



Nov 18, 2025

Version 3

Protocol collection for Kim et al. Cell 2024 "TNF-NF- κ B-p53 axis restricts in vivo survival of hPSC-derived dopamine neurons" V.3

 [Cell](#)

DOI

<https://dx.doi.org/10.17504/protocols.io.kqdg3qmzev25/v3>

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DOI: <https://dx.doi.org/10.17504/protocols.io.kqdg3qmzev25/v3>

External link: <https://doi.org/10.1016/j.cell.2024.05.030>

Protocol Citation: Kim , So Yeon Koo, Markus Riessland 2025. Protocol collection for Kim et al. Cell 2024 "TNF-NF- κ B-p53 axis restricts in vivo survival of hPSC-derived dopamine neurons". **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.kqdg3qmzev25/v3> Version created by **Johannes Jungverdorben**



Manuscript citation:

TNF-NF- κ B-p53 axis restricts *in vivo* survival of hPSC-derived dopamine neurons

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Protocol status: Working

We use this protocol and it's working

Created: October 01, 2025

Last Modified: November 18, 2025

Protocol Integer ID: 228774

Keywords: p53 axi, derived dopamine neuron, dopamine neuron, vivo survival, cell 2024 protocol collection, targets dopamine neuron differentiation, enrichment of dopamine neuron, mda neurons into nu, derived mda neuron, intracranial transplantation high throughput cell surface marker screen, human pluripotent stem cell, transplantation of hpsc, cloning sgrna barcode sequencing, cell transcriptome, ohda mouse model stereological analysis tissue immunohistochemistry, sequencing cell preparation, sgrna barcode sequencing, transcriptome, hesc line generation of tnfa knockout hpsc line

Funders Acknowledgements:

Aligning Science Across Parkinson's (ASAP)

Grant ID: ASAP-00047



Abstract

Protocol collection for the method section for the publication:

"TNF-NF- κ B-p53 axis restricts in vivo survival of hPSC-derived dopamine neurons"

from Kim et al. Cell 2024

The list consists of 13 different protocols:

- Transplantation of hPSC-derived mDA neurons into nu/nu rat and NSG mice
- Amphetamine-induced rotation test
- Generation of a NR4A2 (NURR1)-driven GFP expressing hESC line
- Generation of TNFa knockout hPSC line
- 6-OHDA mouse model
- Stereological analysis
- Tissue immunohistochemistry (IHC), TUNEL, and H&E stain
- Transcriptome sequencing
- Single-cell transcriptome sequencing
- Cell preparation for survival surgery and intracranial transplantation
- High throughput cell surface marker screen and enrichment of dopamine neuron with using cell surface markers
- Single-strand guide RNA (sgRNA) Design and Cloning
- sgRNA barcode Sequencing and Analysis to identify targets
- Dopamine neuron differentiation from human pluripotent stem cells

The full publication can be found here:

DOI: [10.1016/j.cell.2024.05.030](https://doi.org/10.1016/j.cell.2024.05.030)

Safety warnings

- ! Animal experiments need to be approved by the ethical board of your institution and adhere to national guidelines.

Please acquire the necessary permissions before you conduct any animal experiments.

Ethics statement

All procedures were performed following NIH guidelines and were approved by the local Institutional Animal Care and Use Committee (IACUC) under identifier **D16-00199**, the Institutional Biosafety Committee (IBC) and the Embryonic Stem Cell Research Committee (ESCRO).

Transplantation of hPSC-derived mDA neurons into nu/nu rat and NSG mice

1 References:

- Kriks et al., Nature, 2011; Kim et al., Cell Stem Cell, 2021 (for cell preparation and transplantation strategy)
- Stereotaxic Atlas of the Rat Brain (Paxinos & Watson)
- Stereotaxic Atlas of the Mouse Brain (Franklin & Paxinos)

2 Materials

- 6–8 week-old Sprague-Dawley rats or NSG (NOD-SCID IL2R γ ^{-/-}) mice
- Stereotactic frame and micromanipulator
- Electrical infusion pump (e.g., Boston Scientific)
- Heating pad (microwave heat pad)
- Tissue forceps
- Surgical gloves and masks
- Hamilton syringe (10 μ L for rats, 5 μ L for mice)
- 6-OHDA solution for lesioning (rats only)
- Vehicle control solution (sham group)
- Cell suspension: neural progenitor cells, 150,000 cells/ μ L (rat) or 100,000 cells/ μ L (mouse)
- Anesthesia (e.g., ketamine/xylazine or isoflurane)
- Analgesics (e.g., buprenorphine)
- Surgical instruments and sutures
- Sterile PBS

3 Step 1: 6-OHDA Lesioning in Rats

1. Anesthetize 6–8 week-old Sprague-Dawley rats using isoflurane or ketamine/xylazine.
2. Secure the rat in a stereotaxic frame and disinfect the surgical area.
3. Inject **6-OHDA** unilaterally into the **medial forebrain bundle** using standard coordinates (e.g., AP: -4.4, ML: +1.2, DV: -7.8 from dura).
4. Inject 4 μ L of 6-OHDA solution (3–4 μ g/ μ L in 0.02% ascorbic acid) at a rate of **1 μ L/min**.
5. Wait 3–4 weeks for the lesion to stabilize before transplantation.

