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(54) Title: METHODS AND COMPOSITIONS FOR IMPROVING IN VIVO SURVIVAL OF MIDBRAIN DOPAMINE NEURONS

(57) Abstract: The present disclosure provides methods for improving *in vivo* survival of midbrain dopamine (mDA) neurons (*e.g.*, in vitro differentiated mDA neurons) by suppressing p53-mediated apoptosis of mDA neurons. The present disclosure further provides methods for treating a subject (*e.g.*, a subject suffering from neurodegeneration of midbrain dopamine neurons, and/or a neurodegenerative disease), comprising administering to the subject one or more mDAs, wherein p53-mediated apoptosis of the one or more mDA neurons is suppressed.

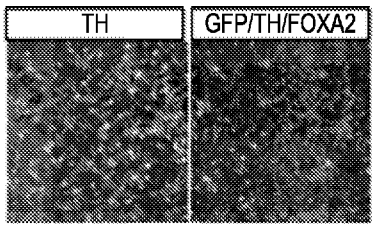

FIGURE 1A

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METHODS AND COMPOSITIONS FOR IMPROVING *IN VIVO* SURVIVAL OF MIDBRAIN DOPAMINE NEURONS

GRANT INFORMATION

The present disclosure was made with government support under Grant No. NS118067-01A1 awarded by the National Institute of Health. The government has certain rights in the disclosure.

PRIORITY

This patent application claims priority to United States provisional application 63/321,507 filed March 18, 2022, the contents of which is incorporated herein by reference in its entirety.

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INTRODUCTION

The present disclosure provides methods and compositions for improving *in vivo* survival of midbrain dopamine (mDA) neurons (e.g., *in vitro* differentiated mDA neurons) by suppressing p53-mediated apoptosis of mDA neurons. The present disclosure further provides methods and compositions for treating a subject (e.g., a subject suffering from neurodegeneration of midbrain dopamine neurons and/or a neurodegenerative disease), comprising administering to the subject one or more mDAs, wherein p53-mediated apoptosis of the one or more mDA neurons is suppressed.

BACKGROUND

Parkinson's disease (PD) remains a major scientific and therapeutic challenge. PD affects an estimated 10 million cases worldwide and brings enormous costs to affected individuals and the greater society in general (Dorsey et al., 2018, *J Parkinsons Dis* 8, S3-S8). The rapid increase in the number of PD cases globally has been referred to as a "Parkinson's Pandemic" by some stressing the urgent need for the development of disease-modifying therapies (Dorsey et al., 2018). Although existing therapies can relieve motor symptoms at early stages of the disease, these symptomatic treatments became gradually less effective, they do not prevent or slow down disease progression

and can trigger side-effects such as dyskinesia, speech deterioration, or depression in some of the patients (Jenner, 2008, *Nat Rev Neurosci 9*, 665-677).

PD patients share a common pathological feature, which is the progressive degeneration of dopamine neurons in the substantia nigra para compacta (Poewe et al., 2017, *Nat Rev Dis Primers* 3, 17013). Cell-based therapy is being considered as a novel therapeutic strategy, as it has the potential to achieve circuit-level restoration of dopaminergic function. Current cell replacement therapy in PD patients was pursued using human fetal midbrain tissue transplantation. While the procedure has shown success in improving motor-related symptoms in a small subset of PD patients (Kefalopoulou et al., 2014, *JAMA Neurol 71*, 83-87; Li et al., 2016, *Proc Natl Acad Sci U S A 113*, 6544-6549), there was huge variability in clinical responses across patients; a two placebo-controlled studies failed to reach their primary endpoints (Freed et al., 2001, *N Engl J Med* 344, 710-719; Olanow et al., 2003, *Ann Neurol 54*, 403-414). Given the challenges of using human fetal tissue as a routine, scalable, and defined source of dopamine neurons, alternative sources such as human pluripotent stem cell (hPSC)-derived dopamine neurons have become the focus for ongoing cell therapy efforts in PD (Piao et al., 2021, *Cell Stem Cell 28*, 217-229 e217; Schweitzer et al., 2020, *N Engl J Med* 382, 1926-1932).

One challenge that has been largely overlooked in all these efforts is the limited survival of grafted dopamine neurons following transplantation surgery. This is a critical factor in determining the success of the procedure given the need to reach a critical dose of dopamine neurons to achieve clinical improvement and given the challenges of predicting effective dose in the patient's brain if there is considerable variability in initial cell survival. Furthermore, injecting a large number of cells to overcome limited *in vivo* survival can pose a risk for triggering a host inflammatory response from the procedure and the associated cell death of the grafted cells (Kriks et al., 2011, *Nature* 480, 547-551.; Tao et al., 2021, *Nat Med* 27, 632-639.).

Therefore, there remains a need for improving the survival of midbrain dopamine neurons following transplantation.

SUMMARY OF THE INVENTION

The present disclosure provides methods and compositions for improving *in vivo* survival of midbrain dopamine (mDA) neurons (e.g., *in vitro* differentiated mDA neurons) by suppressing p53-mediated apoptosis of mDA neurons. The present disclosure further provides methods and compositions for treating a subject (e.g., a

subject suffering from neurodegeneration of midbrain dopamine neurons, and/or a neurodegenerative disease), comprising administering to the subject one or more mDAs, wherein p53-mediated apoptosis of the one or more mDA neurons is suppressed.

In certain embodiments, the present disclosure provides methods for treating a subject. In certain embodiments, the method comprises administering to the subject one or more midbrain dopamine (mDA) neurons, wherein p53-mediated apoptosis of the one or more mDA neurons is suppressed. In certain embodiments, the suppression of p53-mediated apoptosis comprises administering to the subject at least one compound selected from the group consisting of tumor necrosis factor alpha (TNFα) inhibitors, nuclear factor kappa B (NFκB) inhibitors, p53 inhibitors, and combinations thereof. In certain embodiments, the method comprises administering the one or more mDA neurons simultaneously with the administration of the at least one compound. In certain embodiments, the suppression of p53-mediated apoptosis comprises contacting the one or more mDA neurons with at least one compound selected from the group consisting of TNFα inhibitors, NFκB inhibitors, p53 inhibitors, and combinations thereof.

In certain embodiments, the subject suffers from a neurodegenerative disorder and/or neurodegeneration of midbrain dopamine neurons. In certain embodiments, the neurodegenerative disorder is selected from the group consisting of Parkinson's disease, Huntington's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), frontotemporal dementia, and combinations thereof.

In certain embodiments, the present disclosure provides methods of improving *in vivo* survival of one or more midbrain dopamine (mDA) neurons. In certain embodiments, the method comprises suppressing p53-mediated apoptosis of the one or more mDA neurons. In certain embodiments, the suppression of p53-mediated apoptosis comprises contacting the one or more mDA neurons with a compound selected from the group consisting of TNFα inhibitors, NFκB inhibitors, p53 inhibitors, and combinations thereof.

In certain embodiments, the suppression of p53-mediated apoptosis comprises inhibition of tumor necrosis factor alpha (TNFα) signaling, inhibition of nuclear factor kappa B (NFκB) signaling, inhibition of p53 signaling, or a combination of the foregoing.

In certain embodiments, the TNFα inhibitor is selected from the group consisting of anti-TNFα antibodies, TNFα decoy receptors, chemical compounds, nucleic acid

inhibitors, small molecule inhibitors, receptor biologic inhibitors, inactive TNF fragments, TNFα circulating receptor fusion protein, xanthine derivatives, 5-HT_{2A} agonists, and combinations thereof. In certain embodiments, the TNFα inhibitor is an anti-TNFα antibody. In certain embodiments, the anti-TNFα antibody is selected from the group consisting of adalimumab, adalimumab-adbm, adalimumab-adaz, adalimumab-atto, certolizumab pegol, golimumab, infliximab, infliximab-abda, infliximab-dyyb, remtolumab, afelimomab, nerelimomab, ozoralizumab, placulumab, and combinations thereof. In certain embodiments, the anti-TNFα antibody is adalimumab.

In certain embodiments, the NFκB inhibitor is selected from the group consisting of upstream inhibitors of NFκB, inhibitors of IKK activity, inhibitors of IκB phosphorylation, inhibitors of IκB degradation, proteasome inhibitors, protease inhibitors, IκB upregulators, inhibitors of NFκB nuclear translocation and expression, NFκB DNA-binding inhibitors, and NFκB transactivation inhibitors, inhibitors of NFκB directed gene transactivation, antioxidants, and combinations thereof.

In certain embodiments, the p53 inhibitor is selected from the group consisting of JNK inhibitors, p38 MAPK inhibitors, caspase inhibitors, puma/BBC3 inhibitors, BAX inhibitors, CDK inhibitors, MDM2 and MDMX activators, and combinations thereof.

In certain embodiments, the suppression of p53-mediated apoptosis comprises knocking out or knocking down *TP53* gene in the one or more mDA neurons. In certain embodiments, the *TP53* gene is knocked out or knocked down by a gene-engineering system. In certain embodiments, the gene-engineering system is a CRISPR-Cas system.

In certain embodiments, the one or more mDA neurons express a marker selected from the group consisting of EN1, OTX2, TH, NURR1, FOXA2, LMX1A, PITX3, LMO3, SNCA, ADCAP1, CHRNA4, ALDH1A1, SOX6, WNT1, DAT, VMAT2, GIRK2, SATB1, CALB1, CALB2, SNCG, PBX1, and combinations thereof. In certain embodiments, the one or more mDA neurons are post-mitotic mDA neurons.

In certain embodiments, the one or more mDA neurons are *in vitro* differentiated from one or more stem cells. In certain embodiments, the one or more stem cells are selected from the group consisting of human stem cells, nonhuman primate stem cells, rodent nonembryonic stem cells, human embryonic stem cells, nonhuman primate embryonic stem cells, rodent embryonic stem cells, human induced pluripotent stem cells, nonhuman primate induced pluripotent stem cells, rodent induced pluripotent stem cells, and human recombinant pluripotent cells, nonhuman primate recombinant

pluripotent cells, and rodent recombinant pluripotent cells. In certain embodiments, the one or more stem cells are human stem cells. In certain embodiments, the one or more stem cells are one or more pluripotent stem cells or multipotent stem cell. In certain embodiments, the one or more stem cells are selected from the group consisting of embryonic stem cells, induced pluripotent stem cells, and combinations thereof. In certain embodiments, the one or more stem cells are one or more induced pluripotent stem cells.

In certain embodiments, the *in vitro* differentiation comprises contacting the one or more stem cells with at least one inhibitor of Small Mothers Against Decapentaplegic (SMAD) signaling, at least one activator of Sonic hedgehog (SHH) signaling, and at least one activator of wingless (Wnt) signaling.

In certain embodiments, the concentration of the at least one activator of Wnt signaling that is contacted with the cells is increased between about 2 days and about 6 days from the initial contact of the cells with the at least one activator of Wnt signaling. In certain embodiments, the concentration of the at least one activator of Wnt signaling that is contacted with the cells is increased by between about 250% and about 1800% of the initial concentration of the at least one activator of Wnt signaling contacted with the cells.

In certain embodiments, the at least one activator of Wnt signaling comprises an inhibitor of glycogen synthase kinase 3β (GSK3β) signaling. In certain embodiments, the at least one activator of Wnt signaling is selected from the group consisting of CHIR99021, CHIR98014, AMBMP hydrochloride, LP 922056, Lithium, deoxycholic acid, BIO, SB-216763, Wnt3A, Wnt1, Wnt5a, derivatives thereof, and combinations thereof. In certain embodiments, the at least one activator of Wnt signaling comprises CHIR99021.

In certain embodiments, the at least one inhibitor of SMAD signaling comprises an inhibitor of TGFβ/Activin-Nodal signaling, an inhibitor of bone morphogenetic protein (BMP) signaling, or a combination of the foregoing. In certain embodiments, the at least one inhibitor of TGFβ/Activin-Nodal signaling is selected from the group consisting of SB431542, derivatives of SB431542, and combinations thereof. In certain embodiments, derivative of SB431542 comprises A83-01. In certain embodiments, the at least one inhibitor of TGFβ/Activin-Nodal signaling comprises SB431542. In certain embodiments, the at least one inhibitor of BMP signaling is selected from the group consisting of LDN193189, Noggin, dorsomorphin, derivatives of LDN193189,

derivatives of Noggin, derivatives of dorsomorphin, and combinations thereof. In certain embodiments, the at least one inhibitor of BMP comprises LDN-193189.

In certain embodiments, the at least one activator of SHH signaling is selected from the group consisting of SHH proteins, Smoothened agonists (SAG), and combinations thereof. In certain embodiments, the SHH protein is selected from the group consisting of recombinant SHHs, modified N-terminal SHHs, and combinations thereof. In certain embodiments, the modified N-terminal SHH comprises two isoleucines at the N-terminus. In certain embodiments, the modified N-terminal SHH has at least about 90% sequence identity to an un-modified N-terminal SHH. In certain embodiments, the un-modified N-terminal SHH is an un-modified mouse N-terminal SHH or an un-modified human N-terminal SHH. In certain embodiments, the modified N-terminal SHH comprises SHH C25II. In certain embodiments, the SAG comprises purmorphamine. In certain embodiments, the at least one activator of SHH signaling comprises SHH C25II.

In certain embodiments, the *in vitro* differentiation further comprises contacting the one or more stem cells with at least one activator of fibroblast growth factor (FGF) signaling. In certain embodiments, the at least one activator of FGF signaling is selected from the group consisting of FGF18, FGF17, FGF8a, FGF8b, FGF4, FGF2, and combination thereof. In certain embodiments, the at least one activator of FGF signaling comprises FGF18. In certain embodiments, the at least one activator of FGF signaling comprises FGF8.

In certain embodiments, the *in vitro* differentiation further comprises contacting the one or more stem cells with at least one inhibitor of Wnt signaling. In certain embodiments, the at least one inhibitor of Wnt signaling is selected from the group consisting of IWP2, IWR1-endo, XAV939, IWP-O1, Wnt-C59, IWP-L6, and ICG-001, and combinations thereof. In certain embodiments, the at least one inhibitor of Wnt signaling comprises IWP2.

In certain embodiments, the one or more mDA neurons express a detectable level of CD184 and do not express a detectable level of CD49e.

In certain embodiment, the present disclosure provides compositions comprising: (a) one or more midbrain dopamine (mDA) neurons; and (b) at least one compound selected from the group consisting of TNFα inhibitors, NFκB inhibitors, p53 inhibitors,

and combinations thereof. In certain embodiments, the composition is a pharmaceutical composition further comprising a pharmaceutically acceptable carrier.

In certain embodiments, the composition is for treating or ameliorating a neurodegenerative disorder, and/or neurodegeneration of midbrain dopamine neurons. In certain embodiments, the neurodegenerative disorder is selected from the group consisting of Parkinson's disease, Huntington's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), frontotemporal dementia, and combinations thereof.

In certain embodiments, the TNFα inhibitor is selected from the group consisting of anti-TNFα antibodies, TNFα decoy receptors, chemical compounds, nucleic acid inhibitors, small molecule inhibitors, receptor biologic inhibitors, inactive TNF fragments, TNFα circulating receptor fusion protein, xanthine derivatives, 5-HT_{2A} agonist, and combinations thereof. In certain embodiments, the TNFα inhibitor is an anti-TNFα antibody. In certain embodiments, the anti-TNFα antibody is selected from the group consisting of adalimumab, adalimumab-adbm, adalimumab-adaz, adalimumab-atto, certolizumab pegol, golimumab, infliximab, infliximab-abda, infliximab-dyyb, remtolumab, afelimomab, nerelimomab, ozoralizumab, placulumab, and combinations thereof. In certain embodiments, the anti-TNFα antibody is adalimumab. In certain embodiments, the NFκB inhibitor is selected from the group consisting of upstream inhibitors of NFκB, inhibitors of IKK activity, inhibitors of IκB phosphorylation, inhibitors of IκB degradation, proteasome inhibitors, protease inhibitors, IκB upregulators, inhibitors of NFκB nuclear translocation and expression, NFκB DNA-binding inhibitors, and NFκB transactivation inhibitors, inhibitors of NFκB directed gene transactivation, antioxidants, and combinations thereof. In certain embodiments, the p53 inhibitor is selected from the group consisting of JNK inhibitors, p38 MAPK inhibitors, caspase inhibitors, BBC3/PUMA inhibitors, BAX inhibitors, CDK inhibitors, MDM2 and MDMX activators, and combinations thereof.

In certain embodiments, the one or more mDA neurons express a marker selected from the group consisting of EN1, OTX2, TH, NURR1, FOXA2, LMX1A, PITX3, LMO3, SNCA, ADCAP1, CHRNA4, ALDH1A1, SOX6, WNT1, DAT, VMAT2, GIRK2, SATB1, CALB1, CALB2, SNCG, PBX1, and combinations thereof. In certain embodiments, the one or more mDA neurons are post-mitotic mDA neurons.

In certain embodiments, the one or more mDA neurons are *in vitro* differentiated from one or more stem cells. In certain embodiments, the one or more stem cells are selected from the group consisting of human stem cells, nonhuman primate stem cells, rodent nonembryonic stem cells, human embryonic stem cells, nonhuman primate embryonic stem cells, rodent embryonic stem cells, human induced pluripotent stem cells, nonhuman primate induced pluripotent stem cells, rodent induced pluripotent stem cells, and human recombinant pluripotent cells, nonhuman primate recombinant pluripotent cells, and rodent recombinant pluripotent cells. In certain embodiments, the one or more stem cells are human stem cells. In certain embodiments, the one or more stem cells are one or more pluripotent stem cells or multipotent stem cell. In certain embodiments, the one or more stem cells are selected from the group consisting of embryonic stem cells, induced pluripotent stem cells, and combinations thereof. In certain embodiments, the one or more stem cells are one or more induced pluripotent stem cells.

In certain embodiments, the *in vitro* differentiation comprises contacting the one or more stem cells with at least one inhibitor of Small Mothers Against Decapentaplegic (SMAD) signaling, at least one activator of Sonic hedgehog (SHH) signaling, and at least one activator of wingless (Wnt) signaling.

In certain embodiments, the concentration of the at least one activator of Wnt signaling that is contacted with the cells is increased between about 2 days and about 6 days from the initial contact of the cells with the at least one activator of Wnt signaling. In certain embodiments, the concentration of the at least one activator of Wnt signaling that is contacted with the cells is increased by between about 250% and about 1800% of the initial concentration of the at least one activator of Wnt signaling contacted with the cells.

In certain embodiments, the at least one activator of Wnt signaling comprises an inhibitor of glycogen synthase kinase 3β (GSK3β) signaling. In certain embodiments, the at least one activator of Wnt signaling is selected from the group consisting of CHIR99021, CHIR98014, AMBMP hydrochloride, LP 922056, Lithium, deoxycholic acid, BIO, SB-216763, Wnt3A, Wnt1, Wnt5a, derivatives thereof, and combinations thereof. In certain embodiments, the at least one activator of Wnt signaling comprises CHIR99021.

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protein (BMP) signaling, or a combination of the foregoing. In certain embodiments, the at least one inhibitor of TGFβ/Activin-Nodal signaling is selected from the group consisting of SB431542, derivatives of SB431542, and combinations thereof. In certain embodiments, derivative of SB431542 comprises A83-01. In certain embodiments, the at least one inhibitor of TGFβ/Activin-Nodal signaling comprises SB431542. In certain embodiments, the at least one inhibitor of BMP signaling is selected from the group consisting of LDN193189, Noggin, dorsomorphin, derivatives of LDN193189, derivatives of Noggin, derivatives of dorsomorphin, and combinations thereof. In certain embodiments, the at least one inhibitor of BMP comprises LDN-193189.

In certain embodiments, the at least one activator of SHH signaling is selected from the group consisting of SHH proteins, Smoothened agonists (SAG), and combinations thereof. In certain embodiments, the SHH protein is selected from the group consisting of recombinant SHHs, modified N-terminal SHHs, and combinations thereof. In certain embodiments, the modified N-terminal SHH comprises two isoleucines at the N-terminus. In certain embodiments, the modified N-terminal SHH has at least about 90% sequence identity to an un-modified N-terminal SHH. In certain embodiments, the un-modified N-terminal SHH is an un-modified mouse N-terminal SHH or an un-modified human N-terminal SHH. In certain embodiments, the modified N-terminal SHH comprises SHH C25II. In certain embodiments, the SAG comprises purmorphamine. In certain embodiments, the at least one activator of SHH signaling comprises SHH C25II.

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In certain embodiments, the *in vitro* differentiation further comprises contacting the one or more stem cells with at least one inhibitor of Wnt signaling. In certain embodiments, the at least one inhibitor of Wnt signaling is selected from the group consisting of IWP2, IWR1-endo, XAV939, IWP-O1, Wnt-C59, IWP-L6, and ICG-001, and combinations thereof. In certain embodiments, the at least one inhibitor of Wnt signaling comprises IWP2.

In certain embodiments, the one or more mDA neurons express a detectable level of CD184 and do not express a detectable level of CD49e.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1I illustrate derivation and validation of NURR1:GFP sorted DA neurons for CRISPR/Cas9 screening *in vitro* and *in vivo*. Figure 1A shows dopamine neurons population in culture. Figure 1B shows microscopy images of endogenous NURR1::GFP and TH expression in graft 1 month post transplantation of day 25 NURR1:GFP sorted DA neurons. Figure 1C shows flow-cytometry image to generate hPSC line containing pooled lentiviral sgRNAs with MOI 0.35, which indicates single copy sgRNA integration per cell. Figure 1D shows day 40 dopamine neurons co-expressing NURR1::GFP and gRNA::tdTomato post sorting with GFP and Tomato at day25 with/without dox treatment from day16 to day25. Figure 1E shows ablation of the tdTomato signal in hPSC-derived post-mitotic dopamine neurons after dox exposure from day16 to day25. Figure 1F shows graft fluorescence expression 1 month post transplantation. Figure 1G shows PCR analysis for detecting a human PTGER2 gene from dissected tissue around graft region. Figure 1H shows the overall representation of sgRNA across all condition by next-generation sequencing (NGS) from genomic DNA of *in vitro* cultured cells and an *in vivo* grafted cell. Figure 1I shows the overall representations of sgRNAs across all conditions.

Figures 2A-2F illustrate CRISPR screens identifying TP53 as a limiting factor for the survival of post-mitotic dopamine neurons during transplantation. Figure 2A shows a schematic of the *in vivo* CRISPR screen. Figure 2B shows the correlation matrix of all the guide RNAs from different experimental conditions for large pool screen (day 16 *in vitro* vs. day 25 *in vitro* with no dox vs. day 25 *in vitro* with dox vs. day 25 *in vivo* with dox. Scale bar ranging from 0.9 to 1, 1 being the most correlated value. Figure 2C shows volcano plots comparing each experimental condition. Depleted genes are labeled in red and enriched genes in blue. Figure 2D shows enriched sgRNAs in *in vivo* grafted cells vs *in vitro* cultured day 25 cells. Figure 2E shows correlation matrix plot for a small pool screen. A similar scale bar range is used. Figure 2F shows heatmap of all the guide RNAs comparing day 16 progenitors vs. day 25 neurons with dox treatment vs. day 25 neurons without dox treatment (left). Heatmap of the same set of gRNAs for two independent small pool screens (right). Red coloring in the scale bar indicates enrichment of each guide RNAs in the surviving dopamine neurons *in vivo*.

Figures 3A-3F illustrate the characterization of p53-induced dopamine neuron death during transplantation. Figure 3A shows FACS strategy to inject enriched NURR1::GFP and sgRNA-p53-tdTomato post-mitotic DA neuron. Dox treated (D16-D25) or non-treated dopamine neurons sorted by FACS are bilaterally injected into each striatum of the adult NSG mice. Figure 3B shows a representative immunofluorescence image of sorted DA neurons in culture post sorting at day25 expressing NURR1::GFP, gRNA::RFP, and TH at day40. n= 3 independent experiments. Figure 3C shows representative confocal images of dopamine neuron graft at 1-month post-transplantation stained with antibodies against NURR1-GFP. Scale bar = 100 μm. Figure 3D shows stereological analysis for the number (using optical fractionator) and volume (cavalier estimator) of the surviving dopamine neurons at 1-month post-transplantation. *$p<0.05$ (paired t-test). Figure 3E shows representative immunohistochemistry images of the grafted dopamine neurons at different time points (4 hpt, 24 hpt, 72 hpt, 7 dpt) for TP53, cleavage caspase 3 (CC3), and TUNEL assay. Scale bar = 50 μm. Figure 3F shows quantification of the percentage of TP53, CC3, and TUNEL among DA neurons upon engraftment. n = 3 independent experiments.

Figures 4A-4D illustrate increased survival of p53 KO dopamine neuron in graft exhibiting dopamine neuron identity. Figures 4A-4C show immunofluorescence analysis of FOXA2, TH, and NURR1-GFP signal in surviving p53 WT and KO dopamine neurons in the graft (WT;-DOX, KO; +DOX). Figure 4D shows RT-qPCR analysis of p53 and p53 downstream genes before and after needle injection of NURR1:GFP sorted DA neuron in culture.

Figures 5A-5E illustrate time-course analysis of neuroimmune cells' infiltration into the core of the graft. Figure 5A shows H&E staining of the graft. Figure 5B shows immunofluorescence analysis of IBA1. Figure 5C shows immunofluorescence analysis of GFAP and FOXA2. Figure 5D shows H&E staining of IBA1. Figure 5E shows H&E staining of Ly6G.

Figure 6 shows time-course analysis of fiber outgrowth pattern from the graft.

Figures 7A-7H illustrate TNFα-NFκB pathway is an upstream regulator triggering TP53-dependent DA neuron death in the graft. Figure 7A shows a PCA plot of bulk RNAseq data set showing gene expression profiles from sorted dopamine neurons (day 0), *in vitro* cultured neurons for 1 day post sorting (day 1 culture), and *in vivo* grafted neurons for 1 day (day 1 graft). Figure 7B shows differential expression

gene analysis between day 1 culture and day 1 graft. Figure 7C shows hallmark analysis on the upregulated categories in day 1 grafted neurons vs day 1 culture neuron. Figure 7D shows NES analysis of enriched tumor necrosis factor-related genes in day 1 graft than day 1 culture neuron. Figure 7E shows GSEA score from enriched genes in day 1 grafted vs day 1 culture neuron. Figure 7F shows representative immunohistochemistry images of phosphorylated NF kappa B in grafted neurons at distinct time points post-transplantation. % phosphorylated NFkB positive cells from total grafts were quantified. n = 2 independent experiments. Figure 7G shows representative immunofluorescence images of *in vitro* dopamine neurons marked by NURR1::GFP for the induction of p53 and NFκB-p65 comparing mock *vs.* TNFα treatment group *vs.* TNFα and monoclonal antibodies against TNFα called adalimumab treated conditions. n = 3 independent experiments. Figure 7H shows qRT-PCR of gene expression profiles of the three groups listed in Figure 7G for FOXA2, NURR1, P53, P21, and BBC3 (PUMA).

Figures 8A-8F illustrate TNF-NFκB pathway is an upstream regulator triggering TP53-dependent DA neuron death in the graft. Figure 8A shows a dendrogram of the cells among sorted, *in vitro* cultured, and *in vivo* grafted DA neurons from total RNA-seq. Figure 8B shows heat map analysis of TNFα-NFκB related genes enriched in the grafted DA neurons than sorted and in vitro cultured DA neurons from total RNA-seq. Figure 8C shows the clustering distribution of wild-type and p53 knock-out (KO) of grafted DA neurons 1day post transplantation from single cell RNA-seq. Figure 8D shows histograms of clustering distribution of wild-type and p53 knock-out (KO). Figure 8E shows clustering distribution of MAP2 expression for neurons. Figure 8F shows violin plots of dopamine-specific marker and MAP2 expression for neurons (left). Percent of indicated genes from total population of single cell RNA-seq (right).

