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## Title

Loss of Cytoskeletal Transport during Egress Critically Attenuates Ectromelia Virus Infection In Vivo

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# 1 Title: Loss of cytoskeletal transport during egress critically attenuates

- 2 Ectromelia virus infection in vivo
- 3
- 4 Running title: Cytoskeletal transport is critical for ECTV infection
- 5

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#### 24 Abstract

25 Egress of wrapped virus (WV) to the cell periphery following vaccinia virus 26 (VACV) replication is dependent on interactions with the microtubule motor 27 complex kinesin-1 and is mediated by the viral envelope protein A36. Here we 28 report that ectromelia virus (ECTV), a related orthopoxvirus and the causative 29 agent of mousepox, encodes an A36 homologue (ECTV-Mos-142) that is highly 30 conserved despite a large truncation at the C-terminus. Deleting the ECTV A36R 31 gene leads to a reduction in the number of extracellular virus formed and a 32 reduced plaque size, consistent with a role in microtubule transport. We also 33 observed a complete loss of virus-associated actin comets, another phenotype 34 dependent on A36 expression during VACV infection. ECTV  $\Delta$ A36R was severely attenuated when used to infect the normally susceptible BALB/c mouse strain. 35 36 ECTV  $\triangle$ A36R replication and spread from the draining lymph node to liver and 37 spleen was significantly reduced in BALB/c mice and in Rag-1-deficient mice, 38 which lack T and B lymphocytes. The dramatic reduction in ECTV ΔA36R titers 39 early during the course of infection was not associated with an augmented 40 immune response. Taken together, these findings demonstrate the critical role 41 subcellular transport pathways play not only in orthopoxvirus infection in an *in* 42 vitro context, but also during orthopoxvirus pathogenesis in an endemic host. 43 Furthermore, despite the attenuation of the mutant virus, we found that 44 infection nonetheless induced protective immunity in mice, suggesting that 45 orthopoxvirus vectors with A36 deletions may be considered as another safe 46 vaccine alternative.

47

#### 48 Introduction

49 The crowded, densely packed cytoplasm presents a significant hurdle to the 50 subcellular transport of viruses, both in the translocation of viruses to their site 51 of replication following cell entry and the subsequent egress of progeny viruses to the cell periphery (17, 19, 30, 70, 71). This hurdle is most acute for large 52 53 dsDNA viruses such as those belonging to the orthopox genus, which includes 54 variola virus (VARV) and ectromelia virus (ECTV), the causative agents of 55 smallpox and mousepox, respectively, and the prototypical orthopoxvirus, 56 vaccinia virus (VACV). These viruses have a complicated replication cycle that 57 has been best studied at the cellular level with VACV. Vaccinia virus replication 58 produces two infectious forms: mature intracellular virus (MV) that has a single 59 membrane and are generated at the so-called virus factory, and wrapped virus 60 (WV) that is derived from MV and acquires an additional double membrane at 61 the trans-Golgi network or early endosome compartment (34, 59). While it is 62 apparent that microtubule transport plays a critical role at multiple stages 63 during VACV replication, the stage best characterized at the molecular level is 64 the transport of WV from the trans-Golgi network to the cell periphery (28, 33, 58, 76, 77). Here, WV fuse with the plasma membrane and are either released 65 directly or following the transient activation of actin-based motility (9, 58, 78). 66

67 The viral protein A36 (encoded by A36R) is highly conserved across 68 orthopoxviruses and plays an important role in subcellular transport during 69 VACV egress (15, 58, 75). A36 is a type Ib transmembrane protein that is 70 localized to the trans-Golgi network and acquired during wrapping of WV at this compartment and contains a ~190 residue surface in contact with the cytoplasm 71 72 (61, 74). Viruses deleted for A36R display a reduction in transport of WV to the 73 cell surface, a phenotype reminiscent of cells treated with the microtubule 74 destabilising drug nocodazole (14-16, 28, 32, 58). A key role for A36 is to direct 75 the recruitment of the kinesin-1 anterograde motor complex. This interaction 76 appears to via a direct interaction with kinesin-1 light chain (KLC) and has 77 recently been mapped to a bipartite tryptophan motif in A36 at residues 64/65 78 (WE) and 97/98 (WD) (15, 58, 75), with WD playing the dominant role and WE 79 an ancillary role. Conversely, expressing constructs that interfere with kinesin-1 80 function also inhibit WV egress from the trans-Golgi network (58, 66). A simple 81 paradigm that emerges from these findings is that A36 on the surface of WV 82 presents an interaction site for kinesin-1 resulting in transport to the cell 83 periphery. Confounding this paradigm is the viral protein F12 that has also been 84 implicated in the microtubule transport of WV, but recent reports are conflicting as to the exact function of this protein and its mechanism of action remains to be 85 clarified (14, 36, 42, 73). A36 also directs a second transport event following 86 delivery of WV to the cell periphery, whereby phosphorylation of two tyrosine 87 88 residues (112 and 132) initiates a cascade of events leading to recruitment of the 89 Arp2/3 complex beneath extracellular cell-associated WV and the rapid 90 nucleation of actin filaments (27, 41, 44, 45, 55, 65, 78). Polymerisation of actin 91 filaments beneath extracellular WV propels these particles across the surface of 92 the cell thereby facilitating cell-to-cell spread (13, 76).

93 Due to the hazards of working with VARV and the unknown host and origin 94 of VACV, ECTV infection in mice has been used extensively as a model for 95 smallpox infection (3, 24). There is a wealth of knowledge on the pathogenesis of 96 ECTV and the murine immune response to infection. Despite this, there is 97 surprisingly little known regarding the cell biology of infection, with research so 98 far delving into such areas as viral subversion of the immune system (43, 67, 68), 99 ubiquitin proteosome system (35, 79), viral mediated syncytia formation (23) 100 and preliminary studies on intracellular transport and intercellular spread (2, 4). 101 The high level of conservation between VACV and ECTV genomes affords the 102 opportunity to address the contribution of subcellular transport pathways to 103 virulence in an endemic host (8). In the present study, we have demonstrated 104 that both microtubule- and actin-based transport are in operation during ECTV 105 replication. As A36R is required for these events during VACV infection, we 106 identified an ECTV homologue of A36R, annotated as ECTV-Mos-142 or 137.5f 107 (poxvirus.org, referred to henceforth as A36R) that was highly conserved albeit 108 truncated at the C-terminus. Deletion of A36R resulted in ECTV that had a 109 reduction in the appearance of extracellular WV, consistent with reduced 110 microtubule transport, and loss of virus-associated actin comets. Both of these 111 phenotypes were rescued by transient expression of ECTV A36R indicating these 112 phenotypes were specific for the loss of this protein. Infection of ECTVsusceptible BALB/c mice with  $\Delta$ A36R resulted in drastically attenuated 113

114 pathogenesis, including a strong reduction in the spread of virus from the site of 115 inoculation and lower viral load in the major target organs. Virus spread and 116 replication was also significantly curtailed in Rag-1-deficient mice, which lack a 117 functional adaptive immune system. Nonetheless, BALB/c mice that survived 118  $\Delta$ A36R infection effectively generated protective antibody and cytotoxic T 119 lymphocyte (CTL) responses and were able to overcome a subsequent lethal 120 challenge with wild-type ECTV.

121

### 122 Materials and Methods

Ethics Statement. This study was performed in strict accordance with the recommendations in the Australian code of practice for the care and use of animals for scientific purposes and the Australian National Health and Medical Research Council Guidelines and Policies on Animal Ethics. The protocol was approved by the Animal Ethics and Experimentation Committee of the Australian National University (Permit Number: J.IG.75.09).

