Nanoscale Polarization of the Vaccinia Virus Entry Fusion Complex Drives Efficient Fusion

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Introductory paragraph:

To achieve efficient binding and subsequent fusion most enveloped viruses encode between 1 and 5 proteins[1]. For many viruses, the clustering of fusion proteins, and their distribution on virus particles, is crucial for fusion activity[2,3]. Poxviruses, the most complex mammalian viruses, dedicate 15 proteins to binding and membrane fusion[4]. However, their spatial organization and how this influences fusion activity has not been elucidated. Here we show that the vaccinia virus membrane is organized into distinct functional domains which are critical for membrane fusion efficiency. Using super-resolution microscopy and single-particle analysis we found that the vaccinia fusion machinery resides exclusively in clusters at virion tips. Repression of individual fusion complex components disrupts fusion machinery polarization, consistent with the reported loss of fusion activity[5]. Furthermore, we show that displacement of functional fusion complexes from virion tips disrupts fusion pore formation and infection kinetics. Our results demonstrate how the protein architecture of poxviruses directly contributes to membrane fusion efficiency and suggest that nanoscale organization may be an intrinsic property of these viruses to assure successful infection.

Main text:

The *Poxviridae*, including the causative agent of smallpox (variola), monkeypox, and the smallpox vaccine (vaccinia virus) encode 4 proteins for binding and 11 proteins for fusion[4]. The binding proteins (D8, H3, A26 and A27) are not individually essential[6–9]. However, genetic repression of any of the 11 proteins required for fusion, collectively termed the entry fusion complex (EFC)[5], results in formation of morphologically normal virions incompetent for hemifusion (A16, A21, F9, G3, G9, H2, J5 and O3) or full fusion (A28, L1 and L5)[5]. All EFC components are transmembrane proteins and 9 (A16, A21, A28, G3, G9, H2, J5, L5 and O3) form a stable core complex with which L1 and F9 associate[5].

EFCs within MVs are required for fusion of both infectious forms of *Poxviridae*[10]: single-membrane mature virions (MVs), and double-membrane extracellular enveloped virions (EEVs), which shed their outermost membrane allowing for fusion of the underlying MV-like particle[11,12,13]. Here we used a combination of electron microscopy (EM), super-resolution microscopy, single-particle analysis and a large collection of virus mutants to investigate virion binding and fusion orientation, the spatial distribution of binding and fusion proteins on individual vaccinia virus (VACV) particles, and how fusion protein distribution correlates with fusion activity.

Noting an orientation bias in poxvirus EM studies (Supplementary Table 1), we analysed the orientation of VACV MV binding and plasma membrane (PM) fusion using scanning- and transmission- EM, respectively. Quantification of binding orientation showed that >99% of MVs

61   bound to the cell surface on their sides (Fig. 1a). When fusion was forced by lowering the pH[13–

62   15], fusion of virion and cell membranes occurred at MV tips in 96% of cases (Fig. 1b). These

63   results were consistent with the literature in which 98% of MV binding events occurred at virion

64   sides, and 100% of fusion events at tips (Supplementary Table 1).

65

66   These results suggested that viral membrane proteins may be organized into functional domains.

67   To investigate this we applied structured illumination microscopy (SIM)[16] and single-particle

68   averaging to generate models of the distribution of binding and EFC proteins in MVs[17,18]. For

69   this, mCherry-tagged core protein A4 was used to identify virion position and orientation (Fig.

70   1c), and EGFP-tagged protein A13[19] as a viral membrane marker (Fig. 1c). VACV binding (A27

71   & D8) and EFC proteins (A21, A28, F9, J5, H2 and L1) were visualized by

72   immunofluorescence. The localization models showed that MV binding proteins reside at virion

73   sides and EFC components localize to virion tips, independent of virion orientation (Fig. 1c,

74   Supplementary Fig. 1). Polarity factor quantification indicated that binding proteins were

75   enriched 1.2-fold at the sides, and EFC proteins 1.7-fold at the tips of virions (Fig. 1d,

76   Supplementary Fig. 2).

77

78   The EFC is held together by a complex network of interactions[20,21]. Repression of any core

79   component results in disruption of the EFC into sub-complexes without compromising the

80   expression or virion incorporation of other EFC proteins[5]. This allowed us to investigate whether

81   the polarized distribution of binding and EFC components depends upon EFC intactness. MVs

82   lacking EFC core components A28, G9 or O3 were immunolabelled for binding protein D8 and

83   EFC protein L1. Localization models showed that D8 distribution was unaffected by loss of EFC

84   components, while L1 was redistributed evenly around the MV membrane (Fig. 1e). Models of

85   A21, A28, F9, H2 and J5 on A28(-), G9(-) and O3(-) MVs showed that all EFC components

86   were depolarized in these mutants (Supplementary Fig. 3a). No redistribution of D8 or EFC

87   proteins was seen on virions lacking the binding protein H3 (Fig. 1e, Supplementary Fig. 3a, b).

88   Polarity factor quantification confirmed that deletion of EFC core components does not alter D8

89   localization, while shifting EFC distribution from polarized to isotropic (Fig. 1f, Supplementary

90   Fig. 3b). Collectively these results show that VACV binding and fusion machineries are

91   organized as distinct functional domains within the viral membrane (Fig. 1g), and that the

92   polarized distribution of EFCs relies on their intactness.

