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RESEARCH ARTICLE

Efficient Genome and Base Editing in Human Cells Using ThermoCas9

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Abstract

Most genetic engineering applications reported thus far rely on the type II-A CRISPR-Cas9 nuclease from *Streptococcus pyogenes* (SpyCas9), limiting the genome-targeting scope. In this study, we demonstrate that a small, naturally accurate, and thermostable type II-C Cas9 ortholog from *Geobacillus thermodenitrificans* (ThermoCas9) with alternative target site preference is active in human cells, and it can be used as an efficient genome editing tool, especially for gene disruption. In addition, we develop a ThermoCas9-mediated base editor, called ThermoBE4, for programmable nicking and subsequent C-to-T conversions in human genomes. ThermoBE4 exhibits a three times larger window of activity compared with the corresponding SpyCas9 base editor (BE4), which may be an advantage for gene mutagenesis applications. Hence, ThermoCas9 provides an alternative platform that expands the targeting scope of both genome and base editing in human cells.

Introduction

The CRISPR-Cas systems consist of clustered regularly interspaced short palindromic repeats (CRISPR) loci and CRISPR-associated (Cas) proteins. These adaptive immune systems protect their prokaryotic host against invading genetic elements.[1–3] Over the past decade, several CRISPR-Cas nucleases have been repurposed as genetic engineering tools for a broad range of organisms, from bacteria to humans.[3–7] The type II-A Cas9 from *Streptococcus pyogenes* (SpyCas9) was the first Cas nuclease to be characterized in detail,[8] and due to its consistently high genome editing activity, it is most widely used.

Like other Cas9 variants, SpyCas9 requires two RNA molecules, a CRISPR RNA (crRNA) guide and a trans-activating CRISPR RNA (tracrRNA) anchor. For application purposes, a chimeric single guide RNA (sgRNA) molecule that carries an exchangeable sequence (called spacer) is used. The spacer is complementary to the desired DNA target site (called protospacer). Because target specificity relies on the spacer part of the sgRNA, it can easily be reprogrammed to introduce a double-stranded DNA break (DSDB) in any DNA sequence of interest. The only requirement is that the target sequence should be flanked downstream by a protospacer adjacent motif (called PAM), in case of SpyCas9, a 5′-NGG-3′ (or less preferably 5′-NAG-3′).[8–10]

In eukaryotes, the Cas9-mediated DSDBs are predominantly repaired by non-homologous end joining (NHEJ), which generates random insertions and/or deletions (indels) that often result in frameshifts and thus gene inactivation.[8,11,12] Alternatively, precise genetic modifications can be achieved by repairing the DSDB using homology-directed repair (HDR).[13] However, HDR requires a repair template, is restricted to dividing cells (which is an issue for most therapeutically relevant cell types), and presents a strong DSDB-induced NHEJ background.[14,15] To introduce specific genomic alterations with minimum levels of undesired indels and without the need for DSDBs or donor DNA templates, the base editing approach was recently developed. DNA base editors are composed of a partially inactive Cas9 (nickase; nCas9) fused either to a cytidine deaminase (cytidine

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base editors [CBEs]) or to an evolved adenosine deaminase (adenosine base editors [ABEs]).

The initial deamination of C-to-U (CBE) and of A-to-I (ABE) results in precise conversion of C•G to T•A and of A•T to G•C, respectively, through mismatch repair followed by DNA replication or repair. However, if base excision takes place (e.g., by uracil DNA glycosylase) after the initial deamination, it may lead to reversion of deamination via base excision repair, or to indels via AP site cleavage and mismatch repair.[16–18] One of the most efficient base editors to date (known as BE4) is a nSpyCas9-CBE that additionally bears two copies of the uracil DNA glycosylase inhibitor (*ugi*) gene to efficiently block excision of the uracil base and thus increase the base conversion rate.[19] Despite their high efficiencies, the majority of the current genome and base editing tools have been developed based on the SpyCas9 ortholog, providing a range of targetable sites limited to the 5′-NGG-3′ PAM.[8] This is especially problematic in base editing applications, where the PAM needs to be in a certain position adjacent to the targeted base.[20]

Thousands of different Cas9 orthologs have been discovered in the past 10 years. Although only few have been characterized and applied for genome or base editing purposes to date, they present a tremendous untapped potential for alternative genetic engineering capabilities beyond those provided by SpyCas9.[19,21–42] First, these orthologs may provide distinct PAM specificities, expanding the targeting scope and the genome coverage. Second, most of them may show higher fidelity compared with SpyCas9, for example, by recognizing longer PAM and protospacer sequences. Third, certain synthetic Cas9-Cas9 pairs have orthogonal guides, facilitating multiplexed applications.[43–45] Fourth, temporal, spatial, or conditional control of most of these variants can be achieved using natural anti-CRISPR inhibitors.[46] Finally, the type II-C Cas9 orthologs are typically smaller than SpyCas9, allowing for delivery through virus- or mRNA-based vectors, and some of them are resistant to harsh environmental conditions, such as higher stability in human plasma.[42] Hence, there is a plethora of Cas9 orthologs with potentially favorable properties and unexplored genetic engineering capabilities that could serve as alternatives for SpyCas9 guide and protein optimization procedures.

