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We use this protocol and it's working

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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 19 different protocols:

- hPSC culture using E8 and EDTA-based passaging
- Engineering of H9iCAS-APPswe/swe cell lines
- Generation of H9 Nurr1-GFP LRRK2 G2019S knock-in line
- Cortical neuron differentiation
- Whole genome CRISPR Cas9 screen in PSC-derived neurons
- Data analysis for Pooled CRISPR screen
- RNA extraction and qPCR
- Immunocytochemistry
- High content imaging
- A β ELISA
- Western blotting
- Fractionation of protein lysates into sarkosyl soluble/insoluble fractions
- Secondary Validation of CRISPR-screen targets
- Generation of UBA3 and NAE1 overexpression lentiviruses
- Flow Cytometry
- A β 42 neurotoxicity assays
- Proteasome activity assay
- Presto Blue Viability Assay
- CCK8 Viability Assay

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 using E8 and EDTA-based passaging

- 1 **Step 1: Coating Plates with Vitronectin**
 - Dilute Vitronectin 1:100 in DPBS (no Ca^{2+} / Mg^{2+}).
 - Add 1 mL per well of a 6-well plate.
 - Incubate at room temperature for ≥ 1 hour or overnight at 4°C .
- 2 **Step 2: Preparation of E8 Medium**
 - Warm Essential 8 medium to room temperature or 37°C before use.
- 3 **Step 3: Thawing or Maintaining PSCs**
 - Seed thawed engineered PSCs onto Vitronectin-coated plates in E8 medium.
 - Change medium daily.
- 4 **Step 4: Routine Culture Conditions**
 - Maintain cells in 37°C , 5% CO_2 incubator.
 - Keep cells between 30–80% confluency.
- 5 **Step 5: Passaging Frequency**
 - Passage PSCs **twice weekly** (e.g., every 3–4 days).
- 6 **Step 6: Pre-warm Reagents**
 - Warm EDTA solution and E8 medium before passaging.
- 7 **Step 7: EDTA-Based Passaging**
 - Aspirate spent medium.
 - Rinse cells once with DPBS (no Ca^{2+} / Mg^{2+}).
 - Add 1 mL of 0.5 mM EDTA per well.
 - Incubate at room temp for 4–5 minutes.
- 8 **Step 8: Cell Detachment**
 - Gently tap the plate or use pipette to dislodge colonies.
 - Avoid breaking into single cells.
- 9 **Step 9: Reseeding**
 - Transfer cell clumps to new Vitronectin-coated plates with fresh E8 medium.
 - Seed at 1:6 to 1:10 split ratio based on cell density.
- 10 **Step 10: Post-Passage Care**
 - Replace medium the next day and daily thereafter.
 - Monitor cell morphology for healthy PSC characteristics.
- 11 **Notes**
 - Avoid enzymatic dissociation to preserve colony morphology.
 - Routinely test for mycoplasma contamination.

- Maintain consistent passage number for reproducibility.