129 Cells and viruses. Mammalian cell lines used were HeLa, BSC-1, NIH3T3, 130 MEFs, N-WASP null MEFs (69) and virus strains VACV Western Reserve (WR) 131 and  $\Delta A36R$  (52). The murine cell lines P-815 (H-2d) and YAC-1 were obtained 132 from the American Type Culture Collection (Rockville, MD). ECTV-Moscow strain 133 (ECTV-Mos/WT ECTV; ATCC VR1374) was a gift from Professor RM Buller, St. 134 Louis University School of Medicine. Cells were grown in GIBCO Dulbecco's 135 Modified Eagle Medium (DMEM; Invitrogen) supplemented with 5% fetal bovine 136 serum (FBS), 292 µg/mL L-Glutamine, 100 Units/mL penicillin and 100 µg/mL 137 streptomycin and incubated at 37°C with 5% CO<sub>2</sub> atmosphere. For infection, 138 virus was diluted in DMEM not supplemented with FBS and applied to phosphate 139 buffered saline (PBS) washed cells. Cells were incubated at 37°C with 5% CO2 140 atmosphere for 1 hour before being recovered with fresh growth medium. For 141 plaque assays, confluent BSC-1 monolayers were infected as described, but 142 instead of recovery with fresh growth medium, were overlaid with GIBCO 143 Modified Eagle Medium (MEM; Invitrogen) supplemented with 292 µg/mL L-144 Glutamine, 100 Units/mL penicillin, 100 µg/mL streptomycin and 1.5% carboxy-145 methyl cellulose (CMC). Plaques were allowed to form for 5 days before 146 examination.

147 Plasmid construction. To construct a recombination cassette for deletion of 148 A36R, regions of approximately 900 bp of ECTV Mos genomic DNA flanking the 149 gene locus A36R were amplified with primer pairs a36r.ra.for/a36r.ra.rev for the 150 right flanking region and a36r.la.for/a36r.la.rev (Table. 1) for the left flanking 151 region. PCR products were gel purified (Gel Extraction Kit, Promega), digested 152 and ligated into a plasmid vector flanking monomeric red fluorescent protein 153 (mRFP) and a guanosine phospho-transferase (GPT) selection marker. 154 To construct a plasmid encoding protein ECTV A36 fused N-terminally to

155 green fluorescent protein (GFP), the A36R gene was amplified from ECTV Mos 156 genomic DNA with primer pair a36r.for/a36r.rev. PCR products were gel 157 purified (Gel Extraction Kit, Promega), digested with BglII and NotI before 158 ligation into a plasmid vector containing GFP under the control of a synthetic 159 viral early/late promoter (pE/L) (5, 58). A plasmid containing ECTV A36<sup>Y112F</sup> 160 fused to GFP was constructed in the same way, excepting the initial PCR 161 amplification, which instead was a two-step fusion PCR. The first step involved 162 amplification of ECTV genomic DNA with primer pairs a36r.for/a36r.mutrev and 163 a36r.mutfor/a36r.rev which each amplified half of A36R. These PCR products 164 were used as template for a second step of amplification with a36r.for/a36r.rev, 165 resulting in a full-length A36R gene product incorporating a Tyr to Phe mutation 166 at site 112. VACV A36R-GFP plasmid was constructed as described previously 167 (1), with a wild type A36R rather than A36R-YdF.

168 A plasmid encoding Lifeact, a 17 amino acid peptide that binds to 169 filamentous actin (57), fused N-terminally to GFP under pE/L was constructed in 170 a similar process as described above. However, the sequence of Lifeact was 171 designed into the synthesised primer LifeactGFP.for, which was used with the 172 GFPrev primer on a plasmid template of GFP to generate a PCR product spanning 173 all of the GFP sequence. This PCR product was digested with BglII and NotI 174 purified with a QiaexII Gel Extraction Kit (Qiagen) and ligated in to a pE/L GFP 175 vector.

Transient expression of constructs. HeLa or BSC-1 cells grown to 70%
confluency were infected for 1 h, recovered with fresh growth media for an
additional 1 h before being transfected with ECTV A36R-GFP, ECTV A36R<sup>Y112F-</sup>
GFP or VACV A36R-GFP (standard protocol, Lipofectamine 2000, Invitrogen).

180 Recombinant virus construction. HeLa cells grown to 70% confluency 181 were infected with ECTV Mos and transfected with the recombination cassette 182 (standard protocol, Lipofectamine 2000, Invitrogen). Cells were scraped 24 hpi 183 to allow for homologous recombination between genomic DNA and the 184 recombination cassette and then lysed by freeze thawing. Plaque assays were 185 carried out with the addition of GPT selection medium [25  $\mu$ g/mL mycophenolic 186 acid (MPA), 250 µg/mL xanthine, 15 µg/mL hypoxanthine] in the overlay. 187 Plaques able to grow in GPT selection that displayed mRFP fluorescence were 188 purified and verified as  $\Delta A36R$  with sequencing.

189 Antibodies and fluorescent chemicals. The primary antibodies used in this 190 study were:  $\alpha$ -A36 (61),  $\alpha$ -B5 (9),  $\alpha$ -A27 (60),  $\alpha$ - $\beta$ -actin (Sigma-Aldrich),  $\alpha$ -191 kinesin-1 heavy chain (KHC, H-50, Santa Cruz Biotechnology), α-192 phosphotyrosine (4G10, Chemicon) and  $\alpha$ -mRFP (Chemicon).  $\alpha$ -A36-Y112 was 193 raised against the phosphorylated peptide corresponding to residues 105-119 of 194 VACV A36 (APSTEHIpYDSVAGST) and purified as described previously (44), but 195 it is not dependent on phosphorylation of tyrosine 112 of A36. The secondary 196 antibodies used in this study were as follows (all from Invitrogen): Alexa Fluor(R) 197 568 Goat Anti- Mouse IgG, Alexa Fluor<sup>(R)</sup> 488 Goat Anti-Mouse IgG, Alexa Fluor<sup>(R)</sup> 198 350 Goat Anti-Mouse IgG, Alexa Fluor(R) 568 Goat Anti-Rabbit IgG, Alexa Fluor(R) 199 488 Goat Anti- Rabbit IgG, Alexa Fluor<sup>(R)</sup> 488 Goat Anti-Rat IgG, Alexa Fluor<sup>(R)</sup> 350 200 Goat Anti-Rat IgG, Alexa Fluor<sub>(R)</sub> 568 Goat Anti-Rat IgG,  $\alpha$ -rabbit-HRP and  $\alpha$ -201 mouse-HRP. Fluorescent chemicals used were as follows: DAPI (Sigma-Aldrich, 1 202 µg/mL), Alexa Fluor(R) 488 Phalloidin and Alexa Fluor(R) 568 Phalloidin 203 (Invitrogen, 1:300 dilution).

204 **Immunofluorescence analyses.** Cells were grown on glass coverslips, 205 infected with appropriate viruses and fixed at 8 hpi (VACV) or 24 hpi (ECTV) 206 (unless otherwise stated) with 3% paraformaldehyde (PFA) in cytoskeletal 207 buffer (CB) [10 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer, 0.15 M 208 NaCl, 5 mM ethylene glycol tetraacetic acid (EGTA), 5 mM MgCl<sub>2</sub>, 50 mM glucose, 209 pH 6.1] for 10 minutes at room temperature. Before staining, cells were either 210 permeabilized with 0.1% Triton X-100 in CB or not permeabilized, depending on 211 whether the protein of interest was intracellular or extracellular. Cells were 212 blocked in blocking buffer (1% BSA, 2% FBS in CB) for 20 minutes then 213 incubated for 40 minutes with suitable primary antibodies diluted in blocking 214 buffer. After three washes with PBS, secondary antibodies diluted in blocking 215 buffer were applied to cells for 20 minutes. The coverslips were mounted on a 216 glass slide with 0.3-1% (w/v) P-phenylenediamine (Sigma-Aldrich) in mowiol 217 mounting media [10% (w/v) Polyvinyl Alcohol 4-88 (Sigma-Aldrich), 25% (w/v) 218 glycerol, 0.1M Tris, pH 8.5]. Fluorescent and phase-contrast microscopy were 219 performed with an Olympus microscope BX51 with filter sets 31001, 31002 and 220 31013v2 (Chroma) and resulting images analyzed with Photoshop CS3 (Adobe).

