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Phage N15-Based Vectors for Gene Cloning and Expression in Bacteria and Mammalian Cells

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Cite this: *ACS Synth. Biol.* 2023, 12, 4, 909–921
Publication Date: April 6, 2023
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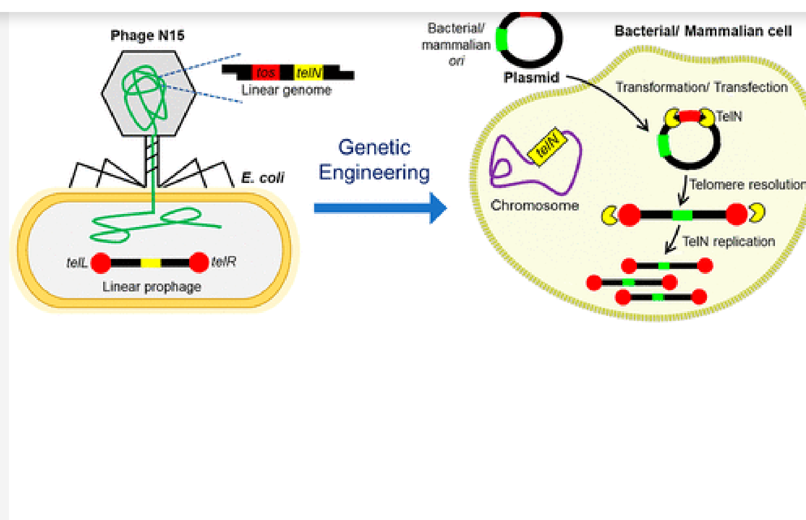

Bacteriophage N15 is the first virus known to deliver linear prophage into *Escherichia coli*. During its lysogenic cycle, N15 protelomerase (TelN) resolves its telomerase occupancy site (*tos*) into hairpin telomeres. This protects the N15 prophage from bacterial exonuclease degradation, enabling it to stably replicate as a linear plasmid in *E. coli*. Interestingly, purely proteinaceous TelN can retain phage DNA linearization and hairpin formation without involving host- or phage-derived intermediates or cofactors in the heterologous environment. This unique feature has led to the advent of synthetic linear DNA vector systems derived from the TelN-*tos* module for the genetic engineering of bacterial and mammalian cells. This review will focus on the development and advantages of N15-based novel cloning and expression vectors in the bacterial and mammalian environments. To date, N15 is the most widely exploited molecular tool for the development of linear vector systems, especially the production of therapeutically useful miniDNA vectors without a bacterial backbone. Compared to typical circular plasmids, linear N15-based plasmids display remarkable cloning fidelity in propagating unstable repetitive DNA sequences and large genomic fragments. Additionally, TelN-linearized vectors with the relevant origin of replication can replicate extrachromosomally and retain transgenes functionality in bacterial and mammalian cells without compromising host cell viability. Currently, this DNA linearization system has shown robust results in the development of gene delivery vehicles, DNA vaccines and engineering mammalian cells against infectious diseases or cancers, highlighting its multifaceted importance in genetic studies and gene medicine.

KEYWORDS: bacteriophage N15, protelomerase TelN, telomerase occupancy site, hairpin telomeres

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Review

Phage N15-Based Vectors for Gene Cloning and Expression in Bacteria and Mammalian Cells

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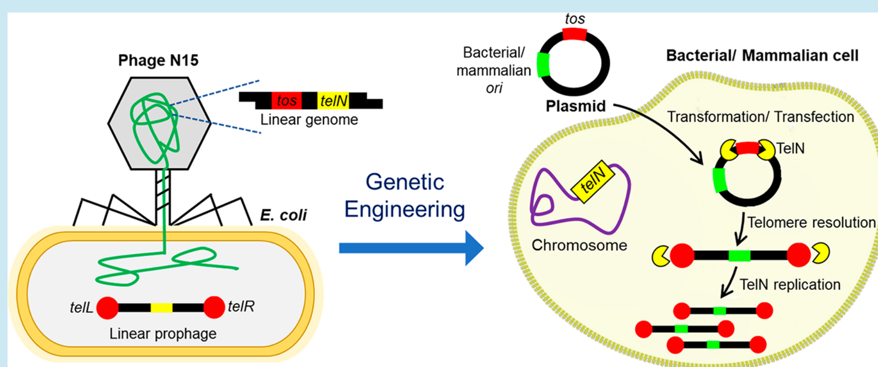

ABSTRACT: Bacteriophage N15 is the first virus known to deliver linear prophage into *Escherichia coli.* During its lysogenic cycle, N15 protelomerase (TelN) resolves its telomerase occupancy site (*tos*) into hairpin telomeres. This protects the N15 prophage from bacterial exonuclease degradation, enabling it to stably replicate as a linear plasmid in *E. coli.* Interestingly, purely proteinaceous TelN can retain phage DNA linearization and hairpin formation without involving host- or phage-derived intermediates or cofactors in the heterologous environment. This unique feature has led to the advent of synthetic linear DNA vector systems derived from the TelN-*tos* module for the genetic engineering of bacterial and mammalian cells. This review will focus on the development and advantages of N15-based novel cloning and expression vectors in the bacterial and mammalian environments. To date, N15 is the most widely exploited molecular tool for the development of linear vector systems, especially the production of therapeutically useful miniDNA vectors without a bacterial backbone. Compared to typical circular plasmids, linear N15-based plasmids display remarkable cloning fidelity in propagating unstable repetitive DNA sequences and large genomic fragments. Additionally, TelN-linearized vectors with the relevant origin of replication can replicate extrachromosomally and retain transgenes functionality in bacterial and mammalian cells without compromising host cell viability. Currently, this DNA linearization system has shown robust results in the development of gene delivery vehicles, DNA vaccines and engineering mammalian cells against infectious diseases or cancers, highlighting its multifaceted importance in genetic studies and gene medicine.

KEYWORDS: *bacteriophage N15, protelomerase TelN, telomerase occupancy site, hairpin telomeres, cloning and expression vectors*

1. INTRODUCTION

The bacteriophage is a valuable tool for genetic engineering owing to the high abundance of genome diversity, which has proven applications in the studies of bacteria[1,2] or mammalian cells,[3,4] food production,[5,6] and water pollution control.[7] In the field of recombinant DNA technology, bacteriophage N15 is identified as one of the two viruses known to deliver linear plasmid prophage into *Escherichia coli*,[8] the other being phage PRD1.[9] In contrast to the linear PRD1 genome which is covalently linked to terminal proteins at its 5′ end,[10] N15 solely replicates as a linear plasmid with covalently closed ends (hairpin telomeres) in *E. coli*,[11] producing an unconventional genomic structure in prokaryotes that is similar to the canonical eukaryotic DNA structure. This capability of N15 has then gained significant attention by researchers pursuing the N15 genome and biology.[8,11]

Several unique properties of the N15 phage have sparked studies and exploitations on its biology. First, it was found that purely proteinaceous N15 protelomerase enzyme (TelN) can

Received: October 27, 2022

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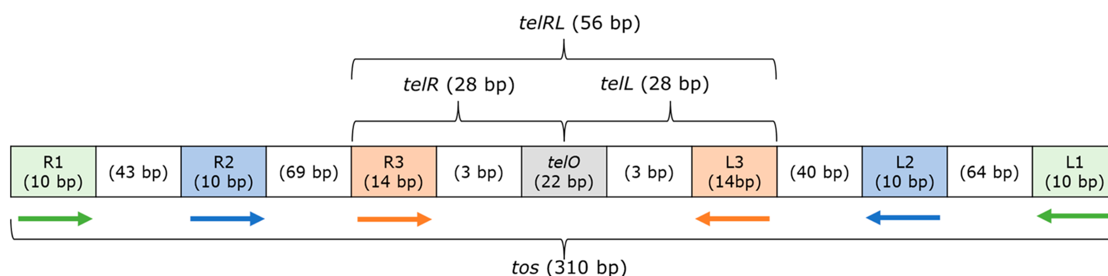

Figure 1. Schematic diagram showing the structure of telomere occupancy site (*tos*). *Tos* consists of three pairs of inverted repeats R1/L1, R2/L2 and R3/L3 flanking a central *telO* palindrome, which is the TelN actual cleavage site. Telomere recognition site, *TelRL* comprising repeats R3/L3 and *telO*. Telomeric ends of TelN-linearized DNA derived from *telL* and *telR*. The size of each feature is labeled in base pairs (bp).

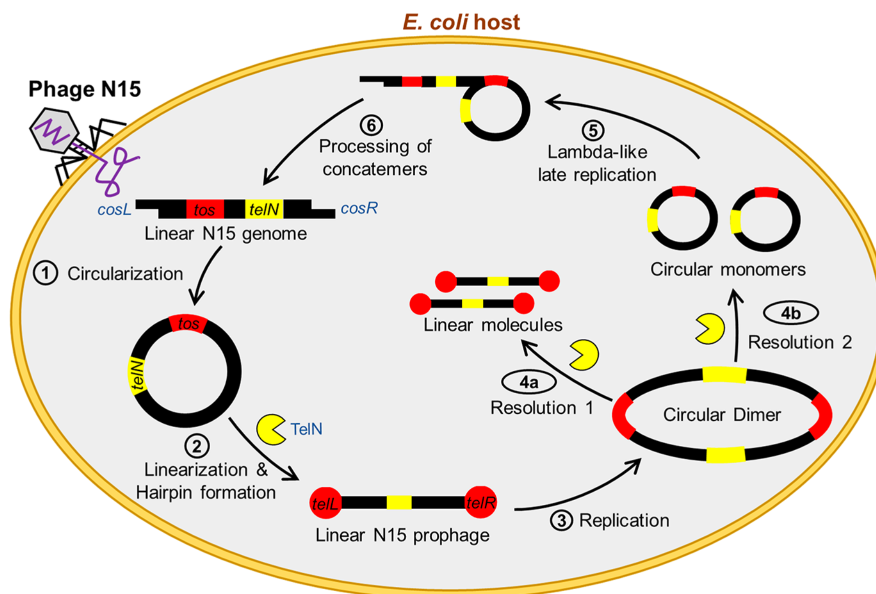

Figure 2. Delivery of linear N15 prophage into *E. coli*. The infecting N15 virion circularizes through its 12 bp single-stranded cohesive ends upon entrance into the bacterial host (1). It is subsequently cleaved at *tos* (red block) by TelN to generate a linear prophage with covalently closed telomeres (2). The linear prophage then undergoes replication to produce a circular head-to-head dimer intermediate (3), which is either resolved into two linear molecules (prophage) (Resolution 1) (4a) during lysogenic cycle or two circular head-to-tail monomers (Resolution 2) (4b) that served as templates for the production of linear virions (6) through lambda-like late replication (5) during lytic development. The roles of TelN in the relevant steps are depicted. Note that the replication of circular monomer is supposed to produce two linear concatemers (only one is shown).

resolve its DNA substrate, telomerase occupancy site (*tos*) into telomeric ends *in vitro* without involving host- or phage-derived intermediate or cofactor[12] as opposed to eukaryotic protelomerases that require the involvement of RNA components. This feature allows the TelN-*tos* module to retain its native functions in the heterologous bacterial and mammalian environments. For example, it was experimentally shown that TelN expressed by bacterial and mammalian cells can fully resolve the *tos* sequence inserted into circular DNA vectors *in vitro* and *in vivo*, resulting in linear DNA with telomeric ends.[13,14] Second, N15 can replicate extrachromosomally as a linear prophage in *E. coli* without causing chromosomal integration,[11] displaying one of the essential features required in the design of episomal DNA vectors. Non-N15 plasmids,[15] *E. coli* chromosome[16,17] and human genomic fragments[15,18] have been linearized using N15 elements without losing the native gene function or compromising host cell viability. These characteristics were then found to provide additional values in the field of microbiology and biotechnology.

