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ADVANCED REVIEW

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Design principles and applications of synthetic self-replicating RNAs

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Abstract

With the advent of ever more sophisticated methods for the in vitro synthesis and the in vivo delivery of RNAs, synthetic mRNAs have gained substantial interest both for medical applications, as well as for biotechnology. However, in most biological systems exogeneous mRNAs possess only a limited half-life, especially in fast dividing cells. In contrast, viral RNAs can extend their lifetime by actively replicating inside their host. As such they may serve as scaffolds for the design of synthetic self-replicating RNAs (srRNA), which can be used to increase both the half-life and intracellular concentration of coding RNAs. Synthetic srRNAs may be used to enhance recombinant protein expression or induce the reprogramming of differentiated cells into pluripotent stem cells but also to create cell-free systems for research based on experimental evolution. In this article, we discuss the applications and design principles of srRNAs used for cellular reprogramming, mRNA-based vaccines and tools for synthetic biology.

This article is categorized under:

- RNA in Disease and Development > RNA in Disease
- RNA in Disease and Development > RNA in Development
- RNA Evolution and Genomics > RNA and Ribonucleoprotein Evolution

KEYWORDS

induced pluripotent stem cells, RNA replication, RNA therapeutics, RNA vaccines, viral RNAs

1 | INTRODUCTION

The canonical flow of genetic information in living organisms is typically unidirectional—from DNA to RNA to proteins (Crick, 1970). Here, RNA takes the role of both carrier of the genetic information (mRNA) and key component of the translation machinery (tRNA and rRNA) (Kaczanowska & Rydén-Aulin, 2007). While the genomic DNA is maintained throughout the whole life of an organism, both through active replication and damage repair (Lenhart et al., 2012), lifetimes of mRNAs and most proteins are typically short as they are continuously removed by enzymatic degradation and lack the ability to replicate (Georgellis et al., 1993; Hawkins, 1991). This transient nature of gene products allows multiple layers of regulation of gene expression but limits applications for which RNA cannot be readily transcribed in vivo such as during DNA-free reprogramming of differentiated cells into induced pluripotent stem cells, which avoids the risks associated with genome integration such as mutagenesis and oncogene activation (Warren et al., 2010; Warren & Lin, 2019). As mRNAs get degraded over time, RNA-based applications usually require frequent transfection events since foreign RNAs trigger antiviral responses through the innate immune defense network, which further reduce the lifetime of exogeneous RNAs (Steinle, Weber, Behring, Mau-Holzmann, von Ohle, et al., 2019; Warren & Lin, 2019). Chemical modifications of mRNAs can drastically reduce the immune response and extend its half-life (Wang, 2021). Alternatively, an increase in lifetime can also be achieved by embedding the gene cargo in self-replicating RNA (srRNA) scaffolds derived from RNA viruses. Here, viral RNA-dependent RNA polymerases (RdRPs) amplify the transformed mRNAs in vivo, leading to a substantially elongated lifetime and level of the respective srRNA.

In this work, we aim to provide a general overview of the potential of srRNAs as tools in cell biology, medicine, and also bioengineering. After a brief overview of some of the current srRNA use cases, we provide general insights into the basic requirements for RNA self-replication as well as the coding potential of synthetic srRNAs depending on their viral origin. We then summarize work on existing srRNA systems and their applications before discussing the current challenges and future prospects of this emerging technology.

2 | APPLICATION SCENARIOS FOR srRNAs

2.1 | Stem cell research

Induced pluripotent stem cells (iPSCs) are one of the most promising prospects for regenerative medicine as they can proliferate indefinitely and replace any other cell type in the body that has been lost due to disease or damage (Doss & Sachinidis, 2019). iPSCs formation can be achieved with the induction of the genes for the "reprogramming" transcription factors Myc, Oct3/4, Sox2 and Klf4 (Jaenisch & Young, 2008; Takahashi & Yamanaka, 2006). Typically, these four genes are introduced by either retro- or lentiviral systems, which integrate the desired genes into the host genome (Jaenisch & Young, 2008). Challenge in reprogramming cells to pluripotency with this approach is the risk of mutational insertion into the target cell's genome, which can have various detrimental effects and even lead to tumorigenicity (Sinn et al., 2005). An attractive alternative for cellular reprogramming that is independent from genome editing "foot-print free" methods is based on RNA (Figure 1). For example, synthetic mRNAs can be used to transiently induce pluripotency in somatic cells (Warren et al., 2010). However, this approach is typically hampered by the limited lifetime of mRNAs and requires daily transfection. In contrast, Steinle et al. showed that srRNAs can induce pluripotency in a less time consuming, faster, and more efficient manner than the daily transfection of cells with synthetic mRNAs making them more suitable for potential therapeutic applications (Steinle, Weber, Behring, Mau-Holzmann, Schlensak, et al., 2019). Cellular reprogramming can, for example, be achieved with RNA vectors derived from *Sendai virus* (SeV) that have been rendered incapable of infectious reproduction (Nishimura et al., 2011, 2017). Based on this principle it was shown that murine and human fibroblasts could be reprogrammed into iPSCs within 14 days after infection with a modified SeV, encoding for the four Yamanaka factors (Nishimura et al., 2011, 2017). Similarly, srRNAs based on the *Venezuelan Equine Encephalitis virus* (VEEV) were shown to successfully reprogram both adult human fibroblasts and renal epithelial cells into iPSCs (Steinle, Weber, Behring, Mau-Holzmann, Schlensak, et al., 2019; Steinle, Weber, Behring, Mau-Holzmann, von Ohle, et al., 2019; Yoshioka et al., 2013; Yoshioka & Dowdy, 2017). Here, a single transfection of the in vitro transcribed srRNA ensured continuous expression of the Yamanaka factors for more than 7 days, and the srRNA could be maintained for over a month in the cells, when being selected for (Yoshioka et al., 2013).

2.2 | Self-amplifying RNA vaccines

The outbreak of the S*evere acute respiratory syndrome coronavirus* (SARS-CoV-2), causing a global pandemic, has accelerated the approval of mRNA-based vaccines to be administered to the public (Szabó et al., 2022). The RNA-vaccines BNT162b2 (BioNTech) and mRNA-1273 (Moderna) have successfully helped to substantially combat the spread and severity of SARS-CoV-2 infections (Dye, 2022; Sikora & Rzymski, 2022). Here, RNA modifications such as the replacement of uridine with N1-methyl-pseudouridine reduced the mRNA induced immune response while allowing for a prolonged and stronger expression of the spike proteins in host cells (Granados-Riveron & Aquino-Jarquin, 2021; Nance & Meier, 2021).

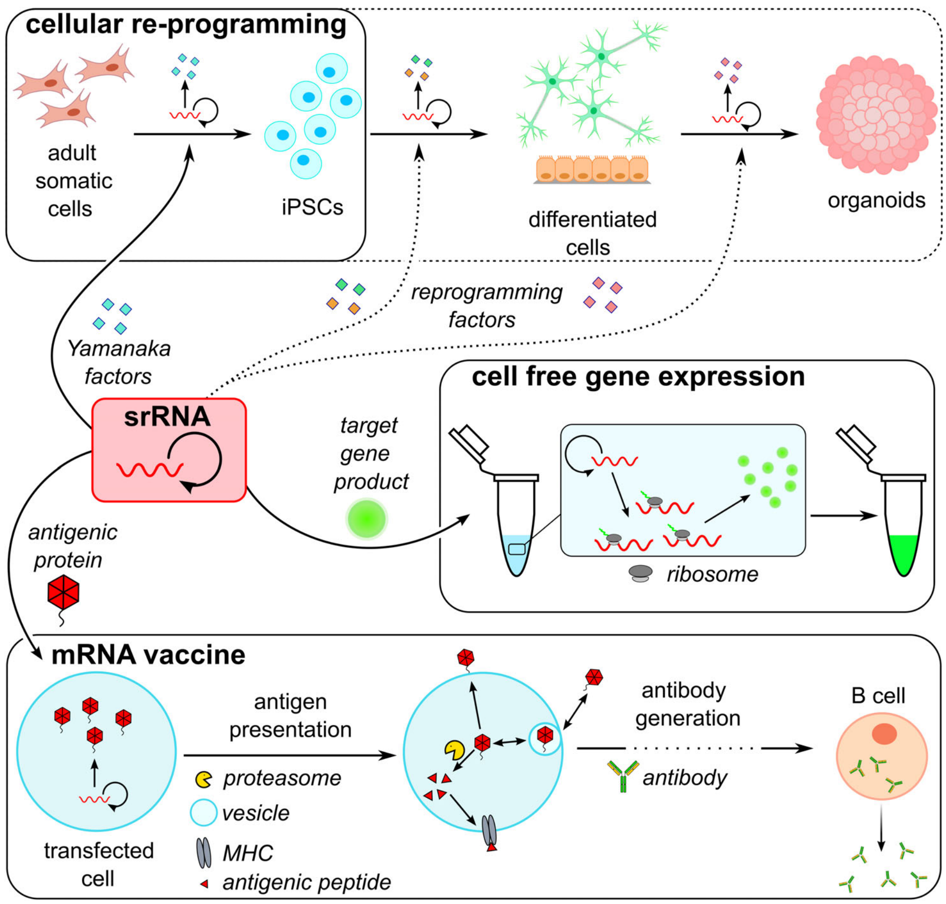

FIGURE 1 Applications for srRNAs: Using srRNAs encoding for the Yamanaka factors, the genome of fully differentiated, postmitotic cells can be reprogrammed to generate induced pluripotent stem cells (iPSC). In theory, srRNAs encoding for the respective reprogramming factors might also be used for the subsequent transdifferentiation into desired cell types, or even the generation of iPSC-derived organoids (dotted arrows and box). In cell free translation systems (or bacterial expression systems, not shown) srRNAs can be used directly as self-amplifying mRNAs for any gene of interest or functional RNAs such as ribozymes and aptamers. srRNAs encoding for antigenic proteins can be harnessed as RNA-based vaccines. Expression of the target protein from srRNAs leads to extracellular presentation of antigenic domains, either via secretion, direct exposition on the outer membrane, or preceding proteolysis and presentation of antigenic peptides via the major histocompatibility complexes (MHC). As a consequence, antigen-specific antibodies will be generated.

Extensive research is also done on srRNA-based (also referred to as self-amplifying) vaccines (Blakney, 2021; Bloom et al., 2020) (Figure 1). For example, VEEV-derived self-amplifying vaccines against SARS-CoV2 are currently in phase I clinical trials (Pollock et al., 2022). Furthermore, Maine et al. designed an anti-cancer srRNA vaccine based on VEEV, which showed potent inhibition of tumor growth in mice (Maine et al., 2021). The same system could also be used to express specific antibodies against the influenza antigen hemagglutinin in macaques (Maine et al., 2021). More detailed information on the use of ssRNA viruses for srRNA vaccine development can be found in several comprehensive reviews (Bloom et al., 2020; Lundstrom, 2021).