In this study, we report the development of a compact, type II-C Cas9 ortholog from *Geobacillus thermodenitrificans* T12 (ThermoCas9) as a genetic engineering platform for human cells. ThermoCas9 is ~25% smaller (1082 aa) than SpyCas9 (1368 aa), and it recognizes a longer (23 nt) protospacer sequence as well as distinct PAMs (5′-N_4CNAA-3′ and 5′-N_4CMCA-3′).[47] We demonstrate that ThermoCas9 is able to induce NHEJ-based editing of human genomes at three different target genes (*dnmt1*, *emx1*, and *vegfa*), with efficiencies similar to SpyCas9. In addition, we create a ThermoCas9-based cytidine base editor (ThermoBE4) for human cells that exhibits a broader window of activity than the SpyCas9 base editor (BE4). Overall, we propose ThermoCas9 as an alternative to SpyCas9 to expand the targeting scope and the repertoire of genome and base editing tools in eukaryotes.

Materials and Methods

Bacterial strains and growth conditions

Escherichia coli DH5α was used in this study for cloning purposes. *E. coli* DH5α cells were cultured in Luria–Bertani (LB) broth or on LB agar plates, supplemented with ampicillin (100 μg/mL), at 37°C for 17 h. Liquid cultures were grown in a shaker incubator at 220 rpm.

HEK293T culturing and growth conditions

HEK293T (ATCC® CRL-3216™) cells were cultured at 37°C and 5% CO_2 in T75 bio-coated flasks containing 10 mL Dulbecco's modified Eagle medium (DMEM) supplemented with D-glucose (1 g/L), L-glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 25 mM), pyruvate, 10% (v/v) fetal bovine serum (FBS), 2% penicillin (10,000 U/mL), and streptomycin (10,000 μg/mL). Cells were passaged at 70–90% confluency every 2–4 days, by washing with 5 mL Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) and adding 1 mL 0.05% Trypsin-EDTA, followed by incubation at 37°C for 5 min. The detached cells were suspended in DMEM growth medium for counting and further processing.

Construction of plasmids

Plasmids, primers or oligonucleotides, genes, and protein sequences used in this study are presented in Supplementary Tables S1, S3, S4, and S5, respectively. For the generation of genome editing plasmids (phTCas9_spT1-T9; phSCas9_spS1-S9), nontargeting plasmids (phTCas9_nt; phSCas9_nt) were first constructed using the NEBuilder HiFi DNA Assembly Cloning Kit (NEB). These plasmids contain a nontargeting spacer that can be excised and replaced using the type IIS restriction enzyme *Sap*I (New England Biolabs). The fragments for assembling the plasmids were obtained through PCR with Q5® High-Fidelity 2X Master Mix (New England Biolabs). The PCR products were run on a 0.8% agarose gel and were subsequently purified using Zymogen Gel DNA Recovery Kit (Zymo Research).

The assembled plasmids were transformed to chemically competent *E. coli* DH5α cells,[48] plated on LB

agar-containing ampicillin (100 μg/mL), and incubated overnight at 37°C. The next day, single colonies were inoculated in LB medium, grown overnight at 37°C (220 rpm), and the plasmids were isolated using the GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific) or the GenElute™ Endotoxin-free Plasmid Midiprep Kit (Sigma–Aldrich). All the constructs were verified using Sanger sequencing (Macrogen). A complementary pair of oligonucleotides was designed for each targeting spacer (spT1-T9; spS1-S9) to replace the nontargeting spacer. For annealing of the complementary oligos, 4 μL oligonucleotide pairs (100 μM each) were mixed in Milli-Q water to a final volume of 100 μL, heated at 95°C for 5 min, and slowly cooled to room temperature. The annealed oligonucleotides were ligated into the *Sap*I-digested nontargeting plasmids following the T4 ligation protocol (NEB), and the assembled plasmids were isolated and verified using Sanger sequencing (Macrogen).

For the generation of base editing plasmids (phThermoBE4_spT1-T9; phBE4_spS1-S9), the human codon-optimized *thermocas9-linker-sv40nls* (Twist Bioscience) and *spycas9-linker-sv40nls* genes in the genome editing plasmids were replaced by the human codon-optimized fusion genes *apobec1-linker-hnthermocas9 (D8A)-linker-ugi-linker-ugi-linker-sv40nls* (Twist Bioscience) and *apobec1-linker-hnspycas9(D10A)-linker-ugi-linker-ugi-linker-sv40nls*, respectively, using the NEBuilder HiFi DNA Assembly Cloning Kit (NEB), and the assembled plasmids were isolated and confirmed using Sanger sequencing (Macrogen).[19] Description of the assembled fragments used for the construction of all plasmids is detailed in Supplementary Table S1.

