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Review

The role of signalling and the cytoskeleton during Vaccinia Virus egress

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ABSTRACT

Viruses are obligate intracellular parasites that are critically dependent on their hosts to replicate and generate new progeny. To achieve this goal, viruses have evolved numerous elegant strategies to subvert and utilise the different cellular machineries and processes of their unwilling hosts. Moreover, they often accomplish this feat with a surprisingly limited number of proteins. Among the different systems of the cell, the cytoskeleton is often one of the first to be hijacked as it provides a convenient transport system for viruses to reach their site of replication with relative ease. At the latter stages of their replication cycle, the cytoskeleton also provides an efficient means for newly assembled viral progeny to reach the plasma membrane and leave the infected cell. In this review we discuss how Vaccinia virus takes advantage of the microtubule and actin cytoskeletons of its host to promote the spread of infection into neighboring cells. In particular, we highlight how analysis of actin-based motility of Vaccinia has provided unprecedented insights into how a phosphotyrosine-based signalling network is assembled and functions to stimulate Arp2/3 complex-dependent actin polymerization. We also suggest that the formin FHOD1 promotes actin-based motility of the virus by capping the fast growing ends of actin filaments rather than directly promoting filament assembly. We have come a long way since 1976, when electron micrographs of vaccinia-infected cells implicated the actin cytoskeleton in promoting viral spread. Nevertheless, there are still many unanswered questions concerning the role of signalling and the host cytoskeleton in promoting viral spread and pathogenesis.

Contents

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1. Introduction

The hijacking of the cytoskeleton is a common strategy employed by viruses infecting virtually all organisms including bacteria, plants and animals (Dodding and Way, 2011; Erb and Pogliano, 2013; Niehl et al., 2013; Taylor et al., 2011). However, one of the most striking examples of viral subversion of the host actin and microtubule cytoskeletons occurs during Vaccinia virus infection (Dodding and Way, 2011; Welch and Way, 2013). Vaccinia is a large double stranded DNA virus, which replicates exclusively in the cytoplasm of infected cells and is the most studied member of the Orthopoxviridae (Moss, 2007). Vaccinia is perhaps best known for its use as the vaccine to protect against smallpox, a deadly human disease caused by its close relative Variola virus (Jacobs et al., 2009; Walsh and Dolin, 2011). Smallpox was eradicated more than 30 years ago. Nevertheless, Vaccinia is increasingly being used as a vaccine vector for a wide range of different diseases as well as for oncolytic therapies (Jacobs et al., 2009; Kirn and Thorne, 2009; Thorne, 2011; Volz and Sutter, 2013; Walsh and Dolin, 2011). The vaccinia virus genome consists of ~200 kbp encoding for some 260 proteins, only about 80 of which, end up in infectious intracellular mature virus (IMV) particles (Chung et al., 2006; Resch et al., 2007; Yoder et al., 2006). This large coding capacity, which allows Vaccinia to infect and replicate in many different cell types, is in part due to its complex replication cycle, which involves the assembly of two morphologically distinct types of cytoplasmic virus particles (Fig. 1). The large genome also reflects the prodigious number of viral proteins Vaccinia uses to inhibit or suppress the antiviral activity of its host at all stages of its replication cycle (Haller et al., 2014). This includes inhibiting apoptosis of infected cells before new viral progeny are assembled and minimizing detection by the host immune system (Bahar et al., 2011; Mohamed and McFadden, 2009; Postigo and Ferrer, 2009).

After binding to the cell membrane, virus entry occurs either by direct fusion with the plasma membrane (Carter et al., 2005; Law et al., 2006) or by low-pH endosomal entry pathway (Huang et al., 2008; Townsley et al., 2006). Moreover, Vaccinia actually promotes its uptake by stimulating actin-dependent macropinocytosis (Mercer and Helenius, 2008; Mercer et al., 2010; Schmidt et al., 2011). Having gained access to the cell cytoplasm, expression of early proteins is initiated allowing viral cores to uncoat and release their DNA (Kilcher et al., 2014; Mercer et al., 2012; Schmidt et al., 2013). This early protein expression is required for viral DNA replication, which occurs in viral factories located in a perinuclear region near the microtubule-organizing centre of the infected cell (Ploubidou et al., 2000; Roberts and Smith, 2008). Only after DNA replication does intermediate and late gene expression start, resulting in the assembly of intracellular mature virus (IMV) particles. IMV which represent the majority of viral progeny are infectious but are only released when the infected cell undergoes lysis (Roberts and Smith, 2008). Alternatively, some IMV can become intracellular enveloped virus (IEV) by being 'wrapped' by membrane cisternae derived from trans-Golgi or endosomal compartments containing a subset of viral proteins (Roberts and Smith, 2008; Smith et al., 2002). The molecular basis for this envelopment remains to be established, but it involves multiple integral viral membrane proteins as well as the Vaccinia E2 and F12 proteins (Dodding et al., 2009; Domi et al., 2008; Roper et al., 1998; Röttger et al., 1999; Sanderson et al., 1998; Smith et al., 2002; Wolffe et al., 1997; Wolffe et al., 1998). Once formed, IEV are transported to the cell periphery on microtubules by kinesin-1 before fusing with the plasma membrane (Fig. 1). Fusion of IEV with the plasma membrane results in two outcomes that have a different impact on the subsequent spread of infection. The extracellular enveloped virus (EEV), which are infectious and released from the cell, promote the long-range spread of vaccinia. Alternatively, after their fusion with the plasma membrane, some virions remain attached to the outside of the cell and are known as the cell-associated enveloped virus (CEV). It is the CEV that are responsible for the local actin-dependent cell-to-cell spread of vaccinia (Fig. 1). In this review, we will discuss our current understanding of how vaccinia uses and manipulates the cytoskeleton of the cell to enhance its spread.

2. Microtubule-based transport of Vaccinia

2.1. IMV and IEV move on microtubules

Microtubule-based transport is the primary way in which cargoes are moved over micron distances in a directed fashion throughout the cell (Franker and Hoogenraad, 2013; Fu and Holzbaur, 2014; Stephens, 2012). It is perhaps not surprising then that viruses have developed numerous strategies to take advantage of this cellular transport system at all stages of their infection cycles (Dodding and Way, 2011; Greber and Way, 2006; Radtke et al., 2006). Moreover, the ability of large viruses, such as Vaccinia, to hijack this rapid and efficient transport system is essential, as their size precludes their movement by diffusion (Greber and Way, 2006; Sodeik, 2000). For the virologist, examining how viruses use the microtubule cytoskeleton and its associated motors is necessary to understand how the infection cycle is established, as well as the mechanisms underlying viral replication, assembly and spread. For the cell biologist, the same analysis promises to uncover fundamental insights into the molecular basis of microtubule motor recruitment and regulation.

Immunofluorescence analysis of vaccinia-infected cells reveals that intracellular mature virions (IMV) are capable of dispersing from their peri-nuclear site of assembly throughout the cell at the latter stages of the viral replication cycle (Fig. 2). This movement to the cell periphery is more apparent in viral backgrounds that result in an absence of IEV formation (Fig. 2). The extent of viral dispersal in fixed samples provided the first suggestion that IMV are capable of moving on microtubules, as it has been calculated that they would only diffuse 10 μm in ~5 h (Sodeik, 2000). Subsequent live-cell imaging of infected cells demonstrated that YFP-tagged IMV are capable of undergoing rapid, linear movements at speeds approaching 3 μm/s (Ward, 2005). IMV movement is saltatory in nature and was abolished when cells were treated with the microtubule depolymerizing agent nocodazole. Direct imaging of IMV moving on microtubules still remains to be performed and the identity of the motor responsible for transporting IMV has yet to be established. In contrast, GFP-tagged IEV have been imaged rapidly moving in a microtubule dependent fashion as well as along microtubules towards the cell periphery prior to their fusion with the plasma membrane (Dodding et al., 2009; Geada et al., 2001; Herrero-Martinez et al., 2005; Hollinshead et al., 2001; Rietdorf et al., 2001; Ward and Moss, 2001a; Ward and Moss, 2001b). IEV movement is dependent on kinesin-1 (also known as Kif5B or conventional kinesin), which uses the power of ATP hydrolysis to transport cargoes towards the plus end of microtubules usually located at the cell periphery (Dodding and Way, 2011; Rietdorf et al., 2001).