2.3 | Biotechnology and directed evolution

srRNAs show also great potential in biotechnological applications such as protein production and evolution. For instance, srRNAs derived from *Qubevirus*, an *Escherichia coli* infecting phage, were able to boost heterologous expression of recombinant genes when being initially transcribed from a plasmid (Yao et al., 2019). The expression enhancement is based on the self-amplifying character of srRNAs, which could improve industrial scale production of recombinant proteins. srRNA-based expression of a target gene could also allow for the rapid directed evolution of said gene, due to the inherently high error rate of viral RNA-dependent RNA polymerases (RdRPs) (Davis & van den

Pol, 2010; Drake & Holland, 1999). Similar to phage assisted continuous evolution, a srRNA-based approach is plausible, where the coding sequence of the gene of interest would be encoded on a rapidly diversifying srRNA species (Miller et al., 2020). Such an approach could leverage the naturally high error rate of RdRPs and bypass the costly synthesis of artificial libraries as any clonal mRNA would quickly diverge into a larger pool of mRNA sequences. srRNA-based evolution could also be carried out exclusively in vitro as artificial compartments such as water-in-oil emulsion droplets, which can provide the required phenotype/genotype coupling (Bansho et al., 2016; Ichihashi et al., 2013; Mizuuchi & Ichihashi, 2018). Using this concept, srRNAs have contributed to basic research approaches that investigate evolutionary principles using simplified experimental model systems. In 2013, Ichihashi et al. developed the first translation-coupled srRNA system capable of Darwinian Evolution under cell-free conditions using *Qubevirus* derived srRNAs (Ichihashi et al., 2013). Later the Ichihashi lab studied the emergence of cooperativity in replicator networks in vitro using the same system (Furubayashi et al., 2020; Mizuuchi et al., 2022). Starting from a single synthetic srRNA, a five-membered network evolved over several generations that exhibits complex and cooperative interactions between the individual members. This situation is similar to the hypercycle proposed by Eigen et al., which serves as a model explanation of how complex life emerged from cooperative interactions of primitive, low-fidelity RNA replicators (Eigen et al., 1991; Eigen & Schuster, 1978). Finally, with the advances of direct RNA sequencing, the transition from screening for a specific phenotype to identifying the corresponding genotype can be substantially facilitated (Harel et al., 2019).

3 | REQUIREMENTS FOR RNA SELF-REPLICATION

3.1 | Natural self-replicating RNAs

srRNAs have the potential to serve as a powerful, nonintegrative alternative to traditional genome engineering approaches and evolutionary tools. However, research into which RNA species can be used to generate functional srRNAs is still in its infancy. To understand which RNA systems could be turned into synthetic RNA replicons, it is therefore necessary to understand the working mechanism of RNAs capable of self-replication in vivo and/or in vitro.

Presumably the largest group of replicating RNAs in nature is represented by RNA viruses and viroids (non-coding RNA pathogens). According to the Baltimore system for virus classification, RNA viruses without DNA intermediates can be separated into three groups (III, IV, V), depending on the overall architecture and polarity of their genome. Thus, group III is made up of all double stranded RNA viruses (dsRNA), group IV and V of single stranded RNA (ssRNA) viruses with positive (+) and negative (−) genome polarity, respectively (Koonin et al., 2021). For +ssRNA viruses, the genomic RNA is at least partially identical to the mRNA required for the expression of their genes, that is it contains the coding sequences. In case of −ssRNA viruses, the genomic strand first must be transcribed to yield the complementary (+)strand, which harbors the coding sequences. Therefore, the viral RdRP is an essential part of the virion of −ssRNA viruses (Luo et al., 2020). The dsRNA virus genome consists of both (+) and (−)strand RNAs that are packaged into the viral capsid together with the viral RdRP (Mertens, 2004). In some cases, the complete genome of RNA viruses is not encoded on a single RNA strand, but may be divided among several segments, similar to the way genomic information may be distributed among chromosomes (Koonin et al., 2021). Importantly, the (+)strand version of the viral genome is not necessarily the only translated mRNA. In several RNA viruses, some of the encoded genes are translated from sub genomic transcripts that were transcribed from the genomic (−)strand using internal or sub-genomic promoters (Conzelmann, 1996; Sztuba-Solińska et al., 2011).

Remarkably, the recently discovered ambiviruses (ssRNA viruses with coding sequences on both strands) as well as some segmented ssRNA viruses with ambisense genomes (combination of segments with sense or antisense polarity) do not fall into either group IV or group V, but rather form their own group (Koonin et al., 2021; Sutela et al., 2020). Furthermore, in case of ribozyviruses, viroids, and potentially also some ambiviruses, the ssRNA genome can be circularized, thus protecting the RNA ends from exo-ribonucleases (Flores et al., 2011; Forgia et al., 2023).

Independent of the classification, the full replication of all these RNA species requires both (+) and (−)strand synthesis (Figure 2a,b). In case of linear +ssRNA and −ssRNA viruses, replication of the genomic strand is preceded by synthesis of the reverse complementary antigenomic strand using the genomic strand as template. In case of dsRNA viruses, replication of the genomic dsRNA commonly starts with synthesis of the (+)strand, also serving as mRNA for the expression of the viral genes (Mertens, 2004). This step is then followed by synthesis of the second strand using the nascent (+)strand as template.

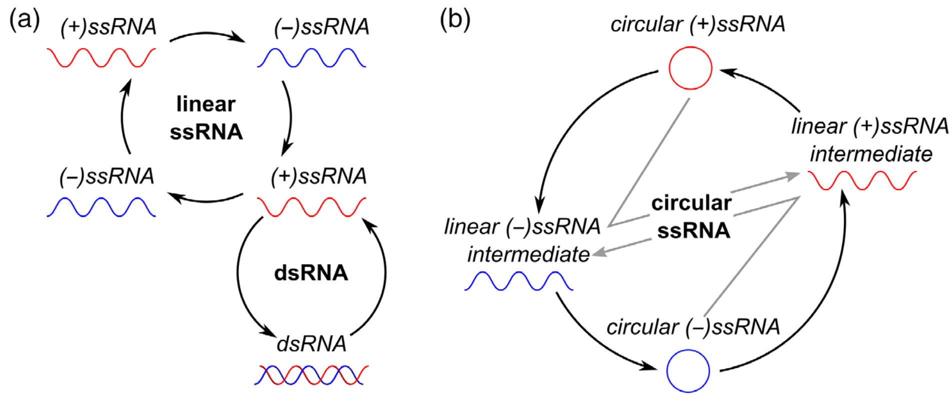

FIGURE 2 Types of replicating RNAs: (a) Linear single stranded RNA (ssRNA), linear double stranded RNA (dsRNA) and circular ssRNA can be replicated. For linear ssRNA viruses with (+) and (−) polarity, respectively, the genomic RNA is replicating via the antigenomic RNA of opposite polarity. In case of dsRNA viruses, the replication starts with +ssRNA, often initially transcribed as mRNA for the expression of viral genes. Synthesis of the complementary (−)strand, using the (+)strand as a template, finishes the dsRNA replication. (b) The circular ssRNA genomes of viroids and circular satellite viruses can be replicated through rolling circle RNA synthesis. This generates linear ssRNA intermediates in the form of antigenomic RNA concatemers. Following cleavage and circularization of these intermediates, rolling circle replication of the antigenomic circular RNA generates linear concatemer precursors of the initial genomic RNA, that in turn can be processed into the final circular ssRNA. Alternatively, the linear intermediate of one strand can function as template for the synthesis of the respective complementary linear intermediate (gray arrows).

The replication of viroids and ribozyviruses has been reviewed in great detail by Flores et al. (2011) and Lee and Koonin (2022). Generally, replication of circular RNAs of ribozyviruses, viroids, and potentially ambiviruses is based on rolling-circle replication (Flores et al., 2011). Here, the circular genomic RNA serves as template for the continuous synthesis of linear repeats of antigenomic strands. These concatemers are subsequently processed into monomeric intermediates, followed by circularization into circular antigenomic RNAs that then in turn can serve as template for an analogous synthesis of new genomic circular RNA. Alternatively, in a hybrid between linear and circular RNA replication the concatemeric linear intermediates already serve as template for the synthesis of the respective complementary strand (Flores et al., 2009, 2011). Generally, the processing of the linear concatemers into circular products requires either the action of co-encoded nucleolytic ribozymes or distinct host factors (Flores et al., 2011).

3.2 | Autonomy and coding potential of srRNAs

RNA viruses, and by extension also viroids, are intracellular parasites (Louten, 2016). As such, they inevitably rely on host factors for the completion of their life cycle. However, the degree of autonomy with regards to the replication of the RNA itself can greatly vary (Lee & Koonin, 2022) and it may be useful to distinguish between passively replicating RNAs that are devoid of self-encoded polymerase enzymes and "active" srRNAs that carry a dedicated RdRP gene and might therefore have a broader host range (Figure 3).

Viroids and satellite RNA viruses (including ribozyviruses) are purely passive replicators as they do not encode for any RNA polymerase. Hence, they are completely dependent on either host polymerases or the polymerases of co-infecting helper viruses to amplify their genomic RNA (Navarro et al., 2021; Palukaitis, 2016). Despite their low independence, viroids and satellite viruses may be re-engineered to encode their cognate RdRP. Indeed, Forgia et al. propose that the acquisition of an RdRP by these short replicators might be the evolutionary origin of the newly discovered ambiviruses, due to their striking similarity to viroids and ribozyviruses like HDV (Forgia et al., 2023). In contrast to these simple RNA pathogens, all non-satellite RNA viruses encode at least the catalytic subunit of an RdRP even though additional host factors are often required for their genome replication. With increasing genome size, the independence of the encoded RdRPs from these host factors increases, and thus also the autonomy of RNA replication. The two members of the *Fiersviridae*, *Emesvirus zinderi* (MS2) and *Qubevirus* (Qβ), two of the smallest known +ssRNA viruses to date, have genome sizes of 3.6 kb and respectively 4.2 kb, thus approximately only twice as large as large satellite viruses like the *Hepatits delta virus* (HDV, 1.7 kb) (Fiers et al., 1976; Flores et al., 2011; Schuppli et al., 2000). They encode for RdRP subunits that, together with three *E. coli* host factors, form the active RdRP complexes capable of

