Supplementary Material for

Structural basis of DNA packaging by a ring-type ATPase from an archetypal viral system

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Figure S1. Biochemical properties of the HK97 DNA packaging system. Dependence on spermidine in the packaging of (A) the HK97 genome and (B) linear cos-containing pUC18 DNA. Dependence on (C) divalent metal ions, (D) pH and (E) monovalent salts in the packaging of linear cos-containing pUC18 DNA. Reaction components were treated with Chelex-100 resin before addition of divalent metal ions. Samples with different salt levels were ethanol-precipitated before analysis by agarose gel electrophoresis. Ethidium bromide staining was used to visualize DNA in the agarose gel.
**Table S1.** TerL crystallographic data collection and refinement statistics.

|                              | SeMet peak | SeMet inflection | KBr soak  |
|------------------------------|------------|------------------|-----------|
| **Data collection**          |            |                  |           |
| Wavelength (Å)               | 0.97949    | 0.97969          | 0.91983   |
| Space group                  | C2         | C2               | C2        |
| Cell dimensions              | a, b, c (Å) | 211.5, 39.3, 66.0 | 211.9, 39.3, 66.1 | 211.8, 39.2, 65.5 |
| β (°)                        | 103.3      | 103.2            | 103.4     |
| Resolution (Å)               | 49.59–2.00 (2.05–2.00) | 49.72–2.40 (2.49–2.40) | 45.53–2.20 (2.27–2.20) |
| Rmerge                       | 0.122 (1.233) | 0.100 (1.249)    | 0.136 (0.842) |
| I / σ(I)                     | 10.1 (1.0) | 13.6 (1.6)       | 7.1 (1.8) |
| CC1/2 (%)                    | 99.7 (36.5) | 99.9 (52.9)     | 99.1 (59.5) |
| Completeness (%)             | 98.3 (85.3) | 99.9 (99.7)    | 99.6 (98.2) |
| Redundancy                   | 6.2 (4.1)  | 6.6 (6.4)       | 3.3 (3.3) |
| Wilson B (Å²)                | 43.3       | 60.5             | 37.6      |
| No. unique reflections       | 35605 (2257) | 21206 (2198)    | 26796 (2242) |
| **Refinement**               |            |                  |           |
| Resolution (Å)               | 45.53–2.20 |                  |           |
| No. reflections              | 25551      |                  |           |
| Free                         | 1244       |                  |           |
| Rwork/Rfree                  | 0.205/0.254 |                  |           |
| No. atoms                    |            |                  |           |
| Protein                      | 3522       |                  |           |
| Water                        | 93         |                  |           |
| Ligand                       | 18         |                  |           |
| B factors                    |            |                  |           |
| Protein                      | 38.9       |                  |           |
| Water                        | 33.3       |                  |           |
| Ligand                       | 53.1       |                  |           |
| R.m.s. deviations            |            |                  |           |
| Bond lengths (Å)             | 0.008      |                  |           |
| Bond angles (°)              | 1.3        |                  |           |
| Ramachandran                 |            |                  |           |
| Favored (%)                  | 97.5       |                  |           |
| Outlier (%)                  | 0.0        |                  |           |
Figure S2. Comparison of viral packaging nucleases. The HK97 TerL nuclease contains an additional ααβ element, colored blue, on the periphery of the central β-sheet. The corresponding region in herpes virus and pac homologues is colored blue. PDB codes are indicated in brackets.
Figure S3. Comparison of ASCE ATPase topologies and Walker A sequences. (A) Topology diagrams of select ASCE (additional strand conserved E) ATPase subfamilies. AAA+, ATPases associated with diverse cellular activities. The consensus fold for viral packaging ATPases across all structurally characterized cos, pac and phi29 systems are shown. The gray helix in the ASCE core with a dotted outline is a random coil in the HK97 TerL. The phi29 ATPase has an additional β-strand between ASCE strand 5 and preceding helix (not shown). The location of confirmed trans-acting arginine fingers are indicated for each ATPase subfamily. (B) Multiple sequence alignment of viral packaging ATPase Walker A sequences. The virus and gene product names are indicated, separated by an underscore. In grey is the classical Walker A lysine; red, critical arginine residue identified in pac phage T4, cos phage lambda and phi29; yellow, critical lysine residue in HK97 and corresponding positive residues in phage T5 and herpes viruses. Lambda gpA has an unusual Walker A sequence with two lysine residues upstream and downstream of the classical position (in bold), both required for ATPase and DNA packaging activities (1, 2).
**Figure S4. Comparison of TerL active sites.** Left, full-length structures of pac phage T4 and Sf6 TerL, phi29 gp16 (PDB 3CPE, 4IDH, 7JQQ), the ATPase and nuclease domains of pac phage P74-26 TerL (PDB 4ZNL, 5TGE), and the tripartite monomer of the herpes simplex virus type 1 (HSV-1) hexameric terminase assembly (PDB 6M5V). The ATPase and nuclease active sites are indicated with a black and white arrowhead, respectively. Active site residues are shown as sticks. Secondary structure elements corresponding to the HK97 protein are colored as in Figure 2 (red, blue, beige).

