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Abstract

This is a protocol collection for the publication

"Genome-wide CRISPR screen identifies neddylation as a regulator of neuronal aging and AD neurodegeneration"

Saurat N et al. Cell Stem Cell 2024

The collection consists of 20 different protocols.

Full manuscript can be found here:

DOI: [10.1016/j.stem.2024.06.001](https://doi.org/10.1016/j.stem.2024.06.001)

hPSC culture

- 1 Human PSCs were maintained on Vitronectin coated plates in E8 medium and passaged twice a week using EDTA according to the manufacturer's (Thermo Scientific) recommendations.

Engineering of H9iCAS-APP^{swe/swe} cell lines

- 2 WA-09 were sequentially engineered to generate the APP^{swe/swe} cell lines for this study.
 - 2.1 First an inducible Cas9 construct was knocked into the AAVS1 locus as described in Gonzalez et al.¹² but with a hygro-resistant Cas9 donor plasmid instead of the puromycin resistant donor.
 - 2.2 After checking for the correct insertion of the iCas9 construct the newly established iCas9 cell line was engineered to insert the APP^{swe/swe} mutation as described in Paquet et al.¹⁵
 - 2.3 The maintenance of an intact karyotype was confirmed after each engineering step.

Generation of H9 Nurr1-GFP LRRK2 G2019S knock-in line

- 3 Generation of the H9 (WA-09) ESC Nurr1-GFP reporter line was described in Riessland et al.⁷⁵
 - 3.1 For the generation of Nurr1-GFP LRRK2 G2019S ESCs, Nurr1-GFP ESCs were dissociated using Accutase and seeded onto Matrigel coated dishes (1:50) at a density of 25k/cm² in the presence of 10 µM Y-27632 in E8 medium.
 - 3.2 Next day, medium was changed to mTeSR1 and a mixture of ssDNA oligo and pX458 LRRK2 gRNA (5:1) was transfected using LipofectamineTM Stem Transfection Reagent (250 ng DNA/25k cells) following the manufacturer's instructions.
 - 3.3 The next day, transfection was repeated with ssDNA oligo only.
 - 3.4 Transfection was repeated with ssDNA oligo only another time the next day.

- 3.5 GFP-positive cells were sorted 24h after the last round of transfection and seeded as single cells into 96well plates in mTeSR1 supplemented with 10 μ M Y-27632.
- 3.6 Medium was changed to E8 after 6 days of plating.
- 3.7 Single clones were expanded and subjected to PCR analysis via digest by BceAI (NEB), where the BceAI cutting site is destroyed by the G2019S mutation, and by Sanger Sequencing.

Oligonucleotide sequences for cloning and validation can be found in **Table S2**.
- 3.8 Karyotype integrity was assessed by metaphase spread karyogram from MSKCC Cytogenetics Core Facility.

Cortical neuron differentiation

- 4 For differentiation to cortical neurons, the PSCs were dissociated to single cells using Accutase and replated onto Matrigel coated dishes at a density of 300,000 per cm^2 in E8 medium supplemented with ROCK inhibitor (Y-27632; 10 μ M).
- 4.1 The following day (DIV=0) culture medium was replaced with E6 containing SB431542 (10 μ M), LDN193189 (100 nM) and XAV939 (2 μ M).
- 4.2 Differentiation media was changed daily and XAV939 removed after 3 days.
- 4.3 At 10 DIV the media was changed to neurobasal supplemented with 1x N2, 1x B27, 2mM L-glutamine and Pen-Strep and the monolayer was maintained for an additional 10 days.
- 4.4 On day 20 after neural induction cells were dissociated using Accutase and replated onto poly-L-ornithine/fibronectin/laminin-coated plates.
- 4.5 Neurons were maintained in Neurobasal medium supplemented with BDNF, ascorbic acid, GDNF, cAMP, L-glutamine and B-27 supplement. DAPT was also added to the culture media until 30 days DIV.

Whole genome CRISPR Cas9 screen in PSC-derived neurons

- 5 The Brunello human CRISPR Knockout Pooled Library⁶⁹ was used for this screen; this library includes 4 gRNAs for 19,114 genes as well as 1000 non targeting controls.