2.2. The basis of kinesin-1 recruitment to IEV

Kinesin-1 is recruited to IEV through an interaction between the tetratricopeptide repeats (TPR) of its light chain (KLC) and A36, an integral IEV membrane protein that has a cytoplasmic domain of ~195 residues exposed on the surface of the virus (Röttger et al., 1999; van Eijl et al., 2000; Ward and Moss, 2004). Fluorescence resonance energy transfer (FRET) experiments have confirmed that

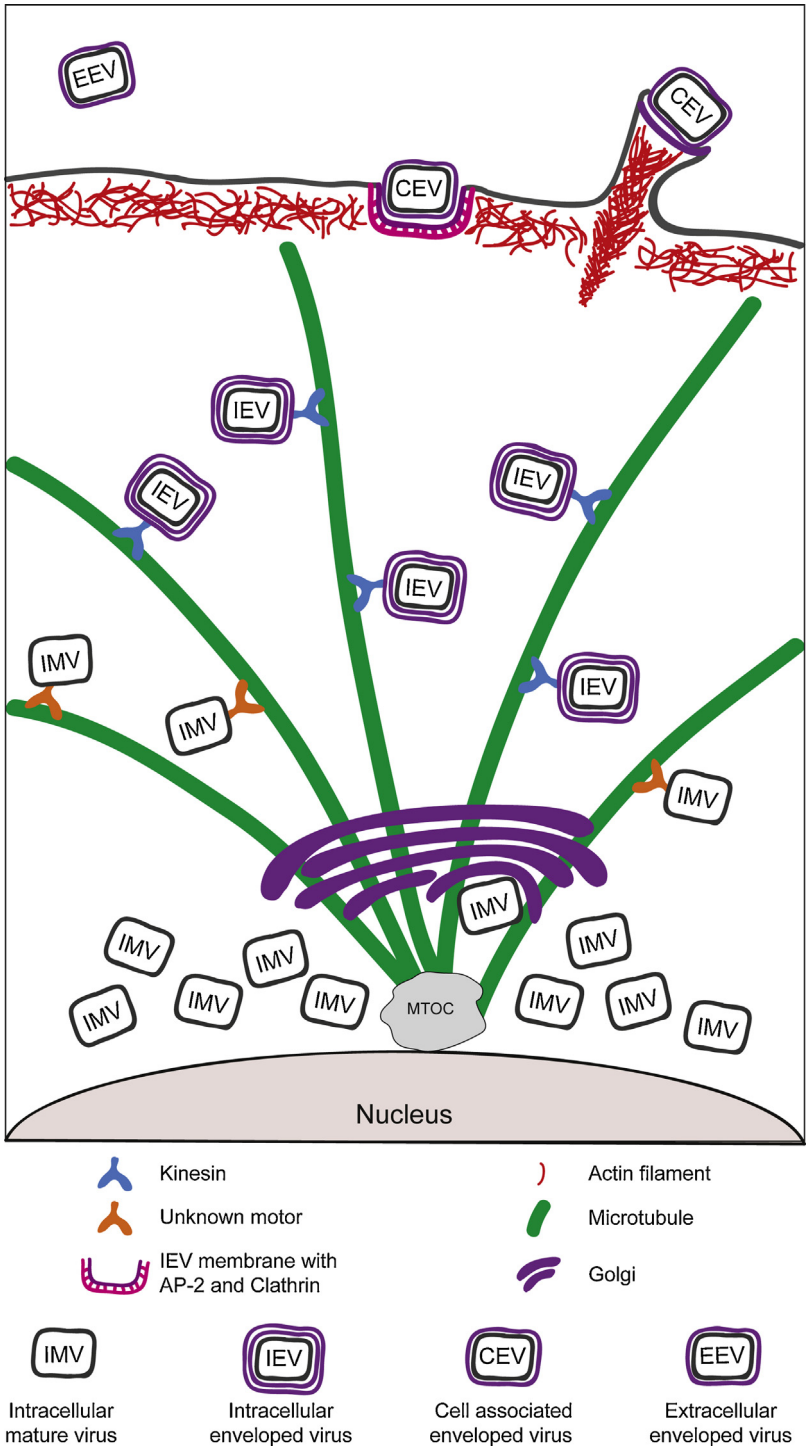


Fig. 1. Schematic representation of viral egress. Once assembled the intracellular mature virus (IMV) can be moved to the cell periphery on microtubules nucleated from the microtubule organizing center (MTOC). The motor responsible for this movement remains to be identified. Alternatively, a proportion of IMV can become enveloped by membrane cisternae derived from trans-Golgi containing a subset of viral proteins. This results in the formation of the intracellular enveloped virus (IEV), which are then transported to the cell periphery on microtubules by kinesin-1. On reaching the cell boundary, IEV traverse the cortical actin network before fusing with the plasma membrane. This fusion results in the formation of the cell associated enveloped virus (CEV). CEV, which remain attached to the cell transiently recruit AP-2 and clathrin prior to inducing actin polymerization and the assembly of an actin tail. Alternatively, CEV can be directly released from the cell to form the infectious extracellular enveloped virus (EEV).

A36 and KLC can interact with each other in infected cells (Jeshtadi et al., 2010). Deletion of the gene encoding A36 from the vaccinia genome has no impact on IEV formation (Röttger et al., 1999; Sanderson et al., 1998; van Eijl et al., 2000), but it does impair viral spread from their perinuclear site of assembly to the plasma membrane (Rietdorf et al., 2001; Ward and Moss, 2001a; Ward et al., 2003). Consequently, an absence of A36 during infection reduces the cell-to-cell spread of vaccinia, as the virus is less likely to reach and fuse with the plasma membrane (Ward and Moss, 2001a). Deletion of the gene encoding ECTV-Mos-42, an A36 orthologue in Ectromelia virus, the causative agent of mousepox, also results in reduced cell-to-cell spread (Lynn et al., 2012).

