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RESEARCH ARTICLE

CRISPR-Directed *In Vitro* Gene Editing of Plasmid DNA Catalyzed by Cpf1 (Cas12a) Nuclease and a Mammalian Cell-Free Extract

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Abstract

Extraordinary efforts are underway to offer greater versatility and broader applications for CRISPR-directed gene editing. Here, we report the establishment of a system for studying this process in a mammalian cell-free extract prepared from HEK-293 human embryonic kidney cells. A ribonucleoprotein (RNP) particle and a mammalian cell-free extract coupled with a genetic readout are used to generate and identify specific deletions or insertions within a plasmid target. A Cpf1 (Cas12a) RNP induces a double-stranded break, and the cell-free extract provides the appropriate enzymatic activities to direct specific deletion through resection and homology directed repair in the presence of single- and double-stranded donor DNA. This cell-free system establishes a foundation to study the heterogeneous products of gene editing, as well as the relationship between nonhomologous end joining and homology directed repair and related regulatory circuitries simultaneously in a controlled environment.

Introduction

The potential to re-engineer human genomes has been heightened by the development of CRISPR*-Cas9 gene editing.[1,2] By repurposing components of this bacterial pathway, molecular geneticists have edited human genes[3–6] at unprecedented frequencies with a simplicity that could enable therapeutic application.[7–9] The precision with which CRISPR-Cas9 acts is being improved by restructuring the Cas9 protein,[10–13] reconfiguration of the composition of the gene editing complex[14,15] and use of alternate nucleases.[16–18] Cpf1 (Cas12a)[19] is one of these nucleases, embedded in a type V CRISPR-Cas system found in microorganisms such as *Acidaminococcus*, *Prevotella*, and *Francisella*.[2,20–22] The active complex structure of Cpf1 differs from Cas9[9,23–25] by associating with single-stranded crRNA without the requirement of a tracrRNA component.[26,27] CRISPR-Cpf1 has already been used extensively in mammalian cell lines for applications such as genome-wide analysis,[28] the generation of knockout mice,[29] and the correction of the human dystrophin gene.[30] CRISPR-Cpf1 exploits homology-directed repair pathways within intact mammalian cells.[31] Additionally, it appears that human cell types and genomic targets respond differently to various types of enzymatic activities,[32–35] suggesting this diversity of function is important.

Previous studies of the mechanism and regulation of gene editing in human cells through the combined activities of CRISPR-Cas9 and single-stranded oligonucleotides (ssODNs)[36,37] unraveled the mechanism of gene editing directed solely by single-stranded DNA[38,39] by developing an experimental system that utilizes a cell-free extract.[40,41] In that system, plasmid DNA is modified in a reaction mixture that contains the extract and ssODN, where the successful outcome of the point mutation repair reaction is visualized by a genetic readout in *Escherichia coli*. The results demonstrated the efficacy of single-agent gene editing *in vitro* and established a role for RNA and double-stranded DNA breakage in the gene editing reaction pathway.[42]

In this work, we describe a system to study the mechanism and regulation of gene editing *in vitro* catalyzed by a ribonucleoprotein complex and enzymatic components of a mammalian cell-free extract. Site-specific deletions

*Clustered Regularly Interspaced Short Palindromic Repeats.

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through DNA resection and site-specific insertion of appropriate donor DNA templates are enabled in the same reaction mixture. These reactions could recapitulate the competing pathways of nonhomologous end joining (NHEJ) and homology directed repair (HDR).[43] The Cpf1 nuclease assembled into a ribonucleoprotein (RNP) particle cleaves plasmid DNA at a prescribed site. A heterogeneous population of plasmid molecules containing deletions, specific insertions, or other forms of genetic modifications is generated in a single *in vitro* gene editing reaction mixture. The development of this system provides an opportunity to study the molecular interactions and the regulatory circuitry controlling CRISPR-directed gene editing in a more defined manner.

Materials and Methods

Cell-free extract preparation

Cell-free extracts were prepared following the technique outlined by Cole-Strauss *et al*.[40] from cell lines synchronized at the G1/S boarder and released. HCT 116-19, HEK-293, and HEL 92.1.7 cell lines (American Type Cell Culture, Manassas, VA) were cultured, and 2×10^6 cells were harvested and immediately washed in cold hypotonic buffer (20 mM of HEPES, 5 mM of KCl, 1.5 mM of $MgCl_2$, 1 mM of dithiothreitol [DTT], and 250 mM of sucrose). Cells were centrifuged based on their respective standard conditions, re-suspended in cold hypotonic buffer without sucrose, and incubated on ice for 15 min before being lysed by 25 strokes of a Dounce homogenizer. Cytoplasmic fraction of enriched cell lysate was incubated on ice for 60 min and centrifuged for 15 min at 12,000 *g* at 4°C. The supernatant was then aliquoted and immediately frozen at −80°C. The cell-free extract concentrations were determined using the Bradford assay.

In vitro reaction conditions

RNP complexes used in *in vitro* reactions consisted of a purified AsCpf1 nuclease (Integrated DNA Technologies, Coralville, IA) and a target-specific crRNA (Integrated DNA Technologies). *In vitro* DNA cleavage reaction mixtures contained 250 ng (0.007566 μM) of pHSG299 plasmid DNA (Takara Bio Company, Shiga, Japan) and 10 pmol of RNP mixed in a reaction buffer (100 mM of NaCl, 20 mM of Tris-HCl, 10 mM of $MgCl_2$, and 100 μg/mL of bovine serum albumin), which was brought to a final volume of 20 μL. Each reaction was incubated for 15 min at 37°C after which DNA was recovered from reaction mixtures and purified using QIAprep Spin Miniprep silica columns (Qiagen, Hilden, Germany). Secondary *in vitro* recircularization reactions contained DNA recovered from the initial cleavage reaction, 20 μg of cell-free extract supplemented with Quick Ligase (New England Biolabs, Ipswich, MA), and a reaction buffer (20 mM of Tris, 15 mM of $MgCl_2$, 0.4 mM of DTT, and 1.0 mM of adenosine triphosphate), which was brought to a final volume of 35 μL. Each secondary reaction was incubated for 15 min at 37°C. For reactions that included single- or double-stranded donor DNA templates (Integrated DNA Technologies), 4.464 μg was added into the secondary reaction mixture. DNA from the secondary *in vitro* recircularization reactions was recovered from reaction mixtures and purified using silica spin columns.

