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Construction of Asymmetrical Hexameric Biomimetic Motors with Continuous Single-Directional Motion by Sequential Coordination

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Abstract

The significance of bionanomotors in nanotechnology is analogous to mechanical motors in daily life. Here we report the principle and approach for designing and constructing biomimetic nanomotors with continuous single-directional motion. This bionanomotor is composed of a dodecameric protein channel, a six-pRNA ring, and an ATPase hexamer. Based on recent elucidations of the one-way revolving mechanisms of phi29 dsDNA motor, various RNA and protein elements were designed and tested by single-molecule imaging and biochemical assays, with that, we have constructed the motor with active components. The motor motion direction was controlled by three operation elements: 1. Asymmetrical ATPase with ATP-interacting domains for alternative DNA binding/pushing regulated by an arginine finger in a sequential action manner. The arginine finger bridges two adjacent ATPase subunits into a non-covalent dimer, resulting in an asymmetrical hexameric complex containing one dimer and four monomers. 2. The dsDNA translocation channel as a one-way valve. 3. The hexameric pRNA ring geared with left-/right-hand loops. Assessments of these constructs revealed that one inactive subunit of pRNA/ATPase is sufficient to completely block the motor function (defined as $K = 1$), implying that these components worked sequentially based on the principle of binomial distribution and Yang Hui's Triangle.

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Keywords

One-way traffic; bionanomotor; single-molecule imaging; real-time recording; inter-subunit communication

Introduction

The ubiquitous intriguing nanomachines in living organisms have inspired numerous biomimetic strategies and human achievements. Biological macromolecules of DNA, RNA and proteins are essential and powerful chemical building blocks of all organisms based on their intrinsically defined features at the nanometer scale.^[1, 2] Construction of self-propelling nanomotors have been a popular subject in the field of nanotechnology.^[3–24] The emerging field of nanotechnology has led to the advancement of biomaterials engineering and synthetic biology.^[25–27] Although the construction of biological nanomotors has been extensively studied,^[28–31] the key step to make these bionanomotors applicable in nanodevices and nanomachines is to functionalize these motors and control their continuous single-directional motion. Here we report one such principle and approach by designing and constructing an artificial biomimetic phi29 dsDNA packaging motor that can move continuously in one direction. This nanomachine is composed of a 3.6 nm dodecameric protein channel and six protein ATPase that are geared by six pRNA (packaging RNA) and driven by ATP (Fig. 1).^[28]

The motion direction of the dsDNA was controlled by ATPase, connector and pRNA systems. In the ASCE (Additional Strand Catalytic E) ATPases superfamily, one well-conserved ATP domain contains one arginine finger motif along with the Walker A and Walker B motifs.^[32–35] Located in proximity to the γ -phosphate of the bound ATP in the adjacent ATPase subunit,^[33, 36–38] arginine finger has been reported to be involved in the formation of the ATP activity pocket.^[35, 38–42] Elucidation of the principle of how arginine finger mediates the sequential action of ATPase has shed light on the realization of controlling motor motion direction. Furthermore, the connector channel as a one-way valve for DNA advancement is another key factor for the control of the uni-directional movement of the biomotor. With the contribution of all three coaxial rings in the motor, the constructed motor, though tiny, is considered as one of the most powerful biological motor ever constructed to date.

Results and Discussion

Screening of pRNA sequences to control motor motion

pRNA is an essential component of the motor.^[43] Six pRNA monomers form a hexameric ring through interactions between four bases in every two adjacent pRNA loops.^[44] A minimum length of 117-nucleotide in each pRNA is required. Each pRNA contains five single-base bulges, one three-base bulge, one bifurcation bulge, one bulge loop and two stem loops (Fig. 2). To select the RNA sequences with activity to gear the motor, 18 different hexamer pRNAs were constructed following the method reported previously.^[44, 45] All these mutant pRNAs were competent in binding to the procapsid with an efficiency equal to that

of the wild type pRNA. However, incorporation of only one mutant, either pRNA^{AdCCA} (with CCA bulge deletion, Fig. 2, red box) or pRNA^{trun} (truncated at the 5'- and 3-ends, Fig. 2, blue box), into the hexameric loop completely obliterated the motor function in DNA packaging activity. The fact that one inactive pRNA is sufficient to block the function of the entire motor has been defined as $K = 1$.^[46–48] In binomial distribution, $K = 1$ for the hexameric RNA ring implies that six RNA molecules worked sequentially.^[48–50]

Screening of connector sequences to control motor motion

The channel of the phi29 motor functions as a dodecameric ring and serves as a pathway for the dsDNA to enter the procapsid during the packaging process. The protein subunit of the connector was modified to screen for active mutants for motor construction. This was achieved by adjusting the inner loop of the connector protein, or by applying terminus extensions or truncations to the connector protein.