Figures 9A-9E illustrate TNF-NFκB pathway is an upstream regulator triggering TP53-dependent DA neuron death in the graft. Figure 9A shows clustering analysis of PCA graphs indicating annotated neuroblasts and floor-plate progenitor. Figure 9B shows heatmap from apoptotic cell-death related genes, enriched in clusters 3, 4, and 7. Figure 9C shows TNFRSF12A positive cells in PCA. Figure 9D shows violin plots of TNFRSF12A positive cells in the clusters. Figure 9E shows violin plots of increased genes, such as BAX, BAD, TNFRSF1A, TNFRSF12A, and TNFRSF10B in p53 WT versus p53 KO DA neurons in each cluster.

Figures 10A-10H illustrate clinically relevant TNFα neutralizing antibodies and CD marker sorting strategies functionally improve the survival of post-mitotic dopamine neurons during transplantation. Figure 10A shows a schematic of the flow-based cell surface marker screen to enrich post-mitotic DA neurons using genetic NURR1::GFP marker. Figure 10B shows FACS plot of the % of NURR1::GFP population corresponding to each sorting strategy (CD49e depletion, CD49e depletion and CD171 enrichment double, CD49e depletion, and CD184 enrichment double), indicating CD49e depletion and CD184 enrichment double CD marker sorting lead to the most enriched DA neuron population expressing NURR1::GFP. Figure 10C shows gene expression of NURR1 via qRT-PCR assay 2 days post sorting using each sorting strategy from Figure 10B. Figure 10D shows representative immunofluorescence images of CD49e-/CD184+ double sorted dopamine neurons at 40 DIV, giving rise to pure dopamine neuron cultures co-expressing NURR1::GFP, FOXA2, and TH. Figure 10E shows 1-month short-term *in vivo* histology analysis of CD49e-/CD184+ double sorted graft compared with unsorted graft. The double CD marker sorted neuron graft exhibits highly compact surviving dopamine neurons than unsorted graft as shown by human NA and TH immunofluorescence-staining. Figure 10F shows representative immunofluorescence images of CD49e-/CD184+ double sorted dopamine neurons either treated with PBS or TNFα blocking antibodies adalimumab. Stereological number and volume quantification of the surviving dopamine neurons using NURR1::GFP, n = 3. Figure 10G shows graphs representing data of Figure 10F. Figure 10H shows D-amphetamine induced rotation assay in 6-OHDA based PD mice model after transplantation of PBS, CD sorted cell, co-injection of CD sorted cell with adalimumab, frozen day16 DA progenitor, and co-injection of frozen day16 DA progenitor with adalimumab.

Figures 11A-11D illustrate high content cell surface marker screening finds a novel double sorting strategy matching NURR1-GFP+ dopamine neurons. Figure 11A shows FACS analysis of NURR1:GFP neurons with indicated CD markers. Figure 11B shows immunofluorescence analysis of CD49e-/CD184+ double sorted dopamine neurons and unsorted neuron with FOXA2 and a proliferation marker (Ki67). Figure 11C shows analysis of survived DA neuron and their volume from CD49e-/CD184+ double sorted dopamine neurons in p53 WT and KO. Figure 11D shows H&E analysis of the graft 1day post transplantation either treated with PBS or TNFα blocking antibodies adalimumab.

Figures 12A-12F illustrate *in vivo* CRISPR/Cas9 screen for identifying TP53 as a limiting factor for the *in vivo* survival of hPSC-derived postmitotic dopamine neurons. Figure 12A shows schematic illustration of the pooled CRISPR/Cas9 screen. Figure 12B shows Pearson correlation abundance matrix of all guide RNAs across the different experimental conditions [day 16 *in vitro* (D16) vs. day 25 *in vitro* with no dox (-D25) vs. day 25 *in vitro* with dox (+D25) vs. day 25 *in vivo* with dox (D25)]. Scale bar range is from 0.9 to 1, with 1 being the most correlated value. Figure 12C shows volcano plots comparing each experimental condition. Depleted sgRNAs are labeled in blue and enriched sgRNAs in red. Figure 12D shows enriched sgRNAs for *in vivo* grafted cells versus day25 *in vitro* cells, both treated with dox. Blue bar displays two sgRNAs and red bar show three sgRNAs targeting for an indicated gene are enriched in grafted cells than *in vitro* cells. Figure 12E shows Pearson correlation matrix plot for the pooled validation screen (library#2). Scale bar range is from 0.8 to 1. Figure 12F shows heatmap of all guide RNAs from pooled library#2 screen comparing day 16 (D16) vs. day 25 neurons without dox treatment (-D25) vs. day 25 with dox treatment (+D25) in culture (left). Heatmap of the same set of sgRNAs comparing +D25 *in vivo* vs +D25 in culture from two independent replicate screens (right). Red versus blue scale indicates enrichment of each sgRNAs in the surviving DA neurons in *in vivo* graft (+D25 *in vivo)* versus day25 cultured cells with dox (+D25 *in vitro*).

Figures 13A-AG illustrate characterization of p53-induced dopamine neuron death during transplantation. Figure13A shows FACS strategy for injecting enriched postmitotic dopamine neurons expressing NURR1::GFP and sgRNA-TP53-tdTomato. Each dot in the scatterplot indicates a single dopamine neuron at 25 DIV. Dox treated from day16 to 25 (+DOX, TP53 knock-out; KO) or non-treated (-DOX, isogenic TP53 wild-type; WT) dopamine neurons are isolated by FACS at day25 based on NURR1::GFP signals (upper panel: P5), followed by sgRNA::tdTomato signal (bottom panel: P6). GFP+/tdTomato+ dopamine neurons (P6 population: -DOX vs +DOX) are bilaterally injected into the striatum of adult NSG mice. Figures 13B-13C show representative confocal images of dopamine neuron grafts at 1 month post transplantation stained with antibodies against GFP (equivalent to NURR1), scale bar = 100 μm. (Figure 13B) and stereological analysis for the number (using optical fractionator, left) and volume (cavalier estimator, right) of the surviving dopamine neurons at 1 month post transplantation n= 5 for each condition (Figure 13C). Figures 13D and 13F show representative confocal images of ALDH1A1 (A9 type dopamine

neuron marker) and CALB1 (A10 type dopamine neuron marker). Scale bar = 100 μm. Figures 13E and 13G show quantification of the percentage of A9 (ALDH1A1, Figure 13E) and A10 (CALB1, Figure 13F) dopamine neurons per NURR1 expressing DA neurons at 1 month post transplantation. *$p<0.05$ (paired t-test). ns. = not significant.

Figures 14A-14F illustrate temporal kinetics of the p53-mediated dopamine neuron death-related pathways post implantation. Figures 14A-14F show representative immunohistochemistry image (Figures 14A-14C) and quantification of the percentages (Figures 14D-14F) of the grafted dopamine neurons at different time points (4 hpt, 24 hpt, 72 hpt, 7 dpt) for TP53, cleaved caspase 3 (CC3), and TUNEL as apoptosis markers in dopamine neuron grafts upon transplantation. All markers show robust induction at 24hr. Scale bar = 50 μm. n = 4 independent experiments.

Figures 15A-15I illustrate TNFa-NFκB pathway is an upstream trigger of p53-dependent dopamine neuron death in the graft. Figure 15A shows PCA plot of bulk RNAseq data for sorted dopamine neurons either immediately post FACS (day 0, D0), *in vitro* cultured for 1 day post sorting (day 1 culture, D1 culture) or *in vivo* grafted for 1 day (day 1 graft, D1 graft). Figure 15B shows differentially expressed gene (DEG) analysis between D1 culture versus D1 graft. Figure 15C shows hallmark pathway analysis on the upregulated genes for functional categories in D1 grafted vs D1 cultured neuron. Figure 15D shows normalized enrichment score (NES) analysis of enriched TNF-related genes in D1 graft *vs.* D1 culture neuron. Figure 15E shows unbiased gene set enrichment analysis (GSEA) identifies Apoptosis, TP53, and TNFa as the GO terms most frequently enriched in D1 graft versus D1 culture neuron. Figure 15F (left panel) shows representative immunohistochemistry images of phosphorylated NFkappaB (p-NFκB) in grafted neurons at distinct time points post transplantation, scale bar = 50 μm. Figure 15F (right panel) shows quantification of the percentages of p-NFκB positive cells among total cells within the graft, n = 3 independent experiments. Figure 15G shows representative immunofluorescence images of NURR1::GFP sorted DA neurons *in vitro* for the induction of p53 and NFkB-p65 comparing mock *vs.* TNFa *vs.* TNFa and monoclonal antibody against TNFa (adalimumab), treated conditions for 1 day. Figures 15H and 15I show western blot and qRT-PCR of gene expression profiles of the three groups listed in Figure 15G for TH and TP53 (Figure 15H) and for the midbrain mDA markers FOXA2 and NURR1, TP53, and PUMA downstream target of TP53 (Figure 15I). N > 3 independent experiments.

Figures 16A-16H illustrate single cell RNA sequencing of grafted neurons identifies JUN-related survival signature and cell death associated dedifferentiation following transplantation. Figure 16A-16C show UMAP plot of scRNA TP53 WT and KO grafted cells at 1 day post transplantation color coded by cell clusters (Figure 16A), by TP53 WT and KO genotypes (Figure 16B), and by annotated cell types including neuroblasts (hNbM), floor-plate progenitor (hProgFPL), and a very small portion of pericytes (hPeric) (Figure 16C). Figure 16D shows heatmap of top enriched gene-set in each cluster of TP53 WT versus TP53 KO cells at 1 day post transplantation, demonstrating highly increased cell death-related genes in clusters 3, 5, and 6 and survival related genes in clusters 2, 4. Red color indicates survival related genes. Figure 16E volcano plots of differentially expressed genes, such as *BAX, CDK1NA, CDKN2B, BBC3 (PUMA),* and *PHPT1* (in red) in TP53 WT versus TP53 KO grafted dopamine neurons from clusters 3, 5, and 6. Figure 16F shows violin plots of BAX, TNFRSF12A, and JUN positive cells among the clusters. The cluster 7 is excluded due to a very small portion of cells. Figures 16G and 16H show HES5 positive cells specifically to clusters 3, 5, and 6 mark de-differentiated cells in UMAP from 1day post graft (Figure 16G) and is not expressed in the sorted cells prior to grafting (Figure 16H).

Figures 17A-17E illustrate high-through flow-based cell surface marker screen identifies novel CD marker to purify NURR1 stage postmitotic dopamine neuron for translational use. Figure 17A shows schematic illustration of the flow-based CD marker screen to enrich for postmitotic dopamine neurons matching genetic NURR1::GFP reporter expression. Figure 17B shows FACS plot of % NURR1::GFP populations corresponding to each sorting strategy (Control, CD49e-low, CD49-low/CD171-high, CD49e-low/CD184-high), indicating CD49e-low/CD184-high double CD marker sorting leads to the most enriched dopamine neuron population expressing NURR1::GFP. Figure 17C shows gene expression of NURR1 via qRT-PCR assay 2 days post sorting using each sorting strategy from Figure 17B**.** Figure 17D shows representative immunofluorescence image of CD49e-low/CD184-high double sorted dopamine neurons at day 40, giving rise to pure dopamine neuron culture co-expressing NURR1::GFP, FOXA2, and TH. Scale bar = 100 μm. Figure 17E shows short term *in vivo* histology analysis at 1-month post grafting of CD49e-low/CD184-high double sorted graft compared with unsorted cells. The double CD49e-low/CD184-high sorted neuron grafts are composed of densely packed dopamine neurons in contrast to unsorted grafts which yield a lower percentage of dopamine neurons as detected by human nuclear antigen

(hNA) and TH immunofluorescence-staining. Figures 18A-18G illustrate clinically relevant TNFa neutralizing antibodies functionally improve the survival of postmitotic dopamine neuron during implantation. Figure 18A shows representative immunofluorescence image of CD 49e-low /CD184-high double sorted dopamine neurons either co-injected with PBS or TNFa blocking antibody, adalimumab. Scale bar = 100 μm. Figure 18B shows stereological cell counts and volume quantification of the surviving dopamine neurons at 1 month post transplantation using NURR1::GFP, n = 5. **p<0.01, *p<0.05 (paired t-test). Figure 18C shows D-amphetamine induced rotation assay in grafted PD mouse model carrying unilateral 6-OHDA lesion. The three treatment groups are: PBS injection (sham), CD sorted neurons, and CD sorted neurons but co-injected with adalimumab. Figure 18D shows representative immunofluorescence images of human grafts that are highly enriched with floor-plated derived dopamine neurons marked by hNA, FOXA2, and TH for each group, scale bar = 50 μm. Figure 18E shows stereological analysis of the number (using optical fractionator) and volume (cavalier estimator) of the surviving dopamine neurons based on TH expression at 6 months post transplantation. . *p<0.05 (paired t-test). Figures 18F and 18G show representative immunofluorescence image and quantification of portion of ALDH1A1 demarking A9 subtype (Figure 18F) and CALB1 demarking A10 subtype (Figure 18G) dopamine neurons population (TH+) in 6 months old graft.

Figures 19A-19H illustrates derivation and validation of NURR1:GFP sorted dopamine neurons for CRISPR/Cas9 screening *in vitro* and *in vivo.* Figure 19A shows immunofluorescent staining of dopamine neuron markers, NURR1:GFP, FOXA2, and TH, in NURR1:GFP sorted cells two weeks post sorting (day 40). Scale bar = 100 μm. Figure 19B shows immunofluorescent staining of a dopamine neuron marker, TH, and NURR1::GFP in graft 1 month post transplantation of day 25 NURR1:GFP sorted DA neurons. Scale bar = 100 μm. Figure 19C shows transduction efficacy and isolate transduced hPSC containing pooled lentiviral sgRNAs expressing Tomato with MOI = 0.35, which indicates single copy sgRNA integration per cell. Figure 19D shows immunofluorescent staining of TH, NURR1::GFP and gRNA::tdTomato at day 40 dopamine neurons post sorting with GFP and Tomato at day25 with/without dox treatment from day16 to day25. Scale bar = 50 μm. Figure 19E shows ablation of the tdTomato signal in hPSC-derived postmitotic dopamine neurons at day25 after dox exposure from day16 to day25 (dox 1ug/ml). Scale bar = 200 μm. Figure 19F shows graft fluorescence expression 1 month post transplantation (upper). PCR analysis for

detecting a human PTGER2 gene from genomic DNA, isolated from dissected tissue around graft region (lower). Scale bar = 500 μm. Figures 19G and 19H show overall representation of sgRNA across all condition by next-generation sequencing (NGS) from genomic DNA of *in vitro* cultured cells (day 16 and day25) and an *in vivo* grafted cell from library (Figure 19G) and more restricted library#2 (Figure 19H).

Figures 20A-20F illustrate increased survival of TP53 KO dopamine neurons in graft exhibit dopamine neuron identity and needle injection of dopamine neuron does not induce TP53 and TP53 downstream genes. Figure 20A shows immunofluorescent staining of NURR1:GFP, sgTP53RNA-Tomato, and FOXA2 and TH at day40 dopamine neuron post sorting with GFP and Tomato at day25 with/without dox exposure from day16 to day25. Scale bar = 100 μm. Figures 20B-20E show representative immunofluorescence image for dopamine markers, such as FOXA2, TH, and NURR1-GFP (Figures 20B-20D) as well as a proliferation marker, hKi67 (Figure 20E) expression in surviving p53 WT and KO dopamine neurons in the graft (WT;-DOX, KO; +DOX). Figure 20F shows qRT-qPCR analysis of TP53 and TP53 downstream gene (p21 and PUMA) before and 1 hour after needle injection of NURR1:GFP sorted dopamine neuron.

Figures 21A-21D illustrate time-course analysis of host neuroimmune cells after transplantation near the graft site. Figure 21A shows immunofluorescence analysis of IBA1 (upper) and GFAP (lower) following transplantation (4hrs, 24hrs, 72hrs, 7days). Scale bar = 50 μm. Figure 21B shows H&E staining of the graft. Scale bar = 100 μm. Figure 21C shows immunofluorescence and immunohistochemistry analysis for Ly6G to examine neutrophils at the graft site. Left panels are examined at 12 hpt and yellow arrows indicate Ly6G positive cells nearby the grafted cells positive for FOXA2. Right panels are examined at 3dpt. Ly6G positive cells marked by dark brown staining infiltrate within the graft. Scale bar = 50 μm. Figure 21D shows immunofluorescence analysis of fiber outgrowth pattern from the graft after implantation using STEM 121. Fiber extension begins at 24 hpt. Scale bar = 50 μm.

Figures 22A-22E illustrate analysis of bulk RNA-seq from sorted cells vs. 1 day cultured cells post sorting vs. 1 day grafted cells post sorting, and characterization of 1 day cultured cells post sorting. Figure 22A shows dendrogram of the cells among sorted (Day 0), in vitro cultured (Day 1 Culture), and in vivo grafted dopamine neurons (Day 1 Graft) from bulk RNA-seq demonstrating agreement among the replicate samples and distinct signature oof the day 1 grafted samples. Figure 22B shows pathway enrichment

analysis identified mTORC1 signaling as upregulated categories in day 1 cultured samples. Figure 22C shows heatmap analysis from total RNA-seq for enriched TNFa-NFκB related genes in the grafted dopamine neurons versus the sorted and cultured dopamine neurons. Figure 22D shows immunofluorescence staining of NURR1::GFP, TP53, and NFkB-p65 at day 26 dopamine neuron in culture 1 day post sorting with GFP shows no nuclear induction of NFkB. Scale bar = 100 μm. Figure 22E shows immunofluorescence staining of TNFa ligand at 1 day grafted neurons, confirming the protein expression of TNFa ligand.

Figure 23A-23D illustrate scRNA-seq analysis from p53 WT and KO dopamine neuron grafts 1 day post implantation. Figure 23A shows clustering distribution of p53 WT and p53 KO of grafted dopamine neurons 1 day post transplantation from scRNA-seq. Figure 23B shows histograms of fraction of cells expressing MAP2, Ki67, and TH positive cells in p53 WT and KO. Figure 23C shows UMAP plots of MAP2, PBX1, and MKI67 in p53 WT and p53 KO neurons from scRNA-seq. Figure 23D shows volcano plot of differentially expressed genes in p53 WT versus p53 KO from scRNA-seq.

Figures 24A-24B illustrate characterization of cell surface (CD) marker sorted cells matching NURR1::GFP *in vitro* and *in vivo*. Figure 24A shows FACS analysis of NURR1::GFP neuron population with indicated CD markers, demonstrating 3 CD markers (49e, 99, and 340) are negatively whereas 2 CD markers (171 and 184) are positively enriched to NURR1::GFP populations. Figure 24B shows immunofluorescence analysis of grafted cells from CD49-low/CD184-high double sorted and unsorted cells with FOXA2 and a human proliferation marker (hKi67) at 1 month post transplantation (left) and quantification of Ki67 positive cells within the grafts (right).

Figures 25A-25C illustrate innervation of grafts from CD marker sorted dopamine neurons co-injected either PBS or TNFa inhibitor, adalimumab, at 6 months. Figures 25A-25B show representative image of extensive innervation of CD marker sorted dopamine neuron grafts co-injected with PBS or adalimumab towards the host striatum as indicated by TH (Figure 25A) and hNCAM immunofluorescent staining (Figure 25B). Scale bar = 500 μm. Figure 25C shows immunofluorescence assay to examine the spread of adalimumab within the brain. Anti-human IgG1 Alexa fluorophore 555 was probed to detect the presence of adalimumab comparing adalimumab co-injected neurons vs. PBS injected neurons at 24 hpt, and red signal

indicates a specific detection of the human monoclonal antibodies only present in adalimumab.

DETAILED DESCRIPTION

The present disclosure provides methods and compositions for improving *in vivo* survival of midbrain dopamine (mDA) neurons (e.g., *in vitro* differentiated mDA neurons) by suppressing p53-mediated apoptosis of mDA neurons. The present disclosure further provides methods and compositions for treating a subject (e.g., a subject suffering from a neurodegenerative disease and/or neurodegeneration of midbrain dopamine neurons), comprising administering to the subject one or more mDAs, wherein p53-mediated apoptosis of the one or more mDA neurons is suppressed. In certain embodiments, the suppression of p53-mediated apoptosis comprises inhibition of TNFα signaling, inhibition of NFκB signaling, inhibition of p53 signaling, or a combination of foregoing. In certain embodiments, the suppression of p53-mediated apoptosis comprises administering to the subject a TNFα inhibitor (e.g., an antagonistic anti-TNFα antibody). In certain embodiments, the suppression of p53-mediated apoptosis comprises contacting the one or more mDA neurons with a TNFα inhibitor (e.g., an antagonistic anti-TNFα antibody).

The present disclosure is at least based on the discovery that the expression of p53 restricted *in vivo* postmitotic dopamine neuron survival following transplantation. Moreover, transcriptomic analysis revealed that TNFα-mediated activation of NFκB is a main upstream regulator of p53-mediated dopamine neuron death. The inventors discovered that knocking out *TP53* gene in midbrain dopamine neurons significantly improved *in vivo* survival of post-mitotic midbrain dopamine neurons. The inventors also discovered that *in vivo* survival of post-mitotic midbrain dopamine neurons after transplantation can be significantly improved by an TNFα antagonist, e.g., adalimumab.

Non-limiting embodiments of the present disclosure are described by the present specification and Examples.

For purposes of clarity of disclosure and not by way of limitation, the detailed description is divided into the following subsections:

5.1. Definitions;

5.2. Methods of Improving *In Vivo* Survival of Dopamine Neurons;

5.3. Compositions; and

5.4. Methods of Treatment.

5.1. Definitions

The terms used in this specification generally have their ordinary meanings in the art, within the context of the present disclosure and in the specific context where each term is used. Certain terms are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner in describing the compositions and methods of the present disclosure and how to make and use them.

The term "about" or "approximately" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, i.e., the limitations of the measurement system. For example, "about" can mean within 3 or more than 3 standard deviations, per the practice in the art. Alternatively, "about" can mean a range of up to 20%, e.g., up to 10%, up to 5%, or up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, e.g., within 5-fold, or within 2-fold, of a value.

As used herein, the term "signaling" in reference to a "signal transduction protein" refers to a protein that is activated or otherwise affected by ligand binding to a membrane receptor protein or some other stimulus. Examples of signal transduction protein include, but are not limited to, tumor necrosis factor alpha (TNFα), nuclear factor kappa B (NFκB), p53. For many cell surface receptors or internal receptor proteins, ligand-receptor interactions are not directly linked to the cell's response. The ligand activated receptor can first interact with other proteins inside the cell before the ultimate physiological effect of the ligand on the cell's behavior is produced. Often, the behavior of a chain of several interacting cell proteins is altered following receptor activation or inhibition. The entire set of cell changes induced by receptor activation is called a signal transduction mechanism or signaling pathway.

As used herein, the term "signals" refer to internal and external factors that control changes in cell structure and function. They can be chemical or physical in nature.

As used herein, the term "ligands" refers to molecules and proteins that bind to receptors.

"Inhibitor" as used herein, refers to a compound or molecule (e.g., small molecules, antibodies, peptides, peptidomimetic, natural compounds, siRNA, anti-sense nucleic acids, or aptamers) that interferes with (e.g., antagonizes, reduces, decreases, suppresses, eliminates, or blocks) the function of the target molecule or pathway. An inhibitor can be any compound or molecule that changes the activity of a named protein (signaling molecule, any molecule involved with the named signaling molecule, a named associated molecule) (e.g., including, but not limited to, the signaling molecules described herein), for one example, via directly contacting TNFα, contacting TNFα mRNA, causing conformational changes of TNFα, decreasing TNFα protein levels, or interfering with TNFα interactions with signaling partners/receptors (e.g., TNFRSF11B, TNFRSF10B, and TNFRSF12A), and affecting the expression of TNFα target genes.

Inhibitors also include molecules that indirectly regulate biological activity, for example, SMAD biological activity, by intercepting upstream signaling molecules (e.g., within the extracellular domain, examples of a signaling molecule and an effect include: Noggin which sequesters bone morphogenic proteins, inhibiting activation of ALK receptors 1,2,3, and 6, thus preventing downstream SMAD activation. Likewise, Chordin, Cerberus, Follistatin, similarly sequester extracellular activators of SMAD signaling. Bambi, a transmembrane protein, also acts as a pseudo-receptor to sequester extracellular TGFβ signaling molecules). Antibodies that block upstream or downstream proteins are contemplated for use to neutralize extracellular activators of protein signaling, and the like. Although the foregoing example relates to SMAD signaling inhibition, similar or analogous mechanisms can be used to inhibit other signaling molecules. Examples of inhibitors include, but are not limited to: LDN193189 (LDN) and SB431542 (SB) (LSB) for SMAD signaling inhibition, and IWP2 for Wnt inhibition. Inhibitors are described in terms of competitive inhibition (binds to the active site in a manner as to exclude or reduce the binding of another known binding compound) and allosteric inhibition (binds to a protein in a manner to change the protein conformation in a manner which interferes with binding of a compound to that protein' s active site) in addition to inhibition induced by binding to and affecting a molecule upstream from the named signaling molecule that in turn causes inhibition of the named molecule. An inhibitor can be a "direct inhibitor" that inhibits a signaling target or a signaling target pathway by actually contacting the signaling target.

"Activators," as used herein, refer to compounds that increase, induce, stimulate, activate, facilitate, or enhance activation the signaling function of the molecule or pathway, e.g., Wnt signaling, SHH signaling, FGF signaling, etc.

As used herein, the term "Wnt" or "wingless" in reference to a ligand refers to a group of secreted proteins (e.g., integration 1 in humans) that are capable of interacting with a Wnt receptor, such as a receptor in the Frizzled and LRPDerailed/RYK receptor family. As used herein, the term "a Wnt or wingless signaling pathway refers to a signaling pathway composed of Wnt family ligands and Wnt family receptors, such as Frizzled and LRPDerailed/RYK receptors, mediated with or without β-catenin. The Wnt signaling pathway include canonical Wnt signaling (e.g., mediation by β-catenin) and non-canonical Wnt signaling (mediation without β-catenin).

As used herein, the term "derivative" refers to a chemical compound with a similar core structure.

As used herein, the term "a population of cells" or "a cell population" refers to a group of at least two cells. In non-limiting examples, a cell population can include at least about 10, at least about 100, at least about 200, at least about 300, at least about 400, at least about 500, at least about 600, at least about 700, at least about 800, at least about 900, at least about 1000 cells. The population may be a pure population comprising one cell type, such as a population of midbrain DA precursors, or a population of undifferentiated stem cells. Alternatively, the population may comprise more than one cell type, for example a mixed cell population.

As used herein, the term "stem cell" refers to a cell with the ability to divide for indefinite periods in culture and to give rise to specialized cells.

As used herein, the term "embryonic stem cell" and "ESC" refer to a primitive (undifferentiated) cell that is derived from preimplantation-stage embryo, capable of dividing without differentiating for a prolonged period in culture, and are known to develop into cells and tissues of the three primary germ layers. A human embryonic stem cell refers to an embryonic stem cell that is from a human embryo. As used herein, the term "human embryonic stem cell" or "hESC" refers to a type of pluripotent stem cells derived from early stage human embryos, up to and including the blastocyst stage, that is capable of dividing without differentiating for a prolonged period in culture, and are known to develop into cells and tissues of the three primary germ layers.

As used herein, the term "embryonic stem cell line" refers to a population of embryonic stem cells that have been cultured under *in vitro* conditions that allow proliferation without differentiation for up to days, months to years.

As used herein, the term "totipotent" refers to an ability to give rise to all the cell types of the body plus all of the cell types that make up the extraembryonic tissues such as the placenta.

As used herein, the term "multipotent" refers to an ability to develop into more than one cell type of the body.

As used herein, the term "pluripotent" refers to an ability to develop into the three developmental germ layers of the organism including endoderm, mesoderm, and ectoderm.

As used herein, the term "induced pluripotent stem cell" or "iPSC" refers to a type of pluripotent stem cell formed by the introduction of certain embryonic genes (such as but not limited to OCT4, SOX2, and KLF4 transgenes) (*see*, for example, Takahashi and Yamanaka *Cell* 126, 663-676 (2006), herein incorporated by reference) into a somatic cell.

