Repulsion of Superinfecting Virions: A Mechanism for Rapid Virus Spread

Virginie Doceul,* Michael Hollinshead,* Lonneke van der Linden,† Geoffrey L. Smith‡

Viruses are thought to spread across susceptible cells through an iterative process of infection, replication, and release, so that the rate of spread is limited by replication kinetics. Here, we show that vaccinia virus spreads across one cell every 75 minutes, fourfold faster than its replication cycle would permit. To explain this phenomenon, we found that newly infected cells express two surface proteins that mark cells as infected and, via exploitation of cellular machinery, induce the repulsion of superinfecting virions away toward uninfected cells. Mechanistically, early expression of proteins A33 and A36 was critical for virion repulsion and rapid spread, and cells expressing these proteins repelled exogenous virions rapidly. Additional spreading mechanisms may exist for other viruses that also spread faster than predicted by replication kinetics.

Mechanisms enhancing the cell-to-cell spread of intracellular pathogens are important for virulence and are targets for development of antimicrobial therapeutics. Vaccinia virus (VACV) is a poxvirus and is the live vaccine used to eradicate smallpox (1). VACV replication is unusual in that it produces both single- and double-enveloped virions (2, 3). The single-enveloped virions, called intracellular mature virus (IMV), remain intracellular until cell lysis and spread slowly from cell to cell. In contrast, the double-enveloped virions, called cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV), are released rapidly and mediate efficient cell-to-cell spread and long-range dissemination (3, 4). VACV spreading mechanisms include virus-induced cell motility (3) and the formation of actin projections (6–8) that propel VACV particles toward other cells late during infection (9). However, we wondered whether either mechanism could explain how VACV Western Reserve (WR) spreads rapidly to form a plaque of diameter 2.90 ± 0.07 mm (SEM, nine experiments, n = 11 to 12 plaques) in 3 days (Fig. 1A). The distance between nuclei of adjacent BSC-1 cells was 37.26 in 3 days (Fig. 1A). The distance between nuclei of adjacent BSC-1 cells, this indicated that VACV crossed one cell every 1.2 hours. This rate of spread is inconsistent with VACV replication kinetics, in which new virions are formed only 5 to 6 hours after infection (12), or virus-induced cell motility, in which cells start to move 5 to 6 hours after infection (5). These observations demonstrated that another mechanism to accelerate spread must exist. Mutants defective in actin tail formation (ΔA33R, ΔA33R, ΔA34R, ΔB5R, ΔF13L, and ΔF12L) spread much more slowly (Fig. 1, G and H), infecting only one cell every 5 to 6 hours, which is consistent with replication kinetics. This reaffirmed the importance of actin tails for VACV spread but did not explain the rapid dissemination because actin tails are produced only late during infection after new virions are formed.

Inspection of plaques formed by vEGFPASL revealed EGFP-positive virions (green dots) several cells away from EGFP-positive cells, where virions are formed, showing that VACV particles spread rapidly to distal cells (Fig. 1F). To investigate this phenomenon, actin was stained with phalloidin, and confocal optical sections revealed virus-tipped actin tails on cells producing new virions but also on distal cells lacking virus factories and so not producing virions (no