Transformation, selection, and DNA isolation and analysis

Plasmid DNA recovered from *in vitro* reactions was transformed into 50 μL of DH5α competent *E. coli* (Invitrogen, Carlsbad, CA) via heat shock transformation. Competent cells were incubated on ice for 30 min after plasmid introduction, heat shocked for 20 s at 42°C, placed on ice for 2 min, brought to a final volume of 1 mL in SOC media and incubated for 1 hr at 37°C, with shaking (225 rpm). Undiluted competent cells were plated on media containing kanamycin antibiotics and incubated overnight at 37°C. Single kanamycin-resistant colonies were selected, and plasmid DNA was isolated via a QIAprep Spin Miniprep Kit (Qiagen). Modifications made to the plasmid DNA selected from bacterial colonies were evaluated via DNA sequencing (GeneWiz, South Plainfield, NJ).

Polymerase chain reaction amplification

Polymerase chain reaction (PCR) amplification of the *lacZ* gene from plasmid DNA isolated from selected bacterial colonies generated a 539 bp amplicon using PCR primers (Integrated DNA Technologies): fwd 5′-GCT TCCGGCTCGTATGTTGTGTGG-3′ and rev 5′-GTTG GACGAGTCGGAATCGCAGA-3′. The PCR conditions involved an initial denaturation of template DNA at 98°C for 30 s, cycle denaturation at 98°C for 10 s, primer annealing at 60°C for 30 s, and extension at 72°C for 10 s for 35 cycles, with a hold at 72°C for 10 min. Each PCR consisted of 10 ng of template DNA, 10 μM of forward and reverse primers, PCR qualified water (Quality Biological, Inc., Gaithersburg, MD), and Phusion High Fidelity PCR Master Mix with HF Buffer (New England Biolabs) in a total reaction volume of 20 μL. PCR products were purified using QIAquick PCR Purification Kit (Qiagen), and modifications were evaluated via DNA sequencing (GeneWiz, South Plainfield, NJ).

Results

The initialization of the *in vitro* gene editing reaction is illustrated in Figure 1A using a Cpf1 nuclease assembled into an RNP particle to direct cleavage. The cell-free

extract provides catalytic activities for DNA resection, DNA replication and repair, phosphorylation, and re-ligation, among others, needed to process the linearized plasmid. After the *in vitro* reaction is complete, purified plasmid is transformed into *E. coli* for colony selection and DNA analyses. In the presence of exogenously added single- and double-stranded donor DNA templates, precise template insertion is readily observable.

DNA cleavage and modification induced by Cpf1 RNP particles

A variety of Cpf1 sites in the *lacZ* gene are depicted with the appropriate guide RNA sequences displayed in Figure 1B. The primary Cpf1 site is surrounded by a red box, with the associated cut site illustrated by a staggered red arrow. The *lacZ* gene embedded in the plasmid pHSG299 provides an attractive template for this assay, as, theoretically, the destruction of the *lacZ* gene through DNA deletion or insertion results in the production of a non-functional β-galactosidase protein. When plasmids containing a disrupted *lacZ* gene are cultured in the presence of X-gal, a change in the color of bacterial colonies, from blue to white, will be visible. The major genetic outcomes of the *in vitro* gene editing reaction following site-specific cleavage by Cpf1 are illustrated in Figure 1C. After Cpf1 cleavage, DNA deletion activity and ligation could occur via NHEJ, while DNA insertion in the presence of a single- or double-stranded donor DNA template could be carried out by HDR.

The cleavage activity of the assembled Cpf1 RNP targeting supercoiled pHSG299 plasmid DNA and the catalytic activity of the cell-free extract on the linearized DNA are demonstrated in Supplementary Figure S1 (Supplementary Data are available online at www.liebertpub.com/crispr). Extracts prepared from different cell sources display varying degrees of DNA resection activity, perhaps reflecting activity from the NHEJ pathway, on the linearized plasmid. The enzymatic activity exhibited by an extract prepared from HEK-293 cells was of particular interest because these cells are understood to have appropriate levels of DNA repair and DNA recombination activities.[44]