93

94   Using stochastic optical reconstruction microscopy (STORM)[22], we extended our investigation

95   to single virions. Immunolabeling of D8 and L1 was performed on WT, EFC [A28(-), G9(-) and

96   O3(-)], and binding protein (ΔH3) mutant MVs. As expected, D8 was distributed to the sides of

97   all virions, while L1 was polarized to the tips of WT and ΔH3 MVs and distributed throughout

98   the membrane in A28(-), G9(-) and O3(-) virions (Fig. 2a, Supplementary Fig. 4a). STORM

99   revealed that D8 and L1 were localized to distinct clusters in WT and ΔH3 MVs (Fig. 2a). D8

100   clusters appeared unaffected in EFC mutants, while L1 clusters were largely disrupted (Fig. 2a).

101   Similar D8 and L1 distributions were observed on WT MVs stained with fluorescently

102   conjugated primary antibodies or Fab fragments, indicating that clustering was not induced by

103   signal amplification through secondary antibodies (Supplementary Fig. 4b, c)[23]. Additionally,

104   STORM showed that both D8 distribution and L1 polarization is maintained on the MV-like

105   particles within EEVs (Supplementary Fig. 5).

106

To analyse D8 and L1 clustering on MVs we applied Voronoi tessellation (SR-Tesseler software[24]). Labelling of A13-EGFP with fluorescently conjugated anti-EGFP nanobodies was used to determine a baseline for clustering (Supplementary Fig. 4d). Images were subjected to localization, segmentation and cluster identification by SR-Tesseler (Supplementary Fig. 6a). The clustering threshold was set for each particle at 3-fold the average density (Supplementary Fig. 6b). This analysis was used to quantify D8 and L1 clustering in WT, EFC [A28(-), G9(-), O3(-)], and binding (ΔH3) mutants (Fig. 2b). The percent of D8 localizations in clusters, and the number of D8 clusters, did not differ between WT and EFC- or binding- mutants (Fig. 2c, d). Conversely, L1 localizations in clusters was reduced from 44% to 3%, and L1 clusters from 8 to ≤4, on WT vs. EFC mutants (Fig. 2e,f, Supplementary Fig. 6c,d). The total cluster area of L1 was reduced from 4,400nm^2/virion on WT and ΔH3 MVs, to below the imaging resolution on A28(-), G9(-) and O3(-) virions (Supplementary Fig. 6e). The loss of fusion machinery polarization and clustering observed in EFC mutants correlated with the fusion defects exhibited by these viruses[5,25].

It was reported by Laliberte *et al.* that unlike other EFC mutants, A28(-) MVs are capable of directing hemifusion[25]. That EFC polarization and clustering were equally disrupted in A28(-) and other EFC mutants, suggested that polarization and clustering may be more important for full fusion than hemifusion. To test this, a viral protein that is not a component of the EFC, whose loss affects MV fusion, was needed. The VACV protein A27 has multiple roles in the virus lifecycle including virus binding, fusion and the intracellular transport and wrapping of MVs[9,26–30]. Direct evidence of A27's involvement in fusion comes from Vazquez and Esteban, who showed that A27(-) virions cannot mediate acid-induced cell-cell fusion[26]. This phenotype

130   was confounded by the fact that the A27(-) virus had no defect in MV production. 24h-yield and

131   cell-cell fusion experiments confirmed that A27(-) MVs display no defect in MV production

132   (Fig. 3a), but are 8-fold less capable of mediating cell-cell fusion than A27(+) virions (Fig. 3b,

133   c).

134

135   Lacking a TM domain, A27 is tethered to the MV surface via the membrane protein A17[27].

136   STORM imaging showed that A27 and A17 are homogeneously distributed on MVs, suggesting

137   no exclusive interaction with EFCs (Fig. 3d, Supplementary Fig. 4e). Yet localization models of

138   D8 and EFC proteins on A27(-) MVs, indicated that their distribution was shifted on these

139   virions (Fig. 3e, Supplementary Fig. 7a). Notably, A27(-) MVs phenocopied EFC mutants,

140   displaying a complete loss of fusion protein polarization (Fig. 3f, Supplementary Fig. 7b).

141   STORM showed that D8 clusters appeared redistributed, and L1 clusters reduced and

142   redistributed on A27(-) MVs (Fig. 3g, h). D8 clusters were reduced from 9 to 7 with no

143   difference in the cluster area covered by D8 (Supplementary Fig. 8a, b). L1 clusters were

144   reduced from 9 to 6, and the total L1 cluster area from $3,015nm^2$ on A27(+) to $1,411nm^2$

145   (Supplementary Fig. 8c, d). Ripley's H-function analysis applied to L1 in WT, A27(-) and G9(-)

146   MVs validated and confirmed (Supplementary Fig. 8e)[31] that A27 is required for polarized

147   clustering of EFCs on MV tips (Fig. 3i). These results show that EFC polarization requires both

148   EFC intactness and A27. As A27 is not an EFC component, these results strongly suggest that

149   the loss of EFC polarization in A27(-) virions underlies their inability to mediate the full fusion

150   reaction required for cell-cell fusion[26].