Genome and base editing

HEK293T cells were seeded on physically surface-treated 24-well plates (Greiner Bio-one) at a seeding density of $0.5–1.0 \times 10^5$ cells per well. After 24 h of incubation, 0.5–1.5 μg of genome or base editing/control plasmid was transfected into the HEK293T cells using Lipofectamine™ 3000 Transfection reagent (Thermo Fisher Scientific), according to the user guide instructions. The transfected cells were cultured either 48 h for flow cytometry-assisted cell sorting (FACS) or 72 h for screening of the population for genome or base editing events.

FACS and generation of single cell-derived knockout clones

After 48 h of incubation, the transfected cells were harvested (with Ca^{2+}/Mg^{2+}-free PBS, 0.05% Trypsin-EDTA, and transfection medium), centrifuged at 250 *g* for 5 min, resuspended in 250 μL cell-sorting buffer (1% FBS in Ca/Mg^{2+}-free PBS), and filtered through Bel-Art Flowmi 40 Micron Cell Strainers (Sigma). Ninety-six-well plates for cell sorting were coated with FBS for 1–2 h. Fluorescent single cells were sorted into the coated 96-well plates containing 50% preconditioned culture medium using the Sony SH800 cell sorter device (Sony) (488 nm laser, FITC detection channel for GFP fluorescence), centrifuged at 100 *g* for 2 min, and cultured for ~3 weeks (37°C; 5% CO_2) by adding fresh medium at least once per week to prevent evaporation. When confluency was reached, the clonally propagated cells were steadily passaged to T25 flasks and screened for indels.

Screening for genome and base editing

HEK293T genomic DNA was isolated either from the population or from the clonal propagated cells, by removing the culture medium, washing with PBS (200 μL per sample), mixing with Lucigen QuickExtract™ DNA Extraction Solution (Lucigen) (100 μL per sample), transferring the cell lysate to PCR tubes, and incubating the lysate in three consecutive steps (65°C for 15 min, 68°C for 15 min, and 98°C for 10 min). Genomic target regions (*dnmt1*, *emx1*, *vegfa*) were PCR amplified with Q5® High-Fidelity 2X Master Mix (New England Biolabs). The PCR products were verified on a 1% DNA agarose gel, and they were subsequently purified with the DNA Clean and Concentrator Kit (Zymo Research). To detect indel formation or cytidine deamination, the purified PCR products were subjected to Sanger sequencing (Macrogen). The sequencing results of the genome and base editing assays were analyzed using the Inference of CRISPR Edits tool (ICE, Synthego) and the free web tool EditR, respectively.[49]

Results

ThermoCas9 induces efficient editing of human genomes

We started by investigating the *in vivo* DNA cleavage activity of ThermoCas9 in human cells. As such, we constructed targeting ThermoCas9 plasmids (called phTCas9) encoding the human codon-optimized *thermocas9-sv40nls* gene and its sgRNA module under the control of the constitutive cytomegalovirus (P_{CMV}) and U6 RNA polymerase III (P_{U6}) promoters, respectively (Fig. 1a; Supplementary Table S1). From the same plasmids, we co-expressed the *egfp* reporter gene under the constitutive elongation factor-1 alpha promoter ($P_{EF\text{-}1\alpha}$) to allow for sorting of the successfully transfected cells. We designed nine spacers that target protospacers in the chromosomal genes *dnmt1*, *emx1*, and *vegfa* (three spacers per gene) (Supplementary Table S2; Supplementary Fig. S1). All protospacers were flanked by a PAM that allows optimal cleavage activity of ThermoCas9 *in vitro* (5′-N_4CCAA-3′).[47]

After transfection of the targeting plasmids in HEK293T cells (up to 70% efficiency), the transfected cells were sorted into single cells and populations of up to 1000 cells for higher heterogeneity, via FACS based on EGFP fluorescence signal (Supplementary Figs. S2 and S3). The cells were cultured and screened for indels at the targeted sites through gene-specific PCR, and subsequent Sanger sequencing.

Successfully transfected populations had average indel frequencies of 87% for the strongest spacer T9 as well as 28% and 18% for the medium strength spacers T3 and T8, respectively (Fig. 1b–d; Supplementary Data). Moreover, indels were found in 25 of 47 HEK293T clonal cell lines, presenting average indel frequencies of up to 99% for spacer T9, 32% for spacer T3, 94% for spacer T8, and 24% for spacer T5 (Fig. 1b–d; Supplementary Data). The location of the indels suggests that ThermoCas9 created blunt-ended DSDB between the third and the fourth nucleotides immediately upstream of the PAM (Supplementary Data), in agreement with our previous *in vitro* study.[47] Targeting of the human genes predominantly generated nucleotide deletions instead of insertions (Fig. 1e, f; Supplementary Data). Overall, these observations suggest that ThermoCas9 is able to introduce DSDBs in human genomes at 37°C and thus to induce indel formation to disrupt targeted genes for the generation of knockout cell lines.