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

- 12 **Cell Line:** WA09 (H9) human embryonic stem cells
Goal: Sequential genome engineering of WA09 to introduce the APP^{swe/swe} mutation via inducible Cas9 system
References: Adapted from Gonzalez et al. (with hygromycin selection) and Paquet et al.
Karyotyping: Performed after each editing step to confirm genomic integrity
- 13 **Step 1: Knock-in of Inducible Cas9 at AAVS1 Locus**
 - Transfect WA09 hESCs with the **AAVS1-targeting sgRNA** and **hygro-resistant iCas9 donor plasmid**.
 - Select for stable integration using **hygromycin B (50–100 µg/mL)** for 7–10 days.
 - Expand surviving colonies and confirm targeted insertion at the AAVS1 locus by **PCR and/or sequencing**.
- 14 **Step 2: Confirmation of iCas9 Cell Line**
 - Induce Cas9 expression with **1–2 µg/mL doxycycline** for 24–48 hours.
 - Confirm Cas9 induction by western blot or RT-qPCR (optional).
 - Perform **karyotyping** to verify genomic integrity before proceeding.
- 15 **Step 3: Insertion of APP^{swe/swe} Mutation**
 - Transfect or electroporate the verified iCas9 line with:
 - **APP-targeting sgRNA**
 - **ssODN or plasmid donor** carrying the APP^{swe/swe} mutation
 - Induce Cas9 with doxycycline for 24–48 hours post-transfection.
 - Allow recovery and expand clones for screening.
- 16 **Step 4: Clone Screening and Validation**
 - Screen colonies by **allele-specific PCR** or **Sanger sequencing** to confirm biallelic APP^{swe/swe} knock-in.
 - Validate absence of off-target edits (optional).
 - Expand positive clones for further use.
- 17 **Step 5: Final Karyotype Check**
 - Perform **karyotyping** on validated APP^{swe/swe} clones to ensure chromosomal integrity.
 - Freeze down multiple validated clones for future experiments.
- 18 **Notes**
 - Always confirm Cas9 induction and cutting efficiency in pilot experiments.
 - Use low-passage WA09 cells to minimize risk of spontaneous karyotypic abnormalities.

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

19 **Step 1: Cell Preparation**

- Dissociate Nurr1-GFP ESCs using **Accutase** for 5–10 minutes at 37°C until single cells.
- Seed cells onto **Matrigel-coated dishes (1:50)** at **25,000 cells/cm²** in **E8 medium + 10 µM Y-27632**.

20 **Step 2: Media Change**

- The following day, replace medium with **mTeSR1** without Y-27632 to promote cell recovery and optimal transfection conditions.

21 **Step 3: Initial CRISPR/Cas9 Transfection**

- Prepare DNA mixture with **pX458 LRRK2 gRNA vector** and **ssODN donor** at **5:1 ratio** (total 250 ng DNA per 25k cells).
- **Transfect using Lipofectamine™ Stem following the manufacturer's protocol.**
- Return cells to mTeSR1 and incubate under standard conditions (37°C, 5% CO₂).

22 **Step 4: Repeat Transfections (Day 2 & 3)**

- On each of the next two days, repeat transfection with **ssODN only** (NO plasmid).
- Use the same Lipofectamine™ Stem protocol with 250 ng ssODN per 25k cells.

23 **Step 5: GFP+ Cell Sorting**

- At **24 hours after the final transfection**, sort **GFP-positive cells** (indicating transfection) using FACS.
- Seed sorted cells as **single cells into 96-well plates** pre-coated with Matrigel in **mTeSR1 + 10 µM Y-27632**.

24 **Step 6: Clone Recovery and Media Switch**

- Allow clones to expand for **6 days** in mTeSR1.
- On day 6, change medium to **E8** and continue expansion.

25 **Step 7: Clone Expansion**

- Once colonies are visible and stable, expand to larger wells (48-well, then 6-well) in E8 medium.
- Monitor morphology and maintain optimal cell density to avoid differentiation.

26 **Step 8: Genotyping via BceAI Digest**

- Extract genomic DNA from clones.
- PCR-amplify the LRRK2 target region and perform **BceAI digestion**.
- **Loss of BceAI site confirms successful introduction of G2019S mutation.**

27 **Step 9: Sanger Sequencing**

- Send PCR products for **Sanger sequencing** to confirm **biallelic or monoallelic editing**.
- Compare sequences against wild-type reference.

28 **Step 10: Karyotype Analysis**



- Submit successfully edited clones for **metaphase spread karyotyping**.
- Only proceed with clones showing normal karyotype.

