**Supplemental Methods**

*RNAseq analysis.* To fully characterize the molecular consequences of our mutant-specific CRISPR-Cas9 strategy, we performed RNAseq analysis. We transfected two HD iPSC lines (iPSC-A and iPSC-B) carrying adult onset CAG repeats with mutant-specific CRISPR-Cas9 (PX551 EFS vector expression SpCas9 and PX552 EFS plasmid expressing our test gRNA; experimental group) or empty vector (PX551 EFS vector for SpCas9 and empty PX552 vector without gRNA; control group). Single cell clones were subsequently developed by limited dilution. 12 clonal lines were developed for each group and further validated by Sanger sequencing and MiSeq analysis of genomic DNA (PCR primers for Sanger sequencing and MiSeq analysis are listed in Supplemental Table 8). Then, genome-wide RNAseq analysis was performed by the Broad Institute. Sequence data were processed by STAR aligner (1) as part of the Broad Institute’s standard RNAseq analysis pipeline.

For allele-specific expression analysis (ASE) of RNAseq data focusing on *HTT*, we counted alleles harboring heterozygous exonic SNPs in targeted and control clonal lines. Our clonal lines derived from two HD iPSC with hap.01/hap.08 diploptype carry 10 heterozygous exonic SNPs including rs363099. Alleles of 10 heterozygous exonic SNPs on the mutant and normal *HTT* in hap.01/hap.08 diploptype were determined based on haplotype sequence data (2). Statistical significance in ASE of *HTT* was judged based on the Student t-test (2-tailed), comparing counts of alleles on normal *HTT* in targeted clonal lines (n=12) to those in controls (n=12) for a given SNP site, followed by Bonferroni multiple test correction (Figure 4B). The same approach was applied to the numbers of alleles on the mutant *HTT* to compare targeted and control clones (Figure 4C).

For differential gene expression (DGE) analysis, we used transcripts per million (TPM) data computed by the TPMCalculator (https://github.com/ncbi/TPMCalculator) (3). Expression levels in 20,260 protein-coding genes based on Ensembl (ftp://ftp.ensembl.org/pub/release-75/gtf/homo_sapiens/) were normalized; 3,420 genes were excluded due to zero TPM values in at least one sample. Subsequently, we analyzed 16,840 genes expressed in all 24 samples. The DGE analysis was performed by the generalized linear model using a library of “glm” in R package v3.3.1 (https://www.r-project.org/) after adjustment for four covariates including sex, batch, and two principal components based on RNAseq data, followed by multiple test correction using a FDR method. A multiple test corrected p-value less than 0.05 was considered statistically significant.

**Off-target prediction and subsequent enrichment analysis using RNAseq data.** Potential off-targets of our primary test gRNA were based on the Cas-OFFinder website (http://www.rgenome.net/cas-offinder/). Parameters for Off-target prediction are maximum 2 mismatches, 1 bulge in DNA, or 1 bulge in RNA. A total of 77 unique potential off-targets were predicted, including 1 exonic and 30 intronic sites of protein coding genes. When a predicted off-target is located between genes, we took the nearest gene to represent the predicted off-target in DGE analysis and gene set enrichment analysis of RNAseq data. As a result, 77 predicted off-target sites were mapped to 52 expressed genes in our RNAseq data. For gene set enrichment analysis of predicted off-targets, we calculated true gene set score by summing the significance values (-log10(FDR)) of predicted
off-targets, and compared to a null distribution of enrichment score based on 1 million random samplings to obtain an empirical p-value.

**GUIDE-seq.** HEK 293T cells (ATCC CRL-3216) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated FBS (HI-FBS) and 1% penicillin/streptomycin. Transfections of HEK 293T cells were performed between 18 and 24 hours following seeding of approximately 20,000 cells per well in 96-well plates. Transfections contained 29 ng of nuclease and 12.5 ng of single guide RNA (sgRNA) expression plasmids, 1 pmol of the GUIDE-seq double-stranded oligodeoxynucleotide (dsODN; oSQT685/686) tag (4), and 0.3 µL of TransIT-X2 (Muris). The transfection components were combined in a total volume of 16 µL supplemented with Opti-MEM, incubated at room temperature for 15 minutes, and then gently added to the media of the plated cells. Genomic DNA from the transfected cells was harvested approximately 72 hours post-transfection by discarding the media and re-suspending the cells in 100 µL of lysis buffer (20 mM Hepes pH 7.5, 100 mM KCl, 5 mM MgCl2, 5% glycerol, 25 mM DTT, 0.1% Triton X-100, and 60 ng/µL Proteinase K). The lysate was heated for 6 minutes at 65 ºC, 98 ºC for 2 minutes, and then stored at -20 ºC until use. To perform the GUIDE-seq assay, the efficiency of on-target editing and GUIDE-seq dsODN tag capture was first assessed by sequencing via a 2-step PCR-based Illumina library preparation method. Genomic loci were amplified by a first PCR using Q5 High-fidelity DNA Polymerase (NEB) and the primers listed in Supplemental Table 9. Products from PCR #1 were quantified via capillary electrophoresis (Qiagen QIAxcel) and purified via paramagnetic beads (5, 6). A second PCR reaction was performed to add Illumina barcodes and adapter sequences using approximately 20 pg of purified PCR #1 as the template. Final sequencing libraries were quantified by qPCR (Illumina Library qPCR Quantification Kit, KAPA Biosystems), then sequenced on a MiSeq sequencer using a 300 cycle Micro Kit v2 (Illumina). On-target tag capture was quantified using CRISPResso2 (https://crispresso.pinellolab.partners.org/submission) (7) by including sequences for the target site spacer, the reference amplicon, the expected forward and reverse orientation dsODN containing amplicons as ‘HDR’ alleles, and the following custom parameters: -w 25 -g GUIDE --plot_window_size 50. Total dsODN capture was quantified by dividing the sum of the forward and reverse dsODN-containing reads (modified and unmodified) reads by the total number of reads for the sample. To analyze potential off-target editing events, GUIDE-seq sequencing libraries were prepared from the samples of genomic DNA (5, 6). Briefly, genomic DNA was purified from the cell lysates using a 1X ratio of paramagnetic beads, quantified via QuantiFluor (Promega), and 400ng of genomic DNA from each sample was normalized to a final volume of 130 µL using 1x TE buffer. Samples were sheared to 500 bp using an ultrasonicator (Covaris E220) and sequencing libraries were prepared as previously described (5, 6) using the sample barcodes listed in Supplemental Table 9. Samples were sequenced on an Illumina MiSeq using a 300 cycle v2 kit with custom Read 2 and Index 1 sequencing primers (Supplemental Table 9). The resulting GUIDE-seq data were analyzed using guideseq v1.0.2 (https://github.com/aryeelab/guideseq) with a mismatch tolerance of 6 (max_mismatches 6).

**Allele-specific CRISPR-Cas9 targeting in differentiated neurons from patient-derived iPSC.** An iPSC line with an adult onset CAG repeat (iPSC-B, 42 CAG) was differentiated into neuronal cells using a previously
described method (8). An iPSC-B line was plated on growth factor reduced Matrigel (Corning) in mTeSR Plus media (STEMCELL Technologies). When cells reached ~ 80% confluence, differentiation was initiated by switching to DMEM-F12/Neurobasal media (2:1) supplemented with N2 and retinol-free B27 (N2B27 RA−; Gibco). For the first ten days, cells were supplemented with SB431542 (10 µM; Tocris), LDN-193189 (100 nM; StemGene), and dorsomorphin (200 nM; Tocris). SB431542 was removed from the media on day 5. Cells were maintained in N2B27 RA− supplemented with activin A (25 ng/ml; R&D) on day 9. On day 22, cells were split using Accutase (STEMCELL Technologies) and seeded on a poly-D-lysine/laminin plate with N2B27 media supplemented with BDNF and GDNF (10 ng/ml each; Peprotech). Media were changed the next day to facilitate neuronal maturation and survival. Cells were fed with new media every two days.