- 5.1 Stem cell culture, transduction, selection, and differentiation of the isogenic stem cell pair was done in parallel.
- 5.2 To perform the screen PSCs were dissociated with Accutase and a total of 250 million cells per line were replated at a density of 150,000/cm² in E8 medium with ROCK inhibitor (Y-27632; 10 µM).
- 5.3 The whole genome lenti-guide RNA library was added during the replating step at an MOI of 0.3-0.5.
- 5.4 The virus was removed 16-18h post transduction and fresh E8 medium added to the culture plate.
- 5.5 The following day transduced cells were selected by adding 0.4ug/ml puromycin to the E8 medium.
- 5.6 After selection, PSCs were dissociated with Accutase, the culture plates were pooled and a total of 116 million cells used for differentiation to ensure that 1000x guide representation was maintained throughout.
- 5.7 PSCs were differentiated as described in the '**hESC culture and differentiation**' subsection.
- 5.8 After differentiation (DIV 20) cultures were dissociated using Accutase to generate a single cell suspension for each cell line and the cells were split to give triplicate samples for the screen with a total of 90 million cells per replicate.
- 5.9 The first set of representation controls (T=0) were harvested at DIV 20 prior to the addition of doxycycline.
- 5.10 The endpoint samples were replated at a density of 200,000/cm² in neurobasal supplemented with N2, B27 and ROCK inhibitor (Y-27632; 10 µM).
- 5.11 Doxycycline (2 µg/ml) was added to half the culture plates to induce Cas9 expression.
- 5.12 Cells treated with doxycycline for a total of 48h before switching to neural maintenance media with DAPT until DIV 30.
- 5.13 From DIV 30 onwards half the culture media was replaced every 2-3 days.

- 5.14 At DIV 65 the second representation control set were harvested, these samples were cultured to the screen endpoint but never received doxycycline and therefore CAS9 was never activated. The endpoint control was harvest in parallel with the +DOX test samples.
- 5.15 To remove any dead cells from the culture the monolayer was washed 2x with PBS followed by a 5min incubation in EDTA at RT.
- 5.16 Then, the neuronal monolayer was scrapped off the culture dish, pelleted and snap frozen.
- 5.17 Cell Pellets from pooled screen were lysed, and genomic DNA was extracted (Qiagen) and PCR amplified to add Illumina adapters and multiplexing barcodes. Amplicons were quantified by Qubit and Bioanalyzer (Agilent) and sequenced on Illumina HiSeq 2500.

Data analysis for Pooled CRISPR screen

- 6 Sequencing reads were aligned to the screened library and the CRISPR screen was analyzed using the MAGECK-MLE pipeline as previously described.¹⁷
- 6.1 To calculate the beta scores for each gene the representation of gRNAs in the endpoint samples (DIV65+Dox) were compared to the gRNA representation in the control samples.
- 6.2 For this screen we generated two possible sample sets that could act as the representation control: the sample set harvested at DIV20 prior to doxycycline addition (T=0) and the sample set that was cultured to the screen endpoint but never received doxycycline (DIV65-Dox). Non targeting sgRNAs were used to normalize read counts between replicates and conditions.
- 6.3 For each genotype, gene essentiality was calculated in using the T=0 control sample as the representation control. This was repeated using the DIV65-Dox samples as the representation control.
- 6.4 Hit genes were identified by comparing the beta scores in the wild type viability screen with the beta scores in the APP^{swe/swe} viability screen. Genes with a beta score<0, FDR<0.3 and Pval <0.05 were considered viability genes. Essential genes met these criteria in both genotypes.
- 6.5 Candidate age regulators met these criteria in the APP^{swe/swe} genotype but not in the WT.
- 6.6 We further filtered hit list to exclude genes that had a viability phenotype in the wild type neurons (Beta score^{WT} was greater than 1.5 standard deviations from the mean or < 0

with an $FDR^{WT} < 0.3$ and $Pval^{WT} < 0.05$).

KEGG pathways

- 7 KEGG pathway analysis was performed using GSEA with an FDR cutoff of 0.01. For essential and hit gene categories, pathway analysis was performed using the top 1000 genes. There were only 4 genes that increased gRNA representation in both genotypes so KEGG analysis for survival/proliferation genes was limited to these 4 genes.