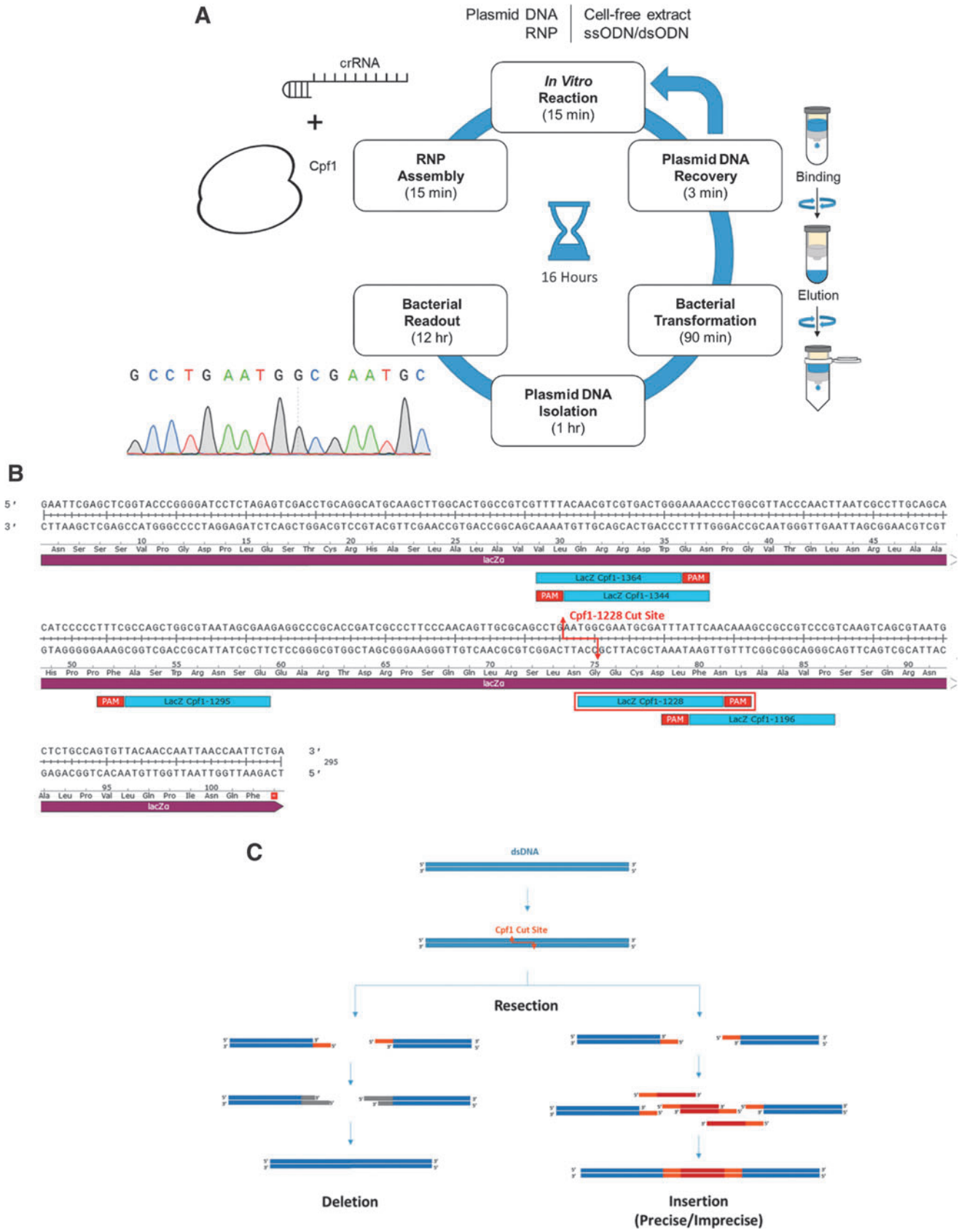
Specific DNA deletion in a cell-free system

We considered the possibility that the *in vitro* CRISPR-based gene editing system could be used to elucidate the mechanism of action of genome modification in human cells by taking advantage of the cleavage activity of Cpf1 RNPs, designed to target pHSG299 (Fig. 2A), and the resection activity in the cell-free extract. The successful gene editing of the *lacZ* gene in pHSG299 after Cpf1 RNP cleavage is displayed in Figure 2B. Bacterial clones transformed with plasmid recovered from the *in vitro* reaction were found to harbor sequence deletions surrounding the targeted site, indicated by the staggered arrow (see Fig. 2A). Representative sequence panels are provided to demonstrate the type of DNA deletions found within the clonal population, including clones in which DNA sequences were found to be unaltered. Of the 41 sequences analyzed, 22 showed DNA alterations surrounding the cleavage site created by the Cpf1 RNP.

Specific DNA insertion promoted by single- and double-stranded donor DNA templates

Next, we attempted to carry out DNA insertion via HDR at the designated Cpf1 cleavage site. Many cellular and animal protocols for HDR utilize single-stranded DNA as the donor template.[45–48] So, initially, we examined the possibility that template insertion could be directed by single-stranded DNA templates. Donor DNA templates were designed to include a cleavage site for the restriction enzyme NotI, a site that is not present in pHSG299. To ensure that any insertion of donor DNA is a result of the exogenously added single-stranded template, we also incorporated a two-base-pair ''barcode'' (TT:AA) included at an upstream position relative to the NotI site in the homology arms. Each template has a 10-base overhang, with a five-base

FIG. 1. *In vitro* gene editing experimental protocol and tools. **(A)** Cpf1 ribonucleoproteins (RNPs) are complexed and added to the first *in vitro* cleavage reaction mixture with plasmid DNA. Plasmid DNA is recovered and added to a second *in vitro* recircularization reaction mixture with cell-free extract and a single- or double-stranded donor DNA template. After the reaction is complete, plasmid DNA is recovered from the reaction and transformed into competent *Escherichia coli*. DNA is then isolated from transformed cells and sequenced to identify modifications made *in vitro*. **(B)** A variety of Cpf1 RNP sites are shown in blue across the *lacZ* gene region of pHSG299. The Cpf1 site used for *in vitro* reactions described in this article is indicated within a red box, and the associated cut site is marked by a staggered red arrow. **(C)** A schematic depiction of the potential outcomes of *in vitro* gene editing reactions following site-specific cleavage by Cpf1 are shown. DNA resection, deletion activity, and ligation could occur via nonhomologous end joining, while DNA insertion could be carried out in the presence of a single- or double-stranded donor DNA template.


