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Review Article

Applications of phage-derived RNA-based technologies in synthetic biology

Wenhui Zhang[a], Qiong Wu[a,b,*]

[a] MOE Key Lab. Bioinformatics, School of Life Sciences, Tsinghua University, Beijing, 100084, China
[b] Center for Synthetic and Systems Biology, Tsinghua University, Beijing, 100084, China

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ABSTRACT

As the most abundant biological entities with incredible diversity, bacteriophages (also known as phages) have been recognized as an important source of molecular machines for the development of genetic-engineering tools. At the same time, phages are crucial for establishing and improving basic theories of molecular biology. Studies on phages provide rich sources of essential elements for synthetic circuit design as well as powerful support for the improvement of directed evolution platforms. Therefore, phages play a vital role in the development of new technologies and central scientific concepts. After the RNA world hypothesis was proposed and developed, novel biological functions of RNA continue to be discovered. RNA and its related elements are widely used in many fields such as metabolic engineering and medical diagnosis, and their versatility led to a major role of RNA in synthetic biology. Further development of RNA-based technologies will advance synthetic biological tools as well as provide verification of the RNA world hypothesis. Most synthetic biology efforts are based on re-constructing existing biological systems, understanding fundamental biological processes, and developing new technologies. RNA-based technologies derived from phages will offer abundant sources for synthetic biological components. Moreover, phages as well as RNA have high impact on biological evolution, which is pivotal for understanding the origin of life, building artificial life-forms, and precisely reprogramming biological systems. This review discusses phage-derived RNA-based technologies terms of phage components, the phage lifecycle, and interactions between phages and bacteria. The significance of RNA-based technology derived from phages for synthetic biology and for understanding the earliest stages of biological evolution will be highlighted.

1. Introduction

Bacteriophages (also known as phages) are viruses that infect bacteria, fungi, algae, actinomycetes or spirochetes. In the early 20th century, phages were isolated independently from *Staphylococcus* by Frederick W. Twort [1] and from *Shigella* by Felix d'Herelle [2]. Phages have simple viral structures consisting of nucleic acids and coat proteins, but in terms of sheer numbers, they are the most abundant biological entities on the planet [3–5]. They progress through fast invasion and propagation in two reproduction modes. The genome size of phages is relatively small, ranging from 5 kb to 500 kb [6], which makes them amenable for genetic manipulation. Because of these characteristics, phages have been recognized as essential models for molecular biology as well as laying the foundation of modern molecular virology and biology including the understanding of DNA self-replication [7], the clarification of the complex structure of microorganisms [8], the molecular mechanism of mutations [9], and research on gene regulation [10]. The deep study of phage biology contributed to the development of important tools and regents that are widely used in fundamental biological research and genetic engineering. The universally used gene regulation tools in synthetic biology such as phage-derived RNA polymerases, transcriptional regulators and integrases stem from phage biology research. Moreover, the interactions between prokaryotes and phages led to the discovery and development of the revolutionary clustered regularly interspaced short palindromic repeats (CRISPR) system. In addition, phage-based applications are widespread in different fields including bacterial detection, drug delivery, novel vaccine design, or nano materials. Owing to the great diversity of phage species, phage research has not yet revealed all their secrets. Accordingly, there is great promise for technique development and further biological breakthroughs based on phage research.

Building on the advances in the capacity of biological systems engineering, synthetic biology requires the development of more tools for gene expression regulation to achieve precise and dynamic control of biological systems. The emerging tools will offer new solutions for the challenges facing medical therapy, energy sources, product

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* Corresponding author. MOE Key Lab. Bioinformatics, School of Life Sciences, Tsinghua University, Beijing, 100084, China.
E-mail address: wuqiong@tsinghua.edu.cn (Q. Wu).

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manufacturing and so on. Most synthetic genetic tools rely on protein-DNA interactions to control gene circuits [11,12]. However, recent progress on RNA biology has brought attention to RNA-based components for understanding and programming biological systems. With highly modular components, such as siRNA, RNA aptamers, riboswitches, ribozymes etc., RNA molecules can be employed to regulate proteins, metabolites, or other nucleic acid molecules [13–16]. The roles of RNA molecules in specific physiological processes can be further identified by manipulation and regulation of RNAs, leading to the discovery of novel functions and design of new RNA regulators. Furthermore, the origin and evolution of life have been inspiring researchers over the years. The advances in research on the RNA world hypothesis provide more clues for the roles of RNA in the central dogma of molecular biology.

Phage research continues to drive the development of broader molecular biological research. The components of phages have been adapted as major parts for synthetic biology, including some key RNA-acting elements. New clues are being discovered for biological evolution based on the phage lifecycle and interactions with host cells. This review highlights RNA-based technologies derived from phages and describes the RNA manipulation tools and RNA-based gene regulation techniques in terms of the phage components for obtaining more functional synthetic modules, the phage lifecycle for understanding and constructing specific artificial systems, and the phage-bacterial interactions for applying widely used technologies to different hosts (Fig. 1). These aspects are closely related and benefit the development of synthetic biology in aspects ranging from genetic circuit construction to chassis cell adaptation. Furthermore, the value of phage research for our understanding of biological evolution will be discussed to illustrate the roles of interacting elements including phages, RNA molecules and biological evolution. The review will further summarize challenges faced by phage-derived RNA-based technologies and prospect novel biotechnological opportunities.

2. RNA-based synthetic biology parts from phage components

Phages divert the ribosomes, various factors required for proteins synthesis, amino acids, nucleic acids and energy production systems of bacterial cells to achieve their own growth and proliferation. Phages have spent a long time evolving different ways to amplify their genetic material, have developed ingenious capsid structures and various essential components to use host cell resources. These elements offer a rich source of RNA-based synthetic biology parts.

2.1. RNA-binding proteins from phages

RNA molecules play key roles in the cell as they take part in many vital cellular processes, from basic protein translation to complex gene expression regulation. Given the importance of specific RNA molecules, it is meaningful to monitor the dynamics of the processing, transport, translation or post-transcriptional degradation of RNA molecules. As RNA-binding proteins (RBPs) are involved in most processes of RNA regulation, it is also very important to confirm the biological roles of RBPs and how they interact with the target RNAs. The progress in RNA biotechnology enables the temporal and spatial tracking of RNAs as well as the RBPs bound to target RNAs in the cell. One of the classic approaches for studying the function of intracellular RBPs is to fuse the RNA-binding domain of a well-characterized RBP with the proteins to be detected. The interaction between the RNA-binding domain and the recognized protein fused with a reporter protein contributes to the effective detection of target proteins. This powerful technique is also widely used in the dynamic detection of different RNAs in the cell, enabling the monitoring of RNA synthesis, RNA transport and RNA localization. Several systems derived from phage RNA motifs and related binding proteins are being used, including the MS2 system, PP7 system and λ_N system.

2.1.1. Introduction of the major RBPs from phages

The ideal RBPs require strong specificity and affinity when they bind to the cognate RNA motif, so that there is no interference from other proteins and RNA motifs in the cell. The MS2 tagging system was derived from the bacteriophage MS2 coat protein (MCP) and its cognate RNA binding sties (MBS). The MCP contains an RNA binding domain that is highly specific for an RNA stem-loop structure which is found only in the genomic RNA of the phage [17]. The MCP and its cognate RNA motif can avoid cross-reactions with different RNAs or proteins present in the host cell via long-term evolution, so as to ensure that the MS2 tagging system can efficiently mark RNAs or proteins with specific functions in the cell. Based on the successful construction of the MS2 tagging system, the functional RNA motif and RBPs were identified in

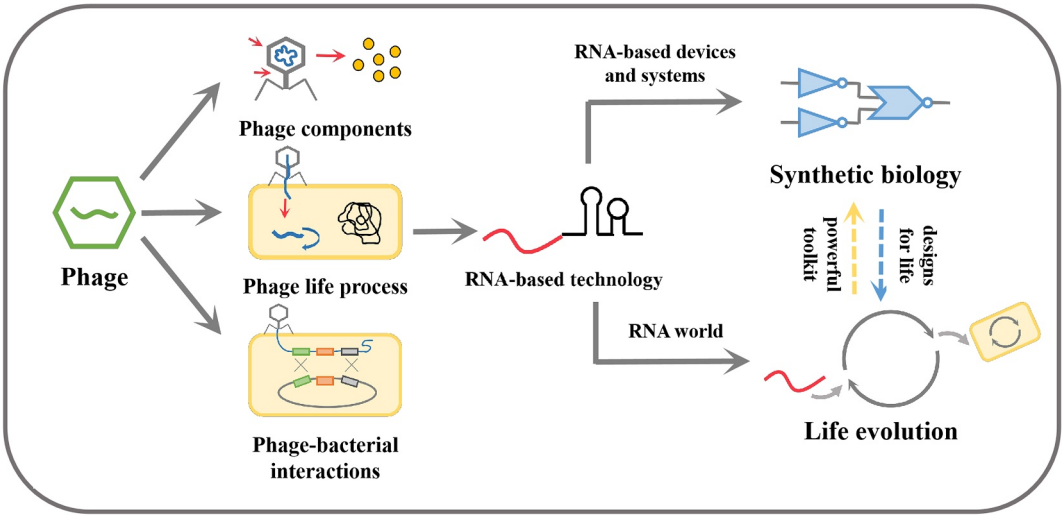

Fig. 1. Phage-derived RNA-based technologies in synthetic biology. The deep study on phage biology contributes to the development of important RNA-based tools or regents, which will be discussed in three aspects including phage components, phage life cycle and phage-bacterial interactions. These RNA-based techniques contribute to the development of functional devices and systems in synthetic biology, while the advances in the research on the RNA world help to further understand the life evolution. Furthermore, the reproduction of life-specific functions for evolution study is highly dependent on synthetic biology and in turn provides new technologies to promote progress of synthetic biology. The interacting elements including phages, RNA molecules, biological evolution and synthetic biology are closely tied, which shows great significance for phage-derived RNA-based technologies.

the phage PP7, which is a distant relative of the phage MS2. The RNA motif located within the PP7 genome is the stem loop bound to the PP7 coat protein (PCP) [18,19]. Compared to MCP, PCP has a higher binding affinity (K_d = 1.6 nM) and specificity (with MS2 RNA binding motif, K_d > 1 μM). The MS2 system and PP7 system can be used in combination to label different target RNAs in the same cell or the resulting chimera can be employed to reduce background noise. The λ_N system is composed of an RNA motif called BoxB and a 22-amino-acid peptide called λ_N, which specifically binds to the BoxB sequence from the DNA phage λ [20]. This cognate pair can be used as an alternative to the MS2 system. Based on the high affinity of the BoxB sequence and the λ_N protein, the introduction of only 4 BoxB sequences is sufficient to bind the λ_N protein to produce detectable fluorescence, while 4 BoxB sequences are only 80 nt long, which is far less than the binding sequence of MS2 or PP7 system. Therefore, using the λ_N system to label target RNAs requires the introduction of only relatively short foreign sequences. Nevertheless, the λ_N system has fallen out of favor in recent years, but there is no direct evidence and this may be due to historical reasons [21]. One study mentioned that the λ_N system was less effective than the MS2 or PP7 system for detecting RNA molecules but no solid data was given [22].