Gene enrichment analysis

- 8 GO ontology analysis was performed using the top 500 (selected to keep the number of genes included constant across screens) essential genes in our screen.
- 8.1 Gene essentiality was ranked based on the combined beta score of WT and APP^{swe/swe} neurons with a $Pval < 0.05$ and $FDR < 0.1$ in both genotypes.
- 8.2 The top 500 essential genes identified in the Tian et al., and Wertz et al., were used for comparison.^{18,19}
- 8.3 Functional analysis was performed using DAVID⁷⁴ (version 6.8) and the top 10 GO terms with a p-value < 0.05 for each screen visualized using ggplot2.

RNA extraction and qPCR

- 9 RNA was extracted using the Zymo RNA Micro Kit and total of 1ug of RNA was used to generate cDNA using iScript (BioRad).
- 9.1 Realtime PCR was performed using SSoFAST EvaGreen Mix (BioRad) in a BioRad CFX96 Thermal Cycler. The manufacturers protocol was used for all steps.
- 9.2 Primers used in this study are listed in **Table S2**.

Immunocytochemistry

- 10 Cells were fixed in 4% paraformaldehyde for 10mins then permeabilized in PBS+0.3% Triton.

- 10.1 Cells were blocked in 5% donkey or goat serum for 1h.
- 10.2 Primary antibody incubation was performed overnight. Primary antibodies used in this study are listed in **STAR Methods**.
- 10.3 For all image quantifications images were taken from 3 individual wells and averaged. This was repeated three times with neurons from independent differentiations.
- 10.4 Min and max adjustments were applied consistently between images for each replicate. Velocity was used to count MAP2+ and Ki67+ cells with pyknotic, DAPI^{bright} nuclei excluded from the cell count.
- 10.5 FIJI was used for TAU quantifications. In brief, images were converted to 8-bit and a threshold was set for the total Tau and pTau (total and 'bright') channels. The "Measure" tool was used to quantify area that met the threshold requirement. Threshold was kept constant across all wells per replicate and a total of 3 wells were imaged and averaged per replicate.

High content imaging

- 11 Neurons were plated, cultured, fixed, and stained as described in section 10.
 - 11.1 Images were acquired on the Operetta high content microscope (PerkinElmer, Waltham, MA).
 - 11.2 Quantifications were performed using the harmony high content analysis software in an automated manner.
 - 11.3 A total of 9 fields were imaged per well and the average value per live cell for each parameter across all 11 fields was calculated.
 - 11.4 Live cells were identified by setting a minimum size threshold and a maximum DAPI intensity.
 - 11.5 A total of 12 wells (3 wells from 4 independent differentiations) were imaged for each assay unless otherwise described.
 - 11.6 Prism's (v 10.1.1) ROUT method with standard parameters was used to identify and exclude outliers. Outlier removal was indicated in the figure legend when used.

- 11.7 Fluorescence intensity was normalized to DMSO, and the one sample t-test used to compare test whether test condition significantly deviated from the DMSO control (hypothetical value = 1).
- 11.8 Nuclear morphology measurements were compared directly using an unpaired two-sided t-test.

A β ELISA

- 12 To quantify amyloid peptide production cell culture media was harvested after 48h and the culture media was briefly centrifuged to remove any cellular debris.
- 12.1 Quantification was performed using the Mesoscale Discovery Assay kit (K15200E-2) according to the manufacturer's instructions. A total of 25ul of culture medium was assayed.
- 12.2 Total A β in this assay refers the sum of the A β 38, A β 40 and A β 42 measurements. For each condition, the reported values represent the average of 3-4 culture wells per differentiation/experiment.
- 12.3 For normalization, protein was extracted and quantified from neurons of matched age/gene knockout. The mean change in total protein between -Dox and +Dox samples from 3 independent differentiations/experiments was used to normalize total A β measurements.

Western blotting

- 13 Samples for western blotting were harvested, pelleted and snap frozen.
- 13.1 Cell pellets were resuspended in RIPA buffer supplemented with Halt protease and phosphatase inhibitors (ThermoFisher) followed by centrifugation to clarify the sample.
- 13.2 Protein concentration was quantified using the Precision Red Advanced Protein Assay according to manufacturer's instructions and equal amounts of protein were mixed with NuPAGE LDS Sample Buffer and NuPAGE Sample Reducing Agent and heated to 72 degrees for 10 mins.
- 13.3 A total of 5-20ug of protein was separated on NuPAGE Novex 4-12% Bis Tris gels and transferred by wet blotting onto PVDF membranes.
- 13.4 Membranes were blocked in 5% milk protein or 5% BSA when probing for the phospho-Tau. Primary antibodies used for this study are listed in **STAR Methods**.

