Developing ABEmax-NG with Precise Targeting and Expanded Editing Scope to Model Pathogenic Splice Site Mutations \textit{In Vivo}

ABEmax-NG: precise targeting and expanded editing scope

Suitable for modulating RNA splicing precisely

Modeling of pathogenic isoform \textit{in vivo}

HIGHLIGHTS

ABEmax performs precise A\textsuperscript{+}T to G\textsuperscript{+}C conversion with an expanded scope

ABEmax-NG covers more splicing sites, resulting in precise RNA splicing modulation

ABEmax-NG efficiently and precisely models pathogenic RNA splicing \textit{in vitro} and \textit{in vivo}
 Applying to precise single-site editing (Lee et al., 2018). ABE (adenine base editor)-based splicing modification has the character of precise single-base editing with expanded editing scope that is suitable for PAM-recognized adenine base editor (hereafter named ABEmax-NG). We demonstrated that ABEmax with ABEmax, which is the most efficient version of ABE (Koblan et al., 2018), to develop the NG modification has been successfully achieved in plant (Kang et al., 2018). Nevertheless, the requirement for the expansion of CBE. First, CBE generates indels, although it is reduced by fusing uracil DNA glycosylase inhibitor (Liu et al., 2018a; Yuan et al., 2018). However, CBE-based splicing modulation is limited by the deficiency of CBE. First, CBE generates indels, although it is reduced by fusing uracil DNA glycosylase inhibitor (Wang et al., 2017). Second, CBE induces high proximal mutations, which makes it impossible to be applied to precise single-site editing (Lee et al., 2018). ABE (adenine base editor)-based splicing modulation has been successfully achieved in plant (Kang et al., 2018). Nevertheless, the requirement for the protospacer adjacent motif (PAM) to be adjacent to the target locus by current Cas9 variants limits the target sites of both ABE- and CBE-based splicing modulation. Therefore BEs suitable for splicing modulation are earnestly needed.

The application of ABE in splice site modulation in plants suggests the potential of ABE in RNA splicing modulation of animals. As ABE induces significantly few unwanted base conversions, indels, and proximal mutations (Lee et al., 2018), and a relaxed-PAM-recognized SpCas9 variant (SpCas9-NG) was developed recently (Nishimasu et al., 2018), we take advantage of them and try to combine the SpCas9-NG with ABEmax, which is the most efficient version of ABE (Koblan et al., 2018), to develop the NG-PAM-recognized adenine base editor (hereafter named ABEmax-NG). We demonstrated that ABEmax-NG has the character of precise single-base editing with expanded editing scope that is suitable for
RNA splicing modulation. Furthermore, we have successfully modeled pathogenic splice site mutation in vivo.

RESULTS
ABEmax-NG Is a Potential Tool with the Expanded Editing Scope that Is Suitable for Modulating RNA Splicing Precisely

Given splice sites only contain highly conserved two bases in various species (Lim and Burge, 2001), BE-based splicing modulation has to be very precise. ABE is considered more precise than CBE because it induces significantly few unwanted base conversions, indels, and proximal mutations (Lee et al., 2018). Also, ABE-mediated A→T and G→C conversion can be used to mutate the SA and SD sites; we hypothesize that ABE may be more suitable for modulating splicing than CBE. To demonstrate this hypothesis, we first explored the potential of ABEmax-NG, a combination of SpCas9-NG and ABEmax, in modulating RNA splicing over the human genome by bioinformatics analysis. As there are relatively conserved guanines near the SD and SA sites (Figure S1), we found that ABEs can precisely edit about twice as many splice sites as CBEs (Figure 1A), indicating that ABEs have advantages over CBEs not only in editing precision but also in the editing scope of the target splice sites. Then we further analyzed the editing scope among different PAMs over all validated ABE variants (Rees and Liu, 2018). As expected, ABEmax-NG can cover almost all editable sites, which is far beyond other variants (Figure 1B). With this, it has the most potential single guide RNAs (sgRNAs), and meanwhile, the most targeting genes (Figure 1C), making the ABEmax-NG a flexible tool to modulate various RNA splicing reactions. As expected, ABEmax-NG is also the most efficient tool that targets most human pathogenic splice sites (Figure 1D).
A genome-wide analysis suggests that ABEmax-NG is a potential tool with expanded editing scope for precise modulation of RNA splicing.

The ABEmax-NG Performs Efficient Base Editing in HEK293FT Cells

To demonstrate the potential of ABEmax-NG, we first constructed it by introducing R1335V/L1111R/D1135V/G1218R/E1219F/A1322R/T1337R mutations for converting SpCas9 nickase to SpCas9-NG nickase on ABEmax (Figure 2A). Then we tested its versatility by checking different NG(N) PAM recognition of ABEmax-NG in HEK293FT cells using an enhanced green fluorescent protein (EGFP) reporter, which was developed by mutating two bases of the EGFP-coding gene, one at the third position of the Thr-63 codon, which does not change the amino acid sequence of EGFP but provides multiple PAM sequences for ABEmax-NG recognition test, and another at the first position of the Gln-69 codon, converting the Gln-69 codon (CAG) into a stop codon (TAG), thus changing EGFP to stopped-EGFP (sEGFP). This sEGFP can be restored when the stop codon is corrected by ABEmax-NG (TAG to CAG), hence the editing efficiency of ABEmax-NG in different PAM sequence can be measured as the frequency of EGFP-expressing cells (Figure 2A). Then we designed an sgRNA to target sEGFP with the mutated T at position 7 of the protospacer, counted from the PAM sequence (Figure 2A; Table S1). The plasmids expressing sgRNA and ABEmax-NG were co-transfected with the EGFP reporter system.
Figure 3. Efficient A-to-G Substitution in Mouse N2a Cells and Embryos by ABEmax-NG
(A) A-to-G editing by ABEmax and ABEmax-NG at 16 endogenous target sites in mouse N2a cells. The target base in the editing window was shown, counting the end distal to the protospacer adjacent motif (PAM) as position 1. Data are represented as the mean ± SEM (n = 3 from three independent experiments).
(B) A-to-G editing efficiency of ABEmax and ABEmax-NG at the four endogenous target sites in mouse embryos. Data are represented as the mean ± SEM (n = 3 for untreated, n = 8 for ABEmax, n = 8 for ABEmax-NG).
EGFP fluorescence could be observed in the cells 48 h after transfection targeting different PAM sequences (Figure 2B). Based on the analysis by flow cytometry, ABEmax-NG can recognize all types of NG(N) PAMs efficiently. In contrast, ABEmax, as expected, only recognized the NGG PAM with high efficiency and the NGA PAM with modest efficiency (Figure 2C). Sanger sequencing results further confirmed that ABEmax-NG induces the target base editing, resulting in the conversion of sEGFP to EGFP with different NG(N) PAMs (Figure 2D). These results demonstrated that ABEmax-NG is a versatile BE with expanded editing scope.