As used herein, the term "neuron" refers to a nerve cell, the principal functional units of the nervous system. A neuron consists of a cell body and its processes - an axon and at least one dendrite. Neurons transmit information to other neurons or cells by releasing neurotransmitters at synapses.

As used herein, the term "differentiation" refers to a process whereby an unspecialized embryonic cell acquires the features of a specialized cell such as a neuron, heart, liver, or muscle cell. Differentiation is controlled by the interaction of a cell's genes with the physical and chemical conditions outside the cell, usually through signaling pathways involving proteins embedded in the cell surface.

As used herein, the term "directed differentiation" refers to a manipulation of stem cell culture conditions to induce differentiation into a particular (for example, desired) cell type, such as midbrain dopamine neurons or precursors thereof. In references to a stem cell, "directed differentiation" refers to the use of small molecules, growth factor proteins, and other growth conditions to promote the transition of a stem cell from the pluripotent state into a more mature or specialized cell fate.

As used herein, the term "inducing differentiation" in reference to a cell refers to changing the default cell type (genotype and/or phenotype) to a non-default cell type (genotype and/or phenotype). Thus, "inducing differentiation in a stem cell" refers to

inducing the stem cell (e.g., human stem cell) to divide into progeny cells with characteristics that are different from the stem cell, such as genotype (e.g., change in gene expression as determined by genetic analysis such as a microarray) and/or phenotype (e.g., change in expression of a protein marker of mDA neurons, such as EN1, OTX2, TH, NURR1, FOXA2, LMX1A, PITX3, LMO3, SNCA, ADCAP1, CHRNA4, ALDH1A1, SOX6, WNT1, DAT, VMAT2, GIRK2, PBX1, SNCG, SATB1, CALB1, and CALB2.

As used herein, the term "cell culture" refers to a growth of cells *in vitro* in an artificial medium for research or medical treatment.

As used herein, the term "culture medium" refers to a liquid that covers cells in a culture vessel, such as a Petri plate, a multi-well plate, and the like, and contains nutrients to nourish and support the cells. Culture medium may also include growth factors added to produce desired changes in the cells.

As used herein, the term "contacting" a cell or cells with a compound (e.g., at least one inhibitor, activator, and/or inducer) refers to providing the compound in a location that permits the cell or cells access to the compound. The contacting may be accomplished using any suitable method. For example, contacting can be accomplished by adding the compound, in concentrated form, to a cell or population of cells, for example in the context of a cell culture, to achieve the desired concentration. Contacting may also be accomplished by including the compound as a component of a formulated culture medium.

As used herein, the term "*in vitro*" refers to an artificial environment and to processes or reactions that occur within an artificial environment. *In vitro* environments exemplified, but are not limited to, test tubes and cell cultures.

As used herein, the term "*in vivo*" refers to the natural environment (e.g., an animal or a cell) and to processes or reactions that occur within a natural environment, such as embryonic development, cell differentiation, neural tube formation, etc.

As used herein, the term "expressing" in relation to a gene or protein refers to making an mRNA or protein which can be observed using assays such as microarray assays, antibody staining assays, and the like.

As used herein, the term "marker" or "cell marker" refers to gene or protein that identifies a particular cell or cell type. A marker for a cell may not be limited to one marker, markers may refer to a "pattern" of markers such that a designated group of markers may identity a cell or cell type from another cell or cell type.

As used herein, the term "derived from" or "established from" or "differentiated from" when made in reference to any cell disclosed herein refers to a cell that was obtained from (e.g., isolated, purified, etc.) an ultimate parent cell in a cell line, tissue (such as a dissociated embryo, or fluids using any manipulation, such as, without limitation, single cell isolation, culture in vitro, treatment and/or mutagenesis using for example proteins, chemicals, radiation, infection with virus, transfection with DNA sequences, such as with a morphogen, etc., selection (such as by serial culture) of any cell that is contained in cultured parent cells. A derived cell can be selected from a mixed population by virtue of response to a growth factor, cytokine, selected progression of cytokine treatments, adhesiveness, lack of adhesiveness, sorting procedure, and the like.

An "individual" or "subject" herein is a vertebrate, such as a human or non-human animal, for example, a mammal. Mammals include, but are not limited to, humans, non-human primates, farm animals, sport animals, rodents and pets. Non-limiting examples of non-human animal subjects include rodents such as mice, rats, hamsters, and guinea pigs; rabbits; dogs; cats; sheep; pigs; goats; cattle; horses; and non-human primates such as apes and monkeys.

As used herein, the term "disease" refers to any condition or disorder that damages or interferes with the normal function of a cell, tissue, or organ.

As used herein, the term "treating" or "treatment" refers to clinical intervention in an attempt to alter the disease course of the individual or cell being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Therapeutic effects of treatment include, without limitation, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastases, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. By preventing progression of a disease or disorder, a treatment can prevent deterioration due to a disorder in an affected or diagnosed subject or a subject suspected of having the disorder, but also a treatment may prevent the onset of the disorder or a symptom of the disorder in a subject at risk for the disorder or suspected of having the disorder.

As used herein, the term "negative", "weak", or "-" when used in reference to any surface marker disclosed herein refer to that the surface marker (e.g., CD49e) is not expressed at a detectable level, or is expressed at a reduced level in a cell as compared to the mean expression of the surface marker in a population of cells of which the cell is

selected or sorted from. As used herein, the term "high", "strong", "+", or "positive" when used in reference to any surface marker disclosed herein refer to that the surface marker (e.g., CD184) is expressed at a detectable level or expressed at an increased level as compared to the mean expression of the surface marker in a population of cells.

In certain embodiments, the cells are distinguished according to their surface marker expression levels based on a readily discernible differences in staining intensity as is known to one or ordinary skill in the art. In certain embodiments, the cut off for designating a cell as a surface marker "weak", "negative", or "-" cell can be set in terms of the staining intensity distribution (e.g., fluorescence intensity distribution) observed for all the cells, with those cells falling below about 50%, about 40%, about 30%, about 20%, about 10%, or about 5% of staining intensity being designated as the surface marker "weak", "negative", or "-" cell. In certain embodiments, the cut off for designating a cell as a surface marker "strong", "high", "+", or "positive" cell can be set in terms of the staining intensity distribution (e.g., fluorescence intensity distribution) observed for all the cells, with those cells falling above about 50%, about 60%, about 70%, about 80%, about 90%, or about 95% of staining intensity being designated as the surface marker "strong", "high", "+", or "positive" cell. In certain embodiments, the frequency distribution of the surface marker staining is obtained for all the cells and the population curve fit to a higher staining and lower staining population, and cells assigned to the population to which they most statistically are likely to belong in view of a statistical analysis of the respective population distributions.

5.2. Methods of Improving In Vivo Survival of Midbrain Dopamine Neurons

The present disclosure provides methods of improving *in vivo* survival of one or more midbrain dopamine (mDA) neurons. In certain embodiments, the methods comprise suppressing p53-mediated apoptosis of the one or more mDA neurons. In certain embodiments, the suppression of p53-mediated apoptosis comprises inhibition of tumor necrosis factor alpha (TNFα) signaling, inhibition of nuclear factor kappa B (NFκB) signaling, inhibition of p53 signaling, or a combination of the foregoing. In certain embodiments, the suppression of p53-mediated apoptosis comprises contacting the one or more mDA neurons with at least one compound selected from the group consisting of TNFα inhibitors, NFκB inhibitors, p53 inhibitors, and combinations thereof. In certain embodiments, the suppression of p53-mediated apoptosis comprises contacting the one or more mDA neurons with a TNFα inhibitor.

In certain embodiments, the one or more mDA neurons express a marker selected from the group consisting of EN1, OTX2, TH, NURR1, FOXA2, LMX1A, PITX3, LMO3, SNCA, ADCAP1, CHRNA4, ALDH1A1, SOX6, WNT1, DAT, VMAT2, GIRK2, SATB1, CALB1, CALB2, SNCG, PBX1, and combinations thereof. In certain embodiments, the one or more mDA neurons are post-mitotic mDA neurons. In certain embodiments, the one or more mDA neurons are *in vitro* differentiated from one or more stem cells. In certain embodiments, the one or more stem cells are human stem cells. In certain embodiments, the one or more stem cells are one or more induced pluripotent stem cells.

5.2.1. Inhibition of TNFα signaling

In certain embodiments, the suppression of p53-mediated apoptosis comprises inhibition of TNFα signaling. The TNFα signaling pathway plays an important role in various physiological and pathological processes, including cell proliferation, differentiation, apoptosis, and modulation of immune responses and induction of inflammation. TNFα is a multifunctional proinflammatory cytokines, with effects on lipid metabolism, coagulation, insulin resistance, and endothelial function. TNFα can be produced by many cell types (e.g., macrophages, lymphocytes, fibroblasts, and keratinocytes) in response to inflammation, infection, and other environmental stresses. TNFα acts by binding to its receptors (e.g., TNFR1, TNFR2, TNFRSF11B, TNFRSF10B, and TNFRSF12A) which in turn recruit and activate complex signaling cascades and networks.

In certain embodiments, inhibition of TNFα signaling is achieved by an inhibitor of TNFα signaling. In certain embodiments, the inhibitor of TNFα signaling can be a molecule (e.g., a chemical compound or an antibody) that interferes with (e.g., antagonizes, reduces, decreases, suppresses, eliminates, or blocks) the function of TNFα and/or its signaling. In certain embodiments, the inhibitor of TNFα signaling is a TNFα inhibitor.

In certain embodiments, the inhibitor of TNFα signaling and/or the TNFα inhibitor can include, without any limitation, interfering ribonucleic acids (e.g., siRNA, shRNA), aptamers, or peptidomimetics.

Non-limiting examples of inhibitors of TNFα signaling and/or the TNFα inhibitors include anti-TNFα antibodies, TNFα decoy receptors, chemical compounds, In certain embodiments, the TNFα inhibitor is selected from the group consisting of anti-TNFα antibodies, TNFα decoy receptors, chemical compounds, nucleic acid inhibitors,

small molecule inhibitors, receptor biologic inhibitors, inactive TNF fragments, TNFα circulating receptor fusion protein (e.g. etanercept, etanercept-szzs), xanthine derivatives (e.g. pentoxifylline), and 5-HT_{2A} agonist (e.g., *(R)*-DOI, TCB-2, LSD, LA-SS-Ac).

In certain embodiments, the inhibitor of TNFα signaling and/or the TNFα inhibitor is an antibody. In certain embodiments, the antibody is an anti-TNFα antibody. In certain embodiments, the antibody is an antagonistic anti-TNFα antibody. In certain embodiments, the anti-TNFα antibody is selected from the group consisting of adalimumab (Humira®), adalimumab-adbm (Cyltezo®), adalimumab-adaz (Hyrimoz®), adalimumab-atto (Amgevita®), certolizumab pegol (Cimzia®), golimumab (Simponi®, Simponi Aria®), infliximab (Remicade®), infliximab-abda (Renflexis®), infliximab-dyyb (Inflectra®), remtolumab, afelimomab, nerelimomab, ozoralizumab, placulumab, and combinations thereof. In certain embodiments, the TNFα inhibitor is adalimumab.

In certain embodiments, the inhibitor of TNFα signaling and/or the TNFα inhibitor is a polypeptide. In certain embodiments, the polypeptide is a TNFα decoy receptor. In certain embodiments, the TNFα decoy receptor is selected from the group consisting of etanercept (Enbrel®), etanercept-szzs (Ereizi®), pegsunercept, onercept, and lenercept.

In certain embodiments, the inhibitor of TNFα signaling and/or the TNFα inhibitor is a chemical compound. Non-limiting examples of chemical compounds that can be used with the present disclosure include apremilast (Otezla®), bupropion (Zyban®), cathechin, cannabinoids, curcumin, lysergic acid 2,4-dimethylazetidide (LA-SS-Az, LSZ), apigenin-7-O-glucuronide, JTE-607 dihydrochloride, MD2-TLR4-IN-1, AUDA, isuzinaxib (APX-115 free base), IQ 3, 3-deazaadenosine hydrochloride, cucurbitacin IIb, lenalidomide (CC-5013), aprepitant (MK-0869), thalidomide (K17), amarogentin, pomalidomide (CC-4047), acetylcysteine (N-acetylcysteine), butoconazole nitrate, CPI-1189, methylthiouracil, UCB-9260, mesaconitine, myrislignan, falcarindiol, gardenoside, demethyleneberberine, stylopine, benpyrine racemate, muscone, ginsenoside Rb1, cepharanthine, QNZ (EVP4593), AX-024 HCl, NE 52-QQ57, resatorvid (TAK-242), apremilast (CC-10004), necrostatin-1, PF-3644022, GSK583, shikonin (C.I. 75535), GSK2982772, mulberroside A, corilagin, 20(S)-ginsenoside Rh1, forsythoside B, 2',5'-dihydroxyacetophenone, geraniin, homoplantaginin, SPD-304, UCB-6876, UCB-5307, UCB-9260, PF-3644022, R-7050, citronellol, and madecassic acid.

5.2.2. Inhibition of NFκB signaling

In certain embodiments, the suppression of p53-mediated apoptosis comprises inhibition of NFκB signaling. NFκB represents a family of inducible transcription factors, which regulates a large array of genes involved in different processes of the immune and inflammatory responses. This family is composed of five structurally related members, including NFκB1, NFκB2, RelA, RelB and c-Rel, which mediates transcription of target genes by binding to a specific DNA element (e.g., κB enhancer). NFκB can regulate inflammatory responses by mediating induction of various pro-inflammatory genes in innate immune cells. NFκB can also regulate the activation, differentiation and effector function of inflammatory T cells.

In certain embodiments, inhibition of NFκB signaling is achieved by an inhibitor of NFκB signaling. In certain embodiments, the inhibitor of NFκB signaling can be a molecule (e.g., a chemical compound) that interferes with (e.g., antagonizes, reduces, decreases, suppresses, eliminates, or blocks) the transcription activity of NFκB and its signaling. In certain embodiments, the inhibitor of NFκB signaling is a NFκB inhibitor.

Non-limiting examples of inhibitors of NFκB signaling and NFκB inhibitors that can be used with the present disclosure include upstream inhibitors of NFκB, inhibitors of IKK activity, inhibitors of IκB phosphorylation, inhibitors of IκB degradation, proteasome inhibitors, protease inhibitors, IκB upregulators, inhibitors of NFκB nuclear translocation and expression, NFκB DNA-binding inhibitors, and NFκB transactivation inhibitors, inhibitors of NFκB directed gene transactivation, and antioxidants. Additional examples of inhibitors of NFκB signaling and NFκB inhibitors that can be used with the present disclosure include, without any limitation, antioxidants, interfering ribonucleic acids (e.g., siRNA, shRNA), antibodies, aptamers, or peptidomimetics.

In certain embodiments, inhibition of NFκB signaling is achieved by an upstream inhibitor of NFκB. Non-limiting examples of upstream inhibitors of NFκB that can be used with the present disclosure include rituximab, pigment epithelium derived factor, betaine, desloratadine, LY29, LY30, MOL 294, pefabloc, rhein, salmeterol, and fluticasone propionate.

In certain embodiments, inhibition of NFκB signaling is achieved by an inhibitor of IKK activity of IκB phosphorylation. Non-limiting examples of inhibitors of IKK activity of IκB phosphorylation that can be used with the present disclosure include heparin-binding epidermal growth factor-like growth factor, hepatocyte growth factor, interleukin-10, anti-thrombin III, chorionic gonadotropin, interferon-α, 2-amino-3-cyano-

4-aryl-6-(2-hydroxy-phenyl)pyridine derivatives, acrolein, AS602868, aspirin, dihydroxyphenylethanol, epoxyquinone A monomer, MLB120, BMS-345541, CYL-19s, CYL-26z, 2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-piperidin-4-yl nicotinonitrile, compound A, compound 5, cyclopentenones, jesterone dimer, PS-1145 (MLN1145), 2-[(aminocarbonyl)amino]-5-acetylenyl-3-thionphenecarboxamides, amino-pyrimidine, benzoimidazole derivative, CDDO-Me (synthetic triterpenoid), CHS 828 (anticancer drug), diaylpyridine derivative, imidazolylquinoline-carboxaldehyde derivatives, indolecarboxamide, LF15-0195 (analog of 15-deoxyspergualine), ML120B, MX781 (retinoid antagonist), N-(4-hydroxyphenyl) retinamide, pyrazolo[4,3-c]quinoline derivatives, pyridooxazinone derivative, scytonemin, sulfasalazine, thalidomide, azidothymidine (AZT), BAY-11-7082 (E3((4-methylphenyl)-sulfonyl)-2-propenenitrile), BAY-11-7083 (E3((4-t-butylphenyl)-sulfonyl)-2-propenenitrile), benzyl isothiocyanate, carboplatin, gabexate mesylate, Gleevec (Imatanib), hydroquinone, ibuprofen, methotrexate, monochloramine, nafamostat mesylate, statins, and THI 52 (1-naphthylethyl-6,7-dihydroxy-1,2,3,4- tetrahydroisoquinoline).

In certain embodiments, inhibition of NFκB signaling is achieved by an inhibitor of IκB degradation. Non-limiting examples of inhibitors of IκB degradation that can be used with the present disclosure include penetratin, vasoactive intestinal peptide, α-melanocyte-stimulating hormone (a-MSH), IL-13, intravenous immunoglobulin, pituitary adenylate cyclase-activating polypeptide (PACAP), SAIF (Saccharomyces boulardii anti-inflammatory factor), acetaminophen, 1-Bromopropane, diamide (tyrosine phosphatase inhibitor), dobutamine, E-73 (cycloheximide analog), ecabet sodium, gabexate mesylate, glimepiride, losartan, pervanadate, phenylarsine oxide, phenytoin, sabaeksan, U0126 (MEK inhibitor), and Ro106-9920 (small molecule).

In certain embodiments, inhibition of NFκB signaling is achieved by a proteasome or protease inhibitor. Non-limiting examples of proteasomes or protease inhibitors that can be used with the present disclosure include N-acetyl-leucinyl-leucynil-norleucynal, MG101, N-acetyl-leucinyl-leucynil-methional, carbobenzoxyl-leucinyl-leucynil-norvalinal, MG115, N-carbobenzoxyl-L-leucinyl-L-leucinyl-L-norleucinal, MG132, ubiquitin ligase inhibitors, boronic acid peptide, bortezomib, salinosporamide A, tacrolimus, deoxyspergualin, disulfiram, N-acetyl-DL-phenylalanine-b-naphthylester, N-benzoyl L-tyrosine-ethylester, 3,4-dichloroisocoumarin, diisopropyl fluorophosphate, N-α-tosyl-L-phenylalanine chloromethyl ketone, and N-α-tosyl-L-lysine chloromethyl ketone.

In certain embodiments, inhibition of NFκB signaling is achieved by an inhibitor of NFκB nuclear translocation and expression. Non-limiting examples of inhibitors of NFκB nuclear translocation and expression for use with the present disclosure include atorvastatin, phallacidin, piperine, pitavastatin, selenomethionine, clarithromycin, cantharidin, neomycin, paeoniflorin, rapamycin, ranpirnase, BMD (N(1)-Benzyl-4-methylbenzene-1,2-diamine), carbaryl, indole-3-carbinol, dehydroxymethylepoxyquinomicin, dipyridamole, disulfiram, diltiazem, fluvastatin, levamisole, rolipram, SC236, omapatrilat, enalapril, and CGS 25462.

In certain embodiments, inhibition of NFκB signaling is achieved by an NFκB DNA-binding or transactivation inhibitor. Non-limiting examples of inhibitors of NFκB DNA-binding or transactivation inhibitors that can be used with the present disclosure include 7-amino-4-methylcoumarin, amrinone, atrovastat (HMG-CoA reductase inhibitor), benfotiamine (thiamine derivative), bisphenol A, caprofen, carbocisteine, celecoxib, germcitabine, flurbiprofen, lovastatinm mercaptopyrazine, monomethylfumarate, moxifloxacin, nicorandil, nilvadipine, pioglitazone, pirfenidone, pyridine N-oxide derivatives, quinadril, raloxifene, raxofelast, ribavirin, rifamides, ritonavir, rosiglitazone, roxithromycin, simvastatin, SM-7368, sulfasalazine, verapamil, dimethylfumarate (DMF), ethyl 2-[(3-methyl-2,5-dioxo(3-pyrrolinyl)) pyrimidine-5-carboxylate, nelfinavir, ritonavir, saquinavir, RO31-8220, SB203580, and troglitazone.

5.2.3. Inhibition of p53 signaling

In certain embodiments, the suppression of p53-mediated apoptosis comprises inhibition of p53 signaling. p53 is a transcriptional factor often associated with cancers. Physiologically, p53 can be disabled either by mutations or by upstream negative regulators, including, but not limited to, MDM2 and MDMX.

In certain embodiments, inhibition of p53 signaling is achieved by reducing the expression of p53. In certain embodiments, reducing the expression of p53 comprises knocking out or knocking down *TP53* gene in the one or more mDAs. In certain embodiments, the *TP53* gene is knocked out or knocked down in the one or more mDA neurons by a gene-editing system.

Non-limiting examples of gene-editing systems for use with the present disclosure include systems utilizing a non-naturally occurring or engineered nuclease (including, but not limited to, Zinc-finger nuclease (ZNFs), meganuclease, transcription activator-like effector nuclease (TALEN)), or a CRISPR-Cas system. Details on the gene-editing systems for use with the present disclosure can be found in Adli et al., *Nat*

Commun. 2018 May 15;9(1):1911 and Maeder & Gersbach, *Mol Ther*. 2016 Mar;24(3):430-46, the content of each of which is incorporated by reference in its entirety.

In certain embodiments, a CRISPR-Cas system is used for knocking out or knocking down the *TP53* gene. The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas (CRISPR Associated) system is an engineered nuclease system based on a bacterial system that can be used for genome engineering. It is based on part of the adaptive immune response of many bacteria and archea. When a virus or plasmid invades a bacterium, segments of the invader's DNA are converted into CRISPR RNAs (crRNA) by the "immune" response. The crRNA then associates, through a region of partial complementarity, with another type of RNA called tracrRNA to guide a CRISPR-Cas nuclease to a region homologous to the crRNA in the target DNA called a "proto spacer". The CRISPR-Cas nuclease cleaves the DNA to generate blunt ends at the DSB at sites specified by a 20-nucleotide guide sequence contained within the crRNA transcript. The CRISPR-Cas nuclease requires both the crRNA and the tracrRNA for site specific DNA recognition and cleavage. This system has been engineered such that the crRNA and tracrRNA can be combined into one molecule (the "single guide RNA"); and the crRNA equivalent portion of the single guide RNA can be engineered to guide the CRISPR-Cas nuclease to target any desired sequence (*see* Jinek et al., *Science* (2012);337:816-821). Thus, the CRISPR-Cas system can be engineered to create a DSB at a desired target in a genome. In certain embodiments, the CRISPR-Cas system comprises a CRISPR-Cas nuclease and a single-guide RNA. Suitable examples of CRISPR-Cas nucleases include, but are not limited to, Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8, Cas9 (also known as Csn1 and Csx12), Cas10, Csy1, Csy2, Csy3, Cse1, Cse2, Csc1, Csc2, Csa5, Csn2. Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csx1, Csx15, Csf1, Csf2, Csf3, Csf4, homologs thereof, or modified versions thereof. These CRISPR-Cas nucleases are known; for example, the amino acid sequence of *S. pyogenes* Cas9 protein may be found in the SwissProt database under accession number Q99ZW2. In some embodiments, the CRISPR-Cas nuclease has DNA cleavage activity, *e.g.*, Cas9. In certain embodiments, the CRISPR-Cas nuclease is Cas9. The CRISPR-Cas nuclease can direct cleavage of one or both strands at the location of a target sequence (*e.g.*, a genomic safe harbor site). Additionally, the CRISPR-Cas nuclease can direct cleavage of one or both strands within about 1, 2, 3, 4, 5, 6, 7, 8, 9,

10, 15, 20, 25, 50, 100, 200, 500, or more base pairs from the first or last nucleotide of a target sequence.

In certain embodiments, inhibition of p53 signaling is achieved by an inhibitor of p53 signaling. In certain embodiments, the inhibitor of p53 signaling can be a molecule (e.g., a chemical compound) that interferes with (e.g., antagonizes, reduces, decreases, suppresses, eliminates, or blocks) the transcription activity of p53 and its signaling. In certain embodiments, the inhibitor of p53 signaling is a p53 inhibitor.

Non-limiting examples of inhibitors of p53 signaling and p53 inhibitors that can be used with the present disclosure include JNK inhibitors, p38 MAPK inhibitors, caspase inhibitors, puma/BBC3 inhibitors, BAX inhibitors, CDK inhibitors, MDM2 and MDMX activators, and combinations thereof. Additional examples of p53 signaling and p53 inhibitors for use with the present disclosure include, without any limitation, interfering ribonucleic acids (e.g., siRNA, shRNA), antibodies, aptamers, or peptidomimetics.

In certain embodiments, inhibition of p53 signaling is achieved by a JNK inhibitor. c-Jun N-terminal protein kinase (JNK) is a subfamily of the mitogen activated protein kinase (MAPK) superfamily. JNK is a key regulator of many cellular events, including programmed cell death (apoptosis). In addition, JNK activates p53, which regulates apoptosis processes. Non-limiting examples of JNK inhibitors that can be used with the present disclosure include SP600125, AS601245, AS602801, JNK-IN-1, JNK-IN-8, ginsenoside Rg1, AV7, BI-78D3, pyridopyrimidione derivates, CC-930, quinazoline, triazolothione 1, XG-102 (D-JNKI-1), 4-fluorophenyl isoxazoles, 4-quinolone analogs, and 4-phenylisoquinolone.

In certain embodiments, inhibition of p53 signaling is achieved by a p38 MAPK inhibitor. p38 mitogen-activated protein kinases are a class of mitogen-activated protein kinases (MAPKs) that are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in cell differentiation, apoptosis and autophagy. p38 MAPK can activate p53. Non-limiting examples of p38 MAPK inhibitors that can be used with the present disclosure include doramapimod, skepinone, ralimetinib, TAK-715, losmapimod, neflamapimod, R1487, VX-702, pamapimod, and adezmapimod.

In certain embodiments, inhibition of p53 signaling is achieved by a caspase inhibitor. Non-limiting examples of caspase inhibitors that can be used with the present

disclosure include Ac - IETD - CHO, Ac-YVAD-CHO, Ac-DEVD-CMK, Z-VAD-FMK, Z-YVAD-FMK, Boc-D-FMK, TRP-601, Q-VD-OPh, VX-765 (belnacasan), VRT-043198, VX-740 (pralnacasan), IDN-6556 (emricasan, PF-034911390), VX-166, M826, M867, QPI-1007 (cosdosiran), NCX-1000, and Isatin sulfonamides.

In certain embodiments, inhibition of p53 signaling is achieved by a nucleic acid targeting a protein regulating the p53 pathway. In certain embodiments, the nucleic acid targets p53. Non-limiting examples of nucleic acids that can be used with the present disclosure include siRNAs and shRNAs. siRNA molecules are polynucleotides that are generally about 20 to about 25 nucleotides long and are designed to bind specific RNA sequence (e.g., p53 mRNA). siRNAs silence gene expression in a sequence-specific manner, binding to a target RNA (e.g., an RNA having the complementary sequence) and causing the RNA to be degraded by endoribonucleases. siRNA molecules able to inhibit the expression of p53 can be produced by suitable methods. There are several algorithms that can be used to design siRNA molecules that bind the sequence of a gene of interest (*see* e.g., Huesken et al., *Nat. Biotechnol.* 23:995-1001; Jagla et al., *RNA* 11:864-872, 2005; Shabalinea, BMC Bioinformatics 7:65, 2005). Additionally or alternatively, expression vectors expressing siRNA or shRNA can be used (*see* e.g., Brummelkamp, *Science* 296: 550-553, 2002; Lee et al., *Nature Biotechnol.* 20:500-505, 2002; Elbashir et al., *Nature* 411:494-498, 2001).