221 Immunoblot analysis. Cells were harvested in sodium dodecyl sulphate 222 (SDS)-reducing sample buffer (62.5 mM Tris-HCl, 0.25 M glycerol, 2% SDS and 223 0.01% (w/v) bromophenol blue, 12.5% (v/v)  $\beta$ -mercaptoethanol) and boiled at 224 95°C for 5 minutes. Proteins were separated by SDS-polyacrylamide gel 225 electrophoresis (SDS-PAGE) (Resolving gel: 10% Acrylamide/Bis Solution, 226 37.5:1, 0.375M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.1% APS and 0.1% TEMED; 227 Stacking gel: 4% of 30% Acrylamide/Bis Solution, 37.5:1, 0.375M Tris-HCl, pH 228 6.8, 0.1%(w/v) SDS, 0.1% APS and 0.1% TEMED). Resolved proteins from SDS-229 PAGE were transferred to nitrocellulose membranes (Hybond-C Extra, 230 Amersham Biosciences) and probed with primary antibodies diluted in PBST-231 milk (5% (w/v) skim milk in PBS with 0.1% Tween-20). The membrane was 232 washed three times in PBST-milk and probed with secondary antibodies 233 conjugated with horseradish peroxidase. Immunoreactive protein bands were 234 visualized with ECL western blotting reagent (GE Health).

Live cell microscopy. 35 mm glass bottom dishes (Mat Tek) were coated in 5  $\mu$ g/cm<sup>2</sup> fibronectin (Sigma-Aldrich) for 2 h before HeLa cells were seeded and grown to 70% confluency. Cells were infected for 1 h, rescued and transfected with pE/L Lifeact-GFP 6 h prior to imaging. Either 8-10 hpi (VACV) or 24-26 hpi (ECTV), cells were imaged on an Olympus FV1000 confocal microscope with a 488nm laser line. Resulting movies were processed with a Manual Tracking plugin in Image J.

242 Mouse experiments. Inbred, specific-pathogen-free female BALB/cAnNCrl 243 (BALB/c), C57BL/6] wild-type mice and Rag-1-deficient B6.129S7-Rag1<sup>tm1Mom</sup>/J 244  $(B6.Rag-1^{-/-})$  (40) mice on a C57BL/6J background at 6-8 weeks of age were 245 obtained from the Australian National University Bioscience Services (Canberra 246 ACT, Australia). Groups of female BALB/c mice were infected subcutaneously in 247 the left hind leg with  $10^2$ ,  $10^3$ , or  $10^4$  pfu WT ECTV or ECTV  $\Delta A36R$  and 248 monitored for survival and clinical signs of disease for 33 days. Separate groups 249 of infected mice were euthanized at 5 and 8 dpi. Rag-1<sup>-/-</sup> mice were inoculated 250 with 10<sup>3</sup> PFU virus and also sacrificed at days 5 and 8 dpi for determination of 251 viral titers in organs. Viral load in livers, spleens and lymph nodes were 252 quantified by viral plaque assay on BSC-1 cell monolayers as described 253 previously (7) and are expressed as  $log_{10}$  PFU/gram tissue or per lymph node.

On day 28 p.i., all surviving BALB/c mice infected with ECTV ΔA36R from the
first group were bled and then challenged with a lethal dose by a subcutaneous
route of 10<sup>4</sup> PFU WT ECTV on day 33. Eight days later, mice were sacrificed and
anti-ECTV antibody in serum and virus in spleen, liver and lymph nodes were
measured.

259 Enzyme-linked immunosorbent assay (ELISA). Serum samples were 260 assayed by ELISA for total ECTV-specific IgG as described earlier (48). Briefly, U-261 bottom 96-well plates (Immulon 2, Dynatech Lab Inc., Alexandria, VA, USA) were 262 coated with purified ECTV. Sera were assayed at 1:200 dilution and ECTV-263 specific antibody detected using horseradish peroxidase-conjugated goat anti-264 mouse IgG (Southern Biotechnology Associates, Birmingham, AL, USA) and color 265 developed with TMB One-Step substrate (Dako Cytomation, Carpinteria, CA, 266 USA).

Plaque reduction neutralization test. The plaque reduction neutralization test, used to determine the neutralizing activity of the antibody present in serum samples to ECTV-WT has been described (48). Serial dilutions of sera, starting at 1:50 dilution were incubated with 100 pfu of ECTV for 1 h before being added to wells of BS-C-1 cell monolayers. The neutralization titer was taken as the reciprocal of the dilution of sera that caused a 50% reduction in the number of virus plaques.

274 Cytotoxic T lymphocyte (CTL) and natural killer (NK) cell assays. CTL 275 and NK cell assays were performed as described elsewhere (7, 37). To measure 276 ex vivo CTL responses, spleen cells from infected animals were assessed for the 277 ability to kill <sup>51</sup>Cr-labelled virus-infected or uninfected syngeneic P815 target 278 cells over a 6 h culture period. To assess NK cell activity *ex vivo*, spleen cells from 279 infected or uninfected BALB/c and C57BL/6 mice were cultured with 51Cr-280 labelled YAC-1 target cells for 4 h and the radioactivity in the supernatant 281 measured in a TopCount NXT<sup>™</sup> scintillation counter.

282

## 283 Results

284 Microtubule and actin-based transport is active during ECTV infection. 285 Orthopoxvirus infection results in the formation of highly compartmentalized 286 replication centers during productive infection. These replication centers can be distinguished using a combination of histological markers and stains. For 287 288 example, the virus factory is positive for DNA stains such as DAPI and viral 289 markers including p14 (A27) (63) but negative for WV markers A36, A33, A34 290 and B5, which localize to the trans-Golgi network where MV are wrapped to form 291 WV (61, 74). Using a combination of stains and antibodies specific to VACV 292 proteins found also to cross-react to ECTV proteins, we analyzed ECTV-infected 293 BSC-1 cells at various time points post-infection. Unlike VACV infected cells, we 294 identified the formation of a virus factory at approximately 4-8 hours post 295 infection (hpi) (Fig. 1A). B5 expression was initiated at approximately the same 296 time and was generally concentrated to a collapsed trans-Golgi network that was 297 localized adjacent to the nucleus (data not shown and Fig. 2C). Overall, ECTV 298 infection closely resembles VACV infection albeit slower by approximately 1.5-299 fold and this was found to be the case in multiple, including murine, cell types 300 (MEFs and NIH3T3, data not shown). Hence, ECTV infection faces a similar 301 transport hurdle of delivery of WV from the perinuclear trans-Golgi network to 302 the cell periphery. As this transport step is dependent on microtubule-dependent 303 transport in VACV replication, we tested whether the appearance of extracellular 304 WV required an intact microtubule cytoskeleton. Treatment of infected cells with 305 nocodazole, an agent that interferes with microtubule polymerization, drastically 306 reduced the appearance of WV at the cell surface confirming that, similar to 307 VACV replication, ECTV requires microtubule transport (Fig. 1B). We also 308 localized the kinesin-1 heavy chain (KHC) of the kinesin-1 motor complex to WV 309 particles suggesting that the mechanism of microtubule transport is also 310 conserved (Fig. 6B). Previous reports have demonstrated that ECTV also 311 undergoes actin-based transport by the stimulation of the nucleation of actin 312 filaments beneath extracellular WV (2). We observed actin comets in 313 approximately 65% of ECTV infected BSC-1 cells at 16 hpi, compared to 314 approximately 50% in VACV at 8 hpi, which is consistent with the delayed ECTV

315 replication cycle (Fig. 2A-B). Induction of actin-based motility was robust and316 was observed in multiple cell types, including murine (data not shown).

317 Actin-based motility of VACV is dependent on recruitment of N-Wasp to 318 phosphorylated tyrosines on A36 (via the adaptor proteins Nck, Grb2 and WIP), 319 which then stimulates activity of the Arp2/3 complex (27, 41, 44, 45, 55, 65, 78). 320 We first observed through immunofluorescence analysis of ECTV-infected cells 321 that phosphorylated tyrosine localized to actin comets (Fig. 2D). We therefore 322 tested whether N-Wasp was required for ECTV induced actin-based motility. 323 Infection of N-Wasp knockout MEFs resulted in cells bereft of virus-associated 324 actin comets, a phenotype rescued by transient expression of GFP-tagged N-325 Wasp (Fig. 2E). Taken together, these results demonstrate that in addition to a 326 high conservation at the genomic level, the replication of ECTV is highly 327 conserved to that of VACV at the cellular level, although substantially slower. 328 Like VACV, replication of ECTV includes transport of WV that appears to be 329 dependent on kinesin-1-mediated microtubule transport and N-Wasp dependent 330 actin-based motility.