A
Plasmid DNA
RNP
Cell-free extract
ssODN/dsODN
crRNA
+
Cpf1
In Vitro Reaction (15 min)
RNP Assembly (15 min)
Plasmid DNA Recovery (3 min)
16 Hours
Bacterial Readout (12 hr)
Bacterial Transformation (90 min)
Plasmid DNA Isolation (1 hr)
Binding
Elution
G C C T G A A T G G C G A A T G C
B
5′ GAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCA
3′ CTTAAGCTCGAGCCATGGGCCCCTAGGAGATCTCAGCTGGACGTCCGTACGTTCGAACCGTGACCGGCAGCAAAATGTTGCAGCACTGACCCTTTTGGGACCGCAATGGGTTGAATTAGCGGAACGTCGT
Asn Ser Ser Ser Val Pro Gly Asp Pro Leu Glu Ser Thr Cys Arg His Ala Ser Leu Ala Leu Ala Val Val Leu Gln Arg Arg Asp Trp Glu Asn Pro Gly Val Thr Gln Leu Asn Arg Leu Ala Ala
lacZα
LacZ Cpf1-1364
PAM
PAM
LacZ Cpf1-1344
Cpf1-1228 Cut Site
CATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGCGATTTATTCAACAAAGCCGCCGTCCCGTCAAGTCAGCGTAATG
GTAGGGGGAAAGCGGTCGACCGCATTATCGCTTCTCCGGGCGTGGCTAGCGGGAAGGGTTGTCAACGCGTCGGACTTACCGCTTACGCTAAATAAGTTGTTTCGGCGGCAGGGCAGTTCAGTCGCATTAC
His Pro Pro Phe Ala Ser Trp Arg Asn Ser Glu Glu Ala Arg Thr Asp Arg Pro Ser Gln Gln Leu Arg Ser Leu Asn Gly Glu Cys Asp Leu Phe Asn Lys Ala Ala Val Pro Ser Ser Gln Arg Asn
lacZα
PAM
LacZ Cpf1-1295
LacZ Cpf1-1228
PAM
PAM
LacZ Cpf1-1196
CTCTGCCAGTGTTACAACCAATTAACCAATTCTGA 3′
295
GAGACGGTCACAATGTTGGTTAATTGGTTAAGACT 5′
Ala Leu Pro Val Leu Gln Pro Ile Asn Gln Phe
lacZα
C
dsDNA
Cpf1 Cut Site
Resection
Deletion
Insertion
(Precise/Imprecise)

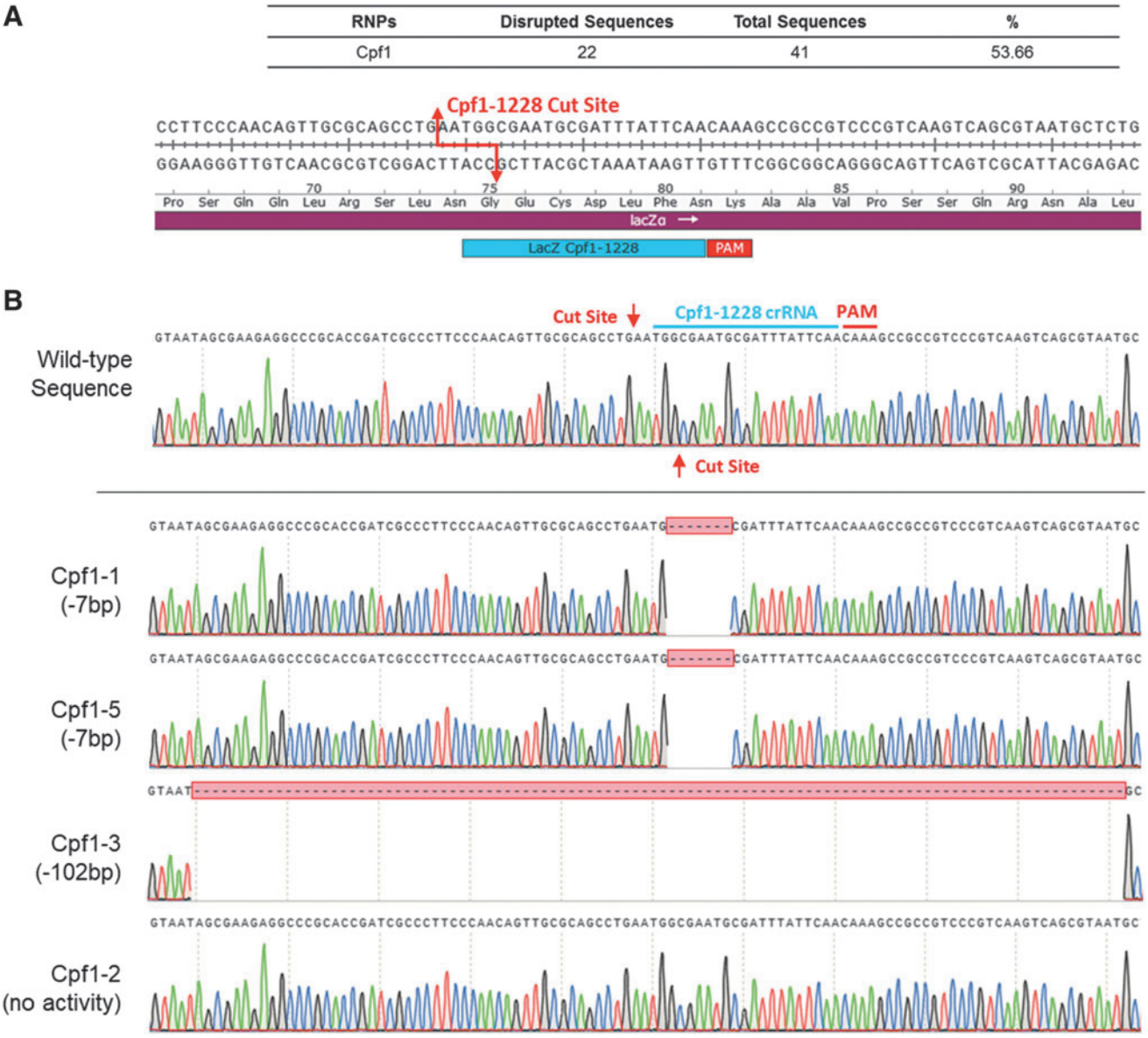


FIG. 2. Cpf1 RNP activity *in vitro*. **(A)** The frequency of DNA disruption by the Cpf1 RNP is shown as a percentage of the number of disrupted DNA sequences detected in relation to the total number of sequences analyzed from bacterial colonies transformed with plasmid DNA recovered from *in vitro* reactions. The Cpf1 site (blue) is shown along the *lacZ* gene region with associated cleavage sites marked by a staggered arrow. The wild-type sequence of the *lacZ* gene region is shown. **(B)** The five sequences shown are representative of the total number of sequences assessed from *in vitro* reactions containing Cpf1 RNPs displaying a variety of DNA disruption around the cleavage site.

region complementary to the overhangs generated at the Cpf1 staggered cut site. A diagram of the single-stranded templates used in this experiment and their orientation relative to the target site are provided in Figure 3A and B. Plasmid DNA from reactions containing ssODNs, designed to integrate into the sense strand, NotI-S, or nonsense strand, NotI-NS, were isolated and treated with NotI (Fig. 3C). RFLP analysis confirms the insertion of both single-stranded templates bearing the newly integrated NotI restriction site in a population of plasmid DNA.