Cortical neuron differentiation

- 29 **Matrigel Coating (for Day -1 plating)**
- Thaw Matrigel on ice at 4°C overnight.
 - Dilute 1:30 in cold DMEM/F12.
 - Add: 1 mL/well (6-well)
 - Incubate at 4°C for up to 1 week or coat at 37°C for 2h before use
- 30 **Step 1: Plate hESCs for Differentiation (Day -1)**
- Dissociate H9 cells into single cells using **Accutase**.
 - Plate at **300,000 cells/cm²** onto **Matrigel-coated plates (1:30 in DMEM/F12)**.
 - Culture in **E8 + 10 µM Y-27632** overnight.
- 31 **Step 2: Neural Induction (Day 0–3)**
- Confirm cells reach **100% confluency** before starting induction.
 - Feed daily with **XLSB in E6**:
 - SB431542 (10 µM)
 - LDN193189 (100 nM)
 - XAV939 (2 µM)
- 32 **Step 3: Neural Patterning (Day 4–10)**
- Replace XLSB with **LSB in E6** (remove XAV).
 - Feed daily:
 - SB431542 (10 µM)
 - LDN193189 (100 nM)
- 33 **Step 4: Early Cortical Maturation (Day 10–20)**
- Switch to **N2/B27 medium (no vitamin A)**.
 - Feed daily with fresh **N2/B27**.
- 34 **PO/Laminin/Fibronectin Coating (for Day 20 onward)**
1. Dilute **Poly-L-ornithine (15 µg/mL)** in DPBS.
 - Incubate **overnight at 37°C**.
 1. Wash **2–3× with DPBS**.
 2. Add **Laminin I + Fibronectin (2 µg/mL each)** in DPBS.

- Incubate **overnight at 37°C**.

35 **Step 5: Passage Cells for Maturation (Day 20)**

- Use **Accutase (30 min at 37°C)** to lift cells.
- Plate at **100,000–150,000 cells/cm²** on **PO/Laminin/Fibronectin-coated plates**.
- Culture in **NB/B27 + L-glutamine + Pen/Strep + 10 µM Y-27632 + DAPT (10 µM)**.

36 **Step 6: Terminal Differentiation (Day 20–30)**

- Maintain cells in **NB/B27 + DAPT** (no Y-27632).
- **Change half the medium every 5 days**.

37 **Step 7: Neuronal Maturation (Day 30 onward)**

- Culture cells in **NB/B27 + L-glutamine + BAGC cocktail**:
 - BDNF, GDNF, cAMP, Ascorbic Acid (1:500 each).
- Change **~½ medium every 2x per week**.

Whole genome CRISPR Cas9 screen in PSC-derived neurons

38 **Step 1: PSC Dissociation and Replating for Transduction**

- Dissociate PSCs using Accutase.
- Plate 250 million cells per line at 150,000 cells/cm² in E8 + 10 µM Y-27632 on Matrigel-coated plates.

39 **Step 2: Lentiviral Library Transduction**

- Add Brunello lentiviral gRNA library at MOI 0.3–0.5 during replating.
- Incubate for 16–18 hours.

40 **Step 3: Media Replacement**

- Replace virus-containing media with fresh E8 medium (no puromycin, no virus).

41 **Step 4: Puromycin Selection**

- At ~24 hours post-transduction, add 0.4 µg/mL puromycin to select for infected cells.
- Continue selection for 48–72 hours until untransduced controls are eliminated.

42 **Step 5: Pooling and Expansion**

- Dissociate selected PSCs with Accutase and pool culture plates.
- Count and plate 116 million cells for differentiation to maintain >1000× gRNA representation during differentiation.

43 **Step 6: Neural Differentiation**

- Differentiate PSCs following the lab's validated hESC cortical differentiation protocol.
- Maintain proper media and coating transitions from Day 0 to Day 20 as previously described.

44 **Step 7: Day 20 – T=0 Harvest and Experimental Setup**



- On Day 20, dissociate cultures using Accutase into single-cell suspensions.
- Split into triplicate samples, 90 million cells per replicate.
- Harvest T=0 representation controls from one set of samples (no doxycycline added).

45 **Step 8: Plating for Induction and Control**

- Plate remaining cells at 200,000 cells/cm² in Neurobasal + N2 + B27 + 10 µM Y-27632.
- Add 2 µg/mL doxycycline to half the culture plates (+DOX condition).
- Leave the other half untreated (-DOX control).