331 ECTV encodes a homologue of VACV protein A36. The WV-specific viral 332 transmembrane protein A36 is a critical mediator of both actin- (26) and 333 microtubule-based transport of VACV (15, 58, 75). We searched the ECTV 334 (Moscow) genome for a homologue of A36 that might be responsible for 335 subcellular transport of WV during ECTV replication. We identified the ECTV 336 open reading frame 137.5f as a candidate A36R homologue, consistent with 337 previous findings (8). ECTV A36R resides at a conserved genomic locus and 338 encodes a hypothetical protein of 160 residues with 92% amino acid identity 339 with the first 157 residues of VACV A36 (Fig. 3A). Homology is lost at position 340 155 of ECTV A36, which appears to be truncated at the C-terminus. Despite the 341 predicted C-terminal truncation, the regions critical for actin-based motility (Tyr 342 112) (26) and the dominant interaction with kinesin-1 (WD 97-98) were 343 conserved (15, 75). The ancillary interaction at residues 64-65, WE in VACV A36, 344 had an amino acid substitution resulting in a second WD motif in ECTV A36. 345 Using antiserum raised against a peptide corresponding to residues 105-119 of 346 VACV A36 we confirmed that ECTV A36 was expressed during ECTV infection, 347 beginning at approximately 8 hpi. ECTV A36 runs at approximately 32 kDa, 348 smaller than VACV A36, which runs at 43-50 kDa (52) (Fig. 3B). Although both 349 proteins run considerably above their predicted molecular weight, the 350 discrepancy is consistent with the deletion in the 3' of ECTV A36R ORF. ECTV 351 A36 also produced a visible band in the presence of cytosine arabinoside (AraC), 352 an inhibitor of DNA replication and late gene expression, which indicates that 353 like VACV A36, ECTV A36 has an early component to its expression (Fig. 3B) 354 (52). Immunofluorescence analysis of ECTV-infected cells demonstrated that 355 A36 localized to the trans-Golgi network and co-localized with B5 at single virus 356 particles, consistent with WV-labelling (Fig. 2C).

357 Deletion of A36R results in defective subcellular transport. As ECTV 358 A36R appeared to be a homologue of VACV A36 based on sequence conservation, 359 expression and localization, we sought to demonstrate that ECTV A36R was also 360 required for actin- and microtubule-based motility. We generated a plasmid 361 construct designed to replace the A36R open reading frame with selectable and 362 screenable markers. Using this construct we selected for recombinant virus able 363 to replicate in the presence of GPT selection media and expressing mRFP from an 364 artificial synthetic early/late promoter. The integrity of the  $\Delta A36R$  recombinant 365 virus was tested by PCR analysis from genomic DNA (Fig. 4A) and sequencing 366 across the insertion site (data not shown). We confirmed that A36R expression 367 was not detectable in ECTV  $\triangle$ A36R-infected lysates by immunoblot (Fig. 4B) and 368 in ECTV  $\triangle$ A36R-infected cells by immunofluorescence assays (Fig. 4C).

369 To analyze the replication dynamics and spread of ECTV  $\Delta A36R$  we 370 performed WV release assays and plaque assays. There was a severe reduction in 371 infectious virus release during replication of ECTV  $\Delta A36R$  although overall 372 production of infectious virus was unaffected (Fig. 5A-B). Plaque size of ECTV 373  $\Delta$ A36R was also greatly reduced compared to the parental strain (Fig. 5C-D). As 374 reduction in plaque size and virus release is consistent with compromised 375 subcellular transport, we next examined ECTV  $\Delta$ A36R-infected cells by 376 immunofluorescence for the hallmarks of actin- and microtubule-based 377 transport. Loss of A36R resulted in a reduction in the appearance of extracellular 378 WV, and those that reached the cell surface were confined to central regions of 379 the cell where the trans-Golgi network lies in close proximity to the plasma 380 membrane (Fig. 6A). Virus particles no longer colocalized with KHC consistent 381 with abrogated microtubule-dependent transport (Fig. 6B). WV that did reach 382 the cell surface were not associated with actin comets (Fig. 6C). To confirm that 383 these phenotypes were due specifically to the loss of A36 expression, we were 384 able to rescue transport by the transient expression of GFP-tagged ECTV A36R 385 and A36R-Y112F. While both constructs were able to rescue motility of WV to 386 the cell surface (Fig. 7A), only A36R-GFP and not A36R-Y112F-GFP was able to 387 rescue actin-based motility (Fig. 7B). This is consistent with previous findings 388 made with VACV, where mutation of the critical Tyr 112 to Phe abrogates actin-389 based motility (27). In summary, ECTV A36 is a functional homologue of VACV 390 A36 and is required for efficient WV intracellular transport and actin-based 391 motility during ECTV infection.

392 We observed that actin comets that localized to extracellular WV during 393 ECTV infection had a divergent morphology being, on average, shorter in length 394 (1.59µm for ECTV and 2.88µm for VACV, Fig. 8A) and this difference was found 395 to be statistically significant (p < 0.0001, Unpaired t-test). The difference in actin 396 comet morphology could be due to differences between the viral initiators (ECTV 397 A36 and VACV A36) of actin polymerisation, in particular the C-terminal 398 truncation, differences in other viral proteins or the time point at which actin-399 based motility was visualized due to the delayed ECTV replication cycle. We 400 determined that the distinct morphologies of ECTV- and VACV-induced actin 401 comets was not due to delayed replication dynamics as VACV infected cells at 24 402 hpi retained the 'VACV-like' morphology (Fig. 2B). We therefore focused on 403 testing the former hypotheses. We examined WV-associated actin comets in 404 VACV  $\Delta A36R$  and ECTV  $\Delta A36R$  infected cells transiently rescued with GFP-405 tagged VACV A36 and ECTV A36. Experiments demonstrated that ECTV  $\Delta$ A36R 406 produced statistically shorter tails than VACV ΔA36R when rescued with either 407 GFP-tagged VACV A36 or ECTV A36 (unpaired t-test, p=0.0006 and p=0.0041 408 respectively) (Fig. 8A). This verified that the difference in WV-associated actin 409 comet morphology is inherent to the background of the virus and not the identity 410 of the viral initiator.

Live cell imaging of ECTV- and VACV-infected cells revealed that differences
in WV-associated actin comet morphology did not lead to changes in the speed of
actin-based motility (Fig. 8B-C, Video. S1-2). Although ECTV-induced actin

414 comets were not significantly different in speed to VACV-induced actin comets, 415 there was more speed variation between individual actin comets, with standard 416 deviations of 0.050  $\mu$ m/s and 0.018  $\mu$ m/s, respectively. Furthermore, 100% of 417 VACV-induced actin comets visible at the start of imaging persisted for the 418 duration of imaging (1 min 13 s) compared to 28% for ECTV virus (Fig. 8D).

419 **ECTV** ΔA36R is attenuated *in vivo*. Having demonstrated that A36R plays a 420 conserved role in directing the subcellular transport of ECTV, we next examined 421 the effects of disabling this motility on infection of the ECTV-susceptible BALB/c 422 mouse strain using three different doses of virus. In the group that received the 423 lowest dose of 10<sup>2</sup> pfu WT ECTV, 10 of 15 mice (66%) succumbed to mousepox 424 and died by 18 days post-infection (dpi) (Fig. 9A). Mice that had survived past 425 day 18 developed pox lesions on their tails that ulcerated with time and were 426 therefore euthanized at 28 dpi for ethical reasons. Animals in the groups infected 427 with  $10^3$  (n=7) or  $10^4$  pfu (n=6) of WT ECTV succumbed to mousepox with 100%428 mortality, with a mean time to death of 11.1 and 8.8 days, respectively. All mice 429 infected with WT ECTV had unkempt hair coat and hunched posture from about 430 day 6 pi and generally became moribund before they died. In contrast, mice infected with the 3 different doses of ECTV  $\Delta$ A36R survived until at least 33 dpi 431 432 (Fig. 9A) with no overt clinical signs of disease. Organism wide dissemination of 433 virus was clearly affected by deletion of A36R. The viral load in the draining 434 lymph nodes, spleen and liver at 5 dpi was 3 to 4  $\log_{10}$  pfu higher in mice infected 435 with  $10^2$  pfu WT ECTV than in those infected with ECTV  $\Delta A36R$  (Fig. 9B). This 436 difference further increased to  $5 \log_{10}$  pfu at 8 dpi due to the increasing viral load 437 in all organs of WT ECTV infected mice and decreasing viral load in all organs of 438 ECTV  $\triangle$ A36R infected mice (Fig. 9C). While WT ECTV infection is lethal in 439 BALB/c mice, ECTV  $\Delta$ A36R infection is readily controlled, showing strong 440 attenuation in vivo. This attenuation was also evident in ECTV-resistant C57BL/6 441 wild type (data not shown) and C57BL/6 Rag-1<sup>-/-</sup> mice, which lack B or T cells, 442 and therefore do not possess adaptive immunity (Fig. 9D). Titers of ECTV  $\Delta A36R$ 443 were between 1 to 4  $log_{10}$  PFU lower than those of WT ECTV at 5 dpi. We were 444 unable to determine viral load in organs of Rag1-/- mice at 8 dpi as all mice 445 infected with WT ECTV had died by this time whereas those infected with ECTV 446  $\Delta$ A36R were still alive with no clinical signs of disease.