In certain embodiments, inhibition of p53 signaling is achieved by a ribozyme that inhibits the expression of p53. Ribozymes are RNA molecules possessing enzymatic activity. One class of ribozymes is capable of repeatedly cleaving other separate RNA molecules into two or more pieces in a nucleotide base sequence specific manner *(see* Kim et al., *Proc Natl Acad Sci USA*, 84:8788 (1987); Haseloff & Gerlach, *Nature*, 334:585 (1988); and Jefferies et al., *Nucleic Acid Res*, 17:1371 (1989). Such ribozymes typically have two functional domains: a catalytic domain and a binding sequence that guides the binding of ribozymes to a target RNA through complementary base-pairing. Once a specifically-designed ribozyme is bound to a target mRNA, it enzymatically cleaves the target mRNA, reducing its stability and destroying its ability to directly translate an encoded protein. Methods for selecting a ribozyme target sequence and designing and making ribozymes are generally known in the art.

5.2.4. Midbrain Dopamine (mDA) Neurons

In certain embodiments, the one or more mDA neurons used with the presently disclosed methods express a marker indicating a mDA neuron. Non-limiting examples of markers indicating a mDA neuron include engrailed-1 (EN1), orthodenticle homeobox 2 (OTX2), tyrosine hydroxylase (TH), nuclear receptor related-1 protein (NURR1), forkhead box protein A2 (FOXA2), and LIM homeobox transcription factor 1 alpha (LMX1A), PITX3, LMO3, SNCA, ADCAP1, CHRNA4, ALDH1A1, DAT, VMAT1, SOX6, WNT1, GIRK2, SATB1, CALB1, CALB2, and PBX1. In certain embodiments, the mDA neurons do not express a detectable level of at least one marker selected from the group consisting of PAX6, EMX2, LHX2, SMA, SIX1, PITX2, SIM1, POU4F1, PHOX2A, BARHL1, BARHL2, GBX2, HOXA1, HOXA2, HOXB1, HOXB2, POU5F1, NANOG, and combinations thereof. In certain embodiments, the one or more mDA neurons express at least one of A9 subtype mDA neuron markers, A10 subtype mDA neuron markers, and mDA neuron maturity markers. In certain embodiments, the one or more mDA neurons express at least one marker selected from the group consisting of TH, EN1, NURR1, and ALDH1A1. In certain embodiments, the one or more mDA neurons express ALDH1A1.

In certain embodiments, the one or more mDA neurons are one or more post-mitotic mDA neurons. A "post-mitotic" cell is a terminally differentiated cell that is no longer able to undergo mitosis and proliferation. In certain embodiments, the one or more post-mitotic mDA neurons do not express a detectable level of CD49e and express a detectable level of CD184. In certain embodiments, the one or more mDA neurons are sorted by not expressing a detectable level of CD49e and expressing a detectable level of CD184.

In certain embodiments, the one or more mDA neurons are *in vitro* differentiated from stem cells. In certain embodiments, the one or more mDA neurons are *in vitro* differentiated from one or more stem cells in accordance to the methods disclosed in International Patent Publication Nos.WO2013067362, WO2016196661, WO2021042027, and WO2021203009, the contents of each of which are incorporated by reference in their entireties.

In certain embodiments, the *in vitro* differentiation comprises contacting stem cells with at least one inhibitor of Small Mothers Against Decapentaplegic (SMAD) signaling (referred to as "SMAD inhibitor"), at least one activator of Sonic hedgehog (SHH) signaling (referred to as "SHH activator"), and at least one activator of wingless (Wnt) signaling (referred to as "Wnt activator"). In certain embodiments, the *in vitro*

differentiation further comprises contacting the cells with at least one activator of fibroblast growth factor (FGF) signaling (referred to as "FGF activator"). In certain embodiments, the *in vitro* differentiation further comprises contacting the cells with at least one inhibitor of Wnt signaling. In certain embodiments, the cells are further contacted with DA neuron lineage specific activators and inhibitors.

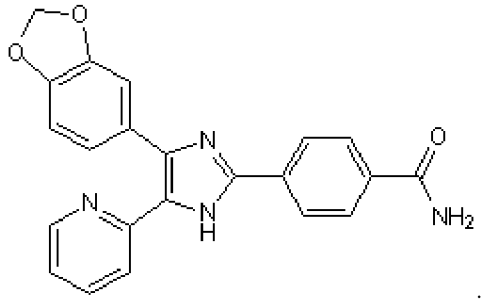
5.2.4.1. Stem Cells

In certain embodiments, the stem cells are pluripotent stem cells. In certain embodiments, the pluripotent stem cells are selected from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and combinations thereof. In certain embodiments, the stem cells are multipotent stem cells. Non-limiting examples of stem cells that can be used with the presently disclosed methods include nonembryonic stem cells, embryonic stem cells, induced nonembryonic pluripotent cells, and engineered pluripotent cells. In certain embodiments, the stem cells are human stem cells. Non-limiting examples of human stem cells include human embryonic stem cells (hESC), human pluripotent stem cell (hPSC), human induced pluripotent stem cells (hiPSC), human parthenogenetic stem cells, primordial germ cell-like pluripotent stem cells, epiblast stem cells, F-class pluripotent stem cells, somatic stem cells, cancer stem cells, or any other cell capable of lineage specific differentiation. In certain embodiments, the stem cell is a human embryonic stem cell (hESC). In certain embodiments, the stem cell is a human induced pluripotent stem cell (hiPSC). In certain embodiments, the stem cells are non-human stem cells. In certain embodiments, the stem cell is a nonhuman primate stem cell. In certain embodiments, the stem cell is a rodent stem cell.

5.2.4.2. SMAD Inhibitors

Non-limiting examples of SMAD inhibitors include inhibitors of transforming growth factor beta (TGFβ)/Activin-Nodal signaling (referred to as "TGFβ/Activin-Nodal inhibitor"), and inhibitors of bone morphogenetic proteins (BMP) signaling. In certain embodiments, the TGFβ/Activin-Nodal inhibitor can neutralize the ligands including TGFβs, BMPs, Nodal, and activins, and/or block their signal pathways through blocking the receptors and downstream effectors. Non-limiting examples of TGFβ/Activin-Nodal inhibitors include those disclosed in WO/2010/096496, WO/2011/149762, WO/2013/067362, WO/2014/176606, WO/2015/077648, Chambers et al., Nat Biotechnol. 2009 Mar;27(3):275-80, Kriks et al., Nature. 2011 Nov 6;480(7378):547-51, and Chambers *et al., Nat Biotechnol.* 2012 Jul 1;30(7):715-20 (2012), all of which are

incorporated by reference in their entireties herein for all purposes. In certain embodiments, the at least one TGFβ/Activin-Nodal inhibitor is selected from inhibitors of ALK5, inhibitors of ALK4, inhibitors of ALK7, and combinations thereof). In certain embodiments, the TGFβ/Activin-Nodal inhibitor comprises an inhibitor of ALK5. In certain embodiments, the TGFβ/Activin-Nodal inhibitor is a small molecule selected from SB431542, derivatives thereof, and mixtures thereof. "SB431542" refers to a molecule with a number CAS 301836-41-9, a molecular formula of $C_{22}H_{18}N_4O_3$, and a name of 4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1H-imidazol-2-yl]-benzamide, for example, see structure below:



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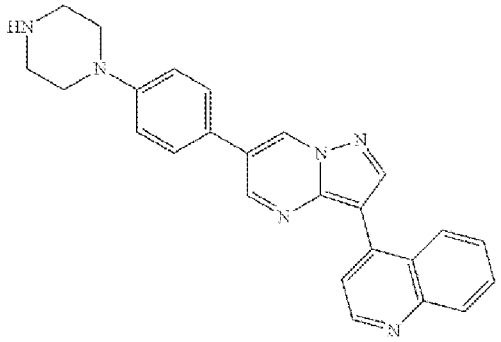
In certain embodiments, the TGFβ/Activin-Nodal inhibitor comprises SB431542. In certain embodiments, the TGFβ/Activin-Nodal inhibitor comprises a derivative of SB431542. In certain embodiments, the derivative of SB431542 is A83-01.

In certain embodiments, the at least one SMAD inhibitor comprises an inhibitor of BMP signaling (referred to as "BMP inhibitor"). Non-limiting examples of BMP inhibitors include those disclosed in WO2011/149762, Chambers *et al., Nat Biotechnol.* 2009 Mar;27(3):275-80, Kriks *et al., Nature*. 2011 Nov 6;480(7378):547-51, and Chambers *et al., Nat Biotechnol.* 2012 Jul 1;30(7):715-20, all of which are incorporated by reference in their entireties. In certain embodiments, the BMP inhibitor is a small molecule selected from LDN193189, Noggin, dorsomorphin, derivatives thereof, and mixtures thereof. "LDN193189" refers to a small molecule DM-3189, IUPAC name 4-(6-(4-(piperazin-1-yl)phenyl)pyrazolo[1,5-a]pyrimidin-3-yl)quinoline, with a chemical formula of $C_{25}H_{22}N_6$ with the following formula.



LDN193189 is capable of functioning as a SMAD signaling inhibitor. LDN193189 is also highly potent small-molecule inhibitor of ALK2, ALK3, and ALK6, protein tyrosine kinases (PTK), inhibiting signaling of members of the ALK1 and ALK3 families of type I TGFβ receptors, resulting in the inhibition of the transmission of multiple biological signals, including the bone morphogenetic proteins (BMP) BMP2, BMP4, BMP6, BMP7, and Activin cytokine signals and subsequently SMAD phosphorylation of Smad1, Smad5, and Smad8 (Yu et al. (2008) Nat Med 14:1363-1369; Cuny et al. (2008) Bioorg. Med. Chem. Lett. 18: 4388-4392, herein incorporated by reference).

In certain embodiments, the BMP inhibitor comprises LDN193189. In certain embodiments, the BMP inhibitor comprises Noggin.

In certain embodiments, the stem cells are exposed to one SMAD inhibitor, e.g., one TGFβ/Activin-Nodal inhibitor. In certain embodiments, the TGFβ/Activin-Nodal inhibitor is SB431542. In certain embodiments, the TGFβ/Activin-Nodal inhibitor is a derivative of SB431542. In certain embodiments, the TGFβ/Activin-Nodal inhibitor is A83-01.

In certain embodiments, the stem cells are exposed to two SMAD inhibitors. In certain embodiments, the two SMAD inhibitors are a TGFβ/Activin-Nodal inhibitor and a BMP inhibitor. In certain embodiments, the stem cells are exposed to SB431542 or A83-01, and LDN193189 or Noggin. In certain embodiments, the stem cells are exposed to SB431542 and LDN193189. In certain embodiments, the stem cells are exposed to A83-01 and LDN193189. In certain embodiments, the stem cells are exposed to SB431542 and Noggin. In certain embodiments, the stem cells are exposed to A83-01 and Noggin.

In certain embodiments, the stem cells are contacted with or exposed to the at least one SMAD inhibitor for about 5 days, for 6 days or for 7 days. In certain

embodiments, the cells are contacted with or exposed to the at least one SMAD inhibitor from day 0 through day 6.

In certain embodiments, the cells are contacted with or exposed to a TGFβ/Activin-Nodal inhibitor. In certain embodiments, the concentration of the TGFβ/Activin-Nodal inhibitor contacted with or exposed to the cells is about 5 μM, or about 10 μM. In certain embodiments, the TGFβ/Activin-Nodal inhibitor comprises SB431542 or a derivative thereof (e.g., A83-01). In certain embodiments, the TGFβ/Activin-Nodal inhibitor comprises SB431542.

In certain embodiments, the cells are contacted with or exposed to a BMP inhibitor. In certain embodiments, the concentration of the BMP inhibitor contacted with or exposed to the cells is about 250 nM. In certain embodiments, the BMP inhibitor comprises LDN193189 or a derivative thereof. In certain embodiments, the BMP inhibitor comprises LDN193189.

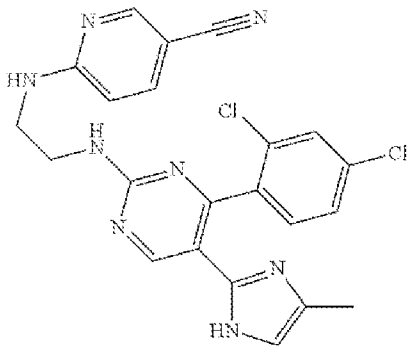
In certain embodiments, the cells are contacted with or exposed to the TGFβ/Activin-Nodal inhibitor and the BMP inhibitor simultaneously. In certain embodiments, the stem cells are contacted with or exposed to the TGFβ/Activin-Nodal inhibitor and the BMP inhibitor for about 5 days, for 6 days, or for 7 days. In certain embodiments, the cells are contacted with or exposed to the TGFβ/Activin-Nodal inhibitor and the BMP inhibitor from day 0 through day 6.

5.2.4.3. Wnt Activators

In certain embodiments, the at least one Wnt activator lowers GSK3β for activation of Wnt signaling. Thus, in certain embodiments, the Wnt activator is a GSK3β inhibitor. A GSK3β inhibitor is capable of activating a WNT signaling pathway, *see e.g.,* Cadigan, *et al., J Cell Sci.* 2006;119:395-402; Kikuchi, *et al., Cell Signaling.* 2007;19:659-671, which are incorporated by reference herein in their entireties. As used herein, the term "glycogen synthase kinase 3β inhibitor" or "GSK3β inhibitor" refers to a compound that inhibits a glycogen synthase kinase 3β enzyme, for example, *see* Doble, *et al., J Cell Sci.* 2003;116:1175-1186, which is incorporated by reference herein in its entirety. Non-limiting examples of GSK3β inhibitors include CHIR99021, BIO ((3E)-6-bromo-3-[3-(hydroxyamino)indol-2-ylidene]-1H-indol-2-one), AMBMP hydrochloride, LP 922056, SB-216763, CHIR98014, Lithium, 3F8, deoxycholic acid, and those disclosed in WO2011/149762, WO13/067362, Chambers *et al., Nat Biotechnol.* 2012 Jul 1;30(7):715-20, Kriks *et al., Nature*. 2011 Nov 6;480(7378):547-51, and Calder *et al., J*

Neurosci. 2015 Aug 19;35(33):11462-81, all of which are incorporated by reference in their entireties.

Non-limiting examples of Wnt activators include CHIR99021, Wnt3A, Wnt1, Wnt5a, BIO ((3E)-6-bromo-3-[3-(hydroxyamino)indol-2-ylidene]-1H-indol-2-one), AMBMP hydrochloride, LP 922056, SB-216763, CHIR98014, Lithium, 3F8, deoxycholic acid, and those disclosed in WO2011/149762, WO13/067362, Chambers *et al., Nat Biotechnol.* 2012 Jul 1;30(7):715-20, Kriks *et al., Nature*. 2011 Nov 6;480(7378):547-51, and Calder *et al., J Neurosci.* 2015 Aug 19;35(33):11462-81, all of which are incorporated by reference in their entireties. In certain embodiments, the at least one Wnt activator is a small molecule selected from CHIR99021, Wnt3A, Wnt1, Wnt5a, BIO, CHIR98014, Lithium, 3F8, deoxycholic acid, derivatives thereof, and mixtures thereof. In certain embodiments, the at least one Wnt activator comprises CHIR99021 or a derivative thereof. In certain embodiments, the at least one Wnt activator comprises CHIR99021. "CHIR99021" (also known as "aminopyrimidine" or "3-[3-(2-Carboxyethyl)-4-methylpyrrol-2-methylidenyl]-2-indolinone") refers to IUPAC name 6-(2-(4-(2,4-dichlorophenyl)-5-(4-methyl-1H-imidazol-2-yl)pyrimidin-2-ylamino) ethylamino)nicotinonitrile with the following formula.



CHIR99021 is highly selective, showing nearly thousand-fold selectivity against a panel of related and unrelated kinases, with an IC50=6.7 nM against human GSK3β and nanomolar IC50 values against rodent GSK3β homologs.

In certain embodiments, the cells are contacted with the at least one Wnt activator for about 15 days, for 16 days, or for 17 days. In certain embodiments, the cells are contacted with the at least one Wnt activator from day 0 through day 16.

In certain embodiments, the concentration of the at least Wnt activator is increased during its exposure to the cells (also referred to as "Wnt Boost"). In certain embodiments, the increase or Wnt Boost is initiated at least about 2 days, at least about 4 days, or at least about 5 days from the initial exposure of the cells to the at least one Wnt

activator. In certain embodiments, the increase or Wnt Boost is initiated about 4 days from the initial exposure of the cells to the at least one Wnt activator. In certain embodiments, the concentration of the at least one activator of Wnt signaling that is contacted with the cells is increased between about 2 days and about 6 days from the initial contact of the cells with the at least one activator of Wnt signaling.

In certain embodiments, the cells are contacted with or exposed to the increased concentration of the at least one Wnt activator for at least about 5 days, or at least about 10 days. In certain embodiments, the cells are contacted with the increased concentration of the at least one Wnt activator for up to about 10 days.

In certain embodiments, the cells are contacted with or exposed to the increased concentration of the at least one Wnt activator for about 5 days, for 5 days or for 6 days. In certain embodiments, the cells are contacted with or exposed to the increased concentration of the at least one Wnt activator from day 4 through day 9. In certain embodiments, the cells are contacted with or exposed to the increased concentration of the at least one Wnt activator for about 10 days, for 12 days, or for 13 days. In certain embodiments, the cells are contacted with or exposed to the increased concentration of the at least one Wnt activator from day 4 through day 16.

In certain embodiments, the initial concentration of the at least one Wnt activator contacted with or exposed to the cells prior to the Wnt boost is about 1 μM or about 0.5 μM. In certain embodiments, the initial concentration of the at least one Wnt activator contacted with or exposed to the cells prior to the Wnt boost is about 0.7 μM.

In certain embodiments, the increased concentration of the at least one Wnt activator post the Wnt Boost is about 3 μM or about 6 μM. In certain embodiments, the increased concentration of the at least one Wnt activator post the Wnt boost is about 7 μM or about 7.5 μM.

In certain embodiments, the concentration of the at least one Wnt activator is increased from the initial concentration contacted with or exposed to the cells by about 200%, about 250%, about 300%, about 350%, about 400%, about 450%, about 500%, about 550%, about 600%, about 650%, about 700%, about 750%, about 800%, about 850%, about 900%, about 950%, about 1000%, about 1050%, or about 1100%. In certain embodiments, the concentration of the at least one activator of Wnt signaling that is contacted with the cells is increased by between about 250% and about 1800% of the initial concentration of the at least one activator of Wnt signaling contacted with the cells.

In certain embodiments, the concentration of the at least one Wnt activator is increased from about 1 μM to about 6 μM. In certain embodiments, the concentration of the at least one Wnt activator is increased from about 1 μM to between about 3 μM and about 5 μM. In certain embodiments, the concentration of the at least one Wnt activator is increased from about 1 μM to about 3 μM.

In certain embodiments, the at least one Wnt activator comprises a GSK3β inhibitor. In certain embodiments, the at least one Wnt activator comprises CHIR99021 or a derivative thereof. In certain embodiments, the at least one Wnt activator comprises CHIR99021.

5.2.4.4. SHH Activators

As used herein, the term "Sonic hedgehog," "SHH," or "Shh" refers to a protein that is one of at least three proteins in the mammalian signaling pathway family called hedgehog, another is desert hedgehog (DHH) wile a third is Indian hedgehog (IHH). SHH interacts with at least two transmembrane proteins by interacting with transmembrane molecules Patched (PTC) and Smoothened (SMO). SHH typically binds to PTC, which then allows the activation of SMO as a signal transducer. In the absence of SHH, PTC typically inhibits SMO, which in turn activates a transcriptional repressor so transcription of certain genes does not occur. When SHH is present and binds to PTC, PTC cannot interfere with the functioning of SMO. With SMO uninhibited, certain proteins are able to enter the nucleus and act as transcription factors allowing certain genes to be activated (*see* Gilbert, 2000 Developmental Biology (Sunderland, Mass., Sinauer Associates, Inc., Publishers). In certain embodiments, an SHH activator refers to any molecule or compound that is capable of activating a SHH signaling pathway, including a molecule or compound that is capable of binding to PTC or a SMO. In certain embodiments, the at least one SHH activator is selected from the group consisting of molecules that bind to PCT, molecules that bind to SMO, and combinations thereof. Non-limiting examples of SHH activators include those disclosed in WO10/096496, WO13/067362, Chambers *et al., Nat Biotechnol.* 2009 Mar;27(3):275-80, and Kriks *et al., Nature.* 2011 Nov 6;480(7378):547-51. In certain embodiments, the at least one SHH activator is selected from the group consisting of a SHH protein, a SMO agonist, or a combination thereof. In certain embodiments, the SHH protein is selected from the group consisting of a recombinant SHH, a modified N-terminal SHH, or a combination thereof. In certain embodiments, the recombinant SHH comprises a N-terminal fragment

and a C-terminal fragment. In certain embodiments, the modified N-terminal SHH comprises two Isoleucines at the N-terminus. In certain embodiments, the modified N-terminal SHH has at least about 80%, about 85%, about 90%, about 95%, or about 99% sequence identity to an un-modified N-terminal SHH. In certain embodiments, the modified N-terminal SHH has at least about 80%, about 85%, about 90%, about 95%, or about 99% sequence identity to an un-modified human N-terminal SHH. In certain embodiments, the modified N-terminal SHH has at least about 80%, about 85%, about 90%, about 95%, or about 99% sequence identity to an un-modified mouse N-terminal SHH. In certain embodiments, the modified N-terminal SHH comprises SHH C25II. In certain embodiments, the modified N-terminal SHH comprises SHH C24II.

Non-limiting examples of SMO agonists (SAGs) include purmorphamine, GSA10, and 20(S)- hydroxy Cholesterol. In certain embodiments, the SAG comprises purmorphamine.

In certain embodiments, the cells are contacted with or exposed to the at least one SHH activator for about 5 days, for 6 days, or for 7 days. In certain embodiments, the cells are contacted with or exposed to the at least one SHH activator from day 0 through day 6.

In certain embodiments, the concentration of the at least one SHH activator contacted with or exposed to the cells is about 400 ng/mL, about 450 ng/mL, about 500 ng/mL, about 550 ng/mL, or about 600 ng/mL.

In certain embodiments, the at least one activator of SHH signaling comprises SHH C25II.

5.2.4.5. FGF Activators

FGF family includes secreted signaling proteins (secreted FGFs) that signal to receptor tyrosine kinases. Phylogenetic analysis suggests that 22 *Fgf* genes can be arranged into seven subfamilies containing two to four members each. Branch lengths are proportional to the evolutionary distance between each gene.

In certain embodiments, the at least one FGF activator is selected from the group consisting of FGF8a, FGF17, FGF18, FGF8b, FGF2, FGF4, and derivatives thereof. In certain embodiments, the at least one FGF activator is selected from the group consisting of FGF8a, FGF17, FGF18, FGF2, FGF4, and derivatives thereof. In certain embodiments, the at least one FGF activator is selected from the group consisting of FGF8a, FGF17, and FGF18.

The FGF8 subfamily is comprised of FGF8a, FGF8b, FGF17, and FGF18. Early patterning of the vertebrate midbrain and cerebellum is regulated by a mid/hindbrain organizer that produces FGF8a, FGF8b, FGF17 and FGF18. It has been shown that FGF8b functions differently from FGF8a, FGF17, and FGF18 (Liu *et al., Development.* 2003 Dec;130(25):6175-85). FGF8b is the only protein that can induce the r1 gene Gbx2 and strongly activate the pathway inhibitors Spry1/2, as well as repress the midbrain gene Otx2 (Liu 2003). Moreover, FGF8b extends the organizer along the junction between the induced Gbx2 domain and the remaining Otx2 region in the midbrain, correlating with cerebellum development (Liu 2003). By contrast, FGF8a, FGF17, and FGF18 cause expansion of the midbrain and upregulating midbrain gene expression (Liu 2003).

In certain embodiments, the at least one FGF activator is capable of causing expansion of the midbrain and upregulating midbrain gene expression. In certain embodiments, the at least one FGF activator is capable of promoting midbrain development. In certain embodiments, the at least one FGF activator is selected from the group consisting of FGF8a, FGF17, FGF18, FGF2, FGF4, derivatives thereof, and combinations thereof. In certain embodiments, the at least one FGF activator is selected from the group consisting of FGF8a, FGF17, FGF18, and combinations thereof. In certain embodiments, the at least one FGF activator comprises or is FGF18.

In certain embodiments, the cells are contacted with or exposed to the at least one FGF activator for about 3 days, about 5 days, or about 8 days. In certain embodiments, the cells are contacted with or exposed to the at least one FGF activator for about 4 days. In certain embodiments, the cells are contacted with or exposed to the at least one FGF activator for 5 days.

In certain embodiments, the contact of the cells with or the exposure of the cells to the at least one FGF activator is initiated about 10 days from the initial contact of the cells with or the initial exposure of the cells to the at least one SMAD inhibitor, and the cells are contacted with the at least FGF activator for about 5 days. In certain embodiments, the contact of the cells with or the exposure of the cells to the at least one FGF activator is initiated 12 days or 13 days from the initial contact of the cells with or the initial exposure of the cells to the at least one SMAD inhibitor, and the cells are contacted with the at least one FGF activator for 4 days or 5 days. In certain embodiments, the cells are contacted with or exposed to the at least one FGF activator from day 12 through day 16.

In certain embodiments, the concentration of the at least one FGF activator contacted with or exposed to the cells is about 100 ng/mL or about 200 ng/mL. In certain embodiments, concentration of the at least one FGF activator contacted with or exposed to the cells is about 200 ng/mL.

In certain embodiments, the at least one FGF activator comprises FGF18.

5.2.4.6. Wnt Inhibitors

Wnt signaling includes canonical Wnt signaling and non-canonical Wnt signaling. In certain embodiments, the at least one Wnt inhibitor is capable of inhibiting canonical Wnt signaling. In certain embodiments, the at least one Wnt inhibitor is capable of inhibiting both canonical Wnt signaling and non-canonical Wnt signaling. Non-limiting examples of Wnt inhibitors that are capable of inhibiting both canonical Wnt signaling and non-canonical Wnt signaling include IWP2, IWR1-endo, IWP-O1, Wnt-C59, IWP-L6, IWP12, LGK-974, IWR-1, ETC-159, iCRT3, IWP-4, salinomycin, Pyrvinium Pamoate, iCRT14, FH535, CCT251545, Wogonin, NCB-0846, hexachrorophene, KY02111, SO3031 (KY01-I), SO2031 (KY02-I), BC2059, PKF115-584, Quercetin, NSC668036, G007-LK, and derivatives thereof. In certain embodiments, the at least one Wnt inhibitor is selected from the group consisting of IWP2, IWR1-endo, XAV939, IWP-O1, Wnt-C59, IWP-L6, LGK-974, IWR-1, Wnt-C59, ETC-159, iCRT3, IWP-4, ICG-001, Salinomycin, Pyrvinium Pamoate, iCRT14, FH535, CCT251545, KYA1797K, Wogonin, NCB-0846, Hexachrorophene, PNU-74654, KY02111, SO3031 (KY01-I), SO2031 (KY02-I), triptonide, IWP12, BC2059, PKF115-584, Quercetin, NSC668036, G007-LK, MSAB, LF3, JW55, isoquercitrin, WIKI4 (Wnt Inhibitor Kinase Inhibitor 4), derivatives thereof, and combinations thereof. In certain embodiments, the at least one inhibitor of Wnt signaling is selected from the group consisting of IWP2, IWR1-endo, IWP-O1, IWP12, Wnt-C59, IWP-L6, LGK-974, IWR-1, ETC-159, iCRT3, IWP-4, Salinomycin, Pyrvinium Pamoate, iCRT14, FH535, CCT251545, Wogonin, NCB-0846, Hexachrorophene, KY02111, SO3031 (KY01-I), SO2031 (KY02-I), BC2059, PKF115-584, Quercetin, NSC668036, G007-LK, derivatives thereof, and combinations thereof. In certain embodiments, the at least one inhibitor of Wnt signaling is selected from the group consisting of XAV939, ICG-001, PNU-74654, Triptonide, KYA1797K, MSAB, LF3, JW55, Isoquercitrin, WIKI4, derivatives thereof, and combinations thereof. In certain embodiments, the at least one Wnt inhibitor comprises IWP2 or a derivative thereof.