46 **Step 9: Cas9 Activation**

- Maintain +DOX samples for 48 hours to induce Cas9 expression.
- After 48 hours, replace medium with neural maintenance medium + DAPT (1 µM) to limit proliferation.

47 **Step 10: Culture Maintenance to DIV 30**

- Maintain cells until DIV 30, replacing half of the medium every 2–3 days.

48 **Step 11: Long-Term Culture to DIV 65**

- From DIV 30 to DIV 65, continue maintenance with BAGC or neurotrophic factors (if applicable).
- Continue 50% media changes every 2–3 days.

49 **Step 12: Endpoint Sample Collection (DIV 65)**

- Collect endpoint control (-DOX) and experimental (+DOX) samples in parallel.
- Wash monolayers 2× with PBS, then incubate in 0.5 mM EDTA for 5 minutes at RT.

50 **Step 13: Cell Harvesting and Freezing**

- Scrape cells off dishes and pellet by centrifugation (300 × g, 5 min).
- Snap freeze the pellets in liquid nitrogen and store at -80°C.

51 **Step 14: Library Prep and sequencing**

- Extract gDNA using following the [manufacturer's instructions](#).
- Quantify DNA using Qubit.
- Amplify integrated gRNA sequences using Illumina-compatible primers
- Confirm amplicon size and quality using Agilent Bioanalyzer.
- Sequence pooled libraries using Illumina HiSeq 2500

Data analysis for Pooled CRISPR screen

52 Sequencing reads were aligned to the screened library and the CRISPR screen was analyzed using the MAGECK-MLE pipeline as previously described.¹⁷

53 **Step 1: Prepare Input Files**

1. Ensure all FASTQ files from the screen are named according to sample identity (e.g., WT_T0, APP_Dox, etc.).



2. Source the reference sgRNA library file corresponding to the Brunello CRISPR library
3. Compile a list of non-targeting sgRNAs for normalization purposes.

54 **Step 2: Align Reads and Count sgRNAs**

1. Align sequencing reads to the sgRNA reference library and generate a raw count matrix.
2. Normalize the count matrix using non-targeting control sgRNAs to account for technical variability between replicates and conditions.

55 **Step 3: Run MAGeCK-MLE Analysis**

1. Perform MAGeCK-MLE analysis separately for each genotype (WT and APP^{swe/swe}), using the DIV20 (T=0) samples as the primary representation control.
2. Repeat the analysis using the DIV65 -DOX samples as an alternative control to confirm reproducibility of hits.
3. For each run, obtain gene-level statistics including:

- Beta score (magnitude and direction of selection)
- P-value and false discovery rate (FDR)

56 **Step 4: Define Viability Genes**

Identify **viability genes** as those meeting all of the following thresholds in **both WT and APP^{swe/swe}** conditions:

- Beta score < 0 (indicating depletion)
- FDR < 0.3
- P-value < 0.05

Candidate age regulators as genes that meet viability criteria **only in APP^{swe/swe}** but **not in WT**.

57 **Notes**

- T=0 and -DOX endpoint controls serve complementary roles; consistency between both strengthens confidence in hits.

58 **Outputs**

- Gene-level beta scores with p-values and FDRs
- Lists of essential genes and candidate age regulators

RNA extraction and qPCR

59 RNA was extracted using the Zymo RNA Micro Kit according to the [manufacturer's protocol](#) and total of 1 µg of RNA was used to generate cDNA using iScript (BioRad).

59.1 Primers used in this study are listed in [Table S2](#).

Immunocytochemistry

60 Fixation:

Note: Volume per well for a 96 well plate: 50 µl

1. Remove medium, wash wells once with PBS
2. Fix in 4% paraformaldehyde for 10mins
3. Wash 3x with PBS

61 Primary antibody

1. Permeabilize in PBS+0.3% Triton at RT for 15min
2. Blocked in 5% donkey or goat serum for 1h at RT.
3. Prepare primary antibody solution. Primary antibodies used in this study and dilutions are listed in [STAR Methods](#)
4. Remove blocking solution and add primary antibody solutions incubate at +4 overnight (on an orbital shaker set to slowest speed).