Fig. 1. VACV spreads more rapidly than predicted. (A) VACV plaques 3 days after infection in BSC-1 cells. Scale bar, 5 mm. (B and C) Live cell imaging recording plaque formation at 0 (B) and 16 (C) hours later. (D and E) Live cell imaging of vEGFPASL-infected cells confirmed the correlation between cytopathic effect (cpe) and virus infection. Yellow lines indicate the boundary between infected and uninfected cells and white arrows indicate the distance this has moved over 16 hours. (F) Confocal image showing the spread of EGFP-tagged virus particles (single green dots) far from the center of infection. (G) Increase in plaque radius formed by VACV WR and mutants with time; n = 6 to 11 plaques. (H) Diagram showing the rate of spread (BSC-1 cell per hour) with indicated viruses. White bars indicate viruses with a defect in actin tail formation. Error bars are SEM, with n = 6 to 11 plaques. Scale bar, (A) 5 mm, (B) to (E) 50 μm, and (F), 10 μm.
EGFP expression) (Fig. 2A). This result was reproduced in different cell lines (BSC-1, RK13, ECV, and CEF) and using different VACV strains (WR and Lister) (figs. S1 and S2). To be certain that actin tails on cells that lack virus factories were not derived from distant virus-producing cells, a lawn of cells was formed in which some cells expressed cherry fluorescent protein fused to actin (cherry-actin) and thus produced red actin tails after virus infection (Fig. 2B and movies S6 and S7). After infection of such monolayers at low multiplicity, cells containing green factories adjacent to a red cell (cherry-actin positive) were studied. This revealed green virions on tips of red actin tails originating from a red cell that lacked any green factory and therefore new virions. Careful examination of z stacks of these cells confirmed no virus factory was present. Therefore, the virions and actin tail originated from different cells. Further examination by means of time-lapse microscopy revealed virus-tipped red actin tails on a cell 5, 20, and 50 min before green factory formation (55 min) (Fig. 2B), confirming that actin tails appeared before virion production. Furthermore, virions on a red actin tail were observed recontacting the same red cell and inducing another actin tail (movie S7). Thus, virions can be repelled repeatedly, thereby accelerating spread until an uninfected cell is found.

To understand this mechanism, we searched for the required VACV proteins. Proteins K2 and A56 inhibit entry of IMV by binding the membrane fusion complex on the IMV surface (13–15), but this is masked on EEV or CEV, and mutants lacking these genes form normal-sized plaques (16, 17). Better candidates would be early, cell-surface proteins that are needed for actin tail formation. Although proteins A33, A34, A36, B5, F12, and F13 are needed for efficient actin tail formation, we focused on A33 and A36 because proteins A33, A34, A36, B5, F12, and F13 are needed for efficient actin tail formation.

Fig. 2. Cells form actin tails before production of new virions. (A) Confocal images showing the edge of vEGFPASL plaque (green) on BSC-1 cells stained for actin (red) or DNA (blue). Bottom panel shows zoomed areas (white squares 1 to 4). Actin tails are on cells with nascent factories (cytoplasmic blue) but that are not producing any virus particles (green) (squares 1 and 2), and on a cell with no virus factory (square 3), whereas square 4 shows a productive virus factory (green). Scale bars, top row, 10 μm; bottom row, 10 μm; and insets 1 to 4, 5 μm. (B) Actin tails (red) present at the surface of a cell expressing cherryFP-actin but with no green virus factory (time (t) = 0, white square and zoomed inset). Bottom panels show zoomed images of this cell with actin tails detected 5, 20, and 50 min later, before the appearance of virus factories at 55 min as indicated by the white square. Scale bars, top row, 10 μm; top right inset, 5 μm; bottom row, 10 μm; and bottom right inset, 1 μm.

Fig. 3. Early expression of A33 and A36 is important for VACV spread. (A) Images of edge of plaque showing A36, but not A34, is expressed early during infection. A36 was detected in cells where no late protein A5 (green) was present, whereas A34 was expressed late during infection in cells that also express A5. (Insets) Zoomed images of virions (single green dots) relative to A36 and A34 distribution. (B) Graph showing the size of plaques formed by recombinant viruses in which A33R, A36R, or B5R are under a late promoter only (4b) or deleted (Δ) as compared with parental viruses WR or vEGFPASL. Error bars are SEM mean values from three experiments with n = 11 to 12 plaques. Scale bars, 20 μm; insets, 5 μm.
formation, only surface proteins B5 (18), A33 (19), and A36 (20) are expressed early (and late). Immunostaining of plaques formed by vEGFP-A5L demonstrated that A33 and A36 are expressed early during infection at the periphery of plaques before expression of late proteins (A34, EGFP-A5, and B5) (Fig. 3A and fig. S3). A34 and B5 were nevertheless detectable on virions spreading toward noninfected cells, as expected. Thus, A33 and A36 seemed candidates for early induction of actin tails.

