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Ultrastable pRNA hexameric ring gearing hexameric phi29 DNA-packaging motor by revolving without rotating and coiling

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Abstract

Biomotors have previously been classified into two categories: linear and rotational motors. It has long been popularly believed that viral DNA packaging motors are rotation motors. We have recently found that the DNA-packaging motor of bacteriophage phi29 uses a third mechanism: revolution without rotation. phi29 motor consists of three-coaxial rings of hexameric RNA, a hexameric ATPase, and a dodecameric channel. The motor uses six ATP to revolve one helical turn of dsDNA around the hexameric ring of ATPase gp16. Each dodecameric segment tilts at a 30°-angle and runs anti-parallel to the dsDNA helix to facilitate translation in one direction. The negatively charged phosphate backbone interacts with four positively charged lysine rings, resulting in four steps of transition. This review will discuss how the novel pRNA meets motor requirements for translocation concerning structure, stoichiometry, and thermostability; how pRNA studies have led to the generation of the concept of RNA nanotechnology; and how pRNA is fabricated into nanoparticles to deliver siRNA, miRNA, and ribozymes to cancer and virus-infected cells.

Introduction

The AAA+ family of proteins is a class of motors with a wide range of functions including chromosome segregation, nucleic acid replication, DNA repair, genome recombination, viral DNA packaging, and translocation of cellular components. Many of these motors display hexameric arrangements that facilitate DNA motion triggered by ATP [1–3]. It has been popularly believed for some time that the AAA+ motor of viral DNA packaging motors use a fivefold/sixfold mismatch rotation mechanism. In 1987, an RNA component was discovered on the packaging motor of bacteriophage phi29 [4] and subsequently, in 1998, this RNA particle was determined to exist as a hexameric ring [5*,6] (featured in *Cell* [7]). On the basis of its hexameric structure, we proposed that the mechanism of the phi29 viral DNA packaging motor is similar to that used by other hexameric DNA tracking motors of the AAA+ family [5*]. Recently, X-ray diffraction, AFM imaging, and single molecule studies have confirmed that the motor consists of three-coaxial rings geared by a hexameric RNA, a hexameric ATPase gp16, and a dodecameric motor channel that only allows dsDNA to move unidirectionally [8,9,10*,11–13,14*,15*]. Concurrently, it has been discovered that the motor utilizes a simple, yet novel revolution mechanism to translocate dsDNA, rather than the perceived rotational mechanism lending to undesirable coiling forces [14**,15**]. This review will discuss how the pRNA molecule meets the requirements of the motor concerning structure, stoichiometry, thermo-stability, and stiffness in an innovative way; and how studies on the novelty of pRNA have led to the generation of the concept of RNA nanotechnology.

Current understanding of the mechanism of phi29 DNA-packaging motor

The phi29 nanomotor consists of an ATPase gp16, a hexameric pRNA ring [4], and a dodecameric connector with a central channel encircled by 12 copies of the protein gp10 that serves as a path for dsDNA translocation (Figure 4a, b). This motor is of particular interest in nanotechnology because it is simple and robust in structure, and is functional when assembled from purified components *in vitro*. The ATPase gp16 converts energy from either entropy transition(s) or ATP hydrolysis into physical motion [16]. It has been found that when the concentration of gp16 is increased, the hexameric band in a native PAGE increases linearly while the concentrations of the smaller oligomers remain constant (Figure 1e). These results suggest that the formation sequence of the gp16 complex begins with a dimer, converts to a tetramer, and then to a hexamer, and the final complex contains three dimers as the ultimate stage in assembly [15^{**}]. The formation of an active hexamer of gp16 during DNA packaging has been further confirmed by well-established binomial distribution assay and by a protein: DNA molar ratio binding assay using both slab and capillary electrophoresis (CE) (Figure 1d and f). Hill constant quantification (Figure 2, Part 1b) and binomial distribution assay (Figure 2, Part 1a) have revealed a novel revolving machine that acts to translocate the dsDNA helix, and avoids the difficulties of DNA supercoiling resulting from rotation [14]. For reference, the definition of ‘revolution’ and ‘rotation’ are analogous to the motion of the Earth: the Earth *revolves* around the sun every 365 days, while the Earth *rotates* along its own axis to face the sun resulting in cycles of day and night.

The connector on the motor is a one way valve [17^{*},18,19^{*}] that only allows dsDNA to move into the procapsid, but not out (Figure 4a). The gp16, which is bridged by pRNA to associate with the connector, is the pushing force. The binding of ATP to one subunit of the connector stimulates gp16 to favorably change its entropy and adopt a conformation with a high affinity for dsDNA, while ATP hydrolysis forces gp16 to assume a new conformation with a lower affinity for dsDNA. This pushes dsDNA away from a subunit, transfers it to an adjacent subunit, and prepares ATPase for the second round of ATP binding. One ATP is hydrolyzed in each transitional step, and six ATPs are consumed in one cycle to translocate dsDNA a complete helical turn of 360° (10.5 base pairs). The binding of gp16 to the same phosphate backbone chain, but at a location 60° different from the last subunit, urges dsDNA to move forward 1.75 base pairs ((10.5 bp/turn)/6 ATP = 1.75 bp/ATP), agreeing with 1.8 bp/ATP that has previously been quantified empirically (see <http://nanobio.uky.edu/movie.html> for our animation) (figure 4c, d.) [14^{**},15^{**}].