The inner loops of the connector are expected to be involved in the one-way traffic of DNA translocation during motor packaging (Fig. 3A, left).^[51, 52] A similar internal loop is also found in the portal protein of SPP1 (Fig. 3A, right). Single-pore conductance assay has been utilized to test the performance of the phi29 connector channels. With the channel embedded and voltage applied across the membrane, the translocation of the negatively charged DNA through the channel can be detected. With the wild-type connector, DNA translocation events occurred only when the channel entrance faced the negative electrode.^[52, 53] In contrast, DNA translocated through the channel from both directions when using the loop-deleted connector.^[52, 54] The results strongly support that the internal loops of the connector are one of the key factor for the one-way traffic of dsDNA,^[52, 54] and that the direction of DNA translocation through the motor can be controlled by tuning the internal loop of the channel protein.

Fusion of a small peptide to the N-terminus or C-terminus of gp10 generated different gp10 extensions named gp10-Nhis, gp10-Nstrephis, and gp10-Cstrep, with the tobacco etch virus (TEV) cleavage site incorporated.^[55] The mutant procapsid with N-terminal extensions (gp10-Nex) demonstrated a 100 fold reduction in virion assembly compared to the wild-type procapsid (Fig. 3B, C). Removal of the extended sequence by TEV cleavage restored the phi29 virion assembly activity. On the other hand, the procapsids with C-terminal extensions (gp10-Cex) was slightly impaired in assembly activity (10-fold drop) (Fig. 3B, C). gp10-Ntrun with N-terminal residues 1–14 truncation and C-terminal strep tag extension was generated. To further define the sequence requirement for building the dodecameric architecture, residues 1–37 or 1–48 of gp10 were removed and tested. Residues 11–37 constitute one entire α -helix running from the narrow end to the wide end of the connector, followed by residues 38–48 which form a β -sheet at the relaxed wider end of the connector. It was obvious that this α -helix was intimately related to connector assembly. Its removal disrupted the balance and self-interaction of gp10 subunits in a connector, or worse, impaired gp10 protein folding which led to insoluble aggregates. In addition, truncation of the flexible region of C-terminal 25 residues generated procapsid-Ctrun with normal morphology but with its virion assembly completely blocked. We speculate that the C-terminal region plays an important role inside the procapsid to facilitate DNA packaging.

Screening of the motor ATPase with different sequences of ATPase pockets to control motor motion

Previously, it has been reported that the phi29 DNA packaging motor adapts sequential action for its coordination among subunits.^[49, 56] More recently, this coordination has been shown to be mediated by the arginine finger motif in the gp16 ATPase of phi29.^[42] Phi29 motor ATPase gp16 shares the common ATP activity domain typical of all members of the ASCE superfamily,^[57, 58] including the very well conserved motifs responsible for ATP binding and ATP hydrolysis,^[59] which have been previously identified as Walker A^[60] and Walker B motifs,^[61] respectively. Sequence alignment studies have further revealed a common arginine residue.^[38, 62–65] The alteration in arginine 146 severely impaired ATP hydrolysis activity and motor DNA binding activity, as confirmed by EMSA (Electrophoresis Mobility Shift Assay) and capillary electrophoresis assay, showing the approximately 4-fold reduction of the binding affinity of gp16 arginine finger mutant with dsDNA compared to that of wild-type gp16 (Fig. 4).

The presence of a non-covalent dimer of the ATPase gp16 has been shown by different approaches including electrophoresis mobility shift assay (EMSA), glycerol gradient ultracentrifugation, and native gel electrophoresis (Fig. 4).^[61] Dimer formation requires that at least one of the adjacent subunits can provide an intact arginine for the inter-subunit interaction, since dimers were produced when the arginine-free ATPase was mixed with the wild type with an intact arginine, but not with the arginine-free ATPase (Fig. 4). Furthermore, it was shown that one single inactive subunit of arginine 146-free ATPase was able to completely, other than partially, block the function of the entire motor,^[42] that is, $K = 1$ in binomial distribution as described above, which implies that six ATPase molecules worked sequentially.^[46–48]

The hexameric structure of gp16 ATPase has been constructed with *P. aeruginosa* FtsK (PDB ID: 2IUU) as a template^[62] based on its predicted monomer structure using I-TASSER^[67] software. The core structure of both N-domain of gp16 and beta-domain of FtsK with the length of 127 residues were aligned. And the RMSD of backbone carbon atoms in these core structure were calculated. Considering of the size of these two proteins and the protein structure after alignment, an RMSD of 3Å can effectively reflect that gp16's N-domain adopts same fold as FtsK's beta-domain.^[68, 69] The model showed that the position of arginine 146 in one ATPase subunit outstretches to the ATPase domain in a neighboring subunit (Fig. 5), which agrees with our data showing the cooperative behavior in the hexameric ATPase ring. As demonstrated above, it is concluded that arginine finger serves as a bridge between single subunits to form non-covalent dimers.