In certain embodiments, the cells are contacted with or exposed to the at least one Wnt inhibitor for about 15 days or about 20 days. In certain embodiments, the cells are contacted with or exposed to the at least one Wnt inhibitor for 4 days, 5 days, 6 days, 7 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, or 23 days.

In certain embodiments, the cells that are contacted with the at least one Wnt inhibitor comprise mDA neuron precursors and mDA neurons.

In certain embodiments, the contact of the cells with or the exposure of the cells to the at least one Wnt inhibitor is initiated about 10 days from the initial contact of the cells with or the initial exposure of the cells to the at least one SMAD inhibitor, and the cells are contacted with the at least Wnt inhibitor for about 5 days. In certain embodiments, the contact of the cells with or the exposure of the cells to the at least one Wnt inhibitor is initiated 12 days or 13 days from the initial contact of the cells with or the initial exposure of the cells to the at least one SMAD inhibitor, and the cells are contacted with the at least one Wnt inhibitor for 4 days or 5 days. In certain embodiments, the cells are contacted with or exposed to the at least one Wnt inhibitor from day 12 through day 16. In certain embodiments, the cells are contacted with or exposed to the at least one Wnt inhibitor from day 12 through day 25. In certain embodiments, the at least one Wnt inhibitor is added every day or every other day to a cell culture medium comprising the cells from day 12 through day 25. In certain embodiments, the at least one Wnt inhibitor is added every day or every other day to a cell culture medium comprising the cells from day 12 through day 30.

In certain embodiments, the concentration of the at least one Wnt inhibitor contacted with or exposed to the cells is about 1 μM.

In certain embodiments, the at least one Wnt inhibitor comprises IWP2.

5.2.4.7. DA neuron lineage specific activators and inhibitors

In certain embodiments, the cells are further contacted with DA neuron lineage specific activators and inhibitors to obtain the mDA neurons (e.g., post-mitotic mDA neurons). Non-limiting examples of DA neuron lineage specific activators and inhibitors include L-glutamine, brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), Cyclic adenosine monophosphate (cAMP), Transforming growth factor beta (TGFβ, for example, TGFβ3), ascorbic acid (AA), and DAPT (which is also known as, N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-l,l-

dimethylethyl ester; LY-374973, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; or N-[N- (3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester).

In certain embodiments, the cells are contacted with the foregoing DA neuron lineage specific activators and inhibitors for about 4 days, about 5 days, about 6 days, about 7 days, or about 8 days.

In certain embodiments, the cells are contacted with L-glutamine at a concentration of about 2 mM. In certain embodiments, the cells are contacted with BDNF at a concentration of about 20 ng/mL. In certain embodiments, the cells are contacted with AA at a concentration of about 200 nM. In certain embodiments, the cells are contacted with GDNF at a concentration of about 20 ng/mL. In certain embodiments, the cells are contacted with cAMP at a concentration of about 500 nM. In certain embodiments, the cells are contacted with TGFβ3 at a concentration of about 1 ng/mL. In certain embodiments, the cells are contacted with DAPT at a concentration of about 10 nM.

5.3. Compositions

The present disclosure provides compositions for treating neurodegeneration of midbrain dopamine neurons and/or for treating neurodegenerative disorders. In certain embodiments, the composition comprises (i) one or more mDA neurons (e.g., mDA neurons disclosed in Section 5.2.4.) and (ii) at least one compound selected from the group consisting of TNFα inhibitors (e.g., the TNFα inhibitors disclosed in Section 5.2.1), NFκB inhibitors (e.g., the NFκB inhibitors disclosed in Section 5.2.2), p53 inhibitors (e.g., the p53 inhibitors disclosed in Section 5.2.3), and combinations thereof.

In certain embodiments, the one or more mDA neurons and the at least one compound are comprised in a composition that further comprises a biocompatible scaffold or matrix, for example, a biocompatible three-dimensional scaffold that facilitates tissue regeneration when the one or more mDA neurons are implanted or grafted to a subject. In certain embodiments, the biocompatible scaffold comprises extracellular matrix material, synthetic polymers, cytokines, collagen, polypeptides or proteins, polysaccharides including fibronectin, laminin, keratin, fibrin, fibrinogen, hyaluronic acid, heparin sulfate, chondroitin sulfate, agarose or gelatin, and/or hydrogel. (*See, e.g.,* U.S. Publication Nos. 2015/0159135, 2011/0296542, 2009/0123433, and 2008/0268019, the contents of each of which are incorporated by reference in their

entireties). In certain embodiments, the composition further comprises growth factors for promoting maturation of the implanted/grafted cells.

In certain embodiments, the composition comprises from about 1 x 10^4 to about 1 x 10^{10}, from about 1 x 10^4 to about 1 x 10^5, from about 1 x 10^5 to about 1 x 10^9, from about 1 x 10^5 to about 1 x 10^6, from about 1 x 10^5 to about 1 x 10^7, from about 1 x 10^6 to about 1 x 10^7, from about 1 x 10^6 to about 1 x 10^8, from about 1 x 10^7 to about 1 x 10^8, from about 1 x 10^8 to about 1 x 10^9, from about 1 x 10^8 to about 1 x 10^{10}, or from about 1 x 10^9 to about 1 x 10^{10} the mDA neurons. In certain embodiments, the composition comprises from about 1 x 10^5 to about 1 x 10^7 the mDA neurons.

In certain embodiments, the composition is frozen. In certain embodiments, the composition further comprises at least one cryoprotectant, for example, but not limited to, dimethylsulfoxide (DMSO), glycerol, polyethylene glycol, sucrose, trehalose, dextrose, or a combination thereof.

In certain embodiments, the composition is a pharmaceutical composition that comprises a pharmaceutically acceptable carrier. In certain embodiments, the compositions can be used for preventing and/or treating a neurodegenerative disorder include Parkinson's disease, Huntington's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), and frontotemporal dementia. In certain embodiments, the compositions can be used for ameliorating neurodegeneration of mDA neurons.

The present disclosure also provides devices comprising the presently disclosed compositions. The devices can be used for administering the presently disclosed compositions. Any suitable administration devices can be used for the present disclosure. Non-limiting examples of devices include syringes, fine glass tubes, stereotactic needles and cannulas.

5.4. Methods of Treatment

The present disclosure provides methods of treating a subject. In certain embodiments, the subject suffers from neurodegeneration of midbrain dopamine neurons. The present disclosure also provides methods for ameliorating neurodegeneration of mDA neurons. In certain embodiments, the subject has a neurodegenerative disorder. Non-limiting examples of neurodegenerative disorders include Parkinson's disease, Huntington's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), and frontotemporal dementia. The present disclosure further provides methods of preventing and/or treating at least a symptom in a

subject having a neurological disorder, and/or suffering from neurodegeneration of midbrain dopamine neurons.

In certain embodiments, the methods comprise administering to the subject one or more mDA neurons, wherein p53-mediated apoptosis of the one or more mDA neurons is suppressed (e.g., those mDA neurons disclosed in Section 5.2.4.). In certain embodiments, the suppression of p53-mediated apoptosis comprises inhibition of tumor necrosis factor alpha (TNFα) signaling (e.g., inhibition of TNFα signaling disclosed in Section 5.2.1), inhibition of nuclear factor kappa B (NFκB) signaling (e.g., inhibition of NFκB signaling disclosed in Section 5.2.2), inhibition of p53 signaling (e.g., inhibition of p53 signaling disclosed in Section 5.2.3), or a combination of the foregoing. In certain embodiments, the suppression of p53-mediated apoptosis comprises administering to the subject at least one compound selected from the group consisting of TNFα inhibitors, NFκB inhibitors, p53 inhibitors, and combinations thereof. In certain embodiments, the method comprises administering the one or more mDA neurons simultaneously with the administration of the compound. In certain embodiments, the one or more mDA neurons are contacted with the at least one compound prior to the administration of the one or more mDA neurons to the subject. In certain embodiments, the method comprises administering the at least one compound shortly after administration of the one or more mDA neurons. In certain embodiments, the method comprises administering the at least one compound before the administration of the one or more mDA neurons.

In certain embodiments, the methods comprise administering to the subject a composition disclosed in Section 5.3.

In certain embodiments, the neurodegenerative disorder is Parkinson's disease. Primary motor signs of Parkinson's disease include, for example, but not limited to, tremor of the hands, arms, legs, jaw and face, bradykinesia or slowness of movement, rigidity or stiffness of the limbs and trunk and postural instability or impaired balance and coordination.

In certain embodiments, the neurodegenerative disorder is a parkinsonism disease, which refers to diseases that are linked to an insufficiency of dopamine in the basal ganglia, which is a part of the brain that controls movement. Symptoms include tremor, bradykinesia (extreme slowness of movement), flexed posture, postural instability, and rigidity. Non-limiting examples of parkinsonism diseases include

corticobasal degeneration, Lewy body dementia, multiple systematrophy, and progressive supranuclear palsy.

In certain embodiments, the one or more mDA neurons and the means to suppress p53-mediated apoptosis (e.g., a TNFα inhibitor, a NFκB inhibitor, a p53 inhibitor, or a combination of the foregoing) can be administered or provided systemically or directly to a subject suffering from a neurodegenerative disorder and/or neurodegeneration of midbrain dopamine neurons. In certain embodiments, the one or more mDA neurons and the means to suppress p53-mediated apoptosis (e.g., a TNFα inhibitor, a NFκB inhibitor, a p53 inhibitor, or a combination of the foregoing) are directly injected into an organ of interest (e.g., the central nervous system (CNS) or peripheral nervous system (PNS)). In certain embodiments, the one or more mDA neurons and the means to suppress p53-mediated apoptosis (e.g., a TNFα inhibitor, a NFκB inhibitor, a p53 inhibitor, or a combination of the foregoing) are directly injected into the striatum.

In certain embodiments, the one or more mDA neurons and the means to suppress p53-mediated apoptosis (e.g., a TNFα inhibitor, a NFκB inhibitor, a p53 inhibitor, or a combination of the foregoing) can be administered in any physiologically acceptable vehicle. In certain embodiments, the one or more mDA neurons and the means to suppress p53-mediated apoptosis (e.g., a TNFα inhibitor, a NFκB inhibitor, a p53 inhibitor, or a combination of the foregoing) can be administered via localized injection, orthotopic (OT) injection, systemic injection, intravenous injection, or parenteral administration. In certain embodiments, the one or more mDA neurons and the means to suppress p53-mediated apoptosis (e.g., a TNFα inhibitor, a NFκB inhibitor, a p53 inhibitor, or a combination of the foregoing) are administered to a subject suffering from a neurodegenerative disorder and/or neurodegeneration of midbrain dopamine neurons via orthotopic (OT) injection.

In certain embodiments, the one or more mDA neurons and the means to suppress p53-mediated apoptosis (e.g., a TNFα inhibitor, a NFκB inhibitor, a p53 inhibitor, or a combination of the foregoing) can be conveniently provided as sterile liquid preparations, e.g., isotonic aqueous solutions, suspensions, emulsions, dispersions, or viscous compositions, which may be buffered to a selected pH. Liquid preparations are normally easier to prepare than gels, other viscous compositions, and solid compositions. Additionally, liquid compositions are somewhat more convenient to

administer, especially by injection. Viscous compositions, on the other hand, can be formulated within the appropriate viscosity range to provide longer contact periods with specific tissues. Liquid or viscous compositions can comprise carriers, which can be a solvent or dispersing medium containing, for example, water, saline, phosphate buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like) and suitable mixtures thereof. Sterile injectable solutions can be prepared by incorporating the compositions of the presently disclosed subject matter (*e.g.*, a composition of Section 5.4), in the required amount of the appropriate solvent with various amounts of the other ingredients, as desired. Such compositions may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, dextrose, or the like. The compositions can also be lyophilized. The compositions can contain auxiliary substances such as wetting, dispersing, or emulsifying agents (*e.g.*, methylcellulose), pH buffering agents, gelling or viscosity enhancing additives, preservatives, flavoring agents, colors, and the like, depending upon the route of administration and the preparation desired. Standard texts, such as "REMINGTON'S PHARMACEUTICAL SCIENCE", 17th edition, 1985, incorporated herein by reference, may be consulted to prepare suitable preparations, without undue experimentation.

Various additives which enhance the stability and sterility of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, alum inurn monostearate and gelatin.

Viscosity of the compositions, if desired, can be maintained at the selected level using a pharmaceutically acceptable thickening agent. Methylcellulose can be used because it is readily and economically available and is easy to work with. Other suitable thickening agents include, for example, xanthan gum, carboxymethyl cellulose, hydroxypropyl cellulose, carbomer, and the like. The concentration of the thickener can depend upon the agent selected. The important point is to use an amount that will achieve the selected viscosity. The choice of suitable carriers and other additives will depend on the exact route of administration and the nature of the particular dosage form, *e.g.*, liquid dosage form (*e.g.*, whether the composition is to be formulated into a

solution, a suspension, gel or another liquid form, such as a time release form or liquid-filled form).

Those skilled in the art will recognize that the components of the compositions should be selected to be chemically inert and will not affect the viability or efficacy of the presently disclosed stem-cell-derived precursors. This will present no problem to those skilled in chemical and pharmaceutical principles, or problems can be readily avoided by reference to standard texts or by simple experiments (not involving undue experimentation), from this disclosure and the documents cited herein.

One consideration concerning the therapeutic use of the cells and the means to suppress p53-mediated apoptosis (e.g., a TNFα inhibitor, a NFκB inhibitor, a p53 inhibitor, or a combination of the foregoing) is the quantity of cells and inhibitor(s) necessary to achieve an optimal effect. An optimal effect includes, but is not limited to, repopulation of CNS and/or PNS regions of a subject suffering from a neurodegenerative disorder and/or neurodegeneration of midbrain dopamine neurons, improved function of midbrain dopamine neurons of the subject, the subject's CNS and/or PNS, and improving the *in vivo* survival of the cells.

An "effective amount" (or "therapeutically effective amount") is an amount sufficient to affect a beneficial or desired clinical result upon treatment. An effective amount can be administered to a subject in at least one doses. In terms of treatment, an effective amount is an amount that is sufficient to palliate, ameliorate, stabilize, reverse or slow the progression of the neurodegenerative disorder or pituitary disorder, or otherwise reduce the pathological consequences of the neurodegenerative disorder. The effective amount is generally determined by the physician on a case-by-case basis and is within the skill of one in the art. Several factors are typically taken into account when determining an appropriate dosage to achieve an effective amount. These factors include age, sex and weight of the subject, the condition being treated, the severity of the condition and the form and effective concentration of the cells administered.

In certain embodiments, an effective amount of the cells is an amount that is sufficient to ameliorate neurodegeneration of midbrain dopamine neurons. In certain embodiments, an effective amount of the cells is an amount that is sufficient to improve the function of midbrain dopamine neurons of a subject suffering from a neurodegenerative disorder and/or neurodegeneration of midbrain dopamine neurons, e.g., the improved function can be about 1%, about 5%, about 10%, about 20%, about

30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98%, about 99% or about 100% of the function of midbrain dopamine neurons of a normal person.

In certain embodiments, an effective amount of the means to suppress p53-mediated apoptosis (e.g., a TNFα inhibitor, a NFκB inhibitor, a p53 inhibitor, or a combination of the foregoing) is an amount that is sufficient to improve the *in vivo* survival of transplanted mDA neurons.

The quantity of cells to be administered will vary for the subject being treated. In certain embodiments, from about 1×10^4 to about 1×10^{10}, from about 1×10^4 to about 1×10^5, from about 1×10^5 to about 1×10^9, from about 1×10^5 to about 1×10^6, from about 1×10^5 to about 1×10^7, from about 1×10^6 to about 1×10^7, from about 1×10^6 to about 1×10^8, from about 1×10^7 to about 1×10^8, from about 1×10^8 to about 1×10^9, from about 1×10^8 to about 1×10^{10}, or from about 1×10^9 to about 1×10^{10} of the cells are administered to a subject. In certain embodiments, from about 1×10^5 to about 1×10^7 of the cells are administered to a subject suffering from a neurodegenerative disorder and/or neurodegeneration of midbrain dopamine neurons. In certain embodiments, from about 1×10^6 to about 1×10^7 of the cells are administered to a subject suffering from a neurodegenerative disorder and/or neurodegeneration of midbrain dopamine neurons. In certain embodiments, from about 1×10^6 to about 4×10^6 of the cells are administered to a subject suffering from a neurodegenerative disorder and/or neurodegeneration of midbrain dopamine neurons.

The precise determination of what would be considered an effective dose may be based on factors individual to each subject, including their size, age, sex, weight, and condition of the particular subject. Dosages can be readily ascertained by those skilled in the art from this disclosure and the knowledge in the art.

EXEMPLARY EMBODIMENTS

A. In certain non-limiting embodiments, the presently disclosed subject matter provides for a method for treating a subject, comprising administering to the subject one or more midbrain dopamine (mDA) neurons, wherein p53-mediated apoptosis of the one or more mDA neurons is suppressed.

A1. The foregoing method of A, wherein the subject suffers from a neurodegenerative disorder and/or neurodegeneration of midbrain dopamine neurons.

A2. The foregoing method of A-A1, wherein the neurodegenerative disorder is selected from the group consisting of Parkinson's disease, Huntington's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), frontotemporal dementia, and combinations thereof.

A3. The foregoing method of A-A2, wherein the suppression of p53-mediated apoptosis comprises a) administering to the subject at least one compound selected from the group consisting of tumor necrosis factor alpha (TNFα) inhibitors, nuclear factor kappa B (NFκB) inhibitors, p53 inhibitors, and combinations thereof; or b) contacting the one or more mDA neurons with at least one compound selected from the group consisting of TNFα inhibitors, NFκB inhibitors, p53 inhibitors, and combinations thereof.

A4. The foregoing method of A-A3, wherein the suppression of p53-mediated apoptosis comprises administering to the subject a TNFα inhibitor.

A5. The foregoing method of A-A4, comprising administering the one or more mDA neurons simultaneously with the administration of the at least one compound.

B. In certain non-limiting embodiments, the presently disclose subject matter provides for a method of improving *in vivo* survival of one or more midbrain dopamine (mDA) neurons, comprising suppressing p53-mediated apoptosis of the one or more mDA neurons.

B1. The foregoing method of B, wherein the suppression of p53-mediated apoptosis comprises contacting the one or more mDA neurons with a compound selected from the group consisting of TNFα inhibitors, NFκB inhibitors, p53 inhibitors, and combinations thereof.

B2. The foregoing method of B-B1, wherein the suppression of p53-mediated apoptosis comprises contacting the one or more mDA neurons with a TNFα inhibitor.

C. The foregoing method of A-A5 or B-B2, wherein the suppression of p53-mediated apoptosis comprises inhibition of TNFα signaling, inhibition of NFκB signaling, inhibition of p53 signaling, or a combination of the foregoing.

C1. The foregoing method of any one of claims A3, A4, B1, and B2, wherein the TNFα inhibitor is selected from the group consisting of anti-TNFα antibodies, TNFα decoy receptors, chemical compounds, nucleic acid inhibitors, small molecule inhibitors, receptor biologic inhibitors, inactive TNF fragments, TNFα circulating receptor fusion protein, xanthine derivatives, 5-HT_{2A} agonist, and combinations thereof.

C2. The foregoing method of C1, wherein the TNFα inhibitor is an anti-TNFα antibody.

C3. The foregoing method of C2, wherein the anti-TNFα antibody is selected from the group consisting of adalimumab, adalimumab-adbm, adalimumab-adaz, adalimumab-atto, certolizumab pegol, golimumab, infliximab, infliximab-abda, infliximab-dyyb, remtolumab, afelimomab, nerelimomab, ozoralizumab, placulumab, and combinations thereof.

C4. The foregoing method of C3, wherein the anti-TNFα antibody is adalimumab.

C5. The foregoing method of A3 or B1, wherein the NFκB inhibitor is selected from the group consisting of upstream inhibitors of NFκB, inhibitors of IKK activity, inhibitors of IκB phosphorylation, inhibitors of IκB degradation, proteasome inhibitors, protease inhibitors, inhibitors of NFκB nuclear translocation and expression, NFκB DNA-binding inhibitors, and NFκB transactivation inhibitors, inhibitors of NFκB directed gene transactivation, antioxidants, and combinations thereof.

C6. The foregoing method of claim A3 or B1, wherein the p53 inhibitor is selected from the group consisting of JNK inhibitors, p38 MAPK inhibitors, caspase inhibitors, puma/BBC3 inhibitors, BAX inhibitors, CDK inhibitors, MDM2 and MDMX activators, and combinations thereof.

C7. The foregoing method of A-A5, B-B2, or C-C6, wherein the suppression of p53-mediated apoptosis comprises knocking out or knocking down *TP53* gene in the one or more mDA.

C8. The foregoing method of C7, wherein the *TP53* gene is knocked out or knocked down by a gene-engineering system.

C9. The foregoing method of C8, wherein the gene-engineering system is a CRISPR-Cas system.

C10. The foregoing method of A-A5, B-B2, or C-C9, wherein the one or more mDA neurons express a marker selected from the group consisting of EN1, OTX2, TH, NURR1, FOXA2, LMX1A, PITX3, LMO3, SNCA, ADCAP1, CHRNA4, ALDH1A1, SOX6, WNT1, DAT, VMAT2, GIRK2, SATB1, CALB1, CALB2, SNCG, PBX1, and combinations thereof.

C11. The foregoing method of A-A5, B-B2, or C-C10, wherein the one or more mDA neurons are post-mitotic mDA neurons.

C12. The foregoing method of A-A5, B-B2, or C-C11, wherein the one or more mDA neurons are *in vitro* differentiated from one or more stem cells.

C13. The foregoing method of C12, wherein the one or more stem cells are selected from the group consisting of human stem cells, nonhuman primate stem cells, rodent nonembryonic stem cells, human embryonic stem cells, nonhuman primate embryonic stem cells, rodent embryonic stem cells, human induced pluripotent stem cells, nonhuman primate induced pluripotent stem cells, rodent induced pluripotent stem cells, and human recombinant pluripotent cells, nonhuman primate recombinant pluripotent cells, and rodent recombinant pluripotent cells.

C14. The foregoing method of C12 or C13, wherein the one or more stem cells are human stem cells.

C15. The foregoing method of C12-C14, wherein the one or more stem cells are one or more pluripotent stem cells or multipotent stem cell.

C16. The foregoing method of C12-C15, wherein the one or more stem cells are selected from the group consisting of embryonic stem cells, induced pluripotent stem cells, and combinations thereof.

C17. The foregoing method of C16, wherein the one or more stem cells are one or more induced pluripotent stem cells.

C18. The foregoing method of C12-C17, wherein the *in vitro* differentiation comprises contacting the one or more stem cells with at least one inhibitor of Small Mothers Against Decapentaplegic (SMAD) signaling, at least one activator of Sonic hedgehog (SHH) signaling, and at least one activator of wingless (Wnt) signaling.

C19. The foregoing method of C18, wherein the concentration of the at least one activator of Wnt signaling that is contacted with the cells is increased between about 2 days and about 6 days from the initial contact of the cells with the at least one activator of Wnt signaling.

C20. The foregoing method of C18 or C19, wherein the concentration of the at least one activator of Wnt signaling that is contacted with the cells is increased by between about 250% and about 1800% of the initial concentration of the at least one activator of Wnt signaling contacted with the cells.

C21. The foregoing method of C18-C20, wherein the at least one activator of Wnt signaling comprises an inhibitor of glycogen synthase kinase 3β (GSK3β) signaling.

C22. The foregoing method of any one of claims C18-C21, wherein the at least one activator of Wnt signaling is selected from the group consisting of CHIR99021,

CHIR98014, AMBMP hydrochloride, LP 922056, Lithium, deoxycholic acid, BIO, SB-216763, Wnt3A, Wnt1, Wnt5a, derivatives thereof, and combinations thereof.

C23. The foregoing method of C22, wherein the at least one activator of Wnt signaling comprises CHIR99021.

C24. The foregoing method of C18-C23, wherein the at least one inhibitor of SMAD signaling comprises an inhibitor of TGFβ/Activin-Nodal signaling, an inhibitor of bone morphogenetic protein (BMP) signaling, or a combination of the foregoing.

C25. The foregoing method of C24, wherein the at least one inhibitor of TGFβ/Activin-Nodal signaling is selected from the group consisting of SB431542, derivatives of SB431542, and combinations thereof.

C26. The foregoing method of C25, wherein the derivative of SB431542 comprises A83-01.

C27. The foregoing method of C24-C26, wherein the at least one inhibitor of TGFβ/Activin-Nodal signaling comprises SB431542.

C28. The foregoing method of C24, wherein the at least one inhibitor of BMP signaling is selected from the group consisting of LDN193189, Noggin, dorsomorphin, derivatives of LDN193189, derivatives of Noggin, derivatives of dorsomorphin, and combinations thereof.

C29. The foregoing method of C28, wherein the at least one inhibitor of BMP comprises LDN-193189.

C30. The foregoing method of any one of C18-C29, wherein the at least one activator of SHH signaling is selected from the group consisting of SHH proteins, Smoothened agonists (SAG), and combinations thereof.

C31. The foregoing method of C30, wherein the SHH protein is selected from the group consisting of recombinant SHHs, modified N-terminal SHHs, and combinations thereof.

C32. The foregoing method of C31, wherein the modified N-terminal SHH comprises two isoleucines at the N-terminus.

C33. The foregoing method of C31 or C32, wherein the modified N-terminal SHH has at least about 90% sequence identity to an un-modified N-terminal SHH.

C34. The foregoing method of C33, wherein the un-modified N-terminal SHH is an un-modified mouse N-terminal SHH or an un-modified human N-terminal SHH.

C35. The foregoing method of C31-C33, wherein the modified N-terminal SHH comprises SHH C25II.

C36. The foregoing method of C30, wherein the SAG comprises purmorphamine.

C37. The foregoing method of C18-C36, wherein the *in vitro* differentiation further comprises contacting the one or more stem cells with at least one activator of fibroblast growth factor (FGF) signaling.

C38. The foregoing method of C37, wherein the at least one activator of FGF signaling is selected from the group consisting of FGF18, FGF17, FGF8a, FGF8b, FGF4, FGF2, and combination thereof.

C39. The foregoing method of C37 or C38, wherein the at least one activator of FGF signaling comprises FGF18 or FGF8.

C40. The foregoing method of C18-C39, wherein the *in vitro* differentiation further comprises contacting the one or more stem cells with at least one inhibitor of Wnt signaling.

C41. The foregoing method of C40, wherein the at least one inhibitor of Wnt signaling is selected from the group consisting of IWP2, IWR1-endo, XAV939, IWP-O1, Wnt-C59, IWP-L6, and ICG-001, and combinations thereof.

C42. The foregoing method of C40 or C41, wherein the at least one inhibitor of Wnt signaling comprises IWP2.

C42. The foregoing method of any one of claims A-A5, B-B2, or C-C42, wherein the one or more mDA neurons express a detectable level of CD184 and do not express a detectable level of CD49e.

D. In certain non-limiting embodiments, the presently disclose subject matter provides for a composition comprising: (a) one or more midbrain dopamine (mDA) neurons; and (b) at least one compound selected from the group consisting of TNFα inhibitors, NFκB inhibitors, p53 inhibitors, and combinations thereof.

D1. The foregoing composition of D, wherein the composition is a pharmaceutical composition further comprising a pharmaceutically acceptable carrier.

D2. The foregoing composition of D or D1, wherein the composition is for treating or ameliorating a neurodegenerative disorder and/or neurodegeneration of midbrain dopamine neurons.

D3. The foregoing composition of D2, wherein the neurodegenerative disorder is selected from the group consisting of Parkinson's disease, Huntington's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), frontotemporal dementia, and combinations thereof.