In order to check the expression levels of marker genes, total RNA of iPSC-B and iPSC-B-derived neuronal cells were extracted using RNeasy Plus Kits (Qiagen), and reverse transcription was performed with SuperScript IV First-Strand Synthesis System (Invitrogen). One μl cDNA samples and following primers were used for the PCR amplification for OCT4 (octamer binding transcription factor 4; 5'-GACACCCCCACCCACCACTAGG-3' and 5'-CTTCCCTCCAACCAGTGTGCCCCAAA-3'), MAP2 (microtubule-associated protein 2; 5'-CTGCTTTACAGGGTAGCACAA-3' and 5'-TTGAGTATGGCAACACGGTCTG-3'), GAD67 (glutamic acid decarboxylase 67; 5'-CGTCTTCGACCCCATCTTC-3' and 5'-CGCAGATCTTGAGCCCATCT-3'), TH (tyrosine hydroxylase; 5'-GAGTACACCGCCGCGAGGAGATTG-3' and 5'-GGGATATCTGGGTGCACTGG-3'), vGLUT1 (vesicular glutamate transporter 1; 5'-TACACCGCTCCTTTTCTGG-3' and 5'-CTGAGGGGATCAGCATGTTT-3'), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; 5'-ATGACATCAAGAAGGTGGTG-3' and 5'-CATACCAGAAATGAGCTTG-3'). Real-time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems; denaturation at 95ºC for 5 min followed by 45 cycles of 95ºC for 10 sec, 60ºC for 10 sec, and 72ºC for 10 sec) by LightCycler 480 System (Roche). The relative expression levels were then determined using the 2^\(-\Delta\Delta\text{Ct}\) method, and fold-changes were calculated subsequently.

For neuronal marker staining, cells were fixed, permeabilized, and blocked using the Image-iT Fix-Perm kit (Invitrogen). Subsequently, cells were stained by Anti-TUBB3 (tubulin beta 3; Biolegend Inc, Cat# 801202) in a blocking solution overnight at 4°C. Then, cells were washed with PBS three times for 5 minutes, followed by incubation with Alexa Flour 594 secondary antibodies (Invitrogen) for 1 hour. Finally, cells were washed with PBS three times for 5 minutes and mounted with Vectorshield mounting medium with DAPI (Vector Laboratories). Images were captured by the Leica fluorescence microscope.

For RNP complex and mRNA transfection in differentiated neurons, Cas9 protein and Cas9 mRNA were purchased from IDT and Invitrogen, respectively. Synthesized gRNA was purchased from Invitrogen. On day 35 of neuronal differentiation, cells were transfected with either RNP complex or mRNA using Lipofectamine CRISPRMAX and Lipofectamine MessengerMAX reagent, respectively, according to the manufacturer’s instructions. After three days, genomic DNA was extracted without puromycin selection, and deep-sequencing of PCR amplicons was performed by MiSeq.
Locations of 91 PAS that are located on the coding exons of \textit{HTT} are shown relative to RefSeq NM_002111 (grey vertical lines, exons). PAS in the top and bottom panels represent SNPs that generate NGG PAM sites on plus (39 PAS) and minus strand (55 PAS), respectively. 3 PAS (rs545932099, rs1065747, rs148032171) generate NGG PAM sites on both strands. Letters and arrows in blue and red represent NGG PAM sites generated by reference (71 PAS) and alternative alleles (23 PAS), respectively.
Among 91 PAS that are located on coding exons of \textit{HTT}, 72 SNPs generate NGG PAM sites in at least one of the 8 common \textit{HTT} haplotypes. By contrast, 19 PAS do not generate NGG PAM sites in any of 8 common \textit{HTT} haplotypes because PAM-generating alleles of those SNPs are rare (below 1\% in KGP all populations). As a result, each common haplotype carries 71 PAM sites. Among 72 SNPs that generate NGG PAM sites on common \textit{HTT} haplotypes, 69 PAS generate NGG PAM sequence on all of 8 common \textit{HTT} haplotypes (black arrows), and therefore they do not permit allele-specific CRISPR-Cas9 in HD subjects carrying common diplotypes. However, 3 PAS generate NGG PAM sites on some haplotypes (red arrows). For example, alternative alleles of \textit{rs1065745} (exon 9) and \textit{rs363099} (exon 29) eliminate NGG PAM site selectively on hap.04 and hap.08, respectively. In contrast, alternative allele of \textit{rs362331} (exon 50) generates NGG PAM sites selectively on hap.04 and hap.08.
Supplemental Figure 3. Targeting efficiency and allele specificity of mutant HTT-specific CRISPR-Cas9 in HD neural precursor cells (NPC) derived from HD patients.

A) NPC lines derived from two juvenile onset HD patients (GM09197 and GM04723) were transfected with lentiCRISPRv2 vector expressing SpCas9 and our primary test gRNA for 72 hours without puromycin selection. Subsequently, targeting efficiency and allele frequency were determined by MiSeq analysis of bulk genomic DNA. Since both lines carry normal HTT on the hap.08 haplotype, each MiSeq sequencing read was determined based on the sequence at rs360399. Percentage values in parentheses are relative to either mutant or normal allele (n=1 for each NPC line). For example, 28.2% in NPC from GM09197 means 28.2% of mutant alleles contained indel modifications.

| Cell | Allele | Description            | Number of reads (%) |
|------|--------|------------------------|---------------------|
| NPCs from GM09197 (180 CAG) | Mutant | Sequence with indel | 8,559 (28.2) |
|       |        | Sequence w/o indel    | 22,209 (71.8)       |
|       | Normal | Sequence with indel   | 41,829 (100)        |
|       |        | Sequence w/o indel    | 0 (0)               |
| NPCs from GM04723 (69 CAG) | Mutant | Sequence with indel | 7,807 (29.2) |
|       |        | Sequence w/o indel    | 18,963 (70.8)       |
|       | Normal | Sequence with indel   | 33,192 (100)        |
|       |        | Sequence w/o indel    | 0 (0)               |

B) Whole cell lysate from NPC cell lines transfected with lentiCRISPRv2 vector expressing SpCas9 and our test gRNA for 72 hours without puromycin selection was analyzed by immunoblot analysis using 1F8 antibody to quantify the levels of mutant HTT protein. Our CRISPR-Cas9 without puromycin selection decreased 66.3% and 32.1% of mutant HTT protein levels in NPC from GM09197 (top) and GM04723 (bottom), respectively (n=1 for each NPC line). EV and CRISPR represent empty vector and CRISPR-Cas9 treatment involving our primary test gRNA, respectively.
Supplemental Figure 4. The levels of allele specificity of alternative CRISPR-Cas9 approach targeting rs363099 as gRNA hybridization.

A) In order to evaluate the levels of allele specificity of an alternative CRISPR-Cas9 approach targeting a SNP as the gRNA hybridization (not using as part of PAM site), we evaluated a different gRNA designed to hybridize to a region harboring rs363099. This gRNA utilizes a PAM site that is present on both mutant and normal *HTT* (red underlines), and therefore the PAM site for this alternative approach does not provide allele specificity. Rather, hybridization target sites in the mutant and normal alleles have zero and one mismatch with the gRNA, respectively. A red arrow and alleles in red represent rs363099. The common PAM site are underlined.

B) A patient-derived iPSC line (iPSC-B) was transfected with Cas9 and an alternative gRNA for Miseq analysis of bulk DNA. Identification of alleles was based on rs363099. Approximately, 18% of mutant *HTT* and 0.9% of normal *HTT* were edited by gRNA designed to hybridize to a region containing rs363099 (n=1).