151

152   Thus we asked if A27(-) MVs can undergo acid-induced hemi- and full- fusion with cells[25,32]

153   using the assay illustrated in Figure 4a[32]. MVs containing an EGFP core were labelled with the

154   self-quenching membrane dye R18. The MVs were bound to cells in medium at 4 °C/pH 7.4, and

155   37 °C/pH 5.0 medium was added to induce MV fusion with the plasma membrane. Proton influx

156   into virions quenches the pH-sensitive EGFP core fluorescence, followed by R18 dequenching as

157   a result of lipid mixing during hemifusion[32]. Upon full fusion, cores are exposed to cytosolic pH

158   allowing for recovery of EGFP core fluorescence. Measurement of the R18 and EGFP

159   fluorescence over time allows for quantitative assessment of MV hemi- and full- fusion[25,32].

160   Comparison of R18 dequenching rates indicated that A27(+) and A27(-) MV hemifusion was

161   comparable (Fig. 4b). While core EGFP quenching occurred in both A27(+) and A27(-) MVs,

162   only A27(+) MVs displayed EGFP recovery (Fig. 4c). These results indicated that A27(-) MVs

163   can undergo hemifusion but are impaired in their ability to mediate full fusion.

164

165   To assess how A27(-) MVs produce equivalent numbers of infectious virus as A27(+) MVs

166   despite being impaired for full fusion, cells infected with A27(+) or (-) MVs expressing EGFP

167   under the control of an early viral promoter were monitored from 2-8 hours post infection (hpi)

168   (Fig. 4d, Supplementary Fig. 9). This assay, employed as a surrogate to assess delayed VACV

169   fusion[25,32], showed detectable early gene expression in 61% of A27(+) infected cells, as opposed

170   to 38% of A27(-) infected cells at 2hpi. The percentage of A27(+) infected cells expressing early

171   genes climbed only slightly over 8h, while the percentage of A27(-) infected cells expressing

172   early genes climbed steadily, reaching the levels of A27(+) within 8h. The ability of A27(-) MVs

173   to overcome delayed full fusion kinetics (Fig. 4c) is consistent with the production of equivalent

174   numbers of MVs in A27(+) and A27(-) infections over a 24h period (Fig. 3a).

175

The experimental data indicates that clustered polarization of the fusion machinery is important for VACV full fusion efficiency. To further explore the impact of EFC clustering on fusion we implemented a simple kinetic model[33]. The contact area between the virus and cell was modelled to contain a variable density of fusion complexes which stochastically activate to drive fast hemifusion followed by rate-limiting full fusion. By varying fusion complex density within the virus-cell contact area, we could assess how fusion complex clustering effects the rate of full fusion. We tested this model at two fusion thresholds[33]: low, which requires 3 activated fusion complexes and high, which requires 5. Simulations of 1,000 viruses indicated that regardless of the fusion threshold, full fusion kinetics slow down when fusion complex density is reduced from high to low (Fig. 4e). Notably, at the high threshold the rate of fusion was more dependent on complex density, indicating that full fusion is highly dependent on complex clustering.

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Based on these collective findings, we propose that the organization of poxvirus membrane proteins into functional domains is important for virus entry, and that EFC polarization and clustering is critical for efficient MV and EEV virus-cell fusion. The only other documented example of polarized virus fusion machinery is on HIV-1[34]. The authors propose that Env clustering is important due to the low number of Env trimers present in the viral membrane[34]. Formation of a single Env cluster on mature virions is required for efficient CD4 engagement and fusion at the PM. While a minority of MVs and EEVs enter by PM fusion, the majority of VACV enter by acid-mediated endocytosis[13,15,35–39]. This, together with our findings, invokes a model whereby VACV MVs bind to the PM in a side-on orientation (see Fig. 4f). This is

197   consistent with MV binding data, the position of the binding machinery on the virus and

198   accounts for the low number of direct PM fusion events observed after low pH-treatment.

199

200   Upon endocytosis - and in the case of EEVs, low pH-mediated rupture of the outer membrane[32] -

201   both MVs and MV-like particles are completely enveloped within cellular membrane

202   compartments. This allows for virus orientation-independent contact between virion tips and the

203   limiting membrane of endosomes in order to drive EFC-mediated fusion and core deposition.

204   Consistent with this model, MV and EEV fusion from endosomes has only been observed to

205   occur at virion tips[15,40,41].

206

207   In sum, by revealing the nanoscale organization of the poxvirus membrane and showing the

208   consequences of its disruption, we demonstrate that virion protein architecture is critical to virus

209   function. We suggest that the organization of the VACV membrane into functionally distinct

210   domains has evolved as a mechanism to maximise virion binding and fusion efficiency for

211   productive infection.

