Supporting Information

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SI Experimental Procedures

Cells and Viruses. Huh-7 (human hepatoma), VeroE6 (African green monkey kidney), and 293T (human embryonic kidney) cells were maintained in Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 10% (vol/vol) FBS (PAN Biotech), 2 mM L-glutamine (Q; Life Technologies), 50 U/mL penicillin, and 50 μg/mL streptomycin (PS; Life Technologies) and grown at 37 °C with 5% CO_2. During live-cell imaging, cells were cultivated in Leibovitz's medium (Life Technologies) with 100 U/mL penicillin, 100 μg/mL streptomycin, 20% (vol/vol) FBS, and 400 μM 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; Sigma). Marburg virus (MARV) (strain Musoke, accession no. DQ217792.1, GenBank) and recombinant MARV were propagated on VeroE6 cells as described earlier (1). All work with infectious viruses was performed in the biosafety level 4 (BSL-4) facility at the Philipps-Universität Marburg.

Treatment of Cells with Cytoskeleton-Modulating Drugs. MARV-infected cells were treated at 24 or 48 h postinfection (p.i.) with 15 μM nocodazole (Sigma) or 0.3 μM cytochalasin D (Sigma) and 0.15% dimethyl sulfoxide (DMSO; Sigma). Chemicals were added to the cell-culture medium 3 h before fluorescence microscopy was performed. Control cells were only treated with 0.15% DMSO.

Molecular Cloning and Plasmids. The plasmids coding for MARV structural proteins and the MARV-specific minigenome were described previously (2–4). For generation of the plasmid pCAGGS-RFP-VP40, the RFP ORF was cloned together with a $(glycine\text{-}serine)_6$ linker in front of the viral protein VP40 ORF using homolog recombination and primer-extension PCR (5, 6). For the construction of the plasmid pCAGGS-VP30-GFP coding for the VP30-GFP fusion protein, the GFP ORF was cloned in-frame to the 3′ end of the VP30 gene using the same technique as above. Recombinant MARV coding for the RFP-VP40 fusion protein was constructed by insertion of an additional RFP-VP40 ORF into an artificial AvrII restriction site between the VP35 and VP40 genes as described earlier (7). Cloning of full-length MARV cDNA was performed as described elsewhere (3). For visualizing cellular proteins in living cells, plasmids encoding mCherry-tubulin (Clontech) or TagRFP-actin (Evrogen) were used. The construction of GFP-tagged myosin 10 (Myo10) was described previously (8). Details of molecular cloning are available on request.

Rescue of Recombinant Virus. A 1:1 mixture of VeroE6 and Huh-7 cells at 60% confluency was transfected using Transit LT1 (Mirus) in a 3-cm cell-culture dish according to the manufacturer's instructions with the following plasmids: 500 ng pCAGGS-NP (encoding nucleoprotein), 100 ng pCAGGS-VP35, 100 ng pCAGGS-VP30, 2,000 ng pCAGGS-L, 500 ng pCAGGS-T7, and 2,000 ng full-length plasmid $pFLMARV_{RFP\text{-}VP40}$. At 24 h posttransfection (p.t.), medium was exchanged, and after 12 d supernatant from these cells was aliquoted and stored at −80 °C if not used for passaging on new cells for generating virus stocks. Viral RNA from virus harvested from passage 1 supernatant was isolated (QIAamp Viral RNA Mini Kit; QIAGEN) and used to generate cDNA fragments for sequencing (Transkriptor One-Step RT-PCR Kit; Roche). The sequence of recombinant MARV encoding additionally RFP-VP40 ($rMARV_{RFP\text{-}VP40}$) was verified by RT-PCR and revealed the expected insertion of the RFP-VP40 gene. Two point mutations were detected in comparison with $pFLMARV_{RFP\text{-}VP40}$. One was located in the VP35 gene (C3217G) and led to amino acid exchange (S to R, position 91); the other was a silent mutation in the gene encoding glycoprotein (G8807A). Mutation in VP35 was located in a region that showed variability among different MARV isolates. Indeed, in MARV strains Popp and Leiden, a mutation at the same amino acid position was detected (accession nos. Z29337.1 and JN408064.1, GenBank). Both mutations were therefore not considered to be significant within the scope of this study.