Middle, the ATPase active site. Structures in complex with ATP, ATP-γ-S or ADP·BeF₃ are shown (PDB 2O0H, 4IEE, 7JQQ, 4ZNL, 6M5V). *Trans*-acting elements from an adjacent subunit are shown in blue, where available. Grey-labelled residues correspond to the unusual lysine K57 of HK97. Right, the nuclease active site. Putative or confirmed residues important for divalent metal ion coordination for DNA cleavage are shown. The phi29 vestigial nuclease domain is shown for comparison.
Figure S5. Molecular weight determination of TerS. (A) Size-exclusion chromatography and multi-angle laser-light scattering analysis (100 μL 2.7 mg/mL) in 20 mM HEPES, 300 mM KCl, 1 mM DTT, pH 7.0. Protein eluting from a Superdex 200 10/300 GL column (GE Healthcare) was analyzed using an on-line Dawn HELEOS-II light scattering detector and Optilab rEX refractometer (Wyatt). Data were processed based on a refractive index increment of 0.183 mL/g in ASTRA 5.3.4 (Wyatt). (B) High resolution Orbitrap native MS measurement showed a single charge state distribution corresponding to a species with a mass of 165.020±0.005 kDa (based on four most intense charge states). The fully deconvoluted spectrum indicated a single species with mass of 165.022 kDa (inset), unambiguously indicating a nonameric assembly based on the known monomer mass of 18.3 kDa.
Table S2. TerS crystallographic data collection and refinement statistics.

| Data collection                           | 500 mM KI soak | 250 mM KI soak |
|------------------------------------------|----------------|----------------|
| Wavelength (Å)                           | 1.6984         | 0.9763         |
| Space group                              | H3             | H3             |
| Cell dimensions                          |                |                |
| a, c (Å)                                 | 123.3, 79.4    | 123.3, 79.0    |
| Resolution (Å)                           | 44.31–1.79 (1.83–1.79) | 44.23–1.40 (1.43–1.40) |
| Rmerge                                   | 0.080 (0.539)  | 0.049 (0.707)  |
| I / σ(I)                                 | 15.2 (1.4)     | 10.3 (1.1)     |
| CC1/2 (%)                                | 99.6 (51.5)    | 99.7 (52.5)    |
| Completeness (%)                         | 95.3 (47.3)    | 98.8 (88.6)    |
| Redundancy                               | 4.4 (1.2)      | 2.7 (2.0)      |
| Wilson B (Å²)                            | 19.0           | 18.6           |
| No. unique reflections                   | 40412 (1184)   | 86871 (3870)   |

Refinement

| Resolution (Å)                           | 44.23–1.40     |                |
| No. reflections                          |                |                |
| Working                                  | 82402          |                |
| Free                                     | 4469           |                |
| Rwork/Rfree                              | 0.159/0.170    |                |
| No. atoms                                |                |                |
| Protein                                  | 2474           |                |
| Water                                    | 338            |                |
| Ligand                                   | 9              |                |
| B factors (Å²)                           |                |                |
| Protein                                  | 21.9           |                |
| Water                                    | 33.0           |                |
| Ligand                                   | 30.9           |                |
| R.m.s. deviations                        |                |                |
| Bond lengths (Å)                         | 0.011          |                |
| Bond angles (°)                          | 1.4            |                |
| Ramachandran                             |                |                |
| Favored (%)                              | 100.0          |                |
| Outlier (%)                              | 0.0            |                |

