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Small Molecules Enhance CRISPR Genome Editing in Pluripotent Stem Cells

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SUMMARY

The bacterial CRISPR-Cas9 system has emerged as an effective tool for sequence-specific gene knockout through non-homologous end joining (NHEJ), but it remains inefficient for precise editing of genome sequences. Here we develop a reporter-based screening approach for high-throughput identification of chemical compounds that can modulate precise genome editing through homology-directed repair (HDR). Using our screening method, we have identified small molecules that can enhance CRISPR-mediated HDR efficiency, 3-fold for large fragment insertions and 9-fold for point mutations. Interestingly, we have also observed that a small

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AUTHOR CONTRIBUTIONS

L.S.Q. and S.D. conceived of the research, designed the study, analyzed the data, and wrote the manuscript; C.Y. and Y.L designed the study, performed the experiments, analyzed the data, and wrote the manuscript; T.M., K.L., S.X., Y.Z., H.L., M.R., and M.X. performed some experiments and commented on the manuscript.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, one table, and can be found with this article online at XX.

The authors declare that there is no conflict of interest.

molecule that inhibits HDR can enhance frame shift insertion and deletion (indel) mutations mediated by NHEJ. The identified small molecules function robustly in diverse cell types with minimal toxicity. The use of small molecules provides a simple and effective strategy to enhance precise genome engineering applications and facilitates the study of DNA repair mechanisms in mammalian cells.

The bacterial adaptive immune system CRISPR (clustered regularly interspaced palindromic repeats)-Cas (CRISPR associated protein) has been used for the sequence-specific editing of mammalian genomes (Barrangou et al., 2007; Cong et al., 2013; Gonzalez et al., 2014; Mali et al., 2013; Smith et al., 2014; Wang et al., 2013; Yang et al., 2013a). The CRISPR system derived from *Streptococcus pyogenes* uses a Cas9 nuclease protein that complexes with a single guide RNA (sgRNA) containing a 20-nucleotide (nt) sequence for introducing sitespecific double-stranded breaks (Hsu et al., 2013; Jinek et al., 2012). Targeting of the Cas9- sgRNA complex to DNA is specified by basepairing between the sgRNA and DNA as well as the presence of an adjacent NGG PAM (protospacer adjacent motif) sequence (Marraffini and Sontheimer, 2010). The double-stranded break occurs 3 bp upstream of the PAM site, allowing for targeted sequence modifications via alternative DNA repair pathways: either nonhomologous end joining (NHEJ) that introduces frame shift insertion and deletion (indel) mutations leading to loss-of-function alleles (Geurts et al., 2009; Lieber and Wilson, 2010; Sung et al., 2013; Tesson et al., 2011; Wang et al., 2014), or homology-directed repair (HDR) for precise insertion of point mutations or a fragment of desired sequence at the targeted locus (Mazón et al., 2010; Wang et al., 2014; Yin et al., 2014).

To date, CRISPR-mediated gene knockout through NHEJ-induced indel mutations has worked efficiently. For example, the efficiency for knocking out a protein-coding gene has been reported to be 20% to 60% in mouse embryonic stem (ES) cells and zygotes (Wang et al., 2013; Yang et al., 2013a). However, precise introduction of a point mutation or a sequence fragment directed by a homologous template has remained inefficient (Mali et al., 2013; Wang et al., 2013; Yang et al., 2013a). A long and tedious screening process via cell sorting or selection, expansion and sequencing is often required to identify correctly edited cells. Improving the efficiency of precise CRISPR gene editing remains a major challenge.

It has been shown that small molecule compounds can effectively activate or block certain DNA repair pathways (Hollick et al., 2003; Rahman et al., 2013; Srivastava et al., 2012). However, it remains unclear whether small molecules could be used to modulate CRISPRinduced genome editing and DNA repair via the HDR pathway. Here we sought to identify new small molecules that can enhance HDR for more efficient and precise gene insertion or point mutations.