The importance of early expression of A33 and A36 was investigated by generating recombinant viruses in which A33R, A36R, or B5R genes were driven only by a late promoter (21). Infection of cells by these viruses (v4b-A33, v4b-A36, and v4b-B5) confirmed expression of these proteins only late during infection (fig. S4) and showed that plaques formed by v4b-A33 and v4b-A36 were much smaller than wild type and closer to those formed by deletion mutants lacking either gene (Fig. 3B). In contrast, v4b-B5 formed plaques similar to wild type. Thus, early expression of A33 and A36, but not B5, is critical for efficient VACV spread. Similar results were obtained with viruses in which 4b-A33R and 4b-A36R were inserted into vEGFP-A5L, allowing direct visualization of spreading virions via EGFP. Virions released from cells infected by these viruses (vEGFP-A5L/4b-A33 and vEGFP-A5L/4b-A36) spread poorly as compared with vEGFP-A5L and induced actin tails only on cells with a virus factory (fig. S5).

Next, we investigated whether A33 and A36 are sufficient to induce actin tails upon contact with an EEV particle. Both proteins are expressed on the plasma membrane of VACV-infected cells (22–24) and interact with each other (25–27). Lentivirus vectors expressing A33 or A36 fused to a C-terminal V5 tag were used to generate HeLa cells expressing cell surface A33-v5, A36-v5, or A36-v5 and A33-v5 (Fig. 4A and fig. S6). These cells were incubated with EEV particles and then stained with phalloidin and a monoclonal antibody to B5. Actin tails were detected on cells expressing A33-v5 and A36-v5 within 15 to 30 min (Fig. 4, B and C). Similar results were obtained with haemagglutinin (HA)–tagged A33. No actin tails were detected if only one protein was expressed or if the cells were incubated with IMV or with GFP-tagged herpes simplex virus 1 (HSV-1). Thus, A33 and A36 are both necessary and sufficient to induce actin tails after binding EEV particles. Currently, we are investigating the effect of ectopic expression of A33 and A36 on VACV spread by measuring plaque size using additional cell lines that form clearer plaques.

Here, we demonstrate that VACV has evolved a mechanism (Fig. 4D) by which infected cells repel superinfecting CEV/EEV particles on actin tails toward neighboring cells. Two outcomes are then possible: (i) If the neighboring cell is uninfected, the virion enters and starts a new cycle of replication; (ii) alternatively, if the cell is already infected then superinfection is blocked, and a new actin tail is formed, propelling the virus further away until it reaches uninfected cells. This mechanism accelerates virus spread and explains how VACV can cross one cell every 1.2 hours as determined by means of live cell imaging. Early expression of proteins A33 and A36 is required, and viruses expressing either protein only late during infection form small plaques. These plaques are closer in size to those formed by the deletion mutants lacking either gene than to wild type, indicating that the formation of actin tails upon superinfection is more important for virus spread than the production of actin tails on cells releasing new virions. All mutations that cause VACV strains to spread poorly and form small plaques also cause dramatic attenuation in vivo, showing the biological importance of rapid spread for VACV virulence (18, 20, 28, 29).

Plaque assays were first described more than 50 years ago (30), and many animal viruses form plaques of size comparable with VACV. Some of these viruses, for instance HSV-1 (31), have replication kinetics similar to VACV, suggesting that other viruses also spread faster than predicted by their replication kinetics. The mechanisms underlying cell-to-cell spread of many viruses remain
poorly understood, and the elucidation of such mechanisms could lead to the discovery of novel therapeutics.

References and Notes
11. Materials and methods are available as supporting material on Science online.
31. G. Elliott, P. O’Hare, J. Randall, University of St. Andrews for the lentivirus vectors. GLS is a Wellcome Trust Principal Research Fellow. This work was supported by the UK Medical Research Council.