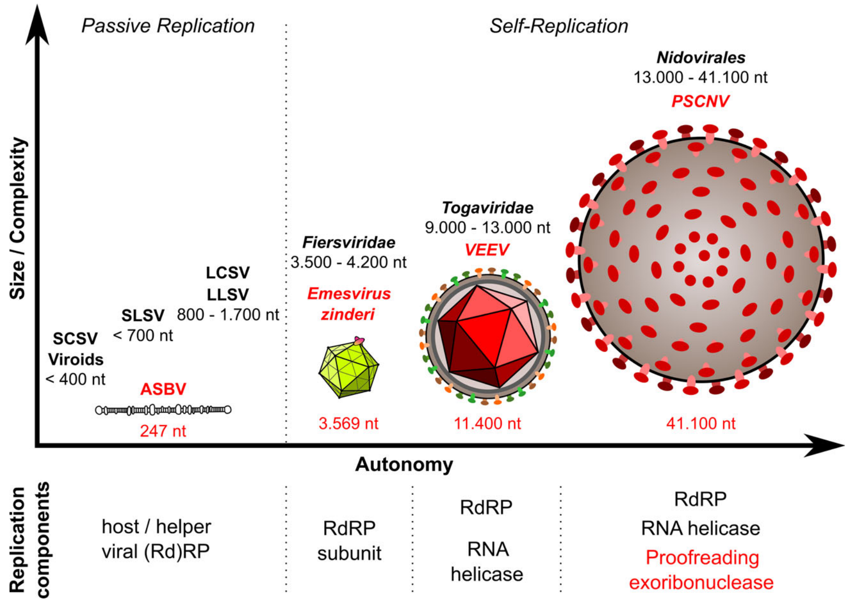

FIGURE 3 Relation of genome size/complexity and autonomy: Comparison of passively replicating and actively self-replicating linear (+) ssRNA species, based on the size/complexity of respective RNA, with examples of the corresponding groups. Passive replicators require either host or helper viral RNA polymerases and include viroids, small circular (SCSV), small linear (SLSV), large linear (LLSV) and large circular (LCSV) satellite viruses. Avocado sunblotch viroid (ASBV) is depicted as an example for viroid RNA. Linear self-replicating RNAs start with the group of *Fiersviridae*, like *Emesvirus zinderi*, that already encode the RdRP subunit and extend over larger RNA viruses like Venezuelan Equine Encephalitis virus (VEEV), encoding for their RdRP and an RNA helicase, to the largest known RNA virus to date, a Planarian secretory cell nidovirus (PSCNV) that in addition also encodes for proofreading exoribonuclease activity. While the concept of RNA self-replicators is here only depicted for (+)ssRNA species, the same principle of course also applies to (−)ssRNA and dsRNA self-replicators.

replication of genomic and synthetic RNAs (Blumenthal & Carmichael, 1979; Kita et al., 2008; Wagner et al., 2022). Larger RNA viruses, for example, from the families of *Togaviridae* (e.g., VEEV) and *Flaviviridae* (e.g., *Hepacivirus* HCV) (9.0–13 kb) encode the fully active RdRP complex in their genome, split across multiple genes in a single polyprotein ORF (Chase, 2022; Weaver et al., 2004). In contrast to the smaller *Fiersviridae*, these larger RNA viruses depend less on host factors as many of them also encode for additional RNA modifying factors such as RNA helicases or enzymes involved in for 5′-capping and 3′-polyadenylation, which are required for translation and potentially also RNA replication (Chase, 2022; Das et al., 2014). Finally, the larger members of the family of *Nidovirales* (e.g., SARS-CoV 19 with 29.9 kb), including the largest known RNA virus to date (*Planarian secretory cell nidovirus* PSCNV with 41.1 kb) even encode genes for proofreading ribonucleases, drastically increasing the fidelity of the genome replication (Gorbalenya et al., 2006; Naqvi et al., 2020; Saberi et al., 2018).

4 | DESIGN PRINCIPLES OF SYNTHETIC srRNAs

Following the previous sections, a simple blueprint of the elements required for the design of a srRNA can be derived (Figure 4a). In the following sections, the currently existing srRNA systems are briefly analyzed in terms of how they were designed and for which systems and applications they can be used (Figure 4b).

4.1 | Phage-derived srRNAs in cell-free systems

Bacteriophage-derived srRNAs can be used for applications in bacteria or thereof derived in vitro translation (IVTxT) systems. Currently only very few srRNA scaffolds for bacteria have been described in literature, all of which were derived from experiments with RdRPs from the two *Fiersviridae* family members *Qubevirus durum* (Qβ) and *Emesvirus zinderi* (MS2), which are both non-segmented +ssRNA viruses. In case of Qβ, there are two classes of engineered RNA

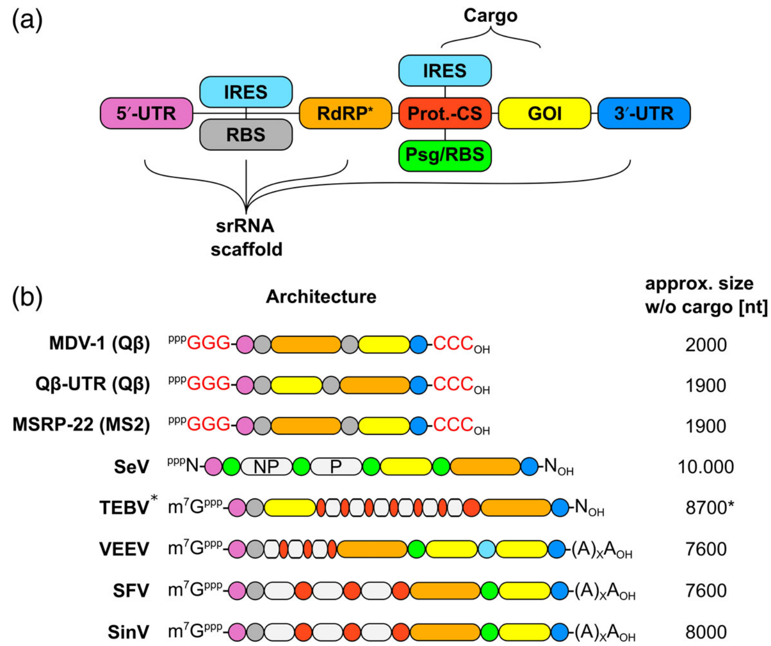

FIGURE 4 Essential and optional elements of self-replicating RNAs: (a) Overview of the typical core modules required by self-replicating RNAs (srRNA scaffold), as well as elements required for exogenic cargo. 5′-/3′-UTR = untranslated regions harboring recognition/regulation motives/sequences and/or ribozymes in case of ambiviruses; IRES = internal ribosome entry site (for eukaryotic srRNAs); RBS = ribosome binding site (for prokaryotic srRNAs); RdRP = RNA-dependent RNA polymerase; Prot.-CS = protease cleavage site; Psg = promoter for subgenomic transcription; GOI = gene of interest. (b) Overview of currently used srRNA vectors with a comparison of their overall architecture and size without any additional genetic cargo. MDV-1 = Midivariant; Qβ-UTR = Qβ-UTR based replicon; MSRP-22 = MS2 RNA Parasite 22; SeV = Sendai virus ((+)-strand equivalent); TBEV = Tick-borne Encephalitis virus; VEEV = Venezuelan Equine Encephalitis virus; SFV = Semliki Forest virus; SinV = Sindbis virus. *The replicon size for TBEV is based on the length of wildtype TBEV without the structural proteins. Colors represent the elements depicted in panel (a), white boxes represent nonstructural proteins.

replicons that can encode additional gene cargo: The first class of Qβ srRNA variants were derived from the short self-replicating "Midivariant RNA" (MDV-1, 218 nt) through the insertion of a ribosome binding site and the coding sequence of the catalytic 586 amino acid long Qβ RdRP subunit on its (+)strand (Kita et al., 2008; Kramer et al., 1974). A derivative of this srRNA has been used in IVTxT systems to also co-replicate and express the *lacZ* gene on the (−) strand (Kita et al., 2008). In later studies, similar designs were used for the in vitro evolution experiments described above (Furubayashi et al., 2020; Ichihashi et al., 2013; Mizuuchi et al., 2022). The second Qβ srRNA variant was used by Yao et al. and consists of the Qβ-RdRP gene embedded in the 5′- and 3′-UTRs of the Qβ genome (~160 nt) (Yao et al., 2019). In this case, up to four genes, encoded in two separate ORFs, could be expressed, and the RNA be replicated in vivo after being transcribed from a plasmid (Yao et al., 2019). In a similar fashion a srRNA derived from in vitro experiments with recombinant MS2 RdRP (544 aa) and RNA replicon MSRP-22 (226 nt) was recently designed (Wagner et al., 2022). Like their Qβ counterparts, these srRNAs have several traits in common: (I) They all have a 5′-triphosphorylated end with a terminal GGG-triplet, as well as a 3′ CCC-triplet with a free 3′-OH, both of which are presumably required for de novo initiation of RNA replication (Küppers & Sumper, 1975; van Dijk et al., 2004). (II) All three srRNAs encode for the respective RdRP subunit only. Thus, they still require three additional host factors—elongation factors EF-Tu, EF-Ts, and ribosomal protein S1—to assemble the fully active RdRP complex, which are readily provided in *E. coli* based in vitro translation systems (Blumenthal & Carmichael, 1979; Wagner et al., 2022). This minimizes the gene load on the srRNA required for self-replication. However, as these RdRPs are missing proper RNA helicase activity, as well as any active proofreading ability, long constructs might be more prone to dsRNA-formation, and/or accumulation of deleterious mutations, eventually leading to the loss of the replication ability (Iranzo et al., 2016; Kun et al., 2015; Tomita et al., 2015).

4.2 | *Paramyxoviridae* based srRNAs

As described above, *Sendai virus* (SeV), a member of the family of *Paramyxoviridae*, has been used as a scaffold for the design of a srRNA to reprogram both murine and human fibroblasts into iPSCs (Nishimura et al., 2017). SeV is a linear, non-segmented −ssRNA virus. Thus, it requires delivery of the (−)strand RNA together with the RdRP, or alternatively, the capped (+)strand for intracellular replication. The SeV-RdRP catalyzes genome replication, capping of the sub-genomic mRNAs with a m^7G-cap as well as 3′-end polyadenylation, and consists of two proteins (L = RdRP subunit, 2228 aa / P = co-factor, 568 aa) (Itoh et al., 1997; Ogino et al., 2005). In addition, the RdRP complex also requires binding of the 524 aa nucleocapsid protein (NP) to the RNA to replicate the genomic RNAs. Nishimura et al. prepared the RNA for delivery by co-expressing the RNA vector with the capsid proteins in vivo and then isolating the packaged RNA from the cells (Nishimura et al., 2011). This is a common method for the generation of packaged viral vectors. However, this approach bears the risk of an immunogenic response when used in multicellular organisms. As this could result in the generation of vector specific antibodies, this approach might be less suitable for vaccines based on srRNAs. Finally, the large size of the replication competent SeV-srRNA scaffold resulting from the more complicated replication mechanism of (−)ssRNA viruses likely limits the coding capacity for additional gene cargo.