4 Step 2: Cell Preparation

1. Prepare suspension of NURR1::H2B-GFP DA neurons that are NURR1-expressing immature DA neurons isolated by FACS with GFP at the following concentrations:
 - **Rat:** 150,000 cells/ μ L (total 450,000 cells per rat)
 - **Mouse:** 100,000 cells/ μ L (total 2 μ L per mouse = 200,000 cells)

1. Keep cell suspensions on ice and protected from light. Resuspend gently before injection to avoid settling.

5 **Step 3: Stereotactic Transplantation in Rats**

1. Anesthetize rats and mount them on a stereotaxic frame.
2. Drill burr holes over the right **striatum** using coordinates:
 - **AP:** +1.0 mm
 - **ML:** +2.5 mm
 - **DV (from dura):** -4.7 mm and -4.4 mm (two sites)
3. Inject **1.5 µL per site** (total 3 µL), using a Hamilton syringe driven by an infusion pump at a rate of **0.5 µL/min**.
4. Wait 5 minutes before slowly retracting the needle to prevent backflow.
5. Sham controls receive vehicle injections following the same procedure.

6 **Step 4: Stereotactic Transplantation in Mice**

1. Anesthetize **6–8 week-old NSG mice** and secure them in a stereotactic frame.
2. Drill a burr hole at:
 - **AP:** +0.5 mm
 - **ML:** +1.8 mm
 - **DV:** -3.4 mm (from dura)
3. Inject **2 µL** of cell suspension at a speed of **0.5 µL/min** using an electrical syringe pump.
4. Leave the needle in place for 5 minutes post-injection to minimize reflux, then retract slowly.

7 **Step 5: Postoperative Care**

1. Suture the scalp and allow animals to recover on a heated pad.
2. Administer analgesics (e.g., buprenorphine 0.05–0.1 mg/kg SC) post-surgery and monitor for 72 hours.
3. House animals individually or in small groups and monitor daily for signs of distress or neurological deficits.

Amphetamine-induced rotation test

8 **Materials**

- 6-OHDA-lesioned rats (pre- and post-transplantation)
- D-Amphetamine sulfate (Sigma-Aldrich or equivalent)
- Sterile 0.9% saline (vehicle)
- Intraperitoneal (IP) injection equipment (1 mL syringe, 25–27G needle)

- Behavioral recording arena (40–60 cm diameter open field or commercial rotation bowl)
- **EthoVision XT 11.5** (Noldus Information Technology Inc., USA) or equivalent automated tracking software
- Timer or stopwatch

9 Timepoints

- **Baseline test:** 1 week before transplantation
- **Post-transplantation tests:** Monthly, until **6 months post-grafting**

10 Step 1: Animal Preparation

1. Acclimate rats to the behavior testing room for at least **30 minutes** before testing to reduce stress.
2. Ensure animals are healthy and weighed on the day of testing to calculate the appropriate drug dose.

11 Step 2: Amphetamine Administration

1. Prepare **D-Amphetamine** solution freshly at the concentration needed to deliver **5 mg/kg** per rat.
2. Administer **D-Amphetamine intraperitoneally (IP)** using a 1 mL syringe with a 25–27G needle.
 - Inject in the lower right or left quadrant of the abdomen.
 - Use sterile saline as the vehicle.

12 Step 3: Behavioral Recording

1. Wait **10 minutes** post-injection before starting the test to allow amphetamine to take effect.
2. Place each rat individually into the center of the behavioral arena or rotation bowl.
3. Record rotational behavior for a total of **40 minutes** using **Ethovision XT 11.5** or similar software:
 - Ensure the tracking software is calibrated and that the rat's position is continuously detected.
 - Use automatic settings for counting full-body turns in both clockwise (ipsilateral) and counterclockwise (contralateral) directions.

13 Step 4: Data Analysis

1. Calculate **net rotations per minute** as:

$$(\text{Ipsilateral rotations} - \text{Contralateral rotations}) / 40 \text{ minutes}$$

2. Export rotation data for each rat from the tracking software for statistical analysis.

14 Step 5: Repeat Testing Over Time

1. Repeat the test **once per month** post-transplantation for up to **5 months** to monitor changes in motor asymmetry and functional recovery.

15 Notes

- Increased **ipsilateral rotations** indicate a strong unilateral dopaminergic lesion.
- A **reduction in rotations over time** post-transplantation suggests graft-mediated recovery.
- Perform the test at the same time of day across sessions to minimize variability.
- Use the same apparatus and settings across all testing time points for consistency.

16 Outputs

- Raw data: number of ipsilateral and contralateral rotations per animal
- Net rotation rate (rotations per minute)
- Time-course analysis of behavioral recovery

Generation of a NR4A2 (NURR1)-driven GFP expressing hESC line

17 Materials

- **Cell Line:** H9 human embryonic stem cells (WiCell)
- **Cas9 / gRNA plasmid:** Addgene plasmid #PX458 (1 µg per reaction)
- **gRNA sequence:** Targeting NR4A2, guide sequence: ATTATTTGTCCAACTGTTGGGG (1 µg)
- **Donor plasmid:** 5'arm-P2A-H2B-EGFP-PgkPuro-3'arm cloned in pUG19 (2 µg)
- **Nucleofector:** Lonza 4D-Nucleofector System
- **Nucleofection kit:** Human Stem Cell Nucleofector Kit 2 (Lonza)
- **Culture substrate:** Vitronectin- or Matrigel-coated plates
- **Media:** E8 medium + ROCK inhibitor (Y-27632, 10 µM)
- **Selection agent:** Puromycin (Thermo Fisher, 1 µg/mL)
- **Reagents for colony picking and clonal expansion**

18 Cell Preparation

1. Culture H9 hESCs under standard conditions in E8 medium on Vitronectin-coated plates.
2. Pre-treat cells with **Y-27632 (10 µM)** for 1 hour prior to nucleofection.
3. Detach cells using **Accutase** to obtain a single-cell suspension.
4. Count and resuspend **1 × 10⁶ cells** per nucleofection in the Lonza nucleofection solution.