ABEmax-NG Can Induce Precise A-to-G Base Conversions In Vitro and In Vivo

To characterize this new tool, we comprehensively examined the editing of ABEmax-NG at 40 endogenous target sites with NG(N) PAMs in mouse-derived Neuro-2a (N2a) cells (Table S1). Compared with ABEmax that only induces A-to-G conversion at the NGG sites, ABEmax-NG performed efficient editing toward all types of PAMs (Figure S2). To further analyze the ABEmax-NG-mediated editing, we next chose 16 NG(N) sites for targeted deep sequencing. As expected, ABEmax-NG induced A-to-G conversion efficiently with few unwanted base conversions (the frequency: 0%–0.54%), indels (the frequency: 0%–2.79%), and proximal mutations (the frequency: 0%–0.32%) (Figures 3A, 3C, S3A, and S3B), indicating that ABEmax-NG is a precise BE.

Then we set out to determine whether ABEmax-NG can also work well in vivo. To this end, four sgRNAs covering all types of NG(N) PAMs have been subjected to test experiments in a total of 64 mouse embryos as previously described (Liu et al., 2018b). As expected, efficient base editing was achieved (Figures S5A and 3B). It is noteworthy that almost no unwanted base conversion, indel, and proximal mutation were detected (Figures 3D, S3C, and S3D), confirming that ABEmax-NG acts as a precise BE in vivo.

ABEmax-NG Can Serve as a Useful Modulator for RNA Splicing

The precision of ABEmax-NG suggests that ABEmax-NG could be a versatile tool for RNA splicing modulation, which is only limited to two editable bases for each SD and SA site. Thus, four splicing sites corresponding to human homologous pathogenic mutations were first tested in N2a cells (Table S1). Interestingly, the targeted deep sequencing showed that all the selected splice sites were edited by ABEmax-NG efficiently and precisely (Figures S4A and S4B). As expected, the corresponding changes to RNA splicing were detected by RT-PCR amplification of the respective cDNAs (Figure S4C). These results were further confirmed by Sanger sequencing (Figure S4D). Then we tested its performance in vivo. As expected, ABEmax-NG also worked well in mouse embryos except one embryo harboring few indels (the frequency is 3.35%; Figures 4A and 4B). Taken together, we demonstrated that ABEmax-NG performed efficient and precise splice site editing and is suitable for modulation of RNA splicing.

The outperformance of ABEmax-NG encouraged us to model pathogenic RNA splicing in mouse. For this purpose, we focused on BBS2 gene, a member of the Bardet-Biedl syndrome (BBS) gene family. It is demonstrated that the splice site mutation of c.472-2A > G for this gene may cause human BBS, which is a developmental disorder that affects multiple systems (Innes et al., 2010). The ABEmax-NG-encoding mRNA together with sgBBS2 mRNA were co-injected into zygotes as previously (Liu et al., 2018b). A total of 40 embryos were transferred into two surrogate mothers, which generated 19 offspring (Figure S5B). Based on the results of deep sequencing, all 19 founder mice carried the expected target site mutation (Figure S6A).

It is known that the target mutation causes skipping of Exon 4 of the BBS2 gene (Figure 4C). Thus, we chose the founder mouse M12 harboring a heterozygous target mutation (Figures 4D and S6A), to further characterize the genotype and RNA splicing in different tissues (heart, liver, spleen, lung, kidney, brain, testis and intestine) at 4 weeks. As expected, all examined tissues carried the heterozygous target mutation and the isoform of the BBS2 RNA transcript skipping Exon 4 (Figures 4E, 4F, S6B, and S6C). These results demonstrated that ABEmax-NG can serve as a useful RNA splicing modulator in vivo.
Off-Target Analysis by WGS

The increased off-target possibility of SpCas9-NG prompted us to comprehensively investigate the off-target effects of ABEmax-NG carefully (Nishimasu et al., 2018). We performed whole-genome sequencing (WGS) on genomic DNA samples isolated from the founder mouse M12 and a wild-type mouse (WT) at a depth of 33 and 30, respectively (Figure 5D). After filtering out mouse dbSNPs, a total of 353,868 and 330,821 single nucleotide polymorphisms were detected over the genomes of WT and M12, respectively. The mutation frequency did not seem to increase significantly in M12 (Figure 5A). The heterozygous target mutation was also confirmed by WGS (Figure 5B). Given the mismatch tolerance of SpCas9 (Cho et al., 2014), we use CasOT (Xiao et al., 2014) to explore potential off-target sites. We used the criteria that up to a mismatch of 2 bp may be included in the seed region and a mismatch of 5 bp in the non-seed region with NG PAM. Among 24,297 potential off-target sites (Figure 5C), two off-target sites that

Figure 4. ABEmax-NG Mediates Pathogenic Exon Skipping In Vivo

(A) A-to-G editing efficiency of ABEmax and ABEmax-NG at four splice sites in mouse embryos. Data are represented as the mean ± SEM (n = 3 for untreated, n = 8 for ABEmax, n = 8 for ABEmax-NG).

(B) Statistical analysis of the A-to-G editing frequency, unwanted base conversions, and indels induced by ABEmax-NG in (A). The median and interquartile range are shown.

(C) Schematic of ABEmax-NG-induced BBS2-long to BBS2-short switch. Top panel: Exon 4 is included in normal BBS2. Bottom panel: ABEmax-NG mutates the invariant A to G at the splice acceptor site, leading to the exclusion of Exon 4.