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Methods:

Cells and viruses

African green monkey kidney (BSC-40) cells and human HeLa cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM Glutamax, 100 units/mL penicillin and 100 μg/mL streptomycin, 100 μM nonessential amino acids and 1 mM sodium pyruvate. Routine mycoplasma tests of the cell culture medium were negative. Recombinant VACV were based on VACV strain Western Reserve (WR). WR mCherry-A4 and WR L4-mCherry/EGFP-F17 were described previously as WR mCherry-A513 and WR EGFP-F17 VP8-mCherry42. WR mCherry-A4 F13-EGFP was described previously as WR mCherry-A5 F13-GFP13. ΔH3, A28(+/-), G9(+/-) and O3(+/-) were described previously as vH3Δ43, vA28-HAi44, vG9i45 and vO3-HAi46 respectively. WRA27(+/-) was previously described as VVIndA27L30. WR A4-mCherry/A13-EGFP, WRA27(+/-) EGFP-A4 and WRA27(+/-) E/L EGFP were constructed as previously described42. For WR A4-mCherry/A13-EGFP, A13 was replaced with A13-EGFP in its endogenous locus within WR A4-mCherry virus. First, primers gcgcctcgagatctcgacattgttgaatcattattac and gcgcggtacccaccagaagtattttggagcc were used to amplify the A13 region from the viral genome with XhoI and KpnI sites, which was inserted in the pBluescript II KS backbone. Then an EGFP tag was inserted with Gibson assembly. To generate WRA27(+/-) EGFP-A4, A4 was replaced with EGFP-A4 in its endogenous locus within WRA27(+/-). First, primers ccatcgatgatgactataggacaagaaccctcctc and cggaattccgcttgaacagcattgc were used to amplify the A4 region from the viral genome with EcoRI and ClaI sites, which was then inserted into the pBluescript II KS backbone. Then, an EGFP tag was inserted using Gibson assembly. For WRA27(+/-) E/L EGFP, EGFP under the control of an early/late viral promoter was inserted

between the H2-H3 loci of the WRA27(+/-) virus. First, primers gcgcggtaccctagccgctggtaaggatga and gcgcggtaccgcagatactggataatgccg were used to amplify the H2-H3 region from the viral genome with KpnI sites, which was then inserted into the pUC/neo backbone. Then, E/L EGFP was introduced into the H2-H3 locus using primers gtacaagtaagaattctgttagataaatgcggtaacgaat and tttagtaatatggaatagaagcttaaaaattgaaatttt to introduce EcoRI and HindIII sites into the H2-H3 region and the primers aagcttaaaaattgaaattttatttttttt and gaattcttacttgtacagctcgtcc to introduce the same sites into E/L EGFP. Briefly, BSC-40 cells were infected the parental viruses and subsequently transfected with linearized plasmid containing the region of genome to be replaced, flanked 5' and 3' by 300bp of genomic sequence for targeted homologous recombination. Recombinant viruses were selected by fluorescence through 4 rounds of plaque purification. All viruses were produced in BSC-40 cells, and MVs purified from cytoplasmic lysates by banded on sucrose gradients, as previously described[35]. For WRA27(+/-) and its derivatives, virus stocks were generated in the presence or absence of 2mM Isopropyl β-D-thiogalactoside (IPTG, Sigma) to obtain A27+ or A27- MVs. WRA28(+), WRG9(+) and WRO3(+) stocks were produced with 100μM, 50μM and 20μM IPTG respectively. EEVs were prepared as previously described[13]. Briefly, RK13 cells were infected with WR F13-EGFP mCherry-A4 at MOI 1 and EEVs purified from the supernatant at 24hpi. Debris was removed by centrifugation at 1,000 x g, and EEVs concentrated by centrifugation at 38,000 x g before being resuspended in 1 mM Tris pH 9.

Antibodies

Anti-L1 mouse monoclonal antibody (clone 7D11) was purified from a hybridoma cell line kindly provided by Bernard Moss (National Institutes of Health, Bethesda, MD) with permission of Alan Schmaljohn (University of Maryland, Baltimore, MD). Anti-D8 rabbit polyclonal

antibody was made by immunizing a rabbit with purified D8 protein and adjuvant. Anti-A17 rabbit polyclonal antibody was a kind gift from Jacomine Krijnse-Locker. Antibodies against viral proteins A21 (R206), A28 (R204), F9 (R192), H2 (R202), J5 (R264), were produced by GHC using purified recombinant baculovirus-expressed proteins as previously described[47].

Structured illumination imaging

High performance coverslips (18 × 18 mm 1.5 H, Zeiss) were washed as previously described[17]. Purified virus was diluted in 1 mM Tris pH 9, bound to the ultra-clean coverslips for 30 min and fixed with 4% Formaldehyde. Samples were washed 3 times with PBS prior to mounting for imaging. To visualize membrane proteins, virus was blocked after fixation using 5% bovine serum albumen (BSA, Sigma) in PBS for 30 min, incubated in 1% BSA in PBS with primary antibody, followed by Alexa Fluor 488-conjugated goat anti-mouse or anti-rabbit secondary antibody for 1 hour each. Samples were washed 3 times with PBS after each staining step. The coverslips were mounted in Vecta Shield (Vector Labs) and sealed with nail polish.

SIM imaging was performed using Plan-Apochromat 63 × /1.4 oil DIC M27 objective, in an Elyra PS.1 microscope (Zeiss). Images were acquired using 5 phase shifts and 3 grid rotations, with the 561 nm (32 μm grating period) and the 488 nm (32 μm grating period) lasers, and filter set 3 (1850–553, Zeiss). 2D images were acquired using a sCMOS camera and processed using the ZEN software (2012, version 11.0.3.190, Zeiss). For channel alignment, TetraSpeck beads (ThermoFisher) were mounted on a slide, imaged using the same image acquisition settings and used for the alignment of the different channels.