ATP binding and hydrolysis triggers conformational entropy changes of ATPase with high or low DNA binding affinity

ASCE proteins undergo a cycle of conformational changes upon ATP binding and hydrolysis with two major states: high or low affinity for the substrate.^[70] In recent publications,^[50, 56, 61, 71] a similar model of sequential action for gp16 has been reported. Partial proteolysis treatment and tryptophan intrinsic fluorescence assays indicated a conformational change in the gp16-ATP complex, more specifically, a more constrained and

less conformational entropy for gp16 before ATP binding. EMSA, capillary electrophoresis assays, and FRET assays showed stronger binding of gp16 to dsDNA when incubated with γ -S-ATP (Fig. 4), suggesting that the gp16/dsDNA complex is stabilized upon addition of the non-hydrolysable ATP substrate.

As a dsDNA-dependent ATPase,^[60, 72, 73] binding of the gp16/ATP complex to dsDNA resulted in ATP hydrolysis, leading to its second round of conformational entropy change to a low DNA-affinity configuration, and subsequently to the release of dsDNA for its concomitant transfer to the adjacent downstream subunit. An increased ATP hydrolysis rate has been observed when the ATPase is bound to DNA, indicating a global structural alternation in the protein upon DNA binding/release.^[50] Taken together, a mechanism of ATPase for dsDNA translocation is proposed (Fig. 6) for constructions of active motors, showing that the energy consumption status corresponds to the inter-subunit communication primed by the arginine finger. The resulting asymmetrical hexameric intermediate was supported by many other hexameric ATPase systems based on structural computation, X-ray diffraction and Cryo-EM imaging (Fig. 6).^[74–77] The results provide clues as to why the hexameric ATPase gp16 of phi29 and gp17 of T4 were previously interpreted as having a pentameric configuration by cryo-EM.^[78, 79] With two adjacent ATPase subunits interacting with each other and forming a closer dimer configuration, the asymmetrical hexamer could be mis-analyzed as a pentamer after reconstruction by averaging numerous images as in Cryo-EM.

Single-molecule real-time imaging of the biological active motor with continuous motion

The motion of the constructed artificial ATPase motor without the dodecameric channel and the RNA ring was confirmed by single-molecule fluorescence imaging (Fig. 7, Movie 1). Purified ATPase was labeled with Cy3 fluorescent dye and incubated with DNA, which is tethered between two polylysine beads. The motion of fluorescent spots representing the ATPase complexes was observed along the DNA chains through real-time recording, which confirmed the translocating activity of ATPase along dsDNA. Next, the successful construction of the artificial motor containing both dodecameric ring and RNA hexamer to drive dsDNA was tested by fluorescence imaging in real time. DNA translocation was recorded through the biomotor constructed with all active motor components. The functional motor was stalled using non-hydrolysable γ -S-ATP, isolated^[66, 81] and then attached to a slide. The distal end of the dsDNA was labeled with a biotin moiety and attached to a fluorescent streptavidin-coated microsphere. After addition of ATP, the continuous motion of the motor was resumed and observed by real-time fluorescence microscopy through epi-illumination (Fig. 8).

Analysis of the motion in x, y, and z dimensions showed that the swing distance of the microsphere was reduced as the motion process was close to the end of packaging. As a control, the microsphere exhibited a mainly linear and static Brownian motion when the stalled motor was not restarted, indicating that the constructed motor was functional in continuous DNA translocation with a one-way direction (Fig. 8, Movie 2). Single directional motion of the motor was also detected in real time using a horizontal setup with biomotors

assembled in the procapsids,^[31] which confirmed the successful construction of the artificial motor with the control of motion direction.

