Programmable C:G to G:C genome editing with CRISPR-Cas9-directed base excision repair proteins

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Many genetic diseases are caused by single-nucleotide polymorphisms. Base editors can correct these mutations at single-nucleotide resolution, but until recently, only allowed for transition edits, addressing four out of twelve possible DNA base substitutions. Here, we develop a class of C:G to G:C Base Editors to create single-base genomic transversions in human cells. Our C:G to G:C Base Editors consist of a nickase-Cas9 fused to a cytidine deaminase and base excision repair proteins. Characterization of >30 base editor candidates reveal that they predominantly perform C:G to G:C editing (up to 90% purity), with rAPOBEC-nCas9-rXRCC1 being the most efficient (mean 15.4% and up to 37% without selection). C:G to G:C Base Editors target cytidine in WCW, ACC or GCT sequence contexts and within a precise three-nucleotide window of the target protospacer. We further target genes linked to dyslipidemia, hypertrophic cardiomyopathy, and deafness, showing the therapeutic potential of these base editors in interrogating and correcting human genetic diseases.
Many human genetic diseases are caused by single-nucleotide polymorphisms (SNPs), in which the disease and healthy alleles differ by a single DNA base. Base editors can correct these SNPs by converting the targeted DNA bases into another base in a controllable and efficient fashion. Until recently, base editing technology is only efficient in converting of C:G base pairs to T:A base pairs using cytidine base editors (CBEs) and A:T base pairs using adenine base editors (ABEs)\textsuperscript{1-3}, which together represent half of all known disease-associated SNPs. CBEs and ABEs are also known to effect some C:G to G:C edits as byproducts\textsuperscript{4,5}. Indeed, two recent reports capitalized on this observation and demonstrated that fusion of APOBEC-nCas9 to uracil DNA glycosylase (UNG) – which induces abasic sites – results in C:G to G:C editing in mammalian cells\textsuperscript{6,7).

In contrast to UNG-mediated base excision initiation\textsuperscript{6,7}, we look to leverage on the cell’s innate base excision repair (BER) pathway, in which DNA polymerase β, DNA ligase III, and XRCC1 are major players\textsuperscript{8}. Previous work suggests that removal of uracil glycosylase inhibitor (UGI) from a CBE might lead to an increase in C:G to G:C levels\textsuperscript{2,6,7}. UGI inhibits UNG, thereby inhibiting downstream BER\textsuperscript{1,2,9}. We speculate that the endogenous BER pathway – in the presence of a bound nCas9 – is linked to the observed C:G to G:C editing and that replacing UGI with BER proteins might increase such editing events. Using a version of CBE – BE3 – as a starting point, we removed UGI, instead fusing rAPOBEC-nCas9 with DNA ligase 3, DNA repair protein XRCC1, or the DNA binding and lyase domain of DNA polymerase β (PB)\textsuperscript{10}.

In this work, we demonstrate a distinct CGBE architecture that manipulates the BER pathway downstream of abasic site creation. We demonstrate that this class of C:G to G:C Base Editors (CGBEs) edits C:G to G:C (Fig. 1a), which potentially opens up treatment avenues to 11% (singular CGBE) to 40% (CGBE with CBE/ABE) of the disease-associated SNPs in ClinVar (Supplementary Table 1). We further characterize the editing preferences of CGBEs, their activity in clinically relevant cell types, and their off-target profiles.

**Results**

**Shortlisting CGBE candidates.** Because the relative orientation of Cas9 fusions may affect the activity of the fusion, we built several versions of our CGBE candidates with the fused rAPOBEC and BER proteins at different orientations with respect to nCas9 (Fig. 1b). We then separately treated HEK293 AA V cells with each candidate along with gRNAs designed to target genomic sites HEK2 and HEK3, both of which were previously used to characterize base editors\textsuperscript{1}. As controls, we also treated cells using BE3 and BE4 with the same gRNAs. Since no effective means of C:G to G:C base editing was known at the time, and BE3 has been observed to effect this reaction as a byproduct of C:G to T:A editing\textsuperscript{1}, we used BE3 as a benchmark for our CGBEs.

High-throughput sequencing of the HEK2 site revealed that our CGBE candidates were able to edit C:G to both G:C and T:A. Out of the 31 candidates tested, 20 candidates showed an increased level of C:G to G:C editing relative to BE3 at position 6 within the protospacer (Supplementary Fig. 1a). The next closest available C was at position 4, where editing levels were lower (<10%, Supplementary Fig. 1b), suggesting a narrower editing window than BE3\textsuperscript{1}. In the best performing candidates, up to 29% C:G to G:C editing and 6% C:G to T:A editing were observed for the CGBE candidates, compared with 14 and 36%, respectively, for BE4, and 16 and 18%, respectively, for BE3.