Single-particle analysis

Individual viral particles were extracted from the SIM images. Seed images were generated with VirusMapper as described previously[17]. VirusMapper models were then created by registration

283   of the entire set of particles according to cross-correlation with the seeds and calculation of a

284   weighted average of a subset of particles. These values of (n) are shown in Supplementary Figs.

285   3 and 7. Models are normalized and intensity therefore does not reflect protein abundance.

286   **Polarity factor**

287   Polarity factors were calculated directly from the sets of particles used to generate the models

288   (Supplementary Fig. 2). Following the cross-validation method of Szymborska *et al.*[48], particles

289   were randomly divided into subsets of 50 particles and separately averaged. Radial profiles were

290   generated from these images by transforming from x - y coordinates to r - θ. The radial profiles

291   were divided into four regions according to a parameter φ and the mean intensity within the viral

292   membrane in these regions was evaluated. The four regions were defined in θ by:

$$I_1: 360° - \varphi < \theta \ or \ 0 < \theta < \varphi$$

$$I_2: \varphi < \theta < 180° - \varphi$$

$$I_3: 180° - \varphi < \theta < 180° + \varphi$$

$$I_4: 180° + \varphi < \theta < 360° - \varphi$$

293   where $0 < \theta \leq 360°$. The images are therefore divided by two lines through the centre of the

294   image that intersect at angle φ and divide the image symmetrically about the horizontal and

295   vertical axes. The polarity factor was then calculated as

$$p = \frac{I_2 + I_4}{I_1 + I_3}$$

296   A value for φ was used that resulted in a mean polarity factor of 1 for the A4 core protein

297   (48.5°). Values quoted in the text were calculated by averaging the mean polarity factors for the

298   sets of binding and fusion proteins.

299   **Labelling of primary antibodies**

Mouse monoclonal antibody (IgG) against L1 from hybridoma supernatant and rabbit polyclonal antibody against D8 from whole serum were purified with a Nab Protein A/G Spin Kit (Thermo Scientific) according to the manufacturer's instructions. Fab fragments were generated from primary antibodies with a Pierce Fab Preparation Kit (Thermo Scientific). Antibodies and Fab fragments were buffer exchanged into 0.1M NaHCO$_3$ pH 8.2 and concentrated to >1mg/ml with Amicon Ultra centrifugal filters 10 kDa MWCO (Merck). Antibodies and Fab fragments were custom-labelled with a 50-fold molar excess with AlexaFluor647-NHS (Invitrogen) for 1h at RT. The reaction was quenched by addition of 2 µl 1M Tris pH 9. Unreacted dye was removed by three passes through Zeba-Spin columns 3.5K MWCO (Thermo Scientific).

STORM imaging

Single-molecule localisation microscopy was performed by direct stochastic optical reconstruction microscopy (dSTORM)[49], a method developed based on STORM[22]. High performance coverslips (18mm, 1.5 H, Zeiss) were washed in ultrapure ethanol (Sigma) and deionized water to clean them and make their surface hydrophobic. Purified MVs or EEVs were diluted in 20 µl 1 mM Tris pH 9, placed in the centre of the clean coverslips for 30 min and bound virus fixed with 4% EM-grade formaldehyde (EMS). Virus was blocked after fixation using 5% BSA (Sigma), 1% FCS, 0.2-1% TritonX-100 in PBS for 30 min. Virus was immunostained in 5% BSA in PBS with primary antibodies at 4 °C overnight and Alexa Fluor 647-conjugated secondary antibodies (Invitrogen) for 1-2 hours at RT. Samples were washed 3 times with PBS after each staining step. Optionally, a post-fixation step with 4% PFA was included. Autofluorescence was quenched by brief incubation in 0.25% (w/v) NH$_4$Cl in PBS. Coverslips were mounted on a Secure-Seal incubation chamber (EMS) in BME-buffer [1% (v/v) β-mercaptoethanol (Sigma), 150 mM Tris, 1% glucose, 1% glycerol, 10 mM NaCl, pH 8] or in

323   MEA-buffer [50 mM cysteamine (Sigma), 3% (v/v) OxyFluor (Oxyrase Inc), 20% sodium

324   lactate (Sigma), PBS, pH 8[50]].

325   Imaging was performed on an Elyra PS.1 inverted microscope (Zeiss) using an alpha Plan-

326   Apochromat 100× /1.46 NA oil DIC M27 objective with a 1.6x tube lense and an iXon 897

327   EMCCD camera (Andor). Images were acquired at 25-30ms exposure time with 642nm

328   excitation at 100% laser power and a 655nm LP filter. Fluorophore activation was dynamically

329   controlled with a 405nm laser at 0-2% laser power. Images were processed in Fiji[51] using

330   ThunderSTORM[52]. Localizations were fitted with a maximum-likelihood estimator, lateral drift

331   corrected by cross-correlation, localizations <20nm apart within ≤1 frames merged, and images

332   rendered using a Gaussian profile with the NanoJ-Orange LUT (NanoJ). Lateral resolution was

333   25 nm, determined by FRC. Dual-colour STORM and SIM images (Supplementary Fig. 4, 5)

334   were registered using NanoJ[53].

