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Nathalie Saurat¹, Johannes Jungverdorben¹, Andrew Minotti¹, Gabriele Ciceri¹

¹Memorial Sloan Kettering Cancer Center



Johannes Jungverdorben

Memorial Sloan Kettering Cancer Center

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



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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 20 different protocols.

Full manuscript can be found here:

DOI: **10.1016/j.stem.2024.06.001**

hPSC culture

- 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 **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 (1:25 in DMEM/F12)**.
- Culture in **E8 + 10 μ M Y-27632** overnight.

30 **Step 2: Neural Induction (Day 0–3)**

- Confirm cells reach **100% confluency** before starting induction.
- Feed daily with **XLSB in E6**:

- SB431542 (10 μ M)

- LDN193189 (100 nM)

- XAV939 (2 μ M)

31 **Step 3: Neural Patterning (Day 4–10)**

- Replace XLSB with **LSB in E6** (remove XAV).
- Feed daily:

- SB431542 (10 μ M)

- LDN193189 (100 nM)

32 **Step 4: Early Cortical Maturation (Day 10–20)**

- Switch to **N2/B27 medium (no vitamin A)**.
- Feed daily with fresh **N2/B27**.

33 **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 μ M Y-27632 + DAPT (10 μ M)**.

34 **Step 6: Mid Differentiation (Day 20–30)**

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

35 **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 of medium every 5–7 days.

36 **Matrigel Coating (for Days -1 to 20)**

- Thaw Matrigel on ice at 4°C overnight.
- Dilute 1:25 in cold DMEM/F12.
- Add:

- 0.5 mL/well (24-well),
- 2 mL/well (6-well), etc.

- Incubate at 4°C for up to 1 week.

37 **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**.

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** 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: Genomic DNA Extraction**

- Extract gDNA using **QIAamp DNA Mini Kit (Qiagen)** following the manufacturer's instructions.
- Quantify DNA using **Qubit**.

52 **Step 15: PCR Amplification of gRNA Sequences**

- Amplify integrated gRNA sequences using Illumina-compatible primers with sample-specific barcodes.
- Use high-fidelity polymerase and follow the recommended number of cycles (e.g., 25–28).

53 **Step 16: Library QC**

- Confirm amplicon size and quality using **Agilent Bioanalyzer**.
- Pool samples based on equimolar concentrations.

54 **Step 17: Sequencing and Data Analysis**

- Sequence pooled libraries using **Illumina HiSeq 2500** (single-end 50 bp recommended).
- Analyze data using tools like **MAGeCK** or **PinAPL-Py** for gRNA enrichment and dropout.

Data analysis for Pooled CRISPR screen

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

56 **Step 1: Prepare Input Files**

1. Ensure all FASTQ files from the screen are demultiplexed and named according to sample identity (e.g., WT_T0, APP_Dox, etc.).
2. Collect or generate the reference sgRNA library file corresponding to the Brunello CRISPR library used in the screen.
3. Compile a list of non-targeting sgRNAs for normalization purposes.
4. Create a sample metadata file that describes experimental groups, treatments, and time points.

57 **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.

58 **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)

59 Step 4: Define Viability Genes

1. Identify **viability genes** as those meeting all of the following thresholds:

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

60 Step 5: Identify Essential and Candidate Age Regulator Genes

1. Define **essential genes** as those that meet viability criteria in **both WT and APP^{swe/swe}** conditions.
2. Define **candidate age regulators** as genes that meet viability criteria **only in APP^{swe/swe}** but **not in WT**.

61 Step 6: Exclude WT Viability Confounders

1. To refine the list of candidate age regulators, exclude genes that show potential viability effects in WT cells:
 - Genes with beta scores in WT that are:
 - Less than 0 with FDR < 0.3 and p < 0.05, or
 - Greater than 1.5 standard deviations above the mean beta score across all genes in WT.

62 Step 7: Compare Gene Lists Across Conditions

1. Compare beta scores between WT and APP^{swe/swe} conditions for each gene.
2. Generate ranked gene lists and Venn diagrams to visualize overlaps between essential, viability, and age regulator gene sets.

63 Step 8: Prepare Data for Visualization and Downstream Analysis

1. Export beta scores and statistical metrics for visualization in R, Python, or other tools.
2. Optional: Perform pathway enrichment analysis or clustering on significant genes to identify functional modules or biological processes affected.

64 Notes

- T=0 and -DOX endpoint controls serve complementary roles; consistency between both strengthens confidence in hits.
- Using multiple biological replicates improves statistical power and reliability of beta score estimation.
- MAGeCK-MLE is designed to model replicate variability and normalize for gRNA-level effects, making it preferable for complex pooled screens.