In bacterial cells, N15-based DNA vectors have been generally developed for two purposes: (i) to understand the genomics of phage N15 and (ii) to clone repetitive or unstable or large DNA sequences. So far, there has not been any focus on expression studies using N15-based DNA vectors in *E. coli*, which we feel will be necessary to further the development of these vectors for wider use.

In this review, we focus on the versatile use of N15 elements as a DNA linearization tool for gene cloning and expression in bacterial and mammalian hosts. We begin with a brief overview of the life cycle and basic genetics of N15, followed by the development of N15-based cloning and expression vectors in bacteria and mammalian hosts. After that, we describe the therapeutic applications of TelN-linearized DNA vectors and conclude with our perspective on the potentials of the N15-based linear vector system.

2. LINEAR N15 PLASMID PROPHAGE

2.1. Telomere Resolution of N15 Prophage. Unlike most temperate phages that integrate their genome into the chromosome of bacterial host during lysogenic infection, lambdoid phage N15 is atypical because they replicate their genome as a non-integrating linear dsDNA prophage with covalently closed-ends in the *E. coli* host.[11] During its life cycle,

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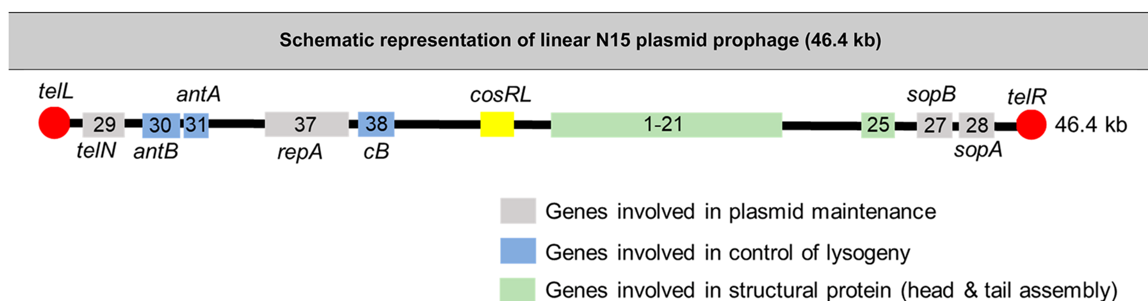
Table 1. Overview of Circular and Linear N15-Based Plasmids



No.	Plasmid (Size)	Vector characteristic	refs
1	Linear pG54	• Comprises 20.1 kb N15 genomic fragment (from *telL* to nt 18,996 and from nt 45,269 to the *telR*) • Includes N15 genes responsible for plasmid replication and lysogeny control • Excludes N15 lytic and structural genes which are not involved in the propagation of linear prophage	27, 31, 32
2	Linear pG59 (13.7 kb)	• Consists N15 gene 29 (*telN*) to gene 38 (*cB*), comprising N15 replicase *repA* (gene 37) • Retains plasmid replication, telomere resolution and copy number control	20, 28
3	Linear pG591 (13.1 kb)	• Derived via the deletion of *sopAB* genes from pG59 • Retains plasmid replication, telomere resolution, and copy number control • Equivalent low-copy number as N15 prophage	20, 28
4	Circular pNC06	• Contains *repA*, repA-cB intergenic region, and last 10 bp of *cB*	20
5	Linear pNL01	• Derived via the addition of *telRL* site and *telN* gene into pNC06 • Has a much higher copy number than pG591 and N15 prophage due to the absence of *cB* repressor gene, which controls N15 copy number via binding to the *repA* promoter	20, 30
6	Circular pNC071	• Cloned into plasmid pBAD24 with deleted *ori* site • Contains *repA* (encompassing N15 *ori* site)	20
7	Circular pNC10	• Contains one of the N15 centromeres, IR1 centromere unit, termed centromere-carrying plasmids	33
8	Circular pN15E vectors (pN15E4 and pN15E6)	• Constructed via joining multiple modules, including a fragment of pG591 containing replication region (genes 34−36, *repA*) and *cB* repressor genes • Low-copy number plasmid	30
9	Linear N15-203 (13.8 kb)	• High-copy (250 copies/cell) plasmid • Vector capacity up to 20 kb	31
10	Linear pN15L (13.8 kb)	• Consists a fragment of N15 phage genome partially digested with *Sau3A*, which contains N15 mini-replicon • High-copy (250 copies/cell) number • Vector capacity of more than 50 kb for nonpalindromic insert • Displays remarkable stability and cloning fidelity of artificial and natural palindromes	34
11	Linear pJAZZ vectors (13.4 kb pJAZZ-OK, 13.4 kb pJAZZ-OC, 8.6 kb pJAZZ-OCmin)	• Low-copy (5 copies/cell) transcription-free cloning vectors derived from pG591 • Consist *repA* (contains *ori*) • Both ends of the cloning site (*lacZ* gene) and vector backbone (antibiotic resistance gene) are separated by transcriptional terminators • Host: *E. coli* TSA strain (chromosomally integrated genes: *telN, sopAB, antA*) • The only difference between pJAZZ-OK and pJAZZ-OC is their antibiotic resistance gene • pJAZZ-OCmin is a minimal linear vector excluding inessential N15 genes in *cis*, i.e., *telN* and antirepressor operon (genes 30−32) • Stable cloning of short tandem repeats (≥2 kb), AT- and GC-rich inserts (≥30 kb)	35
12	Linear pBeloBAC11-*tos* (8.7 kb)	• Insertion of *tos*-Zeo^R cassette into commercial plasmid pBeloBAC11	15
13	Linear pBelo-*tos*-eGFP (11.7 kb)	• Insertion of eGFP-Neo^R cassette from commercial plasmid pEGFP-N2 into pBelo-*tos*[15]	18
14	Linear BAC4396-*tos* (100 kb)	• Insertion of *tos*-Zeo^R cassette into BAC4396[36] comprising 70 kb full-length human β-globin gene cluster	15
15	Linear BAC4396-*tos*- S/MAR/Hyg (110 kb)	• Insertion of S/MAR-eGFP cassette from pEPI-eGFP,[37] and hygromycin cassette from commercial plasmid pSELECT-hygro-mcs into BAC4396-*tos* sequentially • Episomal S/MAR-based human globin BAC	25
16	Linear BAC-GLA-*tos*-S/MAR (101.5 kb)	• Insertion of *tos*-Zeo^R cassette from pBeloBAC11-tos, and S/MAR-eGFP cassette from pEPI-eGFP[37] into human BAC clone RP11−125I15 (BACPAC resources) sequentially • Episomal S/MAR-based BAC containing a fragment of human X chromosome	18

the N15 replicon alternates between linear and circular forms. The replication of N15 linear plasmid is attributed to the cleavage-joining activity of phage-encoded TelN enzyme on 22-bp palindromic *telO* substrate within the 310-bp *tos* site (Figure 1) to form terminal hairpin loops at both ends.[12] The *tos* sequence consists of a series of inverted repeats (R1/L1 to R3/L3) flanking the *telO* cleavage site, which plays roles in the stabilization of TelN-DNA complexes, especially R3/L3.[12] Previous *in vitro* studies found that a 50-fold higher concentration of TelN is required to resolve *telO* into hairpin

https://doi.org/10.1021/acssynbio.2c00580
ACS Synth. Biol. XXXX, XXX, XXX−XXX

telomere in the absence of flanking repeats R3/L3.[12,19] Repeats R3/L3 and *telO* are also known as telomere recognition site, *TelRL*.

Upon infection of *E. coli*, the linear N15 virion circularizes through its single-stranded cohesive ends (Figure 2).[11] It is subsequently cleaved within *tos* by TelN enzyme and reannealed to generate covalently closed telomeres.[8,12,19] The resulting linear plasmid serves as the template for the synthesis of circular head-to-head dimers,[11] which are subsequently cut into linear prophages by TelN during lysogenic cycle[20] (Figure 2, Resolution 1), or resolved into circular head-to-tail monomers by TelN during lytic development (Figure 2, Resolution 2), which then undergoes the lambda-like late replication to produce linear virions.[21] The conversion between lysogeny cycle to lytic development depends on the control of lysogeny regulated by antirepressor protein, AntC.[11]

During lysogenic cycle, the linear prophage replicates as a stable linear low-copy (3−5 copies per bacterial chromosome) plasmid in *E. coli*.[22] The telomeric ends of linear plasmid facilitate their stable retention and replication in the bacterial host by protecting it against degradation from nucleases. In short, TelN is a multifunctional enzyme integral for the recognition of palindromic sequence, cleavage-joining activity, and replication of N15 linear prophage, as well as its lytic development. An inadequate level of functional TelN will cause inefficient DNA replication and accumulation of unresolved circular "head-to-head" dimers and/or circular "head-to-tail" monomers, instead of the formation of linear molecules.[8,21]

2.2. Structural Study of Protelomerase TelN. Structural analysis of TelN protein is important for the understanding of their interaction with its DNA substrate, *tos*. Sequence alignment analysis between TelN, TelK, and other protelomerases revealed that these enzymes share a highly conserved, arginine-rich catalytic core.[23] However, the structure−function relationship and mechanism on how TelN binds to and catalyzes its substrate remain elusive. Liew et al. (2019) reported the structure and function of TelN with the use of *in silico* tools such as I-TASSER and COACH. The homology TelN model was highly identical to the protelomerase TelK from *Klebsiella oxytoca* phage ϕKO2,[13] which has a crystal structure available.[23] More recently, Hao et al. (2022) reported the identification of *telN* gene from a novel linear prophage HMP1 isolated from deep sea bacterium *Halomonas* sp. MT08-1,[24] which further supports the notion of TelN being a highly conserved protein in bacteriophages. However, no observable structural relationship was found in relation to mammalian proteins, suggesting that TelN does not share similar functions to mammalian proteins.