2.1.2. Applications of RBPs from phages

The coat proteins from the systems described above function as the common RBPs that interact with specific RNA motifs and thus offer a platform for manipulating RNAs. The earliest application of these systems consisting of coat proteins and target RNA motifs is RNA imaging. The MS2 system has enabled substantial advancement in the tagging of RNAs since its application for imaging *ASH1* mRNA in yeast [23]. For the MS2 system, two modules are essential to detect a target RNA: the RNA motif and the RBP. Different copies of the phage RNA motif are inserted into the untranslated region of the RNA, while the MCP and a fluorescent protein such as GFP are fused and co-expressed. In general, the copy number of the RNA motif is 6–24, forming a long chimeric RNA to ensure sufficient fluorescence intensity (Fig. 2a). The binding of MCP-GFP to the target RNA enables the dynamic detection of target RNAs [24] (Fig. 2b). The MS2 tagging system is widely applied for the imaging of mRNAs in different biological processes, including the positioning of different functional RNAs in eukaryotic and prokaryotic cells, which has become a powerful tool for real-time imaging. The MS2, PP7 and λ_N systems can be used in combination to label different target RNAs in the same cell, or the resulting chimera can be employed to reduce background noise.

In addition to RNA imaging, the specificity of the binding of coat proteins to the cognate RNA motif provides a tool to tether RNAs or proteins in different biological scenarios. Considering the importance of RNA-interacting molecules, it is critical to identify the RNA-interacting factors by isolating them, while maintaining them in a highly purified and functional form. The MS2 system has been developed as an efficient tool to isolate functional complexes. The essential modules of the MS2 system are the target RNA tagged with several MBS at the 3′UTR, and a chimeric protein consisting of the MCP and an affinity tag. The MS2 purification method was used to purify functional spliceosomal complexes combined with mass spectrometry and electron microscopy for further analysis of their protein components [25,26]. A systematic approach called MS2-tagged RNA affinity purification (MS2-TRAP) has been developed to identify RNA-associated miRNAs and mRNA-interacting factors [27,28]. When the MCP is fused to the cellular components of interest, it is possible to localize mRNAs and their interacting proteins [29,30]. The MS2 system also allows the tethering of proteins to the target RNA, which enables the recruitment of functional proteins fused with the MCP to operate on the target RNA [31–33]. Furthermore, combined use of the MS2 and PP7 systems enabled the orthogonal tethering of proteins to their cognate RNA binding sites, which was used for multiplex detection and control of gene expression [34–36].

The MS2/PP7 systems are best suited to track RNAs in both prokaryotic and eukaryotic cells, offering a well-established platform with confirmed target specificity, mode of action, and accuracy for labeling individual mRNA molecules compared to other tagging systems that detect intracellular RNAs, such as different types of RNA aptamers [37,38] or CRISPR-Cas systems [39]. The RBP and cognate RNA motif of the phage-derived MS2/PP7 systems can be employed as efficient synthetic modules in the field of RNA biology to monitor and control the interaction between RNAs and proteins. Further study of the RNA-motif binding systems from phages is therefore merited. However, these systems also have several shortcomings, including the challenging removal of background noise, interference of the inserted motif sequences with the target RNA, the instability to repeat multiple cognate RNA

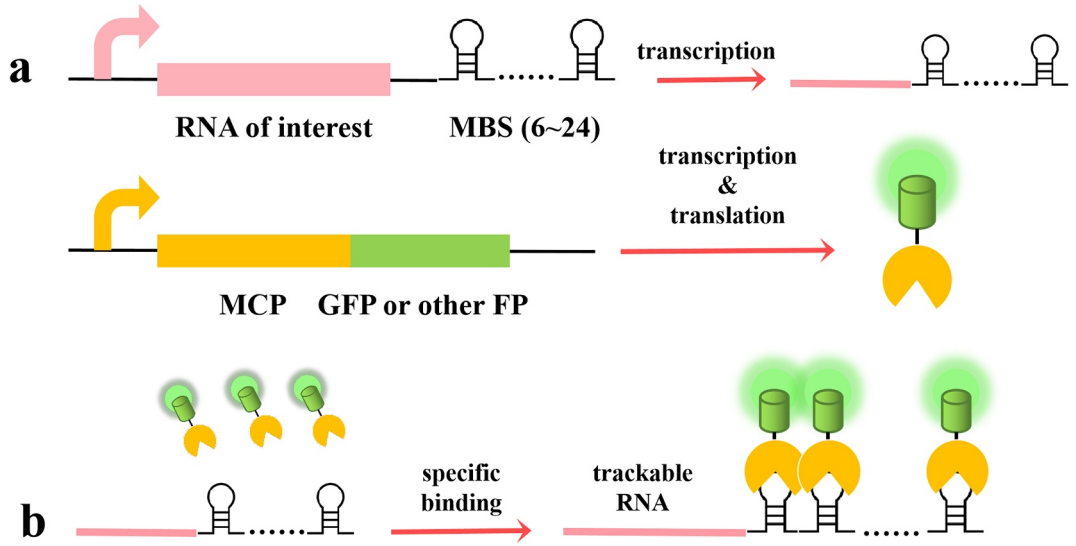

Fig. 2. MS2 system for RNA tagging. a) Two modules are essential to detect a target RNA for the MS2 system. The RNA motif and the RNA binding protein (RBP) are indispensable modules for the MS2 system to function. MS2 RNA motifs are encoded downstream of the RNA of interest. Transcribed RNA contains the binding domain recognized by the MS2 coat proteins (MCPs). While the copy number of the RNA motif is 6–24, it forms a long chimeric RNA to ensure sufficient fluorescence intensity. The MCP is expressed separately and fused to a fluorescent protein such as GFP. b) Specific binding of two modules. The binding of MCP-GFP to the RNA motifs enables the dynamic detection of the target RNA. The binding sites of the MCP are dependent on the copy number of the hairpins, which influences the signal strength.

hairpins, the effectiveness of labeling the targeted RNA and the difficulty of transfecting specific cells or tissues with the functional modules. Current improvements to the MS2 system include the construction of single-chain tandem dimers of MS2 and PP7 coat proteins to reduce their dimerization steps [40], simplifying repeat sequences in the MS2 system [41], or changing the affinity of MCP and MBS in the MS2 system [42]. In addition, the tracking of new RNAs with unknown sequences is relatively difficult with current RNA tagging systems. The optimization of RNA tagging systems can be combined with the rapid development of bioinformatics tools to rationalize the design of the RNA motif in the system, so as to simplify system design, improve the affinity of the RNA-protein interaction, and discover new functions of RNAs.

2.2. RNA processing enzymes

With the discovery of a vast number of ubiquitous RNAs with previously unknown biological functions (e.g., siRNAs, miRNAs, RNA ribozymes) [43–45], the study of RNA *in vitro* or *in vivo* continues to develop. The role of RNA according to the central dogma of molecular biology, which is that of a information transmission intermediate between DNA and proteins, has therefore been greatly expanded, showing great promise in biotechnology and other applications [46–49]. The functional RNA molecules are recognized as highly modular components that can be adapted for building metabolic, regulatory and genetic circuits. Consequently, it is critical to understand and integrate different types of RNA modularity at structural and functional level for further construction of complex biological systems and synthetic devices, while modified RNA molecules can help expand their functions. Some phages produce enzymes that act directly on RNA, which are used to study the structure and function of site-specifically modified RNA molecules.

2.2.1. RNA ligases

In biological systems, RNA molecules can also be modified or repaired. For example, phage T4 repairs key tRNAs that have been cut by host nucleases [50]. These repair pathways depend on the role of RNA ligase [51]. RNA ligases catalyze the formation of a covalent phosphodiester bond between two oligonucleotides (Fig. 3a). In the RNA world hypothesis, RNA ligases offer a crucial explanation for the emergence of longer and more complex RNA molecules from short oligonucleotides [52]. Most research on RNA ligases focused on T4 RNA ligase. Hurwitz et al. [53] first isolated RNA ligase, an enzyme that catalyzes the formation of phosphodiester bonds at the 5' end of oligonucleotides in *E. coli* infected with T4 phage. T4 RNA ligase mainly includes two types of RNA terminal editing enzymes: T4 RNA ligase 1 (Rnl1) [54] and T4 RNA ligase 2 (Rnl2) [55]. The function of Rnl1 *in vivo* is related to host defense, while the function of Rnl2 remains unknown. However, Rnl2 has extremely high RNA ligation activity, which is more than 10 times higher than that of Rnl1 [56].

Rnl1 and Rnl2 are widely used in various *in-vitro* experiments for labeling RNA termini, circularizing RNAs or inter- and intramolecular ligation of RNAs and DNAs, which focus on integrating small pieces into large RNA or DNA molecules (Fig. 3b). The universal application of ligation reactions mediated by T4 RNA ligases can be generalized but not limited to obtaining large molecules that cannot be directly synthesized, site-specifically modifying large RNA molecules for structural and functional analysis, labeling segments of specific RNAs, and constructing mutant RNA libraries. For modifying specific RNA molecules, Harald et al. [57] used Rnl1 to incorporate the fluorescent analogue into the 3′ termini of tRNA for the synthesis of yeast $tRNA^{Phe}$ derivatives. To generate RNA molecules with the aimed structure, Beadudry et al. [58] developed a simple and fast one-test strategy for synthesizing large amounts of pure circular RNA molecules using T4 RNA ligase to ligate the 5′ and 3' termini in close proximity. Sakhabutdinova et al. [59] introduced a new method of synthesizing small circular DNA molecules via the cyclization of ssDNA based on T4 RNA ligase for subsequent rolling circle amplification. There are also many studies focused on constructing RNA libraries for high-throughput sequencing with the help of T4 RNA ligase [60–64]. Taking an RNA molecule as a module, T4 RNA ligase serves as an important biochemical assistant for incorporating specially modified modules to advance the understanding and design of RNA molecules with expanded functionality.

In addition to T4 RNA ligase, some thermostable RNA ligases have been identified in other bacteriophages. For example, Thorarinn et al. [65] found a thermostable homolog of RNA ligase 1 in the thermophilic phage RM378, which has a temperature optimum of 60–64 °C and ligates both RNA and single-stranded DNA. The thermal stability and high-temperature catalytic activity of thermostable RNA ligases makes them suitable for RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) and other RNA or DNA ligation reactions. Based on current research on T4 RNA ligase, more specific and effective RNA ligases were designed, further expanding their function of modifying RNA molecules [66].

