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Cover sheet

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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23

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 Δ A36R was severely
35 attenuated when used to infect the normally susceptible BALB/c mouse strain.
36 ECTV Δ 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 Δ A36R 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
52 to the cell periphery (17, 19, 30, 70, 71). This hurdle is most acute for large
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,
65 58, 76, 77). Here, WV fuse with the plasma membrane and are either released
66 directly or following the transient activation of actin-based motility (9, 58, 78).

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
71 compartment and contains a ~190 residue surface in contact with the cytoplasm
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
85 as to the exact function of this protein and its mechanism of action remains to be
86 clarified (14, 36, 42, 73). A36 also directs a second transport event following
87 delivery of WV to the cell periphery, whereby phosphorylation of two tyrosine
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 proteasome 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 ECTV-
113 susceptible BALB/c mice with Δ A36R resulted in drastically attenuated

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**

123 **Ethics Statement.** This study was performed in strict accordance with the
124 recommendations in the Australian code of practice for the care and use of
125 animals for scientific purposes and the Australian National Health and Medical
126 Research Council Guidelines and Policies on Animal Ethics. The protocol was
127 approved by the Animal Ethics and Experimentation Committee of the Australian
128 National University (Permit Number: J.I.G.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 Δ 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 μ g/mL L-Glutamine, 100 Units/mL penicillin and 100 μ g/mL
137 streptomycin and incubated at 37°C with 5% CO₂ 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 μ g/mL L-
144 Glutamine, 100 Units/mL penicillin, 100 μ g/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^{Y112F}
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 GFPprev 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.

176 **Transient expression of constructs.** HeLa or BSC-1 cells grown to 70%
177 confluency were infected for 1 h, recovered with fresh growth media for an
178 additional 1 h before being transfected with ECTV A36R-GFP, ECTV A36R^{Y112F}-
179 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 µg/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 Δ A36R with sequencing.

189 **Antibodies and fluorescent chemicals.** The primary antibodies used in this
190 study were: α -A36 (61), α -B5 (9), α -A27 (60), α - β -actin (Sigma-Aldrich), α -
191 kinesin-1 heavy chain (KHC, H-50, Santa Cruz Biotechnology), α -
192 phosphotyrosine (4G10, Chemicon) and α -mRFP (Chemicon). α -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^(R) 488 Goat Anti-Mouse IgG, Alexa Fluor^(R)
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^(R) 488 Goat Anti-Rat IgG, Alexa Fluor^(R) 350
200 Goat Anti-Rat IgG, Alexa Fluor^(R) 568 Goat Anti-Rat IgG, α -rabbit-HRP and α -
201 mouse-HRP. Fluorescent chemicals used were as follows: DAPI (Sigma-Aldrich, 1
202 μ g/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 MgCl₂, 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) β -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).

235 **Live cell microscopy.** 35 mm glass bottom dishes (Mat Tek) were coated in
236 5 μ g/cm² fibronectin (Sigma-Aldrich) for 2 h before HeLa cells were seeded and
237 grown to 70% confluency. Cells were infected for 1 h, rescued and transfected
238 with pE/L Lifeact-GFP 6 h prior to imaging. Either 8-10 hpi (VACV) or 24-26 hpi
239 (ECTV), cells were imaged on an Olympus FV1000 confocal microscope with a
240 488nm laser line. Resulting movies were processed with a Manual Tracking
241 plugin in Image J.

242 **Mouse experiments.** Inbred, specific-pathogen-free female BALB/cAnNCrl
243 (BALB/c), C57BL/6J wild-type mice and Rag-1-deficient B6.129S7-Rag1^{tm1Mom}/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 10², 10³, or 10⁴ pfu WT ECTV or ECTV Δ 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^{-/-} mice were inoculated
250 with 10³ 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 log₁₀ PFU/gram tissue or per lymph node.

254 On day 28 p.i., all surviving BALB/c mice infected with ECTV Δ A36R from the
255 first group were bled and then challenged with a lethal dose by a subcutaneous
256 route of 10^4 PFU WT ECTV on day 33. Eight days later, mice were sacrificed and
257 anti-ECTV antibody in serum and virus in spleen, liver and lymph nodes were
258 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).

267 **Plaque reduction neutralization test.** The plaque reduction neutralization
268 test, used to determine the neutralizing activity of the antibody present in serum
269 samples to ECTV-WT has been described (48). Serial dilutions of sera, starting at
270 a 1:50 dilution were incubated with 100 pfu of ECTV for 1 h before being added
271 to wells of BS-C-1 cell monolayers. The neutralization titer was taken as the
272 reciprocal of the dilution of sera that caused a 50% reduction in the number of
273 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 ^{51}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 ^{51}Cr -
280 labelled YAC-1 target cells for 4 h and the radioactivity in the supernatant
281 measured in a TopCount NXT™ 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
287 distinguished using a combination of histological markers and stains. For
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 and
316 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 Δ 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 Δ A36R-infected lysates by immunoblot (Fig. 4B) and
368 in ECTV Δ A36R-infected cells by immunofluorescence assays (Fig. 4C).