Conclusion

Based on the one-way revolving mechanism of the phi29 dsDNA packaging motor, we have successfully constructed phi29 biomimetic nanomotors with altered and modified pRNA, ATPase and protein channel with single-directional motion. Arginine finger bridges two adjacent ATPase subunits to form a non-covalent dimer, resulting in an asymmetrical hexameric complex. Single direction motion of the dsDNA was controlled by three sets of operation elements: 1) the asymmetrical hexameric ATPase containing one dimeric and four monomeric subunits that hold an ATP-interacting domain to regulate alternative DNA binding and also push an arginine finger to control the sequential action; 2) the dsDNA translocation channel that serves as a one-way valve to ensure single direction advancement and to prevent the reversal of the dsDNA; 3) the hexameric pRNA ring geared with their left and right-hand loops. Assessment of the resulting constructs revealed that one inactive subunit of pRNA or ATPase is sufficient to completely, other than partially, block the function of the entire motor. The finding of $K = 1$ implies sequential action with single directional motion, based on the criterion of binomial distribution and Yang Hui's Triangle.

Experimental Section

Cloning, mutagenesis and protein purification

The engineering of eGFP-gp16 and the purification of the gp16 fusion protein have been reported previously.^[82] The eGFP-gp16 mutant R146A and mCherry-gp16 mutant R146A were constructed by introducing mutations in the gp16 gene (Keyclone Technologies).

Glycerol gradient ultracentrifugation

50 μ l of eGFP-gp16 (500 μ g/ml) were dropped onto 5 ml linear 15–35% glycerol gradients in TMS buffer. After centrifuging at 35000 rpm in a SW55 rotor at 4 °C for 22 hr, the gradients were collected into 31 fractions from bottom to top and measured using plate reader under 488 excitation before being applied to *in vitro* assembly assay.

Electrophoretic Mobility Shift Assay (EMSA)

Fluorescently tagged protein that facilitates detection and purification was shown to possess similar assembly and packaging activity as compared to wildtype.^[50, 82] The EMSA method has been described previously.^[56, 61] The gp16 mutants or wild-type were mixed with 33bp Cy5-dsDNA in the presence or absence of ATP and γ -S-ATP. Samples were incubated at ambient temperature for 20 min and then loaded onto a 1% agarose gel (44.5 mM Tris, 44.5 mM boric acid) and electrophoresed at 4°C for around 1 hr at 8 V/cm. The eGFP-gp16, mCherry-gp16, and Cy5-DNA samples were analyzed by a fluorescent LightTools Whole Body Imager using 488 nm, 540 nm, and 635 nm excitation wavelengths for GFP, mCherry, and Cy5, respectively.

CE experiments to determine ratio of gp16 to bound dsDNA

CE (capillary electrophoresis) experiments were performed on a Beckman MDQ system equipped with double fluorescence detectors (at 488 nm and 635 nm excitation wavelengths). A bare borosilicate capillary with a total length of 60 cm and a 50 μ m inner diameter were used. Assay conditions contained separation buffer of 50 mM Tris-HCl, 100 mM sodium borate at pH 8.00, 5 mM MgCl₂, 10% PEG 8000 (w/v), 0.5% acetone (v/v), 3 μ M eGFP-gp16 monomer, and variable amounts of ATP and DNA.

In vitro assembly assay

Purified *in vitro* components, including prohead-connector, pRNA, genome DNA, ATPase, and tail proteins were mixed and subjected to virion assembly assay as previously described.^[29] Briefly, newly assembled infectious virions were inoculated to *Bacillus* bacteria and plated. Activity was expressed as the number of plaques formed per volume of sample (pfu/mL). Different fractions of samples were isolated from the glycerol gradient and added into the system for their activity test.

Observation of gp16 motion

Double-stranded lambda DNA (48 kbp) was tethered between two polylysine coated 4 μ m silica beads.^[83] The dsDNA was bound between beads by back-and-forth infusion of DNA over the beads for 10 min; binding occurred as a result of charge-charge interactions. The back and forth motion of DNA over the polylysine beads stretched the DNA taut and lifted the chain above the surface by the 4 μ m silica beads as visualized under the microscope. The incident angle of the excitation beam in objective-type TIRF (total internal reflection fluorescence) was adjusted to a sub-critical angle in order to image the samples a few microns above the surface, yielding a low fluorescence background.^[83] To-Pro-3 was used to confirm the formation of the DNA tightropes. After the DNA tightrope was formed, a 30 μ L mixture with a final concentration of 1 nM Cy3-gp16 with 100 nM unlabeled gp16 in buffer B (25 mM Tris, pH 6.1, 25 mM NaCl, 0.25 mM MgCl₂) was infused into the sample chamber for binding to the stretched DNA. After 30 min incubation, 30 μ L of a solution containing anti-photobleaching reagents^[81] was infused into the chamber in order to prevent photobleaching of less photostable fluorophores and to detect binding. Movies were taken after the chamber was washed with buffer C (25 mM Tris, pH 8, 25 mM NaCl, 0.25 mM MgCl₂). A comparison was made of washings with buffer C, with and without 20 mM ATP. Since the DNA has been fixed by charge interactions and the protein fixed by binding affinity to the tethered DNA, washing does not remove pertinent material. Sequential images were acquired with a 0.2 s exposure time at an interval of 0.22 s, with a laser of 532 nm for excitation. The movies were taken for about 8 min, or until the Cy3 fluorophores lost their fluorescence due to photobleaching. Image J software was utilized to generate kymographs to show the displacement of the Cy3-gp16 spots along the DNA chains.