Table S3. Intramolecular contacts in the N-terminal helix-turn-helix-like region of HK97 TerS.

| Residue 1 | Residue 2 | Nature of contact                               |
|-----------|-----------|-------------------------------------------------|
| T25       | H43       | hydrogen bond between sidechains                |
| I26       | W42       | hydrogen bond between backbone oxygen and sidechain |
| P28       | W42       | CH/τr stacking interaction                      |
| P29       | A32       | hydrogen bond between backbone atoms            |
| S30       | G33       | hydrogen bond between backbone atoms            |
Figure S6. Control packaging experiment and photobleaching step analyses for GFP-TerL. (A) Packaging of Scal-linearized cos-containing pUC18 DNA by GFP-TerL with and without BOBO-3 DNA staining. A reduction in band intensity was observed with BOBO-3 bound to DNA because the dye interfered with ethidium bromide staining (data not shown). (B) GFP-TerL photobleaching steps observed by total internal reflection fluorescence microscopy. Left column, GFP-TerL in the presence of only DNA; right column, GFP-TerL in the presence of DNA, TerS and procapsids. Top row, events not coincident with BOBO3 DNA staining. Bottom row, events coincident with DNA staining.

Table S4. Event counts for the step-wise photobleaching measurements.

|                          | DNA + TerL | DNA + TerL + TerS + Procapsid |
|--------------------------|------------|-------------------------------|
| Number of DNA spots      | 699        | 367                           |
| with no co-localizing    | 634        | 259                           |
| protein signal           |            |                               |
| with co-localizing protein signal, discarded | 6         | 43                            |
| with co-localizing protein signal, analyzed | 59        | 65                            |
Figure S7. Low-resolution cryo-EM-based model of the HK97 DNA packaging assembly. (A) Cross-section of three reconstructions with icosahedral, C5 and C6 symmetry imposed. The poorly resolved densities corresponding to terminases and DNA indicated a dynamic system. 3D densities depicted on the right side of each cross-section were used to generate a composite map. (B) Fourier shell correlation analysis of the final half-maps during refinement with no symmetry imposed. Inset shows densities from this C1 refinement (whole and cut-through). Scale bar = 100 Å. (C) Composite map showing portal (red) and TerL (orange). Inset, rigid body fitting of a HK97 portal homolog (PDB 3KDR), and model of the HK97 TerL pentamer based on pac phage P74-26 pentameric ATPase model. It is unclear if the distal density corresponds to DNA or TerS.
Figure S8. HK97 TerL ATPase domain docked against homologous TerL assemblies. (A) Pentameric ATPase model of P74-26 TerL (PDB 4ZNK), generated using M-ZDOCK (3) as in (4). (B) Cryo-EM structure of the phi29 DNA packaging assembly (PDB 7JQQ). pRNA was omitted from the cartoon representation for clarity. (C) Hexameric terminase assembly of herpes simplex virus type 1 (HSV-1; PDB 6M5V). The identified trans-acting arginine finger for each assembly is indicated with a blue sphere. Confirmed or putative DNA-binding residues are marked with a yellow sphere for the P74-26 and HSV-1 assemblies. A superposition of the HK97 TerL ATPase domain is shown on the right, with residues K57, R254 and R90 indicated with a blue, red and yellow sphere, respectively.
Figure S9. Molecular weight determination of TerL. (A) Size-exclusion chromatography and multi-angle laser-light scattering analysis (100 μL 1.2 mg/mL) in 20 mM HEPES, 300 mM KCl, 1 mM DTT, pH 7.0 using a Superdex 200 10/300 GL column (GE Healthcare). (B) Sedimentation coefficient distribution by sedimentation velocity analytical ultracentrifugation. Epon double-sector centerpieces were filled with protein (2.5-20 μM in 20 mM HEPES, 300 mM KCl, 1 mM DTT, pH 7.0) and buffer, respectively, and centrifuged in an An-60 Ti rotor at 50000 rpm, 20 °C, for 7.5 h using a Beckman Optima XL/I ultracentrifuge. Absorbance scans were acquired at 280 nm at 7.5 min intervals. A protein partial specific volume of 0.73747 mL/g, buffer density and viscosity of 1.01390 g/mL and 0.01002 P was estimated using Sednterp (5). Data were analyzed under the continuous c(s) distribution model using Sedfit (6). Frictional ratio estimates of 1.32–1.37 and standard sedimentation coefficients of 3.66–3.81 S were obtained, contributing to over 90% of the signal.
Figure S10. Alanine mutagenesis analysis of TerL. (A) ATP hydrolysis rates at 1 mM ATP concentration, based on inorganic phosphate release, of TerL with different combinations of TerS, procapsid and DNA. The mean and range of three initiate rate measurements are reported for each condition. (B) Far-UV circular dichroism spectra of wild-type and mutant HK97 proteins. Spectra were recorded for protein (2–5 μM) in 20 mM Tris sulfate, 50 mM Na₂SO₄, 10 mM MgSO₄, 0.5 mM tris(2-carboxyethyl)phosphine hydrochloride, pH 7.5, at 20 °C. (C) Superposition of the HK97 TerL structure with the arginine finger of pac phage P74-26 TerL (gold, PDB 4ZNK). (D, E) ATPase rescue experiments conducted with TerL Walker B E154A mutant in equimolar amounts at 1 mM ATP concentration in the absence and presence of DNA, TerS and procapsids, respectively. The mean and range of three initiate rate measurements are reported for each condition.
Table S5. DNA oligonucleotides for cloning and mutagenesis. Primers for plasmid linearization for restriction-free cloning are listed. Numbering on cos DNA primers indicates the genome position of the first complementary nucleotide. All sequences are provided in 5’ to 3’ direction.