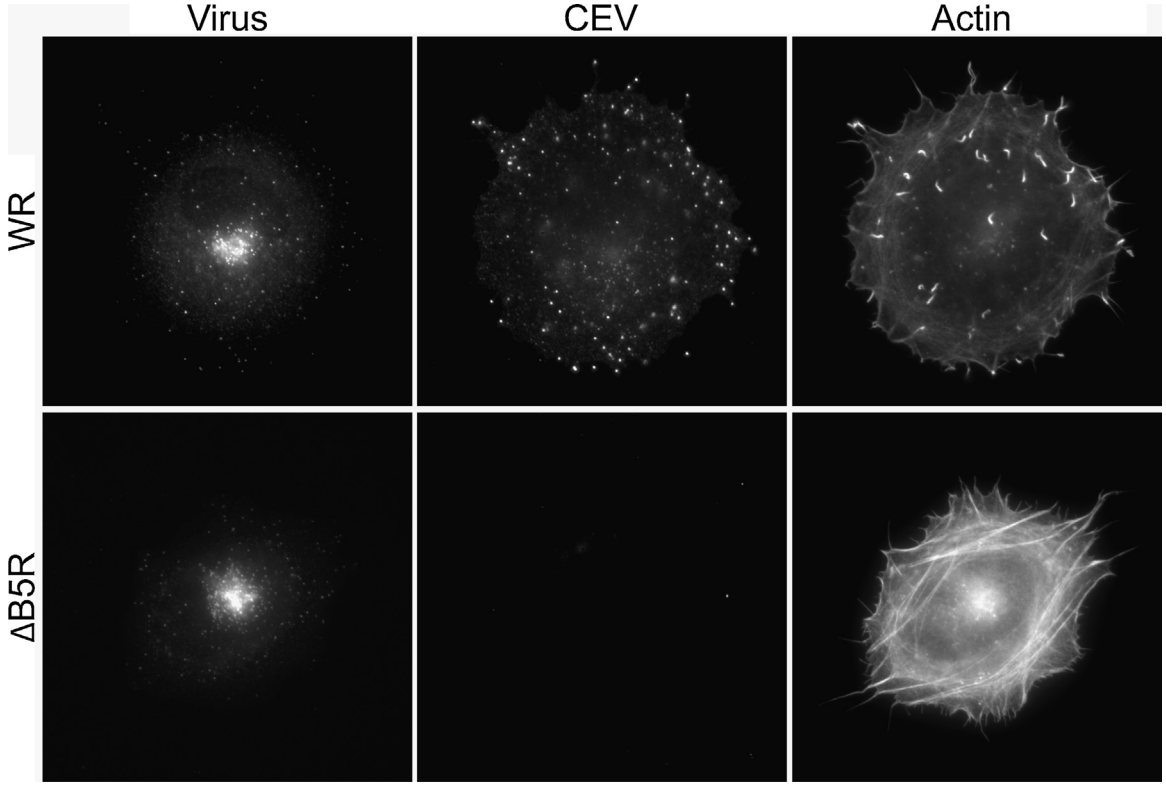


Fig. 2. Immunofluorescence images of HeLa cells infected for 8 h with WR or the recombinant ΔB5R virus, which does not form IEV as it lacks the viral protein B5. All forms of the virus are detected with an antibody against A27 (left). CEV are detected with an antibody against B5 (middle). The actin cytoskeleton is labelled with phalloidin, which only detects actin filaments (right). In WR infected cells, CEV induce the formation of comet shaped actin tails. In contrast, in the ΔB5R-infected cells there are only prominent actin stress fibres. Nevertheless, dispersal of IMV towards the cell periphery still occurs.

Using a combination of yeast two hybrid and *in vitro* GST pull-down assays, it was demonstrated that residues 81–111 of A36 were sufficient to bind the KLC tetratricopeptide repeats (Ward and Moss, 2004). Examination of this region of A36 reveals it contains a short WD motif present in the KLC binding site of Calsyntenin-1 (Araki et al., 2007; Dodding and Way, 2009; Konecna et al., 2006; Morgan et al., 2010). An additional KLC binding WE motif first identified in Caytaxin and γ-BAR (also known as Gadkin) (Aoyama et al., 2009; Schmidt et al., 2009) also occurs at residues 64–65 of A36 (Dodding et al., 2011; Morgan et al., 2010). Mutation of the WE motif in A36 deficient in its ability to stimulate actin polymerization (see Section 4.1), results in a small but significant decrease in IEV spread to the cell periphery at 8 h post infection (Dodding et al., 2011). In contrast, loss of the WD motif leads to a dramatic albeit not total reduction in viral spread at 8 h post infection. Live cell-imaging reveals that this reduction is due to a decrease in the IEV run length. This represents the average distance travelled along the microtubule between pauses, from 6.44 ± 0.37 to $2.58 \pm 0.14\,\mu m$ rather than a change in the velocity of the virus (Dodding et al., 2011). Immunofluorescence analysis suggests this change is due to a decrease in the ability of the virus to recruit and/or retain kinesin-1 (Dodding et al., 2011). Nevertheless, by 11 h post infection, significant numbers of IEV are able to reach the cell periphery (See Fig. 3B Dodding et al., 2011). A similar result was observed by Morgan et al. (2010), who found that over expression of an A36 WD motif alanine mutant in cells infected with the ΔA36R virus, which does not encode A36 allowed IEV to reach the plasma membrane and induce actin tails at 12 h post infection (Morgan et al., 2010). In contrast, mutation of both the WD and WE motifs results in an inability of IEV to recruit kinesin-1 and an absence of viral spread, as seen with the ΔA36R virus (Dodding et al., 2011).

In addition to A36, it has been proposed that the viral protein F12 is also essential for microtubule-mediated transport of IEV (Morgan et al., 2010; van Eijl et al., 2002). F12 associates with IEV when they are moving on microtubules and its loss results in peri-nuclear accumulation of the virus (Dodding and Way, 2009; Morgan et al., 2010; van Eijl et al., 2002; Zhang et al., 2000). Our ultrastructural analyses of ΔF12L infected cells, however, suggests that the loss of IEV spread in the absence of F12 is because the protein is required for their formation (Dodding and Way, 2009). Moreover, live cell imaging reveals that the small numbers of IEV that do assemble can still undergo linear movements at $\sim 1\,\mu m/s$ (Dodding and Way, 2009). While we cannot formally rule out the participation of F12 in microtubule-based transport of IEV, live cell imaging clearly demonstrates F12 is not essential for this process. Furthermore, recent analysis has revealed that F12 has a structural fold of an inactive DNA polymerase (Yutin et al., 2014), rather than structural similarity to the kinesin light chain as predicted by Morgan et al. (Morgan et al., 2010). Nevertheless, F12 does contain a potential kinesin-1 WD binding motif (Morgan et al., 2010). In contrast to A36, it still remains to be demonstrated if this motif directly binds to the TPR of the kinesin-1 light chain. However, over expression of an F12 WD motif mutant in cells infected with the ΔF12L virus does not rescue kinesin-1 recruitment and IEV egress (Morgan et al., 2010). It is still not clear if this phenotype is due to a defect in IEV assembly rather than microtubule transport *per se*. It is also possible that the mutation impacts on the structure of F12 and/or its interaction with E2 and/or A36, although the function of these interactions remains to be elucidated (Dodding and Way, 2009; Johnston and Ward, 2008). Notwithstanding the above, the current overexpression study clearly indicates that the WD motif in F12 is important for the function of the protein during the virus

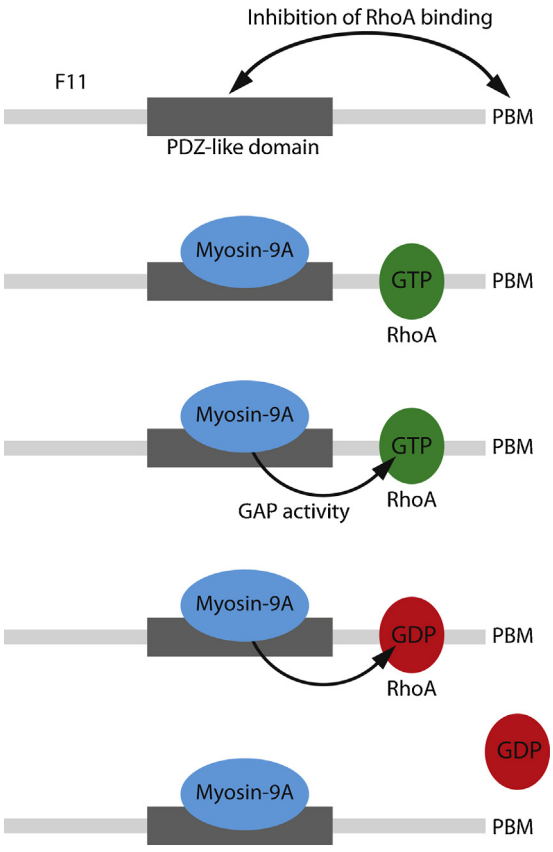


Fig. 3. Schematic representation of the mechanism by which F11 down regulates RhoA signalling. An intramolecular interaction between the central PDZ-like domain and the C-terminal PDZ binding motif (PBM) prevents F11 from interacting with RhoA. In its "open" conformation the PDZ domain interacts with the RhoGAP Myosin-9A, while GTP bound RhoA binds towards the C-terminus of the protein. The GAP activity of Myosin-9A enhances the hydrolysis of GTP to GDP, resulting in the release of RhoA and inhibition of its signalling.

replication cycle. The generation and detailed analysis of a recombinant virus expressing the F12 WD mutant will hopefully help clarify the exact role played by this potential kinesin-1 binding site during IEV assembly and transport.