Measles virus (MeV), another member of the *Paramyxoviridae*, has a genome similar in size to SeV (MeV: 15.9 kb, SeV: 15.4 kb), with an RdRP (2183 aa) slightly smaller than SeV (Itoh et al., 1997; Takeuchi et al., 2000). It has been used by Fujiyuki et al. to induce immunity against *Highly pathogenic avian influenza virus* (HPAIV) in cynomolgus monkeys (Fujiyuki et al., 2017). In contrast to Nishimura et al., Fujiyuki et al. did not remove genes that are not involved in RNA replication but instead used an attenuated MeV as srRNA-scaffold, and the HPAIV hemagglutinin gene was encoded between the genes encoding for NP and P protein. Nonetheless, both approaches presumably could also be deployed to reprogram other members of the *Paramyxoviridae* family into potential srRNA scaffolds.

4.3 | *Flaviviridae* based srRNAs

The +ssRNA Tick-borne Encephalitis virus (TEBV) belongs to *Flaviviridae* family. It encodes three structural and seven nonstructural proteins in a single ORF on its (+)-strand. This polyprotein (3414 aa) matures into the individual proteins by autocatalytic proteolysis (Zheng et al., 2018). The nsP5 (901 aa) carries both RdRP and 5′-capping activity and is thus essential for RNA replication, however, the RdRP does neither polyadenylate the 3′-tail nor synthesize subgenomic transcripts (Mandl, 2005). Kofler et al. repurposed this virus into a srRNA vaccine against TBEV infection by deleting parts of the capsid protein (Kofler et al., 2004). In consequence, the RNA could still be replicated in vivo, and the viral proteins be translated but no infectious progeny virion could be assembled. In their discussion, Kofler et al. suggest that this approach might also work for other flaviviral pathogens (e.g., Hepatitis C virus), due to the substantial similarities between members of this family.

4.4 | *Togaviridae* based srRNAs

Alphaviruses, the sole genus in the *Togaviridae* family, are so far the biggest source of scaffolds for the generation of srRNAs. These vectors include replicons based on VEEV, *Semliki Forest virus* (SFV), and Sindbis virus (SinV). All the three viruses have +ssRNA genomes with 5′-m^7G cap, 3′-poly-A tails, encode their own RdRP subunit in a single ORF together with three additional nonstructural proteins, and their structural proteins in a second ORF under the control of a subgenomic promoter (Raju et al., 1999; Ying et al., 1999; Yoshioka et al., 2013). While the RdRP (nsP4) is the main protagonist during RNA synthesis, the other three nsPs play crucial roles in this process as well (nsP1: capping and RNA synthesis, nsP2: protease and RNA helicase, nsP3: RNA synthesis) (Chase, 2022). After translation, the polyprotein processes itself proteolytically through the action of nsP2. This leads to formation of the RdRP holo-complex that in turn synthesizes the subgenomic mRNAs for expression of the structural genes including a 5′ cap and poly-A tail. For the design of srRNAs, the structural genes can be replaced with the genes of interest. This way, Yoshioka et al. have turned VEEV into a vector for the reprogramming of human fibroblasts into iPSCs (Yoshioka et al., 2013). Interestingly, three of the Yamanaka factors were expressed from a single ORF, separated co-translationally into the individual proteins via the "self-cleaving" 2A-peptide (Szymczak et al., 2004). The fourth factor, and an additional selection marker, were under control of a separate IRES, allowing for the finetuned expression ratios of these genes (Yoshioka et al., 2013). SFV and SinV have been exploited as scaffolds for potential srRNA-vaccines against rabies, cancer and influenza, following a similar approach for incorporating the genes of interest as Yoshioka et al. (Cheng et al., 2001; Ying et al., 1999; Zhang et al., 2021; Zhou et al., 1994). By comparison, SinV has the largest precursor polyprotein and RdRP (2513 aa / RdRP: 616 aa), exceeding those of SFV (2431 aa / RdRP: 614 aa) and VEEV (2493 aa / RdRP: 606 aa), although the absolute differences are only minimal (Kinney et al., 1992; Salonen et al., 2003; Strauss et al., 1984). Noteworthy, although these viruses are substantially larger, their RdRP subunits are of similar size to those of the bacteriophages MS2 and Qβ. Importantly, although the RdRP might not require the m^7G cap for de novo initiation of genome replication, it is a crucial component for efficient in vivo translation and as such necessary for the expression of the RdRP inside a cell. Furthermore, it substantially enhances the in vivo stability by protecting the RNA from ribonucleases (Rampersad & Tennant, 2018). This modification can be introduced into the RNA, using anti-reverse cap analogs during in vitro transcription, which will allow for the proper formation of the m^7G cap (Grudzien-Nogalska et al., 2007).

4.5 | *Rhabdoviridae* based srRNAs

Lastly, while being a direct target of potential srRNA vaccines, the *Lyssavirus rabies* (RV) has also been identified as a potential source for srRNA-based vaccines itself. RV belongs to the group of *Rhabdoviridae*, and as such has a 11.9 kb ssRNA genome of negative polarity encoding five genes (Finke & Conzelmann, 2005b; Tordo et al., 1988). Similar to SeV, the genomic RNA is replicated by the L-protein (RdRP, 2142 aa) in complex with the P-protein, while requiring the template RNA to be tightly packed by viral N-proteins, and thus all three factors are essential components of the mature virion (Finke & Conzelmann, 2005b). Interestingly, transcription of subgenomic mRNAs only depends on L/P-complexes, and a deeper understanding of the underlying mechanisms might enable the design of synthetic RNAs that replicate independently from the N-protein (Finke & Conzelmann, 2005a). Several properties suggest that RV has great potential for the design of srRNAs: (I) The low recombination rate of RV suggest that it might serve as a highly stable RNA vector. (II) The rod-like structure of the RV-RNP does not impose a size limit on the packaged RNA in contrast to the more constrained capsids of many other viruses. (III) The virions can readily be targeted to a desired cell type by modification of the RV-glycoprotein (reviewed in depth by Finke & Conzelmann, 2005a). By inserting antigens against SARS-CoV2 or Middle Eastern Respiratory Syndrome (MERS)-CoV into an attenuated RV it was possible to induce significant immunoprotection against both target viruses in mice (Kurup et al., 2020; Wirblich et al., 2017). In both cases, the respective antigen was inserted between the N- and P-gene. In a very similar fashion, the highly related vesicular stromatitis virus (VSV) could be turned into potential srRNA-vaccines against Ebola virus and HIV with both vaccine candidates eliciting strong protection in monkeys against the respective virus (Marzi et al., 2019; Rose et al., 2001).

In conclusion, while there are not yet that many srRNA systems established, more will probably follow, especially following the global SARS-Cov19 pandemic and the ensuing increased interest in mRNA-based vaccines. Further virus-based replicons as potential RNA vaccines were reviewed by K. Lundstrom, though these in general follow similar design strategies (Lundstrom, 2021).

5 | CHALLENGES

5.1 | srRNA delivery

The wide variety of RNA vectors suitable for srRNA design offers tremendous potential for tailoring synthetic replicons to individual needs. To do this, however, it is crucial to know which vectors are best suited for the respective needs and which requirements must be met in the respective environments. Thus, a srRNA created for applications in bacteria will exhibit different characteristics compared to one designed for the use in eukaryotes, and both will probably differ from those designed for purely synthetic environments.

This already starts with the first step, delivery of the srRNA to the desired target environment. For purely synthetic applications, the naked srRNA can simply be supplied to the system, as has been done many times with Qβ-based replicons in recombinant in vitro translation systems (Bansho et al., 2012; Kita et al., 2008). In contrast, applications in living cells require more sophisticated methods of delivery, as the srRNA must first cross the cell barriers. In the case of many bacterial and some single-cell eukaryotic systems, the srRNA can be directly transformed via electroporation (Chassy, 1988; Chu et al., 1987; H. Liu et al., 2017; Taketo, 1989). For more demanding systems, a variety of transfecting agents and methods has been developed (Chong et al., 2021). Thus, the mRNA vaccines against SARS-Covid19 for example are administered as mRNA-containing lipid nanoparticles (Szabó et al., 2022). In vitro encapsulation of srRNAs in virus capsids may enable tissue and/or cell type specific delivery. Albeit powerful and used in a variety of cases described above, this approach requires the proper and efficient packaging of the target RNA and may cause unwanted immunogenic side effects (Chong et al., 2021). Nonetheless, RNA encapsulation is under extensive evaluation for numerous potential medical applications (Aljabali et al., 2021). A comprehensive overview for the delivery of therapeutic RNAs was recently published by Yan et al. (2022).

5.2 | srRNA modifications and sequence motifs

After their delivery, srRNAs need to successfully establish expression of both their replication machineries and their gene cargos, which may require additional, host-specific elements. In bacterial systems, these elements can be as simple as a 5′-triphosphate end and ribosomal binding sites (RBS) for each encoded open reading frame (ORF) (Figure 4a). This is in fact the simplified architecture of both MS2 and Qβ viruses, as well as Qβ-based synthetic replicons (Fiers et al., 1976; Kashiwagi & Yomo, 2011). Contrary to bacterial mRNA, most eukaryotic gene transcripts contain a m^7G 5′-cap and a 3′ poly(A) tail (Araujo et al., 2012). These two elements are both protecting the mRNA from degradation but also required for efficient translation initiation. Thus, many RNA viruses have evolved methods to either introduce these modifications into their RNA themselves, capture and transfer them from endogenous mRNAs, or circumvent the need for them by deploying special RNA structures/motifs to mimic them (Rampersad & Tennant, 2018). Translation initiation in eukaryotes is also considerably more complex than in bacteria and heavily dependent on a 5′ m^7G-cap of the mRNA template (Hinnebusch & Lorsch, 2012). In fact, several viruses rely on sub genomic transcripts that carry the cap-modification. Alternatively, specific RNA structures, so called internal ribosome entry sites (IRES), allow for the translation from internal ORFs as well by mimicking the translation initiation complex (Filbin & Kieft, 2009). A further viral strategy that minimizes the requirements is encoding genes as long polyprotein precursors that are proteolytically processed into their final form during or after the translation (Hellen et al., 1989). Rampersad & Tennant have written an excellent overview about the underlying strategies (Rampersad & Tennant, 2018).