- 13.5 Band intensity was visualized using BioRad ChemiDoc XRS+ molecular imager.
- 13.6 After imaging the membrane was re-probed with either GAPDH or β -ACTIN antibodies for normalization.
- 13.7 Band intensity was quantified using Fiji.

Fractionation of protein lysates into sarkosyl soluble/insoluble fractions

- 14 Protocol for fractionation of cell lysates from stem cell derived neurons was adapted from Manos et al.⁷⁷
- 14.1 Cortical neurons were cultured in 6 well plates until DIV50 then 1 μ M MLN4924 or DMSO were applied for 10 days before harvesting.
- 14.2 Cell pellets from 1 well of a 6 well plate were resuspended in 100uL RIPA buffer supplemented with Halt protease and phosphatase inhibitors (ThermoFisher) and incubated for 15mins to lyse.
- 14.3 Lysates were spun at 14,000rpm for 1min to remove any debris.
- 14.4 The protein concentration was then measured using the Precision Red Advanced Protein Assay according to manufacturer's instructions.
- 14.5 Equal amounts of total protein were transferred to a fresh tube and sarkosyl added to a final concentration of 1%.
- 14.6 Cell suspensions were incubated at RT for 30mins and spun using ultracentrifuge (150,000g for 30mins).
- 14.7 The soluble fraction was decanted, fresh RIPA buffer + 1% sarkosyl added and the spin step was repeated.
- 14.8 The supernatant was removed and discarded and the pellet was resuspended in Laemmli buffer + reducing agent.
- 14.9 Lysates were boiled at 95C for 5mins before loading.

14.10 Western blots were run as described in section 13.

14.11 Reversible Protein Stain Kit for PVDF membranes (Pierce) was used as described by the manufacturer prior to the blocking step.

Fractionation of protein lysates into sarkosyl soluble/insoluble fractions

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15.3 Lysates were spun at 14,000rpm for 1min to remove any debris.

15.4 The protein concentration was measured using the Precision Red Advanced Protein Assay according to manufacturer's instructions.

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15.8 The supernatant was removed and discarded and the pellet was resuspended in Laemmli buffer + reducing agent.

15.9 Lysates were boiled at 95 C for 5 mins before loading.



- 15.10 Western blots were run as described in section 13 but were stained using the Reversible Protein Stain Kit for PVDF membranes (Pierce) as described by the manufacturer prior to the blocking step.

Viability assays

- 16 Viability assays were performed in 96 well plates using the PrestoBlue Cell Viability Reagent or CCK8.
- 16.1 Presto blue reagent was diluted 1:10 in neural maintenance media and 85ul was applied to each well.
- 16.2 For the CCK8 assay the assay reagent was prepared as described by the manufacturer with 110 ul used per well.
- 16.3 Culture plates were incubated with the assay reagent for 2 h at 37 C before assaying.
- 16.4 For the secondary validation experiments, each well was normalized to the mean absorbance of the no doxycycline control wells (for the genetic experiments) or to the mean absorbance of the DMSO control (for chemical inhibition).
- 16.5 Technical replicates were averaged to give a single value for each differentiation/experiment.
- 16.6 For MG132 and BafA1 viability curves cortical neurons were treated with MG132 for 5 days or BafA1 for 7 days and each well was normalized to the mean absorbance of the DMSO control wells.

Generation of lentiGuide RNA viruses

- 17 gRNAs used for secondary validation were the top scoring gRNAs from the WGS.
- 17.1 The list of gRNA sequences used for this study can be found in **Table S3**.
- 17.2 gRNAs were cloned into the lentiGuide-Puro plasmid (Addgene 52963) or pLKO5.sgRNA.EFS.GFP (Addgene 57822) as described by the Zhang lab.^{71,78}
- 17.3 For viral packaging, the lentiGuide-Puro plasmid and packaging plasmids (psPAX2; Addgene 12260 and pMD2.G; Addgene 12259) were transfected into 293T cells using X-

tremeGENE HP (Sigma) in a 10:10:1 molar ratio, respectively.

17.4 Virus particles were harvested after 48h.

Generation of UBA3 and NAE1 overexpression lentiviruses

18 Human UBA3 (HG16320-G) and NAE1 (HG14282-G) ORF clones were purchased from Sino Biological and the ORF was cloned into the pLV-EF1a-IRES-Puro vector (Addgene: 85132) using standard PCR-based cloning.