Since the contact of the connector with the dsDNA chain is transferred from one point on the phosphate backbone to another point, rotation of neither the hexameric ring nor the dsDNA is required. All 12 subunits of the connector protein tilt at a 30° angle to form the channel in a configuration that runs anti-parallel to the dsDNA helix. This occurrence tends to argue against the nut and bolt mechanism. Instead, this anti-parallel structural arrangement greatly facilitates controlled motion and suggests that dsDNA revolves through the connector channel without producing a coiling or torsion force, touching each of the 12 connector subunits in 12 discrete steps of 30° per helical pitch (360°/12 = 30°). Nature has created and evolved a clever rotating machine to translocate the DNA double helix that actually avoids the difficulties associated with rotation, such as DNA supercoiling, that have been observed in many other processes.

Crystal structure analysis of the phi29 connector [8] has revealed that the prevalingly negatively charged connector interior channel wall is decorated with 48 positively charged lysine residues that exist as four rings derived from the 12 protein subunits that enclose the channel. The four lysine rings (K200, K209, K234, and K235) have been proposed to play a role in DNA translocation [8]. On the basis of the crystal structure, the length of the

connector channel is ~ 7 nm. Vertically, these four lysine layers fall within a 3.7 nm [8] range and are spaced approximately 0.9 nm apart [19^{*}]. Since B-type dsDNA have a pitch of 0.34 nm/bp, ~ 2.6 bp per rise along its axis can be used in translocation ($0.9 \text{ nm}/0.34 \text{ nm/bp} = \sim 2.6 \text{ bp}$) (Figure 4e), agreeing with the aforementioned finding by an optical tweezer measurement that the motor pauses every 2.5 bp during translocation [20]. However, the previous discussion that the four pauses are due to the interaction of the negatively charged phosphate backbone with the four positively charged lysine rings has been interpreted as resulting from the inactivation of one pentameric motor subunit [20].

Current understanding of the structure and folding of pRNA as a hexameric ring

RNA has defined features at the nanometer scale that can serve as powerful building blocks for in the bottom-up fabrication of nanostructures. RNA, a cousin of DNA, has recently emerged as an important nanotechnology platform due to its diversity in both structure and function [21], and can be fabricated with a level of simplicity similar to that of DNA, but possess more versatile tertiary structures and catalytic functions that mimic proteins [22–24]. RNA has also been proven to be unique by virtue of its high thermodynamic stability [25,26]; formation of both canonical and noncanonical base pairs [27–29]; capability of base stacking [25,26]; and distinct *in vivo* attributes in the processes of controlled transcription, splicing, processing, self cleavage, and modification [5^{*},6,30–33]. The remarkable modularity of RNA tertiary motifs can be encoded for specifying complex 3-D architectures such as helices, loops, bulges, stems, hairpins, and pseudoknots at the individual nucleotide level. Single-strand loops are especially suitable for intermolecular and intramolecular interactions as mounting dovetails for self-assembly.

pRNA is 117 nucleotides (nt) that fold into a complex structure consisting of two major domains: a helical domain with an open 5'/3' end and an interlocking domain for function and stability. In the center, a thermostable three way junction (3WJ) motif [34^{*},35^{*}] extends to the branching of the helical domain, as well as to two looped regions deemed the right-hand and left-hand loops (Figure 3b). The loops allow for intermolecular interactions between pRNA monomeric units and the creation of dimeric, tetrameric, and even hexameric rings [68]. pRNA [4,10^{*},36] has been found to serve as a foothold for the ATPase gp16 [37]. It is believed that pRNA dimers form the completed ring within the motor (Figure 2, Part 2a), as revealed through a series of experiments; including concentration-dependent curves [38], binomial distribution of mutant and wild-type pRNA [39], stoichiometric calculations showing a common factor of 2 and 3 [5^{*}], single molecule photobleaching (Figure 2, Part 2) [10^{*}], gold and ferritin labeling of pRNA (Figure 3c) [12], AFM imaging [13,14^{**},15^{**}], and X-ray crystallography (Zhang H, Endrizzi JA, Shu Y, Haque F, Guo P, Chi YI: Crystal structure of 3WJ core revealing divalent-ion-promoted thermostability and assembly of the Phi29 hexameric motor pRNA. *RNA* 2013, submitted for publication).

Current understanding of pRNA chemical and thermodynamic stability *in vitro* and *in vivo*

The unique features uncovered from the studies of phi29 pRNA include: first, the independent folding of two domains: the gp16 interaction domain [37] and the motor binding domain [40–45]; second, the ease of manipulation and production in the lab without degradation, as observed in most RNA constructs; and third, the capacity to harbor and escort therapeutic functional modules without mis-folding or a loss of functionalities [34^{*}, 46–50,51^{*}]. It has a long half-life *in vivo* and can serve as a useful platform for the

construction of RNA nanoparticles [34*]. These special properties of phi29 pRNA have led to the development of pRNA as a novel vehicle for applications in nanotechnology and medicine.

The pRNA 3WJ displays an unusual thermodynamic stability necessary for RNA to gear the strong packaging motor [34*,35*]. The core displays a very low ΔG that drives the folding of the entire pRNA and produces its global structure; the rest of the sequence follows the nearest neighbor folding principle. The pRNA 3WJ core scaffold can be assembled from three pieces of RNA, is stable in serum, remains intact at ultra low concentrations, and is even resistant to denaturation in 8 M urea; its T_m curve is close to 90°. More importantly, various functionalities incorporated into the 3WJ core, such as siRNA, ribozyme, or receptor-binding aptamer, have resulted in the formation of polyvalent particles displaying all the authentic functionalities *in vitro* and *in vivo*, and folding into their authentic structures [34*,35*] (Shu Y, Haque F, Shu D, Li W, Zhu Z, Kotb M, Lyubchenko Y, Guo P: Fabrication of 14 different RNA nanoparticles for specific tumor targeting without accumulation in normal organs. *RNA* 2012, submitted for publication).

