

Supporting Information

Liu et al. 10.1073/pnas.1321940111

SI Materials and Methods

Preparation of the Cre⁺ and Cre⁻ Procapsids. The PCR-amplified *cre* gene was cloned into pJM252 (1) between the HindIII and EcoRI sites, after the internal HindIII site in the *cre* gene was modified by replacing the *cre* residue 552 (AAG) with residue 552 (AAA) so that the encoded amino acid remains lysine, but the HindIII site is removed by site-directed mutagenesis. Phage T4 recombinants containing the *cre* gene from the *cre*-containing pJM252 were selected using the method of gene insertion (phage T4 lysozyme deletion e⁻ to insertion e⁺) described by Mullany and Black (2) and were verified by DNA sequencing. Expression of the cyclic recombination (Cre) protein from the recombinant phage *cre* gene driven by the strong phage T4 IPIII promoter was confirmed by standard-protocol Western blotting using polyclonal anti-Cre antibodies (Novagen) with purified Cre commercial enzyme (Novagen) as control. Cre⁺ procapsids were prepared by growing Cre⁺ T4 phage in the presence of 9 aminoacridine (9AA) as described by Dixit et al. (3). Cre⁺ T4D phage empty procapsids containing the capsid-targeting sequence (CTS)-Cre protein were prepared for packaging in vitro by infection of *Escherichia coli* B40 *supF* so as to insert tyrosine at amber codon 7 in the CTS of pJM252 to allow synthesis and CTS targeting of CTS-Cre protein in the phage procapsid (1). The Cre⁺ and Cre⁻ (wild-type) phage procapsid or proheads were highly purified by centrifugation and column chromatography as previously described (4). *E. coli* DH10B was used for the transformation of plasmids in cloning as well as for site-directed mutagenesis. LB medium was used for growth of bacteria except for phage-infection experiments, in which M9S supplemented with 20% LB medium was used instead. Phage infection in the presence of 9AA was carried out at 30 °C; bacteria were grown at 37 °C and were shifted to 30 °C immediately before infection. Thirty seconds after the first infection, 9AA (3 µg/mL) was added to the culture to block terminase activity (3). After 9AA treatment procapsids were prepared by glycerol gradient centrifugation similar to the purification of proheads as described in ref. 4, but no column purification was done.

Preparation of Dye-Labeled dsDNA. Isolated *Pseudomonas* phage φ6 dsRNA fragments were reverse transcribed using 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, and a 1:2 mixture of 0.1 mM dCTP and 0.2 mM 5-aminoethylacrylamido-dCTP (aha-dCTP) according to the manufacturer's protocol (Life Technologies), with modifications. Three primer pairs, RT-S F1 (5'GTGTTGTTCCCACTAATAAT3')/RT-S R1 (5' ATGCATGAAGGGGCTGGA-C3'); RT-M F1 (5' TCAACCCATAATAAGAGATC3')/RT-M R1 (5' CATCCTATTGGATGCTCGC3'); and RT-L F1 (5'ATCCGACTTTTATAAGGACG3')/RT-L R1 (5'TGATGTTACCAACG-AAGATG3'), were used to reverse transcribe two complementary φ6 S, M, and L cDNAs at 47 °C for 1 h, followed by denaturing at 85 °C and gradual cooling to 4 °C, designed to make three dsDNAs 1,520 bp, 2,030 bp, and 3,320 bp in length, respectively. The resulting dsDNAs (~2 µg) were treated with RNase H (Life Technologies, Inc.), were purified with Qiaquick PCR (Qiagen, Inc.), and were precipitated by ethanol at -80 °C. The precipitate then was resuspended in 5 µL H₂O, followed by the addition of 3 µL of the Alexa 488 dye (30 µg/µL) and 2 µL of sodium bicarbonate (85 mg/mL). The reaction was incubated at 22 °C for 1 h using a Tetrad Thermo Cycler (MJ Research, Inc.). The fluorescent DNA then was purified by passage through an Amicon membrane filter (100-kDa cutoff) (EMD Millipore Corp.), washed three times with

10 mM Tris (pH 7.5), and characterized by mass spectrophotometry and gel electrophoresis.