2.2.2. RNA cyclase ribozymes

In recent years, circular RNAs (circRNAs) have attracted increasing attention as a newly discovered type of non-coding RNA. Although a large number of studies have demonstrated the mechanism of circRNA formation [67,68], their specific biological functions still remain

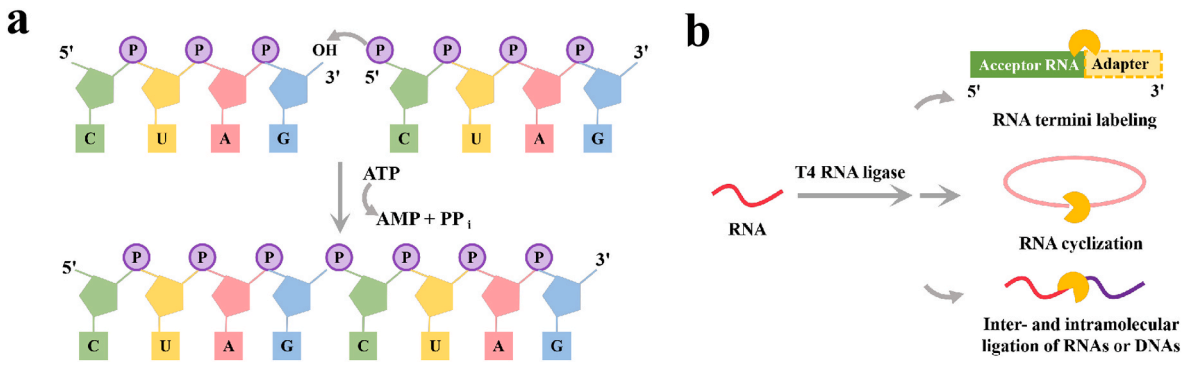

Fig. 3. T4 RNA ligase. a) T4 RNA ligase activity. The T4 RNA ligase catalyzes the ATP-dependent formation of a phosphodiester covalent bond between 5′-phosphate and 3′-hydroxyl termini of RNA or DNA. b) Applications of T4 RNA ligase. T4 RNA liagse is widely used in various *in-vitro* experiments such as labeling termini of RNAs, circularizing RNAs or inter- and intramolecular ligation of RNAs and DNAs. The 3′-hydroxyl termini of the acceptor RNA can be joined by the 5′-phosphate termini of the adapter sequence, which may be radioactive labelled or modified in other ways. Liner RNA strand with 3′ hydroxyl termini and 5′-phosphate termini can be ligated by the enzyme to obtain functional circular products. T4 RNA ligase can also ligate termini of RNA to termini of DNA to produce hybrid or chimeric molecules.

largely elusive. With the rapid development of sequencing methods and bioinformatics, unknown functions of circRNAs can increasingly be predicted through computational analysis. However, new biochemical methods are required for the construction of functional circRNA models *in vitro*. The commonly used strategies include ligation of RNA fragments using enzymes (such as the T4 RNA ligase mentioned above), chemical ligation of nucleic acid chains, and ribozymes for RNA circularization. A circRNA synthesized using phage components was reported in the work of Ethan et al., in which the sequence that drove the reaction was an RNA cyclase ribozyme [69]. This work provides a flexible tool for studying the structure and function of circRNA, but no further research progress has been reported to date.

I*n-vitro* RNA editing makes it possible to regulate the structure and composition of RNAs, to construct RNA libraries for analysis and selection, and to rationally design artificial functional RNAs. These RNA-editing enzymes, which can be used to generate *in-vitro* functional RNA modules, are essential for uncovering new RNAs and elucidating their function. However, even with mature commercial enzymes such as T4 RNA ligase, some limitations still exist when used in specific reactions. The major problem for using T4 RNA ligase is the ligation bias, which may influence the ligation efficiency. The bias is mainly dependent on the enzyme itself, the target RNA sequence, the structure, and the reaction conditions, so several studies have been conducted to reduce the bias, with approaches including enzyme engineering [70,71], target sequence optimization [72,73] and regulation of reaction conditions [74]. Furthermore, based on these naturally occurring enzymes from phages, novel functional enzymes can be isolated and characterized from different phages, as was done with the thermostable RNA ligase, and also more enzymes can be generated by design and selection based on the modular architectures of the known enzymes, which offers promising approaches in biotechnology.

2.2.3. Phage-derived RNA-dependent RNA polymerase

In addition to monitoring the dynamics of RNA molecules in cells and engineering RNA molecules into functional modules for synthetic biology, further studies are focused on reconstituting complex biological systems using RNA self-replication as a model for understanding the principles of the evolution of natural microorganisms. As the molecular basis, the RNA-dependent RNA polymerase (RdRP) is the key enzyme for RNA replication in RNA viruses. One property of RdRP is the lack of exonuclease proofreading activity, which results in a high rate of error during its replication ($\sim 10^{-4}$) [75]. Accumulated mutations during replication help RNA viruses evolve under host defense or other environmental pressures [76]. With the help of divalent metal ions, phosphodiester bonds are formed between nucleic acid molecules by RdRP using RNA as a template [77]. RdRP is a multi-domain protein with an average core domain length of less than 500 amino acids that folds to form three subdomains [77]. The active site of RdRP in different RNA viruses is conserved [78–80]. The complementary RNA strands are synthesized starting from the 3′ in the 3′ to 5’ direction in a primer-dependent or independent manner [81]. RNA phages are mainly divided into two families: Cystoviridae (dsRNA phages) with 1 genus, *Cystovirus* with 7 recognized species; and Leviviridae (ssRNA phages) with 2 genera, *Levivirus* and *Allolevivirus*, each of which contain two species [82,83]. The Qβ phage from the genus *Allolevivirus* and MS2 phage from the genus *Levivirus* are typical models for research.

Qβ phage is a positive-stranded RNA phage with a total genome of approximately 4200 nucleotides that expresses four proteins including A2 protein, A1 protein, coat protein and replicase [84] (Fig. 4a). The complete RNA-directed RNA polymerase of bacteriophage Qβ is composed of four subunits, the Qβ-subunit expressed by the phage genome, the translation elongation factors EF-Ts, EF-Tu, and ribosomal protein S1 from the host [85,86] (Fig. 4b). Qβ replicase can recognize special structures formed by the interaction between 5′ and 3’ end of the phage genome to initiate RNA replication. The phage genome can act as an mRNA to synthesize the resulting protein, and at the same time serve as a template to replicate complementary (−) RNA. Both (+) RNA and (−) RNA can serve as replication templates of the Qβ phage for the next round of RNA replication [87] (Fig. 4c). *In vitro*, Qβ replicase can also use other RNA molecules other than the genome of Qβ phage as a replication template [88,89]. Ideally, *in-vitro* RNA replication is exponentially amplified in an autocatalytic manner [88,90]. With its well-characterized structure and function, Qβ replicase has been used in various studies, including Qβ replicase-mediated “template-free” RNA synthesis [91], the discovery of spontaneous recombination of RNA molecules [92], the ability of Qβ replicase to recognize specific templates [93], and the discovery of new functions of ribosomal protein S1 [94]. These studies laid the groundwork for the application of RNA replication both *in vitro* and *in vivo*. However, although Qβ replicase has a research history of more than 40 years, there are still many unsolved questions surrounding this enzyme. Further studies will be needed to understand and regulate Qβ replicase, and also help clarify the role of RNA as genetic material in the RNA world.

MS2 phage is one of the oldest models in molecular biology. Its genome, which was the first to be sequenced, has a total length of about 3569bp and encodes four proteins: mutation protein, coat protein, lysis gene, and replicase β subunit for RNA replication [95]. Similar to Qβ phage, the (+) RNA genome is used as a template to obtain the complementary (−) RNA intermediate for the next step of RNA replication during the replication process of MS2 phage. MS2 replicase was the first RdRP to be isolated and studied [96,97]. However, due to its instability and challenging purification [96], most applications of MS2 phage were focused on RNA-RNA and RNA-protein interactions, such as the RNA imaging mentioned above. A recent study also reported the use of MS2 phage replicase to construct an *in-vitro* expression system, which provides new ideas for studying the template specificity of MS2 replicase and designing gene circuits with DNA- and RNA-encoded systems [98]. In addition, there are also reports on the replicases of other types of RNA phages, such as R17 and f2 phage belonging to group I [99,100], GA phage belonging to group II [101], SP phage belonging to Group IV [102], and the dsRNA phage ϕ6 belonging to the Cystoviridae family [103]. However, the specific role of these replicases in RNA replication is currently unknown.

As an enzyme for amplifying RNAs that contain specific probe sequences *in vitro*, the RdRP from phages, especially Qβ replicase, can be used as an alternative to polymerase chain reaction to obtain massive amounts of RNA under appropriate *in-vitro* reaction conditions. However, although the RdRP has a long research history, it is still keeping many secrets. In the case of Qβ replicase, the template recognition and conformational changes remain unclear. At the same time, the replication efficiency and fidelity also need to be balanced when large amounts of RNA are amplified. All of these aspects will lead to unpredictable results when the replicase serves as the key enzyme for RNA replication, and these problems will be discussed in detail in the next chapter.

3. RNA-replication-based synthetic systems mimicking the phage lifecycle

From the bottom up, synthetic biology is attempting to design and construct novel functional components, networks, and pathways, aiming to achieve reprogramming and rebuilding of live organisms. The core common feature of living cells is the replication of genetic information, mutation and heredity, enabling them to evolve [104]. To date, a large variety of complex biological phenomena in living organisms has been reconstituted on the basis of several cellular functions. Among them, the RNA replication process derived from RNA phages provides a classical model for exploring the properties of living systems, while potentially also providing insights into the RNA world. The research on synthetic systems based on RNA replication contributes to the development of new technologies and the reprogramming of chassis cells for multiplex synthetic applications.

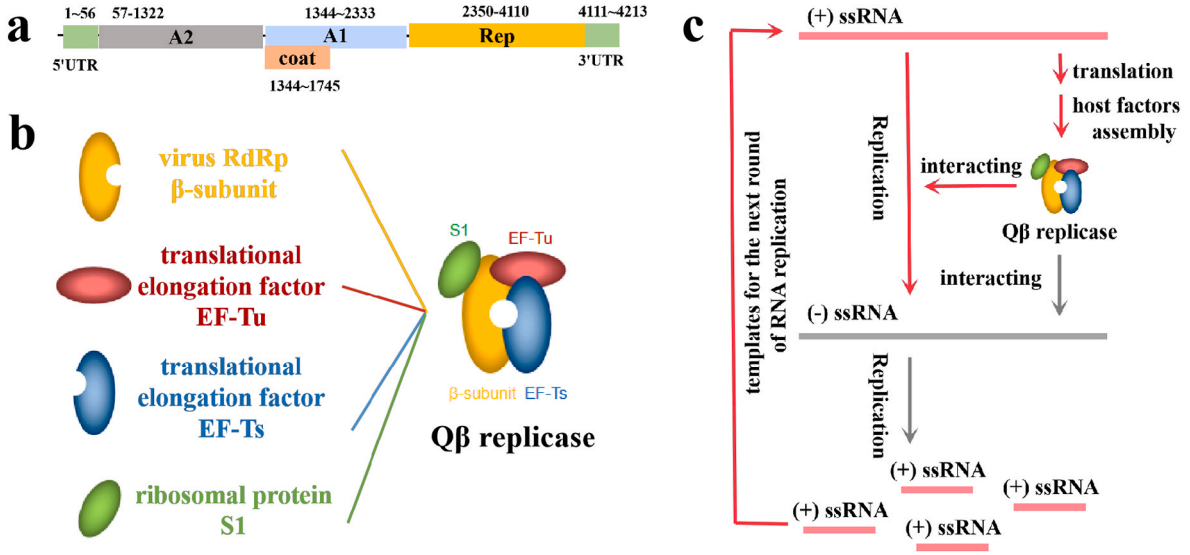

Fig. 4. Schematic representation of Qβ phage. a) Composition of the Qβ phage genome. The whole genome of Qβ phage is approximately 4200 nucleotides and encodes four proteins, including A2 maturation protein, A1 protein with unidentified function, coat protein and replicase β-subunit. b) Composition of the Qβ replicase complex. The complete RNA-directed RNA polymerase of bacteriophage Qβ consists of four subunits: the β-subunit expressed by the phage genome, the translation elongation factors EF-Ts, EF-Tu, and ribosomal protein S1 from the host. The β-subunit is the catalytic active site for RNA replication while the three host-derived factors help initiation and elongation of the RNA strand. c) Replication of Qβ phage RNA. The phage genome can act as a mRNA to synthesize the resulting replicase, and at the same time serves as a template interacted with the replicase to replicate complementary (−) RNA. Then huges amounts of (+) RNA can be synthesized through the interaction between (−) RNA and the replicase. Both (+) RNA and (−) RNA can serve as replication templates of the Qβ phage for the next round of RNA replication.