19 Nucleofection

1. Mix the cell suspension with the following plasmids:
 - 1 µg **Cas9 / gRNA plasmid** (NR4A2-targeting guide)
 - 2 µg **donor plasmid** (pUG19 backbone with EGFP cassette)

2. Transfer the mixture into a certified nucleocuvette.
3. Electroporate using Lonza 4D-Nucleofector program **CA-137** (or optimized setting).
4. Immediately transfer cells into pre-warmed **E8 + Y-27632 (10 μ M)** medium on Matrigel-coated plates.

20 **Puromycin Selection**

1. After 48 hours, start selection by adding **puromycin (1 μ g/mL)** to the medium.
2. Maintain puromycin selection for **15 consecutive days**, changing media daily.

21 **Colony Isolation**

1. At day 15 post-transfection, manually pick surviving individual **colonies** under a fluorescent microscope.
2. Transfer picked colonies into 24-well plates and expand in **E8 medium + Y-27632 (10 μ M)** with routine passaging.

22 **Validation**

1. Confirm targeted integration at the NR4A2 locus by:
 - **Genotyping PCR** across homology arms
 - **EGFP expression** by fluorescence microscopy or FACS after differentiating into DA neuron
 - **Sanger sequencing** to validate junctions
 - Optional: **Off-target analysis** and **karyotyping**

Generation of TNFa knockout hPSC line

23 **Materials**

- **Cell line:** NURR1::GFP human pluripotent stem cells (hPSCs)
- **sgRNA oligos for TNFa targeting:**

Forward: CACCGCCTCTTCTCCTTCCTGATCG

Reverse: AAACCGATCAGGAAGGAGAAGAGGC

- **CRISPR-Cas9 vector:** pX458 (CAG-Cas9-2A-GFP-U6-sgRNA, Addgene)
- **Transfection system:** Lonza 4D-Nucleofector
- **Nucleofection program:** B-016 (for hPSCs)
- **Culture medium:** E8 medium + Y-27632 (10 μ M)
- **Coating:** Matrigel- or Vitronectin-coated plates
- **FACS:** BD Aria III cell sorter
- **PCR primers for TNFa genotyping:**

Forward: 5'-CAGGTTCTCTTCCTCTCACATAC-3'

Reverse: 5'-CCTCTCTTGCGTCTCCATTT-3'

- **Genomic DNA extraction kit**

- **Sanger sequencing reagents or services**

24 **Step 1: sgRNA Cloning into px458**

1. Anneal the oligos targeting **TNF α** :

Forward: CACCGCCTCTTCTCCTTCCTGATCG

Reverse: AAACCGATCAGGAAGGAGAAGAGGC

2. Ligate annealed oligos into **BbsI-digested px458 vector** under U6 promoter.
3. Transformation of ligate into Stbl3 competent cells
4. Picking colony and extracting plasmid DNA
5. Confirm insertion by Sanger sequencing using standard U6 promoter-flanking primers.

25 **Step 2: Cell Preparation and Nucleofection**

1. Culture NURR1::GFP hPSCs in E8 + Y-27632 (10 μ M) on Matrigel-coated plates until 70–80% confluent.
2. Detach cells with Accutase and prepare a single-cell suspension.
3. Resuspend **1 $\times$ 10⁶ cells** in nucleofection solution (Human Stem Cell Kit).
4. Add **5 μ g px458-sgTNF α plasmid** to the cell suspension.
5. Electroporate using program **B-016** on the Lonza 4D-Nucleofector.
6. Plate cells in E8 + Y-27632 onto Matrigel-coated dishes.

26 **Step 3: GFP Sorting and Clonal Expansion**

1. After **48 hours**, dissociate cells and sort **GFP-positive** hPSCs using **BD Aria III FACS**.
2. Plate sorted cells at clonal density into 96-well plates pre-coated with Matrigel in E8 + Y-27632.
3. Monitor and expand individual clones over the next 1–2 weeks.

27 **Step 4: Genotyping and Validation**

1. Extract genomic DNA from expanded clones using a DNA extraction kit.
2. Amplify TNF α target region by PCR using primers:
 - Forward: 5'-CAGGTTCTCTTCCTCTCACATAC-3'
 - Reverse: 5'-CCTCTCTTGCGTCTCCATTT-3'
3. Run PCR products with Q5 hotstart PCR kit (NEB, M0493L) on a gel to confirm amplification and submit for **Sanger sequencing**.
4. Analyze sequences to confirm **indels** or **frameshift mutations** at the target site.

6-OHDA mouse model

28 **Materials**

- **Animals:** Adult (6–12 weeks old) male and female NSG mice (Jackson Laboratory, Cat# 005557, IMSR_JAX:005557)
- **Anesthesia:** Isoflurane (1%–2%) in oxygen

- **Neurotoxin:** 6-Hydroxydopamine (6-OHDA, Sigma)
- **Vehicle:** 0.9% Saline with 1% Ascorbic Acid (freshly prepared)
- **Injection volume:** 1 μ L (3 mg/mL 6-OHDA)
- **Injection rate:** 0.5–1 μ L/min
- **Coordinates (from dura):**
 - AP: –2.9 mm
 - ML: +1.1 mm
 - DV: –4.5 mm
- **Equipment:**
 - Stereotaxic apparatus with digital micromanipulator
 - Hamilton syringe (10 μ L) with 33G needle
 - Heating pad
 - Ophthalmic ointment
 - Tissue forceps
 - Scissors
 - Needle holder

29 **Step 1: Pre-operative Preparation**

1. Acclimate NSG mice for at least 3 days prior to surgery.
2. Prepare fresh 6-OHDA solution at 3 mg/mL in 0.9% saline with 1% ascorbic acid. Keep on ice and protect from light.
3. Sterilize all surgical instruments and prepare surgical area.