Supporting Online Material
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Materials and Methods
Figs. S1 to S6
References
Movies S1 to S7
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Drive Against Hotspot Motifs in Primates Implicates the PRDM9 Gene in Meiotic Recombination
Simon Myers1,2,† Rory Bowden1,2, Afidalina Tumian1, Ronald E. Bontrop3, Collins Freeman2, Tammie S. MacFie4, Gil McVeans1,2,5 Peter Donnelly1,2,6

Although present in both humans and chimpanzees, recombination hotspots, at which meiotic crossover events cluster, differ markedly in their genomic location between the species. We report that a 13-base pair sequence motif previously associated with the activity of 40% of human hotspots does not function in chimpanzees and is being removed by self-destructive drive in the human lineage. Multiple lines of evidence suggest that the rapidly evolving zinc-finger protein PRDM9 binds to this motif and that sequence changes in the protein may be responsible for hotspot differences between species. The involvement of PRDM9, which causes histone H3 lysine 4 trimethylation, implies that there is a common mechanism for recombination hotspots in eukaryotes but raises questions about what forces have driven such rapid change.

In humans and most other eukaryotes, meiotic crossover events typically cluster within narrow regions termed hotspots (1–5). Previously (6), we identified a degenerate 13-base pair (bp) motif, CCNCCNTNNCCNC, that is overrepresented in human hotspots. Both linkage disequilibrium (LD)-based analysis (6) and sperm typing at currently active hotspots (7) implicated this motif in the activity of 40% of hotspots. Despite nearly 99% identity at aligned bases, humans and chimpanzees show little if any sharing of hotspot locations (4, 5), although it has remained undetermined whether the recently identified hotspot motif is also active in the chimpanzee. To resolve this question, we collected chimpanzee genetic variation data at 22 loci where there is both an inferred hotspot at the orthologous location in humans and human-chimpanzee sequence conservation of the 13-nucleotide oligomer: 16 motifs within THE1 elements and 6 within L2 elements, chosen for their high activity of a particular "core" version of the motif in humans (fig. S1). We used the statistical software LDhat to estimate recombination rates separately in each region in different populations of both species (8). For humans, we used the HaploTyp Map (HapMap) Phase II data. For chimpanzees, we genotyped 36 Western, 20 Central, and 17 Vel- lverous chimpanzees at a total of 694 chimpanzee single-nucleotide polymorphisms (SNPs), an average of 31.5 per region.

Because these regions are inferred human hotspots, the average estimated recombination rate surrounding the motif in humans showed a strong peak for both L2 and THE1 elements (Fig. 1A). In contrast, chimpanzees showed no evidence of increased recombination rates for either background. In Western chimpanzees, the THE1 estimated recombination rate around the motif was similar to the regional average, whereas a weak peak in mean rate for the L2 elements was produced solely by a single potential hotspot in one of the six regions (Fig. 1B). Results for the other chimpanzee subspecies were less informative (fig. S2) (8) but did not reveal a different pattern. To ensure that unknown haploptopy phase, smaller sample size, less dense data, and SNP ascertainment in chimpanzees had not compromised the ability to detect hotspots, we repeatedly sampled from the Centre d’Etude du Polymorphisme Humain (CEPH) from Utah (CEU) HapMap population data to produce human data sets comparable with those from chimpanzees in terms of these features (8). We conditioned only on the presence of the 13-nucleotide oligomer in THE1 and L2 elements and not the presence of a hotspot. This bootstrap technique revealed that the differences between human and chimpanzee rates cannot be explained by differences in power (P = 0.00052), although the signal was only significant for THE1 elements when analyzed separately (P = 0.00012) (fig. S3). These results provide evidence that the 13-nucleotide oligomer motif does not recruit hotspots in chimpanzees, implying changes in recombination machinery between humans and chimpanzees. The existence of factors capable of such changes in recombination genome-wide has been demonstrated in Caenahabditis elegans (9) and by the mapping in mice of a trans-acting factor responsible for differences in hotspot location among inbred strain crosses (10, 11).
Supporting Online Material for

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Virginie Doceul, Michael Hollinshead, Lonneke van der Linden, Geoffrey L. Smith*