3.1. Development of new technologies

The amplification of the genetic material of almost all RNA viruses is entirely performed by RNA replication, with the exception of retroviruses. Compared to eukaryotic hosts, the RNA replication process of phages in prokaryotic hosts is relatively simple. Due to their small genomes and simple genetic constitution, the RNA replication and related regulatory factors of phages have a well-established research background. As the simplest (+)-RNA virus, Qβ phage can play a major role in providing the catalytic enzymatic basis for RNA-dependent RNA replication in synthetic systems. Many important results related to Qβ replicase, including the kinetics [105–108], structure [109–111], related host proteins [112–115] and mechanism of template recognition and initiation [116–119], have established Qβ phage as the pioneering model for applications of RNA replication and laid a solid foundation for exploring and employing the RNA replication process. Compared to replicases from other viruses, Qβ replicase has advantages in terms of purification efficiency, activity preservation in cell-free extracts, RNA amplification rate and template specificity [120], which makes Qβ replicase the prime choice for RNA replication in different scenarios.

One of the most practical applications of the RNA replication process is *in-vitro* amplification of specific RNAs. The Qβ replicase system is widely used as a cell-free system able to replicate RNA exponentially as long as there is an excess of RdRP in the reaction system. In fact, it can amplify a single RNA molecule into 1×10^{12} molecules or 122 ng of RNA molecules in about 10 min [121,122]. Based on the ability of RNA replicase to rapidly amplify RNA *in vitro*, Qβ replicase was used to amplify specific RNA probes and applied in diagnostic tests. Pritchard et al. [123] used Qβ replicase as a means of amplifying short RNA probes to rapidly quantify trace probes in solution. Benjamin et al. [124] employed an automated Qβ replicase assay to amplify signal probes to detect the rRNA of four respiratory pathogens. Sanjay et al. [125] constructed a specific and sensitive nucleic acid amplification assay for routine gene detection based on the amplification capabilities of Qβ replicase. The ability of Qβ replicase to exponentially amplify RNA probes targeting nucleic acid molecules of interest provides another option for medical testing.

The replication ability also enabled Qβ replicase to be used in sequencing. Makeyev et al. [126] attempted to directly sequence RNA using the *in-vitro* replication process of RNA replicase. The sequencing principle is similar to the Sanger sequencing method [127], but the DNA polymerase was replaced with RNA replicase, and the template was also replaced with RNA. However, because this sequencing method is limited by the template specificity of RNA replication, it can only detect certain RNAs with known sequences, such as rapid sequencing of some mutant viruses, etc.

Different from traditional DNA-dependent RNA polymerases, which use DNA as the template to initiate transcription and produce a target RNA, the RdRp represented by Qβ replicase can synthesize large amounts of RNA products in a short time, which may produce large amounts of target proteins at a high rate. At the same time, the RNA replication process is not contrary to the RNA synthesis process mediated by traditional RNA polymerases, both of which can be combined. Therefore, RNA replication mediated by Qβ replicase, usually coupled with transcription and translation, was employed to amplify recombinant mRNA and synthesize specific proteins. Wu et al. [128] inserted an mRNA into the sequence of MDV-1 RNA, which recognized and replicated by Qβ replicase. These recombinant mRNAs were amplified exponentially by Qβ replicase and could express enzymatically active proteins. In a study by Ryabova et al. [129], the recombinant mRNAs embedded within the MDV RNA sequence were replicated and the proteins were efficiently synthesized in *in-vitro* reactions. These studies indicate the possibility of utilizing RdRPs such as Qβ replicase to generate large amounts of recombinant mRNAs for protein synthesis. To select mRNA sequences that are more functionally replicable by Qβ replicase, Yumura et al. [130] established a new combinatorial selection method to find an RNA sequence with secondary structures replicable by Qβ replicase while maintaining the gene function. It is extremely appealing to utilize the ability of Qβ replicase to amplify recombinant mRNAs exponentially, which shows great promise for the overexpression of specific proteins.

3.2. Reprogramming chassis cells for multiplex synthetic applications

3.2.1. The in vitro reconstitution of artificial systems based on RNA replication

Reconstitution of artificial living systems can shed light on the basic design rules of evolution and contribute to many new technologies such as *in-vitro* protein modification, engineering cellular behavior, as well

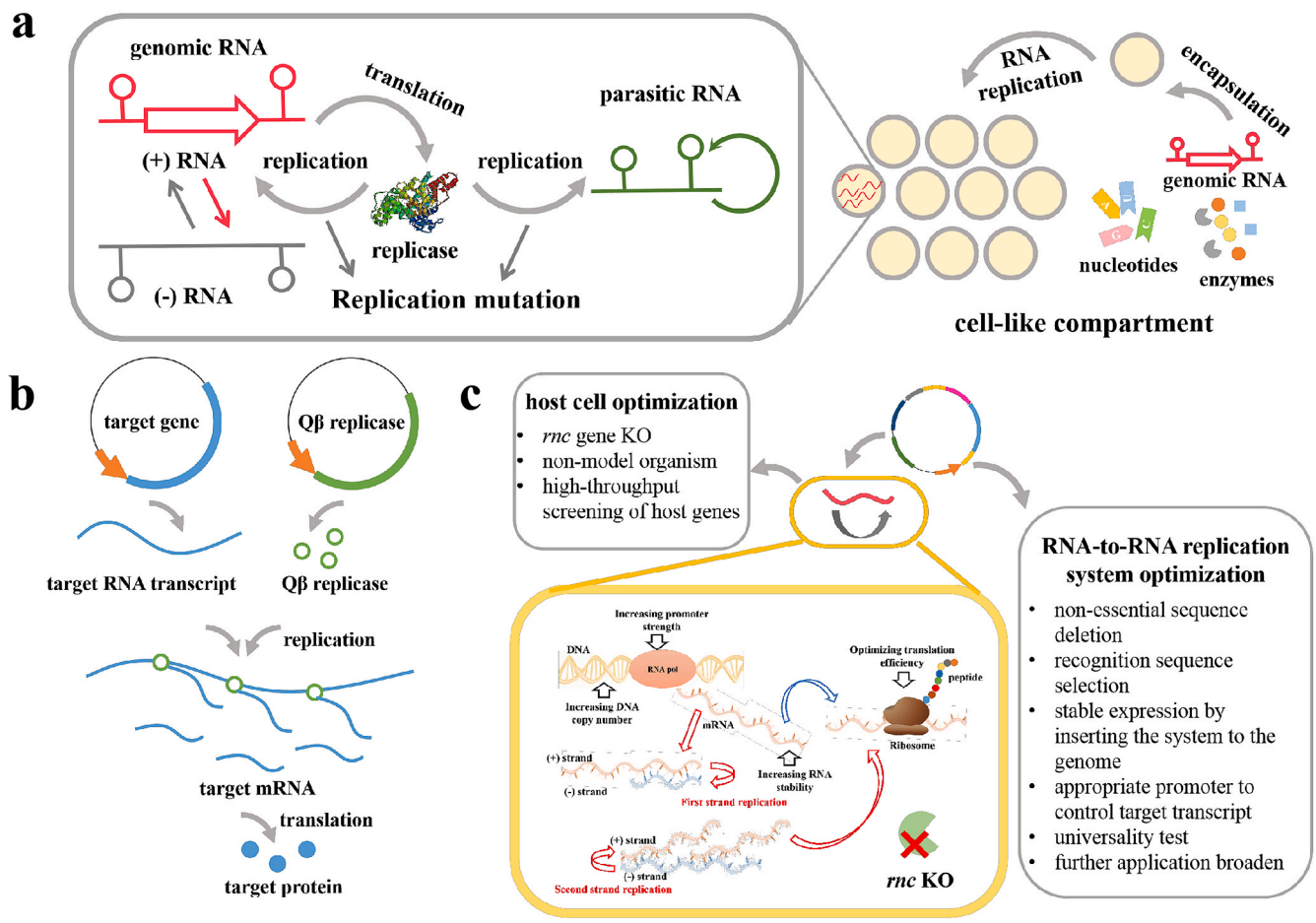

Fig. 5. Reprogramming of chassis cells for multiplex synthetic applications based on RNA replication. a) *In vitro* reconstitution of artificial systems. RNA replication *in vitro* coupled with other biological process is the classic model for the study of rules that guide Darwinian evolution and building the smallest artificial living system. The artificial genomic RNA can replicate via the translation of the replicase. The error-prone replication process results in the occurrence of mutant genomic RNA (parasitic RNA). The genomic and parasitic RNAs compete for the reaction resources and undergo several rounds of error-prone RNA replication to spontaneously introduce mutations into the RNAs, which results in the evolution of the system. The artificial genomic RNA and essential reaction substrates are usually encapsulated in the cell-like compartment to ensure long-term replication. b) The earliest specific RNA replication platform using Qβ replicase *in vivo*. The phage-like RNA sequence was successfully transcribed from a plasmid and replicated by Qβ replicase, producing replicated mRNA which could be translated into functional proteins by Qβ replicase. c) The RNA-to-RNA replication system in bacteria. Based on research on the Qβ phage and RNA replication *in vitro*, Yao et al. constructed a RNA-to-RNA replication system to enhance the expression of genes of interest in bacteria. A simplified replicable sequence containing a 5′UTR, a 3′UTR, the target gene, the replicase β-subunit and other functional elements was inserted into the genome of the bacteria to generate a stable replicable template. The RNA-to-RNA replication system was optimized in different forms, and verified that the system could be used to increase the expression of distinct target genes for further applications. The host cell was optimized by knocking out the host *rnc* gene influencing the RNA replication, conducting high-throughput screening to select endogenous genes and confirming the effectiveness of the system in non-model organism.