A

| Mutant allele | TTGG AGGGTTTCTCCGCTCAGCCT TGG ATGTTCTTTTCTCA |
|---------------|-----------------------------------------------|
| Normal allele | TTGG AGGGTTTCTTCGCTCAGCCT TGG ATGTTCTTTTCTCA |

rs363099

| Allele | Modification | # of reads (%) |
|--------|--------------|----------------|
| Mutant | Sequence with indel | 3,888 (18.0) |
| Mutant | Sequence w/o indel | 17,680 (82.0) |
| Normal | Sequence with indel | 224 (0.9) |
| Normal | Sequence w/o indel | 26,051 (99.1) |
Supplemental Figure 5. MiSeq analysis of cDNA from single cell clones targeted by our mutant-specific CRISPR-Cas9 strategy.

RT-PCR assays designed to detect the CAG repeat in \(HTT\) mRNA revealed low levels of mutant \(HTT\) mRNA in the targeted clonal lines carrying a juvenile onset CAG repeat (Figure 3B). To confirm this phenomenon, we performed MiSeq analysis of cDNA from targeted single cell clones (n=3; iPSC-C1, iPSC-C2, iPSC-C3). Total RNA was isolated from clonal lines developed by our CRISPR-Cas9 strategy utilizing a PAM site generated by rs363099, and cDNA was analyzed by PCR amplification / MiSeq analysis subsequently. Dashed lines represent deletions. T and C allele at rs363099 respectively represent alleles on the normal and mutant \(HTT\). Numbers of sequence reads (Read count) and the proportion of each allele (Proportion) are shown on the right. In this analysis, a proportion value was calculated by dividing the number of mutant allele read count by the sum of mutant and normal alleles, representing the percentage of targeted mutant allele out of all sequence reads. Note, neither untargeted mutant alleles nor targeted normal alleles were detected in the genomic DNA of those clonal lines.

| Targeted clonal line iPSC-C1 (23 nucleotide deletion) | Read count | Proportion |
|--------------------------------------------------------|------------|------------|
| Normal \(TTTGGAGGGT\) \(TTCTTCGCTC\) \(AGCCTTGGAT\) \(GTTCTTTCTC\) \(AGATACTAGA\) | 16,530 | 93.0 % |
| Mutant \(TTTGGAGGG\) \(TTCTTTTCTC\) \(AGATACTAGA\) | 1,237 | 7.0 % |

| Targeted clonal line iPSC-C2 (5 nucleotide deletion) | Read count | Proportion |
|--------------------------------------------------------|------------|------------|
| Normal \(TTTGGAGGGT\) \(TTCTCGCTC\) \(AGCCTTGGAT\) \(GTTCTTTCTC\) \(AGATACTAGA\) | 55,309 | 93.7 % |
| Mutant \(TTTGGAGGG\) \(TTCTCGGC\) \(GTTCTTTCTC\) \(AGATACTAGA\) | 3,697 | 6.3 % |

| Targeted clonal line iPSC-C3 (25 nucleotide deletion) | Read count | Proportion |
|--------------------------------------------------------|------------|------------|
| Normal \(TTTGGAGGGT\) \(TTCTCGCTC\) \(AGCCTTGGAT\) \(GTTCTTTCTC\) \(AGATACTAGA\) | 38,150 | 94.4 % |
| Mutant \(TTTGGAGGG\) \(TTCTC\) \(GTTCTTTCTC\) \(AGATACTAGA\) | 2,251 | 5.6 % |
Supplemental Figure 6. Impacts of PAS-based mutant-specific NMD-CRISPR-Cas9 on the levels of HTT mRNA and protein.

Three independent targeted clonal lines from an iPSC with a juvenile onset CAG repeat (iPSC-C, 72 CAG) were analyzed to determine the impacts of mutant HTT-specific NMD-CRISPR strategy on the levels of HTT mRNA (A) and protein (B). Band intensities of gel analysis for HTT mRNA (Figure 3D) and protein (Figure 3E) are shown (n=3). Mean values of the mutant and normal HTT in empty vector-treated clones (n=3) were set to 1 as the baseline to compare the band intensities of targeted clonal lines (n=3). Each boxplot shows the maximum, upper quarter, median, lower quarter, and minimum based on 3 independent clones/group. **, p-value < 0.001 by Student t-test (2-tailed). A p-value less than 0.05 was considered nominally significant. NS, not significant (nominal p-value > 0.05). ND, not detected.
Supplemental Figure 7. Ablation of mutant HTT protein in clonal lines targeted by our CRISPR-Cas9.

In order to determine whether our CRISPR-Cas9 selectively decreased the levels of mutant HTT protein, we performed SDS-PAGE analysis for non-HD control, EV-treated HD, and targeted clonal lines (refer to Figure 3B for details). We analyzed an iPSC line carrying a juvenile onset repeat (e.g., iPSC-C) to separate mutant HTT protein from the normal counterpart in immunoblot assays. Immunoblot analysis was performed using MAB2166 antibody (A; 4% gel; detecting both mutant and normal HTT protein) and mutant-specific 1F8 antibody (B; 6% gel; detecting polyQ protein). The upper band in EV-treated HD lines (middle lanes in panel A) and unique bands in EV-treated HD lines (middle lanes in panel B) represent full length mutant HTT protein (red arrows). A green arrow in panel A indicates normal HTT. Regions focusing on HTT protein are shown in Figure 3E and 3F. The 1F8 antibody was raised against polyglutamine tract, and therefore, smaller bands in panel B may represent non-specific and/or other polyglutamine-containing proteins. EV represents empty vector-treated control clones. NMD-CRISPR represents our mutant-specific NMD-CRISPR-Cas9 based on rs363099.
Supplemental Figure 8. The lack of fragmented mutant HTT protein in targeted clonal lines by our CRISPR-Cas9 strategy.

In order to determine whether our mutant-specific CRISPR-Cas9 strategy generates fragmented mutant HTT protein, we performed immunoblot analysis of non-HD controls and clonal lines established by empty-vector (EV) or NMD-CRISPR-Cas9 treatment (iPSC-C1, iPSC-C2, iPSC-C3). Focusing on evaluating smaller molecular weight proteins, whole cell extracts were resolved for a relatively short period of time (A, 6% gel for 55 min running; B, 4-20% gradient gel for 55 min running). Therefore, full length mutant and normal HTT proteins were not separated. Membranes were probed using MAB2166 antibody (A) and N-terminal antibody (B, N17). Red arrows indicate full length total HTT protein, representing the sum of mutant and normal HTT protein.
Ten sites are heterozygous in EV-treated (EV; n=12) and targeted clonal lines (CRISPR; n=12). For each heterozygous SNP site, allele-specific analysis (ASE) of RNAseq data generated 4 groups of read count data such as 1) number of counts of alleles on the normal HTT in EV-treated clones (A, EV), 2) counts of alleles on the normal HTT in CRISPR-Cas9-treated clones (A, NMD-CRISPR), 3) counts of alleles on the mutant HTT in EV-treated clones (B; EV), and 4) counts of alleles on the mutant HTT in CRISPR-Cas9-treated clones (B, NMD-CRISPR). We then calculated the mean read count for each group. Each box plot shows the distribution of read counts of 10 sites in 12 samples. P-values represent uncorrected nominal p-values from Student t-test (2-tailed; n=12). An uncorrected p-value less than 0.05 was considered nominally significant.
Supplemental Figure 10. Differential gene expression analysis comparing two groups of randomly assigned samples.

