Supporting Information

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SI Experimental Procedures

Cells and Viruses. BS-C-1 (American Type Culture Collection no. CCL-26) and HeLa (American Type Culture Collection no. CCL-2) cells were grown in Modified Eagle Medium (MEM) with Earle's salt and Dulbecco Modified Eagle Medium, respectively, supplemented with 10% (vol/vol) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (Quality Biologicals). Recombinant vaccinia virus (VACV) expressing firefly luciferase has been described previously (1). Plaque assays were performed in BS-C-1 cell monolayers in 12-well culture plates with 10-fold serial dilutions of virus. The virus was adsorbed for 1 h at room temperature, after which unbound virus was removed and the cells were washed and incubated with medium containing 0.5% (wt/vol) methylcellulose.

High-Throughput Screen. Screening was conducted using the Ambion Silencer Select Human Genome siRNA Library version 4, which targets ~21,500 genes, the vast majority consisting of three nonoverlapping and nonpooled siRNAs, and the Dharmacon siGENOME SMARTpool siRNA, consisting of four unique siRNA duplexes per gene in a single well. For screening, siRNA reagents (0.8 pmol) were spotted into black clear-bottomed 384-well plates (Corning 3712) using a Velocity11 VPrep liquid handling system (Agilent) integrated into a BioCel robotic platform (Agilent). All screening plates had a full column (16 wells) of both negative control (Ambion SilencerSelect Negative Control no. 2) and positive control (Dharmacon STX5A SMARTPool siRNA). The positive control served to assess transfection efficiency and assay performance, whereas the median value of each plate's negative control column was used to normalize corresponding sample wells.

Lipofectamine RNAiMax (0.15 µL; Invitrogen) was added to plate wells in 20 µL of serum-free media using a WellMate dispenser (Thermo Scientific). The transfection reagent and siRNA were complexed for 45 min at ambient temperature before the addition of 1,500 cells in 20 µL of medium containing 20% FBS with the WellMate dispenser. This yielded final transfection mixtures with 20 nM siRNA in media containing 10% serum (standard for the growth of HeLa cells). The cells were then cultured for 48 h at 37 °C in 5% CO_2 before the addition of VACV. Virus was added in 5 µL of medium, and plates were incubated for an additional 18 h at 37 °C in 5% CO_2 before being fixed by the addition of 4% paraformaldehyde in 20 µL of PBS. Fixation was allowed to proceed for 20 min before aspiration of the supernatant and washing with phosphate buffered saline (PBS) (2 × 40 µL). Nuclei were stained with Hoechst 33342 (1 µg/mL in PBS) for 30 min, and the cells washed once with PBS before the plates were sealed for subsequent imaging. All washing and staining steps were performed using a BioTek EL406 washer with BioStack.

Image Acquisition and Processing. Assay plate wells were imaged with a Molecular Devices ImageXpress Micro high- content platform integrated into an Agilent BioCel robotic system. The ImageXpress Micro uses a 1.4-megapixel cooled CCD camera with 100-nm resolution in the *x*, *y*, and *z* planes. Image acquisition was performed with a 4× S Fluor 0.20 NA lens to capture the majority of each well. Semrock DAPI (catalog no. DAPI-5060B) and FITC (catalog no. FITC-3540B) filter sets/dichroic mirrors were installed for the image acquisition. Acquired data were transferred to the Molecular Devices MDCStore data management system database and indexed by plate barcode. High-throughput image analysis was performed with MetaXpress PowerCore server software using the "multi-wavelength cell scoring" module to determine the percentage of VACV-positive cells in each well. The following parameters were used for nuclei segmentation: approximate minimum width, 10 µm; approximate maximum width, 25 µm; intensity above local background, 50 gray levels. For determination of virus-infected cells, the parameters were approximate minimum width, 20 µm; approximate maximum width, 80 µm; and intensity above local background, 50 gray levels.

Gene Network Analysis. Protein–protein networks were generated using Ingenuity Pathway Analysis (IPA; Ingenuity Systems; www.ingenuity.com). Molecules from the dataset that met our criteria, termed high-confidence genes, were considered for analysis. The Gene Ontology (GO) database or the Ingenuity Knowledge Base was interrogated for key terms. Molecules were then graphically represented in a network using Ingenuity Path Designer with the connect feature. The molecules were represented as nodes, and the biological relationship between two nodes was represented as an edge (line). High-confidence genes were overlaid onto the nodes and represented by shading. Nodes that were not present in the high-confidence dataset were removed from the network for visual simplification. Data displayed in a top molecular and cellular functions bar graph were generated from IPA Core Analysis performed on the high-confidence genes to directly link genes from the dataset to cellular processes. Top molecular and cellular functions were defined by the largest −log (*P* value).