Comparison of ThermoCas9 and SpyCas9 *in vivo* cleavage activity in human cells

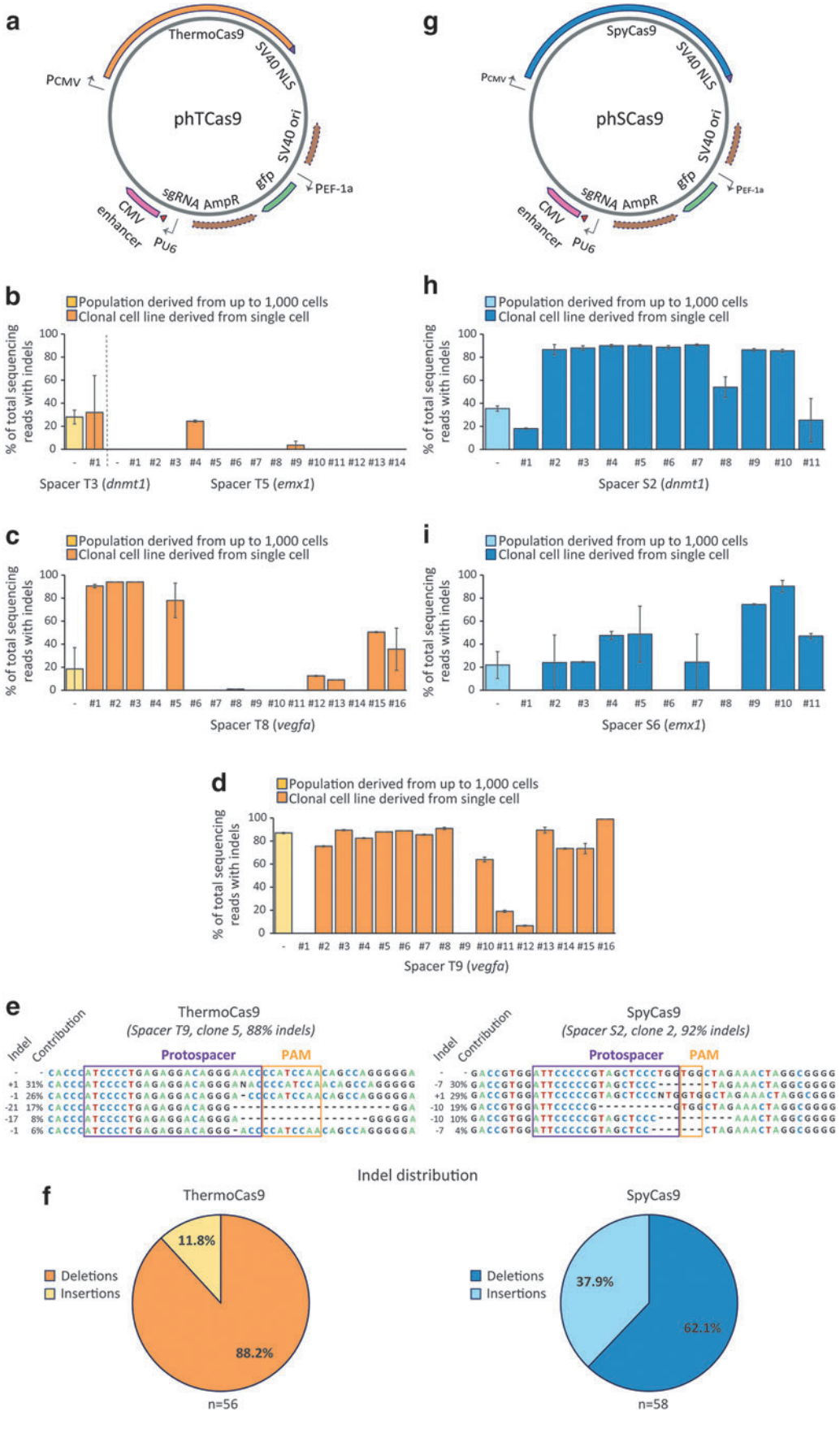
Next, we evaluated the *in vivo* DNA cleavage activity of SpyCas9 using the same setup for a direct comparison with ThermoCas9. For this purpose, we substituted the *thermocas9-sv40nls* gene and its sgRNA module in the phTCas9 plasmids with the human codon-optimized *spycas9-sv40nls* gene and its sgRNA, generating the phSCas9 plasmids (Fig. 1g; Supplementary Table S1). Similar to the ThermoCas9 approach, we selected three protospacers, each flanked by an optimal PAM (5′-NGG-3′) in the chromosomal genes *dnmt1*, *emx1*, and *vegfa* and individually incorporated the corresponding spacers into the phSCas9 plasmids (Supplementary Table S2; Supplementary Fig. S1).[8]

Following the same procedure, in the successfully transfected populations, we observed average indel frequencies of 35% for the stronger spacer S2 and 22% for the weaker spacer S6 (Fig. 1h, i; Supplementary Data; Supplementary Figs. S2 and S3). In addition, SpyCas9-mediated cleavage of the human chromosomes led to the generation of indels in 19 of 22 HEK293T clonal cell lines, presenting average indel frequencies of up to 90% for both spacers S2 and S6 (Fig. 1h, i; Supplementary Data). Nucleotide deletions instead of insertions were mainly observed, as indicated for ThermoCas9 (Fig. 1e, f). Altogether, cleavage activities in HEK293T cells by ThermoCas9 are comparable to SpyCas9.