To quantitatively characterize CRISPR-mediated HDR efficiency, we established a fluorescence reporter system in E14 mouse ES cells. We used ES cells in the screening assay because ES cells exhibit overall better HDR efficiencies compared to somatic cells (Kass et al., 2013), thus providing an easier system for measuring the gene insertion frequency. To create the reporter system, we co-transfected ES cells via electroporation with three plasmids: a Cas9- expressing vector, a sgRNA-expressing vector targeting the stop codon of *Nanog* (sgNanog), and a circular template plasmid containing a promoterless

superfolder GFP (sfGFP) with a Nterminal in-frame 2A peptide (p2A) and two copies of nuclear localization sequence (NLS) (Figure 1A). The template also contains two sfGFP-flanking homology arms to *Nanog*, a 1.8 kilo base (kb) left arm and a 2.4 kb right arm. CRISPR-mediated insertion of the p2A-NLS_{x2}-sfGFP sequence into the endogenous *Nanog* locus was measured by gain of green fluorescence using flow cytometry 3 days after electroporation. Our results showed that only co-delivery of all three plasmids yielded GFP-positive ES cells (~17% of cells showing strong fluorescence), but the controls lacking any of the three plasmids generated almost no GFP-positive cells (Figure 1B). To confirm correct insertion of the template into the *Nanog* locus in GFP-positive cells, we sorted GFP-positive cells, PCR amplified, and sequenced to verify the target locus. We observed correct sfGFP integration in GFP-positive cells (Figure 1C). Furthermore, we detected no fluorescence signal when using a template without homology arms (Figure S1A). Together, the experiments suggested a correlation between gain of fluorescence and HDR-mediated precise gene insertion.

To investigate a broad range of small molecules that could act as enhancers or inhibitors of CRISPR-mediated HDR, we developed a high-throughput chemical screening assay based on the reporter system (Figures 1D & S1B). In this assay, mouse ES cells were co-transfected with Cas9, sgNanog, and the template, and seeded at a density of 2,000 cells/well into Matrigelcoated 384-well plates containing the LIF-2i medium supplemented with individual compounds from our known drug collections (Supplemental Information). After 3 days of culture, cells were fixed, stained with DAPI, and imaged using an automated high-content IN Cell imaging system for the analysis of the numbers of DAPI-positive and GFP/DAPI double-positive nuclei in each well.

From a collection of roughly 4,000 small molecules with known biological activity, we identified and confirmed using flow cytometry that two small molecules, L755507 and Brefeldin A, could improve the HDR efficiency (Figures 1D & 1E). L755507, a β3-adrenergic receptor agonist (Parmee et al., 1998), increased the efficiency of GFP insertion by 3 fold compared to DMSO-treated control cells, which was further verified by PCR amplification and sequencing of the target locus (Figures 1E & 1F). Brefeldin A, an inhibitor of intracellular protein transport from the endoplasmic reticulum to the Golgi apparatus (Ktistakis et al., 1992), also exhibited enhanced insertion efficiency by 2-fold (Figures 1E & 1F).

Interestingly, we also identified that two thymidine analogues, azidothymidine (AZT) and Trifluridine (TFT), could decrease the HDR efficiency (Figure 1D & 1E). AZT, previously used as an anti-HIV drug that inhibits the reverse transcriptase activity (Mitsuya et al., 1985), and TFT that was identified as an anti-herpesvirus drug by blocking viral DNA replication (Little et al., 1968), decreased the HDR efficiency by 3-fold assayed using flow cytometry (Figure 1E), or by more than 10-fold detected by sequencing (Figure 1F).

We further examined the dosage effects, treatment duration, and cytotoxicity of the identified small molecules. We found that HDR enhancers, L755507 and Brefeldin A, achieved their maximal effects at 5 μM and 0.1 μM, respectively (Figure 1G). The HDR inhibitors, AZT and TFT, exhibited maximal effects at 5 μM. In addition, we also examined

compound treatment windows of 0–24 h, 24–48 h, 48–72 h, or 0–72 h post electroporation. All compounds showed optimal activity within the first 24 hours, suggesting that the genome knockin events occurred mostly during the first 24 hours in our system (Figure S1C). Notably, at their optimized concentrations, the compounds exhibited no or very mild toxicity as assayed by both cell counts and MTS cell proliferation assay (Figures S1D & S1E).