369 To analyze the replication dynamics and spread of ECTV Δ A36R we
370 performed WV release assays and plaque assays. There was a severe reduction in
371 infectious virus release during replication of ECTV Δ A36R although overall
372 production of infectious virus was unaffected (Fig. 5A-B). Plaque size of ECTV
373 Δ 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 Δ 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 ($1.59\mu\text{m}$ for ECTV and $2.88\mu\text{m}$ 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 ΔA36R and ECTV ΔA36R infected cells transiently rescued with GFP-
405 tagged VACV A36 and ECTV A36. Experiments demonstrated that ECTV ΔA36R
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.

411 Live cell imaging of ECTV- and VACV-infected cells revealed that differences
412 in WV-associated actin comet morphology did not lead to changes in the speed of
413 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 $\mu\text{m/s}$ and 0.018 $\mu\text{m/s}$, 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 10^2 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
431 infected with the 3 different doses of ECTV ΔA36R survived until at least 33 dpi
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 ΔA36R (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 ΔA36R infected mice (Fig. 9C). While WT ECTV infection is lethal in
439 BALB/c mice, ECTV ΔA36R 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^{-/-} mice, which lack B or T cells,
442 and therefore do not possess adaptive immunity (Fig. 9D). Titers of ECTV ΔA36R
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 ΔA36R were still alive with no clinical signs of disease.

447 The finding that ECTV Δ A36R titers were significantly lower compared to
448 those of WT ECTV even in C57BL/6 Rag-1^{-/-} mice early during the course of
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 Δ 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 Δ A36R infection induces protective immunity.** We determined
460 whether ECTV Δ A36R, which replicated poorly in mice, induced any protective
461 immunity to ECTV. All mice that had survived infection with either 10², 10³ or
462 10⁴ pfu ECTV Δ A36R were challenged with a lethal dose (10⁴ 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 Δ A36R 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 Δ A36R during primary
475 infection and subsequent secondary challenge. ECTV-specific IgG was clearly
476 induced during primary (14 and 28 dpi) infection with ECTV Δ A36R 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 Δ 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
496 either VACV or ECTV is lethal 6-8 days post-infection (52). This interpretation is
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 Δ 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
525 demonstrating A36⁸¹⁻¹⁰⁰ was sufficient to mediate binding to KLC (75). In a
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.

539 Furthermore, although the exact role of F12 in microtubule motility of VACV
540 remains to be clarified, evidence suggests that it may mediate or stabilize the
541 binding of A36 to kinesin-1 (14, 36, 42, 73) with yeast 2-hybrid screens
542 identifying a specific interaction between F12³⁵¹⁻⁴⁵⁸ and A36⁹¹⁻¹¹¹ (14, 36, 42,
543 73). ECTV Mos possesses a highly conserved homolog to VACV WR F12 (96.69%
544 identity) that only bears two amino acid substitutions in region 350-457 which
545 may further suggest a conserved mechanism of microtubule-based motility.

546 Actin-based motility has been observed across the orthopox genus in VACV
547 (9), ECTV (this study and (2, 4, 23)), VARV and monkeypox (56), as well as in
548 more distantly related poxviruses such as myxoma virus (20) and yabalike
549 disease virus (18, 20, 38). In the closely related orthopoxviruses the nucleation
550 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
555 do not encode A36 orthologues, the genomic locus where functional actin
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 Δ 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 Δ 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 Δ A36R 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 Δ A36R was also attenuated in
586 Rag-1^{-/-} 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^{-/-} mice infected with WT ECTV died from the
589 infection at 8 dpi whereas those infected with ECTV Δ A36R 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 Δ A36R was possibly controlled by NK cells, as mutant virus titers were at
606 least 1-4 log₁₀ 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 Δ A36R, it is very likely that cell-mediated immunity
616 contributed to the recovery from primary infection (7). Anti-ECTV antibody was

617 clearly generated during primary infection (Fig. 10B) and this response is vital
618 for complete recovery from a primary infection.

619 Despite the significant attenuation of ECTV Δ A36R, it nonetheless induced
620 protective immunity. BALB/c mice that had recovered from infection with 3
621 different doses of ECTV Δ 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
634 findings in the current study suggest that orthopoxvirus vectors with A36
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
655 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

683 importance of cytoplasmic transport. Our results confirm that cytoplasmic
684 transport is a fundamental barrier to orthopoxvirus transmission in an intact
685 host and therefore an attractive target for inhibiting viral pathogenesis.
686

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695

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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 33 μ M 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 α -
 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 α -B5 (NPC) (red), phalloidin (green).

984 Arrowheads indicate a WV associated with an actin comet. (C) ECTV-infected
 985 cells were also probed with α -A36-Y112 (green), phalloidin (red) and α -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 10 μ m.

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
 998 kinesin-1 and microtubule-based motility. Chevrons represents tyrosine
 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 40µg/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 α -A36-Y112,
 1003 detecting ECTV or VACV A36. Immunoblots were also probed with α - β -actin as a
 1004 loading control. Molecular size markers are indicated on the left.