To explain the atypical shape of the volcano plot in our DGE analysis of RNAseq data, we hypothesized that our RNAseq data represent a distribution of null statistics that do not involve significant differences except for HTT. To test this hypothesis, we performed DGE analysis comparing two groups of samples, each group comprising 12 randomly selected samples (6 EV-treated clones and 6 CRISPR-Cas9-treated clones). Subsequently, a volcano plot was drawn to evaluate the overall distribution of test statistics of random sample comparisons. Y-axis and X-axis represent significance (-log10(FDR)) and log2(fold-change), respectively. A red horizontal line represents the FDR significance (FDR, 0.05).
Supplemental Figure 11. GUIDE-seq assay to detect potential off-targets of our NMD-CRISPR-Cas9 strategy.

A) The GUIDE-seq assay was performed to evaluate genome-wide off-target events in our NMD-CRISPR-Cas9 strategy. HEK-293T cells were treated with Cas9, our test gRNA, and a GUIDE-seq double-stranded oligodeoxynucleotide (dsODN) tag, followed by sequencing to identify dsODN integration as a proxy for CRISPR-Cas9-mediated double-strand breaks. The top line represents the on-target site (HTT, 29th exon); off-target locations with dsODN integration are shown below. Each dot and colored letter represent a perfect match and a mismatch, respectively. Number of sequence reads with tag represent the number of unique MiSeq sequencing reads containing dsODN, indicative of the levels of off-target editing.

B) Potential off-target sites identified in HEK293T cells were further evaluated in patient-derived iPSC lines. The second transfection experiment of iPSC-A and the first transfection experiment of iPSC-B which showed the highest editing efficiencies (refer to Supplemental Table 2) were subjected to PCR amplification using site-specific PCR primers and MiSeq analysis. Percent editing values represent the proportion of sequence reads...
with indels out of all alleles for a given site. Corresponding empty vector-treated controls were also sequenced, showing low levels of modified alleles at off-target site #2 potentially due to sequencing errors.
Supplemental Figure 12. Allele specificity of NMD-CRISPR-Cas9 in differentiated neurons.

A) A patient-derived iPSC line with an adult onset CAG repeat (iPSC-B, 42 CAG) was differentiated into neurons. Subsequently, the expression levels of an iPSC marker (OCT4), neuronal markers (MAP2, GAD67, TH, VGLUT1), and a housekeeping gene (GAPDH) in iPSC-B (blue) and differentiated neurons (orange) were determined by qRT-PCR assays. Fold induction (Y-axis) of an iPSC marker and neuronal markers were calculated relative to the expression levels of those in neurons and iPSC, respectively.

B) A representative neuronal marker staining is shown (magnification, 400X). DAPI (blue) and TUBB3 (red) represent nuclear and mature neurite marker, respectively.

C) Differentiated neurons were treated with RNP complex (RNP delivery; Cas9 protein-gRNA) or mRNA (mRNA delivery; Cas9 mRNA and gRNA), and then subjected to MiSeq analysis to evaluate the levels of editing efficiency and allele specificity.

| Allele     | Description                | Percentage of sequence reads |
|------------|----------------------------|-----------------------------|
|            | RNP delivery | mRNA delivery |
| Mutant HTT | Sequence reads with indels | 4.2                         | 2.5 |
|            | Sequence reads without indels | 95.8                        | 97.5 |
| Normal HTT | Sequence reads with indels | 0                           | 0   |
|            | Sequence reads without indels | 100                         | 100 |
Supplemental Table 1. A complete list of PAS on coding exons of HTT.

To develop allele-specific NMD-CRISPR-Cas9 strategies, PAS that generate or eliminate NGG PAM sites for wild-type SpCas9 on the coding sequence of HTT were identified by analyzing KGP (phase 3) data set. A total of 91 coding SNPs alter PAM sites for SpCas9 (i.e., NGG), representing candidate PAS that may permit allele-specific CRISPR-Cas9. Allele frequency (AF) was based on all populations in the KGP data set.

| SNP ID   | Position (hg19) | Reference | Alternative | AF (%) |
|----------|-----------------|-----------|-------------|--------|
| rs184820567 | 3107140       | C         | T           | 0.019968 |
| rs368339242 | 3107143       | G         | C           | 0.119808 |
| rs567202171 | 3109053       | G         | C           | 0.019968 |
| rs538952960 | 3109056       | A         | G           | 0.019968 |
| rs374267492 | 3109063       | C         | T           | 0.019968 |
| rs548352092 | 3117133       | A         | G           | 0.019968 |
| rs181961779 | 3117891       | C         | T           | 0.019968 |
| rs114476023 | 3122969       | G         | C           | 0.978435 |
| rs371255782 | 3123015       | C         | T           | 0.019968 |
| rs1065745  | 3123074       | C         | T           | 7.22843 |
| rs374296062 | 3123101       | C         | T           | 0.019968 |
| rs189018320 | 3123124       | G         | T           | 0.019968 |
| rs545932099 | 3127303       | C         | G           | 0.019968 |
| rs148032171 | 3129023       | G         | C           | 0.039936 |
| rs536416239 | 3129043       | C         | T           | 0.019968 |
| rs201861325 | 3129129       | G         | A           | 0.019968 |
| rs2301367  | 3129124       | G         | A           | 0.039936 |
| rs537369613 | 3129158       | G         | A           | 0.019968 |
| rs573811022 | 3129205       | C         | T           | 0.019968 |
| rs118005095 | 3129240       | G         | A           | 0.61901  |
| rs576787839 | 3131750       | G         | A           | 0.019968 |
| rs201938386 | 3132088       | A         | G           | 0.019968 |
| rs374016128 | 3132131       | C         | T           | 0.019968 |
| rs34389685  | 3133113       | G         | A           | 0.199681 |
| rs150027738 | 3134406       | T         | C           | 0.079872 |
| rs539073497 | 3136241       | G         | A           | 0.019968 |
| rs192933099 | 3137663       | A         | C           | 0.019968 |
| rs363075   | 3137674       | G         | A           | 2.3762   |
| rs188101620 | 3138036       | C         | T           | 0.119808 |
| rs549028339 | 3142283       | C         | A           | 0.019968 |
| rs527729099 | 3146920       | C         | T           | 0.019968 |
| rs1065746  | 3148624       | G         | C           | 0.019968 |
| rs561719779 | 3148659       | C         | T           | 0.019968 |
| rs1065747  | 3149780       | C         | G           | 0.019968 |
| rs542613235 | 3149831       | G         | A           | 0.019968 |
| rs200581635 | 3149844       | C         | T           | 0.019968 |
| rs528411716 | 3149868       | C         | T           | 0.019968 |
| rs188342053 | 3158882       | C         | A           | 0.019968 |
| rs363099   | 3162056       | C         | T           | 21.3858  |
| rs534902248 | 3174067       | A         | G           | 0.019968 |
| rs                | Position | Allele 1 | Allele 2 | p-value |
|------------------|-----------|----------|----------|---------|
| rs534610900      | 3180138   | G        | A        | 0.019968 |
| rs116293982      | 3182267   | C        | G        | 0.219649 |
| rs150003356      | 3189510   | T        | C        | 0.09984  |
| rs201878423      | 3201532   | C        | G        | 0.019968 |
| rs558887183      | 3201637   | C        | G        | 0.019968 |
| rs201094471      | 3208572   | G        | A        | 0.159744 |
| rs200125263      | 3208575   | C        | G        | 0.019968 |
| rs200077637      | 3208632   | C        | T        | 0.019968 |
| rs540936636      | 3209018   | C        | T        | 0.039936 |
| rs181217572      | 3209071   | G        | A        | 0.039936 |
| rs201646519      | 3210567   | T        | C        | 0.09984  |
| rs201104173      | 3210589   | C        | T        | 0.019968 |
| rs547119123      | 3211676   | G        | T        | 0.019968 |
| rs531260079      | 3213835   | G        | A        | 0.019968 |
| rs565501560      | 3214317   | C        | T        | 0.019968 |
| rs561152154      | 3215801   | C        | T        | 0.019968 |
| rs573397591      | 3215822   | C        | T        | 0.139776 |
| rs362331         | 3215835   | T        | C        | 44.1294  |
| rs191063722      | 3216913   | G        | A        | 0.019968 |
| rs377176195      | 3219553   | G        | A        | 0.019968 |
| rs369228257      | 3221488   | A        | G        | 0.039936 |
| rs182132217      | 3225168   | C        | T        | 0.179712 |
| rs560138731      | 3225216   | G        | A        | 0.09984  |
| rs570565498      | 3227431   | C        | T        | 0.019968 |
| rs201944095      | 3230381   | C        | A        | 0.019968 |
| rs201422922      | 3230431   | C        | G        | 0.039936 |
| rs559860075      | 3230452   | C        | T        | 0.019968 |
| rs547238016      | 3230668   | C        | T        | 0.019968 |
| rs570616330      | 3230736   | C        | T        | 0.019968 |
| rs536877956      | 3231619   | A        | G        | 0.019968 |
| rs142891110      | 3231622   | G        | C        | 0.019968 |
| rs573475907      | 3231643   | G        | C        | 0.019968 |
| rs201467885      | 3231679   | A        | C        | 0.079872 |
| rs200586785      | 3231706   | A        | G        | 0.019968 |
| rs2229985        | 3231757   | C        | T        | 0.379393 |
| rs529410176      | 3234904   | G        | A        | 0.039936 |
| rs71608259       | 3235036   | G        | A        | 0.119808 |
| rs144891713      | 3237100   | C        | T        | 0.019968 |
| rs567166179      | 3237101   | G        | A        | 0.079872 |
| rs573732436      | 3237314   | G        | T        | 0.019968 |
| rs536481227      | 3237315   | A        | G        | 0.019968 |
| rs199535935      | 3237376   | C        | T        | 0.119808 |
| rs564865166      | 3237433   | G        | A        | 0.019968 |
| rs200392606      | 3240187   | T        | C        | 0.019968 |
| rs373377741      | 3240324   | C        | A        | 0.059904 |
| rs371718523      | 3240685   | C        | G        | 0.019968 |
| rs532740914      | 3240700   | G        | A        | 0.019968 |
| rs563636870      | 3241573   | C        | T        | 0.019968 |
| rs542631628      | 3241634   | C        | T        | 0.019968 |
| rs200112468      | 3241720   | C        | T        | 0.039936 |
| rs362308         | 3241735   | T        | C        | 0.059904 |
Supplemental Table 2. Allele specificities of rs363099-based NMD-CRISPR-Cas9.