GO Term Enrichment. Previously assigned GO annotations were used to identify associations between RNAi activity and specific gene function descriptions. GO terms occurring frequently in the descriptions of gene targets for spread-inhibiting RNAi have a *z*-score <0, whereas gene descriptive terms for spread-stimulating RNAi have a *z*-score >0. Only terms with a statistically significant (false discovery rate <0.05) association in both the Dharmacon and Ambion screens were listed. Using the parametric analysis of gene set enrichment method (2), the *z*-score for each gene set was calculated as follows. First, from input data containing RNAi screen score values for each gene, the mean of all score values (µ) and standard deviation of all score values (δ) of a given screen dataset were calculated. Then, with the mean of score values of genes for a given gene set denoted by *Sm* and the size of a given gene set by *m*, the *z*-score was calculated as $z = (Sm - \mu) \times m^{1/2}/\delta$. Raw *P* values were calculated from the standard normal distribution and then adjusted using the Benjamini–Hochberg false discovery rate (3). Ambion screen scores were reduced to one result per gene using the median RNAi activity.

Common Seed Analysis. The common seed analysis plot for the nuclear pore (Nup) protein Nup62 was generated using the methods and code of Marine et al. (4) In brief, a table of siRNA sequences, their intended targets, and the associated assay results were parsed to identify all siRNAs sharing the same seed sequences (bases 2–7 of the guide strand). A plot was then generated depicting assay results for all siRNAs targeting a given gene on the left and strip plots for the assay result of siRNAs targeting different genes but containing the same seed sequences on the right. If an siRNA exhibited roughly the same phenotype as all other siRNAs containing the same seed, then we concluded that the phenotype was due to off-targeting by the seed sequence, not to silencing of the intended target. Conversely, if many siRNAs screened in the assay had the same seed sequence but elicited little

relative activity in the assay, then we concluded that this seed sequence did not have an off-target effect on the assay.

Western Blot Analysis. Proteins of whole-cell lysates and purified virions were separated in 4–12% (wt/vol) NuPAGE Novex acrylamide gels (Life Technologies) with 2-(*N*-morpholino)ethanesulfonic acid buffer and then transferred to nitrocellulose membranes using the iBlot system (Life Technologies). The membrane was blocked with 5% (wt/vol) nonfat milk in PBS with 0.05% (vol/vol) Tween 20 and then incubated for 1 h at room temperature or overnight at 4 °C in the same solution with primary antibodies at appropriate dilutions. Excess antibodies were removed by washing with PBS containing Tween 20, followed by PBS without detergent. IRDye 800-conjugated secondary antibodies against mouse and rabbit antibodies were added, and the mixture was incubated for 1 h at room temperature, washed, and developed using an Odyssey Infrared Imaging System (LI-COR Biosciences). Alternatively, HRP-conjugated secondary antibodies against mouse, rabbit, or rat were used and developed using Supersignal West Dura Substrate (Thermo Scientific). Rat NUp62-expressing plasmid (Open Biosystems clone 8363131) was purchased from Thermo Scientific. Rat anti-human Nup62 was purchased from Sigma-Aldrich.

Analysis of DNA Synthesis and Early and Late Gene Expression. For the entry assay, cells were infected with VACV IHDJ/iFire at a multiplicity of 5 PFU/cell for 1 h at 4 °C. The medium was then replaced, and the cells were incubated at 37 °C for 90 min, after which firefly luciferase activity was measured using Promega Luciferase Assay Substrate. For late gene expression, cells were infected with VACV strain Western Reserve/F17 open reading frame-luciferase (WR/F17-Luc) at 5 PFU/cell for 1 h at room temperature. The medium was then replaced, and cells were incubated at 37 °C for 6 h, after which firefly luciferase activity was measured using Promega Luciferase Assay Substrate. Viral DNA synthesis was assessed as described previously (5).