447 The finding that ECTV  $\triangle$ A36R titers were significantly lower compared to those of WT ECTV even in C57BL/6 Rag-1<sup>-/-</sup> mice early during the course of 448 449 infection suggested that either the virus replicated poorly in vivo or that the 450 innate immune system effectively controlled virus replication. Since NK cells 451 play a key role in controlling ECTV replication early in infection and are activated 452 rapidly following ECTV infection (7, 25, 50), we assessed the cytolytic activity of 453 NK cells in virus-infected BALB/c and C57BL/ wild type mice at 5 dpi, the peak of 454 the NK cell response. The NK cell activity induced by ECTV  $\Delta$ A36R was more than 455 3-fold lower in BALB/c mice (Fig. 9E) and at least 27-fold lower in C57BL/6 mice 456 (Fig. 9F) than the activity that was generated by WT ECTV infection in both 457 strains of mice. The data suggested that it is unlikely that NK cells contributed to 458 the early control of mutant virus replication.

459 ECTV **AA36R** infection induces protective immunity. We determined 460 whether ECTV  $\Delta A36R$ , which replicated poorly in mice, induced any protective 461 immunity to ECTV. All mice that had survived infection with either  $10^2$ ,  $10^3$  or 462 10<sup>4</sup> pfu ECTV  $\Delta$ A36R were challenged with a lethal dose (10<sup>4</sup> pfu) of WT ECTV at 463 33 dpi. Eight days later, mice were sacrificed, their spleen, liver and lymph 464 nodes collected for determination of viral load whereas the serum was used to 465 measure anti-ECTV antibody titers. Splenocytes were used to measure the recall 466 anti-ECTV-specific CTL response.

467 Virus was below the level of detection in spleen, liver and lymph nodes in 468 mice challenged with a lethal dose of WT ECTV (data not shown), indicating that 469 a primary infection with all 3 doses of ECTV ΔA36R induced protective immunity 470 against a subsequent lethal virus challenge. Recall anti-ECTV CTL responses 471 were generated in all groups at near comparable levels in magnitude (Fig. 10A).

472 Since antibody plays a critical role in recovery and virus control during both 473 primary and secondary ECTV infection (48, 49), we measured virus-specific 474 antibodies in sera obtained from mice infected with ECTV ΔA36R during primary 475 infection and subsequent secondary challenge. ECTV-specific IgG was clearly 476 induced during primary (14 and 28 dpi) infection with ECTV ΔA36R and 8 days 477 post-challenge with WT ECTV (Fig. 10B). Antibody titers were augmented as the 478 virus inoculum dose was increased. It was also evident that as the antibody 479 response matured over time (i.e. between 14 and 28 dpi), the anti-ECTV IgG

480 titers also increased. In addition, IgG titers were boosted further in all groups 481 following secondary virus challenge. ECTV  $\Delta$ A36R generated neutralizing 482 antibodies during a primary infection, and neutralizing titers increased 483 substantially after the secondary virus challenge (Fig. 10C).

484

## 485 Discussion

486 The ECTV (Moscow) genome is highly conserved between related members 487 of the orthopox genus, with approximately 90-99% identity at the amino acid 488 level for WV-specific proteins (8). Our analysis of ECTV replication suggests the 489 morphogenesis pathway that gives rise to WV is also conserved. A subset of MV 490 generated at the virus factory acquires B5 and A36 immuno-reactivity and are 491 transported to the cell periphery in a microtubule-dependent manner. We 492 observed a delay in the replication of ECTV in vitro compared to VACV (Western 493 Reserve) that was independent of cell type and origin of host cells. The 494 difference in replication dynamics may reflect adaptation by VACV to replication 495 in cultured cells, as this delay is not apparent *in vivo* where infection of mice with either VACV or ECTV is lethal 6-8 days post-infection (52). This interpretation is 496 497 complicated by the absence of a known endemic host for VACV and differences in 498 virus titer used to achieve a lethal dose.

499 Through immunoblot analyses, we observed that like VACV A36, ECTV A36 500 has an early component to its expression profile and alignments of the promoter 501 region up to 100 nucleotides upstream of the open reading frames of the two 502 genes showed 98% identity. The early expression of VACV A36 is believed to be 503 important in repulsion of super infecting virions, a process which allows viral 504 spread to occur at a rate beyond the speed of its replicative cycle (13). Through 505 cell surface signalling, viral entry into already infected cells is blocked and super 506 infecting virions are directed toward uninfected cells (13). Our results show that, 507 as ECTV A36 is expressed early, it is possible that repulsion of super infecting 508 virions also occurs in ECTV.

509 We have shown that the viral protein ECTV A36 plays a key role in mediating 510 subcellular transport of ECTV, as has been shown previously for VACV A36 511 during VACV replication (27, 33, 52, 75, 76). Divergent orthopoxviruses appear 512 to encode functional homologues of A36 at conserved genomic loci and while 513 these are highly conserved at the N-terminus they lack significant sequence 514 conservation (8-15% amino acid identity) at the C-terminus (53). ECTV A36 is 515 consistent in displaying greater diversity at the C-terminus, in fact possessing the 516 greatest extent of divergence amongst all available orthopoxvirus genomes with 517 its predicted 63 residue C-terminus truncation.

518 Immunoblot analysis confirmed that ECTV A36R encoded a truncated 519 protein. Nonetheless, it retains the essential transport functions of VACV A36 520 and possesses microtubule-based motility (Fig. 6-7). These observations are in 521 agreement with a previous study in which microtubule transport of WV is 522 rescued in a VACV  $\triangle$ A36R strain by the expression of the transmembrane 523 domain fused to A3671-100 suggesting that a KLC-binding site lies within this 524 region (58). This was further supported with yeast 2-hybrid assays demonstrating A36<sup>81-100</sup> was sufficient to mediate binding to KLC (75). In a 525 526 recent study, kinesin-1 recruitment has been narrowed down to a bipartite 527 tryptophan motif at residues 64/65 (WE) and 97/98 (WD) of VACV A36 (15). 528 While ECTV A36 is conserved at the WD site, it has an amino acid substitution of 529 another acidic residue at the WE site, creating a second WD site. Even though the 530 sites are not absolutely conserved, a bipartite tryptophan domain is retained. In 531 their study, Dodding and colleagues demonstrate that substitution of the 532 WE/WD bipartite tryptophan motif with a WD/WD motif from Calsytenin 533 rescues kinesin-1 recruitment, and this substitution is unlikely to have any 534 deleterious effects on microtubule-based motility. ECTV WV particles were still 535 found to partially colocalize with both components of the kinesin-1 motor 536 complex, KHC and KLC (Fig. 6B and data not shown) suggesting that microtubule 537 transport of WV is likely to operate via a conserved mechanism during ECTV 538 replication.

Furthermore, although the exact role of F12 in microtubule motility of VACV
remains to be clarified, evidence suggests that it may mediate or stabilize the
binding of A36 to kinesin-1 (14, 36, 42, 73) with yeast 2-hybrid screens
identifying a specific interaction between F12<sup>351-458</sup> and A36<sup>91-111</sup> (14, 36, 42,
73). ECTV Mos possesses a highly conserved homolog to VACV WR F12 (96.69%
identity) that only bears two amino acid substitutions in region 350-457 which
may further suggest a conserved mechanism of microtubule-based motility.