In terms of TelN's functionality, it was predicted that the protein binds to the palindromic *telO* within *tos*, which is absent in the human genome.[13] This analysis reveals that *tos* is the only binding site for TelN protelomerase without interrupting the human genome, supporting the safety of engineered human cells incorporated with TelN-*tos* module. This was demonstrated by the faithful reiteration of its cleavage-joining activity on circular *tos*-containing DNA substrate in TelN-expressing HeLa and NIH3T3 cells.[13,25] As demonstrated by Liew et al. (2019), the TelN function and structure, albeit predicted, were supported by experimental observations that can lead to further efforts in deciphering the molecular mechanisms of TelN. This was previously shown by Aihara et al. (2007), where the authors proposed the TelK dimerization and complexing mechanism with its palindromic DNA substrate, with the depiction of the crystal structure of TelK-substrate complex.[23] While being able to shed some light on the catalytic behavior of TelN, the predicted protein structure is still a far cry from a complex crystal structure toward deciphering the mechanism of the TelN protein.

3. ADAPTATION OF N15 GENOME AS DNA VECTORS

N15 was not naturally suited as a cloning vector until the modification of the N15 linear prophage, providing it with the basic cloning vector's characteristics, i.e., addition of structural elements such as origin of replication (*ori*), antibiotic resistance gene, reporter gene, and multiple cloning sites.[26] Initially, circular and linear N15 miniplasmids with minimized size of N15 genomic fragments (Table 1, nos. 1−9) were developed to study the N15 genetics as well as to overcome the large N15 genome size that hinders its use as a cloning vector. This was achieved via removing the unnecessary N15 genes from full-length N15 genome (46.4 kb N15 genome, GenBank accession no. NC_001901), such as genes that play roles in lytic function, phage structural proteins, and plasmid partition.[27,28]

The study of N15 miniplasmids has a great influence on the evolution of N15-based cloning vectors. It was discovered that N15 *repA* gene encoding multifunctional replication protein not only plays roles in primase, helicase, and DNA-binding activities during lytic replication of N15[11] but also comprises *ori* site which can initiate the replication of circular or linear N15 miniplasmids in bacterial hosts.[20,29] This means that the *repA* gene can initiate the plasmid replication without relying on the *ori* site from bacterial host system, before proceeding to DNA replication accomplished by bacterial host DNA polymerase. In other words, replication of N15-derived linear plasmid can be initiated by N15 replicon[30] or plasmid bacterial *ori* site.[14,15] Despite the appropriate *ori* site, the research of N15 miniplasmids consistently revealed that only TelN and *tos* elements are essential for the propagation of linear N15-based plasmids in bacterial hosts, highlighting the feasibility of TelN-*tos* module as a molecular tool for genetic manipulation without the necessity of natural phage infection (Table 1, no. 10). Therefore, the later construction of N15-based linear DNA vectors (Table 1, nos. 11−16) was fully based on the addition of *tos* sequence into any circular plasmid DNA or BAC, either via restriction digestion (Table 1, no. 11) or homologous recombination modifications (Table 1, nos. 12−16). The *tos*-containing DNA vectors were then linearized by TelN protein supplied *in vitro* or *in vivo* (will be further discussed in Section 4.2), resulting in hairpin-capped linear DNA.

4. CLONING ADVANTAGES OF N15-BASED LINEAR PLASMID

4.1. Stable Cloning of Repeat-rich DNA Sequences. Several advantages of the TelN-*tos* module have led to its adaptation for the development of linear cloning vectors. Primarily, it was reported that unstable repetitive DNA are more stably propagated in N15-based linear vectors (pN15L[34] and pJAZZ[35] plasmids, Table 1, nos. 10−11) than the conventional cloning vectors (e.g., pUC-based plasmids[38]).

The first generation of linear cloning vector, termed pN15L (Table 1, no. 10), was constructed by ligating the partially digested N15 genome with an antibiotic selective selection marker.[34] pN15L shared the same end structures and replicon

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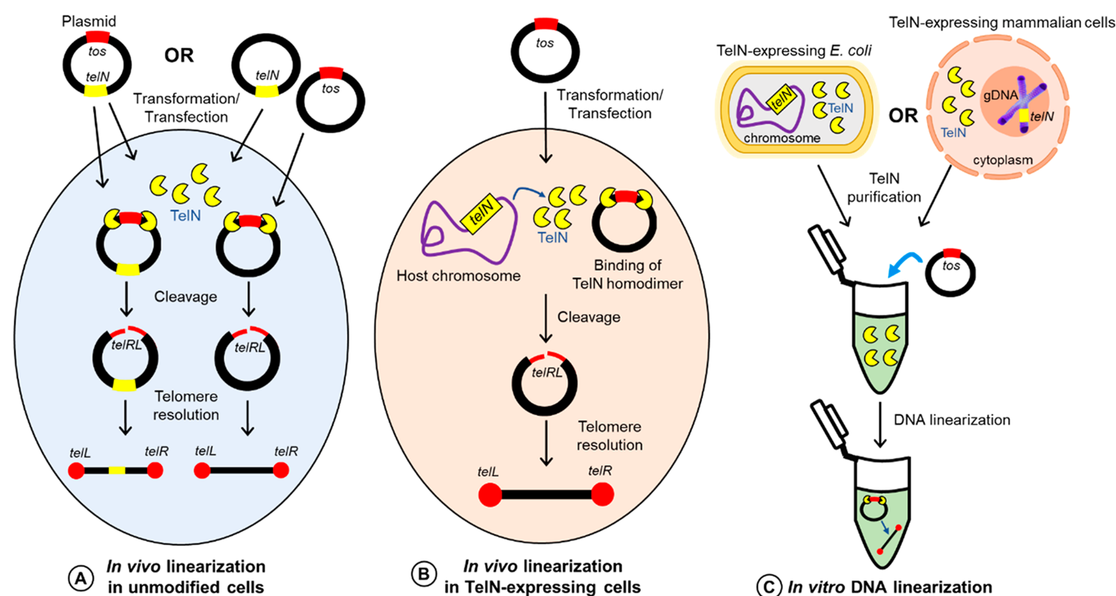

Figure 3. Functionality of TelN-*tos* module in heterologous bacterial or mammalian environments. TelN can retain its cleavage-joining activity on the *tos* sequence to form telomeric ends in transformed bacterial or transfected mammalian cells without relying on N15 replication machinery. Generally, circular *tos*-containing plasmid can be linearized as a linear plasmid with covalently closed ends via three ways: (A) *in vivo* linearization using recombinant TelN expressed *in cis* (on the same plasmid) or *in trans* (carried separately in two coresident plasmids) in unmodified host, (B) *in vivo* linearization in host cell with chromosomally integrated *telN* gene, or (C) *in vitro* linearization using recombinant TelN purified from TelN-expressing host.

as the linear N15 genome. Interestingly, pN15L was found to have a remarkable stability and cloning fidelity in propagating artificial (11 kb) and natural (12.5 kb) palindromic sequences as well as a large, nonpalindromic sequence (50 kb) without hampering its copy number (250 copies/*E. coli*).[34] Unlike typical circular vectors (will be further discussed later), linear cloning vectors do not form cruciform or other secondary structures during plasmid replication, which are prone to deletion, rearrangement or DNA degradation by cellular nucleases in *E. coli*.[34,35]

Later, the second generation of linear cloning vectors derived from N15 miniplasmid pG591 (Table 1, no. 3), termed "pJAZZ" vectors (Table 1, no. 11), were developed.[35] This class of vectors can stably propagate AT- and GC-rich DNA inserts of up to 30 kb and short tandem repeats of up to 2 kb in engineered TelN-expressing *E. coli* TSA strain.[35] Compared to pN15L, pJAZZ vectors have several cloning features that improve the plasmid stability: (i) lower copy number that allows efficient cloning of large insert (>8 kb) or repetitive sequences; (ii) use of transcriptional terminators to prevent transcriptional interference between the vector and insert; (iii) removal of nonessential N15 genes in *cis* (including *telN)* to minimize the vector size for better cloning efficiency; and (iv) propagation of linear plasmids in engineered *E. coli* TSA strain with chromosomally integrated *telN*, *sopAB* (partitioning gene) and *antA* (antirepressor gene).[35] When compared to circular vectors with similar vector characteristics (single-copy and transcription-free), linear pJAZZ vectors were found to have much higher efficiency in cloning large genomic inserts (10−20 kb), which resulted in the formation of more transformed colonies that had robust growth rate.[35] Currently, the pJAZZ vector series and *E. coli* TSA strain are commercially available as a linear cloning kit, named 'BigEasy v2.0 Linear Cloning System' (Lucigen Corporation, USA).

In contrast, insertion of human palindromic sequences as short as 460 bp into the typical circular vector pUC19 had caused unstable propagation (mutations include deletion, rearrangement, or unclonable at all) and drastic reduction of copy number compared to the nonpalindromic insert.[34,35] These undesirable molecular events were also detected in vectors with relatively large transgene capacity, such as cosmids (capacity of 35−45 kb)[26,34,35] and yeast artificial chromosomes (YACs) (capacity of up to 2.3 Mb).[39,40] The difference in stability and copy number between circular and N15-based linear vectors could be explained by the vector topology. Insertion of unstable palindromic or other repetitive DNA sequences into circular plasmids could contribute to the release of superhelical tension and strand slippage during the replication of lagging strand, thereby inducing the formation of cruciform or other secondary structures during the replication event.[41] These structures are prone to deletion, rearrangement, random chromosomal integration or degradation by endogenous nucleases in *E. coli*.[42,43]

There is growing evidence to support the rationale that the N15-based linear cloning vector is preferred over standard circular plasmid to circumvent the cloning of huge, unstable repetitive sequences, or abnormal DNA sequences commonly existing in nature, including the production of viral vectors[42,44] and cloning of human genes.[45,46] The high cloning fidelity of N15-based linear vectors may provide the simpler alternatives for several genetic studies: (i) discovery of novel genes involved in human diseases; (ii) assembly of a more uniform (decreased size bias) DNA libraries containing larger fragments; (iii) complete finishing of large genome sequencing by allowing closure of sequencing gap caused by Sanger or next-generation sequencing; and (iv) establishment of cell culture or animal disease models related to large repetitive sequences. For instance, the N15-based linear vector was used to generate stable cDNA clones of the Zika virus (ZIKV) genome with

https://doi.org/10.1021/acssynbio.2c00580
ACS Synth. Biol. XXXX, XXX, XXX−XXX

inherent instability, allowing the production of recombinant ZIKV in transfected Vero cells for the study of ZIKV pathogenicity in mouse model.[47] This supported that N15-based linear vector can also benefit the study of human genetic diseases such as Fragile X syndrome, a human repeat expansion disorder associated with the mutation of a DNA fragment constituting more than 200 CG-rich repeats.[48]