D4. The foregoing composition of D-D3, wherein the TNFα inhibitor is selected from the group consisting of anti-TNFα antibodies, TNFα decoy receptors, chemical compounds, nucleic acid inhibitors, small molecule inhibitors, receptor biologic inhibitors, inactive TNF fragments, TNFα circulating receptor fusion protein, xanthine derivatives, 5-HT_{2A} agonist, and combinations thereof.

D5. The foregoing composition of D4, wherein the TNFα inhibitor is an anti-TNFα antibody.

D6. The foregoing composition of D5, wherein the anti-TNFα antibody is selected from the group consisting of adalimumab, adalimumab-adbm, adalimumab-adaz, adalimumab-atto, certolizumab pegol, golimumab, infliximab, infliximab-abda, infliximab-dyyb, remtolumab, afelimomab, nerelimomab, ozoralizumab, placulumab, and combinations thereof.

D7. The foregoing composition of D5, wherein the anti-TNFα antibody is adalimumab.

D8. The foregoing composition of any one of D-D7, wherein the NFκB inhibitor is selected from the group consisting of upstream inhibitors of NFκB, inhibitors of IKK activity, inhibitors of IκB phosphorylation, inhibitors of IκB degradation, proteasome inhibitors, protease inhibitors, inhibitors of NFκB nuclear translocation and expression, NFκB DNA-binding inhibitors, and NFκB transactivation inhibitors, inhibitors of NFκB directed gene transactivation, antioxidants, and combinations thereof.

D9. The foregoing composition of D-D8, wherein the p53 inhibitor is selected from the group consisting of JNK inhibitors, p38 MAPK inhibitors, caspase inhibitors, puma/BBC3 inhibitors, BAX inhibitors, CDK inhibitors, MDM2 and MDMX activators, and combinations thereof.

D10. The foregoing composition of D-D9, wherein the one or more mDA neurons express a marker selected from the group consisting of EN1, OTX2, TH, NURR1, FOXA2, LMX1A, PITX3, LMO3, SNCA, ADCAP1, CHRNA4, ALDH1A1, SOX6, WNT1, DAT, VMAT2, GIRK2, SATB1, CALB1, CALB2, SNCG, PBX1, and combinations thereof.

D11. The foregoing composition of D-D10, wherein the one or more mDA neurons are post-mitotic mDA neurons.

D12. The foregoing composition of D-D11, wherein the one or more mDA neurons are *in vitro* differentiated from one or more stem cells.

D13. The foregoing composition of D12, wherein the one or more stem cells are selected from the group consisting of human stem cells, nonhuman primate stem cells, rodent nonembryonic stem cells, human embryonic stem cells, nonhuman primate embryonic stem cells, rodent embryonic stem cells, human induced pluripotent stem cells, nonhuman primate induced pluripotent stem cells, rodent induced pluripotent stem cells, and human recombinant pluripotent cells, nonhuman primate recombinant pluripotent cells, and rodent recombinant pluripotent cells.

D14. The foregoing composition of D12 or D13, wherein the one or more stem cells are human stem cells.

D15. The foregoing composition of D12-D14, wherein the one or more stem cells are one or more pluripotent stem cells or multipotent stem cell.

D16. The foregoing composition of D12-D14, wherein the one or more stem cells are selected from the group consisting of embryonic stem cells, induced pluripotent stem cells, and combinations thereof.

D17. The foregoing composition of D16, wherein the one or more stem cells are one or more induced pluripotent stem cells.

D18. The foregoing composition of D12-D17, wherein the *in vitro* differentiation comprises contacting the one or more stem cells with at least one inhibitor of Small Mothers Against Decapentaplegic (SMAD) signaling, at least one activator of Sonic hedgehog (SHH) signaling, and at least one activator of wingless (Wnt) signaling.

D19. The forgoing composition of D18, wherein the concentration of the at least one activator of Wnt signaling that is contacted with the cells is increased between about 2 days and about 6 days from the initial contact of the cells with the at least one activator of Wnt signaling.

D20. The foregoing composition of D18 or D19, wherein the concentration of the at least one activator of Wnt signaling that is contacted with the cells is increased by between about 250% and about 1800% of the initial concentration of the at least one activator of Wnt signaling contacted with the cells.

D21. The foregoing composition of any one of claims D18-D20, wherein the at least one activator of Wnt signaling comprises an inhibitor of glycogen synthase kinase 3β (GSK3β) signaling.

D22. The foregoing composition of D18-D21, wherein the at least one activator of Wnt signaling is selected from the group consisting of CHIR99021, CHIR98014,

AMBMP hydrochloride, LP 922056, Lithium, deoxycholic acid, BIO, SB-216763, Wnt3A, Wnt1, Wnt5a, derivatives thereof, and combinations thereof.

D23. The foregoing composition of D22, wherein the at least one activator of Wnt signaling comprises CHIR99021.

D24. The foregoing composition of D18-D23, wherein the at least one inhibitor of SMAD signaling comprises an inhibitor of TGFβ/Activin-Nodal signaling, an inhibitor of bone morphogenetic protein (BMP) signaling, or a combination of the foregoing.

D25. The foregoing composition of D24, wherein the at least one inhibitor of TGFβ/Activin-Nodal signaling is selected from the group consisting of SB431542, derivatives of SB431542, and combinations thereof.

D26. The foregoing composition of D25, wherein the derivative of SB431542 comprises A83-01.

D27. The foregoing composition of D25, wherein the at least one inhibitor of TGFβ/Activin-Nodal signaling comprises SB431542.

D28. The foregoing composition of D24-D27, wherein the at least one inhibitor of BMP signaling is selected from the group consisting of LDN193189, Noggin, dorsomorphin, derivatives of LDN193189, derivatives of Noggin, derivatives of dorsomorphin, and combinations thereof.

D29. The foregoing composition of D28, wherein the at least one inhibitor of BMP comprises LDN-193189.

D30. The foregoing composition of D19-D29, wherein the at least one activator of SHH signaling is selected from the group consisting of SHH proteins, Smoothened agonists (SAG), and combinations thereof.

D31. The foregoing composition of D30, wherein the SHH protein is selected from the group consisting of recombinant SHHs, modified N-terminal SHHs, and combinations thereof.

D32. The foregoing composition of D31, wherein the modified N-terminal SHH comprises two isoleucines at the N-terminus.

D33. The foregoing composition of D31 or D32, wherein the modified N-terminal SHH has at least about 90% sequence identity to an un-modified N-terminal SHH.

D34. The foregoing composition of D33, wherein the un-modified N-terminal SHH is an un-modified mouse N-terminal SHH or an un-modified human N-terminal SHH.

D35. The foregoing composition of D31-D34, wherein the modified N-terminal SHH comprises SHH C25II.

D36. The foregoing composition of D30, wherein the SAG comprises purmorphamine.

D37. The foregoing composition of D18-D36, wherein the *in vitro* differentiation further comprises contacting the one or more stem cells with at least one activator of fibroblast growth factor (FGF) signaling.

D38. The foregoing composition of D37, wherein the at least one activator of FGF signaling is selected from the group consisting of FGF18, FGF17, FGF8a, FGF8b, FGF4, FGF2, and combination thereof.

D39. The foregoing composition of D37 or D38, wherein the at least one activator of FGF signaling comprises FGF18 or FGF8.

D40. The foregoing composition of D18-D39, wherein the *in vitro* differentiation further comprises contacting the one or more stem cells with at least one inhibitor of Wnt signaling.

D41. The foregoing composition of D40, wherein the at least one inhibitor of Wnt signaling is selected from the group consisting of IWP2, IWR1-endo, XAV939, IWP-O1, Wnt-C59, IWP-L6, and ICG-001, and combinations thereof.

D42. The foregoing composition of D40 or D41, wherein the at least one inhibitor of Wnt signaling comprises IWP2.

D43. The foregoing composition of D-D42, wherein the one or more mDA neurons express a detectable level of CD184 and do not express a detectable level of CD49e.

EXAMPLES

The present disclosure will be better understood by reference to the following Example, which is provided as exemplary of the presently disclosed subject matter, and not by way of limitation.

Example 1 - Manipulation of TNF-NFκB-p53 axis for the survival of enriched hPSC-derived post-mitotic dopamine neuron in vivo.

One challenge in developing hPSC-based cell therapies in PD is to eliminate contaminating cell types such as non-dopaminergic neurons and a range of non-neuronal lineages within the graft. Studies using fetal tissue transplantation resulted in graft-induced dyskinesia (GID) as an unexpected side effect in some of the patients (Dorsey et al., 2018, J Parkinsons Dis 8, S3-S8), and it has been suggested that GID may be caused by contaminating serotonergic neurons in fetal grafts (Politis et al., 2010, *Sci Transl Med 2*, 38ra46). Recent hPSC-based studies highlighted the presence of potential other contaminants in dopamine neuron grafts, such as in rare instances, contamination with TTR+ positive choroid plexus epithelial cells (Doi et al., 2020, *Nat Commun* 11, 3369) or the presence of perivascular fibroblast-like populations.

Surface marker sorting strategies have been proposed to enrich floor-plate intermediate or late stage of dopamine precursor for transplantation, but none of these markers are specific for dopamine neuron lineage. The first-in-human clinical trials of using hPSC derived dopamine neuron grafts are based on grafting dopamine neuron precursors, rather than postmitotic dopamine neurons as cell type of choice (Doi et al., 2020, *Nat Commun* 11, 3369; Kirkeby et al., 2017, *Cell Stem Cell* 20, 135-148.; Piao et al., 2021, *Cell Stem Cell 28*, 217-229 e217; Schweitzer et al., 2020, *N Engl J Med* 382, 1926-1932). However, given the limited understanding of long-term risks and potential side-effects that could come from "off-target" neuronal or non-neuronal populations, it can be ideal to engraft homogenous post-mitotic dopamine neuron populations to minimize any safety-related concerns.

Pooled genetic screening using CRISPR/Cas9 technology has increasingly become a technology of choice to uncover causal genes driving specific phenotypes in an unbiased manner (Shalem et al., 2015, *Nat Rev Genet* 16, 299-311). This approach has been widely adopted for in vitro studies but has also been vital to discover essential genes for *in vivo* tumor growth and metastasis (Chen et al., 2015, *Cell* 160, 1246-1260.), modulators for macrophage infiltration, and cancer immunotherapy targets (Wang et al., 2021, *Cell* 184, 5357-5374 e5322) and *in vivo* colon tumor suppressors (Michels et al., 2020, *Cell Stem Cell* 26, 782-792 e787.). Here, the presently disclosed subject matter sets out to systematically identify candidate mechanisms driving the death of the grafted cell dopamine neurons using CRISPR-Cas9 technology. Purified Nurr1::H2B-GFP+ postmitotic dopamine neurons were used for this screen to avoid confounding factors such as dopamine neuron precursor proliferation and to identify conditions that may eventually enable the efficient grafting of postmitotic neurons in a translational setting.

Barcode sequencing identified a key role for TP53 in restricting postmitotic dopamine neuron survival following transplantation. Further, the kinetics of p53 induction upon grafting and the subsequent recruitment of host neuroimmune cells to the dying neurons were mapped and examined. Transcriptomic analysis revealed TNFα-mediated activation of NFκB as one of the main upstream regulators of p53-mediated dopamine neuron death. To further exploit those insights towards translational use, a set of two cell surface markers was identified to reliably enrich post-mitotic dopamine neurons and thereby avoiding the need for a genetic reporter system. Furthermore, it was demonstrated that the use of an FDA-approved monoclonal antibody blocking TNFα (adalimumab) was capable of dramatically improving post-mitotic dopamine neuron survival, mimicking the results observed in TP53 null dopamine neurons. The present example offers a better understanding of the mechanisms that drive postmitotic dopamine neuron death upon transplantation and establishes a clinically relevant strategy for future implementation in cell-based therapy approaches for PD.

Methods

Cell line. H9 (WA-09, passage 40-60) human pluripotent stem cell (hPSC) line was employed throughout the study, which engineer to generate *Nurr1::GFP* reporter hPSC and doxycycline-inducible CRISPR/Cas9 expression in the *Nurr1::GFP* hPSC line (iCas9/*NURR1::GFP* hPSC) as well as iCas9/*NURR1::GFP* hPSC lines containing sgRNA-pool libraries and sgRNA for dTomato and p53. hPSCs were grown in feed-free conditions on vitronectin (VTN-N; Thermo Fisher Scientific)-coated dishes in E8-essential medium and maintained at 37°C, 5% CO_2. Tri-I (MSKCC, Weil-Cornell, Rockefeller University) Embryonic Stem Cell Oversight (ESCRO) approved this study.

Construction of Nurr1:GFP and inducible expression of CRISPR/Cas9 in Nurr1:GFP hESC lines. Generation of Nurr1::GFP hESC line was previously described (Riessland et al., 2019, *Cell Stem Cell.* Oct 3;25(4):514-530.e8). Briefly, the stop codon of endogenous NR4A2 (Nurr1) was replaced by EGFP expression cassette (P2A-H2B-PgkPuro) by using a CRISPR/CAS9-mediated knock-in approach. The resulting NURR1:GFP^+ cells almost express TH (a mature mDA marker; 98%) based on single-cell qRT-PCR. To generate doxycycline-inducible CRISPR/Cas9 expression in the *Nurr1::GFP* hPSC line (iCas9/*NURR1::GFP* hPSC line), a pair of TALEN, Neo-M2rtTA donor, and Hygro-Cas9 donor (Addgene#86883), targeting to an *AAVS* locus,

were transfected into the Nurr1::GFP hPSC using Nucleofector (Lonza, B-016 program) in AMAXA machine and stable cell line was generated following a published protocol. Briefly, 2 days after transfection, neomycin and hygromycin (100µg/ml) were treated for 1 week and picked clonal expanded hPSC. Inducible expression of Cas9 in each clone was confirmed by immunofluorescent staining with a Cas9 antibody after Dox (1µg/ml) exposure for 3 days.

Single-strand guide RNA (sgRNA) Design and Cloning. 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).

Lentiviral production and transduction. SGL40C.EFS.dTomato vector containing sgRNA for libraries, dTomato, and p53 was co-transfected with packing vectors, psPAX2 (Addgene#12260) and pMD2.G (Addgene#12259) into HEK293T cell using Xtream Gene 9 transfection reagent (Sigma). The virus was collected after 2 days of transfection, and infected into the iCas9/*NURR1::GFP* hPSC. 2 days post infection, dTomato expressed hPSCs were sorted using flow cytometry associated cell sorting (FACS) in Flow Cytometry Core Facility at MSKCC. The sorted hPSCs were cultured and maintained for subsequent experiments until use.

hESC differentiation toward DA neurons. Midbrain dopaminergic neuron differentiation was performed using H9 hESCs, which include Nurr1:: GFP. hESCs were grown on VTN-N (Thermo Fisher Scientific)-coated 6-well plates in E8-essential medium. Cells were maintained at 37°C, 5% CO_2. hESCs were differentiated with an optimized protocol from a previously reported study (Kim et al., 2021, Cell Stem Cell Feb 4;28(2):343-355.e5; Riessland et al., 2019, *Cell Stem Cell.* Oct 3;25(4):514-530.e8). At day 25 of differentiation from hPSCs iCas9/NURR1::GFP hPSC lines containing sgRNA-pool libraries and sgRNA for dTomato and p53 hPSCs, GFP and dTomato expressed dopamine neurons were sorted using flow cytometry associated cell sorting (FACS). The sorted hPSCs were either injected into mice or cultured for subsequent experiments until use. Furthermore, double sorting approach with CD49e-low and CD184-high was applied to enrich post-mitotic dopamine neuron at day 25

differentiation derived from hPSC using FACS, and sorted cells were used for transplantation and in vitro culture. TNF-a neutralizing antibody was employed either to co-injection (1mg/ml) or in vitro cultured dopamine neuron (10μg/ml).

sgRNA barcode Sequencing and Analysis to identify targets. Cell Pellets at each desired time point were lysed, and genomic DNA was extracted (Qiagen) and quantified by Qubit (Thermo Scientific). A quantity of gDNA covering 1000X representation of sgRNAs was PCR amplified to add Illumina adapters and multiplexing barcodes. Amplicons were quantified by Qubit and Bioanalyzer (Agilent) and sequenced on Illumina HiSeq 2500. Sequencing reads were aligned to the screened library and counts were obtained for each gRNA. The resulting single end reads were checked for quality (FastQC v0.11.5) and processed using the Digital Expression Explorer 2 (DEE2) workflow. Adapter trimming was performed with Skewer (v0.2.2). Further quality control done with Minion, part of the Kraken package. Differential gRNA hits were identified using EdgeR, a Bioconductor package, to identify the primary hits. We used the Trimmed Mean of M-values for normalization and the glmQLFTest / F test for statistic tests. Additionally, we used the camera analysis function from EdgeR for gene-level analysis as previously described. To calculate the correlation between the screen samples, quantifications were normalized by the estimateSizeFactorsForMatrix of DESeq2 using only the non-targeting control and safe harbor probes. Pearson correlation of the pairwise comparison was plotted using an R package pheatmap (https://CRAN.R-project.org/package=pheatmap).

Intracranial transplantation. hESCs-derived DA neurons that were sorted based on either NURR1::H2B-GFP or CD49e low and CD184 high were resuspended in 100,000 ±cells /μL in neurobasal medium with 200 mM L-glutamine and 100 mM ascorbic acid (AA) transplantation medium (without human albumin or kedbumin 25%). Unless specified, 3-4 μL of sorted neurons were injected at the rate of 0.5 – 1 μL per deposit) into the striatum of wild-type (unlesioned) 6 to 8-week-old male NSG mice ([AP] +0.5mm, [ML] +/-1.8mm, [DV] -3.4 to -3.3 mm from dura). Each surgery did not exceed more than 30 minutes per animal and the entire surgery time was within 8 - 10 hours post cell preparation. For the short-term 1-month survival study, p53 WT (-dox) *vs.* KO (+dox) NURR1::GFP+ dopamine neurons or CD + PBS vs. CD + adalimumab was bilaterally engrafted into the striatum of the same mouse brain to reduce variability between animals. For other studies including time course or behavior studies, cells are engrafted unilaterally. For the time-course experiments, the mice were euthanized and

used for immunohistochemistry analysis at designated time points (4hrs, 1day, 3days, and 7days post engraftment). For *in vivo* CRISPR screen, maximum loadable amount of the cells is implanted with cell density of 200,000 ± 10,000 cells/μL (4 μL total in each striatum) in order to have enough representations of the guide RNAs.

DNA extraction from the xenograft sample and detection of human DNA. Grafted dopamine neurons at 1 month were isolated from thick tissue slices based on NURR1::GFP and sgRNA::Tomato expressions and extracted genomic DNAs using the DNeasy Tissue kit according to the manufactural protocol (Qiagen #69556). To detect human DNA from the extracted genomic DNA, PCRs were performed to detect human and mouse cells with human and mouse specific primers for *PTGFR2* and *ptgfr2* subsequently using the Q5 High-Fidelity PCR kit (NEB #M0493S). Primer sequences are below.

Human PTGFR2 primer 5': GCTGCTTCTCATTGTCTCGG (SEQ ID NO.: 539)

Human PTGFR2 primer 3': GCCAGGAGAATGAGGTGGTC (SEQ ID NO.: 540)

Mouse ptgfr2 primer 5': CCTGCTGCTTATCGTGGCTG (SEQ ID NO.: 541)

Mouse ptgfr2 primer 3': GCCAGGAGAATGAGGTGGTC (SEQ ID NO.: 542)

6-OHDA mouse model. Briefly, adult female and male NSG mice (6-12 weeks) were anesthetized with 1%–2% isoflurane mixed in oxygen. 1 μL 6-OHDA (3 mg/ml, in saline with 1% ascorbic acid) was directly injected into the right side of substantial nigra (anterior-posterior [AP] = - 2.9 mm, lateral [ML] = 1.1 mm, vertical [DV] = 4.5 mm, from dura) with rate of 0.5 – 1 μL per minute to generate unilateral toxin Parkinsonian mouse model. Animals with amphetamine-induced rotation at > 6 rotations per min were selected for cell transplantation 4 weeks after 6-OHDA-lesion surgery. Animals were randomly grouped and transplanted with CD sorted neurons vs. CD sorted neurons + adalimumab vs. PBS (sham surgery control).

d-amphetamine induced rotation behavior assays. Amphetamine-induced rotation tests were performed twice before transplantation, and 1, 2, 3, 4, 5, 6 months after transplantation. The mice were injected intraperitoneally with a d-amphetamine in saline (Sigma, 10 mg/kg). After 10 minutes, the rotation

Immunohistochemistry. Histology on tissues from mice was performed on frozen sections from xenografts. Mice were anesthetized with pentobarbital and transcardically perfused using heparinized (10U/mL) PBS (pH 7.4), followed by 4% paraformaldehyde in PBS. The liquid is administered using peristaltic pump to control

the rate of the solution delivery to the system. Tissues were post-fixed in ice cold 4% paraformaldehyde for 18 hours sharp and transferred to 30% sucrose until the tissue sink (typically 3-6 days post-), followed by snap freezing in O.C.T (Fisher Scientific, Pittsburgh, PA) or Neg-50 (Thermo Scientific). Brain tissues are all sectioned in 30 μm thick coronal sections using a cryostat and mounted onto a Superfrost plus microscope slides (Fisher Scientific). All the slides are stored at -80°Cfor long-term until use. To process for immunolabeling, tissues are washed twice with 1x PBS, followed by permeabilization in in 0.5% Triton X-100 in PBS for 10 minutes. Living cells in culture were directly fixed in 4% paraformaldehyde for 18 min, followed by 10 min permeabilization in 0.5% Triton X-100 in PBS. For labeling, cells or tissue sections were immunostained with primary antibodies of interest in 2% BSA in 0.25% Triton X-100 in PBS at 4°C overnight. Next day appropriate Alexa Fluor secondary antibodies are conjugated at room temperature for 1hr at a dilution of 1:1,000. Nuclei were counterstained by DAPI.

Tissue immunohistochemistry (IHC), TUNEL, and H&E stain. H&E and IHC on tissues from mice was performed on FFPE (formalin fixed paraffin embedded) sections from xenografts. Mice were anesthetized with pentobarbital and transcardically perfused using heparinized (10U/mL) PBS (pH 7.4), followed by 10% formalin. The liquid is administered using peristaltic pump to control the rate of the solution delivery to the system. Tissues were post-fixed in 10% formalin 24-48 hours at room temperature (can stay up to a week in 10% formalin) and directly transferred to 70% ethanol. Histology was performed by HistoWiz Inc. (histowiz.com) using a Standard Operating Procedure and fully automated workflow. Samples were embedded in paraffin, and sectioned at 4μm. Immunohistochemistry was performed on a Bond Rx autostainer (Leica Biosystems) with heat-mediated antigen retrieval using Epitope Retrieval Solution 1 (Leica Biosystems). Primary antibodies used were rabbit polyclonal total NFKB (Cell Signaling, CST8242, 1:300), Cleaved Caspase-3 (Cell signaling, CST9661, 1:300), p53 (CM5P, 1:500), and p-NFkB (GTX55113, 1:5000) followed by anti-rabbit HRP conjugated polymer system. Bond Polymer Refine Detection (Leica Biosystems) was used according to the manufacturer's protocol. After staining, sections were dehydrated and film coverslipped using a TissueTek-Prisma and Coverslipper (Sakura). Whole slide scanning (40x) was performed on an Aperio AT2 (Leica Biosystems). For TUNEL: Standardized conditions using the Promega DeadEnd Colorimetric Detection System (G3250), Enzyme Digestion for 10 minutes, using the Leica Bond Polymer Refine

Detection Kit (DS9800). For H&E, staining was performed on Sakura Autostainer. Briefly, deparaffinize the slides in 2 changes of xylene, 2 changes of 100% alcohol, 1 change in 95% alcohol, then wash with water. Place slides in this sequence: hematoxylin, a rinse with water, define solution, a rinse with water, bluing agent solution, rinse with water, 95% alcohol, eosin, and 95% alcohol. Finish with two changes of 100% alcohol and two with xylene.

RNA extraction and Real time quantitative reverse transcription-PCR (qRT-PCR). Total RNA samples were prepared from cells and DNase I treated using TRIzol according to the manufacturer's instructions. Delta-delta-cycle threshold (ΔΔCT) was determined relative to GAPDH levels and normalized to control samples. Error bars indicate the standard deviation of the mean from three biological replicates. The sequences of qRT-PCR primers are shown below.

Human GAPDH primer 5': ATGTTCGTCATGGGTGTGAA (SEQ ID NO.: 543)

Human GAPDH primer 3': AGGGGTGCTAAGCAGTTGGT (SEQ ID NO.: 544)

Human FOXA2 primer 5': CCGACTGGAGCAGCTACTATG (SEQ ID NO.: 545)

Human FOXA2 primer 3': TACGTGTTCATGCCGTTCAT (SEQ ID NO.: 546)

Human NURR1 primer 5': CGCTTCTCAGAGCTACAGTTAC (SEQ ID NO.: 547)

Human NURR1 primer 3': TGGTGAGGTCCATGCTAAAC (SEQ ID NO.: 548)

Human PUMA primer 5': CCTGGAGGGTCCTGTACAATCT (SEQ ID NO.: 549)

Human PUMA primer 3': TCTGTGGCCCCTGGGTAAG (SEQ ID NO.: 550)

Human TP53 primer 5': GTACCACCATCCACTACAACTAC (SEQ ID NO.: 551)

Human TP53 primer 3': CACAAACACGCACCTCAAAG (SEQ ID NO.: 552)

Protein isolation and western-blot analysis. Cells treated with PBS or monoclonal antibodies against TNFα were harvested and lysed in the following lysis buffer (RIPA buffer, 1:1000 Halt™ Protease, and Phosphatase Inhibitor cocktail (Thermo Fisher Scientific)). After cells are resuspended in a lysis buffer, cells are sonicated for 3x30 seconds at 4°C. Supernatant was collected upon 15 minutes centrifugation >15,000 rpm at 4°C and protein concentration was measured using Precision Red Advanced Protein Assay (Cytoskeleton). Equal amounts of protein (20

micrograms) were boiled in NuPAGE LSD sample buffer (Invitrogen) at 95°C for 5 minutes and separated using NuPAGE 4%-12% Bis-Tris Protein Gel (Invitrogen) in NuPAGE MES SDS Running Buffer (Invitrogen). Proteins were electrophoretically transferred to a nitrocellulose membrane (Thermo Fisher Scientific) with NuPAGE Transfer Buffer (Invitrogen). Blots were blocked for 60 minutes at RT in 5% nonfat milk (Cell Signaling) in TBS-T + and incubated with respective primary antibody at 4°C overnight. The following primary antibodies were used: mouse-anti-GAPDH (6C5) (Santa Cruz, 1:1,000); mouse anti-p53 (DO-1) (Santa Cruz, 1:1,000); rabbit anti-tyrosine hydroxylase (Pel Freeze, 1:1000). Primary antibodies were detected using the secondary anti-rabbit IgG HRP-linked (Cell Signaling, 1:1,000) or anti-mouse IgG HRP-linked (Cell Signaling, 1:1,000) with the SuperSignal™ West Femto Chemiluminescent substrate (Thermo Fisher Scientific).

RNA-Seq 1 day post transplantation. One day after the intracranial injection of mDA neurons sorted by NURR1-GFP$^+$ signal, the mice were euthanized, and the injection site was grossly dissected and processed for papain dissociation (Worthington). Dissociated xenograft samples along with *in vitro* cultured neurons (day 1 *in vitro)* were simultaneously subject to FACS for re-isolating dopamine neurons based on the endogenous reporter signal. Total RNA was extracted in TRIzol (Invitrogen) according to the manufacturer's instructions. RNAseq libraries of polyadenylated RNA were prepared using the TruSeq Stranded mRNA Library Prep Kit (Illumina) according to the manufacturer's instructions and sequenced on an Illumina NextSeq 500 platform. The resulting single-end reads were checked for quality (FastQC v0.11.5) and processed RNAseq libraries of polyadenylated RNA were prepared using the TruSeq Stranded mRNA Library Prep Kit (Illumina) according to the manufacturer's instructions and sequenced on an Illumina NextSeq 500 platform. The filtered reads were mapped to human reference genome hg19 using STAR aligner (version 2.5.0a). The expression data at the coding sequence were quantified using the GENCODE version 38 transcriptome and HTSeq version 1.99.2 (Anders et al., 2015, *Bioinformatics*, 31(2):166-9). Differential expression analysis was done with the negative binomial statistical model using DESeq2 version 1.30.1 (Love et al., 2014, *Genome Biol* 15(12):550). Enrichment analysis with the various reference data was done using clusterProfiler version 4.2.1 (Yu et al., 2012, *OMICS* 16(5):284-7; Wu et al., 2021, *Innovation* (N Y) 2(3):100141).