*To whom correspondence should be addressed. E-mail: geoffrey.l.smith@imperial.ac.uk

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This PDF file includes:

Materials and Methods
Figs. S1 to S6
References

Other Supporting Online Material for this manuscript includes the following:
(available at www.sciencemag.org/cgi/content/full/science.1183173/DC1)

Movies S1 to S7
Supporting online materials

Material and Methods

Viruses and infection. VACV strain Western Reserve (WR) (S1) and the deletion mutants vΔA33R (S2), vΔA34R (S3), vΔA36R (S4), vΔAB5R (S5), vΔA56R (S6), vΔF12L (S7), vΔF13L (S8), vEGFP5L (S9) and vΔA36R-EGFP5L (S10) were described previously. vΔA33R-EGFP5L was constructed using vΔA33R (S2) and pEGFP5L (S9) by transient dominant selection as described (S10). Cells were infected with the different VACVs, overlaid with semi-solid medium containing 1.5% carboxymethylcellulose.

Generation of recombinant VACV viruses and plaque assay. DNA coding for A33, A36 and B5 was introduced into the BamHI/EcoRI, BamHI or KpnI restriction sites, respectively, of pRK19, a plasmid containing the VACV 4b late promoter inserted within the VACV thymidine kinase gene. The resultant plasmids were transfected into infected cells to introduce the 4b-A33, 4b-A33-EGFPA5L, 4b-A36 or 4b-B5 genes into the TK locus of vΔA33R (S2) or vΔA33R-EGFP5L, vΔA36R (S4) or vΔA36R-EGFP5L and vΔAB5R (S5), respectively. The resulting viruses, v4b-A33, v4b-A33-EGFPA5L, v4b-A36, v4b-A36-EGFPA5L and v4b-B5, were selected by plaque size and several rounds of plaque purification. The fidelity and purity of the final recombinant virus was confirmed by PCR and cytosine arabinoside (AraC) treatment (Fig. S4) as described (S4). For plaque assays, BSC-1 cells were infected for three days and processed as described (S11). The radius of 11-12 plaques was determined in three independent experiments using the AxioVision Rel. 4.6 software (Zeiss). The relative plaque size is presented as the percentage of the plaque size obtained with WR or vEGFP5L.

Generation of cherryFP-actin cells. DNA coding for EGFP was removed from pEYFP-Actin (BD Biosciences) using Nhel and BsrGI and replaced by DNA coding for CherryFP to generate pCherryFP-Actin. Following the transfection of BSC-1 cells and G418 selection, CherryFP-actin was detected by fluorescence microscopy.

Live cell imaging, immunofluorescence and electron microscopy. For live cell imaging, BSC-1 cells were infected and overlaid with semi-solid medium containing 1.5% carboxymethylcellulose to prevent lateral diffusion of virions. Imaging was performed at 37 °C using a Zeiss Axiovert 200M microscope with a 10x lens and an AxioCam camera (Zeiss). The formation of individual plaques was recorded every h for 12-19 h. The radius of plaque was determined for each time point using AxioVision Rel. 4.6 software (Zeiss). Six to eleven plaques were recorded for each virus and the speed of plaque formation was determined in μm per h using linear regression. For time-lapse confocal imaging, cells were infected with vEGFP5L and transferred to a microscope stage preheated to 37 °C. Images were acquired every h for 16 h using a Zeiss 510 Meta confocal microscope using Zeiss time-lapse software. For immunostaining, coverslips were seeded with cells and fixed with 4% or 8% paraformaldehyde in 250 mM HEPES (pH 7.4) in PBS. The cells were permeabilized with Triton-X-100 (VWR) when required, blocked in 0.5% BSA or 10% FBS and incubated with a mouse anti-v5 mAb (AbD
Serotec), a rabbit polyclonal Ab raised against the cytoplasmic domain of A33 (S12), a rat mAb anti-B5 19C2 (S13) or mouse mAbs directed against A34 (34-1) and A36 (6.3) (S14). A mouse mAb directed against ARPC5 was used to detect the Arp2/3 complex (p16-Arc, clone 323H3, Synaptic system). Secondary Alexa 546-conjugated donkey anti-mouse or rabbit Abs were used to detect bound primary Ab. Alexa 546-conjugated phalloidin (Molecular Probes) was used to stain actin. Samples were mounted in Mowiol–4',6-diamidino-2-phenylindole (DAPI) mounting medium. Microscopy was carried out with a Zeiss 510 Meta confocal microscope (Zeiss).