65 Outputs

- Gene-level beta scores with p-values and FDRs



- Lists of essential genes and candidate age regulators
- Publication-ready plots for volcano, enrichment, and comparison of conditions
- Input files and parameters for reproducibility tracking

KEGG pathways

66 Prepare Ranked Gene Lists

1. From MAGECK-MLE output, rank all genes based on beta scores for each condition (e.g., WT_Dox vs T0, APP_Dox vs T0).
 2. Generate .rnk files containing gene symbols and corresponding beta scores or log-fold changes.
- Use the full list of genes for unbiased GSEA.
 - Create additional filtered files with the **top 1000 genes** for focused analyses of hit and essential categories.

67 Launch GSEA and Set Parameters

1. Open the GSEA GUI or run via command-line interface.
 2. Upload the .rnk files for each condition or category (essential, APP-specific, WT-specific).
 3. Select the appropriate KEGG pathway gene set from MSigDB (e.g., c2.cp.kegg.v7.5.symbols.gmt).
 4. Set analysis parameters:
- Number of permutations: 1000
 - Permutation type: gene_set
 - Collapse dataset: false (if ranked list already uses gene symbols)
 - Metric for ranking: Signal2Noise or user-supplied values
 - FDR cutoff: **0.01** (stringent threshold as used in this analysis)

68 Run GSEA

1. Execute the analysis for each gene category:
- All genes (unfiltered)
 - Top 1000 essential genes (common to WT and APP^{swe/swe})
 - Top 1000 APP-specific hit genes

- WT-specific hit genes (if applicable)
- **Survival/proliferation genes (only 4 total genes)** — note that GSEA may be limited or not informative due to small input size.

69 Review and Interpret Results

1. Examine GSEA output:

- NES (Normalized Enrichment Score)
- Nominal p-value
- FDR q-value

1. Identify KEGG pathways enriched with **FDR < 0.01**.
2. Save results as tables and plots (e.g., enrichment plots, ranked list heatmaps).

70 Visualization and Reporting

1. Visualize top enriched pathways using enrichment plots and summary bar charts.
2. Cross-compare pathways enriched in WT vs APP^{swe/swe} cells to identify genotype-specific biological signatures.
3. For categories with too few genes (e.g., 4 survival/proliferation genes), consider manual pathway mapping or overrepresentation analysis in tools like DAVID or Enrichr as an alternative.

71 Notes

- An FDR < 0.01 ensures high confidence in pathway-level enrichment.
- For gene categories with limited gene numbers (<10), GSEA may lack power. Consider complementary tools like Enrichr or manual inspection.
- Ensure consistent gene symbol formatting across input and KEGG gene sets.

Gene enrichment analysis

72 Step 1: Define and Rank Essential Genes

1. Combine beta scores from MAGeCK-MLE output for both WT and APP^{swe/swe} samples by averaging or summing them.
2. Filter for genes that meet the following criteria in **both genotypes**:
 - P-value < 0.05
 - FDR < 0.1
1. Rank the filtered genes by the combined beta score (strongest depletion).

2. Select the **top 500 essential genes** for downstream analysis to ensure consistency across screens.

73 **Step 2: Prepare Input for Functional Annotation**

1. Format gene lists using official **gene symbols** (HGNC).
2. Prepare equivalent top-500 essential gene lists from:
 - Your current screen (combined WT and APP^{swe/swe})
 - Tian et al. study¹⁸
 - Wertz et al. study¹⁹
1. Save gene lists in plain text format (one gene per line) or as comma-separated values for DAVID upload.

74 **Step 3: Perform GO Analysis Using DAVID**

1. Navigate to **DAVID 6.8**.
2. Upload each gene list separately under "Functional Annotation Tool".
3. Select the appropriate identifier (e.g., **OFFICIAL_GENE_SYMBOL**) and species (**Homo sapiens**).
4. Under "Gene_Ontology", choose the **Biological Process** category.
5. Run enrichment analysis with default background (whole genome).
6. Filter results by:
 - **p-value < 0.05**
 - Limit to **top 10 GO terms** per gene set for consistency and clarity

75 **Step 4: Extract and Organize Results**

1. Download the DAVID output tables for each gene list (Current Screen, Tian et al., Wertz et al.).
2. Retain columns:
 - GO Term
 - Count
 - % of genes
 - P-value
 - Benjamini (optional)

1. Reformat for visualization:

- Add a "Screen" column indicating source (e.g., "This Study", "Tian", "Wertz")
- Combine all GO terms and screens into a single table

76 Step 5: Visualize Enrichment Using ggplot2

1. Load the combined results table into R.
2. Use ggplot2 to create bar plots or dot plots showing the **top 10 GO terms per screen**, ranked by $-\log_{10}(\text{p-value})$ or gene count.
3. Facet or color by screen to compare GO categories between datasets.