3. Getting through the actin cortex

3.1. F11 enhances viral spread by inhibiting RhoA signalling

When IEV reach the cell periphery, they cannot fuse with the plasma membrane until they traverse the cortical actin cytoskeleton, a dense mesh of actin filaments associated with the cytoplasmic face of the plasma membrane (Biro et al., 2013; Charras et al., 2006; Clark et al., 2013; Fritzsche et al., 2013). The actin cortex provides a cell with mechanical resilience (Clark et al., 2013; Fritzsche et al., 2013; Gauthier et al., 2012; Salbreux et al., 2012; Tinevez et al., 2009) and plays an important role in regulating the morphology of the cell during a variety of cellular processes such as migration, mitotic rounding and cytokinesis (Bovellan et al., 2014; Matthews et al., 2012; Sedzinski et al., 2011). However, from a viral perspective, the cortical actin cytoskeleton represents a significant physical barrier, not only to IEV egress, but also to many other viruses during both their entry and exit from the cell (Radtke et al., 2006; Van den Broeke et al., 2014). Fortunately for Vaccinia, it encodes F11, which inhibits the activity of the small GTPase RhoA (Arakawa et al., 2007b; Cordeiro et al., 2009; Valderrama et al., 2006). F11 was originally identified as a viral protein required for vaccinia-induced cell migration (Morales et al., 2008; Valderrama et al., 2006). However, its ability to inhibit RhoA signalling also plays an important role in promoting viral release and spread in cell culture as well as *in vivo* in an intranasal mouse model of infection (Arakawa et al., 2007a; Cordeiro et al., 2009). F11 mediated inhibition of RhoA signalling increases the dynamics and targetting of microtubules to the cell periphery, which enhances the ability of IEV to reach the plasma membrane (Arakawa et al., 2007b). F11 also makes it easier for the virus to get through the cortical actin network by preventing RhoA from stimulating mDia induced actin polymerization (Arakawa et al., 2007a). Lastly, by stimulating infected cell migration and the formation of long neurite-like projections, F11 enhances the spread of infection by increasing viral contact with more non-infected cells (Cordeiro et al., 2009; Valderrama et al., 2006). The ability of F11 to promote viral spread has also been elegantly illustrated using Myxoma virus, which lacks a related homologue (Irwin and Evans, 2012). Irwin et al. found that a recombinant Myxoma virus expressing F11 is more effective in its cell-to-cell spread, as plaques expand ~6 fold faster and are four times larger than controls after 4 days (Irwin and Evans, 2012). The expression of F11 also improves the oncolytic efficacy of Myxoma by increasing viral spread in tumours and prolonging the survival of mice (Irwin et al., 2013). The conservation of F11 homolgues in chordopoxviruses (www.poxvirus.org) suggests that inhibiting RhoA signalling represents a common strategy used by poxviruses to enhance their cell-to-cell spread (Handa et al., 2013).

3.2. F11 is a PDZ scaffolding protein that binds Myosin-9A to inhibit RhoA

F11 binds directly to RhoA using a short sequence motif that is also present in its downstream effector ROCK (Valderrama et al., 2006). Consistent with this, F11 only interacts with active GTP-bound RhoA (Cordeiro et al., 2009). This allows F11 to sequester active RhoA to inhibit its signalling, but does not explain how the protein reduces the steady state level of GTP-bound RhoA during vaccinia infection (Arakawa et al., 2007a; Cordeiro et al., 2009). More recently, the mechanism by which F11 down regulates RhoA signalling has been uncovered (Figure 3). We found F11 contains a central PDZ-like domain that is required to down regulate RhoA signalling and enhance viral spread (Handa et al., 2013). PDZ domains are 80–90 residues in length with ~ 30% sequence identity and are conserved from bacteria to man, with the human genome encoding ~ 270 different PDZ domains (Harris and Lim, 2001; Ivarsson, 2012; Luck et al., 2012; Nourry et al., 2003; Ponting, 1997; Subbaiah et al., 2011). PDZ domains most frequently bind short peptide motifs, known as PDZ binding motifs (PBMs) located at the very C-terminus of proteins. However, they can also interact with internal peptide motifs and lipids as well as hetero- and/or homo dimerize (Harris and Lim, 2001; Ivarsson, 2012; Luck et al., 2012; Nourry et al., 2003; Subbaiah et al., 2011). PDZ containing proteins participate in a wide range of fundamental cellular processes including adhesion, polarity, proliferation, apoptosis and signalling. It is not surprising, given their role in so many cellular processes, that viruses frequently target their function using viral proteins containing PDZ binding motifs (Davey et al., 2011; Javier and Rice, 2011). Curiously though, there is no evidence that viruses manipulate their hosts using virally encoded PDZ domain containing proteins (Ivarsson, 2012; Subbaiah et al., 2011). F11 therefore represents the first example of a viral protein containing a functional PDZ-like domain.

In addition to its PDZ domain, F11 also has a class II PDZ binding motif (PBM) at its extreme C-terminus (Handa et al., 2013). The interaction of this PBM with the central PDZ domain inhibits the ability of F11 to bind RhoA, presumably by inducing a conformation that occludes the RhoA binding site (Figure 3). The C-terminal PBM also has additional roles that go beyond regulating the interaction

of F11 with RhoA, as its deletion reduces virus release to a level that is equivalent to the loss of the complete protein (Handa et al., 2013). This phenotype may be related to a disruption of the localization of F11 within the infected cell, as presence of the C-terminal PBM enhances its association with membranes.

The PDZ domain of F11 is required to reduce the level of active RhoA during infection (Handa et al., 2013). Given this, we wondered if the F11 PDZ domain down regulates RhoA by interacting with a class II PBM containing GTPase activating protein (GAP). GAPs, which have an over representation of PDZ binding motifs compared to other protein families, inactivate Rho GTPases by stimulating the hydrolysis of GTP to GDP (Garcia-Mata and Burridge, 2007; Giallourakis et al., 2006). Pulldown assays demonstrate that the PDZ domain of F11 interacts with the PBM of the GAPs β-Chimaerin and Myosin-9A, which regulate Rac and RhoA respectively (Chieregatti et al., 1998; Handa et al., 2013; Yang and Kazanietz, 2007). Consistent with a possible role in promoting viral spread, Myosin-9A regulates cortical actin in a RhoA-dependent fashion during cell-cell adhesion (Omelchenko and Hall, 2012). Subsequent analysis demonstrated that RNAi mediated loss of Myosin-9A during infection leads to reduced viral release and spread, as RhoA signalling is no longer inhibited at 8 h post infection. The ability of Myosin-9A to enhance viral spread is dependent on its GAP activity as well as its ability to bind the F11 PDZ domain using its C-terminal PBM. Interestingly, it also depends on the ability of F11 to bind RhoA. Furthermore, pulldown assays demonstrate the presence of a tri-molecular complex between F11, RhoA and Myosin-9A during infection. This suggests that F11 is acting as a PDZ scaffolding protein to bring Myosin-9A and RhoA together to inactivate the GTPase (Fig. 3).