To confirm the presence of precise or imprecise insertion, we carried out DNA sequencing across the region of interest in NotI-positive plasmid samples recovered from both sense and nonsense single-stranded template reactions (Fig. 4). Sequencing data confirm our RFLP

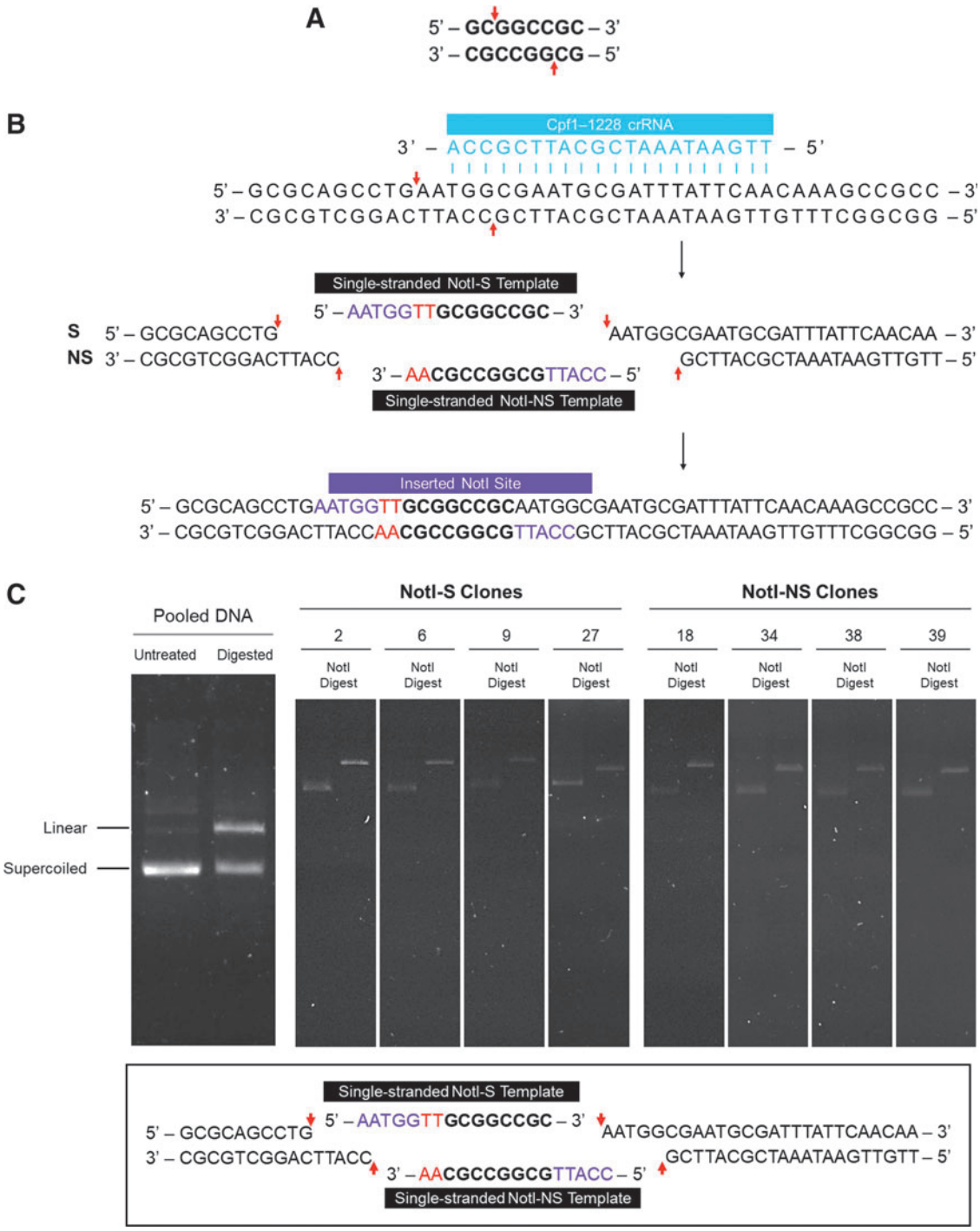


FIG. 3. Proposed mechanism and verification of single-stranded NotI template insertion. **(A)** Illustration of the NotI restriction cut site. **(B)** The Cpf1 RNP staggered double-stranded cleavage site on the *lacZ* gene is indicated by red arrows. The single-stranded NotI templates are inserted into one of two strands, the sense strand (S) or nonsense (NS) strand, by utilizing arms complementary to the overhangs produced by Cpf1 cleavage. **(C)** Pooled and isolated plasmid DNA from selected bacterial colonies transformed with plasmids recovered from *in vitro* single-stranded NotI reactions were subject to NotI enzyme digestion to confirm the integration of the NotI site into the *lacZ* gene region.