Similarly, sequencing at the HEK3 site revealed editing of C:G to both G:C and T:A. 12 out of 31 candidates gave C:G to G:C editing levels at position 5 that were higher than that achieved by BE3, with the best performing candidates effecting up to 13% C:G to G:C editing compared with 4% for BE3 (Supplementary Fig. 2a). Despite an increase in C:G to T:A editing with CGBEs compared to BE3, more than twofold increase in C:G to G:C editing with CGBEs led to an increase in the editing ratio of C:G to G:C compared to C:G to T:A. Compared to position 5, C:G to G:C editing at position 3 and position 4 are significantly lower (Supplementary Fig. 2b, c). From this pilot screen, we shortlisted seven of the best performing candidates for a secondary screen (Fig. 1c).

We further tested the CGBEs for C:G to G:C editing at four genomic sites known to be amenable to BE3-mediated editing – EMX1, HEK4, RNFP2, and FANCF (Supplementary Fig. 3a). With CGBEs, C:G to G:C edits were efficiently induced (17–24%,...

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**Fig. 1 Initial screen of CGBE candidates for C:G to G:C editing.** a CBEs like BE3 and BE4 predominantly convert C:G to T:A while CGBE aims to predominantly convert C:G to G:C. b CGBE candidates were designed in three orientations – ACX, AXC, and XAC, where X denotes the fused BER protein. c Seven candidates were selected for their high C:G to G:C editing at both HEK2 and HEK3. The lower editing at HEK3 is likely due to a disfavored motif (refer to data in Fig. 2a). Targeted C’s are in red. PAMs are underlined. *p < 0.05; **p < 0.01; ***p < 0.001 using one-way ANOVA (Dunn-Šidák) of C:G to G:C editing against ‘Untreated’. Exact p values are available in Source Data. Each dot represents editing of an individual biological replicate; bars represent mean values; error bars represent SEM of three biologically independent replicates.
compared to 8–10% with BE3) as the predominant product (up to 69% purity) at HEK4 and RN2. At sites EMM1 and FANC, up to 9% C-G to G-C editing was observed despite this not being the predominant edit, while BE3 effected up to 3% C-G to G-C editing. Across the sites tested, CGBE candidates exhibit higher indel rates than BE3 (Supplementary Fig. 4a). We suspect that the generation of an abasic site – which is essential for BER – may have led to higher indel rates. Comparable for recently published UN-based CGBS, which leverage abasic site generation, also reported mean indel rates of 10.4%. From this secondary screen, we chose ACX, rXRC1, and ACX, rPB8(kD) for further characterization.

Characterizing CGBE editing preferences. We next determined if target sequence context impacts editing efficiency. In characterizing the two CGBS with four gRNAs targeting four disease-associated sites – including dyslipidemia-associated gene ADRB214, hearing loss-associated gene GJB215, and hypertrophic cardiomyopathy-associated gene MYBPC313 – we noticed that not only did CGBE efficiently interrogate disease-associated genes, but they also gave higher levels of C-G to G-C editing at C’s immediately following an A/T (Supplementary Fig. 3b, Supplementary Fig. 5, and Supplementary Table 2). Hence, we suspected that the difference in editing efficiency between gRNAs might be due to the different motifs within which the targeted C is located (e.g., CA at HEK2, CCA at HEK3; targeted C is underlined). To address such potential differences, we designed 16 gRNAs targeting 16 different HEK2 sites. These gRNA-site combinations collectively cover all possible NCN motifs with targeted C’s at position 6 (Fig. 2a, Supplementary Fig. 3c). Sequencing revealed that the most readily edited motif was ACA, while the least edited motif was GCC. Up to 10% C-G to G-C editing was observed for ACA, ACC, ACT, GCT, TCA, and TCT. This observation applied to both ACX, rPB8(kD) and ACX, rXRC1. From these results, we propose that the favored DNA motifs are WCW, ACC, and GCT (W is either A or T, Fig. 2b, Supplementary Fig. 6). This understanding is consistent with our observation that C-G to G-C editing is more efficient at HEK2, HEK4, and RN2, which all had favored DNA motifs of ACA, ACT, and TCT, whereas EMM1, FANC, and HEK3, with suboptimal DNA motifs of TCC andCCA, are less amenable to C-G to G-C editing. With a preference for WCW, ACC, and GCT, our CGBE most efficiently target 6 out of 16 possible motifs.