Direct observation of DNA translocation

The stalled packaging intermediates containing biotinylated DNA were prepared by using non-hydrolyzable γ -S-ATP.^[66] The intermediates were then immobilized through IgG prohead antibody to perfusion chambers built from glass slides and coverslips. The

fluorescent streptavidin microspheres (0.56 μm) were bound to the protruding, biotinylated DNA end of the intermediates. After restarting the packaging reaction by adding gp16 and ATP,^[66] an individual DNA-packaging event was observed. Epi-illumination was used, and sequential images were recorded.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Shu D, Moll WD, Deng Z, Mao C, Guo P. *Nano Lett.* 2004; 4:1717–1723. [PubMed: 21171616]
2. Shu Y, Haque F, Shu D, Li W, Zhu Z, Kotb M, Lyubchenko Y, Guo P. *RNA.* 2013; 19:766–777.
3. Liaw JW, Chen YS, Kuo MK. *Opt. Express.* 2014; 22:26005–26015. [PubMed: 25401634]
4. Loh IY, Cheng J, Tee SR, Efremov A, Wang Z. *ACS Nano.* 2014; 8:10293–10304. [PubMed: 25268955]
5. Li J, Gao W, Dong R, Pei A, Sattayasamitsathit S, Wang J. *Nat. Commun.* 2014; 5:5026. [PubMed: 25248549]
6. Balk AL, Mair LO, Mathai PP, Patrone PN, Wang W, Ahmed S, Mallouk TE, Liddle JA, Stavis SM. *ACS Nano.* 2014; 8:8300–8309. [PubMed: 25019966]
7. Xuan M, Shao J, Lin X, Dai L, He Q. *Chemphyschem.* 2014; 15:2255–2260. [PubMed: 24740913]
8. Kumar KR, Kamei T, Fukaminato T, Tamaoki N. *ACS Nano.* 2014; 8:4157–4165. [PubMed: 24680285]
9. Ahmad Z, Cox JL. *Scientific World Journal.* 2014; 2014:567398. [PubMed: 24605056]
10. Morozov KI, Leshansky AM. *Nanoscale.* 2014; 6:1580–1588. [PubMed: 24336860]
11. Dong B, Zhou T, Zhang H, Li CY. *ACS Nano.* 2013; 7:5192–5198. [PubMed: 23647410]
12. Khataee HR, Ibrahim MY. *IET. Nanobiotechnol.* 2012; 6:87–92. [PubMed: 22894532]
13. Gao W, Manesh KM, Hua J, Sattayasamitsathit S, Wang J. *Small.* 2011; 7:2047–2051. [PubMed: 21626685]
14. Thakur S, Chen JX, Kapral R. *Angew. Chem. Int. Ed Engl.* 2011; 50:10165–10169. [PubMed: 21433237]
15. Bamrungsap S, Phillips JA, Xiong X, Kim Y, Wang H, Liu H, Hebard A, Tan W. *Small.* 2011; 7:601–605. [PubMed: 21370463]
16. Arai N, Yasuoka K, Koishi T, Ebisuzaki T. *ACS Nano.* 2010; 4:5905–5913. [PubMed: 20923165]
17. Gibbs JG, Zhao Y. *Small.* 2010; 6:1656–1662. [PubMed: 20669163]
18. Klapper Y, Sinha N, Ng TW, Lubrich D. *Small.* 2010; 6:44–47. [PubMed: 19943245]
19. Kang H, Liu H, Phillips JA, Cao Z, Kim Y, Chen Y, Yang Z, Li J, Tan W. *Nano Lett.* 2009; 9:2690–2696. [PubMed: 19499899]
20. Besenbacher F, Nørskov JK. *Science.* 2000; 290:1520. [PubMed: 17771226]
21. Bishop JD, Klavins E. *Nano Lett.* 2007; 7:2574–2577. [PubMed: 17655267]
22. Balzani V, Clemente-Leon M, Credi A, Ferrer B, Venturi M, Flood AH, Stoddart JF. *Proc Natl Acad Sci U S A.* 2006; 103:1178–1183. [PubMed: 16432207]
23. Chen Y, Mao C. *J. Am. Chem. Soc.* 2004; 126:8626–8627. [PubMed: 15250695]