Metabolic Labeling. HeLa cells grown in 24-well plates were infected with 5 PFU/cell of VACV IHDJ/iFire for 1 h at room temperature. Then the inocula were removed, and the cells were incubated with complete DMEM for the indicated times. At 30 min before labeling, the cells were incubated with cysteine- and methionine-free medium at 37 °C and pulse-labeled for 15 min with 100 μCi of [^{35}S]methionine-cysteine (Perkin-Elmer). The cells were washed with cold PBS and lysed in 0.5% Nonidet P-40 containing Complete Protease Inhibitor Mixture (Roche Applied Science). Proteins in the clarified cell lysate were resolved by SDS-PAGE and transferred to nitrocellulose membranes using the iBlot system (Life Technologies). The membrane was blocked with 5% (wt/vol) nonfat milk in PBS with 0.05% (vol/vol) Tween 20 and then exposed to film.

Transmission Electron Microscopy. Specimens were fixed with 2% (vol/vol) glutaraldehyde in 0.1 M phosphate (pH 7.4) buffer. Samples were postfixed for 30 min with 0.5% osmium tetroxide/0.8% (wt/vol) potassium ferricyanide, then transferred to 1% (vol/vol) tannic acid for 1 h and then to 1% (wt/vol) uranyl acetate overnight at 4 °C. Samples were dehydrated with a graded ethanol series and embedded in Spurr's resin. Thin sections were cut with a Leica EM UC6 ultramicrotome, and then stained with 1% (wt/vol) uranyl acetate and Reynold's lead citrate before viewing at 120 kV with a Tecnai BT Spirit transmission electron microscope (FEI). Digital images were acquired with a XR100 side-mount digital camera system (Advanced Microscopy Techniques) and processed using Adobe Photoshop CS5.

1. Bengali Z, Townsley AC, Moss B (2009) Vaccinia virus strain differences in cell attachment and entry. *Virology* 389(1-2):132–140.
2. Kim SY, Volsky DJ (2005) PAGE: Parametric analysis of gene set enrichment. *BMC Bioinformatics* 6:144.
3. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J R Stat Soc, B* 57:289–300.
4. Marine S, Bahl A, Ferrer M, Buehler E (2012) Common seed analysis to identify off-target effects in siRNA screens. *J Biomol Screen* 17(3):370–378.
5. Paran N, De Silva FS, Senkevich TG, Moss B (2009) Cellular DNA ligase I is recruited to cytoplasmic vaccinia virus factories and masks the role of the vaccinia ligase in viral DNA replication. *Cell Host Microbe* 6(6):563–569.

