CRISPR knock-in (endogenous tagging)

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ABSTRACT

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Protocol status: Working
We use this protocol and it's working

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Plasmid construction

1

For the C-terminus knock-in experiment, the sgRNAs and the homology-independent donor templates were generated
following strategies similar to those described previously

CITATION

Ogawa Y, Rasband MN (2021). Endogenously expressed Ranbp2 is not at the axon initial segment..
LINK
https://doi.org/10.1242/jcs.256180

Briefly, the coding sequence of *SNCA1* –the gene encoding α-syn at exon 6 was targeted by a sgRNA, and the oScarlet tag (a variant of mScarlet that is engineered to reduce aggregation; a gift from Karl Deisseroth, Addgene plasmid #137135) was knocked in using a homology-independent mechanism .

2 The donor sequence was designed following the SATI knock-in vector with slight modifications for the intron knock-in experiment. Briefly, the following sequences were directly conjugated as a donor tag; SNCA1 intron 4 (only the sequence after the gRNA targeting site), SNCA1 exon 5 (with wild type or mutation sequences; S129A or S129D), SNCA1 exon 6 (coding site until stop codon), 3X GGGGS linker, oScarlet (without a start codon and with a stop codon at the end), and SNCA1 3' UTR. The *SNCA1* intron 4 was targeted by a sgRNA (TTCTAAGTGTACCAAACCAC),
and the donor tag was homology-independently knocked in. The plasmid PX552 (RRID:Addgene_60958) (a
gift from Feng Zhang) was digested with a NotI restriction enzyme (New England Biolabs, Cat#R3189L) and used as a plasmid backbone.

3 Oligonucleotides were ordered from Sigma. The genomic sequence of *SNCA1* was partially synthesized by Twist Bioscience, and for the rest, it was PCR amplified from a purified genome of a C57BL/6J mouse. DNA fragments were ligated together using the In-Fusion Snap Assembly Master Mix (Takara, Cat# 638947) or DNA Ligation Kit Mighty Mix (Takara, Cat#6023). The sgRNA sequence for knock-in is listed in the supplemental materials. The AAV-SpCas9 plasmid PX551 RRID:Addgene_60957 (a gift from Feng Zhang) was modified by removing the HA tag.

Adeno-associated virus (AAV) production, neuronal transduct...

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Small-scale AAV cell lysates were produced using the AAVpro Purification Kit (All Serotypes) (Takara, Cat# 6666) with slight modifications. Briefly, HEK293T cells (RRID:CVCL_0063) were triple transfected with the AAV targeting plasmid, helper plasmid (Agilent Technologies, Cat # 240071), and serotype PHP.S plasmid pUCmini-iCAP-PHP.eB (RRID:Addgene_103005)(a gift from Viviana Gradinaru) with PEI Max (Polysciences, Cat#24765).

5 The medium was changed the next day of transfection, and cells were incubated for 3 days after transfection. HEK cells were then collected and lysed with the AAV Extraction Solution A plus.

6 The extracted solution was centrifuged at 10,000 x g for 10 min to remove debris and mixed with Extraction Solution B. This small-scale AAV solution was stored at -80 until use. For viral transduction, hippocampal neurons were plated at 60,000 cells/cm^2 density and infected 4 hours later with 10ul of the AAVs expressing SpCas9 and sgRNAs/donor.

7 The media was replaced 2 days after infection. Before imaging or genomic analysis, the transduced neurons were cultured to maturity (DIV-17-DIV-21).

8 For quantification of the knock-in α-syn:o-Scarlet fluorescence at synapses, images were first background-corrected, small ROIs were manually placed over ~20-30 synapses on each image, and average intensities were calculated – all using dropdown menus in MetaMorph Microscopy Automation and Image Analysis Software (RRID:SCR_002368)(https://www.moleculardevices.com/products/cellular-imaging-systems/acquisition-and-analysis-software/metamorph-microscopy#gref). The resulting datasets were statistically analyzed using GraphPad Prism [(RRID:SCR_002798) http://www.graphpad.com/].