24. Kanatani K, Ochi Y, Okamoto A, Saito I. Nucleic Acids Res. Suppl. 2003;161–162. [PubMed: 14510430]
25. Lin C, Liu Y, Yan H. Biochemistry. 2009; 48:1663–1674. [PubMed: 19199428]
26. Aldaye FA, Palmer AL, Sleiman HF. Science. 2008; 321:1795–1799. [PubMed: 18818351]
27. Seeman NC. Annu. Rev. Biochem. 2010; 79:65–87. [PubMed: 20222824]
28. Guo P, Grimes S, Anderson D. Proc. Natl. Acad. Sci. USA. 1986; 83:3505–3509. [PubMed: 3458193]
29. Lee CS, Guo P. Virology. 1994; 202:1039–1042. [PubMed: 8030206]
30. Lee CS, Guo P. J. Virol. 1995; 69:5018–5023. [PubMed: 7609071]
31. Chang C, Zhang H, Shu D, Guo P, Savran C. Appl. Phys. Lett. 2008; 93:153902–153903. [PubMed: 19529792]
32. Karata K, Inagawa T, Wilkinson AJ, Tatsuta T, Ogura T. J. Biol. Chem. 1999; 274:26225–26232. [PubMed: 10473576]
33. Hilbert BJ, Hayes JA, Stone NP, Duffy CM, Sankaran B, Kelch BA. Proc. Natl. Acad. Sci. U. S. A. 2015; 112:E3792–E3799. [PubMed: 26150523]
34. Enemark EJ, Joshua-Tor L. Curr. Opin. Struct. Biol. 2008; 18:243–257. [PubMed: 18329872]
35. Iyer LM, Makarova KS, Koonin EV, Aravind L. Nucleic Acids Res. 2004; 32:5260–5279. [PubMed: 15466593]
36. Joly N, Zhang N, Buck M. Mol. Cell. 2012; 47:484–490. [PubMed: 22789710]
37. Wendler P, Ciniawsky S, Kock M, Kube S. Biochim. Biophys. Acta. 2012; 1823:2–14. [PubMed: 21839118]
38. Iyer LM, Leipe DD, Koonin EV, Aravind L. J Struct Biol. 2004; 146:11–31. [PubMed: 15037234]
39. Zeymer C, Fischer S, Reinstein J. J. Biol. Chem. 2014; 289:32965–32976. [PubMed: 25253689]
40. Yukawa A, Iino R, Watanabe R, Hayashi S, Noji H. Biochemistry. 2015; 54:472–480. [PubMed: 25531508]
41. Elles LM, Uhlenbeck OC. Nucleic Acids Res. 2008; 36:41–50. [PubMed: 17986459]
42. Zhao Z, De-Donatis GM, Schwartz C, Fang H, Li J, Guo P. Mol Cell Biol. 2016 *in press*.
43. Guo P, Erickson S, Anderson D. Science. 1987; 236:690–694. [PubMed: 3107124]
44. Guo P, Zhang C, Chen C, Trottier M, Garver K. Mol. Cell. 1998; 2:149–155. [PubMed: 9702202]
45. Chen C, Sheng S, Shao Z, Guo P. J Biol Chem. 2000; 275(23):17510–17516. [PubMed: 10748150]
46. Fang H, Zhang P, Huang LP, Zhao Z, Pi F, Montemagno C, Guo P. Nanomedicine. 2014; 10:1433–1440. [PubMed: 24650885]
47. Shu D, Pi F, Wang C, Zhang P, Guo P. Nanomedicine. 2015; 10:1881–1897. [PubMed: 26139124]
48. Pi F, Vieweger M, Zhao Z, Wang S, Guo P. Expert Opin. Drug Deliv. 2015;1–14.
49. Chen C, Guo P. J. Virol. 1997; 71:3864–3871. [PubMed: 9094662]
50. Schwartz C, Fang H, Huang L, Guo P. Nucleic Acids Res. 2012; 40:2577–2586. [PubMed: 22110031]
51. Fang H, Jing P, Haque F, Guo P. Biophysical Journal. 2012; 102:127–135. [PubMed: 22225806]
52. Zhao Z, Khisamutdinov E, Schwartz C, Guo P. ACS Nano. 2013; 7:4082–4092. [PubMed: 23510192]
53. Jing P, Haque F, Shu D, Montemagno C, Guo P. Nano Lett. 2010; 10:3620–3627. [PubMed: 20722407]
54. Geng J, Wang S, Fang H, Guo P. ACS Nano. 2013; 7:3315–3323. [PubMed: 23488809]
55. Cai Y, Xiao F, Guo P. Nanomedicine. 2008; 4:8–18. [PubMed: 18201942]
56. Schwartz C, De Donatis GM, Zhang H, Fang H, Guo P. Virology. 2013; 443:28–39. [PubMed: 23763768]
57. Snider J, Houry WA. Biochemical Society Transactions. 2008; 36:72–77. [PubMed: 18208389]
58. Hanson PI, Whiteheart SW. Nat. Rev. Mol Cell Biol. 2005; 6:519–529. [PubMed: 16072036]
59. Walker JE, Saraste M, Gay NJ. Nature. 1982; 298:867–869. [PubMed: 6180323]
60. Guo P, Peterson C, Anderson D. J Mol Biol. 1987; 197:229–236. [PubMed: 2960820]
61. Schwartz C, De Donatis GM, Fang H, Guo P. Virology. 2013; 443:20–27. [PubMed: 23706809]