62 Image Acquisition and Preprocessing

1. Acquire fluorescent images using identical microscope settings (e.g., laser power, gain, exposure time) across all wells within each replicate.
2. Apply consistent minimum and maximum intensity adjustments
3. For all image quantifications images were taken from 3 individual wells and averaged. This was repeated three times with neurons from independent differentiations.

High content imaging

63 Plate and Culture Neurons

Generate and plate out cortical neurons in 96-well plates according to protocol described in the "Cortical neuron differentiation" subsection. a Culture conditions and media changes should follow your established timeline until the desired assay time point is reached.

64 Fixation and Immunostaining

Fix and stain neurons for relevant markers of aging and viability (e.g., DAPI for nuclei, marker antibodies for mitochondrial content, oxidative stress, nuclear morphology, etc.) as previously described in the "Immunocytochemistry" subsection.

65 Image Acquisition

Acquire fluorescent images using the Operetta high-content microscope:

- Image at least 9 fields per well.
- Use consistent settings across wells and replicates.

66 **Image Analysis with Harmony**

Analyze images using Harmony software:

- Automatically segment and quantify cells.
- Identify live cells by applying a minimum nuclear size threshold and a maximum DAPI intensity cutoff to exclude dead or pyknotic nuclei.

67 **Data Normalization and Statistical Testing**

Normalize fluorescence intensity measurements to DMSO control wells (set as value = 1):

- Use one-sample t-test to determine if test conditions deviate significantly from control.
- For direct comparisons (e.g., nuclear morphology), apply an unpaired, two-tailed t-test.

68 **Reporting**

Report data as mean $\pm$ SEM or mean $\pm$ SD as appropriate.

- Clearly specify number of wells and biological replicates.
- Include p-values and statistical test details in figure legends.

69 **Note:**

This protocol can be used to quantify aging hallmarks in both chemical and genomic perturbations

A β ELISA

70 **Collect and Clarify Culture Medium**

1. At 48 hours after media change or treatment, **collect conditioned culture** medium from each well.
2. Centrifuge samples at **~500 $\times$ g for 5 minutes at 4°C** to pellet any cellular debris.
3. Carefully transfer supernatant to a fresh low-bind tube. Keep on ice or store at **-80°C** until assay.

71 **Run MSD A β Assay**

1. Perform the **MSD V-PLEX A β Peptide Panel 1** assay according to the **manufacturer's protocol**:

- Use 25 μ L of culture medium per well.
- Include standard curve, blanks, and technical replicates.
- Incubation times, wash steps, and detection procedures should strictly follow the MSD protocol.

72 **Read and Analyze Plate**

1. Read the plate using an MSD instrument (e.g., MESO QuickPlex) with appropriate software settings.
2. Apply standard curve to generate **A β 38, A β 40, and A β 42** concentrations for each sample
3. Calculate **Total A β** as the **sum of A β 38 + A β 40 + A β 42** per sample.

73 **Normalize A β to Total Protein Content**

1. Harvest matched neuronal cultures (same age/treatment) for protein extraction:
 - Lyse neurons using a standard buffer (e.g., RIPA) with protease inhibitors.
 - Quantify total protein using Precision Red assay according to.
2. Normalize Total A β measurements to the mean total protein content per condition:
 - Use the **average protein content** from 3 independent differentiations/experiments for normalization.

74 **Data Analysis and Replication**

1. Average A β measurements across **3–4 culture wells per experimental replicate**.
2. Represent final data as **normalized Total A β per μ g protein** or as a fold change relative to –Dox control.
3. Perform appropriate statistical analysis (e.g., unpaired t-test or ANOVA) to compare experimental groups.

Western blotting

75 **Harvesting Samples**

1. Remove culture media and wash cells once with ice-cold PBS.
2. Scrape cells into ice-cold PBS and transfer to microcentrifuge tubes.
3. Centrifuge at 600 $\times$ g for 5 min at 4 $^{\circ}$ C.
4. Remove supernatant completely.