335   **SR-Tesseler analysis**

336   Cluster analysis was performed with SR-Tesseler[24]. Localization tables from ThunderSTORM

337   were imported and Voronoi diagrams created. Individual virions were selected as regions of

338   interest and segmented as single objects with a density factor δ of $0.1 - 0.5$. Within individual

339   objects, clusters were identified with $\delta = 3$ which yielded <2% clustering in the non-clustered

340   reference probe A13-EGFP (Supplementary Fig. 6). Statistical analysis was performed in

341   GraphPad Prism (Prism Software). Significance (unpaired t-test): * $p < 0.05$; ** $p < 0.01$; *** p

342   < 0.001.

343   **Ripley's H-function analysis**

344   Additional cluster analysis was carried out as previously described[31]. 10 representative virus

345   particles were selected from STORM images of L1 on WT, A27(+/-), G9(-) VACV

346   (Supplementary Fig. 8e). Localizations within the selected ROIs were separately used to

347   calculate the Ripley's H-function as a function of increasing radius $H(r)$ according to:

$$K(r) = \frac{A}{n(n-1)} \sum_{j}^{n} \sum_{i}^{n} \delta_{ij}(r)$$

348   where A is the approximate area of the particle, n is the number of localizations and

349    $\delta_{ij}(r) = 1$ if $|x_i - x_j| < r$; 0 otherwise

350   where x_i is the spatial location of localization i. Then

$$L(r) = \frac{1}{\pi} \sqrt{K(r)}$$

351   and

$$H(r) = L(r) - r$$

352   so that the expected value of $H(r)$ is 0 for a random uniform distribution, and values of $H(r)$

353   above 0 indicate clustering at a range of approximately r. Custom Python scripts were used to

354   calculate the H-function from STORM localization tables. The mean of $H(r)$ and the 95%

355   confidence interval were evaluated for each case.

356   **24-hour yields**

357   Confluent BSC-40 cells were infected with A27(+/-) virus in DMEM at MOI 1. After 60 minutes

358   the inoculum was removed and DMEM with serum and 2mM IPTG was added for 24 hours.

359   Cells were harvested, resuspended in 100µl 1mM Tris pH 9 and freeze-thawed 3 times in liquid

360   nitrogen. The virus concentration was then measured by plaque assay.

361   **Fusion from without**

362   WRA27(+/-) was bound to HeLa cells grown on coverslips on ice at MOI 10 for 1 hour. Cells

363   were washed and incubated at 37°C with 20mM MES pH 5 or 7.4 for 5 mins, then washed and

364   incubated in full medium for 2 hours. Cells were fixed with 4% formaldehyde in PBS for 20

365 mins, blocked and permeabilized using 0.1% Triton X-100 and 5% BSA in PBS, stained with

366 Alexa Fluor 488 phalloidin and Hoechst 33258 (both Invitrogen) for in PBS for 1 hour. Samples

367 were then mounted on slides with ProLong Gold Antifade Mountant (Thermo Fisher). Samples

368 were imaged on a Leica TCS SP8 STED 3x microscope in confocal mode running LAS X

369 (Version 2.01) acquisition software. Alexa Fluor 488 fluorescence was excited using the 488 nm

370 line and Hoechst was excited with the 405nm LED. Z-stacks were acquired at a scan speed of

371 400 Hz in bidirectional scan mode using a Hybrid Detector (HyD, Standard mode) and an

372 Acousto-Optical Beam Splitter for filtering and time-gating of 0.5-8ns. The fusion index was

373 calculated from maximum intensity projections of the stacks as described previously[54].

374 **Bulk fusion measurements**

375 MVs were labelled by incubating with 22.5µM R18 (ThermoFisher) in 1mM Tris pH 9 at room

376 temperature for 2 hours. Labelled viruses were pelleted by centrifugation at 16,000g for 10 min

377 at 4°C and resuspended in 1mM Tris pH 9 twice to remove excess R18. Labelled viruses were

378 bound to 7×10^5 HeLa cells at MOI 30 in DMEM on ice for 1 hour. Cells were sedimented by

379 centrifugation at 300g for 5 mins at 4°C then resuspended in 100µl PBS. The cell suspension

380 with bound virions was added to 630µl prewarmed PBS in a quartz cuvette. After 2 min, the pH

381 in the cuvette was lowered by addition of 100µl 100mM MES resulting in a pH of 5.0. After the

382 acquisition all R18 was dequenched by the addition of 83µl 10% Triton X-100 in PBS. R18

383 fluorescence was normalized to the signal intensity after Triton X-100 addition. R18

384 fluorescence was measured using a Horiba FluoroMax 4 (Horiba Jobin Yvon)

385 spectrofluorometer with an excitation wavelength of 560 ± 5 nm and an emission wavelength of

386 590 ± 5 nm. To measure EGFP fluorescence, unlabelled viruses were bound to cells and the pH

387   was lowered as above, and fluorescence was recorded using an excitation wavelength of 488 ± 5

388   nm and an emission wavelength of 509 ± 10 nm.

389   **Flow cytometry**

390   HeLa cells were infected with WR A27(+/-) E/L EGFP at MOI 4 in DMEM and full medium

391   containing 10µM AraC (Sigma) was added after 1 hour. At the indicated times cells were

392   washed with PBS, trypsinized and resuspended in PBS and fixed with 4% formaldehyde in PBS.

393   Cells were then sedimented by centrifugation at 300g for 5 mins and resuspended in PBS with

394   2% FBS and 5mM EDTA. Flow cytometry was performed with a Guava EasyCyte HT flow

395   cytometer, recording the EGFP fluorescence with the 488nm laser. Analysis of the flow

396   cytometry data was performed with the FlowJo software package.

