KIF1C. It is plausible that MDV US3, directly and indirectly, contributes to the phosphorylation of these motors during infection. US3 is capable of activating and overlaps the substrate specificity of protein kinase A (PKA), which regulates the motility of dynein and kinesins (Benetti & Roizman, 2004; Reilein et al., 1998; Lee & Hollenbeck, 1995; Sato-Yoshitake et al., 1992).

There is significant evidence that particular serine and threonine residues of dynein subunits are phosphorylated in vivo, and that this is a mechanism of dynein regulation. The interaction between PKA with dynein results in its disassociation of dynein from membranous cargoes (Reilein et al., 1998; Kashina et al., 2004). Similarly, the phosphorylation of dynein heavy chain has been attributed in the release of dynein associated with cellular membranes (Lin et al., 1994). In hepatocytes, increased phosphorylation of dynein intermediate and heavy chains has been seen to result in decreased ATPase activity and subsequent motor function (Runnegar et al., 1999). On the contrary to this, within neurons, increased heavy chain phosphorylation has been reported to increase motor activity (Dillman & Pfister, 1994).

The purpose of the interaction between MDV US3 and dynein remains yet to be determined. One could speculate that similar to the role of PKA in the adenovirus recruitment of dynein, MDV US3 could relocate and activate membrane-bound dynein for the transport of the capsid to the nucleus during the initial stages of infection (Scherer et al., 2014). Further study is required to determine whether MDV US3 could alter the velocity of dynein. The average retrograde speeds of moving capsids of a PRV US3 knockout-mutant have been measured to be slightly slower (1.64 ± 0.08 µm/s) than the wild-type virus.
(1.91 ± 0.07 µm/s), with no significant difference between other tegument knock-out mutants (Antinone et al., 2006). It is not possible to exclude that if US3 did activate and alter the velocity of dynein, there might be redundant mechanisms that use other viral proteins, given the importance of retrograde transport in alphaherpesvirus replication.

While it is well known that dynein and kinesins transport the viral capsid to and from the MTOC during entry and egress, very little is known about the transport between the MTOC and nucleus, where directionality must be reversed. If viral transport occurred through the interaction of single, as opposed to motors within a bi-directional complex, it is expected that kinesins must interact directly with the capsid or with inner tegument proteins. Indeed, it has been found that kinesin-1 and kinesin-2 can bind directly to the HSV-1 capsid (Radtke et al., 2010). US11 is the only HSV-1 tegument so far that has been attributed with kinesin-1 binding properties; however, HSV-1 US11-null mutants were capable of still interacting with kinesin-1, suggesting that alternative mechanisms exist (Diefenbach et al., 2002; Radtke et al., 2010). While MDV US3 is seen to interact with KIF1C, MDV US3-null mutants are still capable of arriving at the nucleus to replicate; suggesting that MDV US3, similarly to HSV-1 US11, does not have a critical role transport (Schumacher et al., 2008). While the interactions of US11 and US3 with kinesins are not crucial, they may indeed be involved in promoting motor recruitment or changing motor behaviour in a way that promotes viral spread.

The experiments here have primarily looked at the interaction of MDV US3, dynein and kinesin motors in isolation. The transport of cargo by dynein or kinesins within a cell often requires adaptor proteins. These proteins can activate motors and tether them to specific cargos (Reck-Peterson et al., 2018; Guo et al., 2016; Schlager et al., 2014b; Setou et al., 2000). In addition to this, adaptor proteins such as Hook3 can act as a scaffold for both dynein and kinesin motors, forming a bidirectional complex (Kendrick et al., 2019; Siddiqui et al., 2019). It is thought that this allows for directional switching if roadblocks are encountered. Previous studies have shown that HSV-1 capsids are transported by dynein in a dynactin dependent manner in vitro
(Wolfstein et al., 2006). Indeed, except for nucleus derived capsids, dynactin alone can bind to capsids through an unknown viral receptor suggesting that it is involved in viral entry (Wolfstein et al., 2006). The extent that this interaction is conserved through the alphaherpesvirus family remains unknown. Dynactin is recruited to dynein through an activating adaptor such as BICD2 (Schlager et al., 2014b; Urnavicius et al., 2015). Dynactin or any activating adaptors were not identified as a candidate interaction partner of MDV US3. However, the identified dynein heavy chain is considerably much larger than any adaptor protein, and depending on the sensitivity of the BioID2 screen, may mask any such interactions. Therefore, it is not possible to exclude that MDV US3 may be involved in adaptor recruitment or alter the behaviour of the dynein-adaptor complex and as such warrants further study.

Work by Schumacher et al. (2008) highlighted that MDV mutants with kinase-inactive MDV US3 displayed impaired growth properties which have been attributed to virions accumulating at the periphery of the nucleus. Given the finding that MDV US3 interacts with dynein and kinesin motors, there is the possibility that MDV US3 may function to enhance recruitment of motors and their activity. It would, therefore, be worthwhile investigating whether the loss of US3 kinase activity contributes to reduced growth by reducing viral transport.

**Conclusion**

This study expands our current knowledge of MDV US3 by exploring its potential interactome to understand its function in actin cytoskeleton remodelling. Non-muscle alpha-actinin has been identified and confirmed as an interaction partner of MDV US3. The kinase-independent interaction has been further characterised to be inhibitive to the crosslinking function of alpha-actinin and is enhanced by the presence of calcium. It is predicted that MDV US3 interacts with alpha-actinin through its N-terminus region that binds to the ABD of alpha-actinin, locking it into a conformation that does not bind to F-actin. From this, two mechanisms for stress fibre disassembly are proposed (Figure 7.1): a kinase-dependent depolymerisation of F-actin by cofilin that is conserved amongst all alphaherpesviruses, and a kinase-independent
inhibition of alpha-actinin mediated F-actin bundling that is specific to MDV. Both of these mechanisms result in the loss of actin stress fibres. Further to this, we propose that the loss of F-actin and inhibition of F-actin binding causes the translocation of alpha-actinin-4 to the nucleus where it may act as transcriptional co-activator that promotes viral replication.

The screen of candidate interactors also highlighted the microtubule motor protein dynein. Using fluorescent single-molecule motility assays, it has been shown that kinase-dead MDV US3 directly interacts with kinesin and dynein motors, highlighting a potential new role for MDV US3 in viral transport. Together, the findings of this study determine the mechanism for kinase-independent removal of stress fibres and identify new host interactors of MDV US3. The study highlights the presence of virus-specific interactors for the alphaherpesvirus US3 kinase and poses the question of whether such virus-specific interactions are more commonplace than initially thought.
Figure 7.1 Proposed kinase- and kinase-independent mechanisms of remodelling the actin cytoskeleton by MDV US3. Both result in the loss of actin stress fibres, but for different reasons. The kinase independent mechanism specific to MDV, US3 directly interacts and inhibits the function of alpha-actinin. The kinase dependent mechanism results in the depolymerisation of F-actin by the activation of cofilin. The lack of F-actin or the inhibition of F-actin binding causes alpha-actinin-4 to translocate to the nucleus where it is proposed to act as a transcriptional co-activator.
8 Bibliography