5. Snap-freeze the pellet in a dry-ice/methanol bath.

6. Store at -80°C if not processing immediately.

76 Cell Lysis

- Resuspend frozen pellets in RIPA buffer + 1:100 Halt protease (Thermo Fisher 87786 and phosphatase inhibitors (Thermo Fisher 78420). Typically 1×6 well of neurons requires 100 μL of RIPA buffer.
- Incubate on ice for 30 min pipetting 3x to help lyse cells.
- Centrifuge lysates to clarify (max speed on tabletop centrifuge, 10 min, 4°C).
- Transfer supernatant to fresh tubes and continue to quantification or store at -80°C until needed

77 Western Blotting

1. Load samples onto NuPAGE Novex 4-12% Bis Tris gels alongside loading control and run according to **manufacturer's instructions**
2. Transfer Protein onto PVDF by electrophoretic transfer in transfer buffer containing 20% methanol
3. 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**.
4. Band intensity was visualized using BioRad ChemiDoc XRS+ molecular imager.
5. After imaging the membrane re-probed with either GAPDH or β -ACTIN antibodies for normalization.

Fractionation of protein lysates into sarkosyl soluble/insoluble fractions

78 Notes

Protocol for fractionation of cell lysates from stem cell derived neurons was adapted from Manos et al.⁷⁷

- 78.1 Cortical neurons were cultured in 6 well plates until DIV50 then $1\mu\text{M}$ MLN4924 or DMSO were applied for 10 days before harvesting.

79 Lysis:

1. Resuspend cell pellets from 1 well of a 6 well plate in 100 μL RIPA buffer supplemented with Halt protease and phosphatase inhibitors (ThermoFisher)
2. Incubate for 15mins on ice
3. Spin at 14,000rpm for 1min then transfer supernatant to a fresh tube
4. Measure protein concentration using the **Precision Red Advanced Protein** Assay according to manufacturer's instructions

80 Sarkosyl Fractionation and Blotting

1. Transfer equal amounts of total protein to a fresh tube
2. Add sarkosyl to a final concentration of 1%.
3. Incubated at RT for 30mins
4. Spin using ultracentrifuge (150,000g for 30mins).

5. Decant the soluble fraction
6. Add fresh RIPA buffer + 1% sarkosyl added to the pellets and repeat the spin step.
7. Remove supernatant and discarded
8. Resuspend the pellet in Laemmli buffer + reducing agent.
9. Boil lysates at 95C for 5mins before loading.
10. Perform Western Blots as described in the "Western Blotting subsection" but use Reversible Protein Stain Kit for PVDF membranes (Pierce; Cat. #24585) as **described by the manufacturer** prior to the blocking step to normalise to total protein.

Secondary Validation of CRISPR-screen targets

81 **sgRNAs:**

- The list of gRNA sequences used for this study can be found in **Table S3**.
- gRNAs were cloned into the lentiGuide-Puro plasmid (Addgene 52963) or pLKO5.sgRNA.EFS.GFP (Addgene 57822) as **described by the Zhang lab** [Sanjana et al. Nat Methods 2014](#), [Shalem et al. Science 2014](#) using standard cloning techniques and following the instructions for the insertion of the nucleotides.
- 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.
- Virus particles were harvested after 48h, pelleted to remove cellular debris then snap frozen.

82 **Experimental Set Up:**

1. Differentiate hESCs into cortical neurons according to the "Cortical neuron differentiation" protocol
2. Using dissociated DIV20 cells plate into 96-well plates at 150,000 cells/cm²
3. Plate 3 technical replicates per condition per independent differentiation.