62. Massey TH, Mercogliano CP, Yates J, Sherratt DJ, Lowe J. Mol. Cell. 2006; 23:457–469. [PubMed: 16916635]
63. Volozhantsev NV, Oakley BB, Morales CA, Verevkin VV, Bannov VA, Krasilnikova VM, Popova AV, Zhilenkov EL, Garrish JK, Schegg KM, Woolsey R, Quilici DR, Line JE, Hiett KL, Siragusa GR, Svetoch EA, Seal BS. PLoS ONE. 2012; 7:e38283. [PubMed: 22666499]
64. Gomis-Ruth FX, Moncalian G, Perez-Luque R, Gonzalez A, Cabezon E, de la CF, Coll M. Nature. 2001; 409:637–641. [PubMed: 11214325]
65. Wallden K, Williams R, Yan J, Lian PW, Wang L, Thalassinou K, Orlova EV, Waksman G. Proc. Natl. Acad. Sci. U. S. A. 2012; 109:11348–11353. [PubMed: 22745169]
66. Shu D, Guo P. Virology. 2003; 309(1):108–113. [PubMed: 12726731]
67. Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y. Nat. Methods. 2015; 12:7–8. [PubMed: 25549265]
68. Zhang Y, Skolnick J. Nucleic Acids Res. 2005; 33:2302–2309. [PubMed: 15849316]
69. Marks DS, Colwell LJ, Sheridan R, Hopf TA, Pagnani A, Zecchina R, Sander C. PLoS ONE. 2011; 6:e28766. [PubMed: 22163331]
70. Spies, M. DNA Helicases and DNA Motor Proteins. New York: Springer; 2016.
71. Zhang H, Schwartz C, De Donatis GM, Guo P. Adv. Virus Res. 2012; 83:415–465. [PubMed: 22748815]
72. Ibarra B, Valpuesta JM, Carrascosa JL. Nucleic Acids Res. 2001; 29:4264–4273. [PubMed: 11691914]
73. Lee TJ, Zhang H, Liang D, Guo P. Virology. 2008; 380:69–74. [PubMed: 18701124]
74. Arai S, Saijo S, Suzuki K, Mizutani K, Kakinuma Y, Ishizuka-Katsura Y, Ohsawa N, Terada T, Shirouzu M, Yokoyama S, Iwata S, Yamato I, Murata T. Nature. 2013; 493:703–707. [PubMed: 23334411]
75. Ye Q, Rosenberg SC, Moeller A, Speir JA, Su TY, Corbett KD. Elife. 2015; 4
76. Stinson BM, Baytshtok V, Schmitz KR, Baker TA, Sauer RT. Nat. Struct. Mol. Biol. 2015; 22:411–416. [PubMed: 25866879]
77. Sun SX, Wang H, Oster G. Biophys. J. 2004; 86:1373–1384. [PubMed: 14990467]
78. Morais MC, Koti JS, Bowman VD, Reyes-Aldrete E, Anderson D, Rossmann MG. Structure. 2008; 16:1267–1274. [PubMed: 18682228]
79. Sun S, Kondabagil K, Draper B, Alam TI, Bowman VD, Zhang Z, Hegde S, Fokine A, Rossmann MG, Rao VB. Cell. 2008; 135:1251–1262. [PubMed: 19109896]
80. Lyubimov AY, Costa A, Bleichert F, Botchan MR, Berger JM. Proc. Natl. Acad. Sci. U. S. A. 2012; 109:11999–12004. [PubMed: 22778422]
81. Shu D, Zhang H, Jin J, Guo P. EMBO J. 2007; 26:527–537. [PubMed: 17245435]
82. Lee TJ, Zhang H, Chang CL, Savran C, Guo P. Small. 2009; 5:2453–2459. [PubMed: 19743427]
83. Kad NM, Wang H, Kennedy GG, Warshaw DM, Van HB. Mol. Cell. 2010; 37:702–713. [PubMed: 20227373]

Figure 1. Illustration of the motor components of the phi29 dsDNA packaging motor. (A) Side view and (B) Top view of the phi29 dsDNA packaging motor composed of the three co-axial rings of the dodecameric connector, hexameric pRNA ring, and hexameric ATPase ring.