Infection of Cells. VeroE6 or Huh-7 cells were infected at a multiplicity of infection (MOI) between 0.1 and 1 50% tissue culture infective dose ($TCID_{50}$) in a volume of 1 mL (six-well plates) or 400 μL (μ-dish chamber) DMEM/PS/Q without FBS for 1 h at 37 °C. After infection the inoculum was removed, the cells were washed once with PBS, and appropriate cell-culture medium was added. VeroE6 cells were used for growth kinetics and Huh-7 cells were used for imaging experiments because of their flat appearance.

Microscopy. For live-cell imaging, Huh-7 cells were seeded onto 35-mm μ-dishes (Ibidi) 24 h before infection. Cells were infected in 400 μL Opti-MEM without phenol red (Life Technologies) for 1 h, inoculum was removed, and, if needed, cells were transfected in a 500-μL final volume of CO_2-independent Leibovitz's medium (Life Technologies). Live-cell time-lapse experiments were recorded with a Leica DMI6000B using a 63× oil objective equipped with a remote-control device to operate the microscope from outside the BSL-4 facility. Confocal images of 4% paraformaldehyde were fixed, and immunolabeled samples were acquired on a DMI6000B TCS SP5 laser scanning microscope using a 63× oil objective (Leica Microsystems), 488-nm argon laser, DPSS 561-nm laser, or helium 633-nm laser. Pictures and movie sequences were processed with the Leica LAS AF software package or Nikon NIS Elements 3.1, respectively.

SDS/PAGE, Immunoblot Analysis, Immunofluorescence Analysis, and Flotation Assay. These methods were performed as described previously (9).

Antibodies. For detection of VP40 or RFP-VP40, we used a VP40-specific mouse monoclonal antibody or a rabbit anti-RFP antibody (BioVision) in immunoblot analysis and immunofluorescence microscopy (1:20), a monoclonal antibody for detection of NP, and a GFP-specific polyclonal antibody from goat (Rockland). As secondary antibodies for Western blot analysis, we used goat anti-mouse conjugated to horseradish peroxidase (Dako), goat anti–mouse-Alexa Fluor 680 and goat anti–rabbit-IRDye800 (LI-COR Biosciences), and goat anti–mouse-Alexa Fluor 488, goat anti–mouse-Alexa Fluor 594, goat anti–mouse-Alexa Fluor 647, goat anti–guinea pig-Alexa Fluor 488, goat anti–guinea pig-Alexa Fluor 594, and goat anti–rabbit-Alexa Fluor 594 (1:300; Life Technologies) for fluorescence microscopy. As secondary antibody for detection of RFP in the immunofluorescence analysis, goat anti–rabbit-aminomethylcoumarin acetate (AMCA) was used (1:100; Vector Laboratories).

iVLP Assay. The MARV infectious virus-like particle (iVLP) assay was performed as described earlier (4).

$TCID_{50}$ Assay. The $TCID_{50}$ assay was performed as described earlier (3).