RNA nanoparticles can be systemically delivered to the body where they will exist in low concentrations due to dilution by circulating blood. Only those RNA particles that do not dissociate at low concentrations are feasible for therapeutic purposes. In order to determine whether a larger structure with three branches harboring multimodule functionalities can be subjected to dissociation at low concentrations, [α - ^{32}P] labeled pRNA nanoparticles have been serially diluted to extremely low concentrations. It has been found that the concentration that induces dissociation is below the detection limit of the [^{32}P]-labeling technology, suggesting that the resulting RNA nanoparticles are extremely stable. The particles are resistant to dissociation under extreme denaturing conditions, such as 6–9 M urea and 2'-F U/C modified RNA nanoparticles extending from the 3WJ are resistant to degradation in cell culture medium with a 10% serum, even after 36 hours of incubation, while unmodified RNA degrades within 10 min. Individual RNA modules fused into nanoparticles retain their original folding after incorporation into RNA nanoparticles; 2'-F U/C modified fluorescent 3WJ-pRNA nanoparticles with folate conjugated onto one of the branches of the RNA complex have been tested for cell binding efficiency. Confocal imaging indicates a strong binding of RNA nanoparticles and an efficient entry into targeted cells. The gene silencing effects of the resulting RNA nanoparticles have also been tested. The gene silencing potency has been found to be comparable to the survivin siRNA-only positive control when tested by transfection.

Two of the key factors that may affect pharmacokinetic (PK) profiles are the stability of the particle itself and renal filtration. It has been reported that regular siRNA molecules have extremely poor PK properties because of a short half-life ($T_{1/2}$) and a fast kidney clearance due to metabolic instability and size (<10 nm) [52]. The half-life ($T_{1/2}$) of pRNA nanoparticles is 6.5–12.6 hours, compared to the control 2'-F-modified free siRNA where $T_{1/2}$ =15 min, as reported in the literature [53–55].

Construction of ultra stable tri-Star and X-shape RNA nanoparticles harboring multiple functionalities for cancer targeting without accumulation in normal organs

The phi29 pRNA 3WJ motif is composed of three RNA fragments, denoted as a_{3wj} , b_{3wj} , and c_{3wj} . Multi-module RNA nanoparticles can be constructed using this 3WJ-pRNA domain as a scaffold (Figure 5a) and to build other nanoparticles with multiple branches. Each branch can carry one RNA module with defined functionality, such as a cell receptor-

binding ligand, an aptamer, an siRNA, a ribozyme, or an miRNA. The presence of the modules or therapeutic moieties does not interfere with the formation of the 3WJ domain, as demonstrated by AFM imaging (Figure 5a, b). If each strand with special functionality or structure were added to the 3'-end of each RNA fragment, the strong affinity of the three or four fragment will self-assemble and drive the fragments to form RNA nanoparticles with all functionalities included.

It has also been found that gene silencing effects are progressively enhanced as the number of siRNA in each nanoparticle is gradually increased from one to four [35^{*}]. The polyvalent X-shape RNA nanoparticles with four siRNA display the gene silencing effect as efficient as its siRNA counterpart, with a 100-fold higher concentration [35^{*}]. Systemic injection of tri-star or X-shaped RNA nanoparticles without folate or other cell receptors into the tail-vein of mice has revealed that RNA nanoparticles remain intact and strongly bind to tumors without accumulating in normal organs or tissues.

Advantages of RNA nanotechnology for *in vivo* applications

Since the development of the emerging field of RNA nanotechnology, the area has received much attention from scientists around the world due to its high potential, especially in therapeutics, and wide variety of functions. RNA nanotechnology provides several advantages over competing technologies and fields. Positive aspects of using RNA nanotechnology for therapeutic delivery include: first, self-assembly and self-processing *in vivo*; second, controlled synthesis and a defined structure and stoichiometry; third, multi-valency (combined therapy, targeting, and detection); fourth, targeted delivery and detection; fifth, advantageous size (10–100 nm); sixth, allowance for receptor-mediated endocytosis; seventh, extended *in vivo* half-life; eighth, disallowance of nonspecific cell entry; ninth, avoidance of antibody induction (protein-free nanoparticle); tenth, allowance of repeated treatment of chronic diseases; eleventh, and the fact that RNA nanoparticles are treated as chemical drugs, rather than biological entities. The classification will facilitate FDA approval. In addition, the utilization of RNA nanotechnology in medical or nanotechnological applications requires addressing the following issues: first, chemical instability; second, thermodynamic instability; third, short *in vivo* half-life; fourth, toxicity, *in vivo* safety, and side effects; fifth, specific delivery and targeting problems; sixth, endosome trapping; seventh, and low yield with high production costs. The issues concerning 1–4 have been more or less solved, as discussed in this review and in another recent review [56^{*}]. Although the issue of delivery has shown great progress through the application of the polyvalent property of phi29 or other RNA nanoparticles, additional targets are still desired in order to improve the specificity of targeting. The cost of RNA production has been reduced substantially, but further improvement is still needed. Endosome trapping, though, remains a major challenge. These issues have also been addressed in the recent review [56^{*}] and readers are encouraged to refer to this article for more details.