(D) The chromatogram of Sanger sequencing of tail confirmed heterozygous genotype of founder mouse M12.

(E) The individual fraction of each base induced by ABEmax-NG in BBS2 target splice site from different tissues of founder mouse M12 at 4 weeks. Data are analyzed by deep sequencing.

(F) Quantification of the rate of exon skipping of BBS2 Exon 4 from different tissues of founder mouse M12 at 4 weeks by deep sequencing of RT-PCR amplification of the cDNA.
are located in the intronic and intergenic regions were detected (Figures 5E, S7A, and S7B). For further demonstration, we performed Sanger sequencing of the two off-target sites, confirming the off-target mutagenesis (Figure S7C).

| SNP Type         | WT       | M12      | Uniquely assigned to M12 |
|------------------|----------|----------|--------------------------|
| total SNPs       | 5,186,902| 4,635,562|                          |
| SNPs after excluding dbSNPs | 353,868  | 330,821  | 16,324                   |
| A>G SNPs         | 49,333   | 45,937   | 1,873                    |
| A>C SNPs         | 18,003   | 16,653   | 886                      |
| A>T SNPs         | 19,848   | 18,538   | 1,055                    |
| T>C SNPs         | 51,209   | 47,579   | 1,765                    |
| T>G SNPs         | 18,154   | 16,532   | 724                      |
| T>A SNPs         | 19,747   | 18,574   | 1,185                    |

WT: Wild type

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**Figure 5. Whole-Genome Analysis of BBS2 Mutant (M12) and Wild-Type (WT) Mice**

(A) Summary of single nucleotide polymorphism (SNP) analysis. After filtering out naturally occurring variants in the mouse SNP database, 330,821 SNPs were obtained over the M12 genome. The number of A/T conversions were shown.

(B) Confirmation of the on-target base editing by analyzing the whole-genome sequencing results of M12.

(C) Summary of on-/off-target sites. A total of 24,298 sites, including 1 on-target site and 24,297 off-target sites were analyzed.

(D) Summary of the whole-genome sequencing.

(E) Summary of off-target analysis.

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are located in the intronic and intergenic regions were detected (Figures 5E, S7A, and S7B). For further demonstration, we performed Sanger sequencing of the two off-target sites, confirming the off-target mutagenesis (Figure S7C).
DISCUSSION

In this study, taking advantage of ABE with few unwanted base conversions, indels, and proximal mutations (Lee et al., 2018), and SpCas9-NG with relaxed PAM (Nishimasu et al., 2018), we introduced R1335V/L1111R/D1135V/G1218R/E1219F/A1322R/T1337R mutation into ABEmax and developed ABEmax-NG to induce A-to-G conversion with expanded editing scope and high precision. The versatility of ABEmax-NG confers it as a useful RNA splicing modulator, which is currently the most suitable tool available targeting most human pathogenic splice sites.

More and more splice isoforms have been identified with the development of sequencing technique. It is important to explore the function of the different splicing isoforms because of their potential important biological roles (Kalsotra and Cooper, 2011). The application of BE in splice site modulation opens the potential of BE in studying RNA splicing (Gapinske et al., 2018; Kang et al., 2018; Liu et al., 2018a; Yuan et al., 2018). Nevertheless, the current BE-based splicing modulation is limited. Here we found that ABES can precisely edit about twice as many splice sites as CBEs (Figure 1A), demonstrating that ABES have more potential for studying splicing isoform. Moreover, with the application of the relaxed-PAM-recognized SpCas9-NG, the ABEmax-NG has the broadest editing scope to modulate RNA splicing.

Considering that both SD and SA sites contain only two bases, the precision of the base editing is critical for splicing modulation. As ABE performs precise base editing (Lee et al., 2018), as expected, ABEmax-NG induced almost no unwanted base conversion and indel when targeted at the splice sites (Figures S4A, S4B, 4A, and 4B). Compared with CBE, which induced unwanted base editing around splice sites (Lee et al., 2018; Yuan et al., 2018), ABEmax-NG obviously is a better tool for modulation of RNA splicing.

In summary, we have developed ABEmax-NG, a versatile BE that is the best available tool to successfully model human pathogenic splice site mutations in vitro and in vivo.

Limitations of the Study

Although ABEmax-NG covers the majority of splice sites, there are still some isoforms that cannot be modulated.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.05.008.

ACKNOWLEDGMENTS

We thank members of Huang lab, Sun lab, and Chen lab for helpful discussions. This work is supported by the National Key R&D Program (2016YFC0905901, 2016YFC1000307), National Natural Science Foundation of China (18130004), National Postdoctoral Program for Innovative Talents (BX201700266), and Local Grants (16JC1420200, 17JC1420103).

AUTHOR CONTRIBUTIONS

X.H., Q.S., and X.M. conceived, designed, and supervised the project. S.H., Z.L., and X.L. performed most experiments with the help of G.L., J.L., Z.L., G.Y., and Y.Z. Z.L. and X.L. provided expert technical assistance. S.H. and X.H. wrote the paper with inputs from all authors. X.L. edited the manuscript. X.H., Q.S., and X.M. managed the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: March 29, 2019
Revised: May 1, 2019
Accepted: May 7, 2019
Published: May 31, 2019
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Supplemental Information

Developing ABEmax-NG with Precise Targeting and Expanded Editing Scope to Model Pathogenic Splice Site Mutations In Vivo

Shisheng Huang, Zhaodi Liao, Xiangyang Li, Zhen Liu, Guanglei Li, Jianan Li, Zongyang Lu, Yu Zhang, Xiajun Li, Xu Ma, Qiang Sun, and Xingxu Huang
Figure S1. Human genomic sequence features in the surrounding area of splice sites, Related to Figure 1.

Human reference genome (hg38) and the annotation from GENCODE version 29 were used for analysis. The font size indicates the probability of bases at each position.
Figure S2. Analysis of ABEmax-NG-induced A-to-G substitution in mouse N2a cells, Related to Figure 3.