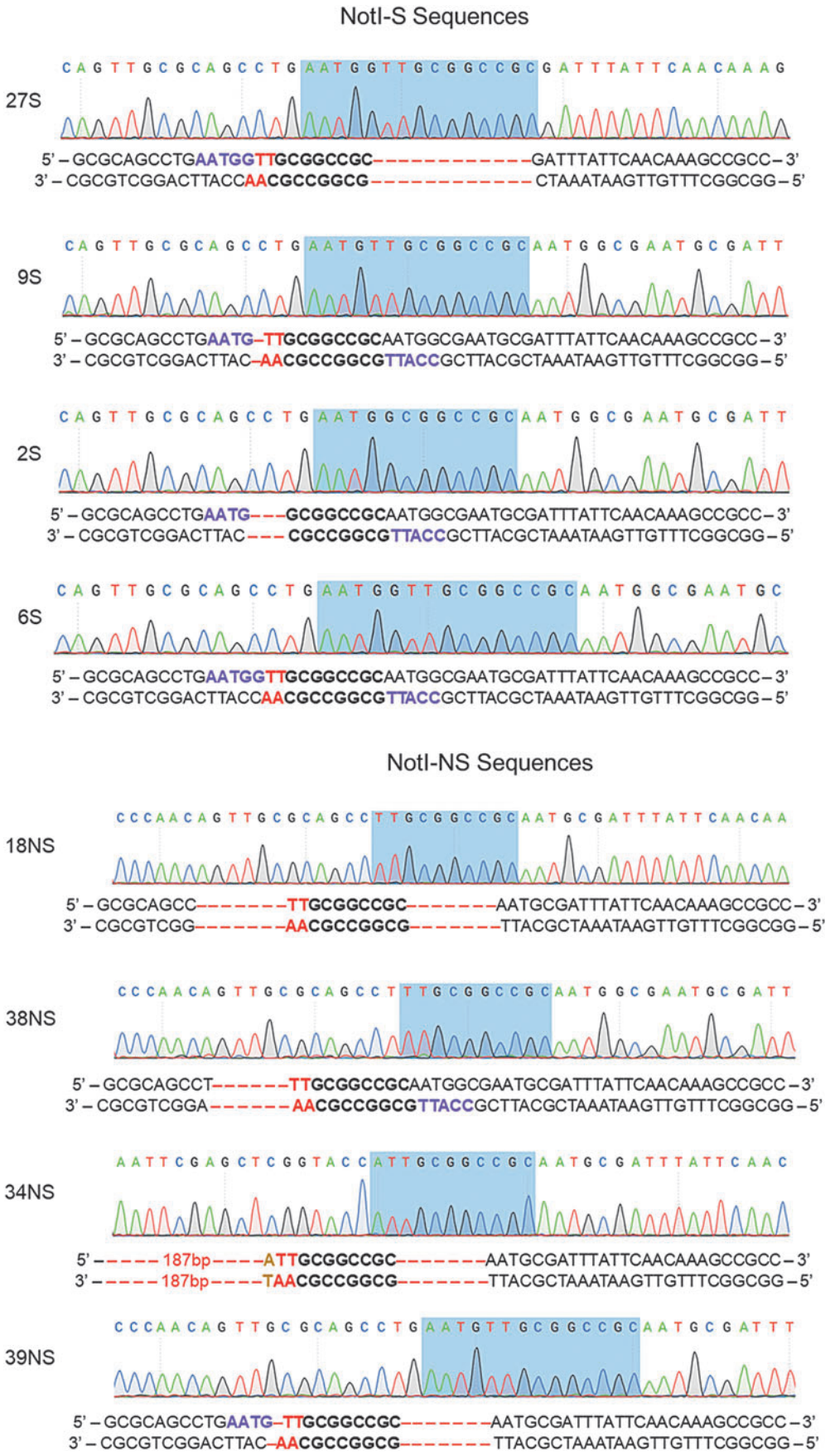


FIG. 4. Single-stranded template insertion sequences. Four representative sequences are shown from plasmid DNA isolated from selected bacterial colonies transformed with plasmid recovered from each of the *in vitro* single-stranded NotI-S and NotI-NS insertion reactions.

analysis and reveal a heterogeneous population of sequence inserts within both categories of clones. Importantly, in 17–18% of the sequences analyzed from reactions driven by NotI-S, a perfect single insertion of the intended template is observed. We did not detect perfect insertion when NotI-NS served as the single-stranded template in all the colonies examined (data not shown). In all cases where no perfect insertion was detected, each clone contained a variable amount of DNA modification, often in the form of a deletion, evidence of resection activity in the cell-free extract. The strand bias exhibited in this HDR directed reaction will likely provide insight into the mechanism of template insertion via the process of HDR in the *in vitro* gene editing reaction. We continue to screen large numbers of colonies generated from multiple experiments to understand the molecular pathways that govern insertion or deletion. At this point, we have never seen a perfect insertion generated by a single-strand donor template of the NS polarity (see Supplementary Fig. S2B). This trend has remained consistent for several single-stranded donor templates of varying lengths, but we will continue to report our results as the studies progress. DNA sequence data in colony results from several screens are provided in Supplementary Fig. S2A and B.

Some experimental protocols utilize a double-stranded template, sometimes in the form of a PCR product.[30] While it is likely that many of the short double-stranded templates are dissociated to single-stranded or partially single-stranded molecules in the cell, we did extend this experimental system by attempting to carry out DNA insertion using a double-stranded donor DNA template at the same designated Cpf1 cleavage site. To do so, we utilized annealed NotI-S and NotI-NS single-stranded templates in order to produce a double-stranded template containing a cleavage site for the restriction enzyme NotI. Figure 5A displays the sequences of several clones that contain a perfect insertion at the designated site of cleavage. Approximately 15% of the plasmid DNA, isolated from colonies transformed with DNA and recovered from *in vitro* reactions containing the duplexed NotI insertion template, contained the perfect insertion. Other insertion patterns included two NotI template insertions accompanied by a single base-pair deletion (Fig. 5B). In all three sequences, the one base deletion occurred at the last nucleotide positioned on the 5′ overhanging end upstream from the insertion site. Figure 5C displays a DNA sequence in which three NotI template insertions accompanied by multiple deletions surrounding the inserted NotI template: a single base-pair deletion upstream from the first inserted template and a seven-base-pair deletion downstream from the third inserted template. Approximately 8% of the plasmid DNA had no alteration in the DNA sequence, as seen in Figure 5D. Additional DNA sequence data are provided in Supplementary Fig. S3.