ThermoBE4 is an efficient base editing tool for human cells

We then set out to develop a ThermoCas9-based base editor for human cells. In the previously described phTCas9 plasmids (Fig. 1a; Supplementary Table S1), we replaced the *thermocas9-sv40nls* gene with its D8A nickase

FIG. 1. Genome editing in human cells by ThermoCas9 or SpyCas9. **(a)** Schematic illustration of the phTCas9 constructs for ThermoCas9-mediated cleavage of three genomic genes (*dnmt1*, *emx1*, and *vegfa*) in HEK293T cells. **(b–d)** Indel frequencies in the targeted genomic genes of HEK293T cell populations (each derived from a sorted population of up to 1000 cells) and clonal cell lines (each derived from a sorted single cell), after successful transfection with the phTCas9 plasmids. The indel frequencies were obtained using the Inference of CRISPR Edits tool (ICE, Synthego) for multiple Sanger sequencing reactions of each target site, and error bars indicate the standard error of the mean. **(e)** Types of insertions or deletions (referred to as "indel") and their relative proportions (referred to as "contribution") in the total number of cells screened via Sanger sequencing. Purple and orange boxes indicate the protospacer sequence and the PAM, respectively, whereas black dotted lines represent the Cas9 cleavage site. **(f)** Indel distribution in the total number of cells edited by ThermoCas9 (left) and SpyCas9 (right). All Sanger sequencing reactions ($n = 56$ for ThermoCas9; $n = 58$ for SpyCas9) were taken into consideration. **(g)** Schematic illustration of the phSCas9 constructs for SpyCas9-mediated cleavage of three genomic genes (*dnmt1*, *emx1*, and *vegfa*) in HEK293T cells. **(h, i)** Indel frequencies in the targeted genomic genes of HEK293T cell populations (each derived from a sorted population of up to 1000 cells) and clonal cell lines (each derived from a sorted single cell), after successful transfection with the phSCas9 plasmids. The indel frequencies were obtained using the ICE tool (Synthego) for multiple Sanger sequencing reactions of each target site, and error bars indicate the standard error of the mean. CRISPR, clustered regularly interspaced short palindromic repeats; PAM, protospacer adjacent motif.



(*nthermocas9*) gene. In addition, we fused the rat Apolipoprotein B MRNA Editing Enzyme Catalytic Subunit 1 (*rapobec1*) gene and two copies of the bacteriophage PBS2 uracil DNA glycosylase inhibitor (*ugi*) gene, followed by the SV40 NLS signal to the N- and C-terminus of the *nthermocas9* gene, respectively.[50,51] We transfected the constructed phThermoBE4 plasmids (Fig. 2a; Supplementary Table S1) to HEK293T cells and screened for cytidine deamination at the targeted sites, via gene-specific PCR followed by Sanger sequencing. We detected C•G to T•A conversions at four of the nine protospacers we targeted with the ThermoBE4 system (Figs. 2b–e, 3a; Supplementary Fig. S5a–d). These protospacers (T3, T5, T8, and T9) are the same that were successfully used to induce indel formation (Fig. 1b–d).

In all cases, the ThermoBE4 base editor was able to edit Cs at multiple (up to seven) positions simultaneously, reaching editing efficiencies of up to 72% for Cs at the PAM-distal end of the protospacer (Figs. 2b–e and 3a; Supplementary Fig. S5a–d). Editing was observed not only within but also immediately upstream of the protospacer regions, presenting an editing window of up to 22 bp (from −5 to −26 positions relative to the PAM) (Figs. 2b–e and 3b; Supplementary Fig. S5a–d). Hence, our results demonstrate that the ThermoCas9 base editor mediates efficient base editing at multiple positions and in a relatively large activity window, providing an alternative approach for targeted gene mutagenesis in eukaryotes.

Comparison of ThermoBE4 and BE4 base editing characteristics in human cells

Finally, we compared the ThermoCas9 base editor with the previously reported SpyCas9 BE4 system.[19] For this reason, we substituted the *spycas9* gene from the phSCas9 plasmids (Fig. 1a; Supplementary Table S1) with the synthetic *rapobec1-nspycas9-ugi-ugi-sv40nls* coding sequence, generating the phBE4 plasmids (Fig. 2f; Supplementary Table S1). After transfection of the base editing plasmids in HEK293T cells, our screening results showed that five of nine protospacers were successfully base edited at multiple (up to six) positions simultaneously (Figs. 2g–k and 3a; Supplementary Fig. S5e–i).