We previously observed a reduction in editing levels at position 4 relative to position 6 of HEK2 (Supplementary Fig. 1). Similarly, editing levels at position 3 and position 4 of HEK3 are lower than that at position 5 (Supplementary Fig. 2). Both observations suggested that our candidates might not inherit the exact base editing window of BE3. To elucidate the editing window of the CGBES, we chose a genomic site that has an alternating 5′-W-C-W-C-W.3′ sequence such that gRNAs can be designed with C’s located either at every odd position or at every even position (Fig. 2c). We assessed BE3 C-G to T-A editing rates between positions 1 and 9, finding higher editing efficiency between positions 4 and 8. For both ACX, rPB8(kD) and ACX, rXRC1, C-G to G-C editing was observed in a nine nucleotide window from positions 2 to 10. However, only at positions 5 and 6 was C-G to G-C editing the predominant and appreciable outcome.

Recognizing that simply removing UGI can potentially increase C-G to G-C editing at the expense of C-G to T-A editing2,6,7,14,15, we next sought to quantify the effect of fusing rXRC1 or rPB8(kD) to rAPOBEC-nCas9. Across 28 independent treatments using a variety of gRNAs (Fig. 3, Supplementary Fig. 7), BE3 was able to effect on average 4.4% C-G to G-C editing. Removing UGI from BE3 raised mean C-G to G-C editing to 11.5% but increased undesired indel byproducts to 5.0% (Supplementary Fig. 4b). Fusing rPB8(kD) did not provide additional significant effect (p < 0.05). However, fusing rXRC1 further raised mean C-G to G-C editing levels to 15.4% (p < 0.01) and decreased undesired indel rates (3.4%), potentially due to an equilibrium shift from abasic site to repaired base. Meanwhile, the major byproduct C-G to T-A editing stayed between 6 and 9%. Our results indicate that UGI removal from BE3 promotes C-G to G-C editing but increases byproducts; rXRC1 fusion further increases C-G to G-C editing and decreases byproducts. Therefore, we narrowed down to rAPOBEC-nCas9-rXRC1 as the preferred CGBE embodiment that effects C-G to G-C editing at 15.4 ± 7% efficiency in human cells, at a 68 ± 14% purity, within a three-nucleotide target window and WCW, ACC, and GCT target sequence contexts.

Off-target activities and activities in other cell types. As with CRISPR-Cas systems, base editors have been reported to exhibit potential DNA and RNA off-target effects16–19. Because CGBEs share the same APOBEC-nCas9 component with BE3, we assessed CGBE and BE3 activities side-by-side on 29 off-target sites using 5 gRNAs. CGBE and BE3 induced >0.1% C-G to T-A editing at all 29 off-target sites (Supplementary Fig. 8). We did not find a site where CGBE induced off-target editing but BE3 did not. These results indicate that CGBE exhibits lower off-target activity at the off-target sites identified for Cas9 and for the BE3 architecture. Nevertheless, there are potentially vast sequence-independent DNA and RNA off-target effects17–19 that would require a detailed dissection in further work before direct translation of CGBES.

One limitation of BE3 is its low efficiency in some cell types20. We suspect that such limitations might apply to our CGBEs. With BE3, we observed low C-G to T-A editing in H9 stem cells at five genomic sites (with a maximum of 1.2% C-G to T-A editing at HEK4; Supplementary Fig. 9). Expectedly, our CGBE exhibit similarly low C-G to G-C editing efficiencies in the H9 stem cells when evaluated side-by-side. The low editing might be due to chromosomal abnormalities21 and different methylation profiles in stem cells22. Since APOBEC is inefficient at deaminating methylated cytidines23, further APOBEC engineering24–26, alongside codon optimization20,27, might be needed to enhance the efficiency of CGBEs in stem cells. In contrast, in the eHAP cell line, we observed moderate levels of editing with the CGBE (ACX, rXRC1) inducing up to 8.5% C-G to G-C editing at the RN2F and VEGFA sites, while BE3 induced 0.9% C-G to T-A editing (Supplementary Fig. 10). The eHAP data suggests that our CGBEs may be moderately efficient in some cell types even if different base editing technology is not. In HTB9 cells – a urinary bladder cancer cell line, both BE3 and our CGBEs can efficiently induce the desired mutations at many sites (up to 17% C-G to G-C editing with CGBE and up to 18% C-G to T-A editing with BE3; Supplementary Fig. 11). ACX, rPB8(kD) appears to consistently outperform ACX, rXRC1 in HTB9 cells, indicating that different CGBE architecture can be employed for optimal performance according to cell types. We demonstrated that CGBEs is functional across multiple cell types, and that absolute efficiency is partially dependent on the cell type and state, features shared with previous base editors.
Here, we developed CGBEs that target cytidine within a specified window and convert it into guanine as a predominant editing product. In separate works, Liu and Koblan designed CGBE candidates by fusing UDG (UNG), UdgX, and polymerases with rAPOBEC-nCas9 \(^{28}\) (Supplementary Fig. 12); Zhao et al. and Kurt et al. developed CGBEs fusing UNG to rAPOBEC-nCas9\(^{6,7}\). All four CGBE studies induce a C to U change via rAPOBEC and envision this U to be further converted to an abasic site; but the strategy involved and downstream resolution of this abasic site are distinctly different. Zhao et al. and Kurt et al. hypothesized that fusing UNG to rAPOBEC-nCas9 increases the amount of C:G to G:C edits by further increasing the generation of abasic sites (Supplementary Fig. 13). In contrast, we focused on manipulating DNA repair downstream of abasic site creation, reasoning that APOBEC-nCas9 is already able to generate a significant amount of C:G to G:C editing (Fig. 3a, BE3 (no UGI)). Under similar reaction conditions used in Kurt et al., UNG-based CGBEs reported similar mean C:G to G:C editing in unselected cells (14.4\% vs. our 15.4\%)\(^{6}\). The distinct approaches suggest the
possibility that a combination of an abasic site creation strategy and a BER-manipulating strategy may lead to the next iterations of enhanced CGBEs.