Atomic Force Microscopy and Data Analysis. A multimode scanning probe microscope (Veeco Instruments) in air under tapping mode using a commercial silicon cantilever (nanoScience Instruments), 125 mm long, with an apex curvature radius of 5–6 nm, a resonant frequency of 300 kHz, and spring constant of 40 N/m, was used for imaging. The scanning rate was 0.5 Hz at an angle of 0°. Research NanoScope II software, version 7.20, was used for imaging processing. All images were filtered using the built-in flattening tool in NanoScope II software. Length and height values of the nanoparticles (NPs) were obtained using the built-in tool for cross-sectional analysis. Height and length values of at least 20 individual T4 NPs were obtained from the section data and were averaged.

Assessment of Inhibition Effects on the Uptake of T4 Capsid by Inhibitors. A549 cells were pretreated with various concentrations (as indicated in *SI Results*) of endocytosis inhibitors including wortmannin, amantadine, and cytochalasin D for 30 min followed by the treatment with Alexa 546-T4 NPs or Alexa488-T4 NPs for 4–6 h at a ratio of 50,000 NPs per cell. Cells were trypsinized and washed with three times with PBS, followed by flow cytometer analysis.

Colocalization of Lysosomes and Dye-T4 NPs in A549 Cells. Lyso-Tracker Blue DND-22 (Life Technologies) at a concentration of 75 nM was added to A549 cells along with the dye-T4 NPs (50,000 T4 NPs per cell), followed by 5-h incubation. The treated cells were fixed with 4% (vol/vol) formaldehyde and subsequently subjected to confocal microscopy.

SI Results

Prediction of Packaged DNA Copy Numbers Within T4 Capsids. Our results indicated that 2 µg of linear pEGFP and plasmid mCherry (pmCherry) driven by a cytomegalovirus promoter were completely packaged into 1.6E+10 T4 procapsids in the in vitro DNA packaging reaction as indicated in Table S1. Based on the calculation, a total length of ~120 kb of dsDNA (24 copies of 5-kb linear DNA) was packaged into each T4 procapsid. However, the efficiency for packaging dye-labeled dsDNA is not 100%, because some fast-migrating smear still appears on the gel (see lanes 3 and 5 in Fig. 2A). Based on the intensity of the leftover DNA smear, the packaging efficiency is estimated to be about 50%, and the total length of the packaged DNA is ~50 kb for each capsid (Table S1).

Uptake of T4 Procapsids Packaged with Linear pEGFP and pmCherry-C1 DNA by A549 Cells. Fig. S1 shows the quantification of the fluorescent cell population resulting from the expression of fluorescent protein from internalized packaged linear DNA using flow cytometry.

Linear pEGFP and pmCherry DNAs were completely packaged into T4 procapsids as indicated in Fig. S1A. Although the change in fluorescence units (FLU) was not as significant in the cells treated with the resulting T4 capsids as in the cells transfected with DNA using Lipofectamine ("lipofected" in Fig. S1 B and C), the median and mean FLU in cells treated with the resulting T4 capsids were higher than those of the negative controls treated with serum-free medium (SFM), as indicated in Table S3.

Uptake of T4 Capsids into A549 Cells via Multiple Pathways. A549 cells were treated with fluorescent A546-T4 and subjected to

inhibitor studies and lysosome colocalization staining. Amantadine, specifically blocking clathrin-mediated endocytosis, and wortmannin, specifically inhibiting PI3K, move the histogram pattern close to that of the SFM control and inhibit the cell uptake in a concentration-dependent manner (Fig. S2 *A* and *B*). PI3 kinase also is involved in other endocytotic pathways besides clathrin-mediated endocytosis (5). The preliminary results suggest uptake is through clathrin-mediated endocytosis; however, the colocalization with LysoTracker Blue indicated that only a small portion of internalized T4 capsids are within lysosomes (Fig. S2 *C* and *D*). Therefore it is likely that uptake of T4 capsids also occurs through other pathways, such as macropinocytosis and caveolae-mediated endocytosis (5, 6).