To test how general these compounds can be used for modulating HDR for different genomic loci and in different cell types, we used another template to insert a t2A-Venus cassette in frame into the Alpha Smooth Muscle Actin (*ACTA2*) locus (Figure 2A), a gene expressed in a wide variety of cancer cell lines and normal cells (Ueyama et al., 1990). The template plasmid contains a left homology arm of 780 bp and a right homology arm of 695 bp that flank the t2A-Venus cassette. We first co-transfected the template plasmid with a single construct expressing both Cas9 and sgACTA2 into HeLa cells. Sequencing results of Venus-positive HeLa cells confirmed that Venus expression represented the correct insertion of Venus into the *ACTA2* locus (Figure 2B). We then tested several other types of human cells. Our flow cytometry results showed that the knockin efficiency was dependent on the cell type, ranging from 0.8% to 3.5%. Treating different types of cells with L755507 showed consistently improved HDR efficiency, with the largest increase of more than 2 fold in human umbilical vein endothelial cells (HUVEC). The fact that L755507 consistently increased the HDR efficiency in diverse cells including cancer cell lines (K562 and HeLa), suspension cells (K562), primary neonatal cells (HUVEC and fibroblast CRL-2097), and human ES cell-derived cells (neural stem cells) (Li et al., 2011) suggested that the mechanism by which L755507 enhances CRISPR-mediated HDR was common in both transformed and primary cells.

Precise editing of single-nucleotide polymorphisms (SNP) through single stranded oligodeoxynucleotide (ssODN) templates is an important application of genome editing in disease modeling and gene therapy. We next sought to test whether the identified small molecules could enhance SNP editing through HDR using a short ssODN. The method for introducing mutations into human pluripotent stem (iPS) cells using CRISPR-Cas9 and ssODN has been established (Ding et al., 2013b; Yang et al., 2013b). Following a similar method, we synthesized a 200-nt ssODN template to introduce an A4V mutation into the human *SOD1* locus (Figure 2D), which is one of the common mutations that cause Amyotrophic Lateral Sclerosis (ALS) in the U.S. population (Rosen et al., 1994). We designed an sgRNA (sgSOD1) in a way that introduction of A4V mutation also disrupted the NGG PAM sequence, thus preventing further targeting by sgSOD1 to the A4V alleles. We co-transfected two vectors that encoded Cas9 and sgSOD1 with or without the ssODN template into human iPS cells (Ding et al., 2013a; Ding et al., 2013b; Zhu et al., 2010). The cells were then treated with DMSO or L755507 followed by genomic DNA extraction, PCR cloning and sequencing of randomly picked E. coli transformants. The sequencing results showed that compared to the DMSO control, L755507 enhanced the frequency of A4V allele mutant by almost 9-fold (Figures 2E & 2F). Our results also showed decreased indel allele mutation frequency with the addition of L755507. These results demonstrated that our small molecules could dramatically enhance SNP editing using a short ssODN template.

We next sought to test if the small molecules repressing HDR also affect NHEJ. We reasoned that if a small molecule directly inhibits the DNA cutting activity of Cas9, it should also inhibit CRISPR-mediated gene deletion without a template. To test this, we generated a clonal mouse ES cell line carrying a monoallelic sfGFP insertion at the *Nanog* locus (Figures S2A and S2B). We designed three sgRNAs (sgGFP-1, 2, 3) that targeted within the sfGFP coding sequence on the same plasmid that encoded Cas9 (Figure 2G). Electroporation of any sgRNA resulted in a population of cells that showed complete loss of GFP expression after 3 days, while ES cells transfected with an sgRNA (sgGAL4) with no targetable sites showed no loss of the GFP signal (Figure 2G). Addition of L755507 immediately after electroporation showed inhibitory effects on GFP knockout. Unexpectedly, the knockin inhibitor, AZT, greatly increased GFP knockout efficiency for all three sgRNAs tested. For example, AZT increased the knockout efficiency by more than 1.8-fold in the case of sgGFP-1 (Figure 2B). This was also consistent with the deep sequencing results for indel detection (Table S1). Together, these results suggested that AZT acted on the NHEJ pathway instead of interacting with the Cas9-sgRNA complex, and also a possible trade-off between the HDR and NHEJ pathways.