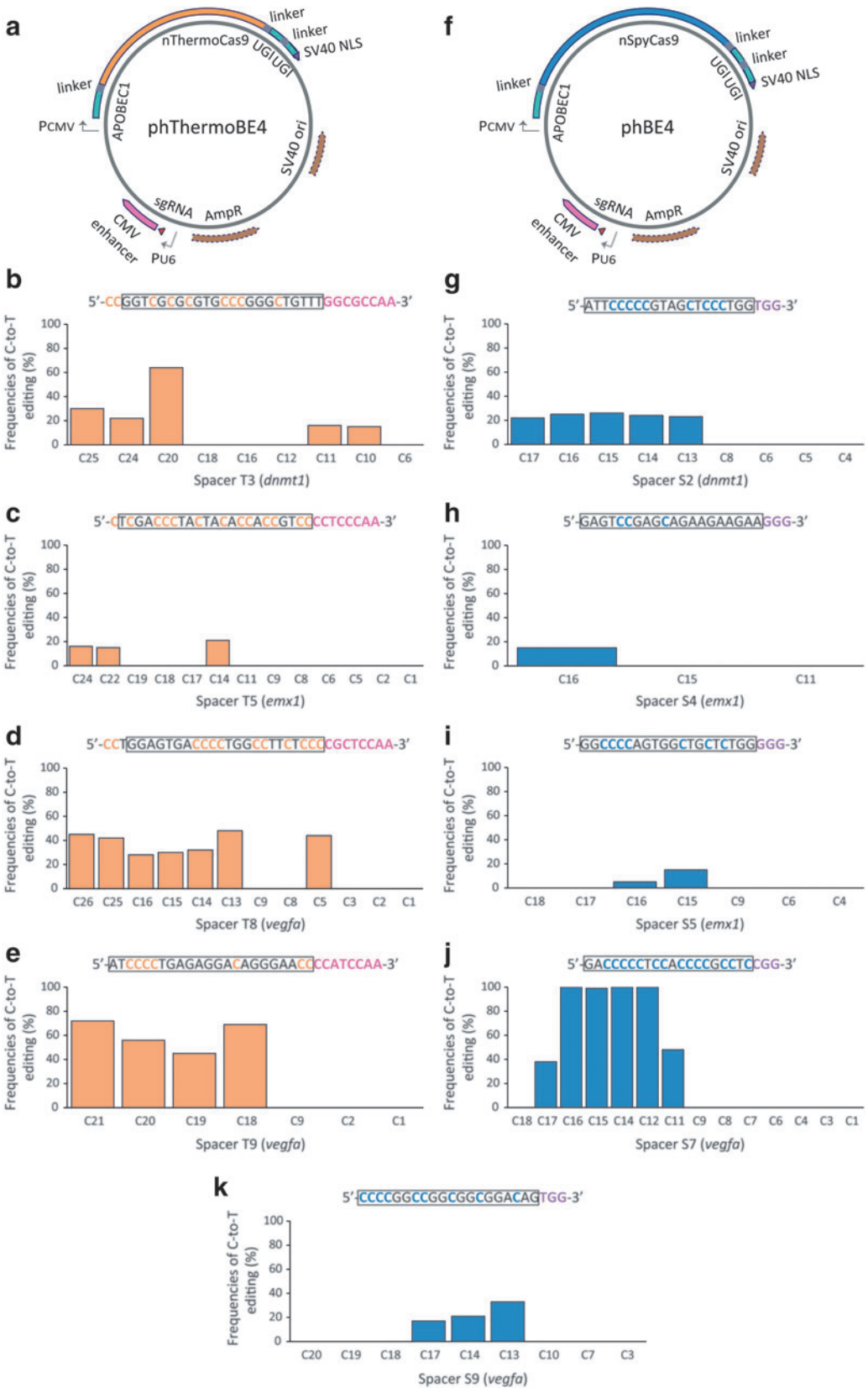
Similar to ThermoBE4 and in agreement with a previous study,[19] nSpyCas9-based BE4 preferentially edited C bases at the PAM-distal end of the protospacer (Fig. 2g–k). However, BE4 exhibited higher (absolute) editing efficiencies and narrower window of activity (7 bp; from −11 to −17 positions relative to the PAM) than ThermoBE4 (Figs. 2g–k and 3b; Supplementary Fig. S5e–i). Remarkably, no editing was observed outside of the protospacer regions. On the whole, our results suggest that BE4 may be a better choice for site-specific mutagenesis, whereas ThermoBE4 could be considered for applications that require a wide activity window, such as gene mutagenesis.

Discussion

In our previous studies, we demonstrated that a type II-C Cas9 endonuclease from *G. thermodenitrificans* T12 is active in a wide temperature range (20–70°C) *in vitro* and that it can be applied for genetic engineering purposes in both mesophilic and thermophilic bacteria.[47] ThermoCas9 can be regulated using an anti-CRISPR off-switch control (manuscript under revision), is considerably smaller than the type II-A SpyCas9, and exhibits naturally higher fidelity (at 37°C) and stability,[47] rendering it ideal for viral delivery and clinical applications. In addition, ThermoCas9 can reach different targets than SpyCas9 and other CRISPR nucleases, due to the recognition of alternative PAMs.[47] However, its activity has never been tested before in eukaryotes.