Demonstration of the Fluorescence Intensity Values for Fluorescent Cells Taking up T4 Cre⁺ and Cre⁻ Procapsids. In Table S2, the measurement of mean and median FLU in cell populations treated with T4 Cre⁻ or Cre⁺ procapsids shows that cells treated with the T4 Cre⁻ procapsids have a slight increase in mean and median fluorescent intensity, but this increase is not as significant as that in cells treated with T4 Cre⁺ procapsids when compared with control cells treated with the highly fluorescent A546-T4 procapsids. The values listed in Table S2 are plotted in Fig. S3, which demonstrates that the uptake of Cre⁺-T4 capsids increases the fluorescent intensity among tested cells (cell counts ranging from 3,000–50,000) and changes the distribution of the cell population in the histogram.

- Mullaney JM, Black LW (1996) Capsid targeting sequence targets foreign proteins into bacteriophage T4 and permits proteolytic processing. *J Mol Biol* 261(3):372–385.
- Mullaney JM, Black LW (2014) Bacteriophage T4 capsid packaging and unpackaging of DNA and proteins. *Methods Mol Biol* 1108:69–85.
- Dixit AB, Ray K, Black LW (2012) Compression of the DNA substrate by a viral packaging motor is supported by removal of intercalating dye during translocation. *Proc Natl Acad Sci USA* 109(50):20419–20424.
- Black LW, Peng G (2006) Mechanistic coupling of bacteriophage T4 DNA packaging to components of the replication-dependent late transcription machinery. *J Biol Chem* 281(35):25635–25643.
- Mercer J, Helenius A (2012) Gulping rather than sipping: Macropinocytosis as a way of virus entry. *Curr Opin Microbiol* 15(4):490–499.
- Pelkmans L, Helenius A (2002) Endocytosis via caveolae. *Traffic* 3(5):311–320.

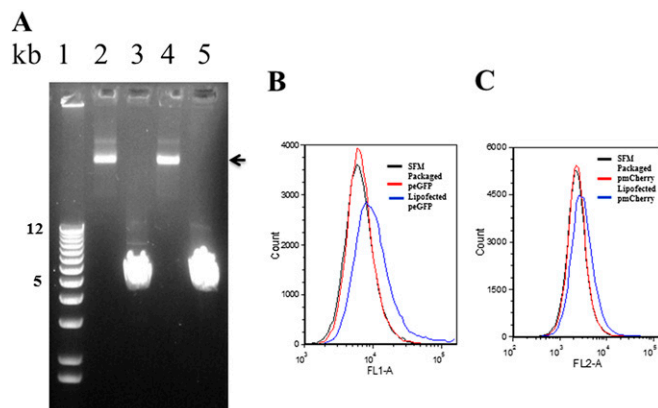


Fig. S1. Histogram of fluorescent cell population resulting from internalized packaged dsDNA. (A) Linear pEGFP and pmCherry-C1 DNA (lanes 3 and 5) were used for the DNA-packaging assay, and 1 μ L of the 20- μ L reaction mixture was examined in agarose gel as shown in lanes 2 and 4. The packaged pEGFP (lane 2) and pmCherry-C1 (lane 4) DNA inside the procapsids were retained on the top (indicated by arrow). The 1-kb+ DNA size marker is shown in lane 1. Approximately 2–3 μ g of linear DNA and 1×10^{11} Cre⁻ procapsids were used in 20 μ L of packaging reaction. The packaging efficiency is high without free unpackaged linear DNA (lanes 2 and 4). The resulting T4 procapsids then were incubated with A549 cells for 6 h, followed by 24-h incubation. The cells then were trypsinized and washed three times with PBS, followed by cell-population analysis using a flow cytometer. (B) There is a slight increase in the fluorescent cell population (right shift of the histogram) resulting from internalized packaged linear EGFP DNA compared with the internalized linear DNA by Lipofectamine. (C) The histogram shows no change in the fluorescence in the cell population from internalized pmCherry-C1 DNA relative that in the control population in SFM.