30 **Step 2: Anesthesia and Positioning**

1. Anesthetize the mouse with **1%–2% isoflurane** in oxygen using an induction chamber.
2. Apply **ophthalmic ointment** to prevent corneal drying.
3. Secure the mouse in the **stereotaxic frame** using ear bars and nose cone to maintain isoflurane anesthesia.
4. Maintain body temperature with a **heating pad**.

31 **Step 3: 6-OHDA Injection**

1. Perform a midline scalp incision and expose the skull.
2. Identify bregma and measure coordinates to target the **right substantia nigra**:
 - AP: –2.9 mm, ML: +1.1 mm, DV: –4.5 mm (from dura)
3. Slowly inject **1 μ L of 6-OHDA solution** at a rate of **0.5–1 μ L/min** using a Hamilton syringe.
4. Wait **3–5 minutes post-injection** before retracting the needle slowly to minimize reflux.

32 **Step 4: Post-operative Care**

1. Suture or close the incision with tissue adhesive.



2. Place the mouse in a clean, warm recovery cage and monitor until fully awake.
3. Administer **analgesics** as per institutional guidelines (e.g., buprenorphine 0.05 mg/kg, s.c.).

33 **Step 5: Behavioral Selection for Transplantation**

1. At **4 weeks post-surgery**, assess lesion efficacy using the **amphetamine-induced rotation test** (5 mg/kg D-amphetamine, i.p.).
2. Select animals that show **>6 ipsilateral rotations/min** over a 40-minute period for inclusion in the transplantation study.

34 **Step 6: Group Assignment and Transplantation**

1. Randomly assign selected animals into one of the following groups:
 - **CD-sorted neurons (CD184 high and CD49E low expressing DA neurons, isolated by FACS)**
 - **CD-sorted neurons + adalimumab (coinjection of CD markers-based isolating DA neurons with adalimumab)**
 - **Sham (PBS vehicle only)**
2. Proceed with stereotaxic transplantation of cells into the **lesioned striatum** as per your cell delivery protocol.

35 **Notes**

- **6-OHDA** is unstable in solution—prepare fresh immediately before surgery and keep protected from light.
- Coordinate accuracy is critical—verify skull alignment before injection.
- The unilateral model mimics Parkinson's motor asymmetry and is suitable for behavioral and histological outcome studies.
- **Amphetamine-induced rotations** are a reliable indicator of dopaminergic neuron loss and motor imbalance.

Stereological analysis

36 **Materials**

- **Transplanted mouse brain tissue**
- **Embedding medium:** O.C.T. or NEG-50
- **Cryostat or freezing microtome**
- **Microscope:** Olympus BX61 with motorized stage
- **Software:** StereoInvestigator (MBF Biosciences)
- **Objective lens:** Olympus 40x oil immersion
- **DA neuron markers:**

GFP (Representing NURR1 expression)
Tyrosine Hydroxylase (TH)

FOXA2 (Floor plate marker)
ALDH1A1 (A9 DA neuron subtype)
CALB1 (A10 DA neuron subtype)

- **Immunostaining reagents**
- **Mouse brain atlas (coronal)**

37 **Step 1: Tissue Preparation**

1. Euthanize mice and perfuse with PBS followed by 4% PFA.
2. Post-fix brains in 4% PFA overnight, then cryoprotect in 30% sucrose.
3. Embed tissue in **O.C.T. or NEG-50** and freeze on dry ice.
4. Cut **30 µm** thick coronal sections using a cryostat.
5. Store sections in antifreeze solution at -20°C until staining.

38 **Step 2: Immunostaining**

1. Perform immunolabeling for **GFP** (NURR1), **TH**, **FOXA2**, **ALDH1A1**, and **CALB1** as required.
2. Mount stained sections on glass slides with appropriate mounting media.

39 **Step 3: Region of Interest (ROI) Selection**

1. Identify the **striatum** using anatomical landmarks (AP +0.5, ML ± 1.8 , DV -3.4 to -3.3 mm from dura).
2. Outline graft area using **NURR1::GFP signal** or reference to the mouse brain atlas.

40 **Step 4: Stereological Sampling Design**

1. Use **optical fractionator probe** within StereoInvestigator to estimate neuron counts.
2. Determine parameters using pilot studies to ensure sufficient sampling and low variance.

- Counting frame: **65 µm x 65 µm**
- Sampling grid: **100 µm x 100 µm** or **10% of ROI**
- Optical dissector height: **20 µm**
- Guard zones: **1 µm** top and bottom

3. Use **Cavalieri estimator probe** to estimate graft volume with:

- Grid spacing: **50 µm x 50 µm**
- Section thickness: **30 µm**
- Grid rotation: **0°**

41 **Step 5: Section Selection**

1. Systematically and randomly select **every 3rd–10th section** (based on graft size) from beginning to end of graft.
2. For each animal, analyze **2–8 sections**, depending on section quality and graft size.

42 **Step 6: Neuron Counting**

1. Acquire images at 40x objective using the BX61 microscope.
2. Use the **optical fractionator** method to count nuclei within the dissector volume.
3. Ensure section thickness is measured at each sampling site.

43 **Step 7: Data Analysis**

1. Calculate total neuron estimates using StereoInvestigator.
2. Ensure **Gundersen coefficient of error (CE) < 0.1** for all conditions.
3. Apply identical parameters for all groups:

- WT vs. **p53 KO** (NURR1::GFP-sorted)
- PBS vs. **adalimumab-treated** (CD-sorted)

44 **Notes**

- Guard zones help eliminate edge artifacts and ensure accurate Z-depth counting.
- Section thickness can vary; always measure it during analysis.
- GFP-labeled nuclei (NURR1::H2B-GFP) enable accurate nuclear quantification for early survival.
- TH staining is used for mature dopaminergic neuron quantification.
- Sample size and section interval selection should be consistent between animals to ensure valid comparisons.