(A-D) A-to-G editing efficiency of ABEmax and ABEmax-NG at endogenous target sites with NGG PAM (A), NGT PAM (B), NGA PAM (C) and NGC PAM (D). Data are represented as the
mean ± s.e.m. (n = 3 from three independent experiments). The editing efficiency was calculated by EditR based on Sanger sequencing chromatograms.

(E) Statistical analysis of the A-to-G editing frequency at a total of 40 endogenous target sites in A-D. The median and interquartile range (IQR) are shown.
Figure S3. Analysis of proximal off-targets and indels for ABEmax and ABEmax-NG in vitro and in vivo, Related to Figure 3.

(A) Proximal off-targets of ABEmax and ABEmax-NG at 16 endogenous target sites in N2a cells were analyzed by deep sequencing. The mutation rates of A-to-G or T-to-C sites ±100 bp
surrounding the protospacer were calculated, designated the side of the protospacer distal to the PAM as negative positions, while the side that includes the PAM as positive numbers, counting against their positions relative to the protospacer.

(B) Analysis of indels at 16 endogenous target sites in N2a cells by deep sequencing. Reads containing at least 1 inserted or deleted nucleotides in the protospacer were calculated as indel-containing reads. Indel frequency was calculated as the number of indel-containing reads among the total number of mapped reads.

(C) Proximal off-targets of ABEmax and ABEmax-NG at 4 endogenous target sites in mouse embryos were analyzed by deep sequencing.

(D) Analysis of indels at 4 endogenous target sites in mouse embryos by deep sequencing.
Figure S4. ABEmax-NG modulated endogenous RNA splicing in N2a cells, Related to Figure 4.

(A) A-to-G editing by ABEmax and ABEmax-NG at 4 splice sites in mouse N2a cells. Data are represented as the mean ± s.e.m. (n = 3 from three independent experiments).

(B) Statistical analysis of the A-to-G editing frequency, unwanted base conversions and indels induced by ABEmax-NG in (A). The median and interquartile range (IQR) are shown.

(C) Different RNA isoforms induced by ABEmax-NG were determined by RT-PCR. ABEmax served as the control. New splicing isoforms were highlighted by red arrowheads.

(D) Sanger sequencing chromatograms of the RT-PCR products confirmed the new splicing isoforms. Exon 2 of OFD1, exon 33 of MYO7A and exon 4 of BBS2 skipped in new splicing isoforms. The target splice site highlighted by red frame was converted from T to C in new splicing isoform of SEPN1 harboring 110 bp extension of exon 5.
| Target gene | sgRNA | Editor   | No. of examined embryos | Mutant ratio (%) | No. of targeted mutants$^a$ | No. of A-to-C/T$^a$ | No. of indels$^a$ |
|-------------|-------|----------|--------------------------|------------------|-----------------------------|------------------|------------------|
| AKR1C19     | sgG3  | ABEmax   | 8                        |                  | 8(100)                      | 0(0)             | 0(0)             |
| EYA1        | sgT10 | ABEmax   | 8                        |                  | 0(0)                        | 0(0)             | 0(0)             |
| SIX6        | sgA2  | ABEmax   | 8                        |                  | 7(87)                       | 0(0)             | 0(0)             |
| BHLHA9      | sgC10 | ABEmax   | 8                        |                  | 0(0)                        | 0(0)             | 0(0)             |
| OFD1        | sgOFD1| ABEmax   | 8                        |                  | 7(87)                       | 0(0)             | 0(0)             |
| MYO7A       | sgMYO7A| ABEmax   | 8                        |                  | 5(63)                       | 0(0)             | 0(0)             |
| SEPN1       | sgSEPN1| ABEmax   | 8                        |                  | 1(13)                       | 0(0)             | 0(0)             |
| BBS2        | sgBBS2| ABEmax   | 8                        |                  | 4(50)                       | 0(0)             | 0(0)             |

$^a$Calculated from the number of examined embryos

| Target gene | sgRNA | Editor   | No. of transferred embryos | No. of offspring | Mutant ratio (%) | No. of targeted mutants$^a$ | No. of homozygous target mutants$^a$ | No. of A-to-C/T$^a$ | No. of indels$^a$ |
|-------------|-------|----------|-----------------------------|------------------|------------------|-----------------------------|----------------------|------------------|------------------|
| BBS2        | sgBBS2| ABEmax-NG| 40                          | 19               | 19(100)          | 0(0)                        | 0(0)                 | 0(0)             |

$^a$Calculated from the number of offspring

Figure S5. Summary of the manipulation and genotyping of mouse embryos and newborn pups, Related to Figure 4.

(A) Summary of the manipulation and genotyping of mouse embryos.

(B) Summary of the manipulation and genotyping of newborn pups.
Figure S6. Verification of genotype and RNA splicing in founder mice, Related to Figure 4.

(A) Alignments of modified sequences from newborn pups after microinjection of ABEmax-NG mRNA and sgRNAs targeting at BBS2 splice site into one-cell embryos. The PAM sequences and substitutions are highlighted in blue and red, respectively. Frequency is calculated from deep sequencing.

(B) Alignments of modified sequences from different tissues of the founder mouse M12.

(C) Different RNA isoforms induced by ABEmax-NG were determined by RT-PCR from different tissues of founder mouse M12. Wild-type mouse served as the control.
Figure S7. Verification of off-target sites, Related to Figure 5.

(A) Integrative Genomics Viewer (IGV) images showing the alignments of sequence reads at the off-target site NC_000077.6: g.115172674T>C.

(B) IGV images showing the alignments of sequence reads at the off-target site NC_000076.6: g.130258627A>G.

(C) Verification of off-target sites by Sanger sequencing chromatograms. Left: NC_000077.6: g.115172674T>C. Right: NC_000076.6: g.130258627A>G.