In this study, we develop a ThermoCas9-based approach for NHEJ-mediated gene editing in HEK293T cells. Similar to SpyCas9, we achieve high indel frequencies both at population level and at clonal cell line level,

FIG. 2. Base editing in human cells by ThermoBE4 or BE4. **(a)** Schematic illustration of the phThermoBE4 constructs for nThermoCas9-mediated base editing of three genomic genes (*dnmt1*, *emx1*, and *vegfa*) in HEK293T cells. **(b–e)** C-to-T editing frequencies induced by the ThermoBE4 system on each cytosine at the targeted genomic genes of HEK293T cells, after transfection with the phThermoBE4 plasmids. The protospacer region is indicated with a gray box, whereas the target Cs and PAM are shown in yellow and pink, respectively. Editing frequencies were obtained using the EditR tool. **(f)** Schematic illustration of the phBE4 constructs for nSpyCas9-mediated base editing of three genomic genes (*dnmt1*, *emx1*, and *vegfa*) in HEK293T cells. **(g–k)** C-to-T editing frequencies induced by the BE4 system on each cytosine at the targeted genomic genes of HEK293T cells, after transfection with the phBE4 plasmids. The protospacer region is indicated with a gray box, whereas the target Cs and PAM are shown in blue and purple, respectively. Editing frequencies were obtained using the EditR tool.



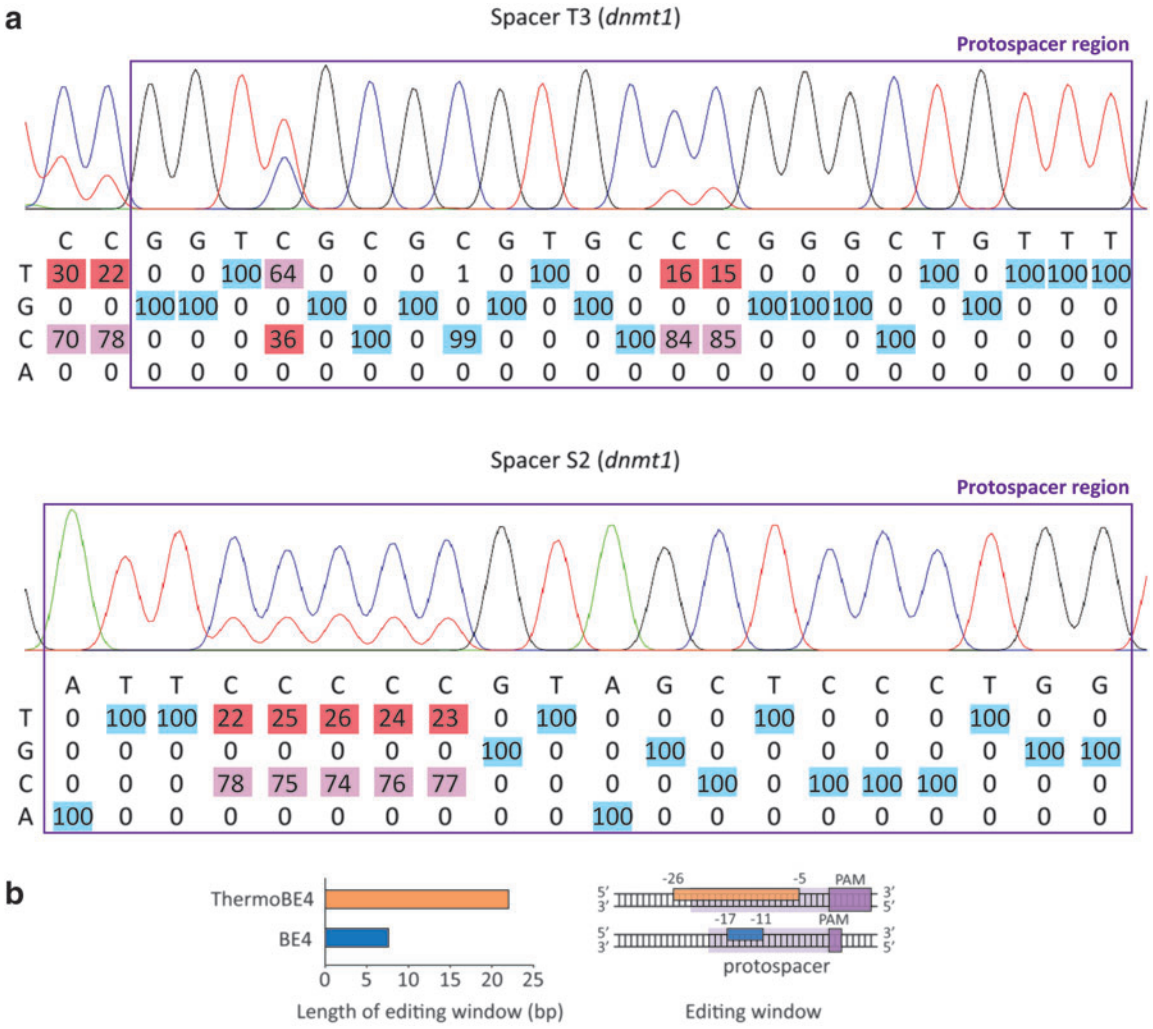


FIG. 3. Characterization of ThermoBE4- and BE4-mediated base editing in human cells. **(a)** Percentages of nucleotide conversions in the targeted protospacers, induced by ThermoBE4 and BE4. The percentages resulted from Sanger sequencing analysis of the tested populations, employing the online tool "EditR." **(b)** Comparison of the length (left) and spectrum (right) of the editing window for ThermoBE4 and BE4. The dark purple boxes indicate the PAM region, whereas the light purple areas depict the protospacer region. Orange and blue boxes represent the overall activity window of the base editors.