Tissue immunohistochemistry (IHC), TUNEL, and H&E stain

45 **Materials**

- **Mice with xenografts**
- **Pentobarbital** (anesthesia)
- **PBS with Heparin (10 U/mL)**
- **10% Neutral Buffered Formalin**
- **70% Ethanol**
- **Xylene, 100%, 95%, 70% ethanol**
- **Automated histology equipment:**
 - Bond Rx autostainer (Leica Biosystems)
 - TissueTek-Prisma + Coverslipper (Sakura)
 - Aperio AT2 slide scanner (Leica)
- **Antibodies:**
 - Total NFκB (Cell Signaling CST8242, 1:300)
 - Cleaved Caspase-3 (CST9661, 1:300)
 - p53 (CM5P, 1:500)
 - p-NFκB (GTX55113, 1:5000)
- **HRP-conjugated secondary (Leica Bond Polymer Refine DS9800)**
- **Promega DeadEnd Colorimetric TUNEL Kit (G3250)**
- **Mounting medium, coverslips**

46 **Perfusion and Tissue Fixation**

1. **Anesthetize** mice with **pentobarbital** following institutional animal care protocols.
2. **Transcardially perfuse** with **heparinized PBS (10 U/mL, pH 7.4)** using a **peristaltic pump**.
3. Continue perfusion with **10% formalin** for complete fixation.
4. Post-fix tissues in **10% formalin** at room temperature for **24–48 hours** (up to 7 days maximum).
5. Transfer tissues to **70% ethanol** for storage prior to embedding.

47 **Embedding and Sectioning**

1. Submit samples to a histology core (e.g., HistoWiz Inc.) or process in-house.
2. Embed tissues in **paraffin** using standard automated embedding systems.
3. Section tissues at **4 µm** thickness using a microtome.

48 **Hematoxylin & Eosin (H&E) Staining**

1. **Deparaffinize slides** in:

- 2x changes of xylene (5 min each)
- 2x changes of 100% ethanol (3 min each)
- 1x change of 95% ethanol (3 min)
- Rinse in distilled water

2. Stain sequence:

- Hematoxylin (5 min)
- Rinse with water
- Differentiate (acid alcohol, ~30 sec)
- Rinse
- Bluing agent (1 min)
- Rinse with water
- 95% ethanol (30 sec)
- Eosin (30 sec)
- Dehydrate in 95% and 100% ethanol
- Clear with xylene (2x)

3. Coverslip using **TissueTek Prisma** and **automated coverslipper**.

49 **Immunohistochemistry (IHC)**

1. Perform IHC using **Bond Rx autostainer (Leica)**:

- Use **Epitope Retrieval Solution 1 (Leica)** for heat-mediated antigen retrieval.

2. Incubate with primary antibodies:

- Total NFκB (1:300)

- Cleaved Caspase-3 (1:300)
 - p53 (1:500)
 - p-NFkB (1:5000)
3. Apply **anti-rabbit HRP-conjugated polymer** from **Bond Polymer Refine Kit**.
 4. Complete staining according to Leica's protocol.
 5. Dehydrate and coverslip using automated workflow.

50 TUNEL Staining

1. Perform **TUNEL staining** using **Promega DeadEnd Colorimetric Kit (G3250)**:
 - Deparaffinize and rehydrate sections as above.
 - Enzyme digestion: **10 min**
 - Follow Promega protocol for terminal labeling and detection.
 - Use **Leica Bond Polymer Refine Detection Kit (DS9800)** for detection.

51 Step 6: Imaging and Analysis

1. Scan slides at **40x resolution** using the **Aperio AT2** digital scanner (Leica).
2. Analyze images using compatible image analysis software or export for downstream quantification.

Transcriptome sequencing

52 Materials

- **Total RNA** (0.5–1 ng; RIN 6.1–10)
- **RiboGreen RNA Quantitation Kit** (Invitrogen)
- **Agilent BioAnalyzer 2100** (Agilent Technologies)
- **SMART-Seq v4 Ultra Low Input RNA Kit** (Takara/Clontech, Cat. #634888)
- **KAPA Hyper Prep Kit for Illumina** (Kapa Biosystems, KK8504)
- **AMPure XP beads** (Beckman Coulter)
- **Qubit dsDNA HS Assay Kit** (Invitrogen)
- **HiSeq 4000 SBS Kit (50 cycles)** or **NovaSeq 6000 S1 Reagent Kit (100 cycles)** (Illumina)
- **Illumina-compatible library barcodes/adapters**
- **Thermocycler**
- **Magnetic rack for 1.5 mL tubes**

53 Step 1: RNA Quantification and Quality Control

1. Quantify total RNA (0.5–1 ng per sample) using **RiboGreen assay** according to **manufacturer's protocol**.
2. Assess RNA integrity using the **Agilent BioAnalyzer 2100**. Proceed only with samples that have a **RIN ≥ 6.1**.

54 Step 2: cDNA Amplification with SMART-Seq v4

1. Use the **SMART-Seq v4 Ultra Low Input RNA Kit** to reverse transcribe and amplify RNA input.
2. Follow the manufacturer's protocol, using **12 PCR cycles** for cDNA amplification.
3. Purify amplified cDNA with **AMPure XP beads** at 1:1 ratio and elute in nuclease-free water.
4. Quantify cDNA using **Qubit dsDNA HS Assay** and assess size distribution with BioAnalyzer or TapeStation.