Blue lines represent the mismatches of sgRNA sequence and red arrows indicate the off-target sites.
### Supplemental Tables

#### Table S1. sgRNAs used in this study, Related to Figure 2-4.

| Site  | Protospacer sequence | PAM  | Target gene | Corresponding human genotype | Associated genetic disease |
|-------|----------------------|------|-------------|-------------------------------|----------------------------|
| sgEGFP| AGCACTACACGCCGTAAGGTGC | AGN  | sEGFP       |                               |                            |
| sgA1  | CAATCCAGACACTGGTGGTGC | AGA  | CHRNA       |                               |                            |
| sgA2  | CGGCCACGGACCATAAGGAAG | CGA  | SIX6        |                               |                            |
| sgA3  | GAGCCTCAGCAGAGGAGTGGC | AGA  | FBN1        |                               |                            |
| sgA4  | ATGGAAGACAGACAGCAGTGGC | TGA  | ITPR1       |                               |                            |
| sgA5  | CAAGATGTATGCGAGTATGAG | TGA  | FGD1        |                               |                            |
| sgA6  | GAACATGAACTCTTTAGACT | CGA  | TMEM67      |                               |                            |
| sgA7  | TTCTATGAGCAGAAAATTAAGA | AGA  | RNF216      |                               |                            |
| sgA8  | ACCTCAGTAAATGACATCAGTGC | AGA  | MLH1        |                               |                            |
| sgA9  | GGATGAAACTATAGCGAGGAG | CGA  | NUP205      |                               |                            |
| sgA10 | ATTCAGCTCCCGGAACATCT | CGA  | TRP53       |                               |                            |
| sgC1  | CTTCAGGGGGAGCGAGGAGGAA | AGC  | COL6A1      |                               |                            |
| sgC2  | TACACAAACCTCACAGCTCT | AGC  | MKKS        |                               |                            |
| sgC3  | CCTCTATTGTGTCTCATGT | TGC  | LMBR1       |                               |                            |
| sgC4  | CGGGAGCCCGCTAGGTGGGCC | AGC  | MEGF8       |                               |                            |
| sgC5  | TAAGAAAGTACCAATCGACAGG | AGC  | MTM1        |                               |                            |
| sgC6  | TACCAGTCCCCCTTGCTCCCC | TGC  | CD207       |                               |                            |
| sgC7  | CAGCAGCTCGTCTTCAGCT | CGC  | NFIX        |                               |                            |
| sgC8  | AGGTCAAGCAGCCTGACACAG | TGC  | NBEAL2      |                               |                            |
| sgC9  | TATTACAGAAAACCCGCCCAG | AGC  | DES         |                               |                            |
| sgC10 | GGCTAAAGTGCAGGGAGGCCAC | AGC  | BHLHA9      |                               |                            |
| sgG1  | ATGTGTATAAGGATGACAG | GGG  | NDUFS1      |                               |                            |
| sgG2  | GTTTCAGAAATCAGGGTAAAGG | AGG  | HOXD13      |                               |                            |
| sgG3  | AGACATATTCCATCTACAGA  | AGG  | AKR1C19     |                               |                            |
| sgG4  | CTTTAGCTGACATGCGGAGC | CGG  | NIPBL       |                               |                            |
| sgG5  | CCCACCAGCTCAAATGCAATG | GGG  | SLC16A2     |                               |                            |
| sgG6  | AGCCAGGTCGGCGGTCTTCT | TGG  | FERMT1      |                               |                            |
| sgG7  | GCCTAGGCGCCACTCTGTGAG | GGG  | LMNA        |                               |                            |
| sgG8  | AATTACAGTAAAGCTGGGAG | AGG  | PTEN         |                               |                            |
| sgG9  | CCCTCAGGGGTACTCTGAGTGA | CGG  | ZEB2        |                               |                            |
| sgG10 | CTACTATGACCTCTATGGTGGC | GGG  | PTPN11      |                               |                            |
| sgT1  | CTTGTATCAGGACCACATGCT | AGT  | WNT5A       |                               |                            |
| sg   | Sequence                        | Strand | Gene    |
|------|---------------------------------|--------|---------|
| sgT2 | TCCGACCGCCACACCAACC             | AGT    | WNT5A   |
| sgT3 | AACGTATGGGCAATGTCGCC             | TGT    | SUFU    |
| sgT4 | AGCCAGACTCTGCAGCGATGAC           | AGT    | GJA1    |
| sgT5 | GAAATGTTCTTGCGCTGTTT            | TGT    | TYR     |
| sgT6 | ATTTACCAAGGAGGCC            | AGT    | PLCB4   |