Finally, the in vivo lifetime and potential unwanted side effects of the srRNA depend on the original RNA vector and the target system. In bacteria, this response can range from simple degradation of srRNAs by ribonucleases up to global unspecific RNA degradation by antiviral immune defenses like CRISPR/Cas13, ultimately leading to cell death (Abudayyeh et al., 2016). In eukaryotes, specific pattern recognition receptors (PRR) can detect both exogenous ssRNA and dsRNA, and in response initiate a reaction from a wide array of options, including the down regulation/inhibition of translation, programmed cell death and, even the activation of an organism-wide interferon response (Bowie & Unterholzner, 2008). While these might be desired effect in some cases (e.g., as anti-cancer therapeutic), they may have detrimental effects in other use cases (e.g., mRNA vaccine). dsRNA, which may occur as an undesired side-product of single-stranded srRNAs, is a potent signaling molecule that causes a protective antiviral state within the cell by triggering type I interferon, leading to the production of interferon-stimulated genes (ISGs) (Bowie & Unterholzner, 2008). Although this might be useful for the immunogenic effect of mRNA vaccines (Pardi et al., 2018), other applications may benefit from a design based on circular ssRNAs as they reduce the immune response by inhibiting the protein kinase PKR, which is involved in the response to viral dsRNA but also structured ssRNAs (C.-X. Liu et al., 2019).

Taken together, the available technologies for the delivery and modifications of non-replicative RNAs should be readily applicable to srRNAs and enable the design of replicating RNA vectors for a variety of different applications.

5.3 | Factors limiting srRNA replicability and coding potential

Despite their promising potential, the coding capacity of srRNAs is considerably lower than for traditional DNA-vectors such as plasmids, cosmids or DNA-viruses. Reasons for the disadvantage of RNA as genetic material compared to DNA are, for example, its lower chemical and enzymatic stability in combination with the much higher tendency to form thermodynamically very stable intra- and interstrand interactions that may hamper its replication. The replication products of ssRNA viruses have an inherent potential to produce double stranded RNA byproducts arising from the annealing of perfectly complementary template and product strands. And while dsRNA viruses can replicate their RNA by copackaging RdRPs and genomic RNAs into subviral particles (Tao & Ye, 2010), naked cytosolic dsRNA is commonly regarded as replication inactive (Cho et al., 1993; Mertens, 2004; Szostak, 2012; Tomita et al., 2015). This is due to the high melting temperature of long RNA duplexes rendering them inaccessible for replication in the absence of strand displacement factors. When designing synthetic srRNAs this characteristic must be kept in mind, as some sequences exhibit higher tendencies for duplex formation than others (Mizuuchi et al., 2019).

A further important factor that limits the coding potential of srRNAs are the high error rates of viral RdRPs, which makes viruses susceptible to "error catastrophes" and prevents the faithful maintenance of genetic information on long genomes due to the accumulation of detrimental mutations (Crotty et al., 2001). The accumulation of such errors may eventually render a srRNA unfit for self-replication or compromise the function of the genetic cargo. An impressive example for the devastating effects of the low fidelity of RdRPs is given by the discovery of the famous "Spiegelman's monster" in 1967 (Mills et al., 1967). In the original experiment, 74 rounds of in vitro replication of the Qβ genome with purified RdRP coupled to serial transfer resulted in the degeneration of the 4.2 kb Qβ wild type genome into a 218 nt long "parasitic" RNA that was devoid of any genetic information. These short replicators can very quickly overtake a replicating system by outcompeting longer ones, when not restrained by compartmentalization (Bansho et al., 2016; Furubayashi et al., 2020). It is foreseeable that such increasing accumulation of mutations over time may also limit the efficacy and versatility of srRNAs in terms of their coding potential and lifespan. However, the increasing accumulation of inactivating mutations that limit therapeutic utility over time can presumably be compensated for via regular re-administration.

Nonetheless, small gene-free srRNAs are still of considerable interest as they are readily generated by simple in vitro replication/serial transfer (Bansho et al., 2012; Mills et al., 1967; Wagner et al., 2022) and can be used as scaffolds for synthetic srRNAs, that is, by adding compatible RdRP genes. As mentioned before, small gene-free srRNAs might also be the evolutionary origin of the newly discovered ambiviruses (Forgia et al., 2023).

6 | CONCLUSIONS

srRNAs show promising potential for multiple applications such as DNA-independent gene expression and evolution, foot-print free genome engineering or more potent RNA vaccines. Notably, srRNA-based approaches are more advantageous with regards to the required dose, number of administrations, and overall efficacy of the RNA agent both for medical and stem cell research compared to non-replicating mRNAs (Fang et al., 2022; Steinle, Weber, Behring, Mau-Holzmann, Schlensak, et al., 2019; Steinle, Weber, Behring, Mau-Holzmann, von Ohle, et al., 2019; Vogel et al., 2018). Although to our knowledge there is no active research in this direction yet, srRNAs could also serve as valuable components of artificial biological circuits, such as switches, logic gates, or signal amplifiers. Finally, even though the focus of this review was on srRNAs in bacteria and eukaryotes, the basic principles presented here might also apply to putative archaeal RNA viruses and their hosts (Bolduc et al., 2012). And with the ever-growing knowledge about the current viral RNAs world due to advancements in metagenomics/metatranscriptomics the toolbox of suitable srRNA scaffolds might expand toward new organisms and even subcellular compartments (Lee et al., 2023; Neri et al., 2022).

AUTHOR CONTRIBUTIONS

Alexander Wagner: Visualization (lead); writing – original draft (equal); writing – review and editing (equal). **Hannes Mutschler:** Conceptualization (equal); funding acquisition (equal); project administration (equal); writing – original draft (equal); writing – review and editing (equal).

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The authors have declared no conflicts of interest for this article.

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Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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REFERENCES

Abudayyeh, O. O., Gootenberg, J. S., Konermann, S., Joung, J., Slaymaker, I. M., Cox, D. B. T., Shmakov, S., Makarova, K. S., Semenova, E., Minakhin, L., Severinov, K., Regev, A., Lander, E. S., Koonin, E. V., & Zhang, F. (2016). C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science*, *353*(6299), aaf5573. https://doi.org/10.1126/science.aaf5573

Aljabali, A. A., Hassan, S. S., Pabari, R. M., Shahcheraghi, S. H., Mishra, V., Charbe, N. B., Chellappan, D. K., Dureja, H., Gupta, G., Almutary, A. G., Alnuqaydan, A. M., Verma, S. K., Panda, P. K., Mishra, Y. K., Serrano-Aroca, Á., Dua, K., Uversky, V. N., Redwan, E. M., Bahar, B., … Tambuwala, M. M. (2021). The viral capsid as novel nanomaterials for drug delivery. *Future Science OA*, *7*(9), 744–2056. https://doi.org/10.2144/fsoa-2021-0031

Araujo, P. R., Yoon, K., Ko, D., Smith, A. D., Qiao, M., Suresh, U., Burns, S. C., & Penalva, L. O. F. (2012). Before it gets started: Regulating translation at the 5′ UTR. *Comparative and Functional Genomics*, *2012*, 1–8. https://doi.org/10.1155/2012/475731

Bansho, Y., Furubayashi, T., Ichihashi, N., & Yomo, T. (2016). Host–parasite oscillation dynamics and evolution in a compartmentalized RNA replication system. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(15), 4045–4050. https://doi.org/10.1073/pnas.1524404113

Bansho, Y., Ichihashi, N., Kazuta, Y., Matsuura, T., Suzuki, H., & Yomo, T. (2012). Importance of parasite RNA species repression for prolonged translation-coupled RNA self-replication. *Chemistry & Biology*, *19*(4), 478–487. https://doi.org/10.1016/j.chembiol.2012.01.019

Blakney, A. (2021). The next generation of RNA vaccines: Self-amplifying RNA. *The Biochemist*, *43*(4), 14–17. https://doi.org/10.1042/BIO_2021_142

Bloom, K., van den Berg, F., & Arbuthnot, P. (2020). Self-amplifying RNA vaccines for infectious diseases. *Gene Therapy 2020 28:3*, *28*(3), 117–129 https://doi.org/10.1038/s41434-020-00204-y

Blumenthal, T., & Carmichael, G. G. (1979). RNA replication: Function and structure of QBeta-Replicase. *Annual Review of Biochemistry*, *48*(1), 525–548. https://doi.org/10.1146/annurev.bi.48.070179.002521

Bolduc, B., Shaughnessy, D. P., Wolf, Y. I., Koonin, E. v., Roberto, F. F., & Young, M. (2012). Identification of novel positive-strand RNA viruses by metagenomic analysis of archaea-dominated Yellowstone hot springs. *Journal of Virology*, *86*(10), 5562–5573. https://doi.org/10.1128/JVI.07196-11

Bowie, A. G., & Unterholzner, L. (2008). Viral evasion and subversion of pattern-recognition receptor signalling. *Nature Reviews. Immunology*, *8*(12), 911–922. https://doi.org/10.1038/nri2436

Chase, C. C. L. (2022). Togaviridae and Flaviviridae. In *Veterinary microbiology* (pp. 552–572). Wiley. https://doi.org/10.1002/9781119650836.ch56

Chassy, B. (1988). Transformation of bacteria by electroporation. *Trends in Biotechnology*, *6*(12), 303–309. https://doi.org/10.1016/0167-7799(88)90025-X

Cheng, W. F., Hung, C. F., Hsu, K. F., Chai, C. Y., He, L., Ling, M., Slater, L. A., Roden, R. B., & Wu, T. C. (2001). Enhancement of sindbis virus self-replicating RNA vaccine potency by targeting antigen to endosomal/lysosomal compartments. *Human Gene Therapy*, *12*(3), 235–252. https://doi.org/10.1089/10430340150218387

Cho, M. W., Richards, O. C., Dmitrieva, T. M., Agol, V., Ehrenfeld', E., & Belozersky, A. N. (1993). RNA duplex unwinding activity of poliovirus RNA-dependent RNA polymerase 3Dpol. *Journal of Virology*, *67*(6), 3010–3018. https://doi.org/10.1128/JVI.67.6.3010-3018.1993

Chong, Z. X., Yeap, S. K., & Ho, W. Y. (2021). Transfection types, methods and strategies: A technical review. *PeerJ*, *9*, e11165. https://doi.org/10.7717/peerj.11165

Chu, G., Hayakawa, H., & Berg, P. (1987). Electroporation for the efficient transfection of mammalian cells with DNA. *Nucleic Acids Research*, *15*(3), 1311–1326. https://doi.org/10.1093/nar/15.3.1311

Conzelmann, K. K. (1996). Genetic manipulation of non-segmented negative-strand RNA viruses. *The Journal of General Virology*, *77*(Pt 3), 381–389. https://doi.org/10.1099/0022-1317-77-3-381

Crick, F. (1970). Central dogma of molecular biology. *Nature*, *227*, 561–563. https://doi.org/10.1038/227561a0

Crotty, S., Cameron, C. E., & Andino, R. (2001). RNA virus error catastrophe: Direct molecular test by using ribavirin. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(12), 6895–6900. https://doi.org/10.1073/pnas.111085598

Das, P. K., Merits, A., & Lulla, A. (2014). Functional cross-talk between distant domains of chikungunya virus non-structural protein 2 is decisive for its RNA-modulating activity. *Journal of Biological Chemistry*, *289*(9), 5635–5653. https://doi.org/10.1074/jbc.M113.503433

Davis, J. N., & van den Pol, A. N. (2010). Viral mutagenesis as a means for generating novel proteins. *Journal of Virology*, *84*(3), 1625–1630. https://doi.org/10.1128/JVI.01747-09

Doss, M. X., & Sachinidis, A. (2019). Current challenges of iPSC-based disease modeling and therapeutic implications. *Cell*, *8*(5), 403. https://doi.org/10.3390/cells8050403

Drake, J. W., & Holland, J. J. (1999). Mutation rates among RNA viruses. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(24), 13910–13913. https://doi.org/10.1073/PNAS.96.24.13910

Dye, C. (2022). The benefits of large scale covid-19 vaccination. *BMJ*, *377*. https://doi.org/10.1136/BMJ.O867

Eigen, M., Biebricher, C. K., Gebinoga, M., & Gardiner, W. C. (1991). The hypercycle: Coupling of RNA and protein biosynthesis in the infection cycle of an RNA bacteriophage. *Biochemistry*, *30*(46), 11005–11018.