4.2. Effective Assembly of Linear Plasmids or BACs. TelN retains its cleavage-joining activity on the *tos* sequence to form telomeric ends independent of the N15 replication machinery, regardless of whether TelN was provided *in vivo* in *cis* from the *tos*-containing plasmid or in *trans* from either of the coresident plasmids in wild-type *E. coli* (Figure 3A). Alternatively, *tos*-containing plasmids can be linearized *in vivo* in engineered *E. coli* with chromosomally integrated *telN* gene (Figure 3B)[14,15,49,50] as well as *in vitro* by recombinant TelN purified from the TelN-expressing *E. coli* (Figure 3C).[12,19,51] These findings had driven the exploitation of the TelN-*tos* module as a versatile molecular tool to generate hairpin-capped linear DNA from the standard circular DNA vectors (e.g., plasmids and bacterial artificial chromosome, BACs).[15,52]

Apparently, linear topology of cloning vectors has no deleterious impact on the cloning capacity of DNA vectors and viability of bacterial hosts. Our group has previously demonstrated that 100 kb *tos*-containing BACs carrying full length human β-globin gene (Table 1, nos. 14−15) can be linearized and stably replicated by engineered *E. coli* with chromosomally integrated *telN* gene.[15] The functionality of hairpin ends derived from the *tos* sequence was confirmed by assessing their resistance to RecBCD exonuclease degradation.[15]

Plasmid linearization has been traditionally performed *in vitro*, either generated with open ends using restriction enzymes[53,54] or covalently closed ends via ligating an enzyme-digested DNA fragment with hairpin-forming oligonucleotides.[55] However, restricted DNA with overhang termini is more likely to accumulate damage and be subjected to nuclease attack than end-capped linear DNA.[55,56] Unlike the one-step *in vivo* production of hairpin-capped linear DNA via TelN-expressing *E. coli*,[14,57] these *in vitro* modifications involve digestion, ligation, and purification steps that contribute to variations in genetic studies while impeding the DNA quality.

In addition, linear DNA can also be synthesized *in vitro* via PCR reaction. Similarly, PCR fragments have blunt ends that are prone to nuclease degradation in the host cells. Also, synthesis of PCR fragments has higher error rates compared to amplifying plasmid DNA or BACs using *E. coli*, especially the cloning of larger gDNA or repetitive transgenes, limiting their used in genetic studies. In contrast, linear N15-based plasmids or BACs have closed ends and are resistant to degradation.

Recently, our group used the same system to generate a linearized 101.5 kb BAC (Table 1, no. 16), which was derived from 183.6 kb human BAC clone RP11-125I15 (BACPAC resources) encompassing a segment of human chromosome X, to study the effect of vector topology on transfection efficiency in multiple human cell lines.[18] It is known that circular DNA generally has a higher transfection efficiency than linear DNA due to its smaller hydrodynamic diameter that could confer resistance to DNA shearing.[18,56] Interestingly, transfection studies had demonstrated that the telomeric ends of TelN-linearized BAC may have contributed to the stability of the large BAC via protecting it from intracellular exonuclease degradation, thereby resulting in transgene expression level similar to its circular counterpart.[18,44] For example, both circular and TelN-linearized BACs up to 100 kb were found to have similar eGFP expressions when delivered into A549 and MCF-7 cell lines.[18] This suggested that TelN-linearized DNA can work as effectively as the standard circular DNA in the transgenic studies without hampering their therapeutic effects (will be discussed in Section 7).

In short, linear vector systems based on the TelN-*tos* module offer a cost- and time-effective method to generate linear DNA vectors by allowing direct purification of end-capped linear DNA from *E. coli* using the standard plasmid extraction protocol. Alternative N15-related products such as TelN protelomerase (NEB, USA) are commercially available to aid the discovery of novel or innovative applications associated with linear DNA vectors. Other than linearizing the existing circular DNA cloning vectors, this linearization strategy is also useful for the construction of linear artificial chromosomes that have several potential applications in synthetic biology: (i) study of genetic requirements for human chromosome segregation and centromere function;[15,25] (ii) functional genomic study of large or complex viral or mammalian genomes in transgenic models without losing their natural linear conformation; and (iii) construction of novel DNA libraries for sequencing of complex genomes, instead of the conventional BAC of YAC libraries.

Although the techniques to construct linear vectors derived from actual human chromosomes, termed human artificial chromosome (HAC), exist,[58,59] the construction method is very laborious and not applicable to existing BAC libraries.[14] A recent example linear cloning platform based on the TelN-*tos* module, named TelN/tos-assisted precise targeting of chromosome segments (TAPE), has emerged to allow direct cloning of large prokaryotic and eukaryotic genomes without the need to purchase genome sequences from companies or conducting lab synthesis.[45] Briefly, TAPE utilizes the homology arms to clone the target genomic segment into the TelN-linearized plasmid *in vitro*, followed by the transformation of the resulting plasmid into the TelN-expressing *E. coli* for vector propagation.[45] This strategy was shown to have effective cloning of 156 kb bacterial choromosomal fragment, 124 kb yeast genomic fragment, and 16 kb mouse mitochondrial fragment without being limited by the homologous sequences of *E. coli* host's genome,[45] proving the TelN-*tos* module is a great prospect for intricate genetic manipulations and cloning procedures.

4.3. Production of MiniDNA Vectors without Bacterial Backbone. Other than using a single *tos* site to produce linear DNA, installing two *tos* sites sandwiching the gene of interest in DNA vectors can generate covalently closed linear DNA vectors with minimal unnecessary DNA sequences. This approach is especially useful for the production of miniDNA vectors devoid of bacterial backbone sequences, such as prokaryotic *ori* site and antibiotic resistance gene(s). It was previously shown that linear miniDNA offers several advantages in gene delivery over conventional plasmids. Generally, miniDNA without bacterial backbone holds two major advantages: (i) smaller size that contributes to better bioavailability and more resistant to DNA shearing, leading to improved gene delivery and transgene expression;[60,61] and (ii) higher immuno-compatibility and less prone to transgene silencing in host cells as the immunogenic bacterial backbone is removed.[60,62] Currently, miniDNA vectors are successfully

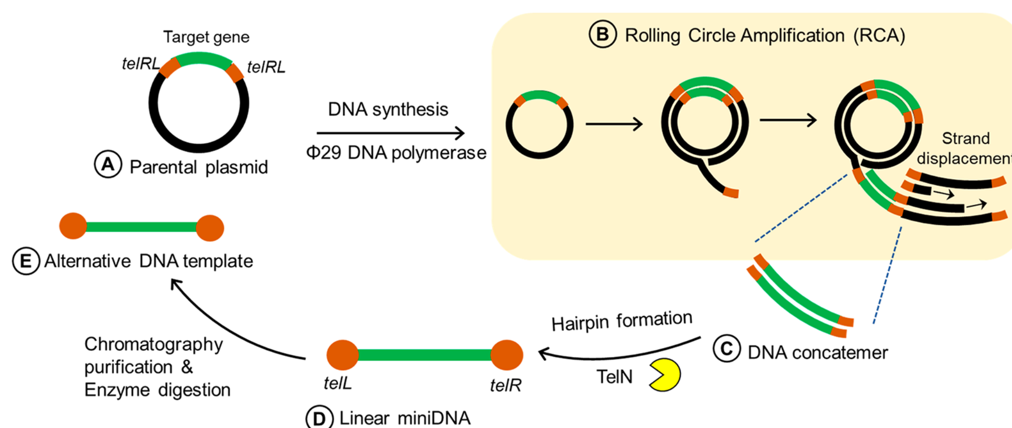

Figure 4. *In vitro* production of TelN-linearized linear miniDNA. First, parental circular plasmid containing two *telRL* sites flanking the gene of interest is purified from *E. coli* (A) and used as a template for DNA synthesis mediated by Phi29 (Φ29) DNA polymerase, termed rolling circle amplification (RCA) process (B). The resulting DNA concatemers (C) will then be cleaved and joined by TelN to generate single units of linear miniDNA capped with telomeric ends (D), which are ultimately subjected to chromatography purification. To remove the TelN-linearized bacterial backbone, the backbone will be digested by a restriction enzyme, followed by exonuclease enzyme that degrades the restricted DNA, and therefore leaving only covalently closed linear miniDNA containing the gene of interest, promoter and polyA tail. Alternatively, the purified linear miniDNA can be used as the template for further amplification reactions (E).

applied in the development of DNA vaccines for viral[63] or parasitic[64] infections, and immunotherapy against cancers[65]

Due to the presence of the closed hairpin ends, TelN-linearized miniDNA offers the additional advantage of having a much lower frequency of natural and targeted genomic integration events than circular or opened-ended linear miniDNA in bacterial[50] and mammalian[66] cells. In mammalian cells, TelN-linearized miniDNA was found to confer higher transgene expression than its circular counterpart due to its more efficient cellular uptake that results in superior transfection efficiency.[66] These features support the use of the TelN-*tos* module as a feasible tool for the development of advanced DNA vectors for gene delivery.

Several other cloning methods are available to generate covalently closed linear miniDNA. This includes minimalistic immunogenically defined gene expression (MIDGE) vectors, which are produced via restriction digestion of gene of interest within parental plasmid, and subsequent ligation of open ends of the resulting fragments to hairpin oligodeoxynucleotides.[67] Alternatively, covalently closed microlinear vectors (MiLV) can be generated via PCR amplification of the gene of interest with subsequent ligation with hairpin-forming oligonucleotides.[55,66] However, these cloning methods are costly and time-consuming as multiple *in vitro* steps are involved.[65]

Instead, the TelN-*tos* module offers a more cost-effective strategy for the high-throughput production of linear miniDNA vectors via a single enzymatic reaction.[50] This is currently available in both *in vivo*[50,66,68] and *in vitro*[42,44,63,69] methods, which were patented by Nafissi and Slavcev in July 2014 (patent no. US20140206037A1) and Touchlight Genetics Ltd. in August 2010 (patent no. EP2391731B1), respectively. In the *in vivo* production system, generation of hairpin-capped linear miniDNA is achieved by passing the parental expression vector containing two *telRL* sites (within a specialized TelN target site flanked by the 76 bp SV40 enhancer sequence, termed "Super Sequence") through a recombinant *E. coli* (R-cell) with temperature-regulated TelN expression.[50,68] Consequently, two linear DNA species with covalently closed ends will be produced: the miniDNA vector with intact gene of interest and the mini plasmid backbone. Standard plasmid extraction and restriction digestion of unwanted plasmid backbone are needed to obtain the purified miniDNA vector. This DNA production system mainly has three unique features: (i) allows rapid and scalable production of miniDNA in R-cell that expresses TelN conditionally;[68] (ii) does not cause undesirable vector integration events into the host chromosome;[50,66] and (iii) confers enhanced nuclear uptake and transgene expression via the insertion of SV40 enhancer sequence at each covalently closed end of the resulting linear miniDNA.[50]