83 **Viral transduction**

For each sgRNA test viability under the following conditions

- WT neurons + sgRNA lentivirus
- WT neurons + sgRNA lentivirus + doxycycline
- App^{swe/swe} neurons + sgRNA lentivirus
- App^{swe/swe} neurons + sgRNA lentivirus + doxycycline

1. Apply unconcentrated lentivirus at a 1:30 dilution on on DIV20 and DIV21 (Two sequential daily applications).
2. Add 2 µg/mL doxycycline simultaneously in the relevant wells
3. On DIV22 remove virus and doxycycline and add **1 µg/mL puromycin** for **48 h** to select for transduced neurons.
4. Maintain neurons afterward in standard neural media.

84 **Viability Assay**

After antibiotic selection (DIV24), maintain neurons as described in the main neuronal culture protocol.

Perform Viability Assay at DIV60 as described in the "Presto Blue Viability Assay" Subsection.

Generation of UBA3 and NAE1 overexpression lentiviruses

- 85 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.
- 85.1 Primers used to amplify to ORFs for cloning and to sequence the resulting clones can be found in [Table S2](#).
- 85.2 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.
- 85.3 Virus particles were harvested after 48h and concentrated using Amicon Ultra-15 Centrifugal Filter Unit (100kDa).

Flow Cytometry

- 86 **Reagents:**
- Zombie UV Fixable Viability Kit (BioLegend, Cat# 423107)
 - CellEvent Senescence Green (Thermo Fisher, Cat# C10840)
 - H3K9me3-PE antibody (Cell Signaling Technology, Cat# 13969S)
 - Proteostat Aggresome Detection Kit (Enzo Life Sciences, Cat# ENZ-51023-KP050)
- 87 **Dissociation**
1. Aspirate media from neuronal cultures, wash with PBS
 2. Add sufficient Accutase to cover cells, supplemented with Neuron Isolation Enzyme (Thermo 88285) at 1:50.
 3. Incubate at 37 °C until cells detach (monitor under microscope).
 4. Gently triturate then pass through a cell strainer to obtain a single-cell suspension.
- 88 **Staining:**
1. Incubate single cell suspensions were stained with Zombie UVTM Fixable Viability Kit (Biolegend 423107) at 1:2500 in PBS for 15 minutes at room temperature.
 2. Fixed cells in 4% Paraformaldehyde for 10 minutes (4°C).
 3. Stain with CellEvent Senescence Green (Thermo C10840) by diluting 1:250 in assay buffer and incubating for 2 hours at 37°C.

4. For intracellular probes: permeabilize cells in 0.5% triton-x for 10 minutes (4°C) then blocked in 5% BSA for 10 minutes
5. Incubate 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.
6. Wash 2x in FACs buffer
7. Analyse on Cytex Aurora FlowCytometer

A β 42 neurotoxicity assays

89 Reagents

AggreSure A β 42 monomers Anaspec, Cat# AS-72216
DMSO, molecular-biology grade
PBS, sterile
PrestoBlue™ Cell Viability Reagent (ThermoFisher, Cat# A13261)
MLN4924 (MedChemExpress, Cat# HY-70062)
CSN5i-3 (Tocris, Cat# 7089)
pLV-EF1a-UBA3-IRES-Puro (Addgene #235660)
pLV-EF1a-NAE1-IRES-Puro (Addgene #235659)
pLV-EF1a-IRES-Puro (Addgene 85132)

90 Before you begin:

Resuspend AggreSure A β 42 (Anaspec) monomers in DMSO then dilute in PBS to generate a 100 μ M working solution. Store in single use aliquots at -80 until needed.

91 A β 42 Neurotoxicity Assay

Add 5 μ M A β 42 or vehicle control (DMSO + PBS) to neurons for 7 days, including one full media change during the treatment period.

At the endpoint, assess viability using PrestoBlue according to section 16

92 Timing of A β 42 Neurotoxicity Assay:

- For loss of function experiments add 5 μ M A β 42 or vehicle control (DMSO + PBS) starting between DIV35-DIV45.
- For chemical manipulations of the neddylation pathway add 1 μ M MLN4924 or 1 μ M CSN5i3 concurrently 5 μ M A β 42 or vehicle control (DMSO + PBS) starting on DIV30.
- For long-term chemical inhibition of the neddylation pathway add 1 μ M MLN4924 starting DIV30 and add 5 μ M A β 42 or vehicle control (DMSO + PBS) the last 7 days of the assay.