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2. Bamberg S, Kolesnikova L, Möller P, Klenk HD, Becker S (2005) VP24 of Marburg virus influences formation of infectious particles. *J Virol* 79(21):13421–13433.
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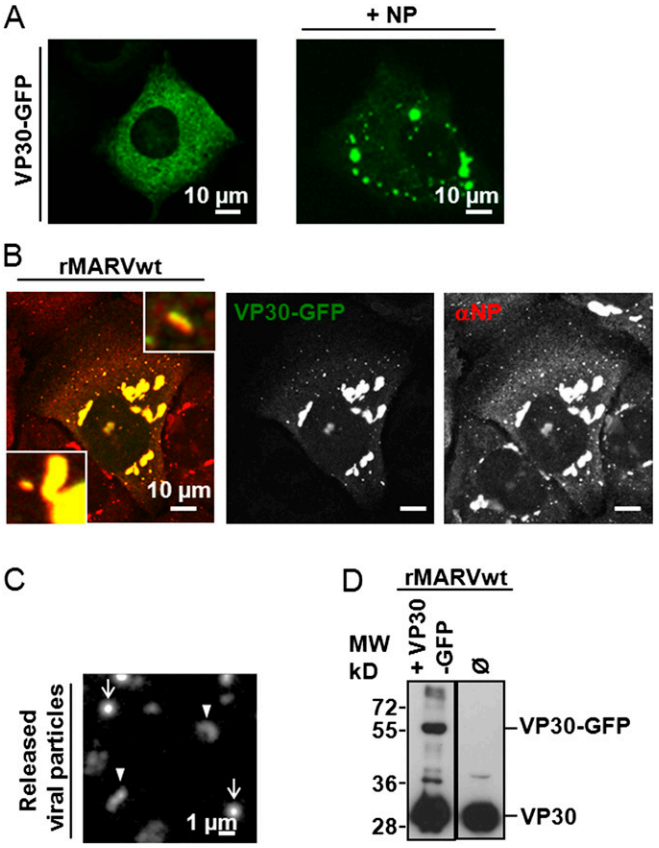


Fig. S1. VP30-GFP is colocalized with NP in transfected and infected cells and recruited into virions. (*A*) NP recruits VP30-GFP into inclusions. Huh-7 cells transiently expressing VP30-GFP (*Left*) or VP30-GFP and NP (*Right*) were analyzed by fluorescence microscopy at 24 h p.t. (*B*) VP30-GFP and NP colocalize in recombinant MARV wild type (rMARVwt)-infected Huh-7 cells at 24 h p.i. Fixed cells were incubated with monoclonal α-NP antibodies followed by a goat α-mouse coupled to Alexa Fluor 647. (*C*) Supernatant from $rMARV_{RFP\text{-}VP40}$–infected cells was analyzed by fluorescence microscopy. Arrows show spherical particles; arrowheads indicate filamentous particles. (*D*) VP30-GFP is incorporated into viral particles. Supernatant of rMARVwt-infected Huh-7 cells transiently expressing VP30-GFP was pelleted after 48 h p.i. and analyzed by Western blot. Blots were incubated with a monoclonal α-VP30 and a secondary goat α-mouse coupled to horseradish peroxidase.