4. Vaccinia induced actin polymerization enhances the spread of infection

4.1. Src and Abl phosphorylation of A36 induces actin polymerization

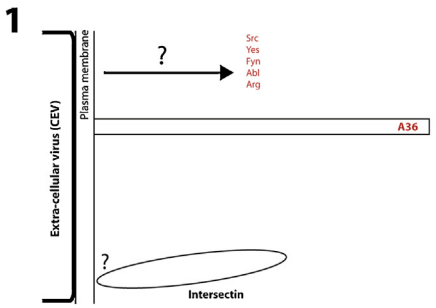
Nearly 40 years ago, analysis of vaccinia infected cells in the electron microscope revealed the presence of virus particles on the tip of large microvilli projecting from the plasma membrane (Stokes, 1976). These projections appeared late in infection and contained actin, α-actinin, fimbrin and filamin but not tropomyosin or myosin (Hiller et al., 1981; Hiller et al., 1979; Krempien et al., 1981). These studies were essentially forgotten until 1995, when it was demonstrated that vaccinia is moved by the power of actin polymerization on the tips of actin tails before extending out into neighbouring non-infected cells (Cudmore et al., 1995; Cudmore et al., 1996). Subsequent studies demonstrated that actin polymerization and tail formation is induced by CEV, which are formed when IEV fuse with the plasma membrane after undergoing kinesin-1 driven microtubule transport (Cudmore et al., 1996; Hollinshead et al., 2001; Humphries et al., 2012; Rietdorf et al., 2001; Ward and Moss, 2001a; Ward and Moss, 2001b) (Fig. 1).

Vaccinia induced actin polymerization is dependent on Src and Abl family kinase mediated phosphorylation of the IEV protein A36 (Frischknecht et al., 1999; Newsome et al., 2004; Newsome et al., 2006; Reeves et al., 2005) (Fig. 4). Deletion of the A36R gene and loss of A36 expression does not affect IEV assembly but does lead to an absence of actin tail formation and a small plaque phenotype, indicative of a defect in viral spread (Röttger et al., 1999; Sanderson et al., 1998; Wolffe et al., 1998). A36 is highly conserved in orthopoxvirus genomes, suggesting that virus-induced actin polymerization at the plasma membrane is widely used by mammalian poxviruses to enhance their cell-to-cell spread. Indeed, monkeypox and variola viruses induce Src and Abl dependent actin tails, while the A36 homologue of ectromelia virus enhances viral spread by inducing actin tail formation (Lynn et al., 2012; Reeves et al., 2011). Moreover, the ability to stimulate actin polymerization is not just restricted to orthopoxviruses, as other divergent vertebrate poxviruses (*Chordopoxviridae*) can also induce actin tails (Dodding and Way, 2009; Duteyrat et al., 2006; Law et al., 2004). In these cases, a divergent family of viral proteins related to YL126 a transmembrane protein of Yaba-Like Disease virus (yatapoxvirus) is used to stimulate actin polymerization (Dodding and Way, 2009). These YL126 orthologues bear little or no sequence homology to A36, but they stimulate actin polymerization in a tyrosine phosphorylation dependent manner via the same Grb2, Nck, WIP and N-WASP signalling network as Vaccinia virus (Dodding and Way, 2009) (see Section 4.2).

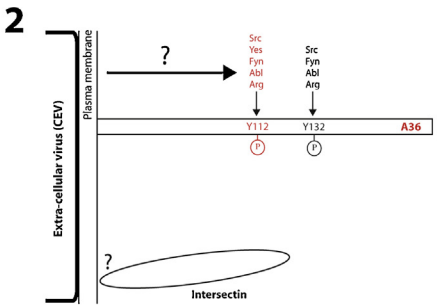
Vaccinia A36 is a type Ib transmembrane protein with a cytoplasmic domain of ~ 195 residues on the IEV surface (Röttger et al., 1999; van Eijl et al., 2000). When IEV fuse with the plasma membrane, A36 becomes localized beneath CEV but retains the same topology with respect to the cytoplasm of the cell (Smith et al., 2002; van Eijl et al., 2000) (Fig. 4). Src recruitment and phosphorylation of A36 only occurs after viral fusion with the plasma membrane, even though its cytoplasmic domain is fully exposed on the IEV surface (Newsome et al., 2004). This implied that one or more of the four integral viral membrane proteins (A33, A34, A56 and B5) exposed on the surface of CEV (Smith et al., 2002), are stimulating an outside-in signal to locally activate Src kinase. We still do not have a molecular understanding of how CEV signal back into the cell, but the SCR4 domain of B5 is essential to activate Src, phosphorylate A36 and induce actin tails (Newsome et al., 2004).

4.2. Phosphorylated A36 recruits a signalling network

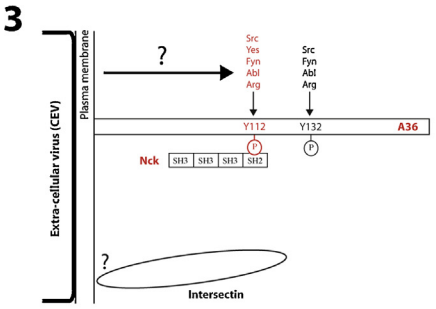
The ubiquitous Src and Abl family kinase members (Src, Fyn, Yes, Abl and Arg) are recruited by CEV and capable of stimulating actin tail formation by virtue of their ability to phosphorylate tyrosine 112 of A36 (Frischknecht et al., 1999; Newsome et al., 2004; Newsome et al., 2006; Reeves et al., 2005) (Fig. 4). Additional tyrosine kinases might also be capable of phosphorylating A36, however, inhibition of Src and Abl family kinases or mutation of Y112 of A36 is sufficient to prevent vaccinia actin tail formation (Frischknecht et al., 1999; Newsome et al., 2004; Reeves et al., 2005; Scaplehorn et al., 2002; Ward and Moss, 2001a). Consistent with this, Abl, Arg, Fyn, Src and Yes can all phosphorylate tyrosine 112 of A36 *in vitro* (Newsome et al., 2006). When phosphorylated, tyrosine 112 of A36 recruits the adapter Nck to the virus by interacting directly with its SH2 domain (Frischknecht et al., 1999; Scaplehorn et al., 2002). Nck is essential for Vaccinia induced-actin polymerization as no actin tails are formed in mouse embryo fibroblasts (MEFs) lacking Nck (Weisswange et al., 2009). The presence of Nck beneath the CEV results in the recruitment of WIP and N-WASP, the latter of which activates the Arp2/3 complex to stimulate actin polymerization (Donnelly et al., 2013; Frischknecht et al., 1999; Moreau et al., 2000; Snapper et al., 2001; Weisswange et al., 2009; Zettl and Way, 2002) (Fig. 5). Nck is not the only SH2/SH3 adapter that is recruited by the virus (Scaplehorn et al., 2002). When phosphorylated by Src, Fyn, Abl and Arg but not Yes, tyrosine 132 of A36 is able to recruit Grb2 to the virus by binding its SH2 domain (Newsome et al., 2006; Scaplehorn et al., 2002) (Fig. 4 and 5). The recruitment of Grb2 is dependent on both the phosphorylation of tyrosine 132 of A36 and the presence of the proline rich region of N-WASP, which presumably interacts with the SH3 domains of Grb2 (Scaplehorn et al., 2002; Weisswange et al., 2009). In contrast to tyrosine 112, phosphorylation of tyrosine 132 is not required for vaccinia-induced actin polymerization, although its presence does enhance actin tail formation (Scaplehorn et al., 2002; Ward and Moss, 2001a; Weisswange et al., 2009). Analysis of the dynamics



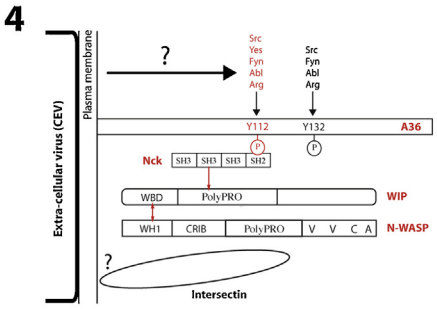
Outside-in signalling by CEV on plasma membrane locally activates Src and Abl family kinases. The Rho-GEF Intersectin is recruited by CEV prior to actin tail formation. The mechanistic basis of these two events remains unknown.