Two independent iPSC lines with adult onset CAG repeats were co-transfected with Cas9 and our primary test gRNA depending on a PAM site generated by rs363099. Subsequently, bulk DNA samples were isolated without Puromycin selection, and subjected to PCR / MiSeq analysis. Three independent transfection experiments were performed for each line. $, percentage of out-of-frame indels; #, percentage of in-frame indels. MiSeq analysis of empty vector-treated patient-derived iPSC lines did not reveal any modification at the target site (not shown).

### A

| Allele       | Description              | 1st experiment | 2nd experiment | 3rd experiment |
|--------------|--------------------------|----------------|----------------|----------------|
| Mutant       | Sequence with indel      | 1668 (16.03 $ / 0 #) | 2390 (16.22 $ / 0 #) | 2097 (15.97 $ / 0 #) |
|              | Sequence w/o indel       | 8737 (83.97)   | 12345 (83.78)   | 11037 (84.03)   |
| Normal       | Sequence with indel      | 0 (0)          | 0 (0)           | 0 (0)           |
|              | Sequence w/o indel       | 11508 (100)    | 16111 (100)     | 13907 (100)     |

### B

| Allele       | Description              | 1st experiment | 2nd experiment | 3rd experiment |
|--------------|--------------------------|----------------|----------------|----------------|
| Mutant       | Sequence with indel      | 4181 (24.98 $ / 0.9 #) | 6421 (22.32 $ / 2.35 #) | 6565 (22.45 $ / 1.12 #) |
|              | Sequence w/o indel       | 11972 (74.12)  | 19607 (75.33)  | 21287 (76.43)  |
| Normal       | Sequence with indel      | 0 (0)          | 0 (0)           | 0 (0)           |
|              | Sequence w/o indel       | 19632 (100)    | 30898 (100)     | 32667 (100)     |
Supplemental Table 3. Characterization of targeted single cell clones for RNAseq analysis.

For RNAseq analysis, we transfected two iPSC lines carrying adult onset CAG repeats with PX551 EFS vector expressing SpCas9 and PX552 EFS vector expressing our primary test gRNA. Subsequently, a total of 12 independent single cell clones were established by limited dilution (without selection) for the experimental group. Twelve targeted clonal lines were then subjected to Sanger sequencing of genomic DNA, MiSeq analysis of genomic DNA, and cDNA to determine targeted alleles and expression levels of mutant and normal \( HTT \) mRNA, respectively. All targeted clones confirmed out-of-frame indels in the mutant \( HTT \) by Sanger sequencing and MiSeq of DNA; MiSeq analysis of cDNA confirmed those modifications. As observed in other clonal lines, targeted clonal lines for RNAseq analysis expressed a small amount of mutant \( HTT \) RNA (see cDNA MiSeq column), suggesting that MiSeq analysis detected newly produced mutant \( HTT \) mRNA that had not been subjected to NMD process. Percent values were relative to the sum of mutant and normal alleles.

| Parental line | NMD-CRISPR-Cas9 Clone # | Allele | Modification | DNA Sanger % | DNA MiSeq % | cDNA MiSeq % |
|---------------|-------------------------|--------|--------------|--------------|-------------|--------------|
| iPSC-A (46 CAG) | 1 | Mutant | 1 nucleotide | 50.57 | 8.4 |
| | | Normal | No change | 49.43 | 91.6 |
| | 2 | Mutant | 2 nucleotides | 54.09 | 7.0 |
| | | Normal | No change | 45.91 | 93.0 |
| | 3 | Mutant | 1 nucleotide | 51.12 | 8.8 |
| | | Normal | No change | 48.88 | 91.2 |
| | 4 | Mutant | 5 nucleotides | 48.33 | 9.1 |
| | | Normal | No change | 51.67 | 90.9 |
| | 5 | Mutant | 1 nucleotide | 50.88 | 9.5 |
| | | Normal | No change | 49.12 | 90.5 |
| | 6 | Mutant | 5 nucleotides | 47.26 | 8.2 |
| | | Normal | No change | 52.74 | 91.8 |
| iPSC-B (42 CAG) | 1 | Mutant | 1 nucleotide | 51.32 | 10.7 |
| | | Normal | No change | 48.68 | 89.3 |
| | 2 | Mutant | 1 nucleotide | 49.71 | 7.0 |
| | | Normal | No change | 50.29 | 93.0 |
| | 3 | Mutant | 70 nucleotides | 24.60 | 6.5 |
| | | Normal | No change | 75.40 | 93.5 |
| | 4 | Mutant | 17 nucleotides | 49.04 | 11.8 |
| | | Normal | No change | 50.96 | 88.2 |
| | 5 | Mutant | 22 nucleotides | 34.34 | 13.3 |
| | | Normal | No change | 65.66 | 86.7 |
| | 6 | Mutant | 1 nucleotide | 50.64 | 12.0 |
| | | Normal | No change | 49.36 | 88.0 |
Supplemental Table 4. Empty vector treated control single cell clones for RNAseq analysis.