Similarly, *in vitro* production of linear miniDNA is also based on parental expression plasmid containing two *telRL* sites flanking the gene of interest (Figure 4A). Instead of transforming parental plasmid into *E. coli* for vector linearization, covalently closed linear miniDNA is produced via the rolling circle amplification (RCA) process using Phi29 (Φ29) DNA polymerase (Figure 4B–D).[44,63,67,69] Φ29 DNA polymerase has high fidelity ($1/10^6$–$1/10^7$) and high processivity (approximately 70 kbp), making the linear miniDNA suitable for the large-scale production of good manufacturing practice DNA vectors.[44] Intriguingly, the synthesized linear miniDNA can also be used as the template for the RCA reaction (Figure 4E). This means that this method can synthesize the linear miniDNA entirely *in vitro* without undergoing a bacterial fermentation step. This bacteria-free enzymatic process offers several advantages: (i) minimizing the variation of plasmid quality between batches;[69] (ii) hindering the carry-over of bacterial contaminants or endotoxin such as lipopolysaccharide;[42,46] and (iii) preventing undesirable recombination events.[46]

The cloning advantages of this system are well exemplified by the functional production of adeno-associated virus and lentiviral vectors as gene delivery vehicles.[42,44] These linear viral miniDNA were comparable to the standard plasmids in terms of viral titers in the transfected HEK-293T cells, which then allowed similar transgene expression in the transduced HEK-293T cells and mice. Notably, this *in vitro* method supported the stable propagation of complex DNA sequences that are prone to deletion in bacterial propagation systems, such as inverted terminal repeats required for viral vector packaging.[42,44] This highlights the potential use of *in vitro*

methods to clone therapeutically relevant genes that are genetically unstable when propagated in *E. coli*, such as human sodium channel coding sequences involved in Dravet syndrome.[70,71]

5. ADAPTATION OF TELN-*TOS* FOR THE GENETIC STUDIES OF BACTERIAL HOST

5.1. Generation of Viable Bacteria with Linear Genome. Other than utilizing the TelN-*tos* module for the construction of DNA vectors, the TelN-*tos* module also allows functional genetic manipulation of natural circular or linear DNA molecules, i.e., bacterial[16,17] and mammalian (will be discussed in Section 6) genomes, respectively. This section focuses on the versatile application of the TelN-*tos* module as a molecular tool to understand the molecular genetic mechanism of bacterial cells.

To investigate the impacts of possessing a linear chromosome in prokaryotic cells which mostly harbor circular genomic DNA in nature, Cui et al. (2007) constructed a linear *E. coli* chromosome capped with N15-derived telomeres.[16] The *tos* sequence was placed within the replication termination region of the *E. coli* chromosome, which was then linearized by TelN expressed from plasmid pBAD24-*telN* or natural N15 prophage.[16] The results revealed that the host cell viability (in respect of growth rate and cellular morphology) and stability of genome structure were not influenced by the topological changes when the linearization of the chromosome was designed at the region apart from the bacterial *ori* site.[16] This is in agreement with a later report which found that an *E. coli* chromosome linearized by TelN at a *tos* sequence inserted near the chromosome dimer resolution site *dif* (located within terminus region of the chromosome[72]) does not delay the growth rate of wild-type *E. coli*.[73] Surprisingly, linearization of the circular *E. coli* chromosome almost did not alter the genome-wide gene expression, as only three genes were found to exhibit different gene expression levels among the 4300 genes analyzed.[16] However, when the *tos* linearization site was placed closer to the *E. coli ori* site, stronger growth defects were observed.[16] The underlying mechanism involved in this correlation remains unknown, but is possibly attributable to the inhibited or unbalanced replication of chromosome arms with different lengths and/or collision between replication and transcription.[16,17,50]

Later, Liang et al. (2013) employed the TelN-*tos* module to demonstrate that circular *E. coli* chromosome (4.64 Mbp) can be separated into two linear, autonomous replicating fragments (3.27 and 1.37 Mbp) which were able to sustain the genetic functions to generate a viable cell.[17] This was achieved by transforming TelN-expressing plasmid pJAZZ-OC into *E. coli* to allow linearization of the circular chromosome at the *tos* sequences inserted individually at two optimized genomic positions, resulting in two linear chromosomal fragments replicated by wild-type *E. coli OriC* and a heterologous *ori* site isolated from *Vibrio cholerae*, respectively.[17] As opposed to the study done by Cui et al. (2007) which claimed that there were no differences between cells with single linear and circular chromosomes with regard to growth rate and cellular morphology,[16] *E. coli* strains with two linear, fragmentized chromosomes retarded the growth rate (1.6-fold slower than wild-type strain) while displaying unusual phenotype in growing culture (10% of elongated cells), although the modified strains were still able to maintain as a genetic and phenotypic stable clone for more than 100 generations.[17] It is noteworthy that viable chromosome fragmentation may not work at other random positions because there are rules related to chromosome replication, segregation, and segmentation, although the underlying mechanisms are yet to be studied. These studies supported the TelN-*tos* module as a promising tool for bacterial genome engineering to gain insights into the chromosome biology and plasticity of genome architecture in prokaryotes.[74]

5.2. Study of Chromosomal Integration of Linear DNA. In a study on genomic integration based on bacteriophage λ integrase (Int)-attP recombination system, maintenance of TelN-linearized plasmid was found to exert a significantly higher genotoxic effect, leading to lower host survival rate than its circular counterpart. A similar lethal effect was observed regardless of whether the integrating plasmid was linearized before or after bacterial transformation, indicating that only nonlinearized plasmids produced viable clones.[50] When the TelN-linearized plasmid is integrated into *E. coli* genome, its covalently closed ends will form chromosomal breakage at the plasmid integration site, causing separation of centromere from the telomere, inability of cellular replication, and cell death.[50,55,68] On the contrary, the parental circular plasmid can generate a significant number of viable integrants because it allows random integration into the nonvital region of the host chromosome without causing chromosomal disruption.[50] Provided that integration of TelN-linearized plasmid into the host genome will be naturally eliminated by arrested cell growth and cell apoptosis, they can hinder the propagation of potentially genotoxic integrant cells in the target cell population.[50,66] Therefore, compared to the standard circular plasmid, which is able to divide in the integrants without breaking the host chromosome, the TelN-linearized plasmid offers an additional safety advantage for the delivery of transgenes into *E. coli*[50] and mammalian cells.[66] This finding also complemented the genetics studies in mammalian cells (will be discussed in Section 6.3), i.e., both studies in bacterial and mammalian cells consistently demonstrated that TelN-linearized DNA can hinder the expansion of undesirable integrant cells via the formation of a chromosomal breakage at the integration site.

6. FUNCTIONALITY OF THE TELN-*TOS* MODULE IN MAMMALIAN CELLS

6.1. *In Vivo* and *In Vitro* Cleavage-Joining Activity. The use of the TelN-*tos* module is not limited to bacterial cells, but also retains its native function in the mammalian environment. Similar to the study in *E. coli*, *tos*-containing DNA vectors up to 110 kb[13,15,18,25] can be stably linearized in TelN-expressing mammalian cells (Figure 3A,B)[13,25] or *in vitro* by TelN purified from TelN-expressing mammalian cells (Figure 3C).[13,49] TelN-expressing mammalian cells can be generated via transient (Figure 3A)[13,49] or stable (Figure 3B,C) transfection of plasmid comprising *telN* gene driven by a mammalian promoter, which expresses TelN that evenly distributed within the cell's cytoplasm.[13,25] The TelN-expressing mammalian cells were proved to stably retain the native cleavage-joining and replication activities of TelN on *tos*-containing plasmid.[13,25]

Further, TelN-linearized DNA vectors, either produced *in vitro* or *in vivo* in bacterial or mammalian cells, were shown to confer functional cDNA and gDNA transgene expressions in a broad range of mammalian hosts, which includes human cells (e.g., HEK-293,[18,52,66] OVCAR-3,[66] HT1080,[15] HeLa[13,25,49]

and Jurket,[46] T lymphocyte,[46] A549[18] and MCF-7[18] cells), mouse cells (e.g., CHO[69] and NIH3T3[13,25] cells) and mice models.[52,69] For example, it was shown that 100 kb TelN-linearized BACs (Table 1, nos. 14−15) comprising full-length human β-globin genomic locus were able to produce accurately spliced transcripts in unmodified HT1080 cells and TelN-expressing NIH3T3 cells, which do not express endogenous β-globin expression.[13,15,25]

6.2. Replication of Linear DNA. By knowing the cleavage-joining activity of microbial TelN-*tos* module remains functional in mammalian cells, the study was extended to interrogate whether TelN-expressing mammalian cells can replicate *tos*-containing DNA in the presence of mammalian *ori* site, as achieved in TelN-expressing *E. coli* that replicates based on bacterial *ori*.[15,35,50] It is known that *tos*-containing β-globin BAC, BAC4396-*tos* contains human *ori* sites within the locus control region of β-globin locus.[75] The mammalian *ori* permits the replication of linear BAC4396-*tos* in TelN-expressing HeLa cells, which was proved via the enzymatic *DpnI*/*Mbo*I/*Sau3AI* assay that distinguished the replicons from the nonreplicons based on the methylation pattern as well as semiquantitative PCR that evaluated the BAC copy number.[25] Further, when linear BAC4396-*tos* was transfected into TelN-expressing NIH3T3 cells, β-globin expression was stably maintained for at least 120 h, in contrast to the decreasing expression in unmodified cells that was not detectable after 96 h.[13] These consistently supported that TelN can facilitate the extrachromosomal replication of *tos*-containing DNA in mammalian cells as long as mammalian *ori* is present.

However, the newly replicated BAC4396-tos copies are believed to retain in mother cells until a mitosis event because there is lack of a mechanism to support the mitotic stability of the replicated linear BAC4396-tos. Human chromosomes achieve mitotic stability with the help of centromere regions that enable the attachment of mitotic spindles during cell division and segregate the newly replicated plasmid copies into daughter cells.[76] Since linear *tos*-containing DNA is centromere-free, it is unlikely to attach to the mitotic spindle and therefore will be diluted out during cell division.

To confer mitotic stability to linear *tos*-containing DNA for long-term retention in mammalian cells, a molecular anchor, human scaffold/matrix attachment region (S/MAR), can be introduced to *tos*-containing DNA to tether the linear plasmid with metaphase chromosome during cell division,[25] thereby facilitating the episomal maintenance and mitotic stability of plasmids in mammalian cells.[77] The resulting BAC4396-tos-S/MAR/Hyg (Table 1, no. 15) successfully achieved more persistent plasmid replication and transgene expression in TelN-expressing NIH3T3 cells for at least 15 days post-transfection, compared to the non-S/MAR control.[25] These showed that the S/MAR element can enhance the retention of linear *tos*-containing plasmid during cell mitosis, thereby allowing their prolonged replication in TelN-expressing mammalian cells.