as drug delivery, therapy and biosensing, all of which greatly promote the development of synthetic biology [131–133]. One of the most important applications of RNA replication *in vitro* is the study of rules that guide Darwinian evolution and building the smallest artificial living system (Fig. 5a). Because the RNA replication process is simple and self-driven, the accumulation of parasitic RNAs from the artificial genomic RNA replication mutation and their competitive interactions provide a good model for studying evolution and constructing artificial life forms. As early as 50 years ago, Spiegelman et al. [134] incubated the Qβ genome with single nucleotides and replicase in a reaction medium. After several transfers to fresh medium, the earliest artificially constructed evolutionary system was tested. Then, more self-replicating RNA systems were constructed and evolution during the replication process was detected [135,136]. There are many challenges to building an evolvable cell-like system entirely from non-living molecules in the field of *in vitro* synthetic biology. Key questions must be answered, including how the system starts the evolution and how the evolution proceeds in real life, which leads to concerns about reaction substrate supply, duration of homeostatic reaction processes, removal of side-reaction products and precise design and prediction of the evolutionary process [137–139]. To solve these problems, many studies explored the design rules for building biological systems and developing new technologies. Oberholzer et al. [140] combined Qβ replicase with self-expanding and spontaneously isolated liposomes to construct liposome microspheres that can grow, isolate, and amplify RNA. This research first purposed an approach that compartmentalized the enzymatic RNA replication substrates into a cell-like self-reproducing membrane to protect the internal microenvironment from external factors, remove the side products influencing reaction activity, and further provide opportunities for segregating complex reactions into units, enabling multi-level *in-vitro* evolution and controllable communication. Kita et al. [141] introduced a translation process and successfully balanced Qβ replication with translation in an *in-vitro* translation system. Ichihashi et al. [142] used a Qβ replication-translation system to mimic equilibrium in Darwinian evolution. This research team also developed a method for *in-vitro* combinatorial screening that can be replicated by Qβ replicase and maintain the function of the encoded protein [130]. They also constructed an automated *in vitro* evolution system based on a

translation-coupled RNA replication system in a droplet flow reactor [143]. These basic research studies and the resulting applications are of great significance for understanding the origin of life and constructing evolvable artificial life forms.

The construction of an artificial cell-like system is a great challenge. Based on the extensive research on the replication process of Qβ phage, the self-replicating RNA derived from Qβ phage coupled with other biological processes like translation can be used to construct an artificial system that performs a continued evolutionary process similar to primitive host species. Previous studies have revealed some possible evolutionary principles of RNA replicators and roles of cell-like structures, which can provide insights into evolutionary scenarios that may have led to the assembly of chemical molecules into the first "living" cells. The combination of RNA replication systems with other gene expression systems contributes to an experimental model of dynamic variation in the RNA-protein world. Furthermore, some interesting questions on artificial self-replication systems remain to be answered, such as the selection and extension of recognition templates for Qβ replicase in the system, the mutation principles of target sequences, and the improvement of the system to survive for long periods of time and to communicate with other systems or living cells. Many attempts are being made to further understand the design principles of biological systems and to develop new technologies for synthesizing life.

3.2.2. The in vivo integration of an RNA replication system

The bottom-up approach to reprogramming chassis cells based on RNA replication focuses on assembling different artificial components into a "living" system. In spite of the simplicity and controllability of the bottom-up strategy for building artificial RNA replication systems, most of the synthetic applications focus on directly engineering chassis cells to perform a specific task. The integration of an RNA replication system *in vivo* adopts a top-down approach to provide an alternative approach for modifying an existing cellular chassis and exploring the RNA replication process in complex cellular environments.

The basic research on Qβ replicase as the prototypical RdRP has provided standards for designing and debugging a feasible RNA replication system to amplify not only specific template sequences but also recombinant mRNAs embedded within the templates [128,129], which proved that the recombinant mRNAs could be regulated at the RNA level as the templates for Qβ replicase, as well as be translated into functional proteins. These preliminary studies demonstrated that the essential components that constitute an RNA replication system include a reaction environment, the replicable template and the RdRP. The earliest study on replicating a specific RNA using Qβ replicase *in vivo* in order to translate the *in vitro* research platform was reported by Mills et al. [144]. The phage-like RNA sequence was successfully transcribed from a plasmid and replicated by Qβ replicase, achieving the first reported replication of an active mRNA by Qβ replicase in the *E. coli* host (Fig. 5b). The authors also preliminarily discussed the evolution of self-replicating RNA in the cellular environment, but many details still needed to be defined. However, subsequent applications of the RNA replication process *in vivo* remain very limited.

With many secrets to be revealed, it has been a desirable goal to develop a general method of amplifying functional RNAs *in vivo*, both for novel tools and cellular evolution research. In 2019, our team [145] constructed an RNA-to-RNA replication system to enhance the expression of genes of interest in bacteria (Fig. 5c). Based on the considerations about RNA replication models and key modules required for RNA replication, a simplified and stable RNA-to-RNA replication system was constructed *in vivo*. Several modules of the system were debugged in the *E. coli* host, including the appropriate promoter to control target gene and replicase transcription levels, the system's expression form, and the inserted target gene length. It was verified that the system could promote the expression of different target genes (long-coding mRNAs) at the RNA level and the protein level. Overall, our study provides an alternative to increase protein expression in bacteria using RNA-to-RNA replication.

The top-down approaches to reprogramming the chassis cells enable tight and predictable control over cellular behavior, while adaptation of the chassis cells and the regulatory tools should be considered. In our research on the RNA-to-RNA replication system, we regulated different modules of the system to make it more applicable in the *E. coli* host, and also found that the *rnc* gene encoding double-strand RNA (dsRNA)-degrading RNase III could interfere with the RNA replication process. To fully understand the interaction between a new cellular behavior and the chassis cell, we further used high-throughput screening methods to select endogenous genes influencing RNA replication (unpublished data). We also used the non-model organism *Lactobacillus casei* Zhang in addition to *E. coli* and identified the applicability of the system across different chassis cells, mainly including bacteria. The results demonstrated the adaptability of the *in-vivo* RNA replication system in more complex chassis cells like yeast or mammalian cells with their corresponding endogenous pathways. Overall, we aim to confirm the essential modules constituting the RNA-to-RNA replication system *in vivo*, coupled with transcription and translation processes, and explore the host factors involved in the chassis cell's adaptation to the foreign system.

In the original transcription-translation pathway, RNA self-replication is introduced as a form of cellular behavior that the chassis cell does not have, which triggers new challenges for the chassis cell that impose requirements on the foreign system. Recent studies on the *in vivo* integration of RNA replication systems are relatively limited, but the existing research basis also provides a platform for studying and utilizing replicating RNA molecules *in vivo*. Previous studies have provided a workable strategy for replicating functional RNAs *in vivo*, offered a preliminary discussion of the cellular evolution of self-replicating RNA, and explored possible interactions between the system and the chassis cells. Very promising approaches include modeling the *in-vivo* RNA replication process, extending the scope of chassis cells, identifying the potential functional endogenous genes, and finally developing a better understanding of general concepts in the evolution of life.

4. RNA-based technology modeled on phage-bacterial interactions

The interactions between phages and their hosts have always been an appealing research field that contributed to many valuable findings. The phage and its bacterial host have evolved together for billions of years. The survival pressure and crisis brought by the phage to the host bacteria has triggered different defense mechanisms against phage infection [146]. Accordingly, phages have also developed various strategies to escape these defense mechanisms [147]. The derived technologies not only function in the interacting host, but can be used to modify various valuable chassis cells.

4.1. Toxin-antitoxin system based on RNA interaction

The toxin-antitoxin (TA) system is a subtype of the abortive infection system (Abi), which is a defense mechanism by which bacterial hosts restrict phage. The TA systems are composed of small genetic modules mainly containing a toxin and an antitoxin neutralizing its effect. By influencing specific gene expression at the transcriptional or post-transcriptional level, TA systems play an important role in various cellular process such as phage defense, stress resistance, cell state regulation, biofilm formation or programmed cell death [148–152]. By exploring the regulatory mechanisms, multiple elements involved in RNA-based regulation of TA systems have been identified, including functional sRNAs and endoribonucleases that may target mRNA, tRNA or rRNA [153–156]. These studies open up new opportunities for developing RNA-based molecular tools and eventually new medical therapies.

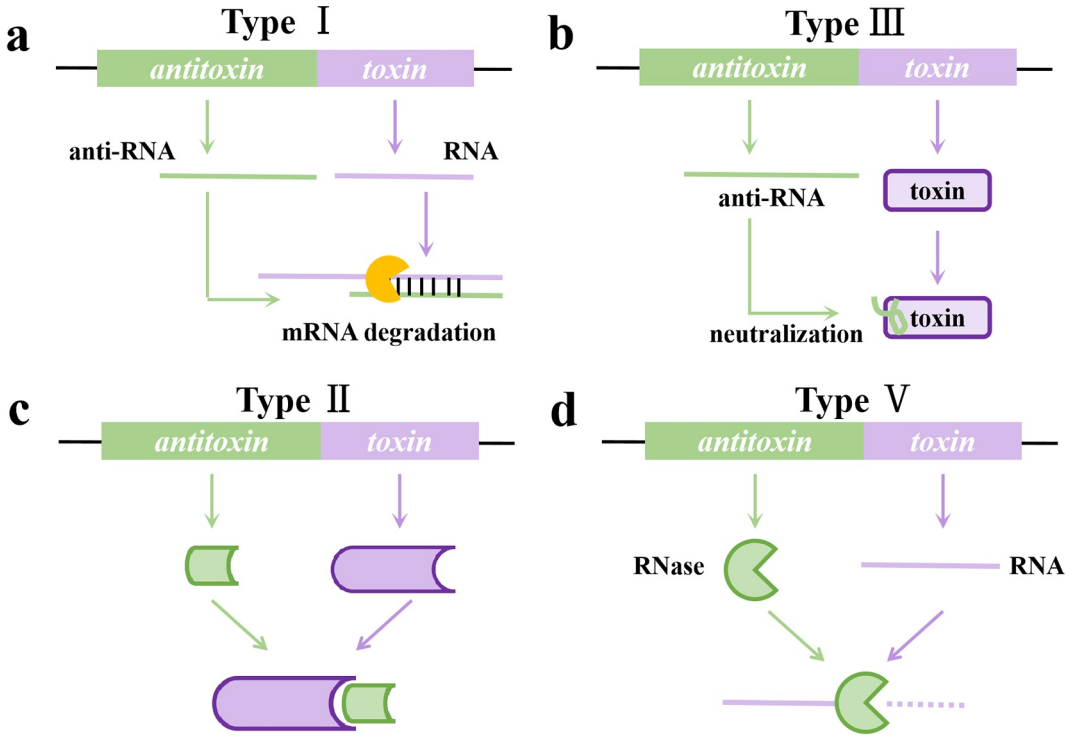

Fig. 6. Different types of TA systems. a) Type I TA system. The RNA duplex formed by the binding of the toxin mRNA and the antitoxin RNA with antisense sequence leads to the degradation of toxin mRNA and thus inhibits toxin production. b) Type III TA system. The antitoxin RNA molecules directly bind the toxin protein to inhibit its function. c) Type II TA system. Antitoxin directly binds and inhibits the toxin via protein-protein interaction, while some toxin proteins are determined to have endoribonuclease activity and target RNAs. d) Type V TA system. The antitoxin acts as a kind of RNase to cleave the toxin mRNA.