| sgT7 | GGTTGATGACACACTGGCC            | AGT    | AH11    |
| sgT8 | GCGAGGGACGGCCACGCACGC           | AGT    | TWIST1  |
| sgT9 | CACCACGCTCTACGCGACG             | AGT    | CKAP2L  |
| sgT10| TTTGGAAGGAAGTGGTATA            | CGT    | EYA1    |
| sgBBS2| GTTCAGGTGTACGAGACAA          | TGT    | BBS2   | NC_000016.10:g.56510923T>C | Bardet-Biedl syndrome 2 |
| sgOFD1| CTGATACCTGAGTTGTGAC            | AGT    | OFD1   | NC_000023.11:g.13733538T>C | Oral-facial-digital syndrome |
| sgMYO7A| CACTCCAAGGACAGCTGGGC         | TGA    | MYO7A  | NC_000011.10:g.77194352A>G | Deafness, autosomal recessive 2 |
| sgSEPN1| CACTCCAAGGACACATCAGG         | TGT    | SEPN1  | NC_000001.11:g.25809152T>C | Eichsfeld type congenital muscular dystrophy |
| Primer name | Primer sequence |
|-------------|----------------|
| sgA1_F      | AGAGCTTAGCCTGTATCACC |
| sgA1_R      | TAGAACAATCTCTGGCAGGCC |
| sgA10_F     | CCTGTAAGTGGAGCCAGCTT |
| sgA10_R     | AAGTCAGTTCTCCTAGGGTG |
| sgA2_F      | GAAACCAACCCACAGCTCTT |
| sgA2_R      | CAGCGGGAAACTTCTTCCTTA |
| sgA3_F      | CACATTGCAGCAGCTAAGGC |
| sgA3_R      | TACCATCACAGCTCTGC |
| sgA4_F      | ATGTCTGACAGATACAGGCC |
| sgA4_R      | TGGTCTGTGCTGATAGGTC |
| sgA5_F      | GGTATTAGCTAGGGTTTCTCA |
| sgA5_R      | AGCCATGAGGAGCTTTAAC |
| sgA6_F      | AGACACACATGCAAGGCAAAG |
| sgA6_R      | AGCCAAGGAAGGTCTGTCT |
| sgA7_F      | GCTTGTTCTTCTAGTGAT |
| sgA7_R      | GCATGTTGGAAGGTCT |
| sgA8_F      | GATGTGCTTTCTTGTACCA |
| sgA9_F      | TGTCAGTGGAGCTTAAAG |
| sgA9_R      | GGTGTTTCCTCTCAGTTCAT |
| sgBBS2_F    | CCGAGGTGCTTGTCTTGCTT |
| sgBBS2_R    | ACAGAGGCAAGGAGCACAGTA |
| sgBBS2_RT_F | TGAACCCCTGAGCTTGGCTAT |
| sgBBS2_RT_R | GTCTGCCACAATCTCTC |
| sgC1_F      | AGGACAACACTCTAGCCAA |
| sgC1_R      | TCAGACTTGTCAGCTG |
| sgC10_F     | TGGACCCAAATAGCAGGAGC |
| sgC10_R     | CGGAACACTGAGCTG |
| sgC2_F      | CATACCTGGAGAACAGTTT |
| sgC2_R      | CTGAGTCAAACCTGGAGT |
| sgC3_F      | ATCCAGCCATCTAGAGT |
| sgC3_R      | CAATGAACGCTCATGGGAC |
| sgC4_F | TCAGTACGGAGGTTTCAGTGA |
|---------|----------------------|
| sgC4_R | GTCATAGGAGGTGGAGACAT |
| sgC5_F | CACTCACAACCAACATGGT |
| sgC5_R | GGGGTAGTCTCAAAGTGAGAT |
| sgC6_F | AGATGCTGCACCCTGCAAATC |
| sgC6_R | GCCATATAAGACACCGGAGGT |
| sgC7_F | GGGGTAGTCTCAAACCGTTC |
| sgC7_R | CCGTGATGGTTAGCAAACAA |
| sgC8_F | ATTCGCCGATCCACAGATGC |
| sgC8_R | CACCCACCAAGCCTATCGAA |
| sgC9_F | AAGACTGGTCCCTCTCTCT |
| sgC9_R | TGGTTGTGCTGTGATGCTTC |
| sgEGFP_F | TCGTTGACCGAATCCGAC |
| sgEGFP_R | CCTTGAAATGCGATCCCTTC |
| sgG1_F | CTGGTAGTAATCAGCAACAG |
| sgG1_R | CCATAAACACATGGGACACA |
| sgG10_F | GCTGTGGGTTGCCATAGTTA |
| sgG10_R | TGGGCTTACAATACACTGCC |
| sgG2_F | GATGTGGCTTTAACCAGCC |
| sgG2_R | CAATGCTTGCTTTCTAGGC |
| sgG3_F | CCATTATGCACCTCTCTTC |
| sgG3_R | TGTTTACCTGAACCTACTGC |
| sgG4_F | TGCAATTGCCGTTCGAACAA |
| sgG4_R | TGCTCTTCAAAGCATAACC |
| sgG5_F | GCAGATAGTGAAGACGGAGA |
| sgG5_R | TGGCCATGTACTCTGTCTAG |
| sgG6_F | AATCGTGACACCTGAGCTAG |
| sgG6_R | GTAAGTGACAGGGGATGTAG |
| sgG7_F | AGACTCCAGCTTACAGAGCA |
| sgG7_R | AATCCAGAACCCTGTCCACT |
| sgG8_F | GAAGACCCATAACCCACCACA |
| sgG8_R | CAGGGATAGGGGATACACTA |
| sgG9_F | AAATGCTGCAAGCGCTTTC |
| sgG9_R | TCTCTCTCATCCCGTATC |
| sgMYO7A_F | CCCATGATTGCGTCTGAAG |
| sgMYO7A_R      | CTGTAGGGCAGAAGACATCA |
|----------------|---------------------|
| sgMYO7A_RT_F   | AAAGGAGGTCTTCACACCCT |
| sgMYO7A_RT_R   | GGGCATAATTGACCACTCC |
| sgOFD1_F       | TAAGCATCTTAGGCTTCC |
| sgOFD1_R       | TCCTGCTACTACATAGACG |
| sgOFD1_RT_F    | AAAGCAGATGAGAGTGCTC |
| sgOFD1_RT_R    | CCAGCTACAGCTCTTTTA |
| sgSEPN1_F      | CAGAGCTGCAAACCCAGC |
| sgSEPN1_R      | CATCCTGGTACCAGCTAT |
| sgSEPN1_RT_F   | GCTATTGTCAAACCCGC |
| sgSEPN1_RT_R   | CATACAGCCACTCCATGC |
| sgT1_F         | TTCAAGCCCCTGAATGGCT |
| sgT1_R         | AGGCTGTAAGCAGACAGC |
| sgT10_F        | CAGCCTACACACTGTAAT |
| sgT10_R        | GAGTCACCTGCAAATGCT |
| sgT2_F         | CCCAGCAAGATTAGGCT |
| sgT2_R         | TGCAAGTTGGGATAATGG |
| sgT3_F         | GTCCTGTAGTAGGAGG |
| sgT3_R         | TGAGGACACAGCACCATA |
| sgT4_F         | TAGTCGGCACAGATGAGC |
| sgT4_R         | GACGAAGATGATCTGCA |
| sgT5_F         | ATGGGCTAGTGCAAACT |
| sgT5_R         | GAAGGATATCCTGGCAGG |
| sgT6_F         | CCGTGCTATCTACCTGCT |
| sgT6_R         | GTATGGCTTCTTGGG |
| sgT7_F         | CCCAAAGTGATAGGAGCA |
| sgT7_R         | TGGTGACATTCCCCATTTC |
| sgT8_F         | AGATGATGACAGCGTTC |
| sgT8_R         | CCTGACACAGGAGTCAG |
| sgT9_F         | AACCTCACAATCGCAG |
| sgT9_R         | ATCAGTGAACCTGCAGA |
| IVT-F          | TCTCGCCGGTTCGGTGATAG |
| IVT-R          | AAAAAGCACCAGACTCGG |