To date, this is the only mammalian study incorporating S/MAR technology to improve the stability of autonomously replicating linear vector systems based on the TelN-*tos* module. In mammals, no TelN-like enzyme has been found with activities to facilitate ligation of nicked DNA ends and replication. Adapting the TelN-*tos* module for recombination in mammalian cells could be a novel tool with wide-ranging applications in chromosome engineering, eukaryotic telomere/telomerase studies, transgene expression studies in mammalian cells.

6.3. Avoidance of Genomic Integration. As mentioned in Section 5.2, upon integration of TelN-linearized DNA into the host chromosomes, their covalently closed ends can form a chromosomal breakage at the integration site, followed by the separation of centromere from telomere and arrested cell division.[66] The resulting genomically unstable integrant cells will be targeted for apoptosis, preventing the expansion of undesirable integrants from the transfected cell population.[50,66] This natural lethal effect prevents the expansion chromosomally integrated mammalian cells in the transfected cell population, thereby providing a better safety profile than isogenic circular DNA vectors for mammalian gene delivery by hindering random genomic integration,[46] which can lead to gene silencing or unpredictable insertional mutagenesis that induces oncogene activation, tumor suppressor deactivation, or destabilization of chromosomal DNA.[78] In contrast, the typical circular DNA or open-ended linear DNA can freely integrate into the nonvital region of the host chromosome without breaking the chromosome, allowing multiplication of integrant cells while displaying normal morphology and growth rate.[46,66] This was proved by the stable transfection study of human cells, which showed that TelN-linearized plasmids had lower site-directed and natural genomic integration frequencies (9-fold and 12-fold, respectively) than isogenic circular plasmids, resulting in dramatically lower number of viable integrants (150-fold).[66]

7. THERAPEUTIC PROSPECTS OF N15-BASED DNA VECTORS

TelN-linearized DNA vectors are potentially adapted for the use of gene medicine, particularly the development of cancer gene therapies and DNA vaccines. This is well exemplified by a study on the antimetastatic activity of interleukin-12 (IL-12) expressed from TelN-linearized plasmid in a metastatic melanoma mice model.[52] The recombinant IL-12 expression stimulated interferon-γ (IFN-γ) secretion from host immune cells to abrogate pulmonary metastasis formation in the mice model without causing cell lesions.[52] More importantly, TelN-linearized plasmid produced higher IL-12-induced IFN-γ secretion and exhibited better antimetastatic activity than the parental circular plasmid and isogenic linear plasmid with open ends, mainly attributed to its highly stable hairpin structure.[52] Similarly, TelN-linearized DNA was applied in the production of DNA vaccine against H1N1 influenza viral infection.[69] The linear DNA vaccine construct comprising of H1N1 gene encoding hemeagglutinin (HA) antigen was able to induce humoral and cell-mediated immune responses equivalent to circular DNA in a mice model, as demonstrated by their similar levels of antigen-specific antibodies and protective hemagglutination inhibition antibodies.[63,69] In short, studies of IL-12 and HA antigen expression in mice models consistently revealed that both circular and linear N15-based vectors are able to confer similar transgene expression in mammalian cells, in concordance to the transfection efficiency study in mammalian cell lines based on the eGFP reporter gene mentioned above (Section 4.2).

Recently, a nonviral *piggyBac* transposon system was successfully developed based on TelN-linearized DNA vectors to produce CD19-specific chimeric antigen receptor (CAR19) T cells that were capable of exerting antitumor effects against B cell malignancies.[46] To permit long-term CAR19 expression on

https://doi.org/10.1021/acssynbio.2c00580
ACS Synth. Biol. XXXX, XXX, XXX−XXX

the T cell surface, the authors integrated CAR transposon into the genome of T cells via the simultaneous delivery of two TelN-linearized constructs carrying either *piggyBac* transposase coding sequence or transposon composed of the gene encoding CAR19 flanked by the transposase recognizable terminal inverted repeats (TIRs).[46] Results showed that TelN-linearized constructs displayed CAR19 surface expression levels similar to those generated using conventional circular plasmids, which has been previously shown to be sufficient for the eradication of patient-derived $CD19^+$ B cell acute lymphoblastic leukemia (B-ALL) xenografts in mice model[79] and $CD19^+$ malignancies in clinical trial.[80] Together, these studies support the feasibility of TelN-linearized DNA for future therapeutic approaches.

8. CONCLUDING REMARKS

Phage N15 elements, TelN and *tos* can function as an independent "units" to resolve any circular DNA into linear DNA capped with hairpin ends in bacterial and mammalian environments. The resulting linearized DNA can be stably replicated by TelN in TelN-expressing *E. coli* or mammalian cells when the respective *ori* site is present in the host cells. These unique features allow the adaptation of the TelN-*tos* module as a viable molecular tool to generate linear cloning and expression vectors.

Currently, the TelN-*tos* module has been successfully used to linearize engineered DNA vectors up to 110 kb[25] and natural *E. coli* genome up to 4.64 Mb.[17] Since linear artificial chromosomes with telomeres more closely represent the structure of natural mammalian chromosomes, the use of *tos* containing DNA vectors may provide an alternative choice for gene therapy and genomic studies.[14,15] It was shown that TelN-linearized DNA vectors can circumvent the unstable cloning of large or repetitive DNA sequences that are unclonable in traditional circular plasmids[35] as well as provide an efficient *in vivo* or *in vitro* production of therapeutically useful miniDNA vectors without bacterial backbone.[61] Additionally, adaptation of TelN-*tos* module into mammalian cells can facilitate the replication of DNA vectors in transfected cells,[13,25] suggesting their possible use in transfection studies for long-term transgene expression. Besides, functional studies of the TelN-*tos* module offer a novel insight into the understanding of basic replication requirements for linear plasmids in heterologous environments.

To improve the long-term stability for future transgenic studies of mammalian cells, the S/MAR-based episomal vector system can be incorporated into TelN-linearized DNA to establish an autonomously replicating linear vector system that hinders random genomic integration. Furthermore, current experimental studies are limited to inserting the *telN* gene and *tos* sequence in *trans* (Figure 3A, right) in engineered bacterial or mammalian cells. Whether the TelN-*tos* module can remain functional when delivered via the same plasmid (Figure 3A, left) remains to be elucidated. This potentially eases the application of TelN-linearized DNA vectors without chromosomally integrating *telN* gene into the host cells, which might result in unpredictable genotoxicity. These vector improvements are particularly important for the development of a transgenesis platform for the creation of cell or animal models for the study of genetic diseases,[81] the assembly of linear artificial chromosomes for transgenic studies,[82,83] and the development of gene[84] or cell-mediated[85] therapy.

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Y.C.W., A.W.R.N., Q.C., and P.S.L. conceived the idea and content. Y.C.W. and A.W.R.N. wrote and proofread the manuscript. Y.C.W. designed and drew the figures. K.N., L.C.W., and E.U.-H.S. conceptualized the project and acquired funding. K.N. revised the manuscript and supervised the project. All authors contributed to the manuscript and approved the submitted version.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We gratefully thank Nikolai V. Ravin for the kind gift of N15 materials. This work was funded by FRGS grant FRGS/1/2016/STG05/MUSM/02/2 from the Ministry of Higher Education, Malaysia to K.N. Y.C.W., Q.C., and P.S.L. were supported by a Higher Degree by Research (HDR) Scholarships from Monash University Malaysia. Y.C.W. was additionally supported by the FRGS grant in part.

REFERENCES

(1) Fernández, L.; González, S.; Campelo, A. B.; Martínez, B.; Rodríguez, A.; García, P. Low-Level Predation by Lytic Phage PhiIPLA-RODI Promotes Biofilm Formation and Triggers the Stringent Response in Staphylococcus Aureus. *Sci. Rep.* **2017**, *7*, 40965.

(2) Chaudhry, W. N.; Concepcion-Acevedo, J.; Park, T.; Andleeb, S.; Bull, J. J.; Levin, B. R. Synergy and Order Effects of Antibiotics and Phages in Killing Pseudomonas aeruginosa Biofilms. *PLoS One* **2017**, *12* (1), e0168615.

(3) Ma, L.; Green, S. I.; Trautner, B. W.; Ramig, R. F.; Maresso, A. W. Metals Enhance the Killing of Bacteria by Bacteriophage in Human Blood. *Sci. Reports* **2018**, *8* (1), 2326.