For control samples in RNAseq analysis, we also transfected same iPSC lines carrying adult onset CAG repeats with PX551 EFS vector expressing SpCas9 and empty PX552 EFS vector. Subsequently, a total of 12 independent clones were developed. All twelve control lines were subject to Sanger sequencing, confirming the lack of modification at the target site. For 6 selected clonal lines, we also performed MiSeq analysis of DNA, confirming the lack of modification and balanced allele counts in DNA. Further, we performed MiSeq analysis of cDNA samples for two selected clonal lines (iPSC-A1 and iPSC-B1), confirming the lack of modification and balanced expression of mutant and normal HTT in RNA. ND, not determined.

| Parental line | Empty vector Clone # | Allele | DNA Sanger Modification | DNA MiSeq % | RNA MiSeq % |
|---------------|----------------------|--------|-------------------------|-------------|-------------|
| iPSC-A (46 CAG) | 1 | Mutant | None | 49.1 | 48.5 |
|              |   | Normal | None | 50.9 | 51.5 |
|              | 2 | Mutant | None | 49.4 | ND |
|              |   | Normal | None | 50.6 | ND |
|              | 3 | Mutant | None | 49.4 | ND |
|              |   | Normal | None | 50.6 | ND |
|              | 4 | Mutant | None | ND | ND |
|              |   | Normal | None | ND | ND |
|              | 5 | Mutant | None | ND | ND |
|              |   | Normal | None | ND | ND |
|              | 6 | Mutant | None | ND | ND |
|              |   | Normal | None | ND | ND |
| iPSC-B (42 CAG) | 7 | Mutant | None | 49.5 | 49.1 |
|              |   | Normal | None | 50.5 | 50.9 |
|              | 8 | Mutant | None | 46.1 | ND |
|              |   | Normal | None | 53.9 | ND |
|              | 9 | Mutant | None | 47.1 | ND |
|              |   | Normal | None | 52.9 | ND |
|              | 10 | Mutant | None | ND | ND |
|             |   | Normal | None | ND | ND |
|              | 11 | Mutant | None | ND | ND |
|             |   | Normal | None | ND | ND |
|              | 12 | Mutant | None | ND | ND |
Supplemental Table 5. DGE analysis of RNAseq for potential off-targets identified by GUIDE-seq analysis.

GUIDE-seq analysis of HEK 293T cells revealed 6 potential off-target sites. To understand the impacts of our CRISPR-Cas9 strategy on the expression levels of potential off-target sites in patient-derived cells, we evaluated RNAseq data. Since 5 potential off-target sites are located in intergenic regions, we analyzed the nearest genes (genes in brackets). FDR represents adjusted p-value by false discovery rate.

| GUIDE-seq analysis | RNAseq analysis |
|-------------------|-----------------|
| Description       | Gene [nearest gene] | Fold-change | FDR   |
| Off-target 1      | [ANPEP]          | 0.97        | 0.9987|
| Off-target 2      | [BTG1]           | 1.10        | 0.9987|
| Off-target 3      | [PABPC4L]        | 0.96        | 0.9987|
| Off-target 4      | TAOK2            | 1.03        | 0.9987|
| Off-target 5      | [ACTR3B]         | 0.91        | 0.9987|
| Off-target 6      | [TMEM132D]       | 1.08        | 0.9987|
Supplemental Table 6. Predicted off-targets.

Potential off-target sites predicted by Cas-OFFinder website are summarized. Genomic coordinates were based on GRCh37/hg19. Genes in brackets represent the nearest genes to the predicted off-target site. Lower case letters in the sequence represent mismatches. Dashed lines represent bulges.