Single-cell transcriptome sequencing. Single cell suspensions were stained with Trypan blue, and the Countess II Automated Cell Counter (ThermoFisher) was used to

assess both cell number and viability. Following QC, the single cell suspension was loaded onto Chromium Chip B (10X Genomics PN 2000060) and GEM generation, cDNA synthesis, cDNA amplification, and library preparation of 3,900-5,300 cells proceeded using the Chromium Single Cell 3' Reagent Kit v3 (10X Genomics PN 1000075) according to the manufacturer's protocol. cDNA amplification included 12 cycles and 88-99ng of the material was used to prepare sequencing libraries with 12 cycles of PCR. Indexed libraries were pooled equimolar and sequenced on a NovaSeq 6000 in a PE28/91 paired end run using the NovaSeq 6000 S1 Reagent Kit (100 cycles) (Illumina). An average of 94 million paired reads was generated per sample.

Single-cell analysis. The samples underwent 10X chromium Single Cell 3' v3 processing. The reads were aligned to human GRCh38 (GENCODE v32/Ensembl 98) using Cell Ranger 5.0.0. The resulting filtered count matrix was further filtered for cells with i) minimum 1000 UMI counts, ii) 500 ≤gene counts ≤ 7000, iii) and mitochondrial gene percentage of less than 25%. Normalization by deconvolution in scran version 1.22.1 was performed and the signal from the gene expression related to the cell cycle was regressed out as directed by Seurat version 4.1. The default 2000 highly variable genes were selected, and the first 50 principal components were extracted from the cell cycle-regressed matrix. Subsequently, the shared nearest neighbors were calculated from the principal components using buildSNNGraph of R software scran using the k parameter of 40. Seven clusters were identified and using the walktrap algorithm, with the function cluster_walktrap of R implementation of the igraph package version 1.3.5. The uniform manifold approximation and projection (UMAP) was performed. Differential gene expression was performed via the Seurat package using MAST. Cluster annotation was performed via clusterProfiler package version 4.2.2, and differential expression visualization using EnhancedVolcano version 1.12.0.

Stereological analysis. For short-term survival studies, unbiased stereological counts of *NURR1::H2B-GFP* DA neurons within the striatum (AP +0.5, ML +/- 1.8, DV -3.4 to -3.3 from dura) were performed using stereological principles and analyzed with StereoInvestigator software (Microbrightfield, Williston, VT, USA), as previously described. For long-term behavioral studies, unbiased stereological counts of TH positive DA neurons within the striatum were performed. The tissue was embedded in O.C.T. or Neg-50 and sections are sliced at 30 µm. Optical fractionator sampling was carried out with an Olympus BX61 microscope equipped with a motorized stage and Olympus 40x objective lens objective. Graft region was outlined on the basis of NURR1::GFP

immunolabeling, with reference to a coronal atlas of the mouse brain. Every 3rd - 10th section (depending on the total thickness of the graft) from the beginning of the graft to the end of the graft was randomly and systematically selected for analysis. For each tissue section analyzed, section thickness was assessed in each sampling site and guard zones of 1 µm were used at the top and bottom of each section. Pilot studies were used to determine suitable counting frame and sampling grid dimensions prior to counting to achieve enough statistical power and low Gunderson coefficient variance. The following stereological parameters were used in the final study: for optical fractionator probe, 65 µm x 65 µm optical dissector, 100 µm x 100 µm (or 10% of ROI) SRS, 20 µm optical dissector height and 1 µm guard zone; for cavalier estimator probe, 50 µm x 50 µm grid spacing, 0-degree grid rotation, and 30 µm section cut thickness. For analysis, at least 2-8 sections were evaluated for analysis. Gundersen coefficients of error for all conditions were less than 0.1. Stereological estimations were performed with the same parameters for all experimental conditions, p53 KO vs. WT (NURR1::GFP sort) or TNFα monoclonal antibodies treatment vs. PBS (CD sort).

High throughput cell surface marker screen and enrichment of dopamine neuron with using cell surface markers. Dopamine neuron differentiated cells at day25 from the NURR1::GFP reporter hESC were single cell suspended in flow cytometer staining buffer (PBS containing 2% bovine serum albumin). The cells were stained with 387 cell surface (CD) markers (0.2M cells per a CD marker) for 30 min on ice in the dark. After 3 times washing with PBS, cells were co-stained with DAPI. All staining for the screen was done in 96 well plates. Data collection using a flow cytometer to identify CD markers to enrich GFP positive population was performed by the MSKCC Flow Cytometry core facility. For enrichment of dopamine neuron with using CD markers, 49e and 184, day25 cells were
stained with 49e and/or 184, followed by isolation of 49e weak, 49e weak and 174 high, and 49e weak and 184 high expressed cells via FACS at the MSKCC Flow Cytometry core facility.

Transcriptome sequencing. After RiboGreen quantification and quality control by Agilent BioAnalyzer, 0.5-1ng total RNA with RNA integrity numbers ranging from 6.1 to 10 underwent amplification using the SMART-Seq v4 Ultra Low Input RNA Kit (Clonetech catalog # 63488), with 12 cycles of amplification. Subsequently, 1.6-10ng of amplified cDNA was used to prepare libraries with the KAPA Hyper Prep Kit (Kapa Biosystems KK8504) using 8 cycles of PCR. Samples were barcoded and run on a HiSeq

4000 or NovaSeq 6000 in a PE50 run, using the HiSeq 3000/4000 SBS Kit or NovaSeq 6000 S1 Reagent Kit (100 Cycles) (Illumina). An average of 56 million paired reads were generated per sample and the percent of mRNA bases per sample ranged from 48% to 76%.

Quantification and Statistical analysis. N=3 independent biological replicates were used for all experiments unless otherwise indicated. n.s. indicates a non-significant difference. *P*-values were calculated by unpaired two-tailed Student's t-test unless otherwise indicated. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Results

In vivo CRISPR/Cas9 screens discovered a regulator of post-mitotic dopamine neuron death. An *in vivo* CRISPR-Cas9 screen was developed to systematically address intrinsic factors that restrict the survival of hPSC derived post-mitotic dopamine neurons. Previously, it was demonstrated that NURR1 can serve as a reliable marker to denote early post-mitotic dopamine neurons derived from hPSCs under the floor-plate differentiation paradigm (Riessland et al., 2019, *Cell Stem Cell* 25, 514-530 e518). FACS-based purification of NURR1::GFP positive cells yielded nearly pure dopamine neurons populations in culture (Riessland et al., 2019, *Cell Stem Cell* 25, 514-530 e518) (Figure 1A; Figure 19A), and gave rise to a highly homogenous and dense dopamine neuron graft *in vivo,* marked by TH, albeit overall poor survival rates less than ~5% derived from an endogenous NURR1::GFP hPSC reporter line (Figures 1B and 1G; Figure 3D; Figures 19B and 19F). Using this established *NURR1::H2B-GFP* reporter hPSC, the presently disclosed subject matter further engineered inducible Cas9 (iCas9) hPSC lines, such as doxycycline-inducible expression of Cas9, integrated into a safe harbor locus, an *AAVS* locus, through TALEN-mediated gene targeting, (iCas9/*NURR1::H2B-GFP* hPSC line) (Figure 2A; Figure 12A). To determine the efficiency of iCas9 for gene knock-out in the presently disclosed culture system, a sgRNA targeting tdTomato was stably incorporated in the iCas9/*NURR1::H2B-GFP* hPSC line. Upon doxycycline treatment from day 16 to day 25 during dopamine neuron differentiation using clinical-grade protocol, efficient ablation of the tdTomato signal (Figure 1E; Figure 19E) was observed without disrupting dopamine neuron induction as shown by NURR1 and FOXA2 expressions, which allows a CRISPR/Cas9 loss-of-function pool screen in hPSC-derived post-mitotic dopamine neurons.

Next, a pooled-lentiviral custom-design library of 550 sgRNAs targeting a total of 150 genes (3 sgRNAs per gene) related to cell death pathways, such as apoptosis,

necroptosis, pyroptosis, ferroptosis, and autophagy as well as 50 non-targeting and 50 safe harbor control guides were stably introduced into the iCas9/*NURR1::H2B-GFP* hPSC line (iCas9/*NURR1::H2B-GFP*/library) (Table 1 and Table 2).

Table 1

APOPTOSIS	FAS
	FASLG
	TNFRSF10A
	TNFRSF10B
	TNFRSF10C
	TNFRSF10D
	TNFRSF11B
	TNFSF10
	TNFRSF1A
	TNF
	FADD
	CFLAR
	CASP1
	CASP2
	CASP3
	CASP4
	CASP5
	CASP6
	CASP7
	CASP8
	CASP9
	CASP10
	CASP14
	NAIP
	BIRC2
	BIRC3
	XIAP
	BIRC5
	BIRC6
	BIRC7
	BCL2
	MCL1
	BCL2L1
	BCL2L2
	BCL2A1
	BCL2L10

	BAX
	BAK1
	BOK
	BID
	BCL2L11
	BMF
	BAD
	BIK
	HRK
	PMAIP1
	BNIP3
	BNIP3L
	BCL2L14
	BBC3
	BCL2L12
	BCL2L13
	APAF1
	CYCS
	DIABLO
	HTRA2
	AIFM1
	ENDOG
	CARD8
	GZMB
	CARD6
	NOX5
NECROSIS	RIPK1
	RIPK3
	MLKL
	CD40LG
	CD70
	PARP2
	FOXI1
	UBR5
	MPG
	CA9
	SLC25A15
	SIRTS
	NPEPL1
	DSC1
	CD40

	COL4A3BP
	DNM1L
	PGAM5
	PARP1
	PTPA(PP2A)
	CYLD
	JUN
	GRB2
	IFNA1
	IFNA7
	IFNA13
	IRGM
PYROPTOSIS	NAIP
	NLRP1
	NLRC4
	AIM2
	NLRP1
	NLRP3
	PYCARD
	GASDMD
FERROPTOSIS	VDAC2
	VDAC3
	NOX4
	TFRC
	CARS
	IREB2
	SLC11A2
	CS
	ACSF2
	TP53
	GPX4
	SLC7A11
	HSPB1
	NEF212
AUTOPHAGY	ULK1
	ULK2
	ATG2A
	ATG2B
	ATG3
	ATG4A
	ATG4B

	ATG4C
	ATG4D
	ATG5
	BECN1
	ATG7
	ATG9A
	ATG9B
	ATG10
	ATG12
	ATG13
	ATG14
	ATG16L1
	ATG16L2
	RB1CC1
	WIPI1
	WIPI2
	SNX30
	SNX4
	ATG101
	MAP1LC3A
	PIK3C3
	TLR7
	TLR4
	DDX58
	IFIH1
	MAVS
	IL1B
	IL18
	IL4

Table 2.

sgRNA Target Sequence	Target Gene	SEQ ID NO.
ATGGTCTCCACGCCCATCGG	ACSF2_1	1
ACGTAGCTGAGGCCTCCGAT	ACSF2_2	2
CGACCAAGGCCTCTCGTTCT	ACSF2_3	3
GGAATACTCACCAGATAACG	AIFM1_1	4
TCCATCCGGGCTCGGGATCC	AIFM1_2	5
ATCCCGAGCCCGGATGGATC	AIFM1_3	6
TTGAAGCGTGTTGATCTTCG	AIM2_1	7
TTCACGTTTGAGACCCAAGA	AIM2_2	8
TCAGCGGGACATTAACCTTT	AIM2_3	9

AGCATTGTAGAATGATACGT	APAF1_1	10
GTTAGTGGAATAACTTCGTA	APAF1_2	11
CCAGGATGGGTCACCATACA	APAF1_3	12
CTATAAGATGCGACTGCTAC	ATG10_1	13
GACACTATTACGCAACAGGT	ATG10_2	14
TGAATTCTGCACAATAACGT	ATG10_3	15
GCCCACGGTGCCAATGGAGT	ATG101_1	16
GTGCCCTGCGCAAGGTTGTT	ATG101_2	17
TGCGCAACTCTGGTGGCGAT	ATG101_3	18
CTCCCCAGAAACAACCACCC	ATG12_1	19
CCTCCAGCAGCAATTGAAGT	ATG12_2	20
AGCAGGTTCCTCTGTTCCCG	ATG12_3	21
GGGTATATCCAAACTCGTCA	ATG13_1	22
AGGGTATATCCAAACTCGTC	ATG13_2	23
TTTACCCAATCTGAACCCGT	ATG13_3	24
TCTACTTCGACGGCCGCGAC	ATG14_1	25
GGCGATTTCGTCTACTTCGA	ATG14_2	26
CGATGCGGAGGGGCTGTACG	ATG14_3	27
CATCATGTCCGGGACTAAAT	ATG16L1_1	28
CGGGACTAAATTGGCAAACC	ATG16L1_2	29
ATTAAGCCGATTGGCTTCCT	ATG16L1_3	30
TACGCAAAAGGCGCTTTTCC	ATG16L2_1	31
CGGACCCACATACACTTACC	ATG16L2_2	32
GCAACCTGCGCAACGAGCGC	ATG16L2_3	33
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GTGACATCTCGGAGACCGCC	ATG2A_2	35
ATCTCGGAGACCGCCGGGCC	ATG2A_3	36
ATGGACTCCGAAAACGGCCA	ATG2B_1	37
GAAACTGCTGACGAATCCTC	ATG2B_2	38
GTGCAGATTGGACGGTTAAT	ATG2B_3	39
TTATAGTGCCGTGCTATAAG	ATG3_1	40
TGTTTGCACCGCTTATAGCA	ATG3_2	41
ACAACCATAATCGTGGAGTC	ATG3_3	42
ATTGGTGGGTATTCGTAGGT	ATG4A_1	43
GGATCCTTCAGTTGCATTGG	ATG4A_2	44
TTAGGATGTTCATTCGCTGT	ATG4A_3	45
AGCAAACCGGAGAGTGTCGT	ATG4B_1	46
TCAGAGCCCGTTTGGATACT	ATG4B_2	47
TCCTGTCGATGAATGCGTTG	ATG4B_3	48
AGAGTCGGGATGTACAATAG	ATG4C_1	49
ATAGAGGATCACGTAATTGC	ATG4C_2	50
ATTGTACATCCCGACTCTGC	ATG4C_3	51
CCTCGCCCTCGAAACGGTAG	ATG4D_1	52

TGGCCGCCGCTACCGTTTCG	ATG4D_2	53
CCCGGCGGTATGTGAGCCAC	ATG4D_3	54
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GAGTGAACATCTGAGCTACC	ATG5_2	56
TCCGATTGATGGCCCAAAAC	ATG5_3	57
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GAAGCTGAACGAGTATCGGC	ATG7_2	59
CTTGAAAGACTCGAGTGTGT	ATG7_3	60
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CCTGTTGGTGCACGTCGCCG	ATG9A_2	62
CCTCGGCGACGTGCACCAAC	ATG9A_3	63
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GCTATGGCCGCGAGCTCCGG	BAD_2	68
GAGCGCGAGCGGCCCCGAAA	BAD_3	69
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GCATGAAGTCGACCACGAAG	BAK1_2	71
GCAGGTGAGCTACAACCGCT	BAK1_3	72
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CAAGCGCATCGGGGACGAAC	BAX_2	74
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TCAACGCACAGTACGAGCGG	BBC3_1	76
GCCGCTCGTACTGTGCGTTG	BBC3_2	77
CAACGCACAGTACGAGCGGC	BBC3_3	78
GGGGCCGTACAGTTCCACAA	BCL2_1	79
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AAGCGTCCCCGCGCGGTGAA	BCL2_3	81
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TTGAAGACGGCATCATTAAC	BCL2A1_3	84
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CCGGTGAATCTGCCGTAACC	BCL2L10_2	89
CTGCCGTAACCTGGCGGCCG	BCL2L10_3	90
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TCCAATACGCCGCAACTCTT	BCL2L11_2	92
CTCCAATACGCCGCAACTCT	BCL2L11_3	93
GGGCCGTCCCATCGGCTTTT	BCL2L12_1	94
AAGCCGATGGGACGGCCCGC	BCL2L12_2	95

AGCCGATGGGACGGCCCGCT	BCL2L12_3	96
GAGGACGCCATTGAATTGGC	BCL2L13_1	97
ATCCGGAACGGTATCCGGAG	BCL2L13_2	98
CTGACATCCGGAACGGTATC	BCL2L13_3	99
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GATCCAGCAGCACGGTGGAT	BCL2L14_2	101
GGCCGTGACGTCTATTACAA	BCL2L14_3	102
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CGTAGATGAAAGCCAGACCC	BIK_3	114
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GTCGCGGCCAATGGGCGACG	BNIP3_3	135
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GGTCTTCGCCGCCGAGATCA	BOK_3	141
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CTGCGCAACAATGGCCACAG	CA9_3	144
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CAGTACCTCCTCAATCTATG	CARD6_2	146
TATGATGACCCAGAGCACGT	CARD6_3	147
TTGTTAGCAAGGCGTCGCTG	CARD8_1	148
TCCTGCTGCGGATCGCCAGT	CARD8_2	149
TCCCACTGGCGATCCGCAGC	CARD8_3	150
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TGAGATGCGTCATAGACGGT	CARS_2	152
TGATGTCAACAGGAGCGCGA	CARS_3	153
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CAAAGCAATCGGCATCTGCG	CASP6_3	177
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TGTAGGCACTCGGTCCCGGG	CASP7_2	179
GGTACAAACGAGGACCGGTC	CASP7_3	180
TCCGGGATATATCTCGTTTG	CASP8_1	181

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GCCTGGACTACATTCCGCAA	CASP8_3	183
CCGCCGATCCGCTTCGTCCA	CASP9_1	184
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ATGACATGTGCCGCAATTTG	CD40LG_2	191
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TGCTACAGTCTTGTGATTCG	DSCC1_3	222
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GCGTCGACGACTTCGAGGCG	FADD_3	228
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ACTCCGAGAGGTAAGCCTGC	FASLG_1	232
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TCTGGTTGCCTTGGTAGGAT	FASLG_3	234
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CACGCCCGATACGCTGAGTG	GPX4_2	239
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GGTGCATAGTCTTACCTTAA	GZMB_3	249
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GTCGCCTAGCGCCTTGAGCC	HRK_3	252
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CGTTCGAGATAGGGACCTCG	HTRA2_1	256
CGCGAGGTCCCTATCTCGAA	HTRA2_2	257
GTCCCTATCTCGAACGGCTC	HTRA2_3	258
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ATGTCTGTCCATCAGACAGG	IFNA1_2	263
TGCTTTACTGATGGTCCTGG	IFNA1_3	264
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GTTCGGTGCAGAATTTGTCT	IFNA13_2	266
CTACCAGCAGCTGAATGACT	IFNA13_3	267

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CATGGCCACAACAACTGACG	IL1B_3	276
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GATGCTTGCCAAAACCGCTG	IRGM_2	284
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CATAAGGTCCGCTCTCGGAC	JUN_3	288
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CCAGCACGGGTTGAGTTGAT	MAVS_2	293
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GGTCACCGATCGAGAGCTAG	NonTargetingControlGuideForHuman_0029	347
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GATGGCGCTTCAGTCGTCGG	NonTargetingControlGuideForHuman_0041	359
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CGTCGCCATATGCCGGTGGC	NonTargetingControlGuideForHuman_0044	362

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CGCCGTTCCGAGATACTTGA	NonTargetingControlGuideForHuman_0046	364
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GCCAAGAGTGTTCGGCACAT	NOX4_1	372
ACACTCTTGGCTTACCTCCG	NOX4_2	373
ACGCCTACAGAATTACACCA	NOX4_3	374
GCTCTTCGAATCGGCCGACG	NOX5_1	375
TTCGAATCGGCCGACGCGGA	NOX5_2	376
CGGCCGACGCGGACGGCAAC	NOX5_3	377
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CGGAGCGCATCGCTGCATTG	NPEPL1_3	380
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GGAACAACGGTTTCGCTCTT	PIK3C3_3	392
TCCAGCAGGTACCGACCCGC	PMAIP1_1	393
CCCAGCGGGTCGGTACCTGC	PMAIP1_2	394
CGCTCAACCGAGCCCCGCGC	PMAIP1_3	395
TAGGCATACGCTGACTACAT	PTPA_1	396
TACCCTCAACGAAGGTGTGA	PTPA_2	397
TTCTCAACACGCTGGACAGG	PTPA_3	398
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CATGTCGCGCAGCACGTTAG	PYCARD_2	400
GTGCCGCTGCGCGAGGGCTA	PYCARD_3	401

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GCGGATCTATCTGATTCACC	RB1CC1_2	403
TGAAGATCGGCTCTACGCCC	RB1CC1_3	404
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CTCGGGCGCCATGTAGTAGA	RIPK1_2	406
GTTGACGTCATTCAGGTGCT	RIPK1_3	407
CGGGCGCAACATAGGAAGTG	RIPK3_1	408
CAGTGTTCCGGGCGCAACAT	RIPK3_2	409
CGGGTTCGGCACAGTGTTCC	RIPK3_3	410
GAAAACAGAATATGGACCTA	SafeHarborchr1_21	411
GAAAAGTGGATGTGTGTCCC	SafeHarborchr10_45	412
GAAAACTGCATTGTGCAGAC	SafeHarborchr11_28	413
GAAAAGACAGTAAACCTTGC	SafeHarborchr11_33	414
GAAAAGACAGTAAACCTTTC	SafeHarborchr11_34	415
GAAAAGCCTGTGTGGAAGAG	SafeHarborchr11_38	416
GAAAAGTAAAACATGCAGAG	SafeHarborchr11_41	417
GAAAAAACTGTTTCTTTGGT	SafeHarborchr12_1	418
GAAAACTATACCTCATGATT	SafeHarborchr12_26	419
GAAAACTTAGAGAAAGCACA	SafeHarborchr12_29	420
GAAAACTTCAGAAATTGTGC	SafeHarborchr12_30	421
GAAAAATCAGACGAGCTCCA	SafeHarborchr13_12	422
GAAAAATGTCTCTACTTGCC	SafeHarborchr13_16	423
GAAAACTAGTAGTGGAAACT	SafeHarborchr13_25	424
GAAAAAGGATACATTTGCAA	SafeHarborchr13_8	425
GAAAACACACCTTATCACAG	SafeHarborchr15_19	426
GAAAACCCTATATATATAGG	SafeHarborchr18_24	427
GAAAAAAGTTAACTTAGACT	SafeHarborchr18_3	428
GAAAAGAATAACAGTAGTAA	SafeHarborchr2_32	429
GAAAACCAGACAAGTTACCT	SafeHarborchr21_23	430
GAAAAGAAATATCATTGGCA	SafeHarborchr21_31	431
GAAAAAAGCTGGAGAGACAC	SafeHarborchr3_2	432
GAAAACAGCTATTCGGTGCT	SafeHarborchr3_22	433
GAAAACTCCCTTGTCTTGGG	SafeHarborchr3_27	434
GAAAAGTTCTATTCAATTTG	SafeHarborchr3_47	435
GAAAAAGAGACAGAGTATGT	SafeHarborchr3_6	436
GAAAAATTGTCCCAGTACAC	SafeHarborchr4_17	437
GAAAAGACCTCATTAGAGTG	SafeHarborchr4_35	438
GAAAAGATCTCTACCAGGAA	SafeHarborchr4_36	439
GAAAAACAGACTTAGGGGAG	SafeHarborchr4_4	440
GAAAAGTAAAACCCTCTCAG	SafeHarborchr4_42	441
GAAAAGTATCTATGATGAAA	SafeHarborchr4_43	442
GAAAAGTCACACTAAAGTGT	SafeHarborchr4_44	443
GAAAAGTTAGAGTGAAGTAG	SafeHarborchr4_46	444

GAAAATAAAGGTAATGGCTG	SafeHarborchr4_48	445
GAAAAAGGCAGGCAATGGTG	SafeHarborchr4_9	446
GAAAAACTCTTAGCTTAATG	SafeHarborchr5_5	447
GAAAAATGACCCATGATTAA	SafeHarborchr6_14	448
GAAAATAAGGCATCAATAAC	SafeHarborchr6_50	449
GAAAAATAGCTTTCTCTCAA	SafeHarborchr7_11	450
GAAAAATCGCTAATATGAAA	SafeHarborchr8_13	451
GAAAAGATGAGTTATATTGG	SafeHarborchr8_37	452
GAAAAAGCAGCACTTCTTAT	SafeHarborchr8_7	453
GAAAAATTTCAGCAGACCTT	SafeHarborchr9_18	454
GAAAACACAGTCTTAATTAT	SafeHarborchr9_20	455
GAAAAGGAAGATTCTGCTGT	SafeHarborchr9_39	456
GAAAAGGTGAGGTTAAGGAG	SafeHarborchr9_40	457
GAAAATAACCACAATTATCA	SafeHarborchr9_49	458
GAAAAATAAGGTGAAGATAG	SafeHarborchrX_10	459
GAAAAATGACTGGTCACATT	SafeHarborchrX_15	460
AACTTGGCCGAGCCATTTTC	SIRT5_1	461
TCAATCGACTTGGGACAATC	SIRT5_2	462
CCACACCCGGGACGGGTTGT	SIRT5_3	463
CCAGCGGCTTGCAGCTAGAC	SLC11A2_1	464
ACTATTATGGCCCTCACATT	SLC11A2_2	465
CGAACATGCCCTTGAGTACC	SLC11A2_3	466
GCGATGTTGGCGATTAGTGC	SLC25A15_1	467
GTTGGCGATTAGTGCTGGAC	SLC25A15_2	468
AGTCGGTGAGGCCCCGGTAC	SLC25A15_3	469
TACCAGCTTTTGTACGAGTC	SLC7A11_1	470
AGCTTTTGTACGAGTCTGGG	SLC7A11_2	471
ACCCAGACTCGTACAAAAGC	SLC7A11_3	472
CCCAACGGTGGTACTCCAGC	SNX30_1	473
ACCTGCTGGAGTACCACCGT	SNX30_2	474
CCTGCTGGAGTACCACCGTT	SNX30_3	475
AGGCACCTCCGGACCCCGAG	SNX4_1	476
GCTCTGGGGTCGACACGGTG	SNX4_2	477
AGGTCAGTTGAACATACCGA	SNX4_3	478
CTGCAGCACGTCGCTTATAT	TFRC_1	479
AATATAAGCGACGTGCTGCA	TFRC_2	480
CTATACGCCACATAACCCCC	TFRC_3	481
CAAAGATACACCAGCGGCTC	TLR4_1	482
ATTCTCCCAGAACCAAACGA	TLR4_2	483
CAATCACCTTTCGGCTTTTA	TLR4_3	484
GCGGGTTTGTTGGCCACTCA	TLR7_1	485
TTATTTTTACACGGCGCACA	TLR7_2	486
AGCGCATCAAAAGCATTTAC	TLR7_3	487