For electron microscopy, samples were labelled with mouse anti-v5 mAb, followed by secondary horseradish peroxidase conjugated anti-mouse Ab, and reacted with DAB / Metal (Pierce) for 30 min. Samples were viewed by widefield microscopy Zeiss Axiovert 200M and then embedded in Epon. Sections were stained with Reynold’s lead citrate and viewed using an FEI Tecnai G2 electron microscope (FEI, Eindhoven, The Netherlands) with a Soft Imaging System Megaview III charge-coupled device camera. Images were collected using ANALYSIS version DOCU software (Olympus Soft Imaging Solutions, Münster, Germany).

**Generation of HeLa cells expressing VACV proteins.** DNA fragments corresponding to A33R or A36R were amplified from pcDNA3-A33 and pcDNA3-A36, two plasmids derived from pcDNA3 (Invitrogen) containing the VACV WR A33R and A36R genes, and cloned into the BamHI or MluI/BamHI sites of the lentivirus vector pdlNot’MCS’R’PK derived from pHR-SIN-CSGW (S15). A control lentivirus (v5) and lentiviruses expressing A33-v5 and A36-v5 were produced as described (S15). HeLa cells were infected with the different lentiviruses and cells were selected using puromycin. A clonal cell line expressing A33-v5 and A36-v5 were produced subsequently to optimise A33 expression and this was transduced with lentiviruses expressing A36-v5 to create the HeLa cells A33-v5/A36-v5.

**Preparation of fresh EEVs, spinoculation and actin tails.** Fresh EEVs from the supernatant of vEGFPA5L-infected cells were clarified by centrifugation for 10 min at 1000 g at 4 °C and spinoculated onto chilled HeLa cells, or derivative cells expressing A33-v5, A36-v5 or both proteins, at 650 g for 30 min at 4 °C. The cells were overlaid with warm medium and incubated for 15 or 30 min at 37 °C. The samples were then fixed in 4% PFA and stained for actin and B5. Actin tails were counted on the entire surface of three independent coverslips (24-mm diameter) for each cell line. A33-v5/A36-v5 HeLa cells were also spinoculated with fresh EEVs (vEGFPA5L), purified IMVs (vEGFPA5L) and virions from a recombinant HSV-1, 166v, expressing GFP-tagged VP-22 (S16). The mean number of virions bound per cell were determined as 27.66 ± 2.072, 22.50 ± 2.88 or 28.78 ± 5.20 for EEVs, IMVs and HSV-1 respectively (SEM, mean from 3 independent experiments with n=5 fields of 4 to 12 cells), indicating the failure to induce actin tails with IMV and HSV-1 was not due to lack of virus binding.

**Preparation of cell lysates and immunoblotting.** Cells were washed with PBS and lysed in RIPA buffer (25 mM Tris HCl pH 8.8, 50 mM NaCl, 0.5% Nonidet P-40 and 0.1% sodium dodecyl sulphate). Insoluble material was centrifuged at 16 000 g for 20
min. Immunoblot analysis of cell lysates was performed using the primary mouse anti-v5 mAb (AbD serotec), a mouse anti-A33 mAb (A33-1), a rabbit polyclonal anti-A36 Ab (S12), the rat anti-B5 mAb 19C2 (S13), a rabbit polyclonal Ab raised against K7, an anti-D8 mAb (AB1.1 (S4)) and a mouse anti-α-tubulin mAb (clone DM1A, Millipore). The secondary Abs used were goat anti-rabbit, anti-mouse (Stratech Scientific) and anti-rat-horseradish peroxidase (GE Healthcare).