(4) Morozova, V. V.; Vlassov, V. V.; Tikunova, N. V. Applications of Bacteriophages in the Treatment of Localized Infections in Humans. *Front. Microbiol.* **2018**, *9*, 1696.
(5) Pujato, S. A.; Quiberoni, A.; Mercanti, D. J. Bacteriophages on Dairy Foods. *J. Appl. Microbiol.* **2019**, *126* (1), 14−30.
(6) Vikram, A.; Woolston, J.; Sulakvelidze, A. Phage Biocontrol Applications in Food Production and Processing. *Curr. Issues Mol. Biol.* **2020**, *40* (1), 267−302.
(7) Ji, M.; Liu, Z.; Sun, K.; Li, Z.; Fan, X.; Li, Q. Bacteriophages in Water Pollution Control: Advantages and Limitations. *Front. Environ. Sci. Eng.* **2021**, *15* (5), 84.
(8) Ravin, N. V. N15: The Linear Phage-Plasmid. *Plasmid* **2011**, *65* (2), 102−109.
(9) Poranen, M. M.; Ravantti, J. J.; Grahn, A. M.; Gupta, R.; Auvinen, P.; Bamford, D. H. Global Changes in Cellular Gene Expression during Bacteriophage PRD1 Infection. *J. Virol.* **2006**, *80* (16), 8081−8088.
(10) Vilen, H.; Aalto, J.-M.; Kassinen, A.; Paulin, L.; Savilahti, H. A Direct Transposon Insertion Tool for Modification and Functional Analysis of Viral Genomes. *J. Virol.* **2003**, *77* (1), 123−134.
(11) Ravin, N. V. Replication and Maintenance of Linear Phage-Plasmid N15. *Microbiol. Spectr.* **2015**, *3* (1), PLAS-0032-2014.
(12) Deneke, J.; Ziegelin, G.; Lurz, R.; Lanka, E. Phage N15 Telomere Resolution. Target Requirements for Recognition and Processing by the Protelomerase. *J. Biol. Chem.* **2002**, *277* (12), 10410−10419.
(13) Liew, P. S.; Chen, Q.; Ng, A. W. R.; Chew, Y. C.; Ravin, N. V.; Sim, E. U. H.; Lee, C. W.; Narayanan, K. Phage N15 Protelomerase Resolves Its Tos Recognition Site into Hairpin Telomeres within Mammalian Cells. *Anal. Biochem.* **2019**, *583*, 113361.
(14) Chen, Q.; Narayanan, K. Recombineering Linear BACs. In *Bacterial Artificial Chromosomes*, 2nd ed.; Springer: New York, 2014; Vol. *1227*, pp 27−54.
(15) Ooi, Y. S.; Warburton, P. E.; Ravin, N. V.; Narayanan, K. Recombineering Linear DNA That Replicate Stably in E. Coli. *Plasmid* **2008**, *59* (1), 63−71.
(16) Cui, T.; Moro-oka, N.; Ohsumi, K.; Kodama, K.; Ohshima, T.; Ogasawara, N.; Mori, H.; Wanner, B.; Niki, H.; Horiuchi, T. Escherichia Coli with a Linear Genome. *EMBO Rep.* **2007**, *8* (2), 181−187.
(17) Liang, X.; Baek, C. H.; Katzen, F. Escherichia Coli with Two Linear Chromosomes. *ACS Synth. Biol.* **2013**, *2* (12), 734−740.
(18) Wong, Y. C.; Osahor, A.; Al-Ajli, F. O. M.; Narayanan, K. Large BACs Transfect More Efficiently in Circular Topology. *Anal. Biochem.* **2021**, *630* (May), 114324.
(19) Deneke, J.; Ziegelin, G.; Lurz, R.; Lanka, E. The Protelomerase of Temperate Escherichia Coli Phage N15 Has Cleaving-Joining Activity. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97* (14), 7721−7726.
(20) Ravin, N. V.; Kuprianov, V. V.; Gilcrease, E. B.; Casjens, S. R. Bidirectional Replication from an Internal Ori Site of the Linear N15 Plasmid Prophage. *Nucleic Acids Res.* **2003**, *31* (22), 6552−6560.
(21) Mardanov, A. V.; Ravin, N. V. Conversion of Linear DNA with Hairpin Telomeres into a Circular Molecule in the Course of Phage N15 Lytic Replication. *J. Mol. Biol.* **2009**, *391* (2), 261−268.
(22) Sengupta, M.; Austin, S. Prevalence and Significance of Plasmid Maintenance Functions in the Virulence Plasmids of Pathogenic Bacteria. *Infect. Immun.* **2011**, *79* (7), 2502.
(23) Aihara, H.; Huang, W. M.; Ellenberger, T. An Interlocked Dimer of the Protelomerase TelK Distorts DNA Structure for the Formation of Hairpin Telomeres. *Mol. Cell* **2007**, *27* (6), 901−913.
(24) Hao, Y.; Wang, S.; Zhang, M.; Tang, Q.; Meng, C.; Wang, L.; Fan, Q.; Yan, Y.; Xiao, X.; Jian, H. Isolation and Characterization of a Novel Linear-Plasmid Phage from the Sediment of the Mariana Trench. *Virol. Sin.* **2022**, *37* (2), 311.
(25) Liew, P. S.; Tan, T. H.; Wong, Y. C.; Sim, E. U. H.; Lee, C. W.; Narayanan, K. A Self-Replicating Linear DNA. *ACS Synth. Biol.* **2020**, *9* (4), 804−813.
(26) Wong, D. W. S. Cloning Vectors for Introducing Genes into Host Cells. In *The ABCs of Gene Cloning*; Springer US: Boston, MA, 2018; pp 93−122.
(27) Ravin, N. V.; Strakhova, T. S.; Kuprianov, V. V. The Protelomerase of the Phage-Plasmid N15 Is Responsible for Its Maintenance in Linear Form. *J. Mol. Biol.* **2001**, *312* (5), 899−906.
(28) Dorokhov, B. D.; Lane, D.; Ravin, N. V. Partition Operon Expression in the Linear Plasmid Prophage N15 Is Controlled by Both Sop Proteins and Protelomerase. *Mol. Microbiol.* **2003**, *50* (2), 713−721.
(29) Mardanov, A. V.; Ravin, N. V. Functional Characterization of the RepA Replication Gene of Linear Plasmid Prophage N15. *Res. Microbiol.* **2006**, *157* (2), 176−183.
(30) Mardanov, A. V.; Strakhova, T. S.; Smagin, V. A.; Ravin, N. V. Tightly Regulated, High-Level Expression from Controlled Copy Number Vectors Based on the Replicon of Temperate Phage N15. *Gene* **2007**, *395* (1−2), 15−21.
(31) Ravin, N. V.; Ravin, V. K. Sverkhkopiinaia Plazmida Na Osnove Replikona Umerennogo Bakteriofaga N15. *Mol. Gen. Mikrobiol. Virusol.* **1994**, No. 1, 37−39.
(32) Ravin, N.; Lane, D. Partition of the Linear Plasmid N15: Interactions of N15 Partition Functions with the Sop Locus of the F Plasmid. *J. Bacteriol.* **1999**, *181* (22), 6898−6906.
(33) Ravin, N. V.; Rech, J.; Lane, D. Mapping of Functional Domains in F Plasmid Partition Proteins Reveals a Bipartite SopB-Recognition Domain in SopA. *J. Mol. Biol.* **2003**, *329* (5), 875−889.
(34) Ravin, N. V.; Ravin, V. K. Use of a Linear Multicopy Vector Based on the Mini-Replicon of Temperate Coliphage N15 for Cloning DNA with Abnormal Secondary Structures. *Nucleic Acids Res.* **1999**, *27* (17), i−iii.
(35) Godiska, R.; Mead, D.; Dhodda, V.; Wu, C.; Hochstein, R.; Karsi, A.; Usdin, K.; Entezam, A.; Ravin, N. Linear Plasmid Vector for Cloning of Repetitive or Unstable Sequences in Escherichia Coli. *Nucleic Acids Res.* **2010**, *38* (6), e88−e88.
(36) Kaufman, R. M.; Pham, C. T. N.; Ley, T. J. Transgenic Analysis of a 100-Kb Human β-Globin Cluster-Containing DNA Fragment Propagated as a Bacterial Artificial Chromosome. *Blood* **1999**, *94* (9), 3178−3184.
(37) Piechaczek, C.; Fetzer, C.; Baiker, A.; Bode, J.; Lipps, H. J. A Vector Based on the SV40 Origin of Replication and Chromosomal S/MARs Replicates Episomally in CHO Cells. *Nucleic Acids Res.* **1999**, *27* (2), 426−428.
(38) Godiska, R.; Patterson, M.; Schoenfeld, T.; Mead, D. A. Beyond PUC: Vectors for Cloning Unstable DNA. *Optim. DNA Seq. Process* **2005**, *1* (36), 55−75.
(39) Lufino, M. M. P.; Edser, P. A. H.; Wade-Martins, R. Advances in High-Capacity Extrachromosomal Vector Technology: Episomal Maintenance, Vector Delivery, and Transgene Expression. *Mol. Ther.* **2008**, *16* (9), 1525−1538.
(40) Kouprina, N.; Earnshaw, W. C.; Masumoto, H.; Larionov, V. A New Generation of Human Artificial Chromosomes for Functional Genomics and Gene Therapy. *Cell. Mol. Life Sci.* **2013**, *70* (7), 1135−1148.
(41) Iyer, R. R.; Pluciennik, A.; Napierala, M.; Wells, R. D. DNA Triplet Repeat Expansion and Mismatch Repair. *Annu. Rev. Biochem.* **2015**, *84*, 199−226.
(42) Karda, R.; Counsell, J. R.; Karbowniczek, K.; Caproni, L. J.; Tite, J. P.; Waddington, S. N. Production of Lentiviral Vectors Using Novel, Enzymatically Produced, Linear DNA. *Gene Ther. 2019 263* **2019**, *26* (3), 86−92.
(43) Kogo, H.; Inagaki, H.; Ohye, T.; Kato, T.; Emanuel, B. S.; Kurahashi, H. Cruciform Extrusion Propensity of Human Translocation-Mediating Palindromic AT-Rich Repeats. *Nucleic Acids Res.* **2007**, *35* (4), 1198−1208.
(44) Karbowniczek, K.; Rothwell, P.; Extance, J.; Milsom, S.; Lukashchuk, V.; Bowes, K.; Smith, D.; Caproni, L. DoggyboneTM DNA: An Advanced Platform for AAV Production. *Cell Gene Ther. Insights* **2017**, *3* (9), 731−738.

(45) Cui, Y. Z.; Zhou, J. T.; Li, B. Z.; Yuan, Y. J. The TelN/Tos-Assisted Precise Targeting of Chromosome Segments (TAPE). *J. Adv. Res.* **2022**, *41*, 169.

(46) Bishop, D. C.; Caproni, L.; Gowrishankar, K.; Legiewicz, M.; Karbowniczek, K.; Tite, J.; Gottlieb, D. J.; Micklethwaite, K. P. CAR T Cell Generation by PiggyBac Transposition from Linear Doggybone DNA Vectors Requires Transposon DNA-Flanking Regions. *Mol. Ther. - Methods Clin. Dev.* **2020**, *17*, 359−368.

(47) Annamalai, A. S.; Pattnaik, A.; Sahoo, B. R.; Muthukrishnan, E.; Natarajan, S. K.; Steffen, D.; Vu, H. L. X.; Delhon, G.; Osorio, F. A.; Petro, T. M.; Xiang, S.-H.; Pattnaik, A. K. Zika Virus Encoding Nonglycosylated Envelope Protein Is Attenuated and Defective in Neuroinvasion. *J. Virol.* **2017**, *91* (23), 1.

(48) Lubala, T. K.; Lumaka, A.; Kanteng, G.; Mutesa, L.; Mukuku, O.; Wembonyama, S.; Hagerman, R.; Luboya, O. N.; Lukusa Tshilobo, P. Fragile X Checklists: A Meta-analysis and Development of a Simplified Universal Clinical Checklist. *Mol. Genet. Genomic Med.* **2018**, *6* (4), 526.

(49) Chen, Q.; Lee, C. W.; Sim, E. U. H.; Narayanan, K. Induction of Protein Expression within Escherichia Coli Vector for Entry into Mammalian Cells. *Hum. Gene Ther. Methods* **2014**, *25* (1), 40−47.

(50) Nafissi, N.; Slavcev, R. Construction and Characterization of an In-Vivo Linear Covalently Closed DNA Vector Production System. *Microb. Cell Fact.* **2012**, *11* (1), 154.

(51) Chen, Q.; Narayanan, K. Crude Protein Extraction Protocol for Phage N15 Protelomerase in Vitro Enzymatic Assays. *Anal. Biochem.* **2011**, *414* (1), 169−171.

(52) Heinrich, J.; Schultz, J.; Bosse, M.; Ziegelin, G.; Lanka, E.; Moelling, K. Linear Closed Mini DNA Generated by the Prokaryotic Cleaving-Joining Enzyme TelN Is Functional in Mammalian Cells. *J. Mol. Med.* **2002**, *80* (10), 648−654.

(53) Lehner, R.; Wang, X.; Hunziker, P. Plasmid Linearization Changes Shape and Efficiency of Transfection Complexes. *Eur. J. Nanomedicine* **2013**, *5* (4), 205−212.