Prospective in RNA nanotechnology

Phi29 pRNA-derived nanotechnology is just one facet of the rapidly emerging field of RNA nanotechnology and therapeutics. New methods are emerging for the construction of RNA nanoparticles, such as *in vivo* assembly of RNA arrays [57]; nanostructures made of bacterial noncoding RNA [58]; RNA triangle assembly guided by proteins [59]; construction of RNA squares [60,61] and hexamers [5^{*},23] (Shu Y, Haque F, Shu D, Li W, Zhu Z, Kotb M, Lyubchenko Y, Guo P: Fabrication of 14 different RNA nanoparticles for specific tumor targeting without accumulation in normal organs. *RNA* 2012, submitted for publication); assembly of multiple RNA aptamers to enhance receptor binding efficiency [62]; building

block fabrication utilizing RNA junctions; construction of tecto-RNA; and the application of HIV kissing loops [63–66]. Despite the fact that great strides have been made in the design and application of RNA nanoparticles, RNA nanotechnology as a field is still in its infancy. The application of RNA nanotechnology necessitates the collective work of an RNA nanotechnology community and an interdisciplinary approach. The finding that RNA nanoparticles can specifically target to cancer for the delivery of siRNA, miRNA, and ribozymes without accumulation in liver, lung and other normal organs [34,35,67] and without toxicity [67] makes RNA nanoparticles ideal reagents for cancer treatment.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
 - of outstanding interest
1. Wang F, Mei Z, Qi Y, Yan C, Hu Q, Wang J, Shi Y. Structure and mechanism of the hexameric MecA-ClpC molecular machine. *Nature*. 2011; 471:331–335. [PubMed: 21368759]
 2. Grainge I, Lesterlin C, Sherratt DJ. Activation of XerCD-dif recombination by the FtsK DNA translocase. *Nucleic Acids Res*. 2011; 39:5140–5148. [PubMed: 21371996]
 3. Lowe J, Ellonen A, Allen MD, Atkinson C, Sherratt DJ, Grainge I. Molecular mechanism of sequence-directed DNA loading and translocation by FtsK. *Mol Cell*. 2008; 31:498–509. [PubMed: 18722176]
 4. Guo P, Erickson S, Anderson D. A small viral RNA is required for *in vitro* packaging of bacteriophage phi29 DNA. *Science*. 1987; 236:690–694. [PubMed: 3107124]
 5. Guo P, Zhang C, Chen C, Trottier M, Garver K. Inter-RNA interaction of phage phi29 pRNA to form a hexameric complex for viral DNA transportation. *Mol Cell*. 1998; 2:149–155. It reports that phi29 DNA-packaging motor is geared by six pRNA, and RNA nanoparticles of dimer, trimer, and hexamer can be assembled from reengineered RNA fragments via hand-in-hand interaction of the interlocking loops. This is a pioneering paper that proves the concept of RNA nanotechnology and proposes that viral DNA-packaging occurs because of a dsDNA mechanism similar to that used by the hexameric AAA+ family. [PubMed: 9702202]
 6. Zhang F, Lemieux S, Wu X, St Arnaud S, McMurray CT, Major F, Anderson D. Function of hexameric RNA in packaging of bacteriophage phi29 DNA in vitro. *Mol Cell*. 1998; 2:141–147. [PubMed: 9702201]
 7. Hendrix RW. Bacteriophage DNA packaging: RNA gears in a DNA transport machine (Minireview). *Cell*. 1998; 94:147–150. [PubMed: 9695942]
 8. Guasch A, Pous J, Ibarra B, Gomis-Ruth FX, Valpuesta JM, Sousa N, Carrascosa JL, Coll M. Detailed architecture of a DNA translocating machine: the high-resolution structure of the bacteriophage phi29 connector particle. *J Mol Biol*. 2002; 315:663–676. [PubMed: 11812138]
 9. Badasso MO, Leiman PG, Tao Y, He Y, Ohlendorf DH, Rossmann MG, Anderson D. Purification, crystallization and initial X-ray analysis of the head–tail connector of bacteriophage phi29. *Acta Crystallogr D Biol Crystallogr*. 2000; 56(Pt 9):1187–1190. [PubMed: 10957642]