**Statistical analysis.** The data in the graphs are represented as mean and standard error of the mean.
Fig. S1. Spread of VACV in chick embryo fibroblasts (CEFs). CEFs were infected with vEGFPASL at low multiplicity of infection (moi) and stained with DAPI and phallolidin, and analysed by confocal microscopy. (A) Merge of DAPI, phallolidin and EGFP fluorescence images. (B) EGFP fluorescence image showing infected cell producing new virions and spread of virions to distant cells. (C and D) The lower panels show a zoomed image of the area indicated by a white box (A) where an actin tail is present on a cell with no virus factory. Panel C shows the EGFP fluorescence showing individual virions. Panel D shows the merge of EGFP and actin staining. Scale bar, 10 μm.

Fig. S2. Spread of VACV strain Lister. BSC-1 cells were infected with VACV strain Lister at low moi and processed for confocal microscopy. Cells were fixed and permeabilized and virus particles were stained with anti-D8 mAb AB1.1 (green), actin
was stained with phalloidin (red) and DNA was stained with DAPI. Confocal microscopy was used to record the edge of a plaque by phase (A) and D8 immunofluorescence (B). A zoomed image (D) of an area at the edge of the plaque indicated by a white box (C) shows the presence of an actin tail in a cell with no virus factory. (E) Merged image. Scale bar, 10 μm, (A to C, E) and 5 μm (D).

**Fig. S3.** A33 is expressed early during infection before EGFP-A5L. BSC-1 cells were infected with vEGFP-A5L at low moi and subsequently the edge of a plaque was visualised by confocal microscopy and immunostaining with anti-A33 (mouse mAb, top row) or anti-B5 (rat mAb, bottom row). The left panel of each row shows the EGFP fluorescence, and the right panel shows the merged image of the left 2 panels. Note the distribution of EGFP and B5 on cells is similar, whereas the A36 stain extends further than the EGFP positive cells, indicating early expression. Scale bar, 20 μm.
Fig. S4. Immunoblot analysis showing that A33, A36 or B5 are made late during infection when these proteins are expressed from the VACV 4b promoter. BSC-1 cells were either mock-infected or infected with VACV WR (A to C), v4b-A33R and v4b-A36R (A), v4b-B5R (B) and v4b-A33R-EGFPA5L and v4b-A36R-EGFPA5L (C) at 5
pfu/cell for 24 h in the presence or absence of cytosine arabinoside (AraC, 40 \mu g/ml). Cell lysates were analyzed by SDS-PAGE and immunoblotting with Abs to VACV proteins A33, A36, B5, D8 and K7 or to alpha tubulin. The images showing expression of A33, A36 and B5 in the presence of AraC were obtained after longer exposure of the X-ray films in comparison to other samples. Note that early during infection, in the absence of A36, A33 is present at lower levels. The same observation was made in cells infected with \(v\Delta A36\) in comparison to WR (D). The level of A33 is also higher in the cells expressing both A33-v5 and A36-v5 in comparison to the parental cell line expressing A33-v5 alone (Fig. 4A).
Fig. S5. Confocal microscopy showing early expression of A33 and A36 is needed for efficient spread of VACV particles. (A) Images (single focal plane) showing the edge of plaques formed by vEGFPΔ5L WR (green, upper left panel) stained with phalloidin (red) and Dapi (blue, upper right panel). Virus particles (green) on red actin tails (white box) were detected 2 to 3 cells away from the cell containing green virus factories (yellow box). Lower panel represents 4 different focal plans of the white box (upper right panel), confirming the presence of red actin tails (white arrows). Scale bar, 10 μm. (B) Confocal
images showing the edge of plaques formed by vEGFPA5L viruses (green) expressing A36 and A33 under a late promoter only (v4b-A33 and v4b-A36) and with the deletion mutants (vΔA36 and vΔA33) stained with phalloidin (red) and Dapi (blue). Virus particles (green) on actin tails (white boxes) were detected on cells producing virus factories (yellow boxes) for v4b-A33 and v4b-A36. Insets show zoomed images of these actin tails as indicated by white arrows. No actin tail was detected for vΔA36 and vΔA33. Scale bar, 10 μm. (C) The spread of virus particles (white dots) away from the edge of the plaque is considerably reduced following infection with the vEGFPA5L viruses v4b-A33 and v4b-A36 and more drastically with vΔA36 and vΔA33 in comparison to WR (white arrows). The panel shows cropped versions of the images presented in figure S5A and B with vEGFPA5L represented in white for clarity. (D) Graph representing the mean number of cells present between productive virus factories and the furthest actin tails detected (n=12). Actin tails were detected 2 to 3 cells away from the closest cells containing green virus factories in plaques formed by vEGFPA5L, whereas actin tails were detected only on cells expressing EGFPA5L or producing new virus particles for vEGFPA5L viruses expressing A33 and A36 under a late promoter only. (E) Graph representing the mean number of actin tails present in cells with productive virus factories in plaques formed by vEGFPA5L WR, v4b-A33 and v4b-A36 (n=12).
Fig. S6. Immunostaining and electron microscopy images showing that A33-v5 and A36-v5 localise to the cell surface when ectopically expressed in HeLa cells. (A) Immunostaining of A36-v5 on HeLa A36-v5 cells either live (top panel) or fixed and permeabilized (middle panel) using an anti-v5 antibody. Note that the v5-specific staining was only detected on fixed and permeabilized HeLa-A36-v5 cells because the v5 tag is fused at the C terminus of A36 that is present in the cytoplasm. In contrast staining of live HeLa A33-v5/A36-v5 cells recognised the v5 tag fused at the C terminus of A33 that is present outside the cell. Scale bar, 10 μm. (B) Immunostaining and electron microscopy. The upper panel shows the localization of A33-v5 and A36-v5 using an anti-v5 antibody by immunoperoxidase/DAB staining. The samples were then processed for electron microscopy as shown in the middle and the lower (zoomed image) panels. Scale bar, 5 μm and 1 μm (bottom left panel only).
Movie legends