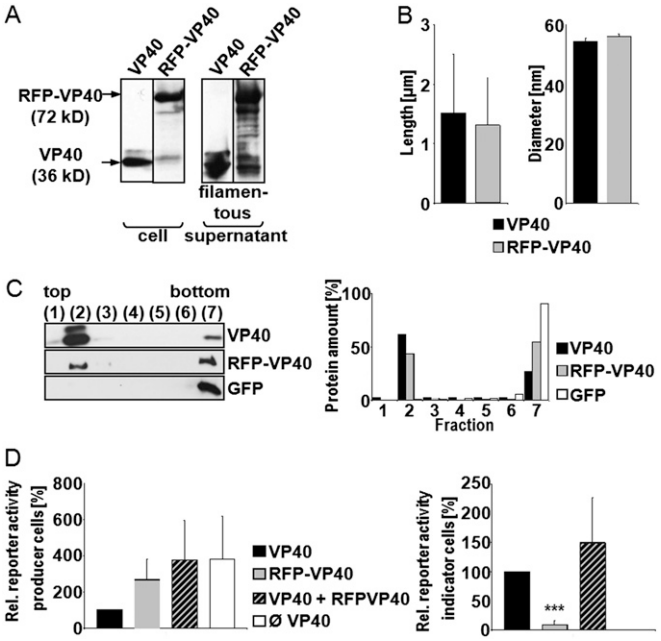


Fig. S2. Characterization of RFP-VP40. (*A*) Recombinant expression of RFP-VP40 leads to the formation of filamentous virus-like particles. Cells expressing VP40 or RFP-VP40 were harvested at 48 h p.t. Cell lysates and filamentous virus-like particles from the supernatants were analyzed by Western blot analysis. VP40 and RFP-VP40 were detected using monoclonal α-VP40 antibody and secondary goat α-mouse antibody coupled to horseradish peroxidase. (*B*) The length and diameter of filamentous virus-like particles from the supernatant of VP40- or RFP-VP40–expressing cells were analyzed on electron micrographs. (*C*) Membrane association of VP40, RFP-VP40, and GFP (a non–membrane-associated protein) via flotation analysis. HEK293T cells expressing VP40 or RFP-VP40 were lysed at 48 h p.t. Soluble proteins (*Bottom*) and membrane-associated proteins (*Top*) were separated by flotation analysis, and fractions of the gradient were analyzed via SDS/PAGE and Western blot. VP40 was detected as described for *A*. (*D*) Effect of RFP-VP40 on reporter gene analysis in an infectious virus-like particle assay. (*Left*) Reporter activity in producer cells. VP40 or RFP-VP40 or both were coexpressed in HEK293T cells together with all other structural MARV proteins and a MARV-specific minigenome. At 48 h p.t., cells were harvested and reporter gene activity (luciferase) was measured. (*Right*) Reporter activity in indicator cells. Virus-like particles purified from the supernatants of producer cells were used to infect indicator cells. At 48 h p.i., cells were harvested and reporter gene activity was measured (***$P \leq 0.0001$).

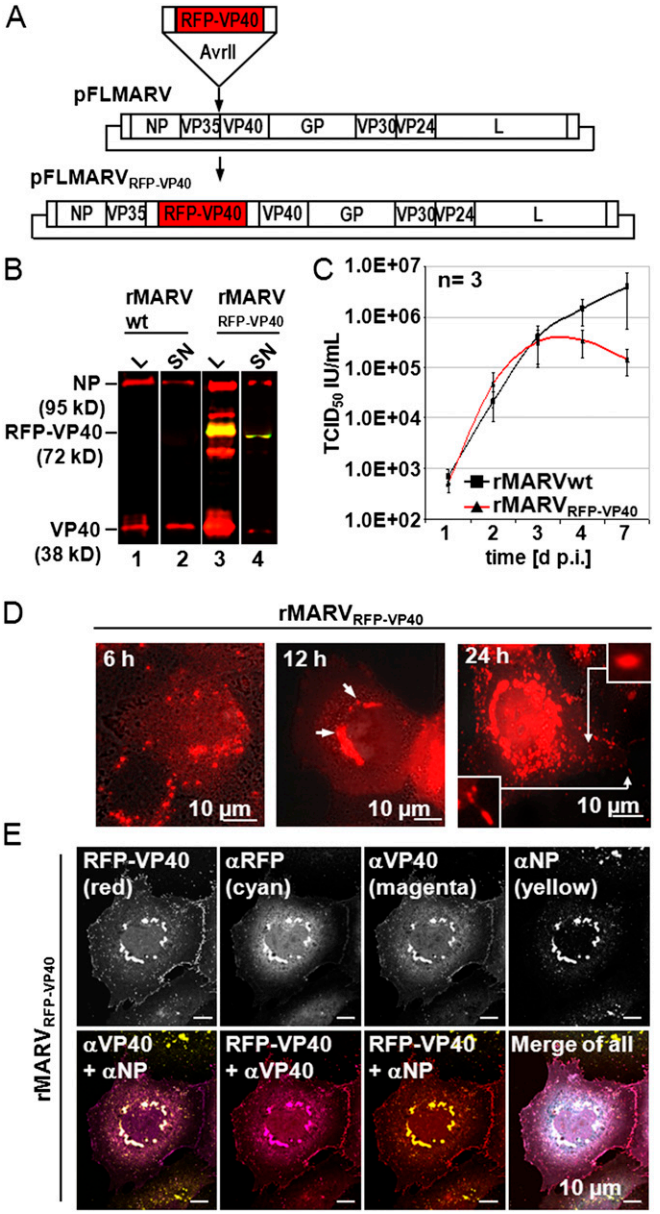
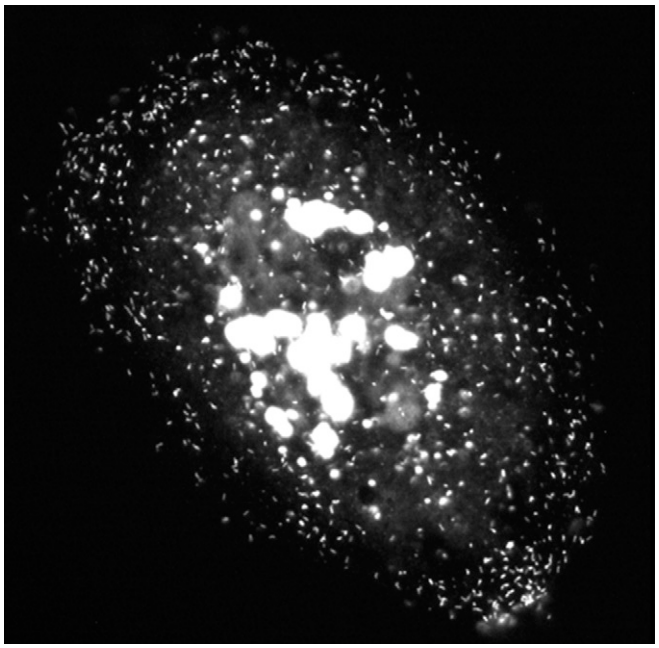
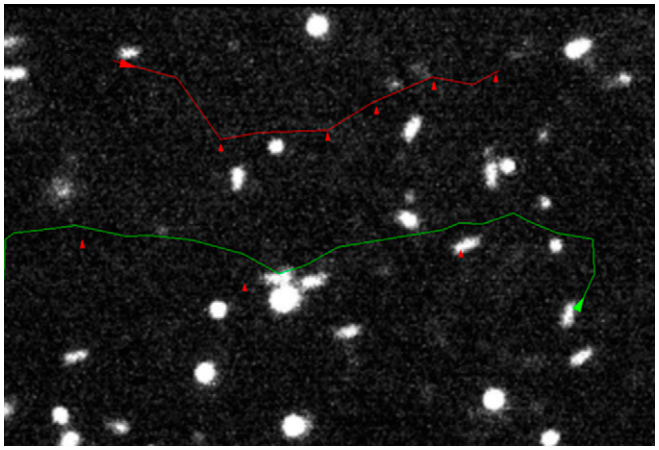