- 10•. Shu D, Zhang H, Jin J, Guo P. Counting of six pRNAs of phi29 DNA-packaging motor with customized single molecule dual-view system. *EMBO J.* 2007; 26:527–537. This manuscript is the first to report a new technique using single molecule photobleaching to determine the stoichiometry of RNA on an active phage. [PubMed: 17245435]
11. Xiao F, Zhang H, Guo P. Novel mechanism of hexamer ring assembly in protein/RNA interactions revealed by single molecule imaging. *Nucleic Acids Res.* 2008; 36:6620–6632. [PubMed: 18940870]
12. Moll D, Guo P. Grouping of ferritin and gold nanoparticles conjugated to pRNA of the phage phi29 DNA-packaging motor. *J Nanosci Nanotechnol.* 2007; 7:3257–3267. [PubMed: 18019159]
13. Zhang H, Schwartz C, De Donatis GM, Guo P. “Push through one-way valve” mechanism of viral DNA packaging. *Adv Virus Res.* 2012; 83:415–465. [PubMed: 22748815]
- 14••. Schwartz C, De Donatis GM, Zhang H, Fang H, Guo P. Revolution rather than rotation of AAA+ hexameric phi29 nanomotor for viral dsDNA packaging without coiling. *Virology.* 2013 in press, Biomotors have previously been classified into two categories: linear and rotational motors. It has long been popularly believed that viral DNA packaging motors are rotation motors. This paper is to provide the first evidence for the presence of third category of biomotors with revolution mechanism without rotation and coiling.
- 15••. Schwartz C, De Donatis GM, Zhang H, Fang H, Guo P. The ATPase of the phi29 DNA-packaging motor is a member of the hexameric AAA+ superfamily. *Virology.* 2013 in press, In the second series submitted to *Virology*, Schwartz *et al.* verified the stoichiometry of the ATPase which directly corresponds to the mechanism of DNA packaging as discussed in the previous manuscript (Ref. [14••]).
16. Guo P, Peterson C, Anderson D. Prohead and DNA-gp3-dependent ATPase activity of the DNA packaging protein gp16 of bacteriophage ϕ 29. *J Mol Biol.* 1987; 197:229–236. [PubMed: 2960820]
- 17•. Schwartz C, Fang H, Huang L, Guo P. Sequential action of ATPase, ATP, ADP, Pi and dsDNA in procapsid-free system to enlighten mechanism in viral dsDNA packaging. *Nucleic Acids Res.* 2012; 40:2577–2586. The group addressed the mechanism of energy production for phage assembly and clearly elucidated the interactions among the ATPase and motor components, including ATP, in order to better understand the mechanism in which ATP hydrolysis linked to the physical movement of DNA within the capsid. [PubMed: 22110031]
18. Jing P, Haque F, Shu D, Montemagno C, Guo P. One-way traffic of a viral motor channel for double-stranded DNA translocation. *Nano Lett.* 2010; 10:3620–3627. [PubMed: 20722407]
- 19•. Zhao, Z.; Khisamutdinov, E.; Schwartz, C.; Guo, P. Mechanism of one-way traffic of hexameric Phi29 DNA packaging motor with four electropositive relaying layers facilitating anti-parallel revolution. *ACS Nano.* 2013. in press, <http://dx.doi.org/10.1021/nn4002775>, It was revealed in this manuscript that the connector portal plays a role in DNA packaging through its interaction with dsDNA. The authors show that DNA revolves around the portal making contact with four positively charged residues to exhibit four pauses during translocation
20. Moffitt JR, Chemla YR, Athavan K, Grimes S, Jardine PJ, Anderson DL, Bustamante C. Intersubunit coordination in a homomeric ring ATPase. *Nature.* 2009; 457:446–450. [PubMed: 19129763]
21. Guo P. The emerging field of RNA nanotechnology. *Nat Nanotechnol.* 2010; 5:833–842. [PubMed: 21102465]
22. Pleij CW, Bosch L. RNA pseudoknots: structure, detection, and prediction. *Methods Enzymol.* 1989; 180:289–303. [PubMed: 2482419]
23. Guo P. RNA nanotechnology: engineering, assembly and applications in detection, gene delivery and therapy. *J Nanosci Nanotechnol.* 2005; 5:1964–1982. [PubMed: 16430131]
24. Isambert H. The jerky and knotty dynamics of RNA. *Methods.* 2009; 49:189–196. [PubMed: 19563894]
25. Sugimoto N, Nakano S, Katoh M, Matsumura A, Nakamuta H, Ohmichi T, Yoneyama M, Sasaki M. Thermodynamic parameters to predict stability of RNA/DNA hybrid duplexes. *Biochemistry.* 1995; 34:11211–11216. [PubMed: 7545436]

26. Searle MS, Williams DH. On the stability of nucleic acid structures in solution: enthalpy-entropy compensations, internal rotations and reversibility. *Nucleic Acids Res.* 1993; 21:2051–2056. [PubMed: 7684832]
27. Leontis NB, Lescoute A, Westhof E. The building blocks and motifs of RNA architecture. *Curr Opin Struct Biol.* 2006; 16:279–287. [PubMed: 16713707]
28. Schroeder KT, McPhee SA, Ouellet J, Lilley DM. A structural database for k-turn motifs in RNA. *RNA.* 2010; 16:1463–1468. [PubMed: 20562215]
29. Li X, Horiya S, Harada K. An efficient thermally induced RNA conformational switch as a framework for the functionalization of RNA nanostructures. *J Am Chem Soc.* 2006; 128:4035–4040. [PubMed: 16551112]
30. Chang KY, Tinoco I Jr. Characterization of a “kissing” hairpin complex derived from the human immunodeficiency virus genome. *Proc Natl Acad Sci USA.* 1994; 91:8705–8709. [PubMed: 8078946]
31. Chen C, Sheng S, Shao Z, Guo P. A dimer as a building block in assembling RNA: a hexamer that gears bacterial virus phi29 DNA-translocating machinery. *J Biol Chem.* 2000; 275:17510–17516. [PubMed: 10748150]
32. Wagner C, Ehresmann C, Ehresmann B, Brunel C. Mechanism of dimerization of bicoid mRNA: initiation and stabilization. *J Biol Chem.* 2004; 279:4560–4569. [PubMed: 14607826]
33. Bindewald E, Hayes R, Yingling YG, Kasprzak W, Shapiro BA. RNAJunction: a database of RNA junctions and kissing loops for three-dimensional structural analysis and nanodesign. *Nucleic Acids Res.* 2008; 36:D392–D397. [PubMed: 17947325]
34. Shu D, Shu Y, Haque F, Abdelmawla S, Guo P. Thermodynamically stable RNA three-way junctions for constructing multifunctional nanoparticles for delivery of therapeutics. *Nat Nanotechnol.* 2011; 6:658–667. This pioneering study showed the self-assembly of thermodynamically stable tri-star nanoparticles with a phi29 pRNA three-way junction core. These nanoparticles are both chemically and thermodynamically stable in serum and remain intact at extremely low concentrations. The modules remain functional *in vitro* and *in vivo*, suggesting that the three-way junction core can be used as a platform to build a variety of multifunctional nanoparticles. [PubMed: 21909084]
35. Haque F, Shu D, Shu Y, Shlyakhtenko L, Rychahou P, Evers M, Guo P. Ultrastable synergistic tetravalent RNA nanoparticles for targeting to cancers. *Nano Today.* 2012; 7:245–257. The authors reported the construction of thermodynamically stable X-shaped RNA nanoparticles to carry four therapeutic RNA motifs by the self-assembly of reengineered small RNA fragments, and proved that each arm of the four helices in the X-motif can harbor different functionalities with synergistic effects. More importantly, systemic injection of ligand-containing nanoparticles into the tail-vein of mice revealed that the RNA nanoparticles remained intact and strongly bound to cancers without accumulating in normal organs or tissues. [PubMed: 23024702]
36. Guo P, Bailey S, Bodley JW, Anderson D. Characterization of the small RNA of the bacteriophage phi29 DNA packaging machine. *Nucleic Acids Res.* 1987; 15:7081–7090. [PubMed: 3116499]
37. Lee TJ, Guo P. Interaction of gp16 with pRNA and DNA for genome packaging by the motor of bacterial virus phi29. *J Mol Biol.* 2006; 356:589–599. [PubMed: 16376938]
38. Shu D, Huang L, Guo P. A simple mathematical formula for stoichiometry quantitation of viral and nanobiological assemblage using slopes of log/log plot curves. *J Virol Methods.* 2003; 115:19–30. [PubMed: 14656457]
39. Trottier M, Guo P. Approaches to determine stoichiometry of viral assembly components. *J Virol.* 1997; 71:487–494. [PubMed: 8985375]
40. Zhang CL, Lee CS, Guo P. The proximate 5′ and 3′ ends of the 120-base viral RNA (pRNA) are crucial for the packaging of bacteriophage phi29 DNA. *Virology.* 1994; 201:77–85. [PubMed: 8178491]
41. Zhang CL, Tellinghuisen T, Guo P. Confirmation of the helical structure of the 5′/3′ termini of the essential DNA packaging pRNA of phage phi29. *RNA.* 1995; 1:1041–1050. [PubMed: 8595559]
42. Zhang CL, Trottier M, Guo PX. Circularly permuted viral pRNA active and specific in the packaging of bacteriophage Phi29 DNA. *Virology.* 1995; 207:442–451. [PubMed: 7533964]