55 **Step 3: Library Preparation with KAPA Hyper Prep Kit**

1. Use **1.6–10 ng** of purified cDNA as input to the **KAPA Hyper Prep Kit** protocol.
2. Perform end repair, A-tailing, and adapter ligation according to the kit instructions.
3. Use **8 cycles of PCR amplification** to enrich adapter-ligated fragments.
4. Clean up libraries with AMPure XP beads and elute in 20 µL of elution buffer.
5. Quantify and check size distribution again using **BioAnalyzer** or **TapeStation**.

56 **Step 4: Sequencing**

1. Pool barcoded libraries equimolarly for multiplexed sequencing.
2. Sequence on **HiSeq 4000** or **NovaSeq 6000** in **paired-end 50 bp (PE50)** mode:
 - **HiSeq:** Use 3000/4000 SBS Kit (50 cycles)
 - **NovaSeq:** Use S1 Reagent Kit (100 cycles)

57 **Step 5: Output and Quality Metrics**

1. Expect an average of **~56 million paired reads per sample**.
2. Verify that **48%–76% of total bases** align to **mRNA** regions, as determined in downstream alignment and QC.

58 **Notes**

- Use RNase-free consumables and work in a clean RNA-dedicated workspace.
- If RIN is <7, expect slightly lower coverage or higher variability.
- For optimal normalization, include ERCC spike-ins if needed.
- Library prep steps can be automated on liquid handling systems (e.g., Bravo or Beckman FX) for high-throughput studies.

Single-cell transcriptome sequencing

59 **Materials and Software**

- **10X Chromium Controller** and **Single Cell 3' v3 Reagent Kits**
- **Illumina sequencing platform**
- **Cell Ranger v5.0.0** (10X Genomics)
- **Reference genome:** GRCh38 (GENCODE v32 / Ensembl 98)
- **R (v4.2 or later)** and **Python (v3.8 or later)**

- R packages:
 - Seurat v4.1
 - scran v1.22.1
 - clusterProfiler v4.2.2
 - EnhancedVolcano v1.12.0
 - monocle3
 - igraph v1.3.5
- Python packages:
 - scanpy
 - scVelo

60 **Step 1: Library Preparation and Sequencing**

Process samples using 10X Genomics Chromium Single Cell 3' v3 kit following the manufacturer's protocol. Sequence libraries on an Illumina platform targeting ~50,000 reads per cell.

61 **Step 2: Alignment and Count Matrix Generation**

Align sequencing reads to GRCh38 (GENCODE v32/Ensembl 98) using Cell Ranger v5.0.0. Use the cellranger count pipeline to generate gene-barcode count matrices.

62 **Step 3: Quality Control Filtering**

Filter the filtered count matrix to retain cells with:

- Minimum of 1,000 UMI counts per cell
- Between 500 and 7,000 detected genes per cell
- Less than 25% mitochondrial gene content

63 **Step 4: Normalization and Cell Cycle Regression**

Normalize data using scran's deconvolution method to account for library size differences. Regress out cell cycle-associated gene expression signals to reduce confounding effects on clustering and downstream analysis.

64 **Step 5: Feature Selection and Dimensionality Reduction**

Select the top 2,000 highly variable genes for analysis. Perform principal component analysis (PCA) and retain the first 50 principal components for downstream clustering and visualization.

65 **Step 6: Graph Construction and Clustering**

Construct a shared nearest neighbor (SNN) graph with $k = 40$ neighbors. Perform clustering using the walktrap community detection algorithm to identify distinct cell populations.

66 Step 7: UMAP Projection

Generate Uniform Manifold Approximation and Projection (UMAP) plots to visualize cellular heterogeneity in two-dimensional space.

67 Step 8: Differential Gene Expression Analysis

Perform differential gene expression analysis between clusters using the MAST statistical framework to identify cluster-specific marker genes.

68 Step 9: Pseudotime and Velocity Analysis

Use Monocle3 for trajectory inference and pseudotime ordering of cells to explore dynamic processes. Apply scVelo for RNA velocity analysis in Python to infer transcriptional dynamics and lineage relationships.

69 Step 10: Cluster Annotation and Visualization

Annotate clusters based on enriched biological pathways and gene ontology terms using clusterProfiler. Visualize differential gene expression results with EnhancedVolcano plots for intuitive interpretation.

70 Notes

- Apply consistent filtering and clustering parameters across all samples to ensure comparability.
- Consider batch correction methods when integrating multiple datasets.
- Adjust UMAP and clustering parameters as needed depending on the complexity of the cellular composition.

71 References

- Satija, R. et al. (2015). Spatial reconstruction of single-cell gene expression data. *Nature Biotechnology*.
- Amezquita, R. et al. (2020). Orchestrating single-cell analysis with Bioconductor. *Nature Methods*.
- Bergen, V. et al. (2020). Generalizing RNA velocity to transient cell states through dynamical modeling. *Nature Biotechnology*.
- Yu, G. et al. (2012). clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS*.

Cell preparation for survival surgery and intracranial transplantation**72 1. Preparation of Cells for Transplantation**

- Resuspend sorted dopamine neurons at a density of **100,000 ± 10,000 cells/mL** in transplantation medium consisting of neurobasal medium with 200 mM L-glutamine and 100 mM ascorbic acid.
- Omit human albumin or kedbumin from the transplantation medium unless otherwise specified.

73 2. Stereotaxic Injection Procedure

- Inject **3–4 μL** of the cell suspension into the striatum at a rate of **0.5–1 $\mu\text{L}/\text{min}$** per deposit.
- Injection coordinates (from dura):
 - Anterior-Posterior (AP): +0.5 mm
 - Medial-Lateral (ML): ± 1.8 mm
 - Dorsal-Ventral (DV): –3.4 to –3.3 mm
- Each surgery should not exceed **30 minutes per animal** and be completed within **8–10 hours** post cell preparation to maintain cell viability.