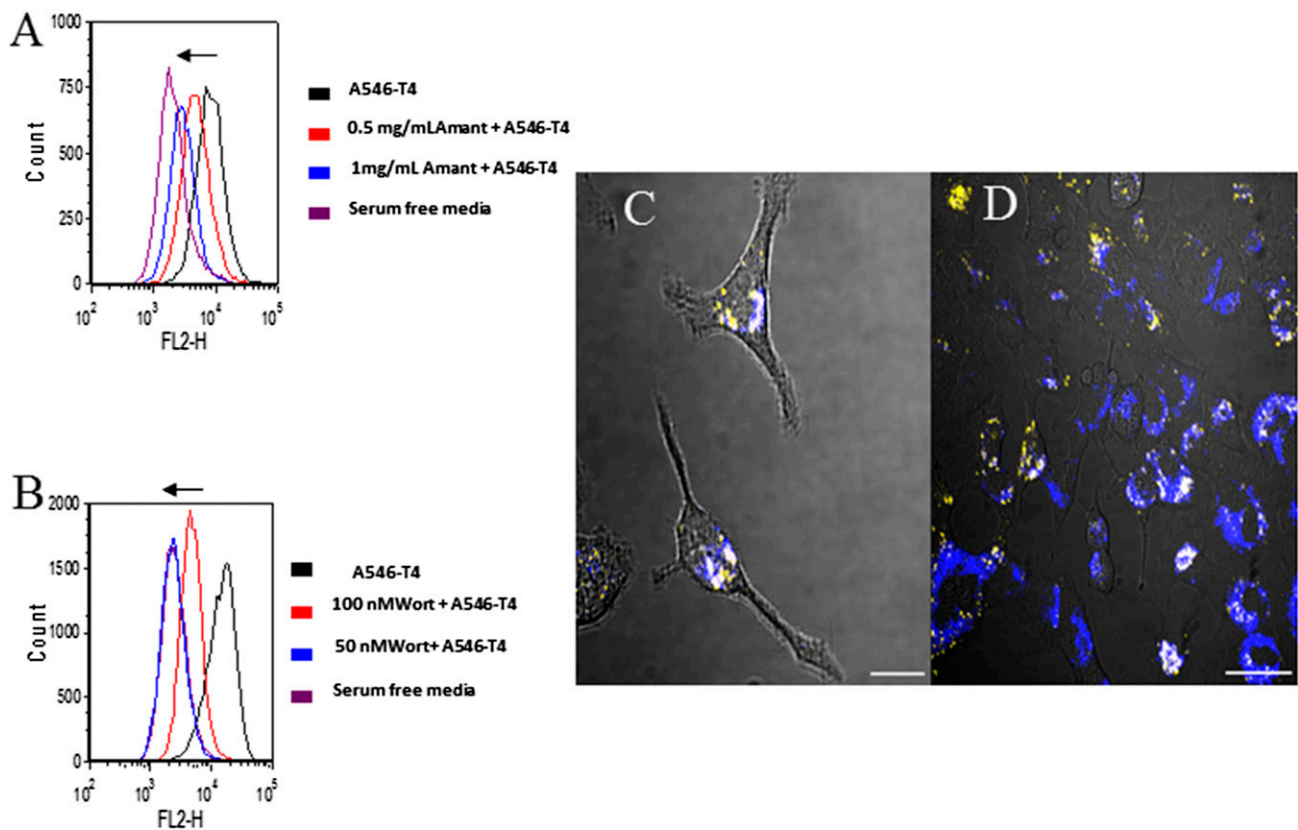


Fig. S2. Measurement of the inhibition by endocytosis inhibitors and colocalization with lysosomes in A546-T4-treated A549 cells. (A) Pretreatment with amantadine, specifically stabilizing the clathrin-coated pits, reduced the uptake of A546-T4 NPs by A549 cells in a concentration-dependent manner. (B) Pretreatment with the PI3 kinase inhibitor, wortmannin, also reduced the uptake of A546-T4 in a concentration-dependent manner. (C) An overlapping confocal cell image obtained with a 60× objective with the internalized A546-T4 procapsids (yellow), lysosomes stained with LysoTracker Blue (blue), and the overlapping spots (white). (Scale bar, 10 μm.) (D) A confocal image shows the broad view of treated cells containing overlapping portions (white spots) of lysosomes (blue) with A546-T4 procapsids (yellow). The image was obtained using a 20× objective. (Scale bar, 50 μm.)