43. Reid RJD, Bodley JW, Anderson D. Identification of bacteriophage phi29 prohead RNA (pRNA) domains necessary for *in vitro* DNA-gp3 packaging. *J Biol Chem.* 1994; 269:9084–9089. [PubMed: 8132646]
44. Reid RJD, Zhang F, Benson S, Anderson D. Probing the structure of bacteriophage phi29 prohead RNA with specific mutations. *J Biol Chem.* 1994; 269:18656–18661. [PubMed: 8034614]
45. Reid RJD, Bodley JW, Anderson D. Characterization of the prohead–pRNA interaction of bacteriophage phi29. *J Biol Chem.* 1994; 269:5157–5162. [PubMed: 8106496]
46. Hoeprich S, ZHou Q, Guo S, Qi G, Wang Y, Guo P. Bacterial virus phi29 pRNA as a hammerhead ribozyme escort to destroy hepatitis B virus. *Gene Ther.* 2003; 10:1258–1267. [PubMed: 12858191]
47. Khaled A, Guo S, Li F, Guo P. Controllable self-assembly of nanoparticles for specific delivery of multiple therapeutic molecules to cancer cells using RNA nanotechnology. *Nano Lett.* 2005; 5:1797–1808. [PubMed: 16159227]
48. Guo S, Tschammer N, Mohammed S, Guo P. Specific delivery of therapeutic RNAs to cancer cells via the dimerization mechanism of phi29 motor pRNA. *Hum Gene Ther.* 2005; 16:1097–1109. [PubMed: 16149908]
49. Guo S, Huang F, Guo P. Construction of folate-conjugated pRNA of bacteriophage phi29 DNA packaging motor for delivery of chimeric siRNA to nasopharyngeal carcinoma cells. *Gene Ther.* 2006; 13:814–820. [PubMed: 16482206]
50. Shu Y, Cinier M, Fox SR, Ben-Johnathan N, Guo P. Assembly of therapeutic pRNA-siRNA nanoparticles using bipartite approach. *Mol Ther.* 2011; 19:1304–1311. [PubMed: 21468002]
- 51•. Shu Y, Cinier M, Shu D, Guo P. Assembly of multifunctional phi29 pRNA nanoparticles for specific delivery of siRNA and other therapeutics to targeted cells. *Methods.* 2011; 54:204–214. In this paper, the authors reported multiple approaches for the construction of longer RNA by using two synthetic RNA fragments with variable modifications. The resulting bipartite RNA was fully competent in folding and function and similar to the intact RNA. The results showed the successful overcoming of the size limitation for chemical synthesis and paved the way to large-scale production of therapeutic RNA nanoparticles. [PubMed: 21320601]
52. Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J, John M, Kesavan V, Lavine G, Pandey RK, Racie T, Rajeev KG, Rohl I, Toudjarska I, Wang G, Wuschko S, Bumcrot D, Koteliansky V, Limmer S, Manoharan M, Vornlocher HP. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature.* 2004; 432:173–178. [PubMed: 15538359]
53. Dassie JP, Liu XY, Thomas GS, Whitaker RM, Thiel KW, Stockdale KR, Meyerholz DK, McCaffrey AP, McNamara JO, Giangrande PH. Systemic administration of optimized aptamer-siRNA chimeras promotes regression of PSMA-expressing tumors. *Nat Biotechnol.* 2009; 27:839–849. [PubMed: 19701187]
54. Behlke MA. Progress towards *in vivo* use of siRNAs. *Mol Ther.* 2006; 13:644–670. [PubMed: 16481219]
55. Li SD, Chen YC, Hackett MJ, Huang L. Tumor-targeted delivery of siRNA by self-assembled nanoparticles. *Mol Ther.* 2008; 16:163–169. [PubMed: 17923843]
- 56•. Guo P, Haque F, Hallahan B, Reif E, Li H. Uniqueness, advantages, challenges, solutions, and perspectives in therapeutics applying RNA nanotechnology. *Nucleic Acid Ther.* 2012; 22:226–245. This review article thoroughly discusses the potential problems of the emerging field of RNA nanotechnology and provides some solutions and perspectives for future clinical applications to problems such as chemical and thermodynamic stability, *in vivo* half-life and biodistribution, yield and production cost, *in vivo* toxicity and side effects, specific delivery and targeting, and endosomal trapping and escape. [PubMed: 22913595]
57. Shu D, Moll WD, Deng Z, Mao C, Guo P. Bottom-up assembly of RNA arrays and superstructures as potential parts in nanotechnology. *Nano Lett.* 2004; 4:1717–1723. [PubMed: 21171616]
58. Cayrol B, Nogues C, Dawid A, Sagi I, Silberzan P, Isambert H. A nanostructure made of a bacterial noncoding RNA. *J Am Chem Soc.* 2009; 131:17270–17276. [PubMed: 19821568]