74 **3. Experimental Group Assignments**

- For **short-term 1-month survival studies**, bilaterally engraft:
 - p53 WT (–dox; left side of brain) vs. p53 KO (+dox; right side of brain) NURR1::GFP+ dopamine neurons into the same NSG mouse brain into the same NSG mouse brain to reduce inter-animal variability, or
 - , ipsilaterally engraft: CD markers-sorted neurons (CD184 high / CD49e low) + PBS vs. CD markers-sorted neurons (CD184 high / CD49e low) + adalimumab into the NSG mouse brain.
- For **short-term 7-day survival studies**, graft NURR1::GFP+ dopamine neurons into 6–8-week-old immunocompetent TNFa WT and KO mice (129S background), with or without co-injection with adalimumab.
- TNFa KO mice were generously provided by Dr. Li Gan's lab (Weill Cornell Medicine).
- Bilateral implantation of TNFa KO human NURR1::GFP+ dopamine neurons with PBS or adalimumab was performed in the same NSG mouse brain for 7-day survival assessment to reduce inter-animal variability.

75 **4. Allograft Transplantation**

- Transplant mouse dopamine neurons (129S background), differentiated from Nurr1-driven-GFP expressing reporter mouse epiblast stem cell at day 12, isolated DA cells using FACS with GFP, into the striatum of immunocompetent, non-isogenic C57BL/6 (B6) mice aged 6–8 weeks.
- Assess graft survival at **7 days post-transplantation**.

76 **5. Time Course and Behavioral Studies**

- For these studies, cells are engrafted **unilaterally**.
- Mice are euthanized at designated time points (**4 hours, 1 day, 3 days, and 7 days**) post-engraftment for immunohistochemical analysis.

77 **6. In Vivo CRISPR Screening**

- Cells are resuspended at a higher density of **300,000 $\pm$ 10,000 cells/ μL** .

- Inject **4 µL** per striatum (totaling approximately up to 800,000 cells per injection site) to ensure sufficient guide RNA representation after in vivo isolation.

High throughput cell surface marker screen and enrichment of dopamine neuron with using cell surface markers

78 **Materials**

- Day 25 differentiated dopamine neurons derived from NURR1::GFP reporter hESCs
- Flow cytometry staining buffer: PBS supplemented with 2% bovine serum albumin (BSA)
- CD surface marker antibodies (387 markers)
- DAPI (for viability staining)
- 96-well plates suitable for flow cytometry staining
- Flow cytometer and cell sorter

79 **1. Single Cell Suspension Preparation**

- Accutase treatment and Harvest day 25 differentiated NURR1::GFP dopamine neurons from NURR1:GFP report H9 hESC
- Prepare a single cell suspension in flow cytometry staining buffer (PBS + 2% BSA).
- Adjust cell concentration to **approximately 0.2 million cells per CD marker**.

80 **2. Surface Marker Staining**

- Aliquot cells into 96-well plates for staining.
- Incubate cells with individual CD antibodies (one antibody per well) for **30 minutes on ice**, protected from light to prevent photobleaching.

81 **3. Washing and Viability Staining**

- Wash cells **3 times** with PBS to remove unbound antibodies.
- Co-stain cells with **DAPI** to exclude dead cells during analysis.

82 **4. Data Acquisition**

- Acquire flow cytometry data to profile the expression of 387 CD markers on the dopamine neuron population derived from NURR1:GFP reporter H9 hESC.
- Analyze data to identify CD markers that enrich for GFP-positive dopamine neurons.

83 **5. Enrichment and Sorting**

- For dopamine neuron enrichment, stain day 25 cells with selected CD markers **CD49e** (49e) and/or **CD184** (184).
- Sort cell populations based on the following gating strategy:
 - CD49e low expression (49e^{low})
 - CD49e low and CD184 high expression (49e^{low}/184^{high})
- Collect sorted populations for downstream applications such as transplantation or in vitro studies, including molecular analysis.

84 Notes

- Perform all staining steps on ice and in the dark to preserve cell viability and fluorochrome integrity.
- Use appropriate controls, including unstained and single-color controls, for accurate compensation and gating.
- Maintain sterile conditions during cell handling to avoid contamination.
- Confirm cell viability post-sorting using viability dyes or trypan blue exclusion.

Single-strand guide RNA (sgRNA) Design and Cloning

- 85**
- sgRNA sequences for pool library were identified by Guidescan (MSKCC) and sgRNA oligos were synthesized on-Chip (Agilent).
 - Synthesized oligos were PCR amplified and amplicons were restriction cloned into SGL40C.EFS.dTomato (Addgene#89395).
 - Library representation was assessed by NGS (Illumina).
 - Individual sgRNA for dTomato and p53, designed by web-based tool (<http://crispor.tefor.net>) and using Guidescan (MSKCC) subsequently, were restriction cloned into SGL40C.EFS.dTomato plasmid vector (Addgene#89395).

sgRNA barcode Sequencing and Analysis to identify targets**86 Materials**

- Cell pellets collected at desired time points
- Genomic DNA extraction kit (e.g., Qiagen)
- Qubit fluorometer and reagents (Thermo Scientific)
- PCR reagents for amplification of sgRNA regions
- Illumina sequencing platform (e.g., HiSeq 2500)
- Quality control instruments (e.g., Bioanalyzer, Agilent)
- Computational tools for sequence alignment, quality control, and statistical analysis

87 1. Genomic DNA Extraction

- Lyse cell pellets collected at each specified time point according to the genomic **DNA extraction kit protocol**.
- Quantify the extracted gDNA using a Qubit fluorometer to ensure sufficient yield and purity.