RNA extraction and qPCR

77 RNA was extracted using the Zymo RNA Micro Kit and total of 1ug of RNA was used to generate cDNA using iScript (BioRad).

77.1 Realtime PCR was performed using SSoFAST EvaGreen Mix (BioRad) in a BioRad CFX96 Thermal Cycler. The manufacturers protocol was used for all steps.

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

Immunocytochemistry

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

78.1 Cells were blocked in 5% donkey or goat serum for 1h.

78.2 Primary antibody incubation was performed overnight. Primary antibodies used in this study are listed in [STAR Methods](#).

78.3 For all image quantifications images were taken from 3 individual wells and averaged. This was repeated three times with neurons from independent differentiations.

79 Image Acquisition and Preprocessing

1. Acquire fluorescent images using identical microscope settings (e.g., laser power, gain, exposure time) across all wells within each biological replicate.

2. Apply consistent **minimum and maximum intensity adjustments** across images for each replicate to ensure comparative quantification.
3. Export images for MAP2/Ki67 quantification into Velocity-compatible format, and Tau quantification images into **.tif format** for FIJI.

80 **MAP2+ and Ki67+ Cell Counting in Velocity**

1. Open DAPI, MAP2, and Ki67 channels in Velocity.
2. Manually exclude cells with **pyknotic or DAPI-bright nuclei**, as these indicate non-viable or apoptotic cells.
3. Use the **cell counting tool** or **region classifier** in Velocity to count:

- Total number of DAPI+ nuclei (viable only)
- Number of MAP2+ cells
- Number of Ki67+ cells

4. Save results and export counts as spreadsheets for further analysis.

81 **Tau and pTau Quantification in FIJI**

1. Open Tau and pTau channel images in FIJI.
2. Convert each image to **8-bit grayscale** (Image > Type > 8-bit).
3. Set a consistent **threshold** for each channel across all wells within the same replicate:

- Use Image > Adjust > Threshold and apply manually or with a saved thresholding macro.
- Two thresholds may be applied if analyzing **total signal** vs. **"bright" (high-intensity) signal** separately.

4. Use the **"Measure"** function (Analyze > Measure) to quantify the **area** (in μm^2 or pixels) above threshold.

5. Ensure the **same threshold values are used** across all images for a replicate.

6. Record total area per image and calculate averages across **3 wells per replicate**.

82 **Step 4: Data Normalization and Averaging**

1. Average counts and measured areas from the 3 wells per replicate.
2. Normalize Tau/pTau area measurements if needed (e.g., to total DAPI+ cells or image field area).
3. Summarize results in a spreadsheet with columns for:

- Replicate ID
- MAP2+ count

- Ki67+ count
- Tau area
- pTau area
- Normalized values

High content imaging

83 Plate and Culture Neurons

1. Plate neurons in 96-well plates and differentiate according to the standard neuronal differentiation protocol. Culture conditions and media changes should follow your established timeline until the desired assay time point is reached.

84 Fixation and Immunostaining

1. 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 your core protocol.

85 Image Acquisition

1. Acquire fluorescent images using the **Operetta high-content microscope**:

- Image **9 fields per well**.
- Use consistent exposure and focus settings across wells and replicates.
- Ensure that all relevant channels (e.g., DAPI, marker-specific) are captured.

86 Image Analysis with Harmony

1. 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.
- Calculate average parameter values per live cell across all **11 total fields per well** (including backup fields if used).

87 Replication and Sample Size

1. Use **12 wells per condition**, sourced from **4 independent differentiations** (3 wells per differentiation) to ensure biological and technical robustness.

88 Outlier Removal and Data Cleaning

1. Use **GraphPad Prism (v10.1.1)** to identify and remove outliers:

- Apply the **ROUT method** with standard parameters.
- Clearly indicate outlier removal in figure legends and data reporting.

89 **Data Normalization and Statistical Testing**

1. 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**.

90 **Reporting**

1. Report data as **mean ± SEM** or **mean ± SD** as appropriate.

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

Aβ ELISA

91 **Collect and Clarify Culture Medium**

1. At 48 hours after media change or treatment, **collect 25 µL** of conditioned culture medium from each well.
2. Centrifuge samples at **~500 × 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.