Fig. S3. Cloning, rescue, and characterization of rMARV$_{RFP-VP40}$. (*A*) Schematic representation of the construction of pFLMARV$_{RFP-VP40}$. (*B*) Western blot of rMARV$_{RFP-VP40}$–infected VeroE6 cells and supernatants. VeroE6 cells were infected with rMARV$_{RFP-VP40}$, harvested at 5 d p.i., and subjected to Western blot analysis. L, lysates; SN, supernatants. Blots were incubated with a mixture of a monoclonal α-NP, monoclonal α-VP40, and rabbit α-GFP antibody. Secondary antibodies were goat α-mouse antibody coupled to Alexa Fluor 680 and goat α-rabbit antibody coupled to IRDye800. Due to the high amount of BSA (69 kD) in the supernatant, the RFP-VP40 (72-kD) band runs slightly faster in those samples than samples from cell lysates. (*C*) Growth kinetics of rMARV$_{RFP-VP40}$ and rMARVwt. VeroE6 cells were infected with an MOI of 0.1 TCID$_{50}$ per cell. Cell supernatants were harvested daily and subjected to TCID$_{50}$ analysis. Error bars show standard deviation from three independent experiments. (*D*) Autofluorescence analysis of RFP-VP40. Huh-7 cells were infected with rMARV$_{RFP-VP40}$ with an MOI of 1 TCID$_{50}$ per cell. Images were acquired at different points of time p.i.: 12 h, arrows indicate inclusion bodies; 24 h, arrows indicate filamentous particles. Magnified pictures show enlarged filamentous particles located in either intracellular (upper right corner) or cellular protrusion (lower left corner). (*E*) RFP-VP40 colocalizes with VP40 and NP at 24 h p.i. Cells treated as in *D* were fixed and stained against RFP (cyan) with a rabbit α-RFP antibody followed by an AMCA-conjugated goat α-rabbit antibody; VP40 (magenta) was detected by a monoclonal α-VP40 antibody and an Alexa Fluor 647-conjugated goat α-mouse antibody. NP (yellow) was detected by an α-NP antibody from guinea pig followed by an Alexa Fluor 488-conjugated goat α-guinea pig antibody. Samples were analyzed by confocal microscopy.