| Restriction-free cloning of His-SUMO-fusion |  |
|--------------------------------------------|-----------------------------------|
| SUMO_GP1_for                               | gaacagattgtgggtgctagataacggaatcgtgccg |
| SUMO_GP1_rev                               | taccctagttctctctgctctggaggaaggcc |
| SUMO_GP2_for                               | gaacagattgtgggtgctagataacggaatcgtgccg |
| SUMO_GP2_rev                               | taccctagttctctctgctctggaggaaggcc |
| Plasmid_for                                | agacaagcttaggtatttacctctctctctcagcag |
| Plasmid_rev                                | accaccaaatctgcggctgctgctgctgctgctgcc |

| Restriction-free cloning of His-fusion      |  |
|--------------------------------------------|-----------------------------------|
| His_GP2_for                                | atcaccacaccacactagctctctgaggaaggcc |
| His_GP2_rev                                | tgggagcagctctctgaggaaggcc |
| Plasmid_for                                | cgcccttctctctcagctctctctctctctctcagcag |
| Plasmid_rev                                | tgggagcagctctctgaggaaggcc |

| Site-directed mutagenesis                   |  |
|--------------------------------------------|-----------------------------------|
| GP2_R3A_for                                | cggcaggtgagcagctgaatagtacgttcctt |  
| GP2_R3A_rev                                | ctcacctgctctctgctctctctcagctctctcagcag |
| GP2_R6A_for                                | gtgggagcagctctctgaggaaggcc |
| GP2_R6A_rev                                | ctcacctgctctctgctctctctcagctctctcagcag |
| GP2_R13A_for                               | tgggagcagctctctgaggaaggcc |
| GP2_R13A_rev                               | ctcacctgctctctgctctctctcagctctctcagcag |
| GP2_R28A_for                               | ctcacctgctctctgctctctctcagctctctcagcag |
| GP2_R28A_rev                               | ctcacctgctctctgctctctctcagctctctcagcag |
| GP2_R56A_for                               | cgcgcgtgagcagtctctctctctcagctctctcagcag |
| GP2_R56A_rev                               | ctcacctgctctctgctctctctcagctctctcagcag |
| GP2_R90A_for                               | ctcacctgctctctgctctctctcagctctctcagcag |
| GP2_R90A_rev                               | ctcacctgctctctgctctctctcagctctctcagcag |
| GP2_R159A_for                              | gacctgcagctctctcagctctctcagctctctcagcag |
| GP2_R159A_rev                              | ctcacctgctctctgctctctctcagctctctcagcag |
| GP2_R226A_for                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_R226A_rev                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_R240A_for                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_R240A_rev                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_R247A_for                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_R247A_rev                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_R254A_for                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_R254A_rev                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_R263A_for                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_R263A_rev                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_R270A_for                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_R270A_rev                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_R270A_for                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_R270A_rev                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_R270A_for                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_K140A_for                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_K140A_rev                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_K204A_for                              | ctcacctgctctctcagctctctcagctctctcagcag |
| GP2_204A_rev   | ttgcgacgagcagcatcatcaagcg gcgtgc | GP2_206A_for   | aatcgccgatccgacatcgtgtg |
| GP2_206A_rev   | gatcgccgatccgacatcgtgc | GP2_T186A_for   | ctagctagctgattgcagcg |
| GP2_T186A_rev  | cctgctactagctgattgcagcg |
| GP2_K60A_for   | ggtgacgtgcacctagctgaaatcgtgc | GP2_K60A_rev   | ccagtgcacccctggtggtgc |
| GP2_E154A_for  | tgcgctcgtcgatctgattgcagcg |
| GP2_E154A_rev  | tgcgctcgtcgatctgattgcagcg |