Actin-based motility has been observed across the orthopox genus in VACV (9), ECTV (this study and (2, 4, 23)), VARV and monkeypox (56), as well as in more distantly related poxviruses such as myxoma virus (20) and yabalike disease virus (18, 20, 38). In the closely related orthopoxviruses the nucleation of actin filaments is likely to be mediated by homologues of VACV A36, as we 551 have demonstrated is the case in ECTV. Significantly, ECTV A36 is also the only 552 orthopoxvirus homologue to lack the second tyrosine (Tyr 132) (53), 553 nonetheless, ECTV A36 is sufficient to promote actin-based motility in ECTV and 554 VACV (data not shown). Interestingly, although the more divergent poxviruses do not encode A36 orthologues, the genomic locus where functional actin 555 556 nucleators are encoded is conserved (18). This study is only the third report 557 defining the loss of actin-based motility in a recombinant virus deleted for the 558 viral nucleator, along with VACV and baculovirus (29, 46).

559 We observed that ECTV-induced actin comets were morphologically distinct 560 from those present during VACV infection, being shorter in length, a difference 561 that was independent of the time point examined. The obvious explanation for 562 these findings was the absence of the Grb2 binding site (Tyr 132), which when 563 mutated to Phe blocks phosphorylation and Grb2 recruitment and results in 564 shorter actin tails, increased virus motility and higher N-Wasp turnover (65, 78). 565 However, although shorter, ECTV-induced actin comets were not faster than 566 VACV-induced actin comets. This inconsistency was clarified through cross-567 rescue experiments with GFP tagged VACV A36 and ECTV A36. Although ECTV 568  $\Delta$ A36R rescued with either construct led to tails statistically longer than ECTV, 569 this was likely an artefact of transient expression and still resulted in tails 570 shorter than those produced by VACV  $\Delta$ A36R rescued with the same constructs. 571 This established that the identity of the infecting virus and not the nucleator is 572 responsible for the distinct actin comet morphology. Infection with 573 orthopoxviruses leads to disruption of many host signaling pathways that could 574 conceivably impact on actin dynamics within the cell. Although there are a 575 number of cellular proteins that are known to affect pathogen actin comet 576 length, for example profilin during Listeria motility (31), elucidation of the 577 mechanism that defines the different rates of actin nucleation between VACV and 578 ECTV must await future studies.

579 The reduced microtubule transport and loss of virus-associated actin comets 580 due to disruption of A36R function in ECTV had a significantly attenuated 581 phenotype *in vivo* in 2 susceptible strains of mice. First, the ECTV-susceptible 582 BALB/c mouse strain did not exhibit any clinical signs of disease and survived 583 doses that were otherwise fatal with WT ECTV. ECTV ΔA36R titers were several 584 orders of magnitude lower in the draining lymph node, spleen and liver than 585 titers of WT ECTV at 5 and 8 dpi. Second, ECTV  $\Delta A36R$  was also attenuated in 586 Rag-1<sup>-/-</sup> mice, which are devoid of an adaptive immune system. Viral load in 587 organs of this strain were also orders of magnitude lower compared with titers 588 of WT ECTV at 5 dpi. All Rag-1<sup>-/-</sup> mice infected with WT ECTV died from the 589 infection at 8 dpi whereas those infected with ECTV ΔA36R were still alive with 590 no clinical signs of disease (data not shown). Although we envisage that Rag-1-/-591 mice would have eventually succumbed to infection with the mutant virus since 592 adaptive immunity is essential for complete recovery (6, 7), this finding further 593 attests to the importance of microtubule transport and actin-based motility for 594 virus replication, spread and ability to cause disease.

595 The BALB/c strain normally generates poor immune response to ECTV 596 infection and depending on the virus dose, succumbs to mousepox between 7-20 597 dpi (Fig. 9A) (7, 62). However, when infected with mutant viruses lacking 598 thymidine kinase activity (49) or immune evasion molecules that target 599 interferon function (62, 80), they are able to generate an antiviral immune 600 response and recover. Thus, reducing the level of virus replication can tip the 601 balance in favor of the BALB/c mouse, allowing it sufficient time to mount CTL 602 and antibody responses to overcome an infection that is otherwise lethal.

603 The NK cell response to ECTV infection generally peaks by 5 dpi and 604 contributes to early virus control (7, 25, 50). We reasoned that replication of the 605 ECTV  $\Delta$ A36R was possibly controlled by NK cells, as mutant virus titers were at 606 least 1-4 log<sub>10</sub> pfu lower than WT ECTV at 5 dpi. However, infection of the ECTV-607 susceptible BALB/c or ECTV-resistant C57BL/6 mice with the mutant virus 608 induced very weak NK cell cytolytic activity that was several fold lower in 609 magnitude than the activity generated by WT ECTV in both mouse strains. 610 Although a role for other innate cell types in rapidly reducing mutant virus titers 611 rapidly cannot be excluded, we believe that the attenuated phenotype of this 612 virus, in contrast to some other ECTV mutants discussed above, is due to its 613 inability to spread efficiently as a consequence of defective subcellular transport. 614 Although we did not measure the primary CTL response in BALB/c mice 615 infected with ECTV  $\Delta$ A36R, it is very likely that cell-mediated immunity 616 contributed to the recovery from primary infection (7). Anti-ECTV antibody was clearly generated during primary infection (Fig. 10B) and this response is vitalfor complete recovery from a primary infection.

619 Despite the significant attenuation of ECTV  $\Delta$ A36R, it nonetheless induced 620 protective immunity. BALB/c mice that had recovered from infection with 3 621 different doses of ECTV  $\triangle$ A36R were protected against challenge with a lethal 622 dose of ECTV. At day 8 post-challenge, we were unable to demonstrate the 623 presence of virus in all organs examined. Recall CTL (Fig. 10A) and antibody (Fig. 624 10B-C) responses were clearly generated, and are indicative of induction of T 625 and B cell memory during primary infection. Although a role for cell-mediated 626 immunity cannot be completely excluded, humoral immunity is known to be 627 absolutely required for recovery from a secondary orthopoxvirus infection. We 628 have previously shown that humoral immunity but not the function of CD4 or 629 CD8 T lymphocyte subsets is required to control virus replication during the 630 acute phase of a secondary ECTV infection (48, 49). This requirement for 631 antibody is not unique to ECTV as elimination of CD4 or CD8 T cells does not 632 affect monkeypox virus clearance or the neutralizing antibody response during 633 the acute phase of a secondary infection in VACV vaccinated macaques (21). The findings in the current study suggest that orthopoxvirus vectors with A36 634 635 deletions may be considered as safe vaccine candidates. A36 deletions generate 636 virions expressing all envelope proteins, so while they have defective 637 transmission and are severely attenuated, all neutralising epitopes are present in 638 their natural state.

639 This is the first ECTV recombinant virus with a WV-specific open reading 640 frame deleted. The significant reduction in the capacity of mutant virus to spread 641 locally and be transmitted to other tissues in vivo highlights the importance of 642 infective WV and the specific roles played by A36 in subcellular transport. 643 Deletion of genes encoding WV-specific proteins in VACV such as A36, B5 and 644 A34 also lead to highly attenuated infections in mice (22, 39, 52, 72). The 645 intranasal route of inoculation used in most of these studies mimics large droplet 646 transmission of smallpox in humans, leading to systemic disease that shares 647 similarities with smallpox disease progression (3, 47, 51). However, intranasal 648 infection of mice with VACV WR require large doses of between  $10^4 - 10^6$  pfu to 649 achieve substantial mortality (22, 39, 52), which may not be reflective of doses of  $^{650}$  variola virus required to cause disease and death in humans. Using lower  $^{651}$  infectious doses ( $10^{-2} - 10^{-4}$  pfu), we have shown that deletion of A36R in ECTV  $^{652}$  attenuates the virus to the extent that even genetically susceptible mice are able  $^{653}$  to survive the infection and generate immunological memory. Our  $^{654}$  characterization of the role and function of A36R with a combination of a host and its natural pathogen offers considerable advantages to elucidate its  $^{656}$  contribution to virulence.