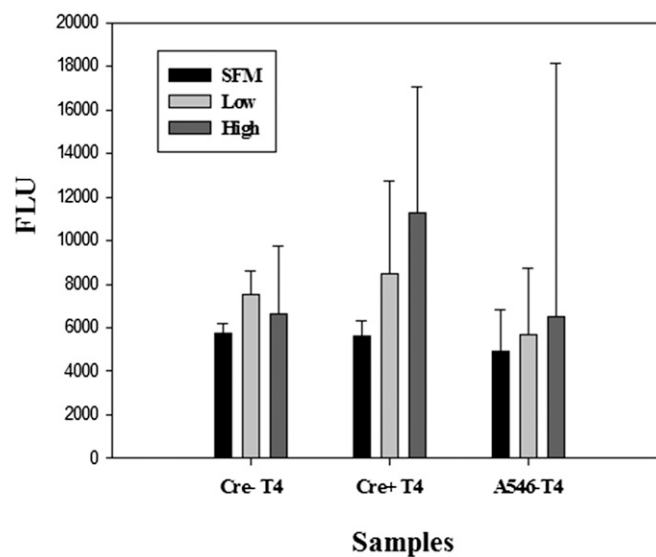


Fig. S3. Comparison of mean FLU values in cells treated with different groups of T4 capsids. Error bars represent SD. SFM, control serum-free medium without the addition of T4 capsids. “Low” and “High” indicate cells treated with low and high amounts of T4 capsids, respectively, as indicated in Table S2.

Table S1. Predicted DNA copy number per T4 capsid in the in vitro DNA-package reaction

Name	Size, bp	Molecular mass, Da	DNA, g	Moles	DNA copy no.	No. of capsids	DNA per capsid
pEGFP-N1	4.73E+03	3.12E+06	2.00E-06	6.40E-13	3.84E+11	1.60E+10	2.40E+01
pmCherry	4.71E+03	3.11E+06	2.00E-06	6.43E-13	3.86E+11	1.60E+10	2.41E+01
φ6 S dsDNA*	1.52E+03	1.00E+06	2.00E-06	1.99E-12	1.20E+12	1.60E+10	3.74E+01
φ6 L dsDNA*	3.32E+03	2.19E+06	2.00E-06	9.13E-13	5.48E+11	1.60E+10	1.71E+01

*Packaging efficiency estimated to be 50% relative to nonlabeled dsDNA.

Table S2. Fluorescent intensity of the treated A549 cells

Samples	Median FLU	Mean FLU	SD
SFM	4,602.0	5,769.3	6,187.6
Cre ⁻ (4.0E+9)	5,623.5	7,509.7	8,612.7
Cre ⁻ (8.0E+9)	5,046.0	6,661.3	9,730.6
SFM	4,155.0	5,769.3	6,187.6
Cre ⁺ (4.0E+9)	6,251.0	8,485.5	12,717.3
Cre ⁺ (8.0E+9)	7,765.0	11,296.3	17,027.7
SFM	4,446.5	4,943.2	6,840.6
A546-T4 (1.0E+9)	5,992.5	5,696.6	8,703.4
A546-T4 (2.0E+9)	7,106.5	6,497.2	18,153.8

Numbers in parentheses indicate the amount of T4 procapsids used in the packaging reaction.

Table S3. Fluorescent intensity of A549 cells treated with Cre⁻ packaged linear plasmids

Samples	Median FLU (channel reading in flow cytometer)	Mean FLU	Standard deviation
SFM	6,212.0 (FL1A)	7,476.1	6,791.5
1.2E+10 Cre ⁻ pEGFP	6,399.0 (FL1A)	7,265.8	9,966.0
Linear pEGFP, % Lipofectamine transfection	9,559.0 (FL1A)	95,930.6	830,410.7
SFM	2,270.0 (FL2A)	2,678.9	2,415.0
1.2E+10 Cre ⁻ pmCherry	2,318.0 (FL2A)	2,728.4	2,186.2
Linear pmCherry, % Lipofectamine transfection	2,957.0 (FL2A)	3,969.8	12,196.7