Figure 2. (A) Sequence of pRNA monomer with CCA bulge (upper panel) or 5'/3' end (lower panel) highlighted in the box. The illustrations of the corresponding 3D structures are shown. The bars symbolize the end-truncation, and the empty squares symbolize the CCA-deletion. (B) Viral assembly activity of pRNA hexamers with different designs. Each color symbolizes one purified dimer or trimer. The uppercase **D** and **T** symbolize the normal dimer and trimer, respectively. The lower case **d** and **t** symbolize the mutant dimer and trimer, respectively.

Figure 3.

(A) Illustration of the internal loop in the phi29 portal protein (left) and SPP1 portal protein (right), showing their function as a ratchet for the one-way translocation of the DNA during genome packaging. (B) Illustration of procapsids with side view of the mutant connectors based on PDB: 1h5w. (C) Comparison of procapsid activity with equal amount of procapsid protein.

A. Capillary Electrophoresis assay

B. EMSA

C. Glycerol Gradient assay combined with EMSA & *in vitro* assembly assay

a. w/o Arginine finger

c. EMSA of fractions from gradient

b. w/ Arginine finger

d. *in vitro* assembly assay of fractions from gradient **Figure 4.**

ATP binding and hydrolysis activity assay of gp16 arginine finger mutant and inter-subunit interactions of ATPase. (A) Capillary Electrophoresis assay for the binding affinity test of different gp16 with dsDNA. (B) Interactions between gp16 arginine finger mutants with gp16 wild-type are shown by the band shift of both ATPase and DNA in the gel. (Green: mCherry channel; Blue: eGFP channel; Red: Cy5 channel). (C) Both dimers and monomers exist in gp16 ATPase rings. In 15%–35% glycerol gradient, one peak for eGFP-gp16 R146A (a) and two peaks for eGFP-gp16 wild-type (b) were observed after parallel

ultracentrifugation, indicating that dimer formation is interrupted by the mutation of arginine finger. The fractions derived from the gradient have been applied to EMSA (c) and *in vitro* assembly activity assay, confirming the formation of dimers mediated by the arginine finger. The isolated gp16 dimer fraction (Fr. 18) showed significantly reduced activity compared to the monomers (Fr. 22) (d), supporting the previous finding that the addition of fresh gp16 monomer is required for re-initiating DNA packaging intermediates.^[66]

Figure 5. Illustration of inter-subunit interaction inside of gp16 ATPase. Gp16 ATPase hexameric ring was constructed (left panel), and the interaction between two adjacent subunits (right panel) has been shown with the arginine finger highlighted in the red sphere and Walker domain (represented by E119 residue in Walker B domain) highlighted in the blue sphere. The interaction of arginine finger with the upstream adjacent subunit is supported by the relative location of the related domains.

A. ATPase sequential action mediated by arginine finger

B. Asymmetrical V1-ATPase

C. Asymmetrical MCM2-7 ATPase

Figure 6.

ATPase coordination with a series of conformational changes during DNA binding and ATP hydrolysis as regulated by the arginine finger, resulting in the asymmetrical configuration of ATPase (A). The asymmetrical structures have also been found in many other biomotors, including V1-ATPase^[74] (B) and MCM2-7 protein (EM accession:EMD-5429)^[80] (C).

Figure 7. Single-molecule detection of the continuous ATPase translocation on dsDNA. (A) The gp16 complex labeled with Cy3^[56] moved along the dsDNA chain tethered between two polylysine coated beads. (B) The motion of the Cy3-gp16 complex was analyzed by *Image J* and (C) a kymograph was generated to demonstrate the motion of the ATPase.

Figure 8.

Single-molecule detection of continuous DNA translocation in the phi29 biomotor.

Comparison of the trajectories and travel distance of microspheres attached to the end of DNA in two different packaging intermediates Beads I (A) and Beads II (B). Beads I showed random Brownian motion. Bead II also showed Brownian motion at the beginning, however, such motion ceased after around 150 seconds, indicating the end of the translocation event. The stalling of the dsDNA serves as evidence of uni-directional translocation. (C) Sequential

images of a fluorescent microsphere attached to DNA, corresponding to when Beads II tends to stop as indicated in the figure.

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