| Chr | Coordinate | DNA            | crRNA                      | Mismatch | Bulge size | Gene       |
|-----|------------|----------------|----------------------------|----------|------------|------------|
| 1   | 94591387   | AAAaAAC-TCCAAGGCTAGTGG | AAAGAACATCCAAGGCTAGNNG    | 1        | 1 (RNA)    | [ABCA4]    |
| 1   | 94591387   | AAA-AaacTCCAAGGCTAGTGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [ABCA4]    |
| 1   | 94591387   | AAAaAACL-TCCAAGGCTAGTGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [ABCA4]    |
| 1   | 94591387   | AAAaAA-cTCCAAGGCTAGTGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [ABCA4]    |
| 1   | 227567892  | AAAGAACACTCTAAGAGCTGIGGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (DNA)    | [CDC42BPA] |
| 2   | 26899611   | tAAGAGg-TCCAAGGCTAGGAGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [KCNK3]    |
| 2   | 26899611   | tAAGAGA-gTCCAAGGCTAGGAGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [KCNK3]    |
| 2   | 60097499   | AAGAACATCAAGGCTAGGAGG  | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (DNA)    | [MIR4432HG]|
| 2   | 60097499   | AAGAACATCAAGGCTAGGAGG  | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (DNA)    | [MIR4432HG]|
| 2   | 64610397   | AAAGAAC-TCCAGGCTAGGAGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [MIR4432B] |
| 2   | 109069020  | AAA-AACaaCAAAGGCTAGGAGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | GCC2       |
| 2   | 110450075  | AAACAAAATCCAA-CTGAGAGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [SOHAHC]   |
| 2   | 110450075  | AAACAAAATCCAA-CTGAGAGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [SOHAHC]   |
| 2   | 193426297  | AAAGAACATCCAA-GaTGGTGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [PCGEM1]   |
| 2   | 193426297  | AAAGAACATCCAA-GaTGGTGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [PCGEM1]   |
| 2   | 193426297  | AAAGAACATCCAA-GaTGGTGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [PCGEM1]   |
| 2   | 218239665  | AAAGAATTTCCAAGGCTAGGAGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (DNA)    | DIRC3      |
| 2   | 218239665  | AAAGAATTTCCAAGGCTAGGAGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (DNA)    | DIRC3      |
| 2   | 227271147  | AAAGAACATCTGAG-AGTAGGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [LOC646736]|
| 3   | 19663504   | AAAG-ACATaCAAAGGCTAGTGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [KCNH8]    |
| 3   | 19663504   | AAAG-ACATaCAAAGGCTAGTGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [KCNH8]    |
| 3   | 101648922  | AAAGAACATGCAAGGCT-AGGAGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [LINC02085]|
| 3   | 121729858  | AAAGAACATGCAAGGCT-AGGAGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | ILDR1      |
| 3   | 121729858  | AAAGAACATGCAAGGCT-AGGAGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | ILDR1      |
| 3   | 126732945  | AcAGAACATCCAGG-TAGTGGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | PLXNA1     |
| 3   | 126732945  | AcAGAACATCCAGG-TAGTGGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | PLXNA1     |
| 3   | 126732945  | AcAGAACATCCAGG-TAGTGGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | PLXNA1     |
| 3   | 135757902  | AAAGAACATCC-AAGGCT-AGGAGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | PPP2R3A    |
| 3   | 135757902  | AAAGAACATCC-AAGGCT-AGGAGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | PPP2R3A    |
| 3   | 195922254  | AAAGAACATCC-AAGGCT-AGGAGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [ZDHHC19]  |
| 4   | 46524862   | AAcAACCITCCA-GGCTAGTGGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [GABRA2]   |
| 4   | 46524862   | AAcAACCITCCA-GGCTAGTGGG | AAAGAACATCCAAGGCTAGNNG    | 2        | 1 (RNA)    | [GABRA2]   |
|    |       |                  |                                                   |    |                      |
|----|-------|-----------------|--------------------------------------------------|----|----------------------|
| 4  | 85525713| tAAGAAaA-CCAAGGCTGAGAGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | CDS1 |
| 4  | 85525713| tAAGAA-AaCCAAGGCTGAGAGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | CDS1 |
| 4  | 185093254| tAAGAA-AaCCAAGGCTGAGAGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | ENPP6 |
| 5  | 185168971| tAAGAA-AaCCAAGGCTGAGAGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | ENPP6 |
| 5  | 12838887 | AAAGAACATCCAAGGCTGAGNGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (DNA)       | [IRX4] |
| 5  | 12838887 | AAAGAACATCCAAGGCTGAGNGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (DNA)       | [SLC27A6] |
| 5  | 12838887 | AAAGAACATCCAAGGCTGAGNGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (DNA)       | [SLC27A6] |
| 6  | 154300413| tAAGAA-AgaCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | ECSCR |
| 6  | 157954509| tAAGAA-AgaCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | GEMIN5 |
| 6  | 157954509| tAAGAA-AgaCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | LOC101927697 |
| 7  | 120098679| AAgGAACcTCCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | KCND2 |
| 7  | 120098679| AAgGAACcTCCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | KCND2 |
| 7  | 120098679| AAgGAACcTCCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | KCND2 |
| 7  | 120098680| A-AgaATCCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | KCND2 |
| 7  | 120098680| A-AgaATCCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | KCND2 |
| 7  | 149550143| AAgGAACcTCCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | CNTNAP3 |
| 7  | 149550143| AAgGAACcTCCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | CNTNAP3 |
| 8  | 39050426| cAcACATCCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | SFRP1 |
| 8  | 39050426| cAcACATCCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | SFRP1 |
| 9  | 65685470| cAAcAACATCCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | CNTNAP3 |
| 9  | 65685470| cAAcAACATCCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | CNTNAP3 |
| 9  | 116590974| tAAGAAaA-gCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | ZNF618 |
| 9  | 116590974| tAAGAAaA-gCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | ZNF618 |
| 9  | 116590974| tAAGAAaA-gCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | ZNF618 |
| 9  | 116590974| tAAGAAaA-gCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | ZNF618 |
| 9  | 133907798| AAgGAACcTCCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | LAMC3 |
| 9  | 133907798| AAgGAACcTCCAAGGCTGAGGG | AAAGAACATCCAAGGCTGAGNGG | 2  | 1 (RNA)       | LAMC3 |
| Chromosome | Position | Gene 1 | Gene 2 | Gene 3 | Gene 4 | Gene 5 | Gene 6 | Gene 7 | Gene 8 | Gene 9 | Gene 10 | Gene 11 | Gene 12 | Gene 13 | Gene 14 |
|------------|----------|--------|--------|--------|--------|--------|--------|--------|--------|--------|---------|---------|---------|---------|---------|
| 10         | 13608321 | AAAGAACccCCAAGGCTG-GGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | [PRPF18] |
| 10         | 61614633 | AAAGAACA-gaAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | CCD6 |
| 10         | 61614633 | AAAGAACAg-aAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | CCD6 |
| 10         | 115830553 | AAAGAACAtaCCAAGGCT-GCGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | [ADRB1] |
| 11         | 17899475 | AAAGAAC-cAacAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | SERGEF |
| 11         | 27318534 | AAAGAACg-AaAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | [CCDC34] |
| 11         | 27407987 | AAAGAACAg-aAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | LGR4 |
| 11         | 27407987 | AAAGAAAg-aAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | LGR4 |
| 11         | 37981053 | cAAAcACATCC-AGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | [LOC105376633] |
| 11         | 37981053 | cAAAcACATCC-AGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | [LOC105376633] |
| 11         | 39246550 | AAAGAACAcCCAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | [LINC01493] |
| 11         | 69708486 | AAAGAACGccCCAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (DNA) | [FGF3] |
| 11         | 89301552 | cAAAcACATCC-AGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | NOX4 |
| 11         | 131203198 | AAAGAACAcCCAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | NTM |
| 11         | 131203198 | AAAGAACAcCCAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | NTM |
| 12         | 92165289 | gAAAGAACATCCAGGCTAGTGG | A-AAAGAACATCCAAGGCTAGNGG | 2 | 1 (DNA) | [LINC01619] |
| 12         | 92165289 | gAAAGAACATCCAGGCTAGTGG | A-AAAGAACATCCAAGGCTAGNGG | 2 | 1 (DNA) | [LINC01619] |
| 12         | 92165289 | gAAAGAACATCCAGGCTAGTGG | A-AAAGAACATCCAAGGCTAGNGG | 2 | 1 (DNA) | [LINC01619] |
| 12         | 92165290 | AAAGAACATCCAGGCTAGTGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (DNA) | [LINC01619] |
| 12         | 92165291 | AA-GAACATCCAGGCTAGTGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (DNA) | [LINC01619] |
| 12         | 92165291 | AA-GAACATCCAGGCTAGTGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (DNA) | [LINC01619] |
| 12         | 92165291 | AA-GAACATCCAGGCTAGTGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (DNA) | [LINC01619] |
| 12         | 92165291 | AA-GAACATCCAGGCTAGTGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (DNA) | [LINC01619] |
| 12         | 92165291 | AA-GAACATCCAGGCTAGTGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (DNA) | [LINC01619] |
| 12         | 99282433 | AAAGAACAcCCAAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | ANK1B |
| 12         | 120161757 | AAAGAACATCCAAgCCTTGGAGGGG | AAAGAACATCCAAGGCTAGNGG | 1 | 1 (DNA) | CIT |
| 12         | 120161757 | AAAGAACATCCAAgCCTTGGAGGGG | AAAGAACATCCAAGGCTAGNGG | 1 | 1 (DNA) | CIT |
| 12         | 120161757 | AAAGAACATCCAAgCCTTGGAGGGG | AAAGAACATCCAAGGCTAGNGG | 1 | 1 (DNA) | CIT |
| 12         | 120161757 | AAAGAACATCCAAgCCTTGGAGGGG | AAAGAACATCCAAGGCTAGNGG | 1 | 1 (DNA) | CIT |
| 13         | 73321278 | AAAGAACAgccCCAAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | BORA |
| 14         | 49144352 | cAAAcACATCC-AGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | [LINC00648] |
| 14         | 49144352 | cAAAcACATCC-AGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | [LINC00648] |
| 14         | 65035930 | AAAGAcitCA-CCAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 (RNA) | PPP1R36 |
| 14         | 102426650 | AAAGAACAAATCCAAGGCT-AGAGG | AAAGAACATCCAAGGCTAGNGG | 1 | 1 (RNA) | [DYNC1H1] |
|   |   |   |   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|---|---|---|
| 14 | 102426650 | AAAGAAaATCCAAGGCTa-GAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [DYNC1H1] |
| 14 | 102426650 | AAAGAAaATCCAAGGCTIaAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [DYNC1H1] |
| 15 | 60445586 | tAAGAAaA-CCAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [FOXB1] |
| 15 | 60445586 | tAAGAAaA-CCAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [FOXB1] |
| 15 | 84243595 | tAAAGcCACTCGa-GCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | SH3GL3 |
| 15 | 84243595 | tAAAGcCACTCGa-GCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | SH3GL3 |
| 15 | 90327253 | t-1GAACATCCAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [ANPEP] |
| 15 | 90327253 | t-1GAACATCCAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [ANPEP] |
| 15 | 90327253 | tT-1GAACATCCAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [ANPEP] |
| 15 | 90327253 | tT-1GAACATCCAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [ANPEP] |
| 16 | 1905010  | AAAGcAcAGCAGAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | DNA | MEIOB |
| 16 | 14500236 | A-AGAACATcAcAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [PARN] |
| 16 | 14500236 | A-AGAACATcAcAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [PARN] |
| 16 | 14604020 | AAAGAAAGCAGcAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | PARN |
| 16 | 24820222 | AAAGAGAaATCCAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | DNA | TNRC6A |
| 16 | 27769986 | A-AGAACATCCAgAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | KIAA0556 |
| 16 | 27769986 | A-AGAACATCCAgAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | KIAA0556 |
| 16 | 27769986 | A-AGAACATCCAgAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | KIAA0556 |
| 16 | 53553406 | AA-AGAACATCCAgAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [AKTIP] |
| 16 | 53553406 | AA-AGAACATCCAgAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [AKTIP] |
| 16 | 73051152 | AAG-AGAACATCCAgAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | ZFHX3 |
| 16 | 73051152 | AAG-AGAACATCCAgAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | ZFHX3 |
| 16 | 10639941 | AAAGAAaATCCAAGGCTAGAGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | TMEM220-AS1 |
| 17 | 63425587 | gAAAGAAC-CCAAGGGTGTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [CRAT40] |
| 18 | 29136616 | AAAGAACATCCAAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [CRAT40] |
| 18 | 32182544 | A-AGAACATCCAgAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | DSG2-AS1 |
| 18 | 45913442 | AAAGAACATCCAgAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | DSG2-AS1 |
| 18 | 45913442 | AAAGAACATCCAgAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | DSG2-AS1 |
| 19 | 767169  | AAAGAACATCTAAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [MISP] |
| 19 | 767169  | AAAGAACATCTAAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [MISP] |
| 19 | 7291791 | AAAGAACATCCAAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | INSR |
| 19 | 7291791 | AAAGAACATCCAAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | INSR |
| 19 | 7291791 | AAAGAACATCCAAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | INSR |
| 19 | 35328785 | AAAGAACATCCAgAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [LINC01801] |
| 19 | 35328785 | AAAGAACATCCAgAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [LINC01801] |
| 23 | 65573412 | AAA-AAAaATCCAAGGCTAGGGG | AAAGAACATCCAAGGCTAGNGG | 2 | 1 | RNA | [HEPH] |
|   |       |          |          |       |       |       |
|---|-------|----------|----------|-------|-------|-------|
| 23| 65573412 | AAAaAA-ATCCcAGGCTGAGAGG | AAAGAACATCCAGGCTGAGNGG | 2 | 1 (RNA) | [HEPH] |
| 23| 78841448 | tAAcAACATCCAAG-CTGAGGGG | AAAGAACATCCAGGCTGAGNGG | 2 | 1 (RNA) | [ITM2A] |
| 23| 78841448 | tAAcAACATCCAAG-CTGAGGGG | AAAGAACATCCAGGCTGAGNGG | 2 | 1 (RNA) | [ITM2A] |
| 23| 147745920 | AAAG-ACAtCiAGGCTGAGGGG | AAAGAACATCCAGGCTGAGNGG | 2 | 1 (RNA) | AFF2   |
| 23| 147745920 | AAAGA-CATCiAGGCTGAGGGG | AAAGAACATCCAGGCTGAGNGG | 2 | 1 (RNA) | AFF2   |
GUIDE-seq analysis and subsequent genomic annotation did not predict significant impacts of rs363099-based CRISPR-Cas9 strategy on the expression levels of protein-coding genes because potential off-target sites reside at intergenic regions or introns. In addition, RNAseq data analysis of targeted single cell clones did not reveal recurrent off-targeting. We further evaluated algorithmically predicted off-target sites (refer to Supplemental Table 6 for full list), focusing on the best predicted off-target site (chr12: 92165291-92165313 with one mismatch). For this, we transfected iPSC-C cells with SpCas9 and our test gRNA, and performed MiSeq analysis of pooled DNA sample. A total of 112,514 MiSeq sequencing reads were analyzed (n=1), but none of them involved indels, indicating this site was not targeted.