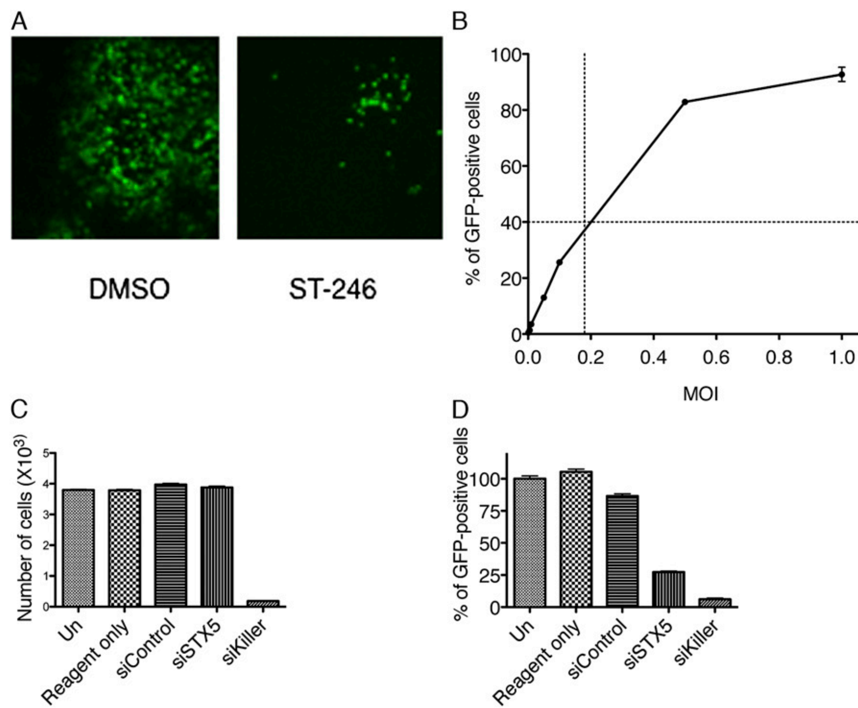

Fig. S1. siRNA pilot experiments. (*A*) Spread of VACV and inhibition by ST-246. HeLa cells were treated with ST-246 or DMSO, infected with 0.2 PFU/cell of VACV IHDJ/GFP, and visualized by fluorescence microcroscopy after 18 h. (*B*) Relationship of multiplicity of infection (MOI) to number of GFP-positive cells. HeLa cells in a 386-well plate were infected with the indicated PFU of VACV and scored for GFP expression after 18 h. (*C*) Total cell numbers after transfection with STX5 siRNA or killer siRNA. HeLa cells were untransfected (Un) or transfected with Lipofectamine (Life Technologies) alone (Reagent only) or with Lipofectamine plus OnTargetPlus (Dharmacon) nontargeting siRNA (siControl), pooled STX5 siRNA (siSTX5), or killer siRNA (siKiller) for 48 h, and then infected with VACV for 18 h. (*D*) Reduction in the number of GFP-positive cells after transfection with siRNA targeting STX5 or killer siRNA and infection with VACV. Labeling is as in *C*.

Fig. S2. Effects of Nup62 depletion on VACV spread. (*A*) Individual siRNAs targeting Nup62. HeLa cells in 384-well plates were transfected with nontargeting siRNA (Cnt) or four different siRNAs targeting Nup62 (Qiagen 1–4) for 48 h and then infected with GFP-expressing VACV. At 18 h after infection, the cells were fixed, stained with Hoechst dye, and imaged by automated microscopy. The mean percentage of GFP-positive cells in control wells was set to 100%. (*B*) Effects of siRNAs to various Nup proteins, Ran protein, and leptomycin B (LMB) on VACV spread. Treatment with Dharmacon nontargeting siRNA (Cnt) or pooled siRNAs targeting Nup62 (N62), Nup93 (N93), Nup98 (N98), Nup133 (N133), RAN protein, or combinations of these siRNA and infection with VACV was carried out as in *A*, with 20 nM LMB added at 1 h before infection.

Fig. S3. Effect of Nup62 knockdown on the integrity and function of nuclear pores. (*Left*) Effect of Nup62 knockdown on size exclusion by pores. OnTargetPlus pooled siRNA targeting Nup62 (siNup62) or nontargeting siRNA (siControl) was transfected into HeLa cells. After 48 h, the cells were permeabilized with 50 μg/mL digitonin and incubated with 10-kDa or 70-kDa dextran conjugated to Oregon Green 488 for 15 min at room temperature. The cells were fixed and then stained with Alexa Fluor 594-phalloidin (red) to visualize actin and DAPI (blue) to visualize nuclear DNA. Cells were imaged by confocal microscopy. Images portray 3D projections. (*Right*) Distribution of 70-kDa dextran between the nucleus and the cytoplasm. Ratios of the green channel in the nucleus and the cytosol are shown. Each point is a randomly selected field with >20 cells. *P* values were calculated using the Student *t* test.

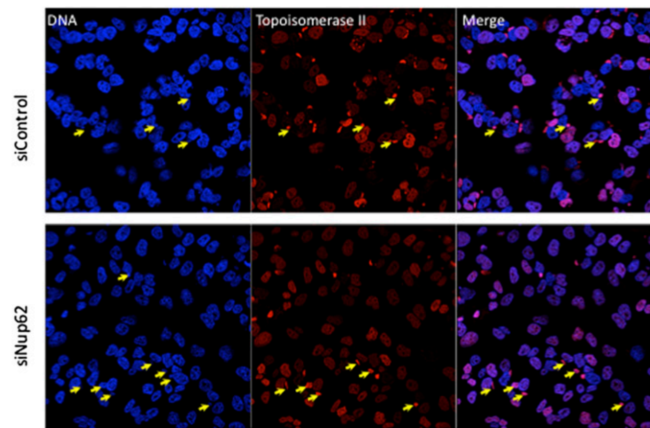

Fig. S4. Recruitment of cellular topoisomerase II to viral factories. HeLa cells were transfected with Lipofectamine plus OnTargetPlus pooled siRNA targeting Nup62 (siNup62) or nontargeting (siControl) and infected with VACV IHDJ/GFP for 7 h. After fixation, cells were stained with mouse antibody to cellular topoisomerase II, followed by Alexa Fluor 594 goat anti-mouse IgG (red) and DAPI (blue) to visualize DNA in nuclei and cytoplasmic viral factories near nuclei. A representative confocal microscopy plane is shown. Yellow arrows indicate viral factories with topoisomerase staining.

Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOCX)
Table S3 (DOCX)
Table S4 (DOCX)