- For UBA3 and NAE1 overexpression experiments add 5 μ M A β 42 or vehicle control (DMSO + PBS) add 5 μ M A β 42 or vehicle control (DMSO + PBS) on DIV30.

93 **Lentiviral Overexpression of UBA3 and NAE1:**

1. Add concentrated virus (pLV-EF1aUBA3-IRES-Puro, pLV-EF1aNAE1-IRES-Puro or pLV-EF1a1-IRES-Puro (control)) to neurons at a 1:500 dilution at DIV 20.
2. On DIV 22 add 1 μ g/mL puromycin to the cultures for 4 days to select for transduced neurons

Proteasome activity assay

94 **Reagents**

Cell-Based Proteasome-Glo™ Assay (Promega, Cat# G8660)

95 **Before you Begin:**

1. Plate neurons into 96-well plates at 150,000 cells/cm².
2. Maintain neurons in standard cortical neuron medium until DIV30.
3. Begin Drug Treatment at DIV30
4. Prepare MLN4924 fresh from stock; avoid freeze-thaw cycles.

96 **Prepare Reagent**

1. Equilibrate Proteasome-Glo™ Cell-Based Buffer and lyophilized Luciferin Detection Reagent to room temperature.
2. In the amber bottle reconstitute the Luciferin Detection Reagent by adding 10mL of Proteasome-Glo™ Cell-Based Buffer.
3. Thaw the Suc-LLVY-Glo™ substrate and equilibrate to room temperature. Mix well by vortexing briefly to remove any precipitate.
4. Prepare the Proteasome-Glo™ Reagent by adding 50 μ l of substrate to 1 bottle of reconstituted luciferin Detection Reagent.

97 **Assay: 96 well plate**

Prepare reagents and equilibrate to RT.

Replace culture media with 100 μ l of room temperature PBS

Include Blank, PBS only wells

Add 100 μ l of Proteasome-Glo™ Cell-Based Reagent to each 100 μ l of sample and control.

Cover the plate using a plate seal

Mix the contents of the wells at 700rpm using a plate shaker for 2 minutes.

Incubate at room temperature for a minimum of 10 minutes.

1. Measure the luminescence of each sample in a plate reader
2. Normalise MLN4924 wells to the DMSO controls.

Presto Blue Viability Assay

98 **Reagents:**

Presto Blue (Thermo Cat# A13261)

99 **Procedure**

1. Dilute PrestoBlue 1:10 in neural maintenance media fresh immediately before use. For one 96-well plate, mix: 0.9 mL PrestoBlue, 8.1 mL neural maintenance media
2. Aspirate all culture media from each well carefully to avoid disturbing cells.
3. Add 85 μ L of the diluted PrestoBlue solution to each well.
4. Avoid introducing bubbles, as they interfere with absorbance readings.
5. Include ≥ 3 blank wells containing PrestoBlue mix only (no cells) to serve as background controls.
6. Return the plate to the **37 °C incubator** for **2 hours**.
7. Measure absorbance (570nm and 600nm) on the plate reader

100 **Analysis:** normalize 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).

CCK8 Viability Assay

101 **Reagents:**

Cell Counting Kit - 8 (Sigma Aldrich Cat# 96992-100TESTS-F)
Manufacturer's Protocol

102 **Procedure**

1. Bring CCK8 solution to RT
2. Prepare CCK8 reagent by adding 100 μ L to every 1mL of neural maintenance media.
3. Aspirate all culture media from each well carefully to avoid disturbing cells.
4. Add 110 μ L of the diluted CCK8 solution to each well.
5. Avoid introducing bubbles
6. Include ≥ 3 blank wells containing CCK8 mix only (no cells) to serve as background controls.
7. Return the plate to the **37 °C incubator** for **2 hours**.
8. Measure absorbance (450nm) on the plate reader

103 **Analysis:** normalize 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).