657 Subcellular transport pathways are critical steps in the replication of many 658 viruses, particularly large DNA viruses that are significantly hindered by the host 659 cytoplasm environment (70). Recruitment of cellular microtubule motors during 660 virus egress is often complex, requiring multiple, redundant interactions with 661 surface viral proteins. These surface viral proteins are often pleiotropic and 662 possess key roles in other stages of morphogenesis, obfuscating straightforward 663 genetic analysis of their function in transport. For example, egress of Herpes 664 Simplex Virus-1 (HSV-1) requires kinesin-1 function and while HSV-1 tegument 665 proteins pUL36 (VP1/2) and pUL37 are good candidate interaction partners 666 (54), null mutants do not produce enveloped virions (10, 11). The effect of these 667 mutations on kinesin-1 recruitment therefore cannot be effectively analysed and 668 in fact, no mutations in HSV-1 have been identified that mimic the loss of kinesin-669 1 function (12, 17). Similarly, ablation of kinesin-2 function blocks Kaposi's 670 Sarcoma-Associated Herpesvirus release and while viral protein ORF45 has been 671 shown to interact with kinesin-2 through Y2H screens, again null mutants are 672 morphologically defective (64, 81).

673 In contrast, the microtubule-dependent egress of VACV is mediated by the 674 well-defined interaction between A36 and the kinesin-1 complex, disruption of 675 which produces a strong defect in virus transport but not other aspects of virus 676 morphogenesis. We have exploited this knowledge and translated it to the 677 related ECTV to demonstrate movement on microtubules and polymerised actin 678 is essential for virus propagation and transmission in its natural host. In an intact 679 animal, cell-to-cell virus transmission occurs in a complex 3-dimensional 680 environment of tissue. This is distinct from the predominantly 2-dimensional 681 surface imposed by virus cell culture techniques such as plaque assays, where 682 virus spread occurs within a cell monolayer and which may overstate the importance of cytoplasmic transport. Our results confirm that cytoplasmic
transport is a fundamental barrier to orthopoxvirus transmission in an intact
host and therefore an attractive target for inhibiting viral pathogenesis.

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## 961 Figure Legends

962

963 **Fig. 1.** ECTV utilises microtubules for egress.

964 (A) BSC-1 cells were infected with ECTV or VACV at MOI 1 and fixed at the 965 indicated time points. Cells were stained with DAPI and assessed for the 966 presence of a virus factory (n=50 per timepoint, in duplicate). The percentage of 967 virus factory containing cells was calculated. Error bars indicate standard error. 968 (B) HeLa cells were infected with ECTV and at 8 hpi 33µM nocodazole (Sigma) 969 was added to wells and either fixed 16 h later or washed three times in cell 970 growth medium as a nocodazole wash-out control. Wash-out controls were 971 allowed to incubate for an additional 24 h post wash out before being fixed. Non-972 permeabilized cells (NPC) were stained for immunofluorescence assays with  $\alpha$ -973 B5 (green) and DAPI (blue). Arrowheads indicate wrapped virus, asterisk 974 indicates B5-positive vesicle, chevron indicates MV and the dotted line indicates 975 the cell periphery, as determined by phase contrast microscopy.

976

977 Fig. 2. Actin-based motility of ECTV is via a N-Wasp dependent pathway.

978 (A) BSC-1 cells were infected with ECTV or VACV at MOI 1 and fixed at the 979 indicated time points. Cells were stained with phalloidin and assessed for the 980 presence of actin comets (n=50 per timepoint, in duplicate). The percentage of 981 virus factory containing cells was calculated. Error bars indicate standard error. 982 (B) HeLa cells were infected with ECTV or VACV, fixed and stained for 983 immunofluorescence assays with  $\alpha$ -B5 (NPC) (red), phalloidin (green). 984 Arrowheads indicate a WV associated with an actin comet. (C) ECTV-infected 985 cells were also probed with  $\alpha$ -A36-Y112 (green), phalloidin (red) and  $\alpha$ -A27 986 (blue) or (D) phalloidin (red), an anti-phosphotyrosine antibody (4G10, green) 987 and DAPI (blue). (E) N-WASP(-/-) MEFs were mock-transfected or transfected 988 with a N-WASP-GFP expression vector (green). Twenty-four h post-transfection, 989 cells were infected with ECTV and fixed. For immunofluorescence assays, fixed 990 cells were stained with phalloidin (red) and DAPI (blue). Arrowheads indicate 991 WV (C) or WV associated with an actin comet (D, E). Scale bars represent 10µm. 992

993 **Fig. 3.** ECTV encodes a highly conserved homologue of A36R.

994 (A) Translations of open reading frames A36R in ECTV Mos (NCBI Genome 995 Accession: AF012825) and A36R in VACV WR (NCBI Genome Accession: 996 AY243312) obtained from www.poxvirus.org and aligned in Geneious 5.4.4 997 (Biomatters Ltd). Bars represent bipartite tryptophan domains for binding of kinesin-1 and microtubule-based motility. Chevrons represents tyrosine 998 999 phosphorylation sites for actin-based motility. (B) BSC-1 cells were infected with 1000 ECTV or VACV at MOI 1 in the absence or presence of  $40\mu g/ml$  AraC (Sigma) at 1001 the time points indicated. Cell lysates were separated with SDS-PAGE and 1002 transferred to nitrocellulose membranes for immunoblotting with  $\alpha$ -A36-Y112, 1003 detecting ECTV or VACV A36. Immunoblots were also probed with  $\alpha$ - $\beta$ -actin as a 1004 loading control. Molecular size markers are indicated on the left.

1005

1006 Fig. 4. Construction and verification of ECTV  $\Delta$ A36R.

1007 (A) BSC-1 cells were infected with ECTV or recombinant ECTV  $\Delta$ A36R and cell 1008 lysates collected at 48 hpi Genomic DNA was extracted and PCR was conducted 1009 with two primer pairs a36r.lafor/a36r.rarev and a36r.for/a36r.rev to confirm 1010 deletion of A36R and insertion of selection cassette. Uninfected cell lysate 1011 genomic DNA and no template control were run simultaneously as negative 1012 controls. Molecular size markers are indicated on the left. Schematic of the PCRs 1013 performed is represented on the right. (B) BSC-1 cells were infected with VACV, 1014 VACV ΔA36R, ECTV or ECTV ΔA36R at MOI 1 and cell lysates collected at 16 hpi 1015 (VACV) or 48 hpi (ECTV). Cell lysates were separated with SDS-PAGE and 1016 transferred to nitrocellulose membranes for immunoblotting with  $\alpha$ -A36 (raised 1017 against the C terminus of VACV A36 that is absent in ECTV A36),  $\alpha$ -A36-Y112,  $\alpha$ -1018 mRFP and  $\alpha$ -ß-actin as a loading control. Molecular size markers are indicated 1019 on the left. (C) HeLa cells were infected with ECTV  $\Delta$ A36R, fixed and stained for 1020 immunofluorescence assays with  $\alpha$ -A36-Y112 (green), phalloidin (red) and  $\alpha$ -1021 A27 (blue). Arrowheads indicate WV. Scale bar represents 10µm.

1022

1023 **Fig. 5.** ECTV ΔA36R results in reduced WV release and virus spread.

BSC-1 cells were infected with ECTV or ECTV ΔA36R at MOI 0.1 and (A)
supernatants were collected at 32 hpi and centrifuged at 6000 rpm for 10 min to
remove cells and cellular debris or (B) all cells were scraped into supernatants at

1027 16 hpi before undergoing three freeze/thaw cycles to release viruses. Plaque 1028 assays were then performed with BSC-1 monolayers overlayed with 1.5% 1029 CMC/DMEM and stained 5 dpi with crystal violet to quantify plaques. Differences 1030 between ECTV and ECTV  $\triangle$ A36R were statistically different (p=0.0018, Unpaired 1031 *t*-test) for infectious WV released, but not for total infectious virus. (C) BSC-1 cell 1032 monolayers were infected with ECTV or ECTV  $\Delta$ A36R and overlayed with 1.5% 1033 CMC/DMEM. Four dpi, plaques were visualised with phase contrast microscopy 1034 (top panels) and fluorescent microscopy (bottom right panel). Scale bar 1035 represents 100µm. The diameter of plaques was determined in Image 1.4.4. as 1036 the widest point at which cytopathic effects were observed (circle). (D) Data 1037 from three experimental replicates was pooled for n = 30. VACV and ECTV were 1038 statistically different (p<0.0001, Unpaired *t*-test).