Staining of pluripotency markers Oct4, Sox2, and Nanog showed that the compounds did not affect pluripotency (Figures S2C & S2D). Furthermore, neither electroporation (Figure S2E) nor compound addition (Figure S2F) affected *Nanog* expression. To rule out that the AZT does not cause more errors in replication that in turn lead to inactivation of GFP, we passaged Nanog-sfGFP ES cells line for 10 passages under AZT treatment without the CRISPR components, and observed no loss of GFP signals (Figure S2G). These results also showed that the compounds identified in the screening system could modulate CRISPR-mediated gene knockout.

In summary, we developed a high-throughput chemical screening platform for CRISPR genome editing and provided proof-of-principle demonstration that small molecules could be used to modulate the efficiency of CRISPR genome editing. We report several small molecules that could enhance or repress HDR-mediated precise gene editing. The identified compounds likely interact with DNA repair pathways via NHEJ or HDR, thus providing a set of useful tools for the mechanistic interrogation of these pathways. The identified chemicals exhibit minimal toxicity and work in diverse cell types, which can be used to enhance both large template-mediated gene insertion and ssODN-mediated SNP editing. We also report small molecules that can enhance gene knockout without a template. The observation that reducing HDR could increase NHEJ may suggest a trade-off between the two DNA repair pathways after DNA cutting by the Cas9 nuclease. Identification of diverse classes of small molecules provides an approach that facilitates precise CRISPR genome editing for both biomedical research and clinical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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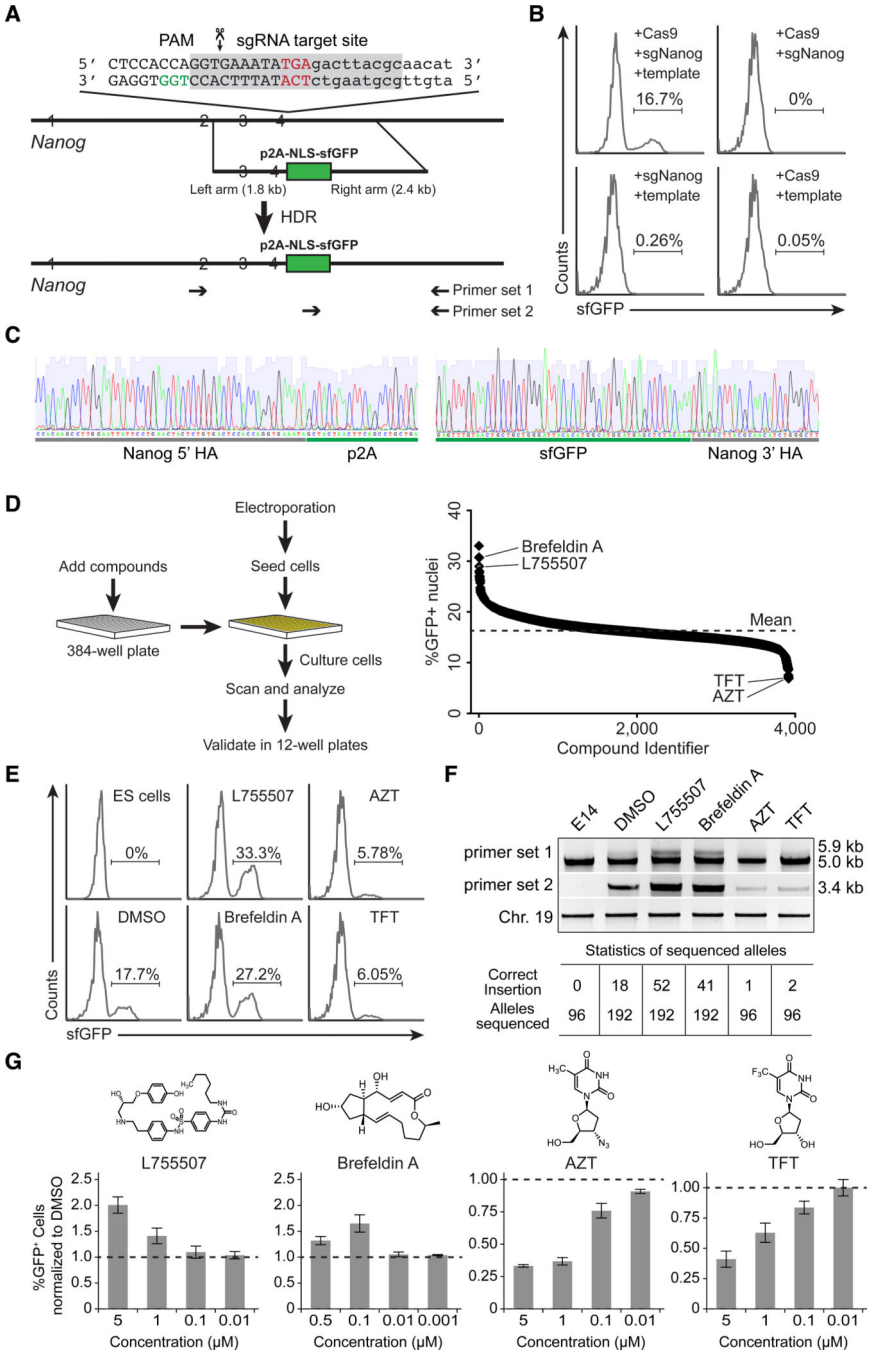