Table S3. Primers used for target deep sequencing, Related to Figure 2-4.

| Primer name   | Primer sequence                  |
|---------------|----------------------------------|
| sgA1_HTS_F    | CTCATCTCACTGGAAGAGAC           |
| sgA1_HTS_R    | ATAGTGTGCCCCAGTTGGTAAG         |
| sgA2_HTS_F    | GTCCAGTCTCCATTTTTGA            |
| sgA2_HTS_R    | TGCGGTGCGATTCTCTAGTA           |
| sgA4_HTS_F    | TGAAGGTCTCTGAGTCGCT            |
| sgA4_HTS_R    | GCTCAAGAAGACAGGTTGCTC          |
| sgA6_HTS_F    | GCTAATGTGCGCTGTCTCTTC          |
| sgA6_HTS_R    | CCAGAATGGAACAGATGCACA          |
| sgBBS2_HTS_F  | GCGTTCACATTGAGGGAACA           |
| sgBBS2_HTS_R  | CCACACACTCTGCTCTCCC           |
| sgBBS2_RT_HTS_F | TCATCGTTGGAAAACTGTCGCT         |
| sgBBS2_RT_HTS_R | CCAAACCGACTGCCATACAT         |
| sgC10_HTS_F   | CTCCTGTCGGAGGCTGGA             |
| sgC10_HTS_R   | GGATAGAGCGAGTGATGCGTG          |
| sgC3_HTS_F    | GAGGTTAGCTCTGTAACCTTC         |
| sgC3_HTS_R    | GCGACATAAACAGGACCA          |
| sgC7_HTS_F    | GGAATGACCTCCACCCGTTTA         |
| sgC7_HTS_R    | CTTCTGCCCGTGATGGA             |
| sgC9_HTS_F    | GACTGTCTCATAAGAAGGTG         |
| sgC9_HTS_R    | CACCCGACTGTTGAAAGACA         |
| sgG1_HTS_F    | GAGGGTTTGAGGCCAACATG         |
| sgG1_HTS_R    | CCTCATTACCTCTCAGTTGTTG       |
| sgG2_HTS_F    | CTTACACAAACTGCAGCTC         |
| sgG2_HTS_R    | TAAACTGTCTTGGCCAACC         |
| sgG3_HTS_F    | TATCCATTTAGCTCTAGGG          |
| sgG3_HTS_R    | TGCAACTAAGAGTTCAACCT         |
| sgG7_HTS_F    | AGCATCGAGGTTGGACAAAG        |
| sgG7_HTS_R    | ATGGAGAGAGCTCTCTCCAT        |
| sgT1_HTS_F    | GCAGGTCTCTAGGATGAAT         |
| sgT1_HTS_R    | CCTACCTATTTGCACTACCC       |
| sgT10_HTS_F   | TTTCCCTGTTGGATAGAG         |
| sgT10_HTS_R   | GGTAAGAAGCTGCCATGGGT         |
| sgT3_HTS_F    | ATGTTGGCTCTTTAGCCCAAG       |
| sgT3_HTS_R       | CTGCGTCTCAGTTGTAACCA       |
|-----------------|--------------------------|
| sgT4_HTS_F      | GGAAAGAGATCATCTCAGAG      |
| sgT4_HTS_R      | ATCGTAGCAGACATTCTCAC      |
| sgT9_HTS_F      | TTCCTGTATCCCAGCTCTCT      |
| sgT9_HTS_R      | TCCAGCGCTCTTAACCTAAG      |
| sgOFD1_HTS_F    | CAGGTCCACGTGACACTAG       |
| sgOFD1_HTS_R    | TAAATGTGGAGCCACCTCGG      |
| sgMYO7A_HTS_F   | GACAGGGAGAACAAGACTAG      |
| sgMYO7A_HTS_R   | ATGTAAGTGCCGACGAGGCT      |
| sgSEPN1_HTS_F   | TCATCCATCGCTGTTAAGC       |
| sgSEPN1_HTS_R   | CCTCTCACAAATCTGTAGG       |
**Transparent Methods**

**Genome-wide analysis.** To identify editable splice sites, human reference genome (hg38) and the annotation from GENCODE version 29 were used. sgRNAs of validated CBE and ABE variants were then designed to target splice sites according to their distinct PAM specificities and corresponding editing windows. The sgRNAs with a single target site in their editing windows were considered as precise editing sgRNAs and used for further analysis. Pathogenic human splice sites were annotated by ClinVar database.

**Animals.** All the experiment protocols involving mice were approved by the Animal Care and Use Committee of the Institute of Neuroscience, Chinese Academy of Sciences, Shanghai, China. Mice were maintained in an Assessment and Accreditation of Laboratory Animal Care credited specific pathogen free facility under a 12 h dark-light cycle. B6D2F1 (C57BL/6 x DBA/2) and ICR mouse strains were used as embryo donors and foster mothers, respectively.

**Plasmid construction.** For construction of sgRNAs, oligos were synthesized, annealed and cloned into BsaI site of the sgRNA expression vector. Plasmids used include pGL3-U6-sgRNA-PGK-puromycin (Addgene, 51133), pGL3-U6-sgRNA-EGFP (Addgene, 107721), pUC57-sgRNA expression vector (Addgene, 51132), pGL3-U6-sgRNA-mCherry.

**Cell culture and transfection.** HEK293FT and Neuro-2a (N2a) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone), supplemented with 10% fetal bovine serum (FBS) (v/v) (Gemini) and 1% Penicillin Streptomycin (v/v) (Gibco). Cells were seeded on poly-D-lysine coated 12-well plates (JETBIOFIL) and transfected at approximately 70% confluence with ABEmax or ABEmax-NG expressing plasmid (1000 ng) and sgRNA-expressing plasmid (500 ng) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Puromycin was added 1 day post-transfection and was maintained in culture until untreated control cells were all died. For deep sequencing, GFP positive cells were harvested from fluorescence-activated cell sorting (FACS) 72 h after transfection. Cells were cultured at 37°C with 5% of CO₂.