Discussion

The evolution and development of CRISPR-related gene editing systems provide geneticists with invaluable tools to examine structure–function relationships of eukaryotic genes. To improve the accuracy and efficacy of gene editing, it will be useful to understand the mechanism of action and the controlling factors of the reaction. Toward this end, we describe an *in vitro* gene editing system for elucidating the mechanics of several Cpf1-directed gene editing reactions.

In vitro CRISPR-Cpf1-directed gene editing can be reduced to a biochemical reaction by coupling the activity of the Cpf1 nuclease and an extract prepared from mammalian cells.[40] The preparation and use of this extract is similar to the strategy of Li and Kelly[49] in their mechanistic and regulatory studies of DNA replication. Herein, the extracts were prepared from HEK-293 cells that had been synchronized at the G1/S border and released 4 h prior to breakage. We[50–52] and others[53–56] have previously established that gene editing activity, directed by ssODNs, occurs at a higher frequency in synchronized and released cells.[44]

We confirmed RNP particles cleave supercoiled plasmid DNA at targeted sites within our *in vitro* system, as described by Zetche *et al.*[16] We then asked if the addition of a cell-free extract could catalyze DNA resection, phosphorylation, replication, homology-directed insertion, and re-ligation at the cleavage site to produce a genetically modified plasmid. To ensure that no additional modifications were made to untargeted sites, we also had large portions of several plasmids isolated from *in vitro* reactions sequenced and aligned to the wild-type plasmid sequence to confirm no off-target effects were occurring (Supplementary Fig. S4). Identification of re-engineered plasmids was done using a genetic readout in *E. coli*, an approach that has been used previously in studies where the objective was to understand the mechanism and regulation of single-agent gene editing.[40,41]

The *in vitro* system presented here can enable precise and imprecise DNA insertion when either single- or double-stranded DNA serves as the donor template. This versatility should afford an opportunity to examine the regulatory pathways that control these insertion reactions independent of which type of template is used. Many donor template reactions produced a perfect insertion, while the rest of the population displayed a variety of genetic modifications, some of which include a

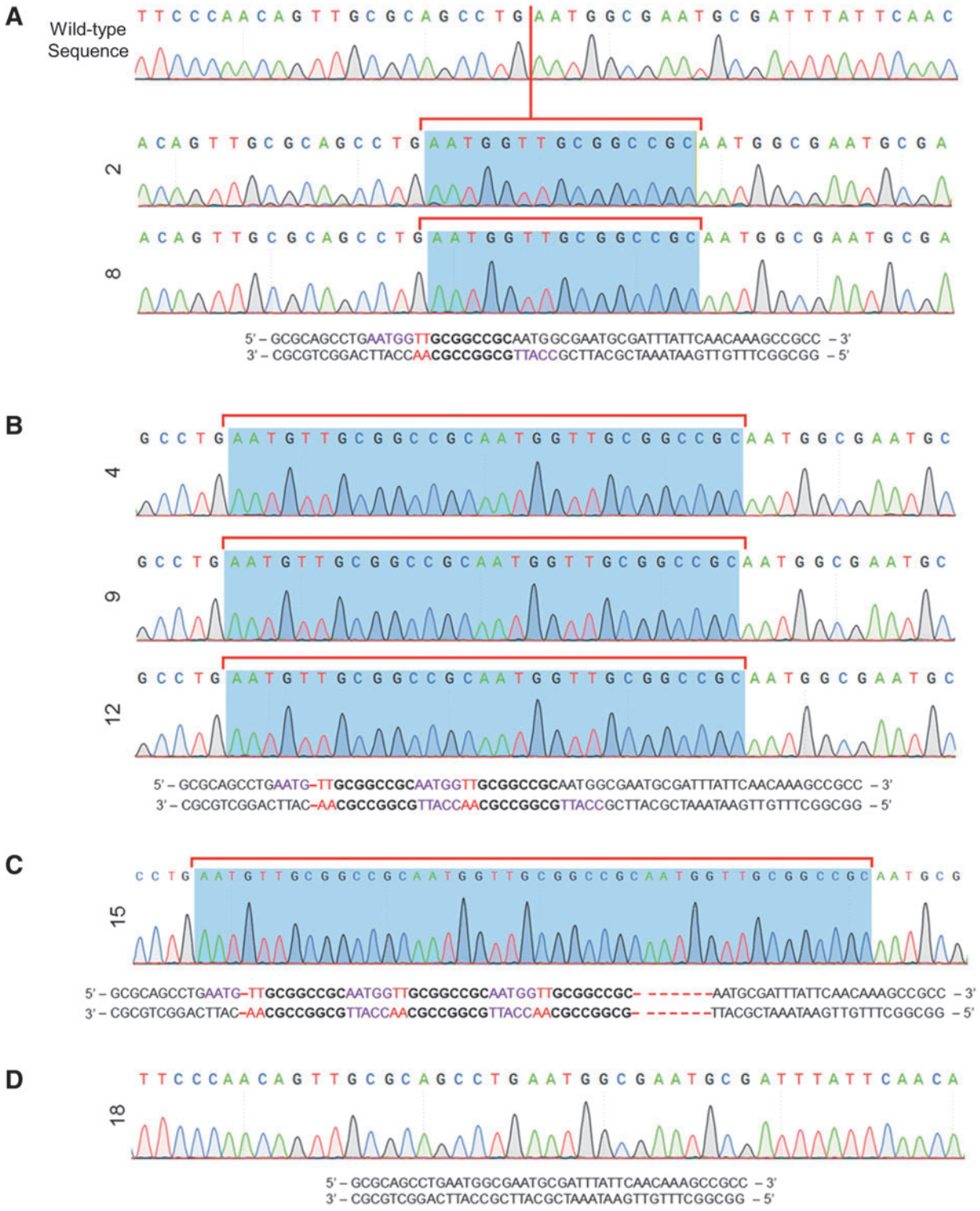


FIG. 5. Duplexed NotI template insertion sequences. The sequence of the wild-type *lacZ* gene region and selected bacterial colonies transformed with plasmid DNA recovered from *in vitro* duplexed NotI template insertion reactions are shown. **(A)** Sequencing analysis revealed two contained perfect NotI template insertion at the cleavage site. **(B)** Three contained two NotI site template inserts accompanied by a 1 bp deletion upstream from the cleavage site. **(C)** One contained three NotI site template inserts accompanied by a 1 bp deletion upstream and a 7 bp deletion downstream from the cleavage site. **(D)** One did not contain a NotI site template insertion at the cleavage site.