92 **Run MSD Aβ Assay**

1. Perform the **MSD V-PLEX Aβ Peptide Panel 1** assay according to the manufacturer's protocol:
 - Use 25 µL of culture medium per well.
 - Include standard curve, blank, and technical replicates if needed.
 - Incubation times, wash steps, and detection procedures should strictly follow the MSD protocol.

93 **Read and Analyze Plate**

1. Read the plate using an MSD instrument (e.g., MESO QuickPlex) with appropriate software settings.
2. Export raw values for **A β 38, A β 40, and A β 42**.
3. Calculate **Total A β** as the **sum of A β 38 + A β 40 + A β 42** per sample.

94 **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 a BCA assay.
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.
 - Normalize **+Dox** and **-Dox** conditions accordingly.

95 **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

96 Samples for western blotting were harvested, pelleted and snap frozen.

96.1 Cell pellets were resuspended in RIPA buffer supplemented with Halt protease and phosphatase inhibitors (ThermoFisher) followed by centrifugation to clarify the sample.

96.2 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.

96.3 A total of 5–20 μ g of protein was separated on NuPAGE Novex 4–12% Bis Tris gels and transferred by wet blotting onto PVDF membranes.

96.4 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**.

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

Fractionation of protein lysates into sarkosyl soluble/insoluble fractions

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



97.10 Western blots were run as described in section 13.

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

Viability assays

98 Viability assays were performed in 96 well plates using the PrestoBlue Cell Viability Reagent or CCK8.

98.1 Presto blue reagent was diluted 1:10 in neural maintenance media and 85ul was applied to each well.

98.2 For the CCK8 assay the assay reagent was prepared as described by the manufacturer with 110 ul used per well.

98.3 Culture plates were incubated with the assay reagent for 2 h at 37 C before assaying.

98.4 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).

98.5 Technical replicates were averaged to give a single value for each differentiation/experiment.

98.6 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

99 gRNAs used for secondary validation were the top scoring gRNAs from the WGS.

99.1 The list of gRNA sequences used for this study can be found in **Table S3**.



- 99.2 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}
- 99.3 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.
- 99.4 Virus particles were harvested after 48h.

Generation of UBA3 and NAE1 overexpression lentiviruses

- 100 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.
- 100.1 Primers used to amplify to ORFs for cloning and to sequence the resulting clones can be found in **Table S2**.
- 100.2 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.
- 100.3 Virus particles were harvested after 48h and concentrated using Amicon Ultra-15 Centrifugal Filter Unit (100kDa).

Flow Cytometry

- 101 Neuronal cultures were dissociated to single cell suspensions using Accutase (Innovative Cell Technologies) supplemented with Neuron Isolation Enzyme (Thermo 88285) solution at 1:50.
- 101.1 Single cell suspensions were stained with Zombie UVTM Fixable Viability Kit (Biolegend 423107) at 1:2500 in PBS for 15 minutes at room temperature.
- 101.2 Cells were fixed in 4% Paraformaldehyde for 10 minutes (4°C).
- 101.3 Cells stained with CellEvent Senescence Green (Thermo C10840) were done so at 1:250 in assay buffer for 2 hours at 37°C.

- 101.4 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).
- 101.5 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.
- 101.6 Cells were analyzed on the Cytex Aurora Flow Cytometer.
- 101.7 Experiments were repeated with cells from 3 independent differentiations. The median intensity indicated on plot for every condition.

A β 42 neurotoxicity assays

- 102 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.
- 102.1 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.
- 102.2 The endpoint neuron viability was assessed using the Presto blue reagent as described in section 16.
- 102.3 For UBA3, NAE1 and SENP8 loss of function experiments knock out was performed at DIV 20.
- 102.4 A β 42 was added at DIV42 for UBA3 and NAE1 and DIV37 for SENP8.
- 102.5 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.
- 102.6 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.
- 102.7 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.



- 102.8 On DIV 22 1ug/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

- 103 Neurons were grown in 96 well plates at 150k/cm.²
- 103.1 1 μ M MLN4924 or DMSO were added to the culture medium from DIV30.
- 103.2 At DIV40 and DIV50 the plates were used to assay for proteasome function using the Cell-Based Proteasome-GloTM Assay kit (Promega G8660) as described by the manufacturer.
- 103.3 Reagents were prepared and left to equilibrate at room temperature.
- 103.4 Culture media was replaced with 100 μ l of room temperature PBS.
- 103.5 Additional PBS only blank wells were also included.
- 103.6 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.
- 103.7 For each differentiation the MLN4924 wells were normalized to the DMSO controls.