despite the lower DNA cleavage rate of ThermoCas9 at 37°C.[47] We speculate that, like its close type II-C Cas9 homolog from *Geobacillus stearothermophilus* (GeoCas9; Supplementary Fig. S4), ThermoCas9 may have a longer half-life in the cells, thus counterbalancing its lower cleavage activity at 37°C.[42] Cell populations generally presented a lower amount of detectable indels than clonally propagated cells, due to their higher heterogeneity and loss thereof during PCR and Sanger sequencing. Moreover, targeting efficiencies were (proto)spacer-specific, suggesting that epigenetic modifications, chromatin structures, DNA supercoiling, or guide/spacer secondary structures may affect the cleavage ability of ThermoCas9, as previously observed for SpyCas9 and other variants.[52] Indeed, negative supercoiling of the target DNA has been previously reported to improve both target accessibility to ThermoCas9,[47] and R-loop formation by a type II-C Cas9 ortholog from *Acidothermus cellulolyticus* (AceCas9).[53]

Furthermore, in agreement to our previous *in vitro* findings and studies on other Cas9 variants,[8,28,47,54,55] analysis of indels in HEK293T cells shows that the cleavage site of ThermoCas9 (and of SpyCas9) is between the third and the fourth nucleotide of the protospacer

upstream of the PAM. The vast majority of generated indels were deletions, as also shown for SpyCas9 and the type II-A Cas9 nuclease from *Staphylococcus aureus* (SauCas9).[31] To date, <10 Cas9 orthologs (mainly of types II-A and II-C) have exhibited the ability to edit eukaryotic genomes.[28,32–34,36–38,41,42] Hence, ThermoCas9 may be a valuable addition to this Cas9 catalog.

To expand the targeting scope also for base editing applications, we create an efficient ThermoCas9 base editor (ThermoBE4), which facilitates C•G to T•A conversions in genomic loci with alternative PAMs. In accordance with the corresponding SauCas9 base editor (SaBE4),[19] we enable editing in a larger window of activity compared with the SpyCas9 editor (BE4), with a preference for Cs located at the PAM-distal end of the protospacer. Intriguingly, ThermoBE4 provides a threefold larger activity window that extends upstream of the protospacer region and may facilitate gene mutagenesis applications in other eukaryotes, for example in plants. SauCas9 has a similar size as ThermoCas9, and they are both more compact than SpyCas9.[31,47] Therefore, we hypothesize that the smaller nuclease size leaves more nucleotide positions exposed for base editing. However, structural analysis is required to fully understand the biochemical mechanism of ThermoBE4.

Conclusions

In summary, we demonstrate that ThermoCas9 can be used as an alternative platform for efficient genome and base editing of human genomes. The compact size, high stability and accuracy, distinct PAM recognition, and anti-CRISPR off-switch control of ThermoCas9 render it a viable tool to complement the current CRISPR toolbox for a wide range of biotechnological and medical applications.

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Authors' Contributions

D.T.: Conceptualization (supporting), methodology (equal), validation (lead), formal analysis (equal), investigation (equal), data curation (lead), writing—original draft (lead), visualization (lead), funding acquisition (supporting). P.B.: Methodology (equal), validation (supporting), formal analysis (equal), investigation (equal), writing—review and editing (supporting). E.B.: Methodology (equal), validation (supporting), formal analysis (equal), investigation (equal). L.d.H.: Writing—review and editing (supporting), supervision (supporting). H.B.: Resources (supporting). R.H.J.S.: Writing—review and editing (supporting), supervision (supporting), funding acquisition (supporting). I.M.: Conceptualization (lead), writing—review and editing (supporting), supervision (lead), project administration (lead), funding acquisition (supporting). J.v.d.O.: Conceptualization (supporting), resources (lead), writing—review and editing (lead), supervision (supporting), project administration (supporting), funding acquisition (lead).

Author Disclosure Statement

J.v.d.O. is cofounder of NTrans Technologies, and member of the Scientific Advisory Board of NTrans Technologies and Hudson River Biotechnology. J.v.d.O. and R.H.J.S. are members of the Scientific Advisory Board of Scope Biosciences. I.M. is employed by the commercial company SNIPR Biome (Copenhagen, Denmark). D.T., J.v.d.O., R.H.J.S., and I.M. are inventors on CRISPR-Cas-related patents/patent applications.

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Supplementary Material

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Supplementary Figure S3
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Supplementary Figure S5
Supplementary Table S1
Supplementary Table S2
Supplementary Table S3
Supplementary Table S4
Supplementary Table S5
Supplementary Data

References

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