18.1 Primers used to amplify to ORFs for cloning and to sequence the resulting clones can be found in **Table S2**.

18.2 For viral packaging, the pLV-EF1aUBA3-IRES-Puro, pLV-EF1aNAE1-IRES-Puro or pLV-EF1a1-IRES-Puro (control) plasmids were transfected into 293T cells using X-tremeGENE HP (Sigma) alongside packaging plasmids (psPAX2; Addgene 12260 and pMD2.G; Addgene 12259) at a 6:3:1.5 molar ratio.

18.3 Virus particles were harvested after 48h and concentrated using Amicon Ultra-15 Centrifugal Filter Unit (100kDa).

Flow Cytometry

19 Neuronal cultures were dissociated to single cell suspensions using Accutase (Innovative Cell Technologies) supplemented with Neuron Isolation Enzyme (Thermo 88285) solution at 1:50.

19.1 Single cell suspensions were stained with Zombie UVTM Fixable Viability Kit (Biolegend 423107) at 1:2500 in PBS for 15 minutes at room temperature.

19.2 Cells were fixed in 4% Paraformaldehyde for 10 minutes (4°C).

19.3 Cells stained with CellEvent Senescence Green (Thermo C10840) were done so at 1:250 in assay buffer for 2 hours at 37°C.

19.4 For intracellular probes, cells were permeabilized in 0.5% triton-x for 10 minutes (4°C) and blocked in 5% BSA for 10 minutes (4°C).

19.5 Cells were stained with H3k9me3-PE antibody (Cell Signaling Technologies #13969S) diluted 1:200, and Proteostat (Enzo Life Sciences ENZ-51023-KP050) diluted 1:2500, in 5% BSA in PBS for 30 minutes at 4°C.



- 19.6 Cells were analyzed on the Cytex Aurora Flow Cytometer.
- 19.7 Experiments were repeated with cells from 3 independent differentiations. The median intensity indicated on plot for every condition.

A β 42 neurotoxicity assays

- 20 AggreSure A β 42 (Anaspec) monomers were resuspended in DMSO then diluted in PBS to generate a 100 μ M working solution which was stored in single use aliquots at -80 until needed.
- 20.1 For viability assays 5 μ M A β 42 or diluent only (DMSO+PBS) was added for a total of 7 days with one media change.
- 20.2 The endpoint neuron viability was assessed using the Presto blue reagent as described in section 16.
- 20.3 For UBA3, NAE1 and SENP8 loss of function experiments knock out was performed at DIV 20.
- 20.4 A β 42 was added at DIV42 for UBA3 and NAE1 and DIV37 for SENP8.
- 20.5 Long-term chemical inhibition of the neddylation pathway 1 μ M MLN4924 was added from DIV30 to DIV53 with A β 42 added the last 7 days of the assay.
- 20.6 For the short-term chemical manipulations of the neddylation pathway 1 μ M MLN4924 or 1 μ M CSN5i3 were added concurrently with A β 42 for 7 days starting on DIV30.
- 20.7 For the UBA3 and NAE1 overexpression experiments concentrated virus (pLV-EF1aUBA3-IRES-Puro, pLV-EF1aNAE1-IRES-Puro or pLV-EF1a1-IRES-Puro (control)) was applied to neurons at a 1:500 dilution at DIV 20.
- 20.8 On DIV 22 1 μ g/mL puromycin was added to the cultures for 4 days to select for transduced neurons and A β 42 was added on DIV30.

Proteasome activity assay

- 21 Neurons were grown in 96 well plates at 150k/cm.²



- 21.1 1 μ M MLN4924 or DMSO were added to the culture medium from DIV30.
- 21.2 At DIV40 and DIV50 the plates were used to assay for proteasome function using the Cell-Based Proteasome-Glo™ Assay kit (Promega G8660) as described by the manufacturer.
- 21.3 Reagents were prepared and left to equilibrate at room temperature.
- 21.4 Culture media was replaced with 100 μ l of room temperature PBS.
- 21.5 Additional PBS only blank wells were also included.
- 21.6 To perform the assay 100 μ l of Proteasome-Glo reagent was added to each well and the plate was incubated at room temperature for 10 min after which the luminescence was measured using a plate reader.
- 21.7 For each differentiation the MLN4924 wells were normalized to the DMSO controls.