4.1.1. The basis of RNA-targeted TA systems

The TA systems can be classified into at least 8 groups according to their binding partners and modes of action with new systems constantly being discovered [157–160]. Most of the known TA proteins are encoded by plasmids, but recent genomic studies found that the chromosomes of many bacteria and archaea also have “TA loci” [161–163]. In type I and III loci, the antitoxin is an RNA, but they function in different ways. Type I antitoxins target the toxin mRNA to promote its degradation or inhibit its translation (Fig. 6a), while the antitoxin RNA of type III system directly binds to the toxin protein and inhibits its function (Fig. 6b). In the most researched type II system, the antitoxin directly binds and inhibits the toxin via protein-protein interactions, while some toxin proteins were identified to have endoribonuclease activity and target RNAs [164] (Fig. 6c). The antitoxin of type V system acts as an RNase to cleave the toxin mRNA [165] (Fig. 6d). There was also newly identified TA system reported to function via anti-sense binding of the antitoxin to the toxin small RNA [166]. Studies of RNA interactions in these TA systems can improve our understanding of the functions of TA loci in biological systems and are potential sources of RNA-related tools.

4.1.2. The applications of RNA-targeted TA systems

Molecular biology tools derived from TA systems mainly focus on positive selection for cloning and protein expression stability maintenance [167,168]. The toxins with endoribonuclease activity for functional RNA sequences were engineered to construct various positive or negative systems and to select modified chassis cells based on lethal effects brought by the toxin and their repression by toxin-antitoxin interaction. The RelE toxin, which is an mRNAse, was developed into a chromosomal manipulation selection system in *E. coli* and further optimized as a negative selection marker for genetic constructs [169,170]. The Kid toxin and its homologues such as MazF and ChpBK, which were demonstrated to bind and cleave specific RNAs [171], were also reported to maintain the function of engineered genes [172–174]. These selection platforms can avoid the cost and inconvenience of antibiotic selection. For stable protein expression, tools such as the Kid-Kis TA system or the RelE-RelB TA system and MazF-MazE TA system were engineered to provide a selection platform for enriching cells with strong heterologous gene expression in eukaryotic cells [175–177]. At the same time, several research teams demonstrated that TA systems could also be applied to program targeted cell death or survival with the help of other synthetic regulatory elements in plant cells or other higher eukaryotic cells [178–181]. These basic molecular tools derived from TA systems based on targeting essential RNAs hold great promise in synthetic biology due to their wide host range and versatile usage.

The application of TA systems in medical therapies is also appealing due to their RNA-cleavage and cell-killing function. The diversity of TA systems’ ribonuclease activity has enabled their use as defense against RNA viruses (Fig. 7a). The well-studied MazF toxin that can specifically cleave ACA-containing mRNA [182] was used to prevent human immunodeficiency virus-1 (HIV-1) replication in human T lymphoid cells [183]. The *mazF* gene was controlled by the TAR promoter from HIV-1, and its expression was induced by the viral Tat protein resulting from virus infection, leading to the cleavage of HIV-1 mRNA containing several ACA sequences. The antiviral process was a typical combination of an RNA-targeted TA system and synthetic gene circuit for medical treatment. The MazF-MazE TA system was also explored to interfere with Hepatitis C virus (HCV) infection using an expression cassette consisting of the system and HCV-encoded protease [184]. This kind of antiviral mode can be applied to other RNA-targeted TA systems and RNA viruses by constructing a virus-responsive genetic circuit, which reduces the negative effect of TA systems on host cells. As many TA systems have been identified to control the fate of eukaryotic cells [179,180], TA systems can also be engineered to kill tumor cells (Fig. 7b). A synthetic system based on the Kid-Kis TA system autonomously functioning by oncoprotein induction was constructed to distinguish between oncogenic cells and normal cells [180]. The MazF-MazE TA system was demonstrated to selectively eradicate cancer cells

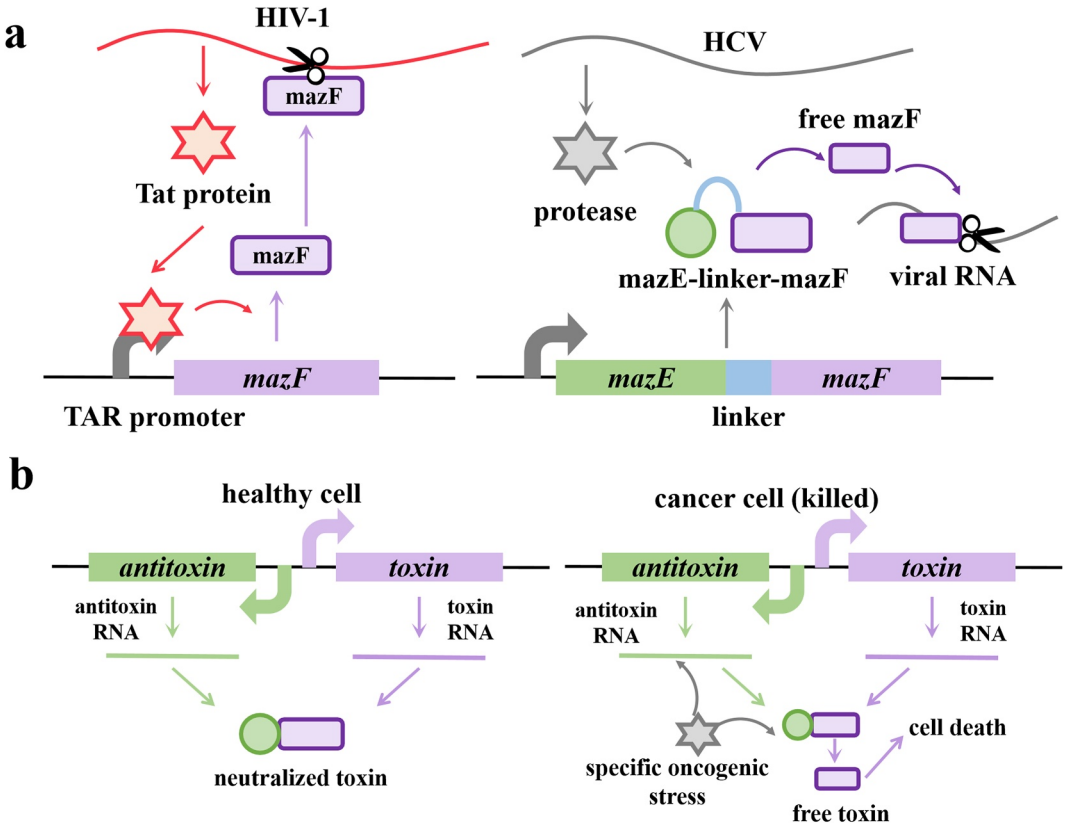

Fig. 7. Applications of RNA-targeted TA systems. a) Typical models for TA systems to defense against RNA viruses. The *mazF* gene was controlled by the TAR promoter from HIV-1 and the expression was induced by the viral Tat protein resulting from virus infection, leading to the cleavage of HIV-1 mRNA. The MazF-MazE TA system was also applied to interfere with HCV infection by using HCV-encoded protease to cleave the linker between the toxin and antitoxin and thus release the neutralized toxin mazF to target the virus. b) TA systems for killing cancer cells. TA systems can be engineered to distinguish between oncogenic cells and normal cells in a synthetic system. In healthy cells, the toxin is neutralized by its antitoxin and keeps the inactive state. In cancer cells induced by specific oncogenic stress, the antitoxin is disrupted in mRNA- or protein-level to trigger the function of the toxin, thus selectively killing cancer cells.

by an innovative cassette for controllable toxin expression [185]. Different RNA-targeted TA systems for the selective killing of cancer cells continue to be developed and optimized using rationally designed programmed synthetic genetic devices [186,187]. TA systems for selective cancer cell removal show great promise, but the interaction among functional TA modules, cancer cells and normal cells should be better evaluated.

At present, the understanding of TA systems is still very limited, and their mechanisms of functioning as well as the involved host signaling pathways appear to be far more complicated than initially thought. These systems constitute a conserved family with similar regulatory properties and provide new models for RNA-targeted action. The TA systems have several advantages, including their small functional module size, the ability to select efficient toxins in bacteria, and their wide range of potential applications. Previous studies showed that the tools derived from TA systems could be applied in different bacterial species and be further adapted to overcome drawbacks in eukaryotic cells [168]. In future development, factors to be considered should include the influence of the secondary structure of RNA molecules involved in the TA system, the influence of host regulatory factors, the hierarchical role of the TA system module, and the design of TA systems.

4.2. RNA-targeted CRISPR-Cas technology

Clustered regularly interspaced short palindromic repeats (CRISPRs) in conjunction with a Cas-nuclease constitute the CRISPR-Cas adaptive immune system, which are widespread in bacteria and archaea to defend themselves against infection by viruses or foreign plasmids [188,189]. In recent years, the tools developed based on the CRISPR-Cas system are widely used in biotechnology, production, and medicine. The chassis cell is varied from the model microorganism *E. coli* to the cell that has difficulty in genome editing like cyanobacteria [190]. Typical gene editing tools such as the CRISPR-Cas9 and -Cas12 systems mainly work on DNA sequences. To expand CRISPR-related tools based on RNA-level gene expression regulation, various studies were conducted to construct CRISPR-based tools that recognize and cleave RNA molecules, which is also a further step in introducing the CRISPR-Cas system into the RNA world.

4.2.1. Diverse RNA-targeted CRISPR-Cas systems

Among the six subtypes of CRISPR-Cas systems (type I-VI), type VI systems can target RNA [191–193], while type III systems can target both DNA and RNA [194,195]. Type II system have also been found to have three subtypes targeting RNAs [196–198] (Fig. 8a). In type III systems, the RNP complex consists of 4–6 different Cas proteins, and these effector proteins can have both DNase and RNase activity [199,200]. The pairing of the crRNA with the region complementary to the target RNA is sufficient for type III systems to recognize and destroy the target RNA through RNase activity internally located on the crRNP backbone in the effector complex. As the most widely used gene editing tool, the CRISPR-Cas9 system (type II) usually recognizes and cleave dsDNA, but analysis of different Cas9 protein families revealed that some proteins can target RNA in addition to DNA [197,198,201], and these are called RNA-targeting Cas9 (RCas9). Among the currently known prokaryotic adaptive CRISPR-Cas immune systems, the type VI is the only one that exclusively targets RNA. Similar to the Type III CRISPR-Cas systems, the type VI-Cas13 system functions by targeting RNA based on conformational changes of the effector complex [202,203]. The application of these RNA-targeted CRISPR-Cas systems has been explored in a number of studies, which is discussed in detail below.