| **GFP and linker insertion** |
|-----------------------------|
| GFP_for                     | atcaacccacccactgtgagcaagggc |
| GFP_rev                     | ctagctagctgattgcagcg |
| Plasmid_for                 | ctagctagctgattgcagcg |
| Plasmid_rev                 | tgcgctcgtcgatctgattgcagcg |

| **Overlap-extension and restriction cloning of HK97 cos region** |
|---------------------------------------------------------------|
| cos-312BamHI_for                                            | gtagctagctgattgcagcg |
| cos1strev                                                   | ctagctagctgattgcagcg |
| cos2ndrev                                                   | gtagctagctgattgcagcg |
| cos+472EcoRIrev                                             | gtagctagctgattgcagcg |

| **Generation of 5' biotinylated DNA** |
|---------------------------------------|
| cos-80for                              | atcaaaatgagaatcgcatacag |
| cos+150rev                             | aacacatgggagcag-BIO |
SUPPLEMENTARY NOTE

Analysis of photobleaching events

DNA particles were identified by extracting connected components from an average of the first 500 frames in the red channel. The intensity-weighted centroids of these components were taken to be DNA coordinates. Coordinates fewer than 10 px apart were rejected. For each coordinate in the red channel, given the translation vector from calibrating with TetraSpeck microspheres (ThermoFisher) emitting in both channels, the corresponding pixel in the green channel was found. A circle 3.5 px in radius, large enough only to enclose an event, was drawn around the center of this pixel. Mean intensity of the circle as a function of time was extracted and analyzed for photobleaching steps using the PIF algorithm (7) with Chung-Kennedy filtering (8). Over-fitted steps were identified by generating a counter-fit with steps between the original steps and comparing chi-squared statistics, as introduced by Kerssemakers et al. (9), and subsequently rejected.

For more robust analysis, a moving two-sample t-test (10) and a chi-squared minimization-based step-finding algorithm (9) were also applied. However, this resulted in a systematic under-fitting or over-fitting of steps, owing to the low signal-to-noise ratio of the experiment.

To build a distribution of the number of photobleaching steps per protein event co-localizing with DNA, an event was accepted only if steps could be fitted and if it had an intensity-weighted centroid within experimental error of the centroid of the DNA particle. A centroid-based measure was used because the density of events in the green channel was high and in turn background pixels surrounding each event could not be reliably defined for the fitting of Gaussian distributions. Adapting from Gelles et al. (11), experimental error was estimated to be the precision (two standard deviations) with which the x- and y-position of one GFP-LT monomer could be assigned relative to another, in time. We calculated one standard deviation to be 40.4 nm in both directions.

We note that the number of photobleaching events could be underestimated due to the presence of a dark GFP population as a result of misfolding or photobleaching prior to the experiment; and due to mis-fitting of two photobleaching events as one because the two events occurred within the same video frame, as described in detail by Ulbrich and Isacoff (12). The observed photobleaching rate of the GFP fusion protein was 0.10 s⁻¹, typical of GFP proteins (13). We also acknowledge the possibility of overestimating the number of photobleaching steps when the event monitored was coincident with other passively absorbed GFP molecules.

If one assumed all molecules observed in the DNA-bound motor experiment were pentamers, a binomial distribution with 72% probability of GFP being fluorescent could be fitted. However, it was likely that lower-order species as observed in the absence of prohead and small terminase also contributed to the distribution. Given the complexity of the experiment, it was reasonable to conclude only that the upper limit for the oligomeric state of TerL in a motor assembly was 5.
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