**Flow cytometry.** Cells were harvested and subjected to flow cytometry 48 hours after transfection. sgRNAs were annealed and cloned into pGL3-U6-sgRNA-mCherry. GFP signal was detected with flow cytometry. ABEmax-NG/ABEmax, sgRNA and hPGK-sEGFP/hPGK-EGFP plasmids were transfected simultaneously. At total of 10,000 cell events were collected and analyzed using FlowJo.
**Genomic DNA extraction and genotyping.** Genomic DNA of cells was extracted using QuickExtract™ DNA Extraction Solution (Lucigen) according to manufacturer’s protocols, genomic DNA of mouse was extracted by phenol-chloroform method, and genomic DNA of zygotes was amplified according to methods described below. The isolated DNA was PCR-amplified with Phanta® Max Super-Fidelity DNA Polymerase (Vazyme). Primers used were listed in Table S2.

**Whole genome random amplification.** After developing to blastocyst stage *in vitro*, single embryo was transferred to 200 μl tube containing 5 μl of an alkaline lysis solution (200 mM KOH/50 mM dithiothreitol). After an incubation of 10 min at 65°C, 5 μl of neutralization solution (900 mM Tris-HCl, pH 8.3/300 mM KCl/200 mM HCl) was added. The lysed and neutralized sample was added with 5 μl of a 400 μM solution of random primers (Genscript, Nanjing, China), 6 μl of 10x PCR buffer (Takara, Dalian, China), 3 μl of a mixture of the 4 dNTPs (each at 2.5 mM), and 1 μl of Taq polymerase (Takara, Dalian, China), and brought to 60 μl with water. Fifty primer-extension cycles were carried out in a MyCycler thermo-cycler (Bio-Rad, US). Each cycle consisted of a 1 min denaturation step at 92°C, a 2 min annealing step at 37°C, a programmed ramping step of 10 sec/degree to 55°C, and a 4 min incubation at 55°C for polymerase extension. Then the products were used as the PCR templates.

**In vitro transcription.** In brief, ABEmax/ABEmax-NG vector was linearized by BbsI enzyme (NEB) and *in vitro* transcribed using T7 Ultra Kit (Ambion) according to the manufacturer’s protocols. mRNA was purified by Mini Kit (Qiagen). sgRNA oligos were annealed into pUC57-sgRNA expression vectors with T7 promoter. Then sgRNAs were amplified and transcribed *in vitro* by MEGASHortscript Kit (Ambion). The sgRNAs were purified by MEGAClear Kit (Ambion) according to the manufacturer’s protocols. Primers used for transcription *in vitro* were listed in Table S2.

**Microinjection of mouse zygotes and embryo transfer.** B6D2F1 female mice at 4 weeks of age were superovulated and mated with B6D2F1 male mice. Fertilized one-cell embryos were collected from the oviducts. For microinjection, mRNA mixtures containing sgRNA (50 ng/μl) and ABEmax-NG/ABEmax (100 ng/μl) were injected into the cytoplasm of zygotes in a droplet of M2 medium containing 5 μg/ml cytochalasin B (CB) using a piezo (Primetech) microinjector. The injected
zygotes were cultured in KSOM mediums at 37°C under 5% of CO₂ in air and transferred to oviducts of pseudopregnant females at 0.5 dpc.

**RNA analysis.** RNA was immediately extracted from cultured cells or mouse tissues by using TRizol reagent (Invitrogen), according to the manufacturer’s instructions. Complementary DNA (cDNA) was generated using the HiScript II Q RT SuperMix (Vazyme), and was PCR-amplified with Phanta® Max Super-Fidelity DNA Polymerase (Vazyme). The PCR-amplified fragments were separated by agarose gel electrophoresis. Products with abnormally size were purified from the gel and sequenced using the amplification primers.

**Targeted deep sequencing.** Target sites were amplified from extracted genomic DNA using Phanta® Max Super-Fidelity DNA Polymerase (Vazyme). PCR products with different barcodes were pooled together for deep sequencing on Illumina HiSeq X Ten (2 × 150 PE) at the Novogene Bioinformatics Institute, Beijing, China. Primers used for deep sequencing were listed in Table S3. The adapter pair of the pair-end reads were removed using AdapterRemoval version 2.2.2, and pair-end read alignments of 11 bp or more bases were combined into a single consensus read. All processed reads were then mapped to the target sequences using the BWA-MEM algorithm (BWA v0.7.16). For each site, the mutation rate was calculated using bam-readcount with parameters -q 20 -b 30. Indels were calculated based on reads containing at least 1 inserted or deleted nucleotide in protospacer. Indel frequency was calculated as the number of indel-containing reads/total mapped reads.

**Whole genome sequencing.** Whole genome sequencing of mouse genomic DNA extracted from the tail was sequenced using an Illumina HiSeq X Ten (2 × 150 PE) at the Novogene Bioinformatics Institute, Beijing, China. The WT control mouse has the same genetic background with the Founder mouse and from our previous study (SRR8263608). All cleaned reads were mapped to the mouse reference genome (GRCm38/mm10) using BWA v0.7.16 with default parameters. Sequence reads were removed for duplicates using Sambamba v0.6.7 and realigned using Genome Analysis Toolkit (GA TK v3.7) IndelRealigner. Variants were identified by GATK HaplotypeCaller and the following criteria were applied to all SNPs: (1) sequencing depth (for each individual) > 1/3× and < 3×; (2) variant confidence/quality by depth > 2; (3) RMS mapping quality (MQ) > 40.0; (4) Phred-scaled P value using Fisher’s exact test to detect strand bias < 60; (5) Z-score from the Wilcoxon rank sum test of Alt vs. Ref read MQs (MQRankSum) > −12.5; and (6) Z-score from the Wilcoxon rank sum
test of Alt vs. Ref read position bias (ReadPosRankSum) > −8. After filtering out variants in the SNP database (dbSNP) and also found in the wild-type genome, potential off-target sites were predicted by CasOT-1.0 considering up to 2-bp mismatch in seed region and 5-bp mismatch in non-seed region with NG PAM.

**Data and Software Availability.** High-throughput sequencing data will be deposited in the NCBI Sequence Read Archive database under accession code (PRJNA527206). All other data are available upon reasonable request.