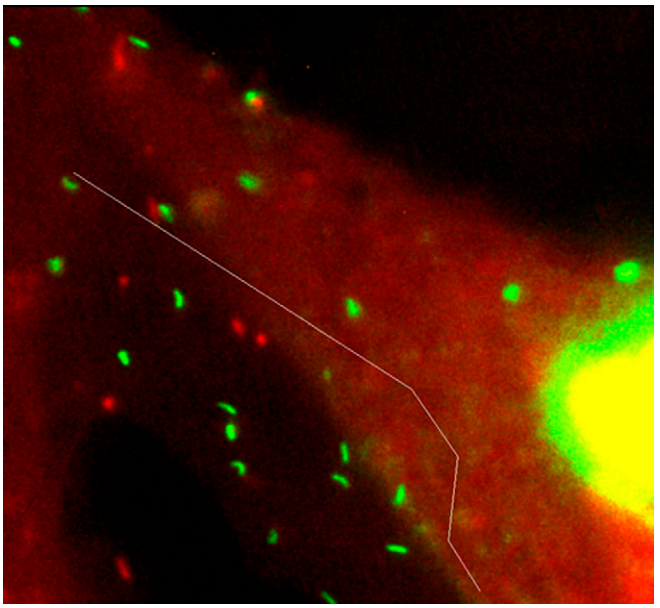
Movie S1. NCs labeled with VP30-GFP move with different velocities and different directions. Huh-7 cells expressing VP30-GFP were infected with rMARVwt and analyzed by time-lapse microscopy at 24 h p.i. Acquisition: Sequence displays 6 min; one frame was taken every 4 s. (Scale bar, 10 μm.)

Movie S1



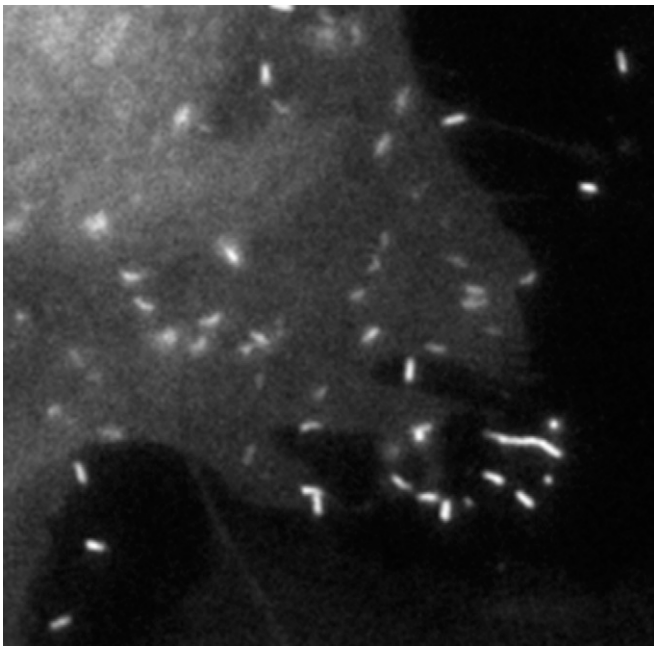
Movie S2. NCs in stop-and-go movements. Huh-7 cells expressing VP30-GFP were infected with $rMARV_{RFP\text{-}VP40}$ and analyzed by time-lapse microscopy at 29 h p.i. Sequence shows signal for VP30-labeled NCs. Red line: track of an NC moving from left to right. Green line: track of a second NC moving from the right to the left. Red arrowheads: temporary stops. Acquisition: Sequence corresponds to 1 min and 28 s; one frame was taken every 1.8 s. (Scale bar, 2 μm.)