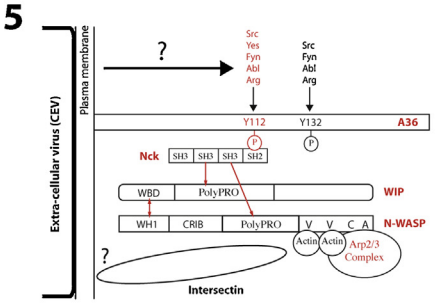
Activated Src and Abl family kinases phosphorylate tyrosine 112 and 132 of the integral transmembrane viral protein A36. Phosphorylation of tyrosine 112 is essential for actin tail formation.



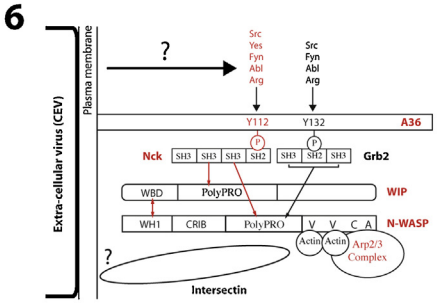
Phosphorylation of tyrosine 112 of A36 results in the recruitment of Nck via its phosphotyrosine binding SH2 domain. Nck is essential for actin tail formation.



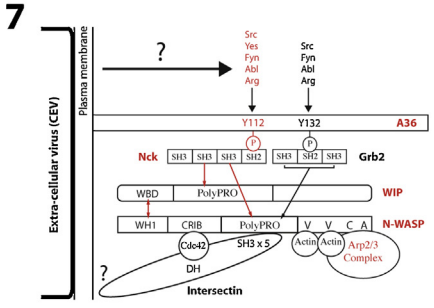
The second SH3 domain of Nck recruits a complex of WIP:N-WASP by interacting with one of two PxxPXRXL motifs in the poly-proline rich region of WIP.



The third SH3 domain of Nck interacts with one of two PxxPXRG motifs in the poly-proline rich region of N-WASP. This interaction allows N-WASP to bind the Arp2/3 complex and stimulate its ability to induce actin polymerization.



Grb2 interacts with phosphorylated tyrosine 132 of A36 and the poly-proline rich region of N-WASP. Grb2 is only recruited when N-WASP is present and helps enhance actin tail formation.



The SH3 domains of Intersectin can interact with the poly-proline rich region of N-WASP. The DH domain of Intersectin activates the RhoGTPase Cdc42, allowing it to bind the CRIB domain of N-WASP. This interaction also enhances the ability of CEV to induce actin tails.

Fig. 4. Schematic of the steps involved in assembling the signalling network responsible for stimulating Arp2/3 complex dependent actin polymerization beneath CEV.

of GFP-tagged Nck, WIP and N-WASP during vaccinia actin-based motility reveals that Grb2 helps stabilize the signalling complex, which may in part account for the enhanced actin tail formation (Weisswange et al., 2009).

4.3. Molecular dissection of the A36 signalling network

The robust recruitment of Nck, Grb2, WIP and N-WASP to the virus, coupled with the ability to quantify both the number and speed of actin tails as well as the dynamics of proteins in the system make vaccinia an excellent model to understand how a phosphotyrosine-based signalling network activates Arp2/3-dependent actin polymerization (Donnelly et al., 2013; Weisswange et al., 2009; Welch and Way, 2013) (Fig. 5). Another strength of the system is the ability of vaccinia to infect mouse embryo fibroblasts (MEFs) derived from knockout mice lacking individual proteins such as Nck, WIP or N-WASP (Donnelly et al., 2013; Snapper et al., 2001; Weisswange et al., 2009). An initial over expression study in HeLa cells suggested that WIP and N-WASP are recruited to the virus as a complex (Moreau et al., 2000). Subsequent analysis using MEFs derived from mice lacking N-WASP confirmed that its presence is required for the recruitment of WIP to the virus (Donnelly et al., 2013; Snapper et al., 2001; Weisswange et al., 2009). The presence of WIP and/or its homologue WIRE is also required for the recruitment of N-WASP (Donnelly et al., 2013). Collectively, these data demonstrate that vaccinia recruits WIP and N-WASP as a complex (Fig. 4). In contrast, Nck is still localized on the virus in the absence of WIP or N-WASP, indicating it is upstream of the WIP:N-WASP complex (Donnelly et al., 2013; Snapper et al., 2001; Weisswange et al., 2009). We envisage that Nck engages with tyrosine 112 of A36 when it is phosphorylated and then recruits a WIP:N-WASP complex to the virus, which subsequently associates with Grb2 interacting with phosphorylated tyrosine 132 of A36. This explains why Grb2 is not recruited to the virus in the absence of N-WASP and why Nck is essential for vaccinia actin tail formation (Weisswange et al., 2009).

A combination of biochemical approaches and expression of mutants in cells lacking endogenous Nck, WIP or N-WASP, demonstrate that an interaction of the second SH3 domain of Nck with WIP is essential for actin tail formation, as it is required to recruit the WIP:N-WASP complex (Donnelly et al., 2013). Consistent with this, the recruitment of N-WASP to the virus depends on its interaction with WIP rather than Nck. The first and third SH3 domains of Nck are essential to stimulate actin tails even though they are not required to recruit the WIP:N-WASP complex. Curiously, however, an interaction between N-WASP and Nck is not required for virus induced actin polymerization even in the absence of Grb2 (Donnelly et al., 2013). Taken together, these data suggested that an additional factor is also able to stimulate the ability of N-WASP to activate the Arp2/3 complex and induce actin polymerization beneath CEV. This additional component may also explain why the turnover dynamics of N-WASP is ~3.4 times slower than that of Nck and WIP (half life of turnover is 2.7 rather than 0.8 s), even though they are responsible for its recruitment to the virus (Weisswange et al., 2009).