59. Ohno H, Kobayashi T, Kabata R, Endo K, Iwasa T, Yoshimura SH, Takeyasu K, Inoue T, Saito H. Synthetic RNA–protein complex shaped like an equilateral triangle. *Nat Nanotechnol.* 2011; 6:116–120. [PubMed: 21240283]
60. Dibrov SM, McLean J, Parsons J, Hermann T. Self-assembling RNA square. *Proc Natl Acad Sci USA.* 2011; 108:6405–6408. [PubMed: 21464284]
61. Severcan I, Geary CVECA, Jaeger L. Square-shaped RNA particles from different RNA folds. *Nano Lett.* 2009; 9:1270–1277. [PubMed: 19239258]
62. Huang Z, Pei W, Han Y, Jayaseelan S, Shekhtman A, Shi H, Niu L. One RNA aptamer sequence, two structures: a collaborating pair that inhibits AMPA receptors. *Nucleic Acids Res.* 2009; 37:4022–4032. [PubMed: 19417060]
63. Pattabiraman N, Martinez HM, Shapiro BA. Molecular modeling and dynamics studies of HIV-1 kissing loop structures. *J Biomol Struct Dyn.* 2002; 20:397–411. [PubMed: 12437378]
64. Laughrea M, Jette L. HIV-1 genome dimerization: kissing-loop hairpin dictates whether nucleotides downstream of the 5' splice junction contribute to loose and tight dimerization of human immunodeficiency virus RNA. *Biochemistry.* 1997; 36:9501–9508. [PubMed: 9235995]
65. Paillart JC, Westhof E, Ehresmann C, Ehresmann B, Marquet R. Non-canonical interactions in a kissing loop complex: the dimerization initiation site of HIV-1 genomic RNA. *J Mol Biol.* 1997; 270:36–49. [PubMed: 9231899]
66. Chang KY, Tinoco I Jr. The structure of an RNA “kissing” hairpin complex of the HIV TAR hairpin loop and its complement. *J Mol Biol.* 1997; 269:52–66. [PubMed: 9193000]
67. Abdelmawla S, Guo S, Zhang L, Pulukuri S, Patankar P, Conley P, Trebley J, Guo P, Li QX. Pharmacological characterization of chemically synthesized monomeric pRNA nanoparticles for systemic delivery. *Mol Ther.* 2011; 19:1312–1322. [PubMed: 21468004]
68. Shu Y, Haque F, Shu D, Li W, Zhu Z, Kotb M, Lyubchenko Y, Guo P. Fabrication of 14 different RNA nanoparticles for specific tumor targeting without accumulation in normal organs. *RNA.* 2012 in press.

Figure 1.

Demonstration of the hexameric structure of bacteriophage phi29 DNA-packaging motor. (a) AFM images of hexameric phi29 motor pRNA containing 7-nt interlocking loops [15^{••}]. (b, c) Illustrations of the phi29 DNA packaging motor and a pRNA hexamer for side view (b) and bottom view (c). (d) Slab gel electrophoresis qualification and (f) capillary electrophoresis quantification by varying the molar ratio of [protein]:[DNA]. The concentration of bound DNA plateaus at a molar ratio of 6:1 as [protein] remained constant, proving that the ATPase gp16 binding to dsDNA is the hexamer. (e) Native PAGE reveals distinct bands characteristic of six oligomeric states of phi29 motor ATPase gp16; the hexamer increases as the concentration of protein is increased. Oligomeric states were assigned based on the mobility of marker proteins in the Native PAGE Mark kit. Adapted from [15^{••}] with permission from Elsevier.

Figure 2.

Part I. (a) Viral assembly inhibition assay using a binomial distribution revealing that phi29 motor ATPase gp16 possesses a sixfold symmetry in the DNA packaging motor [39,15**]. (b). Demonstration that one inactive gp16 mutant in the hexamer completely blocked the motor function in DNA packaging. Theoretical plot of percent Walker B mutant gp16 versus yield of infectious virions in *in vitro* phage assembly assays. Predictions were made with equation

$$(p+q)^Z = \binom{Z}{0} p^Z + \binom{Z}{1} p^{Z-1} q + \binom{Z}{2} p^{Z-2} q^2 + \dots + \binom{Z}{Z-1} p q^{Z-1} + \binom{Z}{Z} q^Z = \sum_{M=0}^Z \binom{Z}{M} p^{Z-M} q^M$$

, where p is the percent of wild type eGFP-gp16; q is the percent of eGFP-gp16/ED; Z is the total number of eGFP-gp16 per procapsid or gp3-DNA; M is the number of mutant eGFP-gp16 in the phi29 DNA packaging motor; and $p + q = 1$ [39]. The minimum number (y) of Walker B mutant eGFP-gp16 in the hexameric ring to block the motor activity was predicted with equation

$$(p+q)^6 = \binom{6}{0} p^6 + \binom{6}{1} p^5 q + \binom{6}{2} p^4 q^2 + \binom{6}{3} p^3 q^3 + \binom{6}{4} p^2 q^4 + \binom{6}{5} p q^5 + \binom{6}{6} q^6$$

where p and q represent the ratio of wild type and mutant eGFP-gp16, respectively, and $p +$