Koblan and Liu’s work seeks to induce base excision and perform a translesion polymerization across the targeted abasic site (Supplementary Fig. 13). A translesion polymerization hypothesis was also put forth by Gajula29. This proposed mechanism is unlikely to be the case for our CGBEs. Instead, we envisage that upon creation of an abasic site in the Cas9-induced R-loop, cellular UNG is displaced by APE1, after which XRCC1 recruits various BER components8,30 to repair the abasic site independently of the unedited opposite strand, giving rise to guanine as the major product. Subsequent DNA repair converts the G:G mismatch to G:C. Such a hypothesis would be consistent with (a) the tight binding of Cas9 on its target strand31, which renders that strand less accessible to other enzymes; (b) the accessibility of the deaminated strand as a single-strand of the R-loop32,33; (c) the detrimental effect of UDG and UdgX on C:G to G:C editing28 (Supplementary Fig. 12), which suggests that the persistence of an UDG-bound site or abasic site might act against the C:G to G:C reaction; and (d) the C:G to G:C editing effected by our CGBEs with domains that do not have intrinsic polymerase activity but are key drivers of abasic site repair. While further mechanistic studies and development continue, CGBEs expand the growing suite of precise genome-editing tools that include CBEs1,2, ABEs3, CGBEs6,7,28, and prime editors32 (Supplementary Fig. 12), together enabling the precise and efficient engineering of DNA for research, biological interrogation, and disease correction.

Methods

Constructs and molecular cloning. The BE3 (Addgene plasmid #73021), prime editor 2 (Addgene plasmid #132775), pegRNA-HEK3_CTT_ins (Addgene plasmid #132778) plasmids used in our study are gifts from David R. Liu. The BE3 plasmid is a mammalian expression plasmid with BE3 being driven by a CMV promoter. hXRCC1 (pTXG-hXRCC1) and hLIG3 (pGEX4T-hLIG3) are gifts from Primo Schaer (Addgene plasmid # 52283 and # 81055, respectively). The mutation R400Q is

Fig. 3 CGBE induces efficient C:G to G:C editing as the predominant product. a Removal of UGI from BE3 increases C:G to G:C editing (blue); fusion of rXRCC1 further increases C:G to G:C editing. The major byproduct is C:G to T:A editing (orange). b Mean C:G to G:C editing/C:G to T:A editing ratio. For both plots, p values were obtained via Mann-Whitney tests between the indicated editors. Each dot represents editing of an individual biological replicate. gRNAs chosen have C’s within WCW, ACC, or GCT motifs. Black lines represent mean values of 43 (BE3), 29 (BE3 (no UGI)), or 46 (ACX, rPB(8KD) and ACX, rXRCC1) biologically independent replicates.
introduced to hXRCC1 and N628K is introduced to hLIG3 via blunt-end ligation.

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Author contributions
L.C. made the initial discovery. L.C., J.E.P., and W.L.C. designed the research. L.C., J.E.P., and P.P. performed the experiments. P.D.R. performed biological replicates. Y.T.C. and S.N.M. cloned plasmids. L.C., J.E.P., P.P., H.-T. P., and W.L.C. analyzed data. L.C., H.-T. P., and W.L.C. wrote the manuscript with input from other authors. W.L.C. supervised the research.

Competing interests
L.C. and W.L.C. are named inventors on a patent application based on this work. Patent applicant: Agency for Science, Technology and Research. Application number: 10201913340Q. Status of Application: PCT submitted; Specific aspect of manuscript covered in patent application: design, applications, and uses of base editors with base excision repair proteins (Figs. 1a, 1b, 3, SI Fig. 3, 5, 7, 9, 10, 11, 13) although all data presented here are included in patent application. The remaining authors declare no competing interests.

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