duplication of the donor template. This heterogeneity is important, since it is likely to recapitulate multiple activities going on in cells and in animal models. Our DNA insertion reactions, which utilized single-stranded DNA donor templates, generated precise insertions in 17–18% of the isolated plasmid molecules when the sense strand single-stranded NotI-S was used. Once again, when perfect insertion was not observed, a heterogeneous population of genetically modified templates was found, including those bearing sequence deletions. No perfect insertions were observed in plasmid targets emanating from reactions bearing the single-stranded DNA donor template of nonsense polarity, NotI-NS. These observations suggest a strand bias for precise template insertion when driven by the single-stranded DNA templates. Strand bias has been reported previously for gene editing in cell lines,[57–59] and again we have translated the *in vitro* observations into a mammalian cell culture system that aims to correct, by insertion, a point mutation in an integrated reporter gene. Therefore, this *in vitro* prediction of the strand bias in the use of ssDNA templates has been reproduced by our own laboratory and by others.[37,60] *In vitro* reactions also recapitulate the importance of the double-stranded DNA breakage prior to successful template insertion. Previous work in the area of single-agent gene editing[61] demonstrated point mutation repair, likely through template insertion, but at an extremely low frequency. However, when DNA cleavage preceded the addition of the template, gene editing frequencies rose substantially.[48,51] Thus, the *in vitro* system appears to reflect the reaction mechanics of gene editing seen in mammalian cells. The cell-free system can also be used to identify the factors that regulate HDR and NHEJ in reactions initialized by CRISPR-directed gene editing in a more methodical fashion with a validated, quantifiable genetic readout. Ongoing experiments aimed at understanding the mechanism of action in the *in vitro* system will likely provide further guidance in the design of CRISPR-directed gene editing reactions in eukaryotic cells.

In terms of optimization, we have begun using plasmids that carry no phenotypic readout. Instead, these plasmids are randomly selected from the bacterial plate, which allows us to obtain a true measure of the plasmid molecules containing deletions or insertions. Thus, one can utilize the methodology described above as a screen for testing the efficiency of gene editing. In other words, this system provides an opportunity to carry out biochemical reactions on the gene of interest using the mammalian cell type used in the reactions as a source of the extract. Despite our concerted efforts and analyses of 18 clones, our attempts to generate DNA deletions within *in vitro* reactions containing Cas9 RNP particles, wild-type, nickase, or dCas9 did not generate any genetically modified plasmids, necessitating additional optimization of this reaction (data not shown). We are presently testing other sources of the Cpf1 enzyme such as the Lba Cpf1 from *Lachnospiraceae* bacterium.

Many studies have demonstrated that single-stranded DNA molecules are more proficient in executing homology-directed repair in cells and in animals.[30,58,60,62,63] Pioneering work by Storici *et al*. offered an influential model for how single-stranded DNA could bridge across a double-stranded DNA break and act to repair the break.[64] The pathway of repair requires a two-step annealing process as opposed to strand invasion or strand assimilation. These same authors reported a strand bias in the process of double-stranded break repair. These results align closely with our observation that the NotI-S ssODN, and not the NotI-NS ssODN directs precise repair at the target site. Recent data suggest that the mechanism of DNA template insertion is a *Rad51*/*BRCA2* independent pathway,[65] yet demonstrates something unique, as double-stranded DNA templates can also catalyze precise insertion events. These results differ from previous studies that found relatively low levels when duplexed DNA was used as the invading template.[48] With the development of this system, we are now well positioned to carry out experiments aimed at discerning the dominant pathway(s) within the gene editing reaction, the optimal donor template attributes for precise insertion, and the factors that influence the imbalance between NHEJ and HDR. We are also developing this cell-free reaction as a novel, innovative, and broad-based approach for site-directed mutagenesis. This new system enables the simultaneous creation of frameshift and point mutations at multiple sites within a target gene, or even multiple genes within the same plasmid. Using such a CRISPR-based site-directed mutagenesis system will overcome the need of current site-directed mutagenesis protocols to amplify the target gene by one or more rounds of PCR amplifications, which may cause false priming and undesired off-target mutations. Using this *in vitro* method, numerous point mutations can be introduced into a plasmid simultaneously and perhaps at different sites. Examining the mechanism of action of donor template replacement will also yield important insights into the overall process of HDR initialized by CRISPR-directed DNA cleavage.

Conclusion

Here, we report the establishment of an *in vitro* system for CRISPR-directed gene editing. A RNP particle and a mammalian cell-free extract coupled with a genetic

readout are used to direct specific deletion through resection and HDR in the presence of single- and double-stranded donor DNA templates within a plasmid target. Site-specific deletions through DNA resection and site-specific insertion of appropriate donor DNA templates are enabled in the same reaction mixture. These reactions could recapitulate the competing pathways of NHEJ and HDR. A heterogeneous population of plasmid molecules containing deletions, specific insertions, or other forms of genetic modifications is generated in a single *in vitro* reaction mixture. Insertion of single-stranded donor templates has also shown a strand bias in favor of precise insertion of the sense strand template and imprecise insertions of the nonsense strand template during *in vitro* reactions. The development of this system provides an opportunity to study the molecular interactions and the regulatory circuitry controlling CRISPR-directed gene editing, as well as the relationship between the NHEJ and HDR regulatory pathways, simultaneously in a controlled environment.

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Author Disclosure Statement

E.N. and G.T. are employees of NovellusDx (Jerusalem, Israel). B.M.S., A.M.W., and E.B.K. have no conflicts of interest or financial interests in associated companies.

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