| Locus | Type                  | Number of reads (%) |
|-------|-----------------------|---------------------|
| chr12: 92165291-92165313 | Sequence w/o indels  | 112,514 (100)       |
|       | Sequence with indels  | 0 (0)               |
| Name | Sequence | Description |
|------|----------|-------------|
| **Oligo 1** | ccgAAAGAACATCCAAGGCTGAG | Cloning of gRNA into lentiCRISPRv2 vector |
| **Oligo 2** | aaacCTCAGCCTTGGATGTTCTTTc | Cloning of gRNA into PX552 vector |
| **Oligo 3** | accAAAGAACATCCAAGGCTGAG | Cloning of gRNA into PX552 vector |

| **For** | **Rev** | | |
|---------|---------|-------------------|
| **Forward 1** | **Reverse 1** | CCAGTAACCCTGTTCCTC | DNA and RNA Miseq |
| **Forward 2** | **Reverse 2** | ATGAAGGCCTTGGATGTTCTTTc | HTT CAG region PCR |
| **Forward 3** | **Reverse 3** | GGGTGGAGAGCTCCTGAGGGA | MiSeq analysis of the best predicted off-target |
| **Forward 4** | **Reverse 4** | TGCTGGAGCTCCATGATTGC | Miseq analysis of off-target #1 |
| **Forward 5** | **Reverse 5** | TAGAGTCAGCAGAGATAGGGA | Miseq analysis of off-target #2 |
| **Forward 6** | **Reverse 6** | TAGTGGCAGAACATTTGTCAGCA | Miseq analysis of off-target #3 |
| **Forward 7** | **Reverse 7** | GTTATCTCGGTCTGATTC | Miseq analysis of off-target #4 |
| **Forward 8** | **Reverse 8** | GCTATGGTCTTCTTGGTCTGAGG | Miseq analysis of off-target #5 |
| **Forward 9** | **Reverse 9** | AGATCAATTATAGGTATCTTCTTCTCTCTCT | Miseq analysis of off-target #6 |
### Supplemental Table 9. Oligos and primers for GUIDE-seq assays.

| Oligos required for next-generation sequencing library prep PCR #1 |
|---|
| **oligo ID** | **sequence** | **description** |
| oBK8995 | GTGTGTCTCTCCCTACCCTCC | P5 primer for HTT exon 29 SpCas9 target site |
| oBK8996 | GCACCCTGGAGAACACAGGC | P7 primer for HTT exon 29 SpCas9 target site |

| Oligos required for next-generation sequencing library prep PCR #2 |
|---|
| **oligo ID** | **description** | **i5** | **i5 barcode** | **sequence** |
| oRW1116 | Nextera DNA index 501 | A501 | TAGATCGC | AATGATACGGCGACCACCGAGATCTAC |
| oRW1118 | Nextera DNA index 503 | A503 | TATCCTCTCT | ACTATCTCTCTCTCTCTCTACACG |
| oRW1120 | Nextera DNA index 505 | A505 | G TAAGGAG | GCACCTCTCTCCGATCT |
| oRW1092 | Nextera DNA index 701 | A701 | TCGCCTTA | CCGAAGACGCGCATACGAGATCTAC |
| oRW1093 | Nextera DNA index 702 | A702 | TCTCCGATCT | CTCTCTCCGATCT |
| oRW1094 | Nextera DNA index 703 | A703 | TTCTGCACTG | CTCTCTCCGATCT |
| oRW1098 | Nextera DNA index 707 | A707 | GTAGAGAG | CTCTCTCCGATCT |
| oRW1099 | Nextera DNA index 708 | A708 | CCTCTCTCTC | CTCTCTCCGATCT |
| oRW1100 | Nextera DNA index 709 | A709 | AGCGTAGC | CTCTCTCCGATCT |

| Oligos required for GUIDE-seq library sequencing |
|---|
| **oligo ID** | **sequence** |
| Index1 | ATCACCCTGCCCCTAGAGAGCTCCAGTCAC |
| Read2 | GTGACTGGAGCTCTCTCTATGGCATGATGGCTCAGTGAT |

| Sample information for GUIDE-seq libraries |
|---|
| **Sample Description** | **P7 Sequence (Barcode 1)** | **Adaptor Sequence** | **SpCas9 target site sequence with PAM** |
| HEK 293T cells - gRNA targeting HTT exon 29 | TAAGGCGA | TAGATCGC | AAAGAACATC|
| HEK 293T cells - no gRNA, dsODN control | CGTACTAG | CTCTCTAT | n/a |
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