TTGGAGTGATCGGCCCCCAG	TNF_1	488
AGAGCTCTTACCTACAACAT	TNF_2	489
GGAGCTGAGAGATAACCAGC	TNF_3	490
CTTCAAGTTTGTCGTCGTCG	TNFRSF10A_1	491
TGAGCTAGGTACGACCTGTG	TNFRSF10A_2	492
CTGAGCTAGGTACGACCTGT	TNFRSF10A_3	493
CCGCGGCGACAACGAGCACA	TNFRSF10B_1	494
GAGCGGCCCCACAACAAAAG	TNFRSF10B_2	495
ATAGTCCTGTCCATATTTGC	TNFRSF10B_3	496
GATGACGACGACGAACTTTA	TNFRSF10C_1	497
CGGCGTCGGGAACCATACCA	TNFRSF10C_2	498
CGATGACGACGACGAACTTT	TNFRSF10C_3	499
GCTCGAGCAGGGCGCTATCC	TNFRSF10D_1	500
AACTTCGTCCTGCCGGGGGA	TNFRSF10D_2	501
TGGGGAACTTCGTCCTGCCG	TNFRSF10D_3	502
CTTCCTTGCATTCGCACACG	TNFRSF11B_1	503
CAACCGCGTGTGCGAATGCA	TNFRSF11B_2	504
GCATTCGCACACGCGGTTGT	TNFRSF11B_3	505
ATATACCCCTCAGGGGTTAT	TNFRSF1A_1	506
ATTGGACTGGTCCCTCACCT	TNFRSF1A_2	507
CACTCCAATAATGCCGGTAC	TNFRSF1A_3	508
GAAGATCACGATCAGCACGC	TNFSF10_1	509
ACTCCGTCAGCTCGTTAGAA	TNFSF10_2	510
CGTCAGCTCGTTAGAAAGGT	TNFSF10_3	511
CCCCGGACGATATTGAACAA	TP53_1	512
CCATTGTTCAATATCGTCCG	TP53_2	513
CCATTGCTTGGGACGGCAAG	TP53_3	514
CGGGTGAACCACGAAATGGA	UBR5_1	515
CCAATACATTCAAAGGGGGG	UBR5_2	516
CCACCCCCCTTTGAATGTAT	UBR5_3	517
CGAAGGCGCCGTGGCCGATC	ULK1_1	518
GCCCTTGAAGACCACCGCGA	ULK1_2	519
GTGGCCGATCAGGTCCTTGC	ULK1_3	520
TCGCCGTGGTCTTCCGGGGG	ULK2_1	521
GACCTCGCAGATTATTTGCA	ULK2_2	522
GTCCCTTTCGCTGGTACAAA	ULK2_3	523
CGCGCGTCGTAAGTAAAGCT	VDAC2_1	524
GCGCGCGTCGTAAGTAAAGC	VDAC2_2	525
TGTTAGGAATTTTCAACGTC	VDAC2_3	526
CCTTTACTTACCTGGTCGAA	VDAC3_1	527
CCCTTCGACCAGGTAAGTAA	VDAC3_2	528
TCCTTTACTTACCTGGTCGA	VDAC3_3	529
TGATACACGTTCATCTGCCG	WIPI1_1	530

TACTTGCCGGTTCAGCCTTA	WIPI1_2	531
CCTTATGGACAAGATGTTGC	WIPI1_3	532
CCCGGCCTTACGTGTTGTCC	WIPI2_1	533
CAGGACAACACGTAAGGCCG	WIPI2_2	534
ACCAGGACAACACGTAAGGC	WIPI2_3	535
GGACTCTACTACACAGGTAT	XIAP_1	536
CATCAACACTGGCACGAGCA	XIAP_2	537
TATCAGACACCATATACCCG	XIAP_3	538

Pooled lentiviral screening requires a multiplicity of infection (MOI) less than 1 (Figure 1C; Figure 19C) for single-copy sgRNA integration per cell. Then, the iCas9/NURR1::H2B-GFP/library hPSC was directed differentiated into dopamine neurons using an established protocol. At day 25 of differentiation, 800,000 homogenous post-mitotic dopamine neurons were grafted into the bilateral striatum of the eight NOD/SCID IL2Rgnull (NSG) mice using FACS with NURR1-GFP and gRNA-tdTomato (Figure 2A; Figure 12A). Additional 2 weeks in vitro culture post sorting gave rise to highly homogenous dopamine neuron identity co-expressing NURR1::GFP and gRNA::tdTomato with a mature dopamine marker, TH, regardless of doxycycline treatment (Figure 1D; Figure 19D). The use of sorted NURR1 positive cell population allowed to cleanly examine the survival rate of post-mitotic dopamine neurons *in vivo* without any confounding factors from proliferating cells in the graft. After 1-month post-transplantation, the implanted cells were re-isolated by dissecting the graft based on fluorescence expression, which genomic DNA was extracted (Figure 1F; Figure 19F). Human-specific PCR reaction for detecting a human PTGER2 gene indicated the presence of the human cells in a xenograft sample (Figure 1G; Figure 19F). Next-generation sequencing (NGS) from genomic DNA of *in vitro* cultured cells and an *in vivo* grafted cell to detect sgRNA barcode indicated consistent sgRNA library representation *in vitro* and *in vivo* independent of Dox treatment (Figure 1H; Figure 19G).

Next, the correlation of each sgRNA incorporation ratio from all samples was compared(*in vitro* cells; day 16, day 25 no dox, day 25 plus dox, and *in vivo* grafted cells; 1-month post grafted cells from day 25 plus dox). Clustering dendrogram showed comparable sgRNA incorporation ratio regardless of dox treatment and cell stage in culture, except the grafted cell (Figure 2B; Figure 12B). In-depth analysis of each sgRNA ratio in the conditions indicated that, without dox treatment in culture, enriched

or depleted sgRNAs could not be detected. However, in dox treatment conditions, all 3 sgRNAs for BCL2L (BCLXL) were significantly depleted during the directed differentiation (Figure 2C; Figure 12C), demonstrating that BCLX is an essential gene for proper dopamine neuron induction from hPSC in the culture system. Previously, it was noted that Bcl-x knock-out mice had less dopamine neuron generation and overexpression of BCLX enhanced the derivation of dopamine neurons from neural stem cells, emphasizing hPSC-based iCas9 system with pooled sgRNA library clearly worked in dopamine neuron differentiation. Most importantly, several significantly enriched sgRNAs were identified in surviving dopamine neurons compared to the cells before grafting, based on two criteria: 1) sgRNAs above a threshold [fold change; more than 0.3 (log_2 value), and p-value; $p < 0.05$], 2) multiple different guide RNA hits targeting the same gene [fold change; more than 0.25 (log_2 value), and p-value; $p < 0.05$]. Using these criteria, surviving dopamine neurons in the graft had enriched sgRNAs corresponding to 9 genes, implying apoptosis and inflammation were major limiting pathways for the survival of post-mitotic dopamine neurons in the graft (Figures 2C and 2D; Figures 12C and 12D).

To further validate the hits, 9 genes, from the screen, stable hPSC line were generated containing only 33 sgRNAs targeting a sum of 9 genes (3 sgRNAs per a gene) with 6 non-targeting and safe harbor control guides in the iCas9/*NURR1::H2B-GFP* hPSC line (iCas9/*NURR1::H2B-GFP*/library#2). Similarly, the overall representations of sgRNAs were comparable across all conditions (Figure 1I; Figure 19H), and sgRNA incorporation was only the most distinct in the grafted cells than *in vitro* cultured cells as indicated by clustering dendrogram (Figure 2E; Figure 12E). Two-independent pooled screens with small-pooled sgRNA library using the iCas9/*NURR1::H2B-GFP*/library#2 hPSC line consistently identified p53 as the most significant hit with two or more independent sgRNAs enriched in the surviving dopamine neurons in the graft (Figure 2F; Figure 12F). Overall, these results demonstrate that post-mitotic dopamine neuron death upon transplantation would be driven by the p53-mediated cell death pathway.

p53 knockout (KO) resulted in improved dopamine neuron survival. To examine the p53 role on dopamine neuron survival *in vivo* graft, iCas9/*NURR1::H2B-GFP* hPSC line were generated with a stably incorporated sgRNA targeting p53 gene. While p53 pathway has been previously implicated in the death of exogenous fetal ventral-mesencephalic graft in rats, the p53 effect on the survival of human post-mitotic dopamine neuron in *in vivo* engraft remains unknown. Furthermore, kinetics of p53-

mediated graft death during the time-course of the transplantation is poorly investigated. Directed differentiation towards midbrain dopamine neuron lineages and purification strategy via FACS was similarly performed as the pooled-sgRNA screens for this individual p53 KO studies. Either dox treated (p53 KO) or non-treated dopamine (isogenic p53 WT) neurons were engrafted bilaterally into striatum to reduce any variability between mice (Figure 3A; Figure 13A). Notably, dopamine neurons in the p53 KO had no obvious survival benefit or excessive proliferation in culture but rather gave rise to highly pure dopamine neuron identity expressing TH and FOXA2 when examined 2 weeks post-sorting with NURR1::GFP and sgRNA::tdTomato (Figure 3B; Figure 20A).

After 1-month post-transplantation, the overall graft composition and size were determined using immunofluorescence assay followed by stereological quantification (Figure 3C-3D;Figures 4A-4C; Figures 13B-13C; Figures 20A-20D). All the surviving p53 KO dopamine neurons expressed floor-plate identity expressing FOXA2, a mature dopamine neuron marker TH, and an endogenous NURR1-GFP signal (Figures 4A-4C; Figures 20A-20D) without obvious detection of a hKi67, which marks proliferating cells, in TP53 KO or in isogenic control grafts (Figure 20E). The stereological method measured the total number and volume of surviving *NURR1::GFP* positive neurons per 100,000 cells at 1 month after grafting, and found that p53 KO graft contains 13,666.78 (mean) ± 7,797.59 (stdev) *NURR1::GFP* dopamine neurons compared to 2,775.99 (mean) ± 1,178.21 (stdev) in wild-type neurons (Figure 3D; Figure 13C). The volume of the p53 KO graft was 0.086 ± 0.060 mm^3 while the wild-type graft was 0.022 ± 0.010 mm^3 per 100,000 cells (Figure 3D; Figure 13C). Taken together, the single p53 gene KO had augmented a significant ($p < 0.05$, paired t-test, $n = 5$) survival benefits for post-mitotic dopamine neurons in the transplant, emphasizing the functional role of p53 in neuronal survival in *in vivo* implant. In addition to the increased volume and increased numbers of NURR1+ neurons, p53 KO grafts also showed an increased proportion of ALDH1A1+ A9-type dopamine neurons. In contrast, the proportion of CALB1 positive (A10) neuron was comparable between p53 KO and p53 WT grafts (Figures 2D-2G) suggesting a particular vulnerability of A9 but not A10 dopamine neuron subtype upon grafting, which is alleviated in p53 KO grafts.

Temporal analysis of p53-mediated dopamine neuron death and host response. Next, experiments were performed to address the kinetics of the p53-dependent neuronal cell death during the transplantation procedure. There would be three main stages of cell

death in the grafting paradigm: 1) Mechanical damage stress during loading cells onto the needle 2) short-term survival factors immediately post-transplantation 3) longer-term effects in established graft. First, p53 and p53 downstream genes were not induced using RT-qPCR assay during cell preparation and loading cells into the needle (Figure 4D; Figure 20F), which was consistent with a previous report demonstrating that there were no signs of apparent cell death after passaging through the needle. Next, p53 and cleaved caspase 3 (CC3) inductions were mapped using immunofluorescence assays at different time points immediately post grafting, observing a strong induction of p53 and CC3 in 30-40% of the dopamine neurons at 1-day post-transplantation (dpt) (Figures 3E and 3F; Figures 14A-14F). Such inductions were diminished at 3 dpt with signs of many apoptotic pyknotic nuclei within the graft, implying that p53-induced neurons at 1 dpt already triggered intrinsic apoptotic "suicide" program. Additionally, the rapid engagement of cell death resulted in a dramatic decrease in the grafted cell density from 1 to 3 dpt (Figures 3E and 3F; Figures 14A-14F). At 7 dpt and beyond, this dynamic programmed cell death cascade appeared to be completely absent. Furthermore, a TUNEL assay to probe for any apoptotic DNA fragmentation and detect the last phase of apoptosis showed a strong TUNEL positive expression among 60% of grafted neurons starting at 1 dpt and the peak TUNEL positive signals at 3 dpt in the graft (Figures 3E and 3F; Figures 14A-14F). Again, the TUNEL signal was completely abolished at 7 dpt, implying this apoptotic program might no longer continue beyond 7 dpt.

Next, neuroimmune cells near the graft site were characterized since published studies indicated apoptotic neurons recruit neighboring macrophages which clear apoptotic cells. Together with the finding that high induction of apoptosis appears in the graft a 1 dpt, both astrocyte and microglia rapidly and concurrently polarized their processes towards the transplants (Figure 5B and 5C). At 4 hours and at 1 dpt, most glial cells were located outside the graft core, suggesting that they are respond to rather than driving the initial wave of dopamine neuron cell death (Figure 21A). At 3 dpt, immune response peaked, but the extent of polarization was more obvious in microglia than in astrocyte where microglia migrated further and fully encapsulated apoptotic cell bodies by infiltrating into the graft core by day 3 dpt (Figure 21A, upper panels), which was further corroborated by existing literature, reporting the coordinated behaviors of astrocytes and microglia (Figures 5B-5D). GFAP staining, more specific to A1-activated type of astrocytes, were also more strongly induced in the host cells at 3 dpt, suggesting that activated microglia could further recruit neurotoxic reactive astrocytes (Figure 5C).

Strong GFAP staining persisted by 7 dpt creating an "astrocyte border" at the graft host interface (Figure 21A, lower panels). Also, evidence of vascular recruitment to the graft at 3 dpt by H&E staining (Figure 5A; Figure 21B) and neutrophil invasion marked by Ly6G present prior to 24 hours post grafting (Figure 5E; Figure 21C). Despite the transplanted neurons undergoing apoptosis and engulfed by immune cells, simultaneously surviving neurons began to extend axons at 1 dpt as indicated by SC121 positive fiber outgrowth from the implants (Figure 6; Figure 21D). Overall, these data revealed that p53-dependent apoptotic cell death of hPSC-derived post-mitotic dopamine neurons *in vivo* post grafting occurred rapidly in a very narrow window during the first 3 dpt in which brain immune cells were heavily recruited towards the graft.

TNFα-NFκB pathway was an upstream regulator triggering TP53-dependent dopamine neuron death in the graft. Given a well-known p53 role as a tumor suppressor and high induction of p53 in the transplant at 1 dpt, it was sought to identify upstream factors of p53 induction. To gain a molecular mechanistic insight on the potential trigger of p53, a bulk RNA seq was performed to compare grafted neurons that were isolated 1 dpt from mouse brain with FACS-based sorted neurons at day 0 and *in vitro* culture neurons at 1-day post sorting. Principal component analysis (PCA) and dendrogram demonstrated that the grafted dopamine neurons exhibited a distinct transcriptional pattern compared to either sorted or *in vitro* cultured dopamine neurons (Figure 7A; Figure 15A). In particular, gene ontology analysis of the 279 differentially expressed genes (DEG) upregulated in 1 day *in vivo* grafted neurons versus 1-day *in vitro* cultured neurons showed that TNF alpha signaling via NF kappa B, apoptosis, hypoxia, and p53 pathways were significantly upregulated in the grafted neurons (Figures 7B and 7C; Figures 15B and 15C) whereas the 374 DEG downregulated in the *in vivo* grafted versus cultured neurons were associated with apical junction, mTORC1 signaling, and cholesterol homeostasis (Figure 22B). Gene Set Enrichment Analysis (GSEA) of enriched genes in day 1 grafted neurons *vs.* day 1 *in vitro* plated neurons further confirmed increased signatures, including apoptosis and tumor necrosis factor signaling pathways (Figures 7D and 7E; Figures 15D and 15E).

Next, the phosphorylated form of nuclear factor kappa B (pNFκB) as an indication of active form of NFκB under TNF stimulation during the time course upon transplantation was examined. Time-course immunofluorescence assay during the transplantation indicated that pNFκB expression was heavily increased within the grafted

neurons at 4 hours and 1 dpt, marking nearly 80% of the grafted neurons, and such induction was decreased within the graft beyond 3 dpt (Figure 7F; Figure 15F). At 1 day post plating, nuclear NFκB expression was not induced in dopamine neurons replaced *in vitro* (Figure 22D). This result demonstrated that TNFα-NFκB signaling cascade immediately occurred after dopamine neuron engraftment prior to the p53-dependent apoptosis, suggesting a potential upstream role of TNFα-NFκB pathway on p53.

To validate the causal link of TNFα-mediated NFκB and p53 induction, treatments with a TNFα-blocking monoclonal antibody called adalimumab were administered at day 25 *NURR1::H2B-GFP* sorted neurons for 24 hours (Figure 7G; Figure 15G). Upon TNFα treatment, dopamine neurons increased p53 expression, which exhibited co-positive with NFκB-p65. In addition, p53 and p53 downstream genes, such as p21 and PUMA mRNA were upregulated in the TNFα exposure conditions while co-treatment with TNFα and adalimumab abolished such inductions (Figure 7G and 7H; Figure 15G-15I), confirming a TNFα-NFκB-p53 axis in this established *in vitro* model system.

Single-cell RNA sequencing of grafted neurons revealed p53-BAX axis is a key driver for neuronal death during transplantation. In order to gain a molecular landscape of grafted dopamine neurons at a single cell level, a single cell mRNA sequencing from p53 WT and KO grafted neurons from the mouse brain at 1 dpt was performed. Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) analysis of wild-type and p53 knock-out (KO) samples showed a highly overlapping clustering distribution (Figures 8B and 8C). Based on PBX1 for a dopamine-specific marker and MAP2 expression for neurons, >90% of the cells exhibited post-mitotic dopamine neuron identity with very low expression of the proliferation marker, *MK167*, in both p53 WT and KO neurons (Figures 8D and 8E). By comparing these data to an available human fetus dataset, the majority of these cells, which occupy one large cluster, were annotated as neuroblasts, which indicated NURR1 expressing stage, and a small cluster as a floor plate progenitor (Figure 9A).

Then, it was sought to determine whether any specific clusters showed molecular signatures of neuronal death. Heatmap from apoptotic cell-death related genes identified that clusters 3, 4, and 7 (Figures 9B-9D) showed increased genes, such as BAX, BAD, TNFRSF1A, TNFRSF12A, and TNFRSF10B. Violin plots of these genes from each cluster further demonstrated that these genes were more significantly expressed in WT

than p53 KO neurons, supporting that TNF induced p53-BAX genes triggering p53 dependent apoptosis pathway in the graft (Figure 9E).

Questions remain regarding the source of the TNFα ligands. This dataset points out that grafted dopamine neurons expressed TNFα superfamily ligands, potentially hinting that dying neurons secreted TNFα themselves upon acute post-traumatic injury (data not shown).

High throughput cell surface marker screen defined novel CD markers to enrich post-mitotic dopamine neurons. Given the complication of using genetically engineered hPSC for translational application, it was sought to define surface markers to capture post-mitotic dopamine neurons obviating the need for genetic selection. A high-throughput flow-based cell surface marker screen with 385 validated antibodies identified the candidate CD markers with three depletion hits (CD49e, CD99, CD340) and two enrichment hits (CD184, CD171) to match the GFP signal from *NURR1::H2B-GFP* hPSC derived dopamine neurons at day 25 (Figures 6 and 10A; Figures 21D and 17A). After extensively characterizing sorted cells either using a single and/or double CD markers via flow analysis, qRT-PCR, and immunofluorescence assay to examine dopamine neuron identity, it was found that CD49e low and CXCR4/CD184 high expressing cell showed the most highly enriched pure post-mitotic dopamine neurons, expressing FOXA2, NURR1::GFP, and TH 2 weeks post sorting *in vitro* (Figures 10B-10D; Figures 17B-17D). Moreover, the short-term histology analysis at 1-month post-transplantation demonstrated that CD-sorted dopamine neurons using this novel double sorting strategy exhibited graft survival expressing TH homogeneously without any human Ki67 proliferating cells, emphasizing on translation benefits to use enriched post-mitotic dopamine neurons via the novel CD marker strategy for transplantation (Figures 10E and 11B; Figures 17E and 24B).

TNFα neutralizing monoclonal antibodies improved the survival of CD marker sorted post-mitotic dopamine neurons in the graft. Next, it was examined whether a publicly available TNFα neutralizing antibody called adalimumab (Humira) could have functional significance on CD-sorted dopamine neuron survival. The adalimumab has been reported to bind to soluble and transmembrane bound TNFα (McCoy and Tansey, 2008) and is widely used to treat arthritis and encephalitis to subdue the inflammation. Co-injection with adalimumab significantly improved the survival of CD marker sorted dopamine neurons *in vivo* post-transplantation (Figures 10F and 10G; Figure 18A and 18B). Stereological counts of NURR1::GFP+ cells at 1 month post grafting found 12,423

(mean) ± 1,859 (S.E.M) in adalimumab treated grafts per 100,000 cells injected versus 6,057 (mean) ± 378 (S.E.M) in PBS treated neurons ($p = 0.01$, paired t-test, $n = 5$) (Figure 18B). The volume of the adalimumab treated graft was 0.1175 mm^3 (mean) ± 0.003 mm^3 (S.E.M) per 100,000 cells in PBS-treated grafts ($p = 0.0085$, paired t-test, $n = 5$). Furthermore, it was examined whether CD sorted postmitotic dopamine neurons, with or without adalimumab treatment, are functional in rescuing amphetamine-induced rotation behaviors in 6-OHDA treated mice, which represents a widely used preclinical model for treating PD motor symptoms. Robust functional recovery, regardless of adalimumab exposure, was observed at 6 months post grafting. Adalimumab-co-injected mDA neurons showed a trend towards an earlier and more complete functional rescue (Figure 10H; Figure 18C). Histological analyses showed that both groups give rise to highly enriched neuronal population *in vivo* at 6 months post implantation (Figures 25A-25B). However, TNFα inhibition via adalimumab resulted in significantly increased total numbers of surviving dopamine neurons as well as overall graft size (Figure 18D and 18E). Similar to the results with p53 KO cells, we observed that adalimumab treatment results in significant increase in the proportion of ALDH1A1 + A9 mDA neuron subtype without affecting the fraction of CALB1 expressing A10 dopamine neuron subtype (Figures 18F and 18G). These results implied the CD marker sorting and TNFα neutralizing antibody strategies could be directly applied for clinical translation.

Discussion

The main gene identified in presently disclosed *in vivo* Crispr screen for dopamine neuron survival was *TP53*. TP53 is a master regulator of diverse cellular processes ranging from tumor suppression to serving as a guardian of cell fate identity and reprogramming to sensing cellular stresses related to DNA damage, oxidative stress, or ischemic injury among others. TP53 has been recently implicated as a candidate factor in driving the vulnerability of human substantia nigra dopamine neurons during PD pathogenesis based on selective expression patterns by single cell analysis. Furthermore, there has been interest in the role of TP53 as a general signaling hub of neuronal cell death across neurodegenerative disorders such as the p53-mediated regulation of C9ORF72-mediated neuronal death in ALS (Maor-Nof et al., 2021). The presently disclosed initial screen identified several genes in addition to TP53 as limiting *in vivo* dopamine neuron survival including *TNFRSF11B, BBC3, BCL2L11, CASP2, CASP9,* genes that are all linked to the TNFα/TP53/apoptotic pathway. Alternative hits such as

SLC7A11, IL18 or *TLR4* are associated with either ferroptosis or neuroinflammatory responses respectively.

The present disclosure focused on cell intrinsic factors driving dopamine neuron death by screening for genes limiting survival directly in purified dopamine neurons and without manipulating any host-related responses. Histological analyses were used to describe a cascade of host cell-related responses at the graft site including the recruitment of neutrophils, microglial and vascular cells that enter the graft core by 3 dpt, and the activation of inflammatory programs in both astrocytes and microglia. The presently disclosed temporal analysis suggests that host responses occur in response to rather than mediating dopamine neuron death. The grafting-related dopamine neuron death had subsided completely by 7 dpt.

The presently disclosed gene expression data demonstrates that TNFα is produced by grafted dopamine neurons post transplantation, possibly in response to injury-related damage. TNFα is known as an inflammatory cytokine secreted in response to hemorrhagic, ischemic or traumatic injury (Tuttolomondo et al., 2014), events commonly associated with cell transplantation.

The use of a genetic reporter line to purify postmitotic dopamine neurons allowed to address the survival without any confounding factors related to cell proliferation or non-autonomous factors. This approach also sets the stage for grafting postmitotic dopamine neurons for translational applications. The presently disclosed CD surface marker-based strategy enables dopamine neuron enrichment at the NURR1 stage without the need for establishing genetic reporter lines. Therefore, this strategy is compatible for translational applications for dopamine neuron replacement therapy in PD. It can also facilitate the use of purified dopamine neuron preparations in human iPSC-based disease modeling given the challenge in reliably generating specific neuron subtypes across many cell lines and laboratories when modeling neural disorders in a dish.

The presently disclosed study demonstrates that co-injection of a TNF neutralizing antibody can greatly reduce neuronal death post grafting when combined with the CD marker strategy to enrich for post-mitotic dopamine neurons. This technology could result in cell therapy approach that maximizes safety for PD patients by avoiding the transplantation of dopamine neuron precursors that retain at least a short-term potential for in vivo cell proliferation (Kim et al., 2021, Cell Stem Cell Feb 4;28(2):343-355.e5; Kirkeby et al., 2017, Cell Stem Cell 20, 135-148.; Piao et al., 2021,

Cell Stem Cell 28, 217-229 e217; Schweitzer et al., 2020, N Engl J Med 382, 1926-1932; Takahashi, Neuron. 2017 Sep 13;95(6):1395-1405.e3).

Example 2 – Single cell RNA sequencing of day 1 grafted neurons identifies molecular signature of surviving dopamine neurons and de-differentiation signature in apoptotic dopamine neurons.

To further define the transcriptional landscape of grafted dopamine neurons, single cell mRNA sequencing was performed from p53 wild-type (WT) and p53 knock-out (KO) grafted neurons, re-isolated from the mouse brain at 1 dpt. Combined clustering of p53 WT and p53 KO cells showed highly overlapping distribution of clusters, implying that the cell state is not changed in dopamine neuron upon p53 KO (Figure 16A and 16B; Figure 23A-23C). Based on *PBX1*, a marker particularly highly expressed in dopamine neurons, and on the expression of the neuronal marker *MAP2*, >90% of the cells exhibited features of postmitotic, dopamine neuron identity with very low expression of the proliferation marker, *MKI67*, in both p53 WT and KO neurons (Figures 23B). By comparing our data to an available dataset from human fetal dopamine neuron development, most of the cells isolated at 1dpt were annotated as neuroblasts, compatible with early NURR1 positive identity while a small fraction of the cells was annotated as floor plate progenitors (Figure 16C).

Clusters were identified that displayed molecular signatures of apoptosis or survival. Differential analysis of p53 WT versus p53 KO identified clusters 3, 5, and 6 with significantly increased expression of p53 downstream genes, such as *BAX*, *BBC3*, *CDKN1A*, and *PHPT1* (Figures 16D-16F). In these clusters, TNFRSF12A was more robustly expressed in both p53WT and p53KO cells relative to the other clusters, supporting our findings that TNFα mediates a TP53-dependent apoptosis pathway in the graft (Figure 16F). Interestingly, apoptosing clusters showed expression of SOX2 and HES5 and were annotated as floor plate progenitors. However, evidence of proliferation based on *MKI67* expression was not observed, suggesting that this cluster may reflect a dedifferentiation state rather than a bona fide, proliferating floor plate progenitor signature (Figures 16G and 16H). Furthermore, the progenitor signature was specific to immediately grafted neurons (1 dpt) and not observed in matched cells immediately pre-engraftment cells (Figure 16H). These data argue that the progenitor-like phenotype is induced in those clusters within 24 hours post-engraftment and likely does not represent a contamination from imperfect sorting.

Conversely, clusters 1, 2, and 4 show high expression of survival signature characterized by the expression of ATF4, JUN, FOS, HSPA6, HSPA1A, HSPA1B, and DNAJB1 (Figure 16D), highlighting that hPSC-derived dopamine neurons are under high cellular stress during engraftment procedure such as due to axotomy, endoplasmic reticulum stress, or DNA damage. Differential analysis between p53 WT and KO identