Methods xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Base editing the mammalian genome

Emma M. Schatoff[a,d,1], Maria Paz Zafra[a,b,1], Lukas E. Dow[a,b,c,*]

[a] Sandra and Edward Meyer Cancer Center, United States
[b] Department of Medicine, United States
[c] Department of Biochemistry, Weill Cornell Medicine, New York 10021, United States
[d] Weill Cornell/Rockefeller/Sloan Kettering Tri-I MD-PhD Program, New York 10065, United States

ARTICLE INFO

Keywords:
BE
Base editing
CRISPR
APOBEC

ABSTRACT

Base editing is a powerful technology that enables programmable conversion of single nucleotides in the mammalian genome. Base editors consist of a partially active Cas9 nuclease ($Cas9^{D10A}$) tethered to a natural or synthetic DNA modifying enzyme. Though only recently described, BE has already shown enormous potential for basic and translational research, allowing the creation or repair of disease alleles in a variety of cell types and model organisms. In the past 2 years, a vast array of new and modified base editor variants have been described, expanding the flexibility and usefulness of the approach. Though simple in concept, effective implementation of base editing requires an understanding of the advantages and limitations of each of these tools. Here, we provide an overview of the concepts of DNA base editing, and discuss the recent progress toward the development of optimized base editing systems for mammalian cells. In addition, we highlight key technical aspects of designing and executing BE experiments, and provide detailed experimental examples of successful base editing in cell lines and organoids to help guide the effective use of these tools for genome modification.

1. Introduction

1.1. Base editing basics

CRISPR/Cas9 technology has revolutionized basic, biomedical, and agricultural research by enabling simple targeting and manipulation of user-defined locations in the eukaryotic genome. In its simplest form, CRISPR is an RNA-guided DNA endonuclease that catalyzes DNA double-strand breaks (DSBs) that often lead to error-prone repair and permanent genetic scars. Broad application and innovation of CRISPR tools has now made it relatively straight-forward to knock out a gene of interest in any given cell type. Though targeting specific genome sites is relatively simple, the introduction of defined genetic alterations at these sites has remained challenging. In 2016, two groups simultaneously described the development of base editing (BE) enzymes that couple the genome-targeting features of Cas9, with direct DNA modifying activity of the APOBEC or AID cytidine deaminases (Fig. 1a) [1,2]. In this context, Cas9 engagement of the target locus and DNA strand separation position the deaminase enzymes to induce cytosine-to-thymine (C > T) transitions within a small ~4–5 nucleotide window at the 5′ end of the sgRNA target sequence (Fig. 1b). Further, the recent evolution of non-natural DNA editing enzymes has broadened the scope of base editing to include adenine-to-guanine (A > G) transitions. The chemistry underlying these specific reactions has been well-reviewed elsewhere [3].

1.2. Advantages

Unlike standard CRISPR knockout approaches, base editing allows the creation of both missense and nonsense mutations to knockout, truncate or mutate genes. For instance, the same C·T (or G·A) mutation can lead to the activation of an oncogene (e.g. $PIK3CA^{E545K}$) or a disruption of a tumor suppressor gene (e.g. $TP53^{R270C}$). Hence, compared to other CRISPR methods, BE is much more suited to the creation of disease alleles, that are overwhelmingly caused by single nucleotide variants (SNVs). Using existing enzymes, theoretically more than half of all cancer-associated mutations could be engineered using base editors [4], though the precise numbers will require extensive empirical testing.

Prior to the advent of BE, the most effective strategy for creating targeted SNVs was via homology-directed repair (HDR)-mediated editing. This approach coopts the endogenous DNA repair machinery to copy an exogenous template DNA into the genome. However, HDR is inefficient in most cell types because it is cell cycle-dependent (more

* Corresponding author at: Sandra and Edward Meyer Cancer Center, United States.
E-mail address: lud2005@med.cornell.edu (L.E. Dow).
[1] Equal authorship.

https://doi.org/10.1016/j.ymeth.2019.02.022
Received 30 November 2018; Accepted 27 February 2019

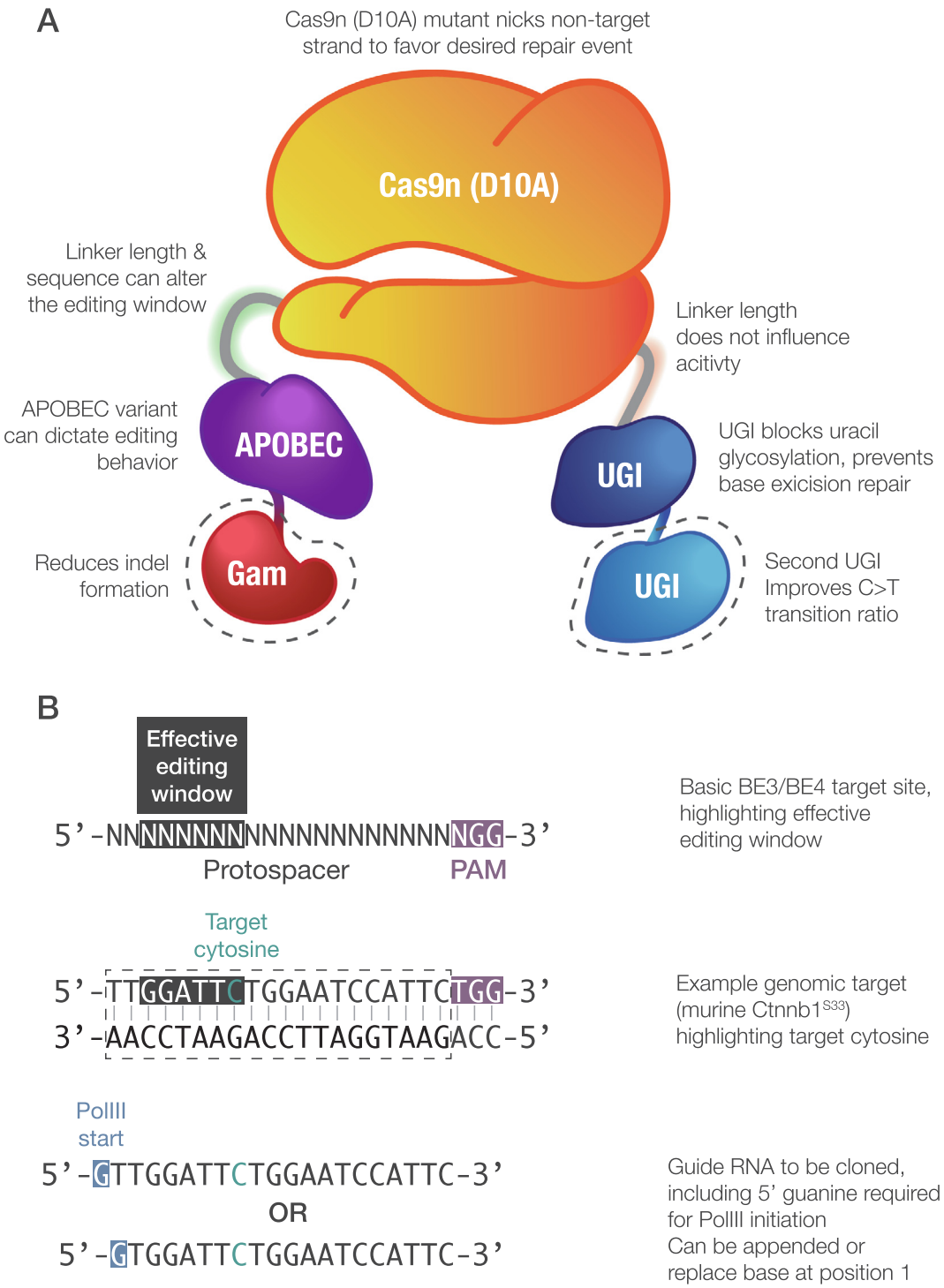


Fig. 1. A. The schematic depicts the core components of current cytidine deaminase base editors: Cas9n, APOBEC, and UGI; alongside potential modifications that have been introduced to improve editing such as additional Gam or UGI domains. The Cas9n (D10A), also known as "Cas9 nickase", associates with an sgRNA (shown in B) to bind and partially unwind a ~20 bp sequence of interest in the genome allowing the APOBEC component is the cytidine deaminase to induce a C > U change within the 3–8 base pair window of the target sequence; this modification is subsequently repaired to C > T. APOBEC is tethered to Cas9n via a linker sequence, and changing the length and composition of the linker can expand the range of the editing window. The UGI, or uracil glycosylase inhibitor, serves to block the enzyme uracil DNA glycosylase from reverting the U:G mismatch to a C:G. A second UGI can be added to the complex to increase the frequency of C > T edits (as opposed to other non-desired edits, e.g. C > A or C > G). Addition of a bacteriophage Mu Gam protein to the N-terminus reduces the frequency of insertions/deletions at the target site. B. The sgRNA target site is comprised of a 20 bp protospacer and PAM sequence, highlighted in purple. The PAM sequence requirement is dictated by the specific base editor being used. The effective editing range of BE3/BE4-based enzymes (base pairs 3–8), are highlighted in black, and the target cytosine (highlighted in green) should fall within this window. To facilitate PolIII initiation, a guanine should be at the 5′ end (position 1) of the guide sequence—it can either be added as an additional base (top) or replace the first base (bottom). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

active in G2/M) and requires efficient delivery of single or double-stranded donor DNA. In contrast, BE requires no template DNA for target mutagenesis and is effective in both dividing and non-dividing cells [5]. Further, since genome targeting by BE enzymes is driven by a single guide RNA (sgRNA), it is possible to engineer multiple mutations in parallel simply by delivering multiple sgRNAs. Multiplexed BE editing has been demonstrated in *ex vivo* cultured organoids and in fertilized zygotes for the production of complex genetic models [4,6,7]. The simultaneous introduction of multiple alterations using HDR is exceedingly difficult, and even in settings where it has been reported, the efficiency is too low to be practically useful [8,9].

In addition to the creation of mutant alleles, BE can also be used to correct disease alleles or augment normal gene function. These potential applications make BE not only a powerful tool for understanding the contribution of specific gene mutations in disease phenotypes, but also an exciting clinical technology. In fact, BE-based therapeutic promise has already spawned new biotechnology enterprises aimed at curing genetically driven diseases. While there is a long road ahead for the development of specific BE medicines, proof-of-concept gene correction has already been demonstrated in cell lines [10–12] and *in vivo* in genetically engineered mice [13–15].

1.3. Limitations

BE is a powerful tool, but it is not without limitations. First, using existing enzymes, it is currently possible to induce C·T (or G·A) and A·G (or T·C). These changes represent nucleotide transitions (i.e. purine-to-purine and pyrimidine-to-pyrimidine), but there are no systems yet described that directly catalyze nucleotide transversions. Overcoming this chemistry and protein engineering roadblock would significantly broaden BE technology and provide important flexibility in genome engineering.

Second, the genome targeting range of Cas9-based BE enzymes is limited by the presence of a protospacer adjacent motif (PAM) sequence. For *Streptococcus pyogenes* Cas9 (SpCas9), this is a triplet sequence with a guanine-dinucleotide (NGG). While this restriction is common to all CRISPR-based tools, it is particularly important for BE, as effective editing occurs only in a small window at the 5′ end of the protospacer (sgRNA target site) (Fig. 1b). Hence, moving the sgRNA target to the nearest PAM can shift the target nucleotide outside the editing window. We recently reported the generation of a BE variant (2X) that expands the targeting window from 3–8 bp to 3–12 bp [4]; however, this also increases the possibility of collateral or bystander editing of non-target cytosines. An alternate approach is to develop BE variants with altered PAM specificity, thus expanding the possible genome targeting range. In 2015, Kleinstiver et al. reported the generation of multiple PAM variant Cas9 enzymes that recognize NGA or NGCG PAMs [16], and these have been used to generate PAM variant BE enzymes [17]. More recently, the Liu and Nureki groups described xCas9 and Cas9-NG [18,19], that reportedly only require a single guanine for docking and sgRNA target recognition. While both variant enzymes do recognize PAM sites other than NGG, our own published [4] and unpublished data (Foronda et al.) indicate that BE activity is significantly compromised at these sites. Further iteration and optimization of these new enzymes will hopefully provide highly active and flexible BE tools.

Finally, like all genome engineering systems, BE carries the possibility of off-target effects. To date, it seems that most off-target activity is due to imperfect binding of the sgRNA/Cas9 complex to similar sequences throughout the genome. In many cases, this can be reduced or eliminated through careful sgRNA design or the use of high-fidelity enzymes (HF1 or HiFi) that weaken binding to imperfect matches [20–22]. What has not yet been explored in detail is whether the enforced expression of DNA modifying enzymes (i.e. APOBEC) has any unintended non-sequence dependent consequences. While such events can be effectively controlled in research applications by using neutral non-targeting sgRNAs, this possibility will need to be addressed during pre-clinical and clinical development.

1.4. Optimizing base editing

Since the initial description of BE enzymes in 2016, several studies have demonstrated its potential in cultured cells and the creation of germline heritable mutations in plants and a wide range of animal species. To date, BE has been used to modify rice, wheat, tomatoes, frogs, fish, mice, rabbits, monkeys, and human embryos [11,23–28], and likely many more by the time of publication of this article. In general, BE has worked well in settings where enzyme or DNA delivery is not a limiting factor, such as easily transfected cells or microinjected zygotes. We and others had struggled to implement BE in *ex vivo* and *in vivo* systems where transfection is difficult or in systems that require viral-based delivery. Through the optimization of codon usage and inclusion of nuclear targeting motifs at the N-terminus of BE enzymes, Zafra et al. and Koblan et al. reported significantly increased activity of multiple BE enzymes, driving editing efficiencies in cultured cells over 90% and enabling efficient editing in organoids and in mouse hepatocytes *in vivo* [4,29].

In addition to improving the potency of base editing, numerous other studies have focused on enhancing various aspects of BE tools (Table 1). For instance, through inclusion of additional protein domains, Komor et al. both improved the fidelity of cytosine conversion (BE4) and reduced non-desired insertions and deletions at the target locus (BE4Gam) [30]. As mentioned, Zafra et al. reported an enzyme that expands the editing window (2X) [4], while Kim et al. developed variants that narrow the editing window [17]. Along a similar theme, but using a distinct approach, Gehrke et al. exchanged and mutated the N-terminal APOBEC domain to derive enzymes that are highly selective toward TC dinucleotides. Further, as mentioned above, various PAM specificity and high-fidelity options have been produced to improve the flexibility and specificity of genome targeting.

In all, the generation and optimization of base editors has created unique and powerful tools for precision genome editing. However, the sheer number of options now available and the rate at which new versions are developed can be daunting for those unfamiliar with the field. Below, we discuss some key practical aspects of base editing and provide experimental examples to guide the effective use of BE technology for research applications.

2. Practical considerations

2.1. Guide RNA design & delivery

The guide RNA consists of two components: a crispr RNA (crRNA) and tracrRNA. The crRNA is a unique sequence that confers genomic targeting, and the tracrRNA is a scaffold RNA common to all guides. These components can be produced independently, but are most often combined as a single (or synthetic) guide RNA (sgRNA); Key features of the sgRNA are shown in Fig. 1b. sgRNA design is a critical step in achieving efficient base editing and there are several features that distinguish effective sgRNAs.

First, as mentioned above, efficient base editing occurs within a small 5–6 bp window as the 5′ end of the sgRNA (Fig. 2). Aim to position the target base within this window, ideally closer to positions 5–7 of the protospacer. If the target cytosine falls within positions 9–12 of the protospacer, the RA-2X editor can be used (Table 1). Second, appropriate positioning of the sgRNA depends on the presence of a PAM in the gDNA. As described, numerous Cas9 variants have been developed that recognize distinct PAM sequences, yet the most efficient is NGG. To date, there is no description of what sequence features dictate effective base editing, so if there are a multiple PAM options that position the target cytosine appropriately, we recommend testing each possibility empirically. The final feature of the sgRNA/target sequence that

Table 1
Overview of Current Cytidine Base Editors.

	Editor	Efficiency	Editing fidelity	Target specificity	PAM flexibility	Editing window	Refs
FIRST GENERATION Preferred first generation base editor	BE3					3-8	[1]
EDITING FIDELITY Second generation editors designed to improve C>T transitions and reduce indels	BE4					3-8	[30]
	BE4Gam					3-8	
EDITING WINDOW Base editor variants that alter the size or prefered sequence context of the base editing window	YEE					4-7	[17]
	2X					3-12	[4]
	A3A					TC	[12]
OPTIMIZED ACTIVITY Optimized base editors that improve the expression and genome targeting of the enzyme.	FNLS					3-8	[4]
	RA-2X					3-12	[4]
	BE4GamRA					3-8	[4]
	BE4max					3-8	[29]
PAM VARIANTS Adapted and optimized base editors that alter the specificity of the PAM sequence for Cas9.	FNLS-VQR					3-8	[*]
	xFNLS/xBE4max					3-8	[4,29]
	FNLS-NG					3-8	[*]
HIGH FIDELITY Adapted and optimized base editors that increase the fidelity of genome targeting and/or alter PAM specificity (i.e. HiFi-NG).	FNLS-HF1					3-8	[*]
	FNLS-HiFi					3-8	[*]
	FNLS-HiFi-NG					3-8	[*]

* Foronda et al, unpublished

impacts editing is the dinucleotide context. APOBEC enzymes have a preference to edit cytosines immediately downstream of thymine ('TC' dinucleotides). The BE3/BE4/FNLS editors show different efficiencies for each dinucleotide, whereby TC > CC ≅ AC > GC. Recently, APOBEC3A variants have been developed that are either less selective (W98Y variant) or more selective (N57G variant) toward TC sequences, and can be used if these features provide more editing flexibility [12].

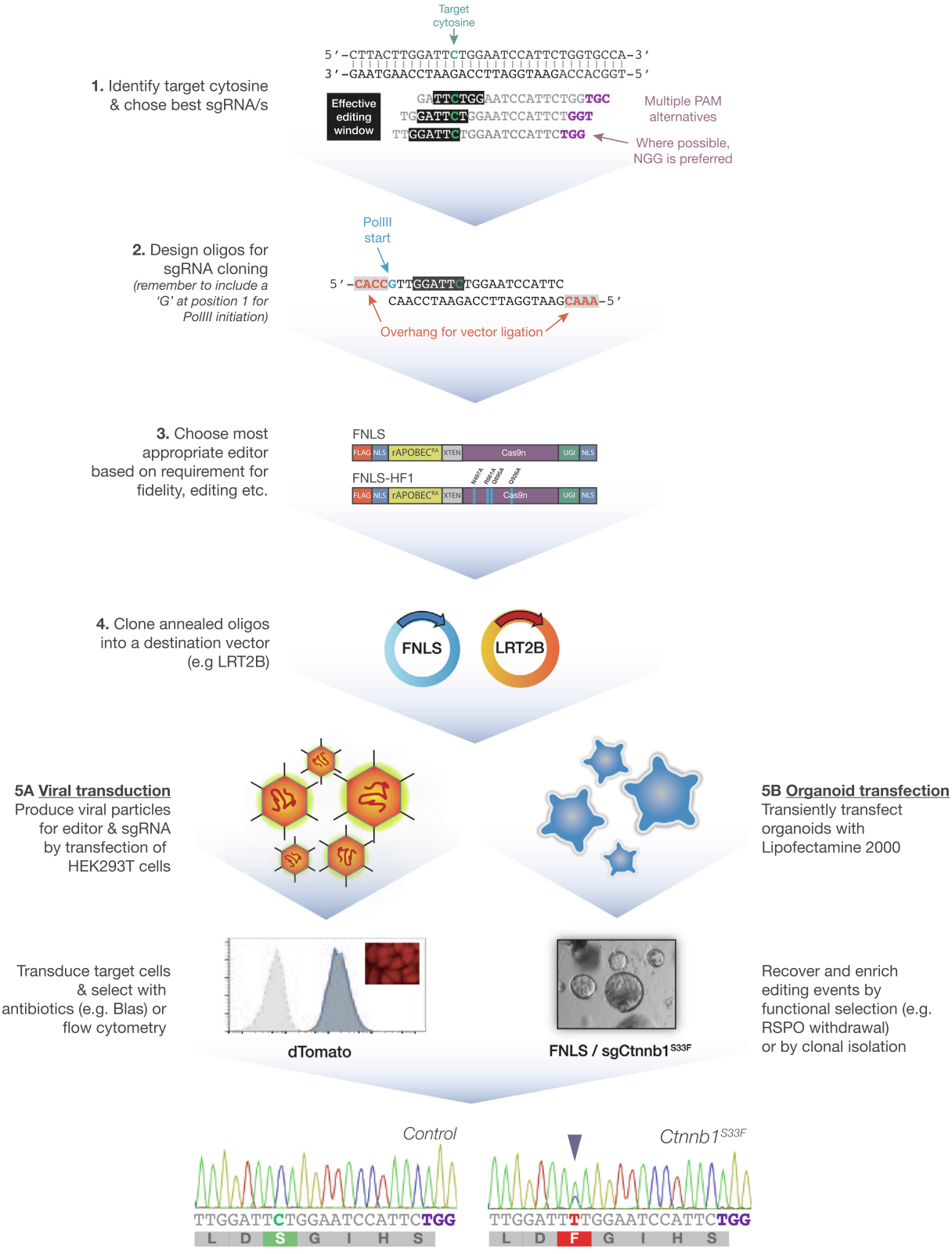
Guide RNAs can be delivered to target cells by numerous routes. *In vitro* transcribed or chemically synthesized sgRNAs can be transfected efficiently with minimal toxicity. For example, we have used chemically stabilized synthetic sgRNAs from Synthego to induce target editing in intestinal organoids, embryonic stems cells and microinjected mouse zygotes. For plasmid-based delivery, sgRNAs can be cloned into a variety of expression and viral constructs available at Addgene (www.addgene.org/crispr/); a detailed cloning protocol is available on the genome engineering website maintained by the Zhang lab (www.genome-engineering.org/crispr), and outlined below (see Section 3). We commonly use a lentiviral construct, LRT2B ([4]; Addgene, Plasmid#110854) that contains an expression-optimized sgRNA scaffold, tdTomato fluorescent reporter, and Blasticidin S antibiotic resistance gene. Of note, this (and other) lentiviral plasmids can also be used for expression of sgRNAs by transient transfection. If required, multiple sgRNAs can be delivered simultaneously via tandem sgRNA vectors. Having multiple sgRNAs in the same vector ensures that all transfected/transduced cells receive both guides and can theoretically enable multiplexed editing. To avoid recombination during lentiviral production, Vidigal et al. described a simple cloning strategy for heterologous U6 promoters [31].

2.2. Editor choice & delivery

A variety of editing enzymes are now available that each allow control of different factors. Namely, the 1) type, 2) positioning, and 3) fidelity of base conversion. While editing type is dictated by the SNV to be created (C > T or A > G), the choice of enzyme positioning and fidelity must be determined for each experimental situation. Table 1 provides an overview of the efficiency, specificity, and fidelity of various BE enzymes. Each enzyme carries unique features that make it most appropriate for specific editing contexts. In general, there is not one enzyme that has maximal efficiency, fidelity, and PAM flexibility. As such, the need for efficiency or fidelity must be considered separately for each experimental application. For instance, in most cases, we opt for the most efficient editor (FNLS), however, some human cell lines (e.g. DLD1s) show a higher than usual rate of insertions and deletions, which can be partially avoided through the use of the BE4GamRA variant [4]. Similarly, if a specific sgRNA shows are high number of predicted off-target sites, it would be more prudent to use a high-fidelity variant such as FNLS-HF1 or FNLS-HiFi (Foronda et al, unpublished). In summary, prioritize what is critical to your experiment to design your strategy.

Editing enzymes can be delivered to target cells by transfection of ribonucleoprotein complexes (RNPs), mRNA or naked plasmid DNA, or by viral transduction (Table 2) [1,4,20].Transfection introduces a high level of enzyme/sgRNA for a short period of time, which may help maintain on-target activity while limiting the off-target effects that accumulate over time [32]. Plasmid transfection is cost-effective and convenient, however RNP and mRNAs are usually a better choice as they are more transient, less toxic, and avoid the potential complication of genomic integration of plasmid DNA.

In contrast to transfection, lentiviral transduction provides stable



6. Isolate genomic DNA and confirm target editing by Sanger sequencing or NGS

(caption on next page)

and long-term expression of the editing enzyme, allowing selection/isolation of transduced cells. In cell types that are difficult to transfect or cannot be clonally derived, viral transduction may be the most effective (or only) option. In our experience, the expression level of the enzyme is a primary determining factor for editing efficiency, and thus choice of expression vector is critical. We strongly recommend using codon optimized editors and a selective marker (e.g. antibiotic resistance) translationally linked to the enzyme (i.e. Enzyme-P2A-

Fig. 2. The schematic outlines the workflow to achieve base editing by viral transduction or plasmid-based transfection. 1. To design the sgRNA, first search for a PAM sequence near the target cytosine; if possible select an 'NGG' PAM sequence. Select the 19 base pairs prior to the PAM (not including the PAM), this will be the sgRNA sequence. 2. Make the base pair at Position 1 a 'G' as this will allow PolIII initiation, and add 'CACC' to the 5′ end of the guide sequence ('CAAA' to the 5′ end of its complement) to create an overhang for subsequent ligation. 3. Next, select the most appropriate editor based on the desired editing (see Table 1). 4. Clone the sgRNA into a destination vector. 5A. For transduction, generate editor and guide virus by transfecting HEK293T cells, then use the editor virus to create stable or inducible editor cell lines before introducing the guide virus. Select for the guide positive editor cells by culturing in antibiotics or by flow cytometry. 5B. For transfection, such as in organoids, editor and guide are introduced simultaneously and expressed for ~3–4 days. Selection for edited cells can be achieved by altering the culture conditions (e.g. removing a growth factor or adding a drug), or by isolating clones. In the case of activating b-catenin mutations, edited organoids can be selected by removing the WNT co-agonist RSPO1 from the media. 6. After generating an edited population either by transduction or transfection, collect genomic DNA and amplify the target region by PCR. Analyze by Sanger or Next Generation Sequencing.

marker). Expressing the selection marker from an independent promoter will lead to the isolation of a sub-population of cells (5–20% depending on the cell line) that are marker-positive, but have silenced expression the upstream promoter controlling the editor.

It is important to note that sustained expression may increase the editing activity of relatively inefficient editors, but may also increase the incidence of off-target editing. Further, it is not known if there are long-term consequences of enforced APOBEC/AID/ABE expression. Thus, it is critical to include appropriate enzyme only and neutral sgRNA controls. To enable temporal control of editor expression in lentiviral transduced lines, we recently developed doxycycline-regulated vectors that show efficient editing with as little as 2 days of enzyme induction.

2.2.1. Technical note: bacterial subculture of base editing vectors

We have observed that lentiviral constructs containing optimized base editors (but not Cas9 only) grow very poorly in bacteria (STBL3 and Stellar). Following transformation, colonies will be very small, and require an additional 6–10 h at 37 °C to reach a reasonable size for picking. After clonal isolation on agar, liquid cultures should be grown at 30–32 °C to maximize yield. For unknown reasons, storage of liquid starter culture at 4 °C for extended periods frequently reduces plasmid yield in midi or maxipreps derived from this starter.

2.3. Editing analysis

The most direct method to assess the level of base editing is by directly sequencing the region of interest in targeted cells. Sanger sequencing provides a semi-quantitative readout of base conversion, which can be analyzed by software tools such as EditR [33]. In our experience, Sanger-based methods are a fast and cost-effective 'first look', but do not provide a true quantitative assessment of editing efficiency. The current gold-standard for quantifying BE frequency is targeted deep sequencing, most often on the Illumina MiSeq platform. For this, we design a PCR between 220 and 270 bp, containing the target base within 100 bp of either primer and perform paired-end 150 bp sequencing. Analysis of MiSeq data and SNV calling can be performed using commercial or custom software packages or via online web portals such as CRISPResso2 [34].

3. Experimental examples

To provide a detailed and practical example of the steps involved in base editing, the following sections describes a typical workflow in our laboratory for achieving editing in immortalized cell lines or primary mouse intestinal organoids (Fig. 2). As mentioned above, there are many factors that influence the choice of sgRNA and editor enzyme. These examples are intended to serve as a template that can be tailored for individual experiments, rather than a step-by-step protocol.

3.1. Lentiviral transduction of cell lines

3.1.1. Guide design and cloning

1. Fig. 2 shows multiple possible sgRNAs targeting the Serine 33 codon in mouse *Ctnnb1*, and identifies key features that influence sgRNA choice.
2. To clone the guides, resuspend the guide oligos at 100 μM in DNase-free water and anneal. Mix:
   - 9 μl oligo_A.
   - 9 μl oligo_B.
   - 2 μl T4 ligase buffer (NEB #) *Note: T4 ligase buffer should be aliquoted to avoid thaw/freeze cycles. Ensure any precipitate is re-suspended before use.*

   Run the annealing protocol in a thermal cycler:

   - 95 °C for 5 min.
   - Ramp down to 25 °C (5 °C/min).
   - Hold at 12 °C.

   Once annealed, dilute the oligos in PCR-grade water at 1:500 to get a 100 nM working solution.

3. Digest recipient vector (LRT2B) with BsmBI (3 h at 55 °C) or Esp3I (3 h at 37 °C) and gel extract and purify the linearized vector backbone. Do not treat with a phosphatase such as CIP. *Note: If using this vector, a filler sequence (~2 kb) will be excised from the empty vector. This helps confirm digestion of the vector and the identification of clones with inserts.*
4. Ligate annealed oligos and linearized vector

Table 2
Overview of Strategies for Editor and Guide Delivery.

		Advantages	Limitations
Transfection	Plasmid DNA	• Simple, safe, cost-effective • Minimal cloning • Transient delivery to minimize OTs	• Not feasible for difficult-to-transfect systems • Potential toxicity • Difficult to selected edited cells.
	mRNA/RNP	• No cloning required (can order reagents) • Minimal toxicity (i.e. zygote infections) • Transient delivery to minimize OTs.	• Difficult to selected edited cells • Not all systems compatible • More costly to purchase or IVT components
	Viral Transduction	• Relatively simple, cost-effective • Easy selection • Stable or inducible expression possible	• Sustained expression may cause problems • Not all cells easily transduced • Need to consider safety for human-tropic virus

- 4 µl of 100 nM annealed and diluted oligos.
- 50–100 ng (1 µl) of linearized vector.
- 1 µl of T4 ligase buffer.
- 0.5ul T4 DNA ligase.
- 3.5 µl DNase-free H_2O.

Incubate at room temperature for 1 h (or overnight at 16 °C) and transform 5 µl of the ligation into 50 µl of STBL3 competent bacteria. *Note: Always transform linearized LRT2B vector without annealed oligos as a negative control.*

5. Correctly ligated clones can be confirmed by Sanger sequencing using a primer at the end of the U6 promoter (U6F_Seq: GACTATC ATATGCTTACCGT).

3.1.2. Virus production

6. Seed HEK293T cells (5×10^5) in a 6 well-plate in DMEM + 10% FBS + penicillin/streptomycin.
7. Transfect 12–24 h later when cells are 90–95% confluent using the following DNA:PEI mixture:
   - 150 µl Opti-MEM or DMEM.
   - 2.5 µg lentiviral backbone.
   - 1.25 µg PAX2 (Addgene plasmid #12260).
   - 1.25 µg VSV-G (Addgene plasmid #8454).
   - 15 µl of PEI (1 mg/ml).

Note: First add the DNA, vortex, then add the PEI. The packaging components will be the same (e.g. Pax2, VSV-G) for generating editor virus and guide virus as both of them are lentiviral constructs.

8. Change culture medium 12–18 hrs following transfection.
9. Replace culture medium with target cell medium 36 hrs following transfection and collect supernatant carrying the viral particles every 12 h for 3–4 cycles, each time replacing with fresh target cell media.
10. After the final collection, clear the supernatant of cell debris by passing through a 0.4 µM sterile filter, or centrifuging for 10 mins at 800*g* and discarding the pellet. Viral supernatants can be stored up for 1–2 weeks at 4 °C without significant loss of titer. Alternatively, if longer storage is needed, we recommend concentrating and freezing in single use aliquots at −80 °C.

3.1.3. Viral transduction

After generating virus, it can be used to transduce Murine Embryonic Fibroblasts (MEFs), cancer cell lines, embryonic stem cells, etc. Depending on the viral titer, the volume of virus used for transduction will vary. A ratio of 1:2–1:20 virus to media is a good starting point. For NIH3T3 transduction.

11. Plate 7.5×10^4 cells/well on a 6-well plate.
12. 24 h following plating, add viral supernatant (1:2–1:20, depending on titer) in the presence of polybrene (8 µg/µl).
13. Two days later, change media to selection media, (e.g. cell media + Puromycin (2 µg/ml)) for 4 days. *Note: To maintain puromycin resistant cells over passages keep them in Puromycin (1 µg/ml).*
14. Once stable or inducible editor cell lines have been established and selected, the cells can be re-transduced with sgRNA vector exactly as described above.

3.1.4. Editing analysis

15. Two days post-transduction, measure transduction efficiency by flow cytometry, calculating the percentage of tdTomato + cells.
16. Select cells in media containing Blasticidin S (4 µg/ml for NIH3T3 cells). *Note: Blasticidin S is not stable for long periods at 4 °C and should be used within 1–2 weeks. The minimal lethal dose for each cell line should be determined empirically.*
17. Collect cells at appropriate time point/s for evaluation of editing by Sanger sequencing/deep sequencing. Base editing can be detected 24–48 hrs after transduction, although it can be highly dependent on the sgRNA chosen. In our experience, base editing reaches saturation between days 4 and 6. If there is no detectable editing by day 6, it is unlikely to increase over time (see Section 4).

3.2. Organoid transfection

3D organoid culture is an increasingly popular platform for cell-based studies as they better mimic the biology of *in vivo* tissues, and in many cases, enable the continuous growth of genetically normal cells. Similar to cell lines, base editing can be induced in organoids by transduction or transfection. We frequently opt for transfection-based delivery, to prevent the long-term expression of editing enzymes that can induce immune responses following transplant of cells in immune-competent animals [35,36]. Outlined below is our standard workflow for base editing by transfection in organoids; Detailed protocols for organoid isolation and culture have been described previously [37].

1. Four days prior to transfection, plate organoids in ENR media [37] at 75% confluency in **n** number of 12 wells, where **n** = number of transfections.
2. Two days prior to transfection, change media to ENCY (EN containing GSK3 inhibitor (CHIR99021 – 5 µM) and ROCK inhibitor (Y-27632 – 10 µM)). *This cocktail hyperactivates WNT signaling to increase the proportion of stem and progenitor cells in the culture, and blocks anoikis-mediated cell death.*
3. For transfection, collect organoids in a 15 mL conical tube and mechanically break down the Matrigel/organoid mixture by pipetting vigorously.
4. Add 5 mL 1X PBS to dilute the Matrigel. Pellet at 250 g × 4 min at 4 °C.
5. Dissociate the organoids in the pellet using 60 µl × **n** transfections TrypLE™ Express Enzyme for 5 min at 37 °C.
6. While the cells are trypsinizing, prepare the DNA and Lipofectamine solutions.

To tube A, add:

- 50 µl of Opti-MEM.
- 500 ng editor plasmid.
- 500 ng guide plasmid.

To tube B, add:

- 50 µl Opti-MEM.
- 2 µl Lipofectamine™ 2000.

Vortex both tubes separately, then combine the Lipofectamine solution to the DNA solution. Incubate 5 min at RT.

7. Inactivate the TrypLE by adding 5 mL of F12 media, then pellet the organoids (250*g* × 4 min).
8. Resuspend the pellet in 300 µl × **n** transfections of ENCY media, and transfer to a well of a 48 well plate.
9. Add the 100 µl DNA-Lipofectamine solution to the 300 µl dissociated organoids in the 48 well plate.
10. Centrifuge the plate at 600*g* at 32 °C for 60 min, then incubate for 4–6 h at 37 °C.
11. Transfer the organoids to a 1.7 mL tube, (pipette to resuspend the organoids). Pellet at 800*g* × 3 min.
12. Resuspend the organoids in 100 µl of Matrigel.
13. Culture the organoids in ENCY for 2 days post-transfection.

14. If possible, select organoids by changing the media. For example: *Apc* mutations, withdraw exogenous Noggin and R-Spondin factors; Kras mutations, withdraw all growth factors and culture in F12; p53 mutations, add 10 μM Nutlin 3A; Pik3ca mutations, add 25 nM Trametinib.