397   **Fusion kinetics mathematical model**

398   We modelled the process of fusion undergone by a single fusion complex as a fast irreversible

399   hemifusion step followed by a rate-limiting, irreversible full fusion step.

$$I \xrightarrow{k_{hf}} HF \xrightarrow{k_{fuse}} F$$

400   Following similar work on influenza[33], for each virus we required that T_f (*fusion threshold*)

401   fusion complexes transition to state F before the virus is considered fused. We simulated the

402   contact area with the cell as containing an initial density ρ_0 (high density), $\frac{\rho_0}{3}$ (medium density)

403   and $\frac{\rho_0}{10}$ (low density) of fusion complexes. We then modelled the effects of fusion complex

404   density on fusion kinetics at two different values of T_f: low ($T_f = 3$) and high ($T_f = 5$). The true

405   value of T_f is unknown for vaccinia but for influenza is thought to be in this range.

406   **Electron Microscopy**

407   For binding orientation experiments, VACV was bound to HeLa cells for 15 min on ice at MOI

408   50. Unbound virus was removed, and samples fixed for 20 min using (final concentration: 1.5%

409   formaldehyde, 0.1%glutaraldehyde, both EM grade). Fix was replaced with 2% glutaraldehyde

410   in 0.1M sodium cacodylate for a further 20 min. Samples were washed in 0.1M sodium

411   cacodylate, incubated 1h at 4˚C in reduced osmium tetroxide. After additional washes, samples

412   were dehydrated through an increasing ethanol series and critically point dried (Leica Auto

413   CPD). Samples were Au/Pd coated and imaged using a FEI Quanta 200 FEG ESEM (Thermo

414   Fisher Scientific) operated at 5kV.

415   For fusion orientation experiments VACV was bound to HeLa cells for 30 min on ice at MOI

416   100. Unbound virus was removed, and samples incubated for 7.5 min at 37 °C in DMEM with

417   100 mM MES adjusted to pH 5. Samples were fixed with 1.5% glutaraldehyde 2%

418   paraformaldehyde (EM-grade) in 0.1M sodium cacodylate for 45 min at RT and prepared for

419   transmission electron microscopy (TEM). Transmission electron micrographs were obtained on a

420   Tecnai T12 FEI equipped with a charge-coupled device camera (SIS Morada; Olympus).

421

422   **Code availability**

423   All custom code used for analysis in the current study is available on Github at

424   https://github.com/HenriquesLab/fusiontools.

425   **Data availability:**

426   The datasets generated during and/or analysed during the current study are available from the

427   corresponding author on reasonable request.

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R.G., D.A., R.H. and J.M. conceived the project, designed the experiments and wrote the manuscript. R.G., D.A., C.B., and M.H. performed the experiments. The Cohen (G.H.C.) and Eisenberg members of the poxvirus research group produced and purified all VACV EFC antibodies. I.J.W. and J.J.B. performed electron microscopy. R.G., D.A. R.H and J.M analysed the data. R.G., D.A., R.H. and J.M. discussed the results and implications of the findings. All authors discussed and provided comments on the manuscript.

Competing interests:

Authors declare no competing interests.

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Figure legends

566
567

568 **Fig. 1. VACV binding and fusion show orientation bias reflecting distinct binding and**

569 **fusion domains on virions. a,** SEM images of VACV MV binding to the PM of HeLa cells.

570 Quantification of binding orientation from >50 SEM images. **b,** TEM images of low pH-induced

571 fusion between VACV MVs and the PM of HeLa cells. Arrows indicate regions of continuity

572 between virus and cell membranes. Quantification of fusion orientation from >200 cell sections.

573 **c,** Localization models of binding and fusion proteins on MVs. **d,** Quantification of the polarity

574 factor of proteins visualized in (c). A polarity factor >1 corresponds to concentration at the tips

575 of MVs; <1 corresponds to concentration at the sides of MVs. **e,** Localization models of D8 and

576 L1 in EFC and binding mutant MVs. **f,** Polarity factors for the models shown in (e) compared to

577 WT. **g,** Illustration of VACV membrane protein organisation in WT/ΔH3 and fusion mutant

578 MVs. Data represent 3 or more biological replicates (**a-f**). Models represent n>260 virions (**c,e**).

579 Scale bars, 200nm (**a-c,e**). Bars represent means ± SEM of n=50 virions (**d,f**).

580

581 **Fig. 2. VACV binding and fusion proteins are organized into nanoscale clusters.**

582 **a,** Distribution of D8 (binding protein; first row) and L1 (fusion protein; second row) on

583 individual virions imaged by STORM in WT, EFC mutant [A28(-), G9(-), O3(-)], and binding

584 protein mutant (ΔH3) MVs. **b,** Voronoi diagrams and cluster identification of D8 and L1 in WT,

585 EFC mutant, and binding mutants in (a). Voronoi tessellation was performed on individual

586 virions with SR-Tesseler software (first row) and clusters identified with a density factor δ=3

587 (second row). **c,** Percentage of D8 localizations within clusters on individual WT and mutant

588 MVs. **d,** Number of D8 clusters identified per virion. **e,** Percentage of L1 localizations within

589   clusters on individual WT and mutant MVs. **f,** Number of L1 clusters identified per virion.