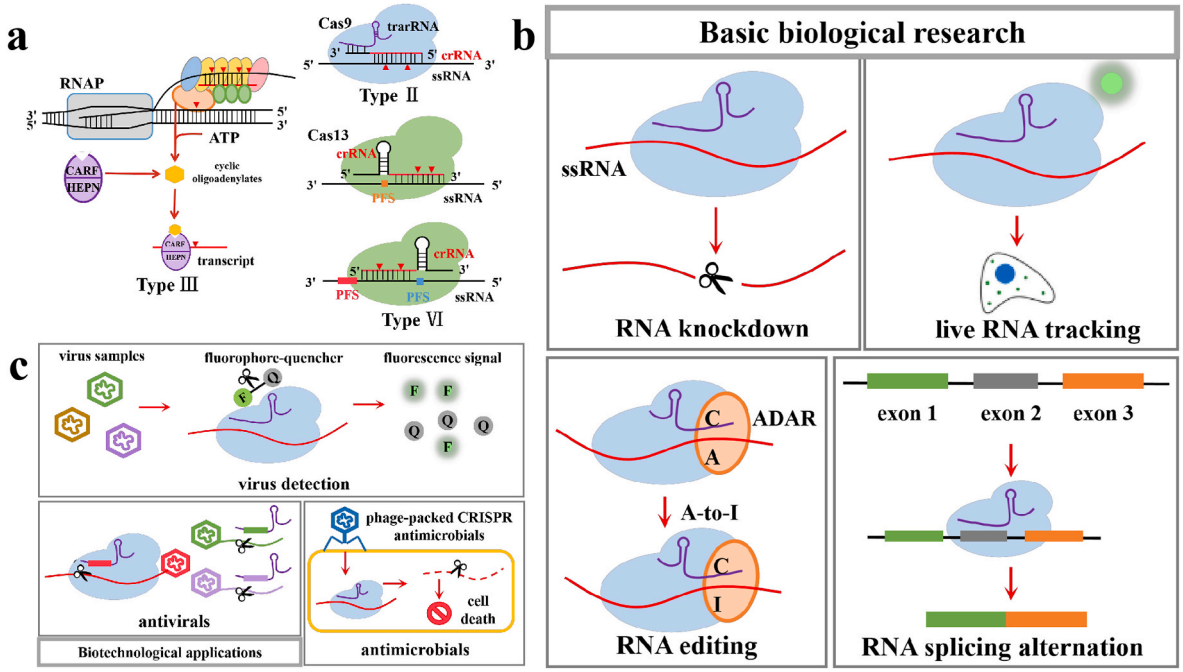

Fig. 8. RNA-targeted CRISPR-Cas systems and applications. a) Diverse RNA-targeted CRISPR-Cas systems. Type III CRISPR systems are composed of multi-subunit crRNP effector complexes, which can trigger both DNase and RNase activity. The pairing of the crRNA with the region complementary to the target RNA can enable the system to recognize and destroy the target RNA. Some types of CRISPR-Cas9 system (type II) can also target RNA. The type VI-Cas13 system functions by targeting RNA based on conformational changes of the effector complex with protospacer flanking site (PFS) preference for Cas13a and Cas13b. b) Basic biological research. The ability of CRISPR-Cas systems to target and cleave RNA contributes to RNA knockdown in cells, while the catalytically inactivated RNA-targeted Cas proteins can be developed into efficient RNA-binding platforms. RBPs derived from dCas9 or dCas13 fused with fluorescent proteins or other tags are powerful tools for RNA tracking. In addition, the fusion of dCas13 orthologs to diverse effector proteins enabled the targeted RNA sequence editing such as A-to-I editing and splicing alteration. c) Biotechnological applications. RNA-targeted CRISPR-Cas systems can be used to detect specific RNAs of viruses by cleavage of the RNA linking the fluorophore and quencher to release the fluorescence signal. The systems also enable programmable RNA virus inhibition by targeting the conserved genomic sequences. Based on the function of specifically targeting transcripts of antimicrobial resistance genes, the CRISPR-Cas13a system delivered by phages has been developed to combat antimicrobial resistance.

4.2.2. *Applications of RNA-targeted CRISPR-Cas technology*

4.2.2.1. Basic biological research. The rapid development of diverse RNA-targeted CRISPR-Cas systems that can selectively bind and manipulate RNA molecules has broadened the range of molecular regulation at the post-transcriptional and translational level. The emerging CRISPR-Cas-based RNA-targeted tools show great potential for application including RNA knockdown, RNA imaging and RNA editing (Fig. 8b).

The ability of these CRISPR-Cas systems to target and cleave RNA contributes to the most common usage of these systems for RNA knockdown. As the most well-characterized CRISPR-Cas system, the type III system was first discovered to recognize and cleave specific RNAs [204]. The system has been applied to interfere with the function of non-essential genes in prokaryotes, similar to the use of RNAi in eukaryotes [205,206]. However, the Cas protein involved in the type III system is complex, which limits the heterologous expression of the functional effector complex. Consequently, the type II-RCas9 and type VI-Cas13 systems were explored more for targeted RNA knockdown. Cas9 from *Streptococcus pyogenes* (SpyCas9), as well as other Cas9 proteins and variants have been found to specifically cut RNA substrates [196,198,201,207], and the mechanisms, design rules and prospective applications of these systems were explored in numerous studies. Due to its efficiency and simplicity, the Cas13 system has been rapidly developed for reporter or endogenous transcript knockdown in a wide range of hosts including bacteria, mammalian cells, insects and plant cells [208–210]. Compared to the traditional RNAi knockdown method, the Cas13 system shows advantages in terms of efficiency and specificity, which makes it an attractive alternative in many cases.

The catalytically inactivated RNA-targeted Cas proteins (dCas9 and dCas13) still keep their RNA-binding affinity and can be developed into efficient RNA-binding platforms. RBPs derived from dCas9 or dCas13 were applied for target RNA capture and RNA-protein interactions [201,210,211]. The practicality of dCas9-or dCas13-based RBPs fused with fluorescent proteins or other tags also make them powerful tools for RNA tracking and live-cell RNA imaging. David et al. established RCas9 as a platform to track RNA in living cells in a programmable manner, and achieved real-time imaging of endogenous mRNA trafficking to stress granules [212]. Orthogonal dCas13 proteins were engineered and screened to achieve efficient and controllable RNA imaging [210,213,214]. Compared to phage-derived RBPs for RNA imaging such as the widely used MS2-MCP system, the RNA-targeted CRISPR-Cas system targets specific RNA sequences using a crRNA, and may simultaneously track different targets by designing different crRNAs, which can be simplified and programmed without genetic manipulation or influence from exogenous insertion of hairpins. The CRISPR-Cas systems can also be orthogonally applied or combined with MS2-MCP or dCas9 to achieve simultaneous visualization of genomic DNA and RNA transcripts in living cells [214]. In addition, the fusion of dCas13 orthologs to diverse effector proteins enabled targeted RNA sequence editing. Cox et al. constructed an RNA editing system called REPAIR (RNA Editing for Programmable A to I Replacement) by fusing ADAR2 (adenosine deaminase acting on RNA type 2) to dCas13, which enabled highly specific A-to-I RNA editing in mammalian cells [215]. The most efficient RNA-cleaving proteins developed from several engineered Cas13d orthologs were used to mediate splice regulation [195]. Other effector domain fusions such as biotin, eukaryotic RNA-modifying enzyme N6-methyladenosine (m6A) or RNA endonuclease further broadened the toolbox of RNA editing platforms [201,216–218].

4.2.2.2. Biotechnological applications

The emerging RNA-targeted CRISPR-Cas systems provide new approaches for exploring the RNA world, enabling great advances in biotechnological applications for understanding and regulating

essential life processes.

These systems have shown great advantages for constructing fast and sensitive nucleic acid detection platforms and powerful diagnostic tools for clinical and environmental samples (Fig. 8c). The SHERLOCK nucleic acid detection platform derived from the Cas13 system can quickly, sensitively and accurately detect specific RNAs and DNAs in mixed samples [219–221]. Diverse viruses, including Zika, Dengue, Ebola and avian influenza A (H7N9) can be efficiently detected using these platforms [222–224]. In the recent outbreak of a new coronavirus, research teams quickly developed protocols suitable for purified RNA, which may help develop CRISPR-based diagnostic methods for the clinical detection of COVID-19, and establish a SHERLOCK-based test method for rapid test strips [225]. Furthermore, a platform for scalable, multiplexed pathogen detection named Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids (CARMEN) was developed to simultaneously differentiate all 169 known human-associated viruses and rapidly detect the causative agent of the 2020 COVID-19 pandemic [226]. These studies have provided a basic framework for diagnosing the new SARS coronavirus, with great significance for medical treatment.

In addition to diagnostic assays, the RNA-targeted CRISPR-Cas systems can also enable programmable inhibition of RNA viruses, making them into antiviral agents (Fig. 8c). The Cas9 from *Francisella novicida* (FnCas9) was shown to confer resistance against several RNA viruses in mammalian and plant cells [227,228]. The Cas13 systems were also demonstrated to target RNA viruses or DNA virus intermediates. Freije et al. identified numerous potential Cas13 target sites located in ssRNA viral genomes and tested the developed system's ability to defend against three ssRNA viruses in cell cultures [229]. A recent study demonstrated a CRISPR-Cas13-based strategy for effective viral RNA inhibition and degradation for SARS-CoV-2 and live influenza A virus (IAV) [230], which could help solve this ongoing medical crisis. The Cas13-based antiviral methods were also extended from mammalian cells to plant cells to combat specific plant viruses, which provides new strategies for controlling plant diseases [231,232].

The RNA-targeted CRISPR-Cas systems may directly assist in treating diseases related to RNA abnormalities. Researchers purposed an RCas9-based platform to reverse the molecular pathology associated with adult onset muscular dystrophy in adult mouse muscles [233]. This CasRx system was rapidly developed into an efficient tool for modulating metabolic functions in mouse hepatocytes [234] and for functional conversion of neurons [235]. The systems were also extended to cancer therapies, which was summarized in detail in a recent report [236].

Furthermore, it is an attractive strategy to use the CRISPR-Cas systems as a novel tool to combat emerging antibiotic-resistant bacteria (Fig. 8c). Most of the CRISPR-Cas systems applied to combat antimicrobial resistance are based on their function of specifically targeting and cleave DNA sequences encoding antibiotic resistance genes, thereby reducing the bacterial antibiotic resistance [237–239]. A recent study aimed to develop a CRISPR-Cas13a system targeting transcripts of antimicrobial resistance genes to inhibit bacterial growth [240]. This RNA-targeted antimicrobial system can solve the problem of Cas9-based systems for target gene location selection and can be used to detect bacterial genes easily, showing the flexibility of RNA targeting. However, the delivery of the antimicrobial CRISPR-Cas system to the target bacteria is another key challenge. Most of the functional CRISPR-Cas-based antimicrobial systems developed to date are encoded on phage genomes [238,241,242], which is a typical combined usage of phages and phage-derived technologies. The development of programmable CRISPR-based antimicrobial platforms for multiple targeting and more efficient delivery may be essential strategies to overcome problems such as off-target effects and indiscriminate killing of bacteria.