Figure 1. Establishment of a high-throughput chemical screening platform for modulating CRISPR-mediated HDR efficiency

(A) A fluorescence reporter system in E14 mouse ES cells to characterize the HDR efficiency. An sfGFP-encoding template is inserted at the *Nanog* locus. The PAM is labeled in green, the stop codon is shown in red, and the sgRNA target site is shaded in grey. The cutting site (scissors) is 3 bp downstream of CCA in this case. The binding sites of two sets of primers are shown by arrows. Primer set #1 binds to the sequences outside of the homology arms, and primer set #2 contains a forward primer binding to the sfGFP sequence

and a reverse primer binding outside of the 3' homology arm. (B) Fluorescence histograms of mouse ES cells transfected with different plasmid combinations using flow cytometry analysis. (C) Sequencing results of the *Nanog* locus in GFP-positive cells. (D) A scheme of the chemical screening platform and a waterfall plot of 3,918 small molecules screened for their activity of CRISPR-mediated gene insertion. Highlighted dots are validated compounds that showed increased or decreased insertion efficiency. The dotted line showed the mean value of all screened compounds. (E) Validation of two enhancing and two repressing compounds using flow cytometry analysis. (F) Efficiency of sfGFP insertion into the *Nanog* locus. Gel pictures showing sfGFP tagging using two sets of primers as shown in Figure 1A. The PCR products of primer set #1 were purified and cloned to a modified pUC19 backbone vector and sequenced. (G) Dose-dependent effects of four compounds for modulating CRISPR gene editing. All data are normalized to the knockin efficiency of DMSO-treated control cells (dotted lines). Error bars represent the standard deviation of three biological replicates.