$q = 1$. If $y = 1$, then the motor activity will be $\binom{6}{0} p^6$; if $y=2$ then the motor activity will be $\binom{6}{0} p^6 + \binom{6}{1} p^5 q$; if $y=3$, then the motor activity will be $\binom{6}{0} p^6 + \binom{6}{1} p^5 q + \binom{6}{2} p^4 q^2$;

if $y=4$ then the motor activity will be $\binom{6}{0} p^6 + \binom{6}{1} p^5 q + \binom{6}{2} p^4 q^2 + \binom{6}{3} p^3 q^3$; if $y=5$

then the motor activity will be $\binom{6}{0} p^6 + \binom{6}{1} p^5 q + \binom{6}{2} p^4 q^2 + \binom{6}{3} p^3 q^3 + \binom{6}{4} p^2 q^4$; if

$y=6$ then the motor activity will be

$$\binom{6}{0} p^6 + \binom{6}{1} p^5 q + \binom{6}{2} p^4 q^2 + \binom{6}{3} p^3 q^3 + \binom{6}{4} p^2 q^4 + \binom{6}{5} p q^5$$

Part II. Single molecule TIRF reveals hexameric pRNA on phi29 DNA packaging motor. Dual-labeled fluorescent pRNA dimer with green (cy3) and red (cy5) (a) was co-localized on a single motor revealed by TIRF microscope (b) and analyzed by a histogram (c). Photobleaching assays exhibited 3 discrete steps for each fluorophore wavelength green (cy3) and red (cy5) (d).

Adapted from [15**] for Part I and [35*] for Part II with permission from NPG and Elsevier.

Figure 3.

Phi29 possesses a sixfold symmetry by hand-in-hand interactions. (a) Hand-in-hand interactions modeled by cartoon characters [5]; (b) the structure of pRNA in which the right handed loop can bind to the left handed loop to form the hexameric structure; (c) negative stained EM images with gold conjugated pRNA reveals six pRNA molecules on the vertex of phi29 procapsids; (d-f) Journal covers illustrating the pRNA hexameric structure. Covers are from *Molecular Cell* where [5*,6] were published (Figure with permission from Dr F. Major and Cell Press); *Human Gene Therapy* where [48] was published with permission of the publisher. (c) is from *ScienceNow* with permission from the publisher.

Figure 4.

Mechanisms of phi29 DNA packaging motor using the revolution without rotation or coiling. **(a)** Structure of the phi29 DNA packaging motor, showing the four lysine rings – K200 (red), K209 (green), N231 (blue), with N246 (yellow) [8,9] scattered inside the inner wall of the connector [19]. One-twelfth of the inner diameter of helical dsDNA translates to a 30° angle, **(b)**. Contact at every 30° transition leads to the translocation of one helical turn of dsDNA through the connector [14^{••},19^{*}]. **(c, d)** Schematic of the mechanism of sequential revolution in translocating genomic dsDNA [14^{••},19^{*}]. The binding of ATP to one gp16 subunit stimulates it to adapt to a conformation with a higher affinity for dsDNA. ATP hydrolysis forces gp16 to assume a new conformation with a lower affinity for dsDNA, thus pushing dsDNA away from the subunit and transferring it to an adjacent subunit. DsDNA moves forward 1.75 base pairs when gp16 binds at a location 60° different from the last subunit on the same phosphate backbone chain. Rotation of the hexameric ring or the dsDNA is not required since the dsDNA chain is transferred from one point on the phosphate backbone to another. In each transitional step, one ATP is hydrolyzed, and in one

cycle, six ATPs are required to translocate dsDNA one helical turn of 360° (10.5 base pairs). Please see attached website <http://nanobio.uky.edu/movie.html> for animations. (e) A planar view of DNA revolving through 30° tilted connector subunits facilitated by anti-parallel helices between dsDNA helix and connector protein subunits [14**]. DNA is advanced along the circular wall of the connector channel with no torsion or coiling force. (f) Elucidation of four-step transition and pause for the packaging of one helical patch of 10.5 bp of dsDNA [19*]. Two rounds of DNA revolution across 12 connector subunits. In the first round, a 21-bp DNA helix shown in blue (10.5 bp) and green (10.5 bp) colors revolves through each subunit, making electrostatic interaction by its negatively charged phosphate group with positively charged amino group of lysine residues (shown in red spheres in each subunits). The interaction of DNA and lysine is shown in black spheres. During the movement, a dsDNA strand passes all four lysine layers. The first round ends (blue strand inside the channel) upon DNA makes 360° rotation equivalent to 10.5 bp and moves up to one DNA pitch ~ 3.4 nm (subunit #12). This follows by the second round of rotation. In the same manner, the same DNA strand colored by green moves through four lysine layers until whole helical turn is achieved. The phosphate backbone of the dsDNA contacted the same layer of the lysine for every three subunits (12 subunits/4 layers = 3 subunits/layer), and shifts to the next layer then revolve through three subunits.

Adapted from [15**] (Schwartz *et al.*, *Virology*, 2013, in press, [14**] and [19*] Zhao *et al.*, *ACS Nano*, 2013, in press).

Figure 5.

Schematic (left) and AFM images (right) of RNA nanoparticles derived from the thermodynamically stable phi29 motor pRNA 3WJ harboring three 117 RNA molecules. **(a)** Therapeutic modules **(b)**, and extending into X-shape with four functional modules **(c)**. Schematic (left) and AFM images (right) of RNA nanoparticles derived from the thermodynamically stable phi29 motor pRNA 3WJ harboring three 117 RNA molecules (a), therapeutic modules (b), and extending into X-shape with four functional modules (c). Adapted from [34*,35*] with permission from Nature Publication group.