590   Images are representative of 3 biological replicates (**a,b**). Scale bars, 200nm (**a,b**). Data represent

591   3 or more biological replicates (**c-f**). Bars represent means ± SEM of n=15 virions (**c-f**). An

592   unpaired two-tailed *t* test was applied (*** P<0.001, ns P>0.05). See supplementary table 2 for

593   exact statistics.

594

595   **Fig. 3. A27 regulates the protein architecture of MV membranes. a,** 24h yields from BSC40

596   cells infected with either A27(+) or A27(-) virus. **b,** Confocal images of A27(+) or A27(-) MV

597   fusion from without experiments (pH 5.0, MOI 50). Cell nuclei (magenta) and actin (green). **c,**

598   Fusion index calculated for fusion from without experiments in (b) **d**, STORM images of A27 on

599   individual MVs. **e,** VirusMapper models of D8 and L1 localization on A27(+) and A27(-) MVs.

600   **f,** Polarity factor of models in (e). **g,** STORM images of D8 and L1 on A27(+) and A27(-) MVs.

601   **h,** Percent of D8 and L1 clustered localizations on A27(+) and A27(-) MVs. **i,** Illustration of

602   VACV membrane protein organization in WT/A27(+) vs. A27(-) MVs. Data represent 3 or more

603   biological replicates (**a,c,f,h**). Images are representative of 3 biological replicates (**b,d,g**). Models

604   represent n>206 virions (**e**). Bars represent means ± SEM of n=3 replicates (**a,c**) or n=50 (**f**) or

605   15 (**h**) virions. Scale bars, 50μm (**b**) and 200nm (**d,e,g**).

606

607   **Fig. 4. VACV fusion machinery polarization is required for full fusion efficiency.**

608   **a,** Schematic of VACV bulk fusion assays to quantify MV hemifusion by R18 dequenching, and

609   full fusion by core EGFP quenching and recovery. **b,** Comparison of A27(+) and A27 (-) MV

610   hemifusion rates by R18 dequenching assay. **c,** Comparison of A27(+) and A27 (-) MV full

611   fusion rates using the EGFP core recovery assay. **d,** Analysis of the kinetics of A27 A27(+) and

612   A27 (-) MV early gene expression by flow cytometry. **e,** Model of VACV density dependent

613   fusion kinetics. Virus-cell contact area was simulated with three different fusion complexes

614   densities (low, medium and high) using two different fusion thresholds (low and high). The

615   fusion rate (% fused viruses/time) was modelled under these conditions. **f,** Model of VACV MV

616   and EEV protein architecture-dependent binding, entry and fusion. Data represent 3 biological

617   replicates (**b-d**). Lines represent mean ± SD (**b,c**) or mean ± SEM (**d**) of n=3. Models were

618   generated using n=1000 viruses/condition (**e**).

619

a

Binding orientation [#]

tip / side

b

Fusion orientation [#]

tip / side

c

Core — A4

Membrane — A13-EGFP

Fusion — A21, A28, F9

Binding — A27, D8

Fusion — J5, H2, L1

d

Polarity factor

A4, A13-EGFP, A27, D8 (Binding), A21, A28, F9, H2, J5, L1 (Fusion)

e

A28(-) — D8, L1

G9(-) — D8, L1

O3(-) — D8, L1

ΔH3 — D8, L1

f

Polarity factor

D8, L1

WT, A28(-), G9(-), O3(-), ΔH3

Virus

g

A16, A28, G9, J5, F9, O3 / A21, G3, H2, L5, L1 — Fusion

D8 — Binding

A27 — Binding

A17 — Structural

A13 — Structural

Lipid bilayer

WT / ΔH3

A28(-) / G9(-) / O3(-)

a

24h yield [pfu/ml]
A27(+) A27(−)

b

A27(+) A27(−)

c

Fusion index
A27(+) A27(−)
pH 5.0 pH 7.4

d

WT A27

e

A27(+) D8 L1
A27(−) D8 L1

f

Polarity factor
A27(+) A27(−)
D8 L1

g

A27(+) D8 L1
A27(−) D8 L1

h

L1/D8 in clusters [%]
A27(+) A27(−)
D8 L1

i

A16 A21
A28 G3
G9   H2   Fusion
J5   L5   machinery
F9   L1
O3

D8   Binding
     proteins
A27

A17 Structural
A13

Lipid bilayer

WT / A27(+)   A27(−)

a Binding (pH 7.4) → pH drop EGFP quenching (pH 5) → Hemifusion R18 dequenching (pH 5) → Full fusion EGFP recovery (pH 5)

b R18 fluorescence [a.u.] vs Time [min]; A27(+), A27(−)

c GFP fluorescence [a.u.] vs Time [min]; A27(+), A27(−)

d % early gene expressing cells vs Hours post infection; A27(+), A27(−)

e % fused vs Time [a.u.]
Low fusion threshold: high density, medium density, low density
High fusion threshold: high density, medium density, low density

f MV (endosomal, fusion); EEV (fusion, endosomal)

Fusion machinery: A16 A21, A28 G3, G9 H2, J5 L5, F9 L1, O3

Binding proteins: D8, A27
Structural: A17, A13
Lipid bilayer
F13 EEV membrane