Eigen, M., & Schuster, P. (1978). The hypercycle: A principle of natural self-organization. Part B: The abstract hypercycle. *Naturwissenschaften*, *65*, 7–41.

Fang, E., Liu, X., Li, M., Zhang, Z., Song, L., Zhu, B., Wu, X., Liu, J., Zhao, D., & Li, Y. (2022). Advances in COVID-19 mRNA vaccine development. *Signal Transduction and Targeted Therapy*, *7*(1), 1–31 https://doi.org/10.1038/s41392-022-00950-y

Fiers, W., Contreras, R., Duerinck, F., Haegeman, G., Iserentant, D., Merregaert, J., Min Jou, W., Molemans, F., Raeymaekers, A., Van den Berghe, A., Volckaert, G., & Ysebaert, M. (1976). Complete nucleotide sequence of bacteriophage MS2 RNA: Primary and secondary structure of the replicase gene. *Nature*, *260*(5551), 500–507. https://doi.org/10.1038/260500a0

Filbin, M. E., & Kieft, J. S. (2009). Toward a structural understanding of IRES RNA function. *Current Opinion in Structural Biology*, *19*(3), 267–276. https://doi.org/10.1016/j.sbi.2009.03.005

Finke, S., & Conzelmann, K. K. (2005a). Recombinant rhabdoviruses: Vectors for vaccine development and gene therapy. *Current Topics in Microbiology and Immunology*, *292*, 165–200. https://doi.org/10.1007/3-540-27485-5_8

Finke, S., & Conzelmann, K.-K. (2005b). Replication strategies of rabies virus. *Virus Research*, *111*(2), 120–131. https://doi.org/10.1016/j.virusres.2005.04.004

Flores, R., Gas, M.-E., Molina-Serrano, D., Nohales, M.-Á., Carbonell, A., Gago, S., de la Peña, M., & Daròs, J.-A. (2009). Viroid replication: Rolling-circles, enzymes and ribozymes. *Viruses*, *1*(2), 317–334. https://doi.org/10.3390/v1020317

Flores, R., Grubb, D., Elleuch, A., Nohales, M.-Á., Delgado, S., & Gago, S. (2011). Rolling-circle replication of viroids, viroid-like satellite RNAs and hepatitis delta virus: Variations on a theme. *RNA Biology*, *8*(2), 200–206. https://doi.org/10.4161/rna.8.2.14238

Forgia, M., Navarro, B., Daghino, S., Cervera, A., Gisel, A., Perotto, S., Aghayeva, D. N., Akinyuwa, M. F., Gobbi, E., Zheludev, I. N., Edgar, R. C., Chikhi, R., Turina, M., Babaian, A., di Serio, F., & de la Peña, M. (2023). Hybrids of RNA viruses and viroid-like elements replicate in fungi. *Nature Communications*, *14*, 2591. https://doi.org/10.1038/s41467-023-38301-2

Fujiyuki, T., Horie, R., Yoneda, M., Kuraishi, T., Yasui, F., Kwon, H.-J., Munekata, K., Ikeda, F., Hoshi, M., Kiso, Y., Omi, M., Sato, H., Kida, H., Hattori, S., Kohara, M., & Kai, C. (2017). Efficacy of recombinant measles virus expressing highly pathogenic avian influenza virus (HPAIV) antigen against HPAIV infection in monkeys. *Scientific Reports*, *7*(1), 12017. https://doi.org/10.1038/s41598-017-08326-x

Furubayashi, T., Ueda, K., Bansho, Y., Motooka, D., Nakamura, S., Mizuuchi, R., & Ichihashi, N. (2020). Emergence and diversification of a host–parasite RNA ecosystem through Darwinian evolution. *eLife*, *9*, 1–15. https://doi.org/10.7554/ELIFE.56038

Georgellis, D., Barlow, T., Arvidson, S., & von Gabain, A. (1993). Retarded RNA turnover in *Escherichia coli*: A means of maintaining gene expression during anaerobiosis. *Molecular Microbiology*, *9*(2), 375–381. https://doi.org/10.1111/J.1365-2958.1993.TB01698.X

Gorbalenya, A. E., Enjuanes, L., Ziebuhr, J., & Snijder, E. J. (2006). Nidovirales: Evolving the largest RNA virus genome. *Virus Research*, *117*(1), 17–37. https://doi.org/10.1016/J.VIRUSRES.2006.01.017

Granados-Riveron, J. T., & Aquino-Jarquin, G. (2021). Engineering of the current nucleoside-modified mRNA-LNP vaccines against SARS-CoV-2. *Biomedicine & Pharmacotherapy*, *142*, 111953. https://doi.org/10.1016/J.BIOPHA.2021.111953

Grudzien-Nogalska, E., Stepinski, J., Jemielity, J., Zuberek, J., Stolarski, R., Rhoads, R. E., & Darzynkiewicz, E. (2007). Synthesis of anti-reverse cap analogs (ARCAs) and their applications in mRNA translation and stability. *Methods in Enzymology*, *431*, 203–227. https://doi.org/10.1016/S0076-6879(07)31011-2

Harel, N., Meir, M., Gophna, U., & Stern, A. (2019). Direct sequencing of RNA with MinION Nanopore: Detecting mutations based on associations. *Nucleic Acids Research*, *47*(22), e148. https://doi.org/10.1093/NAR/GKZ907

Hawkins, A. J. S. (1991). Protein turnover: A functional appraisal. *New Directions in Physiological Ecology*, *5*(2), 222–233.

Hellen, C. U., Kräusslich, H. G., & Wimmer, E. (1989). Proteolytic processing of polyproteins in the replication of RNA viruses. *Biochemistry*, *28*(26), 9881–9890. https://doi.org/10.1021/bi00452a001

Hinnebusch, A. G., & Lorsch, J. R. (2012). The mechanism of eukaryotic translation initiation: New insights and challenges. *Cold Spring Harbor Perspectives in Biology*, *4*(10). https://doi.org/10.1101/cshperspect.a011544

Ichihashi, N., Usui, K., Kazuta, Y., Sunami, T., Matsuura, T., & Yomo, T. (2013). Darwinian evolution in a translation-coupled RNA replication system within a cell-like compartment. *Nature Communications*, *4*, 2494. https://doi.org/10.1038/ncomms3494

Iranzo, J., Puigbò, P., Lobkovsky, A. E., Wolf, Y. I., & Koonin, E. V. (2016). Inevitability of genetic parasites. *Genome Biology and Evolution*, *8*(9), 2856–2869. https://doi.org/10.1093/gbe/evw193

Itoh, M., Isegawa, Y., Hotta, H., & Homma, M. (1997). Isolation of an avirulent mutant of Sendai virus with two amino acid mutations from a highly virulent field strain through adaptation to LLC-MK2 cells. *The Journal of General Virology*, *78*(Pt 12), 3207–3215. https://doi.org/10.1099/0022-1317-78-12-3207

Jaenisch, R., & Young, R. (2008). Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell*, *132*(4), 567–582. https://doi.org/10.1016/j.cell.2008.01.015

Kaczanowska, M., & Rydén-Aulin, M. (2007). Ribosome biogenesis and the translation process in *Escherichia coli*. *Microbiology and Molecular Biology Reviews: MMBR*, *71*(3), 477–494. https://doi.org/10.1128/MMBR.00013-07

Kashiwagi, A., & Yomo, T. (2011). Ongoing phenotypic and genomic changes in experimental coevolution of RNA bacteriophage Qβ and *Escherichia coli*. *PLoS Genetics*, *7*(8), e1002188. https://doi.org/10.1371/journal.pgen.1002188

Kinney, R. M., Tsuchiya, K. R., Sneider, J. M., & Trent, D. W. (1992). Genetic evidence that epizootic Venezuelan equine encephalitis (VEE) viruses may have evolved from enzootic VEE subtype I-D virus. *Virology*, *191*(2), 569–580. https://doi.org/10.1016/0042-6822(92)90232-e

Kita, H., Matsuura, T., Sunami, T., Hosoda, K., Ichihashi, N., Tsukada, K., Urabe, I., & Yomo, T. (2008). Replication of genetic information with self-encoded replicase in liposomes. *Chembiochem*, *9*(15), 2403–2410. https://doi.org/10.1002/cbic.200800360

Kofler, R. M., Aberle, J. H., Aberle, S. W., Allison, S. L., Heinz, F. X., & Mandl, C. W. (2004). Mimicking live flavivirus immunization with a noninfectious RNA vaccine. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(7), 1951–1956. https://doi.org/10.1073/pnas.0307145101

Koonin, E. V., Krupovic, M., & Agol, V. I. (2021). The Baltimore classification of viruses 50 years later: How does it stand in the light of virus evolution? *Microbiology and Molecular Biology Reviews*, *85*(3), e00053–21. https://doi.org/10.1128/MMBR.00053-21

Kramer, F. R., Mills, D. R., Cole, P. E., Nishihara, T., & Spiegelman, S. (1974). Evolution in vitro: Sequence and phenotype of a mutant RNA resistant to ethidium bromide. *Journal of Molecular Biology*, *89*(4), 719–736. https://doi.org/10.1016/0022-2836(74)90047-3

Kun, Á., Szilágyi, A., Könnyu, B., Boza, G., Zachar, I., & Szathmáry, E. (2015). The dynamics of the RNA world: Insights and challenges. *Annals of the New York Academy of Sciences*, *1341*(1), 75–95. https://doi.org/10.1111/NYAS.12700

Küppers, B., & Sumper, M. (1975). Minimal requirements for template recognition by bacteriophage Qbeta replicase: Approach to general RNA-dependent RNA synthesis. *Proceedings of the National Academy of Sciences of the United States of America*, *72*(7), 2640–2643. https://doi.org/10.1073/pnas.72.7.2640