1039

**Fig. 6.** Deficient actin- and microtubule-based motility of ECTV ΔA36R.

1041 (A) HeLa cells were infected with ECTV or ECTV ΔA36R, fixed and probed for 1042 immunofluorescence assays with  $\alpha$ -B5 (NPC) (green) and DAPI (blue). The 1043 dotted line is the cell periphery, as determined by phase contrast microscopy. 1044 (B) Infected cells were also probed with  $\alpha$ -B5 (NPC) (red), KHC antisera (green) 1045 and DAPI (blue) or (C)  $\alpha$ -B5 (NPC) (red), phalloidin (green) and DAPI (blue). 1046 mRFP expressed by ECTV  $\triangle$ A36R was bleached by the addition of a high 1047 concentration of P-phenylenediamine [1% (w/v)] into mounting solution. 1048 Arrowheads indicate WV. Scale bars represent 10µm.

1049

1050 Fig. 7. Transport defects of ECTV △A36R are restored by transient A361051 expression.

1052 (A) HeLa were infected with ECTV  $\Delta A36R$  and transfected with plasmid 1053 constructs of A36R-GFP and A36Y112F-GFP (green) for transient expression 1054 under pE/L, fixed then stained for immunofluorescence assays with  $\alpha$ -B5 (NPC) 1055 (red) and DAPI (blue). The dotted line indicates the cell periphery, as determined 1056 by phase contrast microscopy. (B) Infected and transfected cells were also 1057 probed with phalloidin (red) and DAPI (blue). Scale bars represent 10 $\mu$ m.

1058

1059 Fig. 8. Characteristics of ECTV actin-based motility.

1060 (A) BSC-1 cells were infected with VACV or WR, VACV ∆A36R, ECTV Mos, ECTV. 1061 ΔA36R and transfected with plasmid constructs containing VACV A36R-GFP or 1062 ECTV A36R-GFP for transient expression under pE/L. Cells were fixed and 1063 stained for immunofluorescence assays with  $\alpha$ -B5 (NPC) (blue) and phalloidin 1064 (red) to visualise actin tails. Tail lengths of 6 actin tails from at least 6 different 1065 cells in three experimental replicates (n=118) were measured in Imagel (1.4.4)1066 and pooled. There were significant differences in tail lengths (n/s means not 1067 significant, \*\*p<0.005, \*\*\*p<0.001 and \*\*\*\*p<0.0001, Unpaired *t*-test). (B) HeLa 1068 cells were infected with VACV or ECTV, transfected with pE/L Lifeact-GFP and 1069 imaged 8-10 hpi (VACV) or 24-26 hpi (ECTV). Arrowhead indicates tip of actin 1070 comet, asterisk indicates a stationary object. (C) Average speeds of actin comets 1071 in 5 different cells (n=25) calculated over 6 consecutive frames (3.7 s apart), 1072 with the Manual Tracker Plugin in ImageJ. (D) Number of frames that actin 1073 comets in 5 different cells (n=25) visibly persisted over 60 consecutive frames 1074 (1.2 s apart) of image acquistion, VACV and ECTV were statistically different 1075 (p<0.0001, Log-rank [Mantel-Cox] Test).

1076

#### 1077 **Fig. 9.** Virulence of ECTV $\triangle$ A36R in mouse infections.

1078 Groups of 6-15 female BALB/c mice were infected subcutaneously in the left hind leg with  $10^2$ ,  $10^3$  or  $10^4$  pfu ECTV or ECTV  $\Delta A36R$ . Mice were bled on 0, 14 1079 1080 and 28 dpi to measure antibody titers in sera (see Fig 10) and monitored for (A) 1081 survival over 33 days. There were significant differences in survival of mice 1082 infected with mutant virus or WT virus (p<0.0001 at 10<sup>2</sup> and 10<sup>3</sup> pfu and 1083 p<0.001 10<sup>4</sup> pfu, Log-rank [Mantel-Cox] Test). Data shown for the 10<sup>2</sup> dose of 1084 virus has been combined from 2 different experiments. Separate groups of mice 1085 were infected with 10<sup>2</sup> pfu virus and euthanized at (B) 5 or (C) 8 dpi and the 1086 viral load in lymph nodes, spleens and livers was quantified by viral plaque 1087 assays. Groups of 5 Rag-1<sup>-/-</sup> female mice were infected with 10<sup>3</sup> pfu ECTV or 1088 ECTV  $\Delta A36R$  and euthanized at (D) 5 dpi and the viral load in lymph nodes, 1089 spleens and livers was quantified by plaque assays. Titers of ECTV and ECTV 1090  $\Delta$ A36R in lymph nodes, livers and spleens were significantly different (\*p<0.05, 1091 \*\*p<0.01, Mann-Whitney test). Data shown in B, C and D are viral load ± SEM. 1092 The horizontal dotted lines indicate the limit of detection of the viral plaque

1093 assay, which is 1  $\log_{10}$  pfu. Splenic NK cell responses were measured by the

- <sup>51</sup>chromium release assay in groups of 3 (E) BALB/c and (F) C57BL/6 mice that
  been infected with 10<sup>3</sup> pfu of WT or mutant virus.
- 1096

1097 Fig. 10. ECTV  $\triangle$ A36R infection induces protective immunity.

1098 At 33 dpi, all BALB/c mice inoculated with  $10^2$ ,  $10^3$  or  $10^4$  pfu ECTV  $\Delta$ A36R mice 1099 that survived were challenged with a lethal dose of WT ECTV by the sub-1100 cutaneous route. Mice were sacrificed at day 8 post-challenge and sera, spleen, 1101 liver and lymph nodes were collected. The recall CTL response (A) in the spleen 1102 was similar in all groups. ECTV-specific IgG was measured by ELISA in sera (B) 1103 during primary (0, 14 and 28 dpi) and 8 days after secondary challenge. ECTV-1104 specific IgG titers increased with increased doses of virus inoculum, over time 1105 post-infection and after secondary challenge (\* p<0.05, \*\* p< 0.01, Mann Whitney 1106 test) Serial dilutions of sera, starting at a 1:50 dilution, obtained from mice 1107 during primary infection with varying doses of ECTV  $\Delta A36R$  (broken lines) and 1108 after secondary challenge with WT ECTV (solid lines) were used to determine 1109 neutralizing activity (C). ECTV  $\triangle$ A36R induced neutralizing antibody during 1110 primary infection, albeit, levels were below the 50% neutralizing titer 1111 (horizontal dotted line). Virus was not detected in any of the organs following a 1112 secondary virus challenge (data not shown).

1113

1114 **Table 1.** Primers used for constructing plasmids.

Gene	Primer name	Primer sequence (5'-3')
A36R-RA	a36r.rafor	ACCGCGGCCGCCAGATAATGCAGTTTATCAGTGTCG
A36R-RA	a36r.rarev	ACAGGATCCGCTCAATATACGTACTACTAGTTC
A36R-LA	a36r.lafor	AAAAAGCTTCTGTTGAAGTACTTAATGAAGATACC
A36R-LA	a36r.larev	AAAGTCGACCGGATGCTCGAGGTTACAAACATGG
A36R	a36r.for	GGGAGATCTACCATGATGCTGGTACCACTTATCACG
A36R	a36r.rev	TTTGCGGCCGCCGAAAGGATTGGATGAAAGTTAGG
$A36R^{Y112F}$	a36r.mutfor	AGCACGGAACATATTTTCGATAGTGTTGCCGGA
$A36R^{Y112F}$	a36r.mutrev	TCCGGCAACACTATCGAAAATATGTTCCGTGCT
Lifeact	LifeactGFP.for	AAAGATCTACCATGGGTGTCGCAGATTTGATCAAGAA

		ATTCGAAAGCATCTCAAAGGAAGAAGCGGCCGCCAGC
		AAGGGC
Lifeact	GFPrev	TTTGGATCCAACTCCAGCAGGACCATGTGA

1115













ECTV

Α

DAR G-B mRFI

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ECTV ∆A36R

Α



A36R-GFP





A36RY112F-GFP





Liver

Liver

100:1