1005

1006 **Fig. 4.** Construction and verification of ECTV Δ A36R.

1007 (A) BSC-1 cells were infected with ECTV or recombinant ECTV Δ 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 Δ A36R, ECTV or ECTV Δ A36R 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 α -A36 (raised
 1017 against the C terminus of VACV A36 that is absent in ECTV A36), α -A36-Y112, α -
 1018 mRFP and α - β -actin as a loading control. Molecular size markers are indicated
 1019 on the left. (C) HeLa cells were infected with ECTV Δ A36R, fixed and stained for
 1020 immunofluorescence assays with α -A36-Y112 (green), phalloidin (red) and α -
 1021 A27 (blue). Arrowheads indicate WV. Scale bar represents 10µm.

1022

1023 **Fig. 5.** ECTV Δ A36R results in reduced WV release and virus spread.

1024 BSC-1 cells were infected with ECTV or ECTV Δ A36R at MOI 0.1 and (A)
 1025 supernatants were collected at 32 hpi and centrifuged at 6000 rpm for 10 min to
 1026 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 overlaid with 1.5%
1029 CMC/DMEM and stained 5 dpi with crystal violet to quantify plaques. Differences
1030 between ECTV and ECTV Δ 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 Δ A36R and overlaid 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 100 μ m. The diameter of plaques was determined in ImageJ 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

1040 **Fig. 6.** Deficient actin- and microtubule-based motility of ECTV Δ A36R.

1041 (A) HeLa cells were infected with ECTV or ECTV Δ A36R, fixed and probed for
1042 immunofluorescence assays with α -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 α -B5 (NPC) (red), KHC antisera (green)
1045 and DAPI (blue) or (C) α -B5 (NPC) (red), phalloidin (green) and DAPI (blue).
1046 mRFP expressed by ECTV Δ 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 10 μ m.

1049

1050 **Fig. 7.** Transport defects of ECTV Δ A36R are restored by transient A36
1051 expression.

1052 (A) HeLa were infected with ECTV Δ 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 α -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 μ m.

1058

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

1060 (A) BSC-1 cells were infected with VACV or WR, VACV Δ A36R, ECTV Mos, ECTV.
1061 Δ A36R 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 α -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 ImageJ (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 acquisition, VACV and ECTV were statistically different
1075 (p<0.0001, Log-rank [Mantel-Cox] Test).

1076

1077 **Fig. 9.** Virulence of ECTV Δ A36R in mouse infections.

1078 Groups of 6-15 female BALB/c mice were infected subcutaneously in the left
1079 hind leg with 10^2 , 10^3 or 10^4 pfu ECTV or ECTV Δ A36R. Mice were bled on 0, 14
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 10^2 and 10^3 pfu and
1083 p<0.001 10^4 pfu, Log-rank [Mantel-Cox] Test). Data shown for the 10^2 dose of
1084 virus has been combined from 2 different experiments. Separate groups of mice
1085 were infected with 10^2 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^{-/-} female mice were infected with 10^3 pfu ECTV or
1088 ECTV Δ 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 Δ 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 \pm SEM.
1092 The horizontal dotted lines indicate the limit of detection of the viral plaque

1093 assay, which is 1 log₁₀ pfu. Splenic NK cell responses were measured by the
 1094 ⁵¹chromium release assay in groups of 3 (E) BALB/c and (F) C57BL/6 mice that
 1095 been infected with 10³ pfu of WT or mutant virus.

1096

1097 **Fig. 10.** ECTV ΔA36R infection induces protective immunity.

1098 At 33 dpi, all BALB/c mice inoculated with 10², 10³ or 10⁴ pfu ECTV ΔA36R 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 ΔA36R (broken lines) and
 1108 after secondary challenge with WT ECTV (solid lines) were used to determine
 1109 neutralizing activity (C). ECTV ΔA36R 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	ACCGCGCCGCCAGATAATGCAGTTTATCAGTGTCG
A36R-RA	a36r.rarev	ACAGGATCCGCTCAATATACGTACTACTAGTTC
A36R-LA	a36r.lafor	AAAAAGCTTCTGTTGAAGTACTTAATGAAGATACC
A36R-LA	a36r.larev	AAAGTCGACCGGATGCTCGAGGTTACAAACATGG
A36R	a36r.for	GGGAGATCTACCATGATGCTGGTACCACTTATCAGG
A36R	a36r.rev	TTTGCGGCCGCCGAAAGGATTGGATGAAAGTTAGG
A36R ^{Y112F}	a36r.mutfor	AGCACGGAACATATTTTCGATAGTGTTGCCGGA
A36R ^{Y112F}	a36r.mutrev	TCCGGCAACACTATCGAAAAATATGTTCCGTGCT
Lifect	LifectGFP.for	AAAGATCTACCATGGGTGTCGCAGATTTGATCAAGAA

		ATTGAAAGCATCTCAAAGGAAGAAGCGGCCGCCAGC
		AAGGC
Lifeact	GFP _{Prev}	TTGGATCCAACCTCCAGCAGGACCATGTGA

1115



