4.4. Cdc42 enhances Vaccinia actin tail formation

Our previous observations demonstrated that Cdc42, a well-known activator of N-WASP, was recruited to vaccinia inducing actin tails (Moreau et al., 2000). However, expression of dominant negative Cdc42 appeared to have no impact on actin tail formation, based on assessing for the presence of a single actin tail or not in infected cells (Moreau et al., 2000). Given this stringent quantification criterion and in light of the results of Donnelly et al. (2013), we re-examined the potential role of Cdc42 in vaccinia actin tail formation. We found there was a significant decrease in the number of actin tails in cells either lacking Cdc42 or expressing dominant negative Cdc42 or when N-WASP was unable to interact with Cdc42 (Humphries et al., 2014). Moreover, the inability of N-WASP to bind Cdc42 has a greater impact on actin tail formation than the loss of Nck binding, suggesting Cdc42 is more important than Nck in stabilizing and/or activating N-WASP. Consistent with this, a loss of Cdc42 binding results in a significantly faster turnover of N-WASP than that observed when its interaction with Nck is abolished (half life of turnover is 1.18 rather than 2.41 s) (Donnelly et al., 2013; Humphries et al., 2014). However, Nck still contributes to vaccinia induced actin polymerization, as far fewer actin tails are formed when N-WASP cannot bind both proteins compared to Cdc42 alone. FRAP analysis also reveals that the turnover of N-WASP is essentially the same as Nck and WIP when it cannot interact with Nck and Cdc42 (Humphries et al., 2014). Moreover, actin tail formation is largely abolished in the absence of Grb2 recruitment, indicating that Arp2/3-dependent actin based motility of vaccinia is principally driven by Cdc42 and Nck-dependent activation of N-WASP (Humphries et al., 2014).

In contrast to Nck, Cdc42 is not recruited to the virus in the absence of N-WASP (Humphries et al., 2014). This suggested that activation and recruitment of Cdc42 is downstream of the presence of the WIP:N-WASP complex (Figure 4). Consistent with this, live cell imaging reveals that the accumulation of Cdc42 on the virus is delayed by ~5 s compared to N-WASP, which begins to be recruited ~10 s before actin polymerization is initiated (Humphries et al., 2014). This delay presumably reflects the activation of Cdc42 by a RhoGEF beneath the virus, as N-WASP can only interact with GTP-bound Cdc42 (Miki et al., 1998). The identification of the RhoGEF responsible for activating Cdc42 was greatly facilitated by the observation that immediately following fusion with the plasma membrane, vaccinia transiently recruits AP-2 and clathrin prior to actin tail formation (Humphries et al., 2012). Clathrin appears to have an organizational role, as its recruitment enhances polarization of A36 and N-WASP beneath the CEV making it easier for the virus to induce and sustain actin polymerization (Humphries et al., 2012; Humphries and Way, 2013). We found that the virus activates Cdc42 by recruiting intersectin-1, a RhoGEF that interacts with both AP-2 and N-WASP (Humphries et al., 2014; Hunter et al., 2013; Hussain et al., 2001). Furthermore, the recruitment of intersectin-1 to CEV is upstream of actin tail formation, as it occurs in the absence of N-WASP. Loss of intersectin-1 or its GEF activity results in a similar reduction in actin tail number as expressing dominant negative Cdc42 (Humphries et al., 2014). Moreover, this decrease is not additive, suggesting that intersectin-1 and Cdc42 are acting in the same pathway to enhance vaccinia actin tail formation. In contrast to Nck, however, the recruitment of Cdc42 is not essential for vaccinia actin tail formation. Nevertheless, Cdc42 does collaborate with Nck to enhance the ability of the virus to induce actin polymerization by stabilizing and/or activating N-WASP (Humphries et al., 2014). We now have to understand how CEV recruit intersectin-1 and whether it is essential for AP-2 and clathrin recruitment prior to actin tail formation.

4.5. The role of FHOD1 in actin tail assembly

Cdc42 is not the only Rho GTPase that has been shown to enhance actin tail formation and viral spread. By performing a siRNA screen against actin cytoskeletal regulators Alvarez and Agaisse (2013) demonstrated that the formin, FHOD1 promotes the cell-to-cell spread of vaccinia. Loss of FHOD1 results in ~2.5 fold reduction in the number of tails induced by CEV as well as a 30% decrease in their velocity, suggesting the protein enhances formation and elongation of actin tails (Alvarez and Agaisse, 2013). The ability of FHOD1 to enhance actin tail formation is dependent on

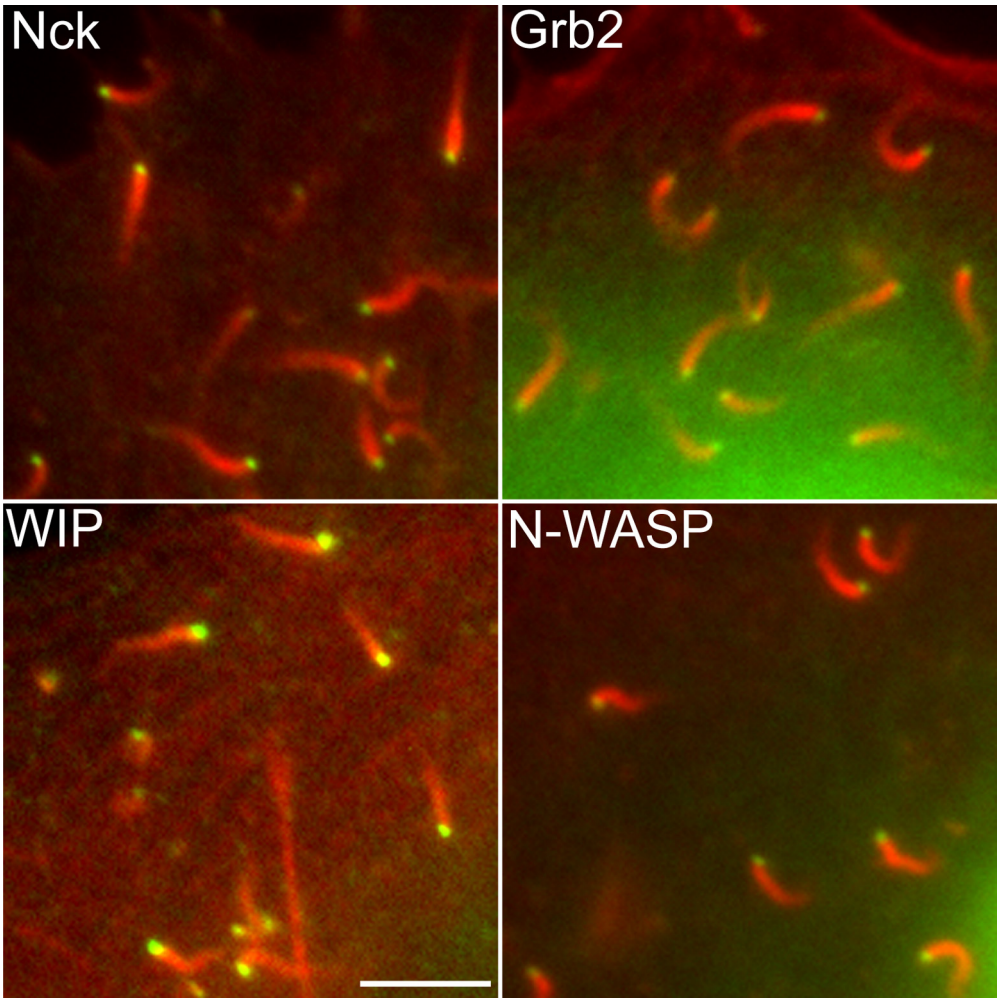


Fig. 5. Immunofluorescence image showing the recruitment of GFP-tagged Nck, Grb2, WIP and N-WASP (green) to the tip of actin tails (red) in a HeLa cell infected for 8 h with WR. The scale bar is 5 μm.

its FH1, FH2 and GBD domains, which interact with profilin:actin complexes, the fast growing end of actin filaments and the RhoGTPase Rac respectively (Schonichen et al., 2006; Schulte et al., 2008). Consistent with a role for the FH1 and GBD domains, RNAi mediated knockdown of profilin or Rac phenocopied the impact of FHOD1 depletion on actin tail number and speed (Alvarez and Agaisse, 2013). The authors also found that active Rac was enriched in the membrane surrounding projecting actin tails in an A36-independent fashion. Furthermore, FHOD1 recruitment to vaccinia actin tails was dependent on Rac as well as N-WASP (Alvarez and Agaisse, 2013). In contrast to Nck and N-WASP, but in line with intersectin-1 and Cdc42, Rac and FHOD1 enhance but are not essential for vaccinia actin tail formation.