Note: If the SNV/stop codon created confers a negative/neutral selection to the culture keep the organoids in complete media. For selection that requires removal of growth factors, ex. Apc or Kras, continue to culture in the absence of growth factors (additional growth factors will require more frequent passaging). For selection in drug, ex. p53 or Pik3ca, remove the drug after 2 passages in drug. Edited organoids should be visible as late as 1 week after transfection. Split organoids when they are confluent. By the second split post-transfection, any surviving organoids should be edited.

15. To pellet the organoids, wash the well once with 1X PBS, then add Cell Recovery Solution to the well (400 μl per 12 well) and use a P1000 pipette tip to scrape the Matrigel droplet(s) from the well into a 1.7 mL tube. Note: 50 μl of Matrigel confluent with organoids should yield sufficient gDNA.
16. Incubate on ice for 20 min, then pellet at 4 °C for 5 min at 300 g.
17. Isolate genomic DNA and analyze by PCR and sequencing.

4. Troubleshooting

4.1. No survival after transduction

- Titrate the amount of Puromycin or Blasticidin S to identify the minimal concentration that eliminates untransduced cells.
- Confirm that you have produced viral particles from the transfection. We recommend using a simple quantitative PCR-based kit (LV900; Applied Biological Materials Inc.). The concentration of viral particles can be calculated relative to standards included in the kit.

4.2. No virus

- Confirm that the transfection worked by including a fluorescent reporter as a positive control.
- Confirm the quality of the viral backbone and packaging components (e.g. Pax2, VSV-G) by restriction digest and gel electrophoresis. DNA quality will dramatically affect transfection efficiency.

4.3. No editing after sgRNA transduction

- Confirm lentiviral integration. If the vector harbors a fluorescent marker, check expression by microscopy or flow cytometry. If there is no marker, confirm the genomic integration of the lentiviral backbone. We recommend using a Taqman copy number qPCR assays (Invitrogen) to detect the Pac (puroR) gene (or other marker). Our custom-designed Pac primers and probe are: (forward-5′GCGG TGTTCGCCGAGAT; reverse-5′GAGGCCTTCCATCTGTTGCT; probe (FAM) CCGGGAACCGCTCAACTC).
- If there is lentiviral integration, confirm expression of the editor by western blot or immunostaining for Cas9.
- If there is detectable Cas9 protein, yet still no editing, confirm that the sgRNAs work in other cell lines or with Cas9 (see below). As guide design for base editing has not been well defined, it is possible many sgRNAs will not work, or your target gene may not be readily accessible in your cell line of interest.
- The simplest and fastest way to check if a human sgRNA works is by plasmid co-transfection in HEK293Ts; for an 'NGG' PAM, use Cas9, for another PAM, use the appropriate editor (Table 1). If using LRT2B for the sgRNA, confirm that tdTomato is detectable by microscopy. Similarly, Cas9 or base editors can be linked to GFP to confirm successful transfection. If there are any insertions/deletions (indels) for Cas9 or C > T changes (base editors) detectable by Sanger sequencing 2 days post-transfection, the guide works. If there is still no editing, select a different guide sequence.

4.4. No survival after transfection

- Confirm DNA quality and concentration by restriction digest and gel electrophoresis.
- Increase the number of organoids/cells per transfection.
- Transfect cells with purified mRNA or protein for the editor and in vitro transcribed or synthetic sgRNAs (to avoid DNA-related toxicity). For mRNA production of FNLS, we use the mMESSAGE mMACHINE T7 Transcription Kit (Invitrogen, #AM1344) with pCMV-FNLS (RA) (Addgene plasmid #112671). For synthetic sgRNAs, we use 5′ chemically stabilized sgRNAs from Synthego (Synthego Inc.).

5. Equipment and reagents

5.1. Equipment

- T100™ Thermal Cycler (Bio-Rad, #1861096).
- QuantStudio 6 Real-Time PCR system (Applied Biosystems, 4485694).

5.2. Cloning reagents

- T4 DNA Ligase (New England Biolabs, #M0202L).
- BsmBI (New England Biolabs, #R0580L).
- One-shot Stbl3 chemically competent bacteria (Thermo Fisher Scientific, # C737303).
- LB Agar plates with 100 μg/mL Carbenicillin (Teknova, #L1010).

5.3. Tissue culture reagents

- Phosphate-Buffered Saline (PBS) 1X (Corning cellgro, #21-040-CV).
- TrypLE™ Express Enzyme (1X), no phenol red (Thermo Fisher, 12604-013).
- Lipofectamine™ 2000 (Thermo Fisher Scientific, # 11668027).
- PEI (Polysciences, # 23966).
- Polybrene (Sigma-Aldrich, # 107689-10G).
- Puromycin (Fisher Scientific, AC227420500).
- Blasticidin S HCl (Thermo Fisher Scientific, # A1113903).
- Hygromycin B (Cellgro, # MT 30-240-CR).
- TRIzol™ Reagent (Invirogen, #15596026).
- Matrigel (Corning, #354253).
- Cell Recovery Solution (Corning, #CB-40253).
- CHIR99021 (Cayman Chemical, # 13122).
- Y-27632 2HCl (Selleck, # S1049).

5.4. Media

- DMEM (Corning cellgro, #10-013-CV).
- Fetal Bovine Serum (FBS) (VWR, #89510-186).
- Advanced DMEM/F12 (Thermo Fisher Scientific, #12634028).
- KnockOut™ DMEM (Thermo Fisher Scientific, 10829018) – 90 mL.
- Opti-MEM™ I Reduced Serum Medium (Life Technologies, 31985062).
- Penicillin/Streptomycin (Corning, #30-002-Cl).
- Glutamax (Thermo Fisher Scientific, #35050-061).
- Recombinant Murine Noggin (PeproTech, #250-38).
- Recombinant Murine EGF (PeproTech, #315-09).
- Recombinant Murine R-Spondin 1 (R&D Systems, # 3474-RS) or conditioned media.
- HEPES (Sigma-Aldrich, H3375-500G).

5.5. Other reagents

- Taqman Mastermix (Applied Biosystems, # 5407966-1).
- Cas9 antibody (BioLegend, #844301).