Movie S2



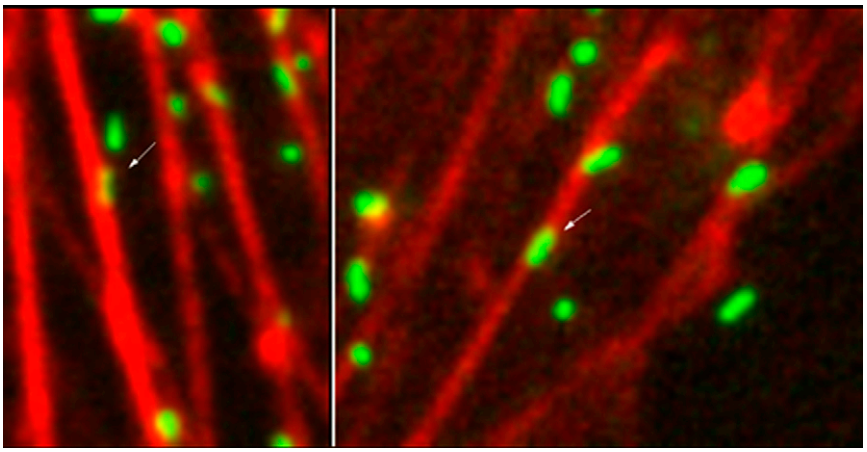
Movie S3. MARV NCs can move over distances of several micrometers with velocities of more than 400 nm/s. Conditions were the same as in Movie S2. A part of an infected cell is shown. Travel distance of one NC (indicated by a white line) was 27 μm. Velocity was higher than 400 nm/s. Acquisition: Sequence corresponds to 1 min and 15 s; one frame was taken every 2 s. (Scale bar, 10 μm.)

Movie S3



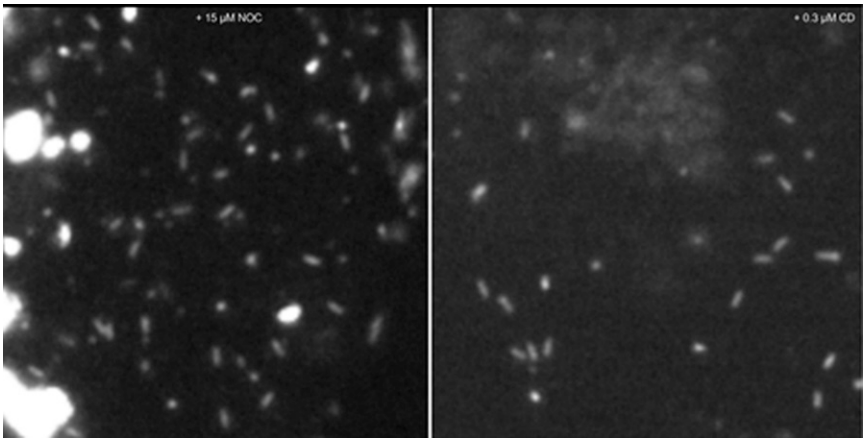
Movie S4. NCs associated with VP40 move in parallel to the cell border with a speed of ~100 nm/s. Conditions were the same as in Movie S2. Only signal for RFP-VP40 is displayed. Acquisition: Sequence corresponds to 2 min and 4 s; one frame was taken every 2 s. (Scale bar, 10 μm.)