88 2. Library Preparation

- Use an amount of gDNA sufficient to provide at least **1000X coverage** of the sgRNA library [550sgRNAs for 150 genes (3 sgRNAs per 1 gene) and 100 sgRNA controls] representation for PCR amplification.

- Perform PCR amplification to add Illumina adapters and multiplexing barcodes, enabling sample pooling for sequencing.
- Quantify the resulting amplicons using Qubit and assess quality and fragment size distribution using a Bioanalyzer.

89 **3. Sequencing**

- Pool the libraries and sequence on an Illumina platform capable of high-throughput single-end reads (e.g., HiSeq 2500).
- Ensure sequencing depth sufficient to maintain library coverage and statistical power.

90 **4. Sequence Data Processing and Quality Control**

- Align sequencing reads to the reference sgRNA library to obtain counts for each guide RNA.
- Perform quality assessment of raw reads to identify issues such as low-quality bases or adapter contamination.
- Trim sequencing adapters and remove low-quality reads.
- Conduct further quality control analyses to confirm data integrity before downstream analysis.

91 **5. Data Normalization and Analysis**

- Normalize sgRNA counts using methods such as Trimmed Mean of M-values (TMM) to adjust for sequencing depth and composition bias.
- Identify significantly enriched or depleted sgRNAs using appropriate statistical tests to detect primary hits.
- Perform gene-level analyses to consolidate sgRNA-level data and identify candidate genes affecting the phenotype of interest.
- Calculate correlations between replicate samples to assess reproducibility and consistency of the screen.
- Visualize data quality and results using heatmaps or other suitable graphical representations.

Dopamine neuron differentiation from human pluripotent stem cells

92 **Day 0**

Plate 400,000~600,000 cells/cm² dissociated with Accutase from hPSC/hiPSC at single-cells on Geltrex (diluted 1:30 with DMEM/F12 coated plates for 3-4h at RT) with **media1** as following **including Y-drug**

o Etc; Feed 4ml in 6well plate and 1ml in 24well plate

Media1 composition

- o Neurobasal media
- o N2 supplement
- o B27 supplement
- o Pen-Strep

- o L-Glutamine
- o SB (10uM)
- o LDN (250nM)
- o SHH (500ng/ml)
- o CHIR (1uM)

Day 1 to Day 2

- Check cell confluence. Cell should be at 100% confluent
- Double feed cells with **media1**

Day 3

- Feed cells with **media1**

Day 4

- Feed cells with **media2** as following
- o (CHIR-Boost; **Change CHIR concentration from 3uM to 6uM for WA-09 hESC line-mediated differentiation*** (*this can be slightly vary depending on hPSC/hiPSC lines)

Media2 composition

- o Neurobasal media
- o N2 supplement
- o B27 supplement
- o Pen-Strep
- o L-Glutamine
- o SB (10uM)
- o LDN (250nM)
- o SHH (500ng/ml)
- o CHIR (6uM)

Day 5 to Day 6

- Double feed cells with media 2

Day 7

- Feed cells with **Media3** as following

Media3 composition

- o Neurobasal media
- o N2 supplement
- o B27 supplement
- o Pen-Strep
- o L-Glutamine
- o CHIR (6uM)



Day 8 to Day 9

- Feed cells with media3 daily

Day 10

- Feed cells with **media4** as following **with CHIR 3uM**

Media4 composition

- o Neurobasal media
- o B27 supplement
- o Pen-Strep
- o L-Glutamine
- o CHIR (3uM)
- o BDNF (20ng/ml)
- o AA (0.2mM)
- o GDNF (20ng/ml)
- o dcAMP (0.5mM)
- o TGF-B3 (1ng/ml)

Day 11 – passage

- Accutase 30min in 37°C incubator
- Plate 800,000~1,000,000cells/cm2 in **PO/L/FN coated dish with media4.**

Day 12

- Check the cell confluence. Cell should be at 100% confluent
- Feed cells with **Media5** as following

Media5 composition

- o Neurobasal media
- o B27 supplement
- o Pen-Strep
- o L-Glutamine
- o BDNF (20ng/ml)
- o AA (0.2mM)
- o GDNF (20ng/ml)
- o dcAMP (0.5mM)
- o TGF-B3 (1ng/ml)

Day 12 to Day 16

- Feed the cells with **media5** everyday
- Cells should be more than 90% of LMX1A/FOXA2+ and FOXA2+/EN1+ double positive by ICC and flow based analysis at day16

Day 16 (Passage) to Day 25



- Accutate 30min in 37°C incubator
- Plate 800,000~1,000,000cells/cm² in **media6**
- Feed cells with **Media6**

Media6 composition

- o Neurobasal media
- o B27 supplement
- o Pen-Strep
- o L-Glutamine
- o BDNF (20ng/ml)
- o AA (0.2mM)
- o GDNF (20ng/ml)
- o dcAMP (0.5mM)
- o TGF-B3 (1ng/ml)
- o DAPT (10uM)

Day 25 to end point

- Accutate 30min in 37°C incubator
- Plate 200,000~250,000cells/cm² in PO/L/FN coated dish with **media6**
- Feed cells with PO/L/FN coated dish with **Media6**

Coating plates with PO/Lam/FN

- Dilute Poly-L ornithine hydrobromide (PO) to **15 mg/ml** in DPBS1X (**1:1000**). Add solution to the plates and incubate at 37°C
- Next day, Remove PO solution
- 2-3 x rinse in DPBS1X Dilute Laminin I and Fibronectin at **2 mg/ml** in DPBS1X (1:500 of the stock). Add solution to the plates and incubate at 37°C for at least 5h.