Acknowledgements

MPZ is supported in part by National Cancer Institute (NCI) Grant NIH T32 CA203702. EMS was supported by a Medical Scientist Training Program grant from the National Institute of General Medical Sciences of the National Institutes of Health under award number T32GM07739 to the Weill Cornell/Rockefeller/Sloan-Kettering Tri-Institutional MD-PhD Program, and an F31 Award from the NCI/NIH under grant number 1 F31 CA224800-01. LED was supported by a K22 Career Development Award from the NCI/NIH (CA 181280-01). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Competing financial interests statement

The authors declare no competing financial interests

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2019.02.022.

References

[1] A.C. Komor, et al., Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage, Nature 533 (7603) (2016) 420–424.
[2] K. Nishida, et al., Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems, Science 353 (6305) (2016).
[3] H.A. Rees, D.R. Liu, Base editing: precision chemistry on the genome and transcriptome of living cells, Nat. Rev. Genet. 19 (12) (2018) 770–788.
[4] M.P. Zafra, et al., Optimized base editors enable efficient editing in cells, organoids and mice, Nat. Biotechnol. 36 (9) (2018) 888–893.
[5] W.H. Yeh, et al., In vivo base editing of post-mitotic sensory cells, Nat. Commun. 9 (1) (2018) 2184.
[6] Z. Liu, et al., Efficient generation of mouse models of human diseases via ABE- and BE-mediated base editing, Nat. Commun. 9 (1) (2018) 2338.
[7] H. Zhang, et al., Simultaneous zygotic inactivation of multiple genes in mouse through CRISPR/Cas9-mediated base editing, Development 145 (20) (2018).
[8] H. Yang, et al., One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering, Cell 154 (6) (2013) 1370–1379.
[9] C. Gurumurthy et al., Re-evaluating One-step Generation of Mice Carrying Conditional Alleles by CRISPR-Cas9-Mediated Genome Editing Technology, 2018.
[10] N.M. Gaudelli, et al., Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage, Nature 551 (7681) (2017) 464–471.
[11] Y. Zeng, et al., Correction of the Marfan syndrome pathogenic fbn1 mutation by base editing in human cells and heterozygous embryos, Mol. Ther. 26 (11) (2018) 2631–2637.
[12] J.M. Gehrke, et al., An APOBEC3A-Cas9 base editor with minimized bystander and off-target activities, Nat. Biotechnol. 36 (10) (2018) 977–982.
[13] A.C. Chadwick, X. Wang, K. Musunuru, In vivo base editing of PCSK9 (proprotein convertase subtilisin/kexin type 9) as a therapeutic alternative to genome editing, Arterioscler. Thromb. Vasc. Biol. 37 (9) (2017) 1741–1747.
[14] L. Villiger, et al., Treatment of a metabolic liver disease by in vivo genome base editing in adult mice, Nat. Med. 24 (10) (2018) 1519–1525.
[15] S.M. Ryu, et al., Adenine base editing in mouse embryos and an adult mouse model of Duchenne muscular dystrophy, Nat. Biotechnol. 36 (6) (2018) 536–539.
[16] B.P. Kleinstiver, et al., Engineered CRISPR-Cas9 nucleases with altered PAM specificities, Nature 523 (7561) (2015) 481–485.
[17] Y.B. Kim, et al., Increasing the genome-targeting scope and precision of base editing with engineered Cas9-cytidine deaminase fusions, Nat. Biotechnol. 35 (4) (2017) 371–376.
[18] J.H. Hu, et al., Evolved Cas9 variants with broad PAM compatibility and high DNA specificity, Nature 556 (7699) (2018) 57–63.
[19] H. Nishimasu, et al., Engineered CRISPR-Cas9 nuclease with expanded targeting space, Science 361 (6408) (2018) 1259–1262.
[20] H.A. Rees, et al., Improving the DNA specificity and applicability of base editing through protein engineering and protein delivery, Nat. Commun. 8 (2017) 15790.
[21] C.A. Vakulskas, et al., A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells, Nat. Med. 24 (8) (2018) 1216–1224.
[22] B.P. Kleinstiver, et al., High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects, Nature 529 (7587) (2016) 490–495.
[23] Y. Zong, et al., Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion, Nat. Biotechnol. 35 (5) (2017) 438–440.
[24] Z. Shimatani, et al., Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion, Nat. Biotechnol. 35 (5) (2017) 441–443.
[25] D.S. Park, et al., Targeted base editing via RNA-guided cytidine deaminases in Xenopus laevis embryos, Mol. Cells 40 (11) (2017) 823–827.
[26] Y. Zhang, et al., Programmable base editing of zebrafish genome using a modified CRISPR-Cas9 system, Nat. Commun. 8 (1) (2017) 118.
[27] K. Kim, et al., Highly efficient RNA-guided base editing in mouse embryos, Nat. Biotechnol. 35 (5) (2017) 435–437.
[28] Z. Liu, et al., Highly efficient RNA-guided base editing in rabbit, Nat. Commun. 9 (1) (2018) 2717.
[29] L.W. Koblan, et al., Improving cytidine and adenine base editors by expression optimization and ancestral reconstruction, Nat. Biotechnol. (2018).
[30] A.C. Komor, et al., Improved base excision repair inhibition and bacteriophage Mu Gam protein yields C:G-to-T: a base editors with higher efficiency and product purity, Sci. Adv. 3 (8) (2017) eaao4774.
[31] J.A. Vidigal, A. Ventura, Rapid and efficient one-step generation of paired gRNA CRISPR/Cas9 libraries, Nat Comm (2015).
[32] J. Tycko, V.E. Myer, P.D. Hsu, Methods for optimizing CRISPR-Cas9 genome editing specificity, Mol. Cell 63 (3) (2016) 355–370.
[33] M. Kluesner et al., EditR: A Novel Base Editing Quantification Software Using Sanger Sequencing, 2017.
[34] K. Clement et al., Analysis and Comparison of Genome Editing using CRISPResso2, 2018.
[35] W. Xue, et al., CRISPR-mediated direct mutation of cancer genes in the mouse liver, Nature 514 (7522) (2014) 380–384.
[36] S. Annunziato, et al., Modeling invasive lobular breast carcinoma by CRISPR/Cas9-mediated somatic genome editing of the mammary gland, Genes Dev. 30 (12) (2016) 1470–1480.
[37] K.P. O'Rourke, et al., Isolation, culture, and maintenance of mouse intestinal stem cells, Bio Protoc. 6 (4) (2016).