Compared with typical DNA-level regulation, the regulation of RNA levels based on CRISPR systems further expands the scope of gene expression control. As more and more functions of RNA are explored, CRISPR-based RNA-related engineering will have a wider application space. The available chassis cells for RNA-targeted CRISPR-Cas systems are being expanded from bacteria and archaea to mammalian and plant cells, and the research scope is not limited to basic biological science but extends to therapeutic and clinical fields. However, some general problems still exist for RNA-targeted CRISPR-Cas technology. Firstly, the effector proteins are too large, which limits their applications in specific tissues, especially when fused with functional tags or domains. Therefore, truncation of the effector protein without influencing its activity is being attempted, while smaller RNA-targeting Cas effectors and related RNA binding domains are also under exploration. Secondly, possible off-target activity of the effector proteins should still be taken into consideration when targeting RNA. Aims for future development therefore include Cas variants with better specificity, as well as different modalities of crRNAs for enhanced specific targeting and optimal gRNAs selection, which are still facing challenges. Thirdly, the secondary structure of the target RNA affects the editing efficiency, which determines the choice of the target sequence. Thus, it is very promising to investigate the principles that guide the interaction between RNA secondary structure and effector proteins and to develop bioinformatic tools for predicting complex interactions. Last but not least, further application of RNA-targeted CRISPR-Cas technology for therapeutics is facing a major challenge in the form of potential immunogenicity. It is therefore highly desirable to reduce the cytotoxicity and inflammation caused by the system by engineering the target cells or improving the Cas proteins, which is being explored. RNA-targeted CRISPR-Cas technology is still in its infancy, and future research will provide new opportunities for understanding the fundamental RNA biology and treating different diseases.

5. Summary and prospects

Research on phages and their hosts has been continuing for many decades, and has received a renewal of interest due to the development of novel genetic engineering tools even in recent years. In this review, we summarized and discussed the RNA-based technologies related to phages from different aspects. Firstly, RBPs and RNA processing enzymes from phages have been important sources of synthetic biological elements. Secondly, self-replicating RNA systems mimicking the phage life cycle are critical for understanding biological evolution and building artificial life. Finally, RNA-targeted TA systems and CRISPR-Cas systems modeled on phage-bacteria interactions provide important technologies for basic biological research and medical therapy in a wide range of chassis cells.

Recent advances in viral metagenomics have led to the discovery of more unknown phages that exist in different environments including the intestines of animals and the ocean. These advances offer a better understanding of the rich diversity of phages, but they also indicate that the current knowledge and use of phages is very limited. A recent study found that 351 new giant phages carrying multiple translation complexes were found in different environments of the earth, blurring the boundary between acellular viruses and bacterial or archaeal cells [243]. The largest phage genomes, some with lengths of more than 200 kilobases (kb), were demonstrated to encode tRNAs, tRNA synthetases, tRNA-modification enzymes, ribosomal proteins, translation-initiation and elongation factors, and diverse novel CRISPR-Cas systems. The newly found CRISPR-Cas systems can regulate host transcription factors and translational genes to reallocate biosynthesis resources, as well play roles in defense against competing phages. These findings may advance the research on the origins of life and provide new tools for nucleic acid manipulation. Illustrating the great value of these little-known megaphages, it was discovered that the small Casϕ (Cas12j) protein encoded in the genomes of megaphages was able to edit the genome [244]. Thus, megaphages hold great promise for discovering more novel biotechnological tools. With increasingly comprehensive metagenomic studies of phages, RNA phages as less-studied RNA viruses are

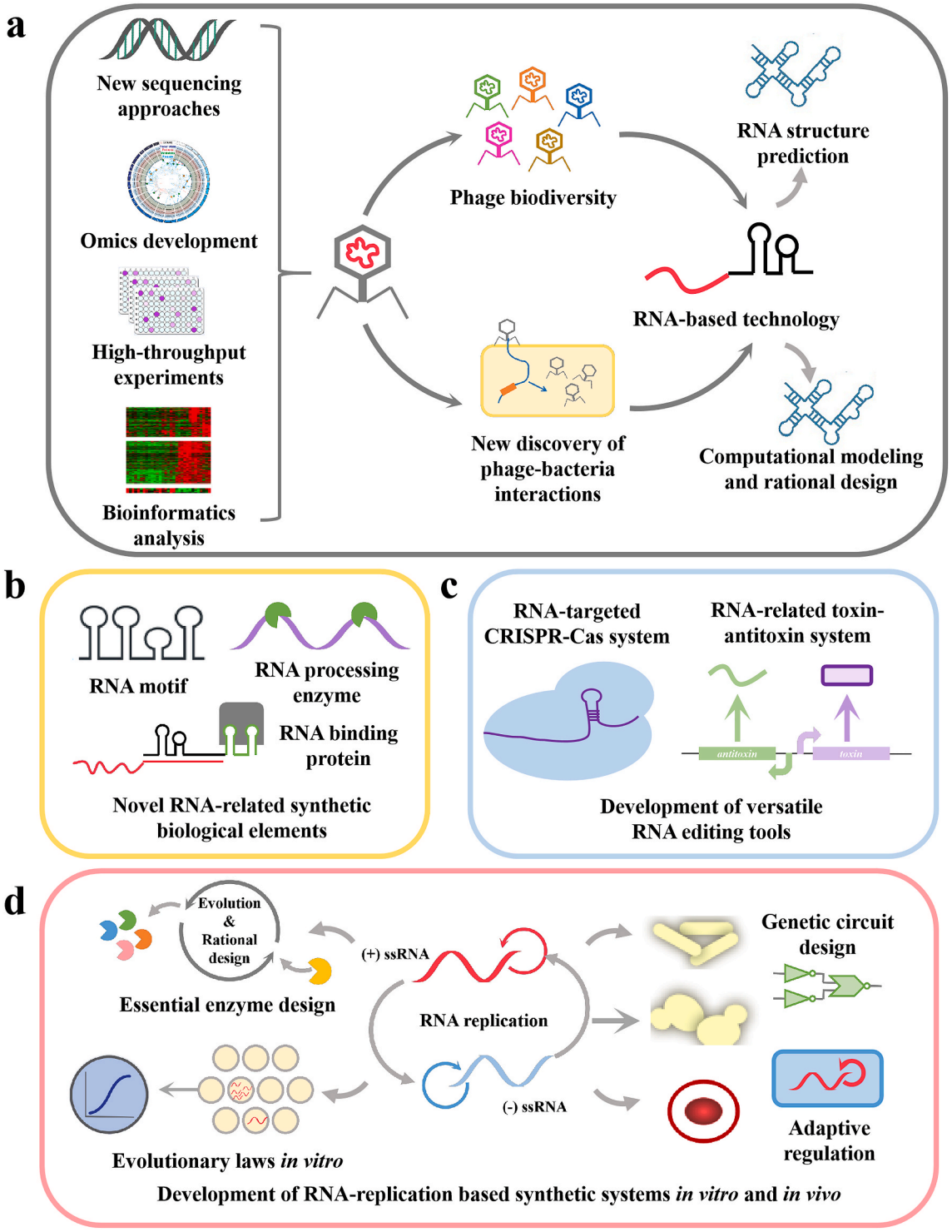

Fig. 9. Prospects for the phage-derived RNA-based technologies. a) The diversity of phages shows great promise. Recent developments in bioinformatics analysis and sequencing approaches allow further understanding of the rich diversity of phages and uncovered mechanisms of phage-bacterial interactions. Combined with the prediction and rational design of RNA molecules, phage-derived RNA-based technologies can be further optimized. b) Exploitation of novel RNA-related synthetic biological elements. c) Development of versatile RNA editing tools. The new discoveries in diverse phages coupled with rational design of the functional RNA-targeted elements and the RNA targets can contribute to developing new novel RNA-related elements such as RNA motifs, RNA processing enzymes and RNA-binding proteins b), and meanwhile new RNA-targeted tools based on TA systems and CRISPR-Cas systems c). d) Development of synthetic systems derived from RNA replication *in vitro* and *in vivo*. To establish more mature platforms for understanding biological evolution based on RNA replication, subsequent issues should be considered including the structural design of the recombinant RNA replication system, confirmation of host factors necessary for RNA replication, and determination of dynamic changes in RNA during the replication process.

garnering more attention and are being increasingly exploited for biotechnology [245] (Fig. 9a). The novel molecular parts and manageable model systems derived from phages that are being discovered in recent years are great inspiration for synthetic biology. However, the diversity of phages also poses challenges for researchers, including incompletely characterized bacterial phage-defense systems, lack of analysis of the

interactions between hosts and RNA phages, as well as the functional and regulatory complexity of phages, which need in-depth studies combined with transcriptomic, proteomic and metabolomic analyses. The biological theories and molecular tools that phages can provide to researchers are far greater than anyone could have considered possible.

With the deepening understanding of the different roles of RNA, more roles have been discovered. These new functions have made RNA into an important component in the regulation of complex gene circuits in synthetic biology as the RNA-based regulatory elements can be easily programmed and rationally designed [246]. Phages have always been an important source of synthetic biological elements. The elements such as RNA processing enzymes, RNA binding sequences, and RNA editing tools provided by phages have been constantly exploited, which can greatly supplement the toolbox of components for complex gene regulatory pathways. At the same time, multiple RNA design tools, such as large databases focused on RNA structure [247] and various algorithms for RNA secondary structure prediction [248], have been developed over the decades, which may greatly benefit the development of novel RNA-based tools, contributing to the construction of feasible regulatory networks from RNA-based devices and expanding their applications in different fields. With more phage genome information becoming available, the rational design and engineering of functional RNA processing elements and RNA targets may become a focus for the development of new RNA-targeted tools (Fig. 9b & c). These new technologies will accelerate the development of RNA-based synthetic biology.

An important step for confirming the plausibility and possibility of the RNA world is to build a self-replicating RNA system, in which phage-derived RNA replication behavior is a critical source for understanding biological evolution and building artificial life. Coupling RNAs with different biological reactions can expand the scope of synthetic genetic circuits and systems *in vitro*, and even achieve functions that cannot be achieved *in vivo*. However, although these research approaches provide access to new technologies and have potential for developing artificially designed life forms, *in-vitro* experiments also have their limitations. Some specific biological processes must be performed in cells, but the research on phage-based RNA replication *in vivo* is very limited, which is influenced by the lack of mechanistic analysis of how RNA phages evade host defenses. If effective RNA replication can be achieved *in vivo,* RNA replication rules in different hosts can be more accurately evaluated, and new technologies can be developed as well. Subsequent issues include the structural design of a recombinant RNA replication system, confirmation of host factors necessary for RNA replication, and determination of dynamic changes in RNA during the replication process (Fig. 9d). The improvement of RNA replication systems *in vitro* and *in vivo* will provide new possibilities for studying molecular evolution and developing new molecular tools.

The goal of engineering and reconstructing biological systems through synthetic biology depends on functional elements and systems. RNA-based technologies are important sources of tools for synthetic biology. Phages provide a rich source of elements for synthetic biology, and RNA-based regulation is an important way for phages to complete life processes and avoid host defenses. RNA-related tools derived from phages will further advance the development of synthetic biology, and accordingly provide more information on the role of phages and RNAs in evolution. The discovery of a large number of new phages will bring more possibilities for the precise regulation of biological systems, hopefully also leading to the development of more robust, scalable and modular tools applicable to a wide range of organisms.

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