**Movie S1.** Movie showing VACV plaque formation. BSC-1 cells were infected with VACV strain WR at low moi and the formation of a plaque was recorded by phase microscopy every h for 16 h after a small plaque first became visible. Note that the motility of infected cells is restricted to within the area showing cytopathic effect (cpe). Virus-induced cell motility is therefore not increasing the rate of spread across the cell monolayer.

**Movie S2.** Movie showing plaque formation by a VACV strain vEGFP-A5L. This virus expresses EGFP fused to the A5 core protein late during infection. BSC-1 cells were infected with vEGFP-A5L at low moi and the progression of infection on one side of a plaque was recorded by phase microscopy every h for 16 h.

**Movie S3.** Movie as for movie 2 except that the growth of the vEGFP-A5L plaque was visualized by the EGFP fluorescence.

**Movie S4.** Movie as for movies 2 and 3 showing merge of phase and fluorescence images. Note that the spread of virus-induced cpe (an early event) precedes that of expression of EGFP-A5L, which is made only late during infection.

**Movie S5.** Movie showing the spread of the edge of a plaque formed by vEGFP-A5L at higher rmagnification to show individual virus particles (green dots). Cells were visualised by confocal microscopy using EGFP fluorescence. Note cells must express EGFP prior to production of new virus particles and yet there are numerous virus particles on cells distal to those cells expressing EGFP.

**Movie S6.** Movie showing the formation of actin tails (red) in BSC-1 cells expressing cherryFP-actin that have been infected with vEGFP-A5L (green). The movie shows a single focal plane at the periphery of an infected cell where actin tails are formed following the transport of virus particles to the cell periphery (duration, 5 min).

**Movie S7.** Movie showing green virus particles moving on the tip of red actin tails on a cell expressing cherry-actin and lacking green virus factories. Note that a virus-tipped red actin tail produced by this cell induces the formation of another actin tail after re-contacting the cell surface.

References