Kurup, D., Wirblich, C., Ramage, H., & Schnell, M. J. (2020). Rabies virus-based COVID-19 vaccine CORAVAX™ induces high levels of neutralizing antibodies against SARS-CoV-2. *Npj Vaccines*, *5*(1), 98. https://doi.org/10.1038/s41541-020-00248-6

Lee, B. D., & Koonin, E. V. (2022). Viroids and viroid-like circular RNAs: Do they descend from primordial replicators? *Life*, *12*(1), 103. https://doi.org/10.3390/LIFE12010103

Lee, B. D., Neri, U., Roux, S., Wolf, Y. I., Camargo, A. P., Krupovic, M., Consortium, R. V. D, Simmonds, P., Kyrpides, N., Gophna, U., Dolja, V. v., & Koonin, E. v. (2023). Mining of metatranscriptomes reveals a vast world of viroid-like circular RNAs. *Cell*, *186*(3), 646–661.e4 https://doi.org/10.1016/j.cell.2022.12.039

Lenhart, J. S., Schroeder, J. W., Walsh, B. W., & Simmons, L. A. (2012). DNA repair and genome maintenance in Bacillus subtilis. *Microbiology and Molecular Biology Reviews*, *76*(3), 530–564. https://doi.org/10.1128/MMBR.05020-11

Liu, C.-X., Li, X., Nan, F., Jiang, S., Gao, X., Guo, S.-K., Xue, W., Cui, Y., Dong, K., Ding, H., Qu, B., Zhou, Z., Shen, N., Yang, L., & Chen, L.-L. (2019). Structure and degradation of circular RNAs regulate PKR activation in innate immunity. *Cell*, *177*(4), 865–880.e21. https://doi.org/10.1016/j.cell.2019.03.046

Liu, H., Jiao, X., Wang, Y., Yang, X., Sun, W., Wang, J., Zhang, S., & Zhao, Z. K. (2017). Fast and efficient genetic transformation of oleaginous yeast *Rhodosporidium toruloides* by using electroporation. *FEMS Yeast Research*, *17*(2), 17. https://doi.org/10.1093/femsyr/fox017

Louten, J. (2016). Virus structure and classification. *Essential Human Virology*, *19*, 19–29. https://doi.org/10.1016/B978-0-12-800947-5.00002-8

Lundstrom, K. (2021). Self-replicating RNA viruses for vaccine development against infectious diseases and cancer. *Vaccine*, *9*(10), 1187. https://doi.org/10.3390/vaccines9101187

Luo, M., Terrell, J. R., & Mcmanus, S. A. (2020). Nucleocapsid structure of negative strand RNA virus. *Viruses*, *12*(8), 835. https://doi.org/10.3390/V12080835

Maine, C. J., Richard, G., Spasova, D. S., Miyake-Stoner, S. J., Sparks, J., Moise, L., Sullivan, R. P., Garijo, O., Choz, M., Crouse, J. M., Aguilar, A., Olesiuk, M. D., Lyons, K., Salvador, K., Blomgren, M., DeHart, J. L., Kamrud, K. I., Berdugo, G., de Groot, A. S., … Aliahmad, P. (2021). Self-replicating RNAs drive protective anti-tumor T cell responses to Neoantigen vaccine targets in a combinatorial approach. *Molecular Therapy*, *29*(3), 1186–1198. https://doi.org/10.1016/j.ymthe.2020.11.027

Mandl, C. W. (2005). Steps of the tick-borne encephalitis virus replication cycle that affect neuropathogenesis. *Virus Research*, *111*(2), 161–174. https://doi.org/10.1016/j.virusres.2005.04.007

Marzi, A., Reynolds, P., Mercado-Hernandez, R., Callison, J., Feldmann, F., Rosenke, R., Thomas, T., Scott, D. P., Hanley, P. W., Haddock, E., & Feldmann, H. (2019). Single low-dose VSV-EBOV vaccination protects cynomolgus macaques from lethal Ebola challenge. *eBioMedicine*, *49*, 223–231. https://doi.org/10.1016/j.ebiom.2019.09.055

Mertens, P. (2004). The dsRNA viruses. *Virus Research*, *101*(1), 3–13. https://doi.org/10.1016/J.VIRUSRES.2003.12.002

Miller, S. M., Wang, T., & Liu, D. R. (2020). Phage-assisted continuous and non-continuous evolution. *Nature Protocols*, *15*(12), 4101–4127. https://doi.org/10.1038/s41596-020-00410-3

Mills, D. R., Peterson, R. L., & Spiegelman, S. (1967). An extracellular Darwinian experiment with a self-duplicating nucleic acid molecule. *Proceedings of the National Academy of Sciences of the United States of America*, *58*(1), 217–224.

Mizuuchi, R., Furubayashi, T., & Ichihashi, N. (2022). Evolutionary transition from a single RNA replicator to a multiple replicator network. *Nature Communications*, *13*(1), 1–10. https://doi.org/10.1038/s41467-022-29113-x

Mizuuchi, R., & Ichihashi, N. (2018). Sustainable replication and coevolution of cooperative RNAs in an artificial cell-like system. *Nature Ecology and Evolution*, *2*(10), 1654–1660. https://doi.org/10.1038/s41559-018-0650-z

Mizuuchi, R., Usui, K., & Ichihashi, N. (2019). Structural transition of replicable RNAs during in vitro evolution with Qβ replicase. *RNA*, *26*(1), 83–90. https://doi.org/10.1261/RNA.073106.119

Nance, K. D., & Meier, J. L. (2021). Modifications in an emergency: The role of N1-Methylpseudouridine in COVID-19 vaccines. *ACS Central Science*, *7*(5), 748–756. https://doi.org/10.1021/ACSCENTSCI.1C00197

Naqvi, A. A. T., Fatima, K., Mohammad, T., Fatima, U., Singh, I. K., Singh, A., Atif, S. M., Hariprasad, G., Hasan, G. M., & Hassan, M. I. (2020). Insights into SARS-CoV-2 genome, structure, evolution, pathogenesis and therapies: Structural genomics approach. *Biochimica et Biophysica Acta. Molecular Basis of Disease*, *1866*(10), 165878. https://doi.org/10.1016/J.BBADIS.2020.165878

Navarro, B., Flores, R., & di Serio, F. (2021). Advances in viroid-host interactions. *Annual Review of Virology*, *8*(1), 305–325. https://doi.org/10.1146/annurev-virology-091919-092331

Neri, U., Wolf, Y. I., Roux, S., Camargo, A. P., Lee, B., Kazlauskas, D., Chen, I. M., Ivanova, N., Zeigler Allen, L., Paez-Espino, D., Bryant, D. A., Bhaya, D., Krupovic, M., Dolja, V. V., Kyrpides, N. C., Koonin, E. V., Gophna, U., Narrowe, A. B., Probst, A. J., … Wang, Z. (2022). Expansion of the global RNA virome reveals diverse clades of bacteriophages. *Cell*, *185*(21), 4023–4037.e18. https://doi.org/10.1016/j.cell.2022.08.023

Nishimura, K., Ohtaka, M., Takada, H., Kurisaki, A., Tran, N. V. K., Tran, Y. T. H., Hisatake, K., Sano, M., & Nakanishi, M. (2017). Simple and effective generation of transgene-free induced pluripotent stem cells using an auto-erasable Sendai virus vector responding to microRNA-302. *Stem Cell Research*, *23*, 13–19. https://doi.org/10.1016/j.scr.2017.06.011

Nishimura, K., Sano, M., Ohtaka, M., Furuta, B., Umemura, Y., Nakajima, Y., Ikehara, Y., Kobayashi, T., Segawa, H., Takayasu, S., Sato, H., Motomura, K., Uchida, E., Kanayasu-Toyoda, T., Asashima, M., Nakauchi, H., Yamaguchi, T., & Nakanishi, M. (2011). Development of defective and persistent Sendai virus vector: A unique gene delivery/expression system ideal for cell reprogramming. *The Journal of Biological Chemistry*, *286*(6), 4760–4771. https://doi.org/10.1074/jbc.M110.183780

Ogino, T., Kobayashi, M., Iwama, M., & Mizumoto, K. (2005). Sendai virus RNA-dependent RNA polymerase L protein catalyzes cap methylation of virus-specific mRNA. *The Journal of Biological Chemistry*, *280*(6), 4429–4435. https://doi.org/10.1074/jbc.M411167200

Palukaitis, P. (2016). Satellite RNAs and satellite viruses. *Molecular Plant-Microbe Interactions*, *29*(3), 181–186. https://doi.org/10.1094/MPMI-10-15-0232-FI

Pardi, N., Hogan, M. J., Porter, F. W., & Weissman, D. (2018). mRNA vaccines—A new era in vaccinology. *Nature Reviews Drug Discovery*, *17*(4), 261–279. https://doi.org/10.1038/nrd.2017.243

Pollock, K. M., Cheeseman, H. M., Szubert, A. J., Libri, V., Boffito, M., Owen, D., Bern, H., McFarlane, L. R., O'Hara, J., Lemm, N.-M., McKay, P., Rampling, T., Yim, Y. T. N., Milinkovic, A., Kingsley, C., Cole, T., Fagerbrink, S., Aban, M., Tanaka, M., … Wilkins, M. (2022). Safety and immunogenicity of a self-amplifying RNA vaccine against COVID-19: COVAC1, a phase I, dose-ranging trial. *eClinicalMedicine*, *44*, 101262. https://doi.org/10.1016/j.eclinm.2021.101262

Raju, R., Hajjou, M., Hill, K. R., Botta, V., & Botta, S. (1999). In vivo addition of poly(A) tail and AU-rich sequences to the 3′ terminus of the Sindbis virus RNA genome: A novel 3′-end repair pathway. *Journal of Virology*, *73*(3), 2410–2419. https://doi.org/10.1128/JVI.73.3.2410-2419.1999

Rampersad, S., & Tennant, P. (2018). Replication and expression strategies of viruses. In *Viruses* (pp. 55–82). Elsevier. https://doi.org/10.1016/B978-0-12-811257-1.00003-6

Rose, N. F., Marx, P. A., Luckay, A., Nixon, D. F., Moretto, W. J., Donahoe, S. M., Montefiori, D., Roberts, A., Buonocore, L., & Rose, J. K. (2001). An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. *Cell*, *106*(5), 539–549. https://doi.org/10.1016/s0092-8674(01)00482-2

Saberi, A., Gulyaeva, A. A., Brubacher, J. L., Newmark, P. A., & Gorbalenya, A. E. (2018). A planarian nidovirus expands the limits of RNA genome size. *PLoS Pathogens*, *14*(11), e1007314. https://doi.org/10.1371/JOURNAL.PPAT.1007314