Movie S4



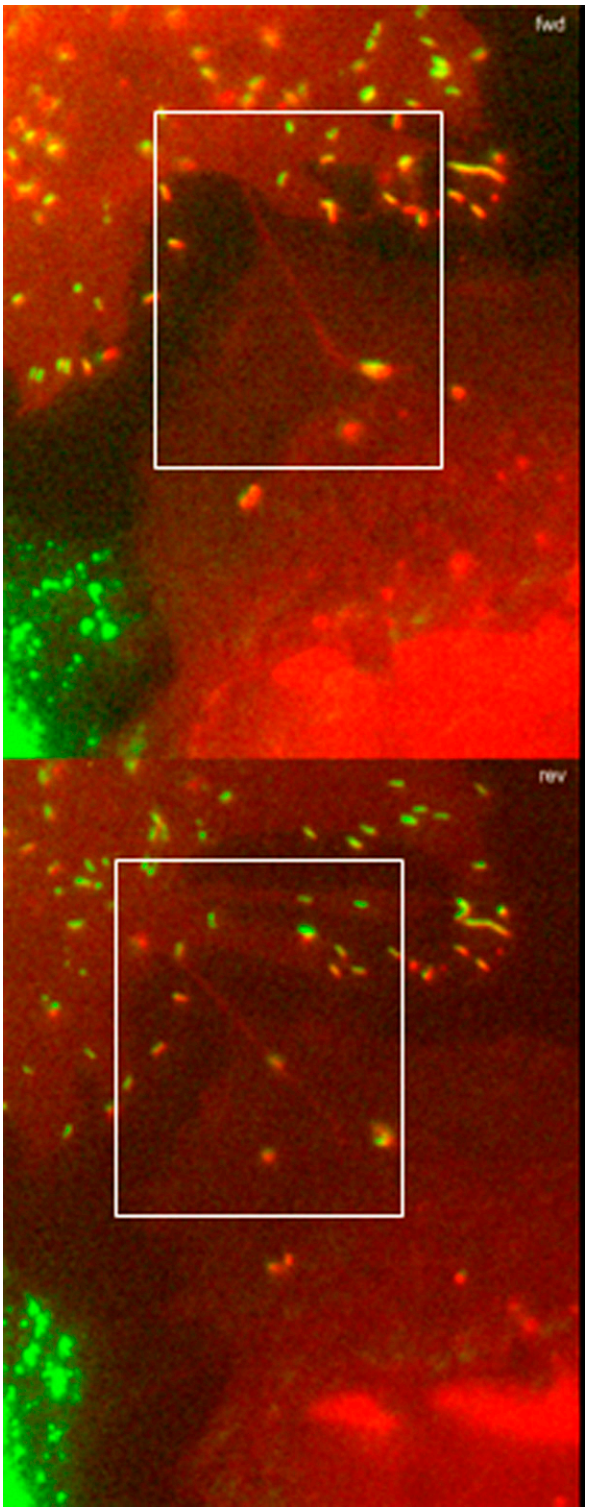
Movie S5. NCs are transported along actin filaments. Displayed are parts of two MARVwt-infected Huh-7 cells transiently expressing VP30-GFP (green) and TagRFP-actin (red) at 27 h p.i. (*Left*) An NC switching between actin filaments (arrow). (*Right*) An NC transported along actin filaments. Acquisition: Sequence displays 31.5 s; one frame was taken every 1.5 s. (Scale bar, 2 μm.)

Movie S5



Movie S6. Cytochalasin D treatment but not nocodazole treatment stops movement of MARV NCs. Huh-7 cells transiently expressing VP30-GFP and TagRFP-actin or mCherry-tubulin were infected with MARVwt. Cells were treated with cytoskeleton-modulating drugs at 24 h p.i. for 3 h. TagRFP-actin–expressing cells were incubated with 0.3 μM cytochalasin D; mCherry-tubulin–expressing cells were incubated with 15 μM nocodazole. Displayed are two rMARVwt-infected Huh-7 cells transiently expressing VP30-GFP at 27 h p.i. Acquisition: Sequence displays 1 min and 13 s; one frame was taken every 1.8 s. (Scale bar, 2 μm.)

Movie S6



Movie S7. NCs associated with VP40 are recruited into filopodia, where they move bidirectionally. Huh-7 cells expressing VP30-GFP were infected with $rMARV_{RFP\text{-}VP40}$ and analyzed by time-lapse microscopy at 29 h p.i. (*Upper*) Recruitment of an NC into a filopodium that is attached to another cell. NC moves forward (fwd). (*Lower*) Reverse movement (rev) of an NC that finally leaves the filopodium. Both movements have speed values of around 100 nm/s. Acquisition: Sequence corresponds to 12 min and 16 s; one frame was taken every 3 s. (Scale bar, 10 μm.)

Movie S7