Based on their observations, Alvarez and Agaisse suggest that FHOD1 enhances actin tail formation and speed by recruiting profilin:actin complexes and enhancing the nucleation and elongation of the barbed end of actin filaments. They propose that FHOD1 might enhance the growth of actin filaments nucleated by the Arp2/3 complex or provide the initial actin filaments from which Arp2/3 induces new filament assembly. We favour a different mechanism. Ground breaking *in vitro* reconstitution experiments with pure proteins from the Carlier laboratory have demonstrated that Arp2/3 driven actin based motility is critically dependent on the presence of an actin filament severing protein such as ADF/cofilin as well as a barbed end capping protein (Loisel et al., 1999). In contrast, the presence of profilin in the *in vitro* motility assays is not essential, although it does enhance the rate of movement. More recently, *in vitro* assays have shown that FHOD1 caps the fast growing or barbed end of actin filaments in an FH2 dependent fashion but does not nucleate or promote filament elongation (Schonichen et al., 2013). Results from *in vitro* biochemical assays may not always reflect the situation in more complex *in vivo* setting. However, we think that the observations of Alvarez and Agaisse are consistent with the hypothesis that FHOD1 is enhancing vaccinia actin tail formation by capping actin filaments and providing profilin:actin complexes for filament growth induced by the Arp2/3 complex. First, FHOD1 is found throughout the actin tail and not immediately beneath the CEV as seen for N-WASP. Such a localization is consistent with FHOD1 binding the barbed ends of actin filaments within the tails. Second, the deletion of the FH2 domain, which is responsible for its actin filament capping activity, results in an absence of FHOD1 recruitment to actin tails. Third, fluorescent actin incorporation experiments have demonstrated that actin polymerization only occurs immediately beneath the virus and that the filaments in the tail do not grow as they are capped (Cudmore et al., 1996). Nevertheless, our analysis using both antibodies and GFP-tagged clones has failed to detect capping protein (also known as CapZ) or Eps8 in vaccinia induced actin tails (Way lab unpublished). Lastly, the presence of FHOD1 in actin tails is dependent on N-WASP. However, this does not demonstrate that the interaction is direct or rule out that the recruitment of FHOD1 is actually dependent on the Arp2/3 complex or the actin filaments

it nucleates. Future experiments are clearly required to determine the exact function of FHOD1 in vaccinia actin tail formation and uncover how the virus activates Rac.

4.6. Actin polymerization powers viral spread

We now know the main players required for CEV-induced actin tail formation. However, we are still a long way from having a complete molecular understanding of exactly how the actin cytoskeleton promotes the release and spread of Vaccinia. More than 30 years ago it was demonstrated that inhibition of actin polymerization with cytochalasin D inhibits release of vaccinia from infected cells (Payne and Kristensson, 1982). More recently, it was found that A36 phosphorylation and actin polymerization promotes virus release by driving CEV out of invaginations in the plasma membrane (Horsington et al., 2013). The activity of Abl and Arg but not Src family kinases also promotes the release of CEV from infected cells (Reeves et al., 2005). Consistent with this, treatment of mice with the Abl-family kinase inhibitor Gleevec/STI-571/Imatinib reduces viral spread and promotes their survival from an otherwise lethal infection (Reeves et al., 2005; Reeves et al., 2011). The activity of Abl and Arg clearly enhances A36-dependent release of Vaccinia from infected cells (Horsington et al., 2013). In addition, both kinases also promote viral release independently of their ability to phosphorylate A36 (Horsington et al., 2013). The identity of this additional Abl and Arg substrate that presumably promotes actin-dependent viral release remains to be established. However, an attractive candidate is N-WASP, as its ability to activate Arp2/3-dependent actin polymerization is enhanced by Arg and Abl mediated phosphorylation (Burton et al., 2005; Miller et al., 2010). Another reason to think N-WASP might be the target is because the release of Vaccinia from infected cells has strong parallels with exocytosis, which also involves N-WASP (Gasman et al., 2004). Furthermore, the recruitment of the phosphoinositide 5-phosphatase SHIP2, a negative regulator of viral release that is not involved in actin tail formation depends on its SH2 domain and the presence of N-WASP (McNulty et al., 2011).

A36 induced actin tails help propel Vaccinia into neighbouring cells (see Fig. 4 (Cudmore et al., 1995) to enhance the spread of infection (Röttger et al., 1999; Sanderson et al., 1998; Ward and Moss, 2001a; Wolffe et al., 1998). However, it was unclear how Vaccinia-induced actin polymerization promoted viral spread in a cell monolayer faster than the virus replication cycle. This mystery has now been solved (Doceul et al., 2010). Live cell imaging of viral spread during plaque formation reveals that if a virus (CEV or EEV) lands on a recently infected neighbouring cell, it is not internalized but rather induces an actin tail. The power of actin polymerization then propels the virus across the outer surface of the infected cell and onto adjacent non-infected cells, where internalization can occur. This mechanism allows the virus to ignore already infected cells, thus enhancing the rate of viral spread through the cell monolayer. The ability of the virus to surf across the surface of an infected cell is dependent on the presence of A33 and A36 in the plasma membrane (Doceul et al., 2010). The SCR4 domain of B5, which is involved in activating Src (Newsome et al., 2004), is also required for viral surfing (Doceul et al., 2012). These observations suggest that the surfing virus is effectively mimicking the normal process of actin tail formation by inducing Src and Abl kinase activation and tyrosine phosphorylation of A36 when it interacts with the extracellular domain of A33 on plasma membrane of the infected cell.

4.7. Conclusions

Studies from many different labs have collectively demonstrated that the ability of Vaccinia to stimulate actin polymerization at the plasma membrane plays an important role in promoting the spread of infection. Moreover, unravelling exactly how Vaccinia stimulates actin polymerization has provided unprecedented insights into how a co-operative phosphotyrosine-based Nck and N-WASP signalling network activated by Src and Abl family kinases is assembled, regulated and functions at the molecular level to stimulate Arp2/3 dependent actin polymerization. Nevertheless, there are still many outstanding questions, including how the ability of Vaccinia to stimulate actin polymerization contributes to viral spread and pathogenesis in animal models. Furthermore, mechanistic basis of Src and Abl family kinase activation at the plasma membrane is yet to be understood. Actin is not the only component of the cytoskeleton that is usurped by Vaccinia. In the absence of cell lysis, microtubule-based transport plays an essential role in viral egress, as it facilitates the virus reaching the plasma membrane. The importance of microtubule transport during Vaccinia infection was realised more than a decade ago, and yet our molecular understanding of this process lags far behind actin-based motility of the virus. This is probably in part because of the speed and transient nature of microtubule-based movement as compared to the robust actin-based motility of Vaccinia, which is some 5–10 times slower, making live imaging less problematic. Nevertheless, in recent years, studies have begun to uncover the molecular basis of kinesin-1 recruitment to IEV. Further analysis of the role of microtubule transport in infected cells promises to provide important fundamental insights into mechanisms regulating the recruitment and activity of kinesin-1. Moreover, such studies have the potential to uncover how additional motors participate in other aspects of virus replication including IMV transport and IEV assembly. Such analyses will also undoubtedly uncover general principals underlying the recruitment and regulation of motors by cellular cargoes outside the context of infection. The next phase of research into the role of signalling and the host cytoskeleton in promoting the spread of Vaccinia clearly promises to provide interesting insights for the virologist and cell biologist alike.

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