Salonen, A., Vasiljeva, L., Merits, A., Magden, J., Jokitalo, E., & Kääriäinen, L. (2003). Properly folded nonstructural polyprotein directs the semliki forest virus replication complex to the endosomal compartment. *Journal of Virology*, *77*(3), 1691–1702. https://doi.org/10.1128/jvi.77.3.1691-1702.2003

Schuppli, D., Georgijevic, J., & Weber, H. (2000). Synergism of mutations in bacteriophage qβ RNA affecting host factor dependence of qβ replicase. *Journal of Molecular Biology*, *295*(2), 149–154. https://doi.org/10.1006/JMBI.1999.3373

Sikora, D., & Rzymski, P. (2022). COVID-19 vaccination and rates of infections, hospitalizations, ICU admissions, and deaths in the European economic area during autumn 2021 wave of SARS-CoV-2. *Vaccines*, *10*(3), 437. https://doi.org/10.3390/VACCINES10030437

Sinn, P. L., Sauter, S. L., & McCray, P. B. (2005). Gene therapy progress and prospects: Development of improved lentiviral and retroviral vectors—Design, biosafety, and production. *Gene Therapy*, *12*(14), 1089–1098. https://doi.org/10.1038/sj.gt.3302570

Steinle, H., Weber, M., Behring, A., Mau-Holzmann, U., Schlensak, C., Wendel, H. P., & Avci-Adali, M. (2019). Generation of iPSCs by non-integrative RNA-based reprogramming techniques: Benefits of self-replicating RNA versus synthetic mRNA. *Stem Cells International*, *2019*, 7641767. https://doi.org/10.1155/2019/7641767

Steinle, H., Weber, M., Behring, A., Mau-Holzmann, U., von Ohle, C., Popov, A.-F., Schlensak, C., Wendel, H. P., & Avci-Adali, M. (2019). Reprogramming of urine-derived renal epithelial cells into iPSCs using srRNA and consecutive differentiation into beating cardiomyocytes. *Molecular Therapy. Nucleic Acids*, *17*, 907–921. https://doi.org/10.1016/j.omtn.2019.07.016

Strauss, E. G., Rice, C. M., & Strauss, J. H. (1984). Complete nucleotide sequence of the genomic RNA of Sindbis virus. *Virology*, *133*(1), 92–110. https://doi.org/10.1016/0042-6822(84)90428-8

Sutela, S., Forgia, M., Vainio, E. J., Chiapello, M., Daghino, S., Vallino, M., Martino, E., Girlanda, M., Perotto, S., & Turina, M. (2020). The virome from a collection of endomycorrhizal fungi reveals new viral taxa with unprecedented genome organization. *Virus Evolution*, *6*(2), veaa076. https://doi.org/10.1093/VE/VEAA076

Szabó, G. T., Mahiny, A. J., & Vlatkovic, I. (2022). COVID-19 mRNA vaccines: Platforms and current developments. *Molecular Therapy*, *30*(5), 1850–1868. https://doi.org/10.1016/j.ymthe.2022.02.016

Szostak, J. W. (2012). The eightfold path to non-enzymatic RNA replication. *Journal of Systems Chemistry*, *3*(1), 1–14. https://doi.org/10.1186/1759-2208-3-2

Sztuba-Solińska, J., Stollar, V., & Bujarski, J. J. (2011). Subgenomic messenger RNAs: Mastering regulation of (+)-strand RNA virus life cycle. *Virology*, *412*(2), 245–255. https://doi.org/10.1016/J.VIROL.2011.02.007

Szymczak, A. L., Workman, C. J., Wang, Y., Vignali, K. M., Dilioglou, S., Vanin, E. F., & Vignali, D. A. A. (2004). Correction of multi-gene deficiency in vivo using a single "self-cleaving" 2A peptide-based retroviral vector. *Nature Biotechnology*, *22*(5), 589–594. https://doi.org/10.1038/nbt957

Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, *126*(4), 663–676. https://doi.org/10.1016/j.cell.2006.07.024

Taketo, A. (1989). RNA transfection of *Escherichia coli* by electroporation. *Biochimica et Biophysica Acta*, *1007*(1), 127–129. https://doi.org/10.1016/0167-4781(89)90142-5

Takeuchi, K., Miyajima, N., Kobune, F., & Tashiro, M. (2000). Comparative nucleotide sequence analyses of the entire genomes of B95a cell-isolated and vero cell-isolated measles viruses from the same patient. *Virus Genes*, *20*(3), 253–257. https://doi.org/10.1023/a:1008196729676

Tao, Y. J., & Ye, Q. (2010). RNA Virus Replication Complexes. *PLoS Pathogens*, *6*(7), e1000943. https://doi.org/10.1371/journal.ppat.1000943

Tomita, K., Ichihashi, N., & Yomo, T. (2015). Replication of partial double-stranded RNAs by Qβ replicase. *Biochemical and Biophysical Research Communications*, *467*(2), 293–296. https://doi.org/10.1016/j.bbrc.2015.09.169

Tordo, N., Poch, O., Ermine, A., Keith, G., & Rougeon, F. (1988). Completion of the rabies virus genome sequence determination: Highly conserved domains among the L (polymerase) proteins of unsegmented negative-strand RNA viruses. *Virology*, *165*(2), 565–576. https://doi.org/10.1016/0042-6822(88)90600-9

van Dijk, A. A., Makeyev, E. v., & Bamford, D. H. (2004). Initiation of viral RNA-dependent RNA polymerization. *The Journal of General Virology*, *85*(Pt 5), 1077–1093. https://doi.org/10.1099/vir.0.19731-0

Vogel, A. B., Lambert, L., Kinnear, E., Busse, D., Erbar, S., Reuter, K. C., Wicke, L., Perkovic, M., Beissert, T., Haas, H., Reece, S. T., Sahin, U., & Tregoning, J. S. (2018). Self-amplifying RNA vaccines give equivalent protection against influenza to mRNA vaccines but at much lower doses. *Molecular Therapy*, *26*(2), 446–455. https://doi.org/10.1016/j.ymthe.2017.11.017

Wagner, A., Weise, L. I., & Mutschler, H. (2022). In vitro characterisation of the MS2 RNA polymerase complex reveals host factors that modulate emesviral replicase activity. *Communications Biology*, *5*(1), 264. https://doi.org/10.1038/s42003-022-03178-2

Wang, A. Y. L. (2021). Application of modified mRNA in somatic reprogramming to pluripotency and directed conversion of cell fate. *International Journal of Molecular Sciences*, *22*(15), 8148. https://doi.org/10.3390/IJMS22158148

Warren, L., & Lin, C. (2019). mRNA-based genetic reprogramming. *Molecular Therapy*, *27*(4), 729–734. https://doi.org/10.1016/J.YMTHE.2018.12.009

Warren, L., Manos, P. D., Ahfeldt, T., Loh, Y.-H., Li, H., Lau, F., Ebina, W., Mandal, P. K., Smith, Z. D., Meissner, A., Daley, G. Q., Brack, A. S., Collins, J. J., Cowan, C., Schlaeger, T. M., & Rossi, D. J. (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell*, *7*(5), 618–630. https://doi.org/10.1016/j.stem.2010.08.012

Weaver, S., Zulay Garcia Mendez, C., Carlos Navarro, J., Navarro, J.-C., Alfonzo, D., & Eugenia Grillet, M. (2004). Venezuelan equine encephalitis. *Annual Review of Entomology*, *49*, 141–174. https://doi.org/10.1146/annurev.ento.49.061802.123422

Wirblich, C., Coleman, C. M., Kurup, D., Abraham, T. S., Bernbaum, J. G., Jahrling, P. B., Hensley, L. E., Johnson, R. F., Frieman, M. B., & Schnell, M. J. (2017). One-health: A safe, efficient, dual-use vaccine for humans and animals against Middle East respiratory syndrome coronavirus and rabies virus. *Journal of Virology*, *91*(2), 2040–2056. https://doi.org/10.1128/JVI.02040-16

Yan, Y., Liu, X.-Y., Lu, A., Wang, X.-Y., Jiang, L.-X., & Wang, J.-C. (2022). Non-viral vectors for RNA delivery. *Journal of Controlled Release*, *342*, 241–279. https://doi.org/10.1016/j.jconrel.2022.01.008

Yao, Y., Zhang, W., Zhang, M., Jin, S., Guo, Y., Zu, Y., Ren, K., Wang, K., Chen, G., Lou, C., & Wu, Q. (2019). A direct RNA-to-RNA replication system for enhanced gene expression in bacteria. *ACS Synthetic Biology*, *8*(5), 1067–1078. https://doi.org/10.1021/acssynbio.8b00521

Ying, H., Zaks, T. Z., Wang, R. F., Irvine, K. R., Kammula, U. S., Marincola, F. M., Leitner, W. W., & Restifo, N. P. (1999). Cancer therapy using a self-replicating RNA vaccine. *Nature Medicine*, *5*(7), 823–827. https://doi.org/10.1038/10548

Yoshioka, N., & Dowdy, S. F. (2017). Enhanced generation of iPSCs from older adult human cells by a synthetic five-factor self-replicative RNA. *PLoS One*, *12*(7), e0182018. https://doi.org/10.1371/journal.pone.0182018

Yoshioka, N., Gros, E., Li, H.-R., Kumar, S., Deacon, D. C., Maron, C., Muotri, A. R., Chi, N. C., Fu, X.-D., Yu, B. D., & Dowdy, S. F. (2013). Efficient generation of human iPSCs by a synthetic self-replicative RNA. *Cell Stem Cell*, *13*(2), 246–254. https://doi.org/10.1016/j.stem.2013.06.001

Zhang, C., Tian, Y., Chen, C., Wang, Z., Pei, J., Lin, C., Zhou, M., Fu, Z. F., & Zhao, L. (2021). Virus-like vesicles based on Semliki forest virus-containing rabies virus glycoprotein make a safe and efficacious rabies vaccine candidate in a mouse model. *Journal of Virology*, *95*(20), e0079021. https://doi.org/10.1128/JVI.00790-21

Zheng, Z., Yang, J., Jiang, X., Liu, Y., Zhang, X., Li, M., Zhang, M., Fu, M., Hu, K., Wang, H., Luo, M.-H., Gong, P., & Hu, Q. (2018). Tick-borne encephalitis virus nonstructural protein NS5 induces RANTES expression dependent on the RNA-dependent RNA polymerase activity. *Journal of Immunology*, *201*, 53–68. https://doi.org/10.4049/jimmunol.1701507

Zhou, X., Berglund, P., Rhodes, G., Parker, S. E., Jondal, M., & Liljeström, P. (1994). Self-replicating Semliki Forest virus RNA as recombinant vaccine. *Vaccine*, *12*(16), 1510–1514. https://doi.org/10.1016/0264-410x(94)90074-4

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