(54) Poulain, A.; Perret, S.; Malenfant, F.; Mullick, A.; Massie, B.; Durocher, Y. Rapid Protein Production from Stable CHO Cell Pools Using Plasmid Vector and the Cumate Gene-Switch. *J. Biotechnol.* **2017**, *255*, 16−27.

(55) Sum, C. H.; Wettig, S.; Slavcev, R. A. Impact of DNA Vector Topology on Non-Viral Gene Therapeutic Safety and Efficacy. *Curr. Gene Ther.* **2014**, *14* (4), 309−329.

(56) Sum, C. H.; Nafissi, N.; Slavcev, R. A.; Wettig, S. Physical Characterization of Gemini Surfactant-Based Synthetic Vectors for the Delivery of Linear Covalently Closed (LCC) DNA Ministrings. *PLoS One* **2015**, *10* (11), e0142875.

(57) Narayanan, K.; Sim, E. U. H.; Ravin, N. V.; Lee, C. W. Recombination between Linear Double-Stranded DNA Substrates in Vivo. *Anal. Biochem.* **2009**, *387* (1), 139−141.

(58) Satoh, D.; Abe, S.; Kobayashi, K.; Nakajima, Y.; Oshimura, M.; Kazuki, Y. Human and Mouse Artificial Chromosome Technologies for Studies of Pharmacokinetics and Toxicokinetics. *Drug Metabolism and Pharmacokinetics*. Japanese Society for the Study of Xenobiotics, 2018; pp 17−30.

(59) Hasegawa, Y.; Ikeno, M.; Suzuki, N.; Nakayama, M.; Ohara, O. Improving the Efficiency of Gene Insertion in a Human Artificial Chromosome Vector and Its Transfer in Human-Induced Pluripotent Stem Cells. *Biol. Methods Protoc.* **2018**, *3* (1), 1−10.

(60) Munye, M. M.; Tagalakis, A. D.; Barnes, J. L.; Brown, R. E.; McAnulty, R. J.; Howe, S. J.; Hart, S. L. Minicircle DNA Provides Enhanced and Prolonged Transgene Expression Following Airway Gene Transfer. *Sci. Rep.* **2016**, *6*, 1.

(61) Wong, S.; Lam, P.; Nafissi, N.; Denniss, S.; Slavcev, R. Production of Double-Stranded DNA Ministrings. *J. Vis. Exp.* **2016**, *2016* (108), 53177.

(62) Lu, J.; Zhang, F.; Fire, A. Z.; Kay, M. A. Sequence-Modified Antibiotic Resistance Genes Provide Sustained Plasmid-Mediated Transgene Expression in Mammals. *Mol. Ther.* **2017**, *25* (5), 1187−1198.

(63) Scott, V. L.; Patel, A.; Villarreal, D. O.; Hensley, S. E.; Ragwan, E.; Yan, J.; Sardesai, N. Y.; Rothwell, P. J.; Extance, J. P.; Caproni, L. J.; Weiner, D. B. Novel Synthetic Plasmid and Doggybone DNA Vaccines Induce Neutralizing Antibodies and Provide Protection from Lethal Influenza Challenge in Mice. *Hum. Vaccines Immunother.* **2015**, *11* (8), 1972−1982.

(64) Riede, O.; Seifert, K.; Oswald, D.; Endmann, A.; Hock, C.; Winkler, A.; Salguero, F. J.; Schroff, M.; Croft, S. L.; Juhls, C. Preclinical Safety and Tolerability of a Repeatedly Administered Human Leishmaniasis DNA Vaccine. *Gene Ther. 2015 228* **2015**, *22* (8), 628−635.

(65) Hardee, C. L.; Arévalo-Soliz, L. M.; Hornstein, B. D.; Zechiedrich, L. Advances in Non-Viral DNA Vectors for Gene Therapy. *Genes (Basel)* **2017**, *8* (2), 65.

(66) Nafissi, N.; Alqawlaq, S.; Lee, E. A.; Foldvari, M.; Spagnuolo, P. A.; Slavcev, R. A. DNA Ministrings: Highly Safe and Effective Gene Delivery Vectors. *Mol. Ther. - Nucleic Acids* **2014**, *3* (6), e165.

(67) Shafaati, M.; Saidijam, M.; Soleimani, M.; Hazrati, F.; Mirzaei, R.; Amirheidari, B.; Tanzadehpanah, H.; Karampoor, S.; Kazemi, S.; Yavari, B.; Mahaki, H.; Safaei, M.; Rahbarizadeh, F.; Samadi, P.; Ahmadyousefi, Y. A Brief Review on DNA Vaccines in the Era of COVID-19. *Future Virol.* **2022**, *17* (1), 49−66.

(68) Nafissi, N.; Sum, C. H.; Wettig, S.; Slavcev, R. A. Optimization of a One-Step Heat-Inducible In Vivo Mini DNA Vector Production System. *PLoS One* **2014**, *9* (2), e89345.

(69) Walters, A. A.; Kinnear, E.; Shattock, R. J.; Mcdonald, J. U.; Caproni, L. J.; Porter, N.; Tregoning, J. S. Comparative Analysis of Enzymatically Produced Novel Linear DNA Constructs with Plasmids for Use as DNA Vaccines. *Gene Ther.* **2014**, *21* (7), 645−652.

(70) DeKeyser, J. M.; Thompson, C. H.; George, A. L. Cryptic Prokaryotic Promoters Explain Instability of Recombinant Neuronal Sodium Channels in Bacteria. *J. Biol. Chem.* **2021**, *296*, 100298.

(71) Mora-Jimenez, L.; Valencia, M.; Sanchez-Carpintero, R.; Tønnesen, J.; Fadila, S.; Rubinstein, M.; Gonzalez-Aparicio, M.; Bunuales, M.; Fernandez-Pierola, E.; Nicolas, M. J.; Puerta, E.; Miguelez, C.; Minguez, P. G.; Lumbreras, S.; Gonzalez-Aseguinolaza, G.; Ricobaraza, A.; Hernandez-Alcoceba, R. Transfer of SCN1A to the Brain of Adolescent Mouse Model of Dravet Syndrome Improves Epileptic, Motor, and Behavioral Manifestations. *Mol. Ther. - Nucleic Acids* **2021**, *25*, 585−602.

(72) Goodall, D. J.; Jameson, K. H.; Hawkins, M.; Rudolph, C. J. A Fork Trap in the Chromosomal Termination Area Is Highly Conserved across All Escherichia Coli Phylogenetic Groups. *Int. J. Mol. Sci.* **2021**, *22* (15), 7928.

(73) Rudolph, C. J.; Upton, A. L.; Stockum, A.; Nieduszynski, C. A.; Lloyd, R. G. Avoiding Chromosome Pathology When Replication Forks Collide. *Nat. 2013 5007464* **2013**, *500* (7464), 608−611.

(74) Dimude, J. U.; Stockum, A.; Midgley-Smith, S. L.; Upton, A. L.; Foster, H. A.; Khan, A.; Saunders, N. J.; Retkute, R.; Rudolph, C. J. The Consequences of Replicating in the Wrong Orientation: Bacterial Chromosome Duplication without an Active Replication Origin. *MBio* **2015**, *6* (6), 1.

(75) Wang, L.; Lin, C.-M.; Brooks, S.; Cimbora, D.; Groudine, M.; Aladjem, M. I. The Human β-Globin Replication Initiation Region Consists of Two Modular Independent Replicators. *Mol. Cell. Biol.* **2004**, *24* (8), 3373−3386.

(76) Ohzeki, J.-i.; Larionov, V.; Earnshaw, W. C.; Masumoto, H. Genetic and Epigenetic Regulation of Centromeres: A Look at HAC Formation. *Chromosom. Res.* **2015**, *23* (1), 87−103.

(77) Bozza, M.; Green, E. W.; Espinet, E.; De Roia, A.; Klein, C.; Vogel, V.; Offringa, R.; Williams, J. A.; Sprick, M.; Harbottle, R. P. Novel Non-Integrating DNA Nano-S/MAR Vectors Restore Gene Function in Isogenic Patient-Derived Pancreatic Tumor Models. *Mol. Ther. - Methods Clin. Dev.* **2020**, *17*, 957−968.

(78) Mulia, G. E.; Picanço-Castro, V.; Stavrou, E. F.; Athanassiadou, A.; Figueiredo, M. L. Advances in the Development and the Applications of Nonviral, Episomal Vectors for Gene Therapy. *Hum. Gene Ther.* **2021**, *32* (19−20), 1076−1095.

(79) Bishop, D. C.; Xu, N.; Tse, B.; O'Brien, T. A.; Gottlieb, D. J.; Dolnikov, A.; Micklethwaite, K. P. PiggyBac-Engineered T Cells Expressing CD19-Specific CARs That Lack IgG1 Fc Spacers Have Potent Activity against B-ALL Xenografts. *Mol. Ther.* **2018**, *26* (8), 1883−1895.
(80) Bishop, D. C.; Clancy, L. E.; Burgess, J.; Mathew, G.; Atkins, E.; Advic, S.; Maddock, K.; Street, J.; Moezzi, L.; Simms, R.; Stephen, K.; O'Brien, T.; Shaw, P. J.; Gottlieb, D.; Blyth, E.; Micklethwaite, K. Matched Sibling Donor-Derived Piggybac CAR19 T Cells Induce Remission of Relapsed/Refractory CD19+ Malignancy Following Haematopoietic Stem Cell Transplant. *Cytotherapy* **2019**, *21* (5), S9.
(81) Gurumurthy, C. B.; Lloyd, K. C. K. Generating Mouse Models for Biomedical Research: Technological Advances. *Dis. Model. Mech.* **2019**, *12* (1), 1 DOI: 10.1242/dmm.029462.
(82) Kouprina, N.; Petrov, N.; Molina, O.; Liskovykh, M.; Pesenti, E.; Ohzeki, J. I.; Masumoto, H.; Earnshaw, W. C.; Larionov, V. Human Artificial Chromosome with Regulated Centromere: A Tool for Genome and Cancer Studies. *ACS Synth. Biol.* **2018**, *7* (9), 1974−1989.
(83) Ikeno, M.; Hasegawa, Y. Applications of Bottom-up Human Artificial Chromosomes in Cell Research and Cell Engineering. *Exp. Cell Res.* **2020**, *390* (1), 111793.
(84) Makhija, H.; Roy, S.; Hoon, S.; Ghadessy, F. J.; Wong, D.; Jaiswal, R.; Campana, D.; Dröge, P. A Novel λ Integrase-Mediated Seamless Vector Transgenesis Platform for Therapeutic Protein Expression. *Nucleic Acids Res.* **2018**, *46* (16), e99−e99.
(85) Weber, E. W.; Maus, M. V.; Mackall, C. L. The Emerging Landscape of Immune Cell Therapies. *Cell* **2020**, *181* (1), 46−62.

https://doi.org/10.1021/acssynbio.2c00580
ACS Synth. Biol. XXXX, XXX, XXX−XXX