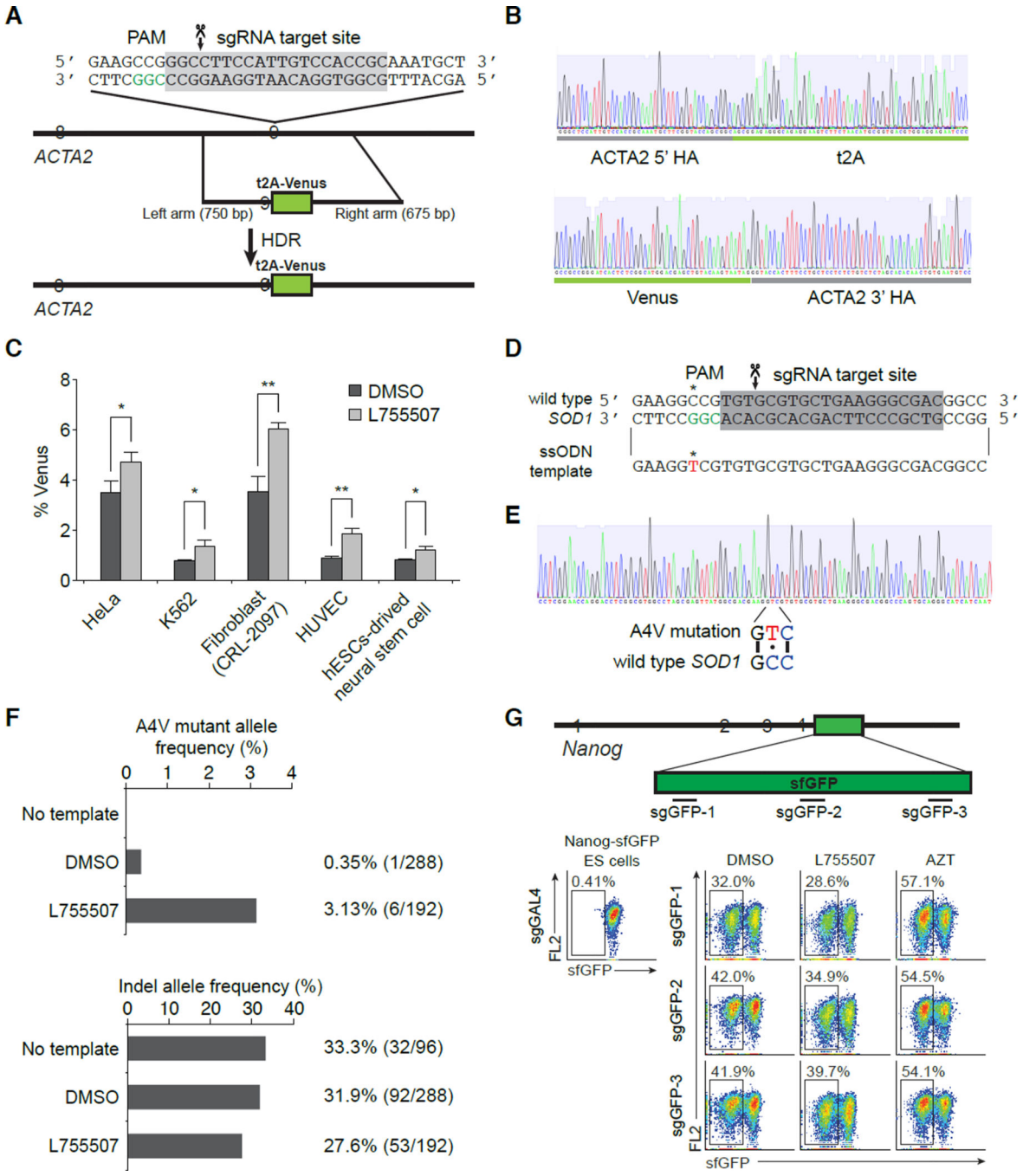

Figure 2. Different identified small molecules could enhance HDR or NHEJ-mediated CRISPR genome editing

(A) A scheme of insertion strategy at the human *ACTA2* locus (top). The PAM is labeled in green, and the sgRNA target site is shaded in grey. (B) Sequencing results of the *ACTA2* locus in Venus-positive HeLa cells. (C) Efficiency of Venus insertion measured by flow cytometry analysis. The error bars indicate the standard deviation of three samples, and the p values are calculated using two-tailed student t-test. *, $p < 0.05$; **, $p < 0.01$. (D) The strategy for introducing the A4V point mutation at the human *SOD1* locus in human iPS

cells. The PAM is labeled in green, and the sgRNA target site is shaded in grey. The point mutation is labeled in red. (E) Sequencing results of the *SOD1* locus. (F) Comparison of A4V allele mutant frequency and indel allele frequency in human iPS cells assayed by PCR cloning and bacterial colony sequencing with no template, DMSO or L755507. (G) Test of knockout efficiency using a clonal mouse ES cell line carrying a monoallelic sfGFP insertion at the *Nanog* locus in the presence of L755705 and AZT. The dot plots of cells transfected with a non-cognate sgRNA (sgGAL4) is shown on the top. The panel shows cells transfected with three different sgRNAs (their target sites shown in the scheme) in the presence of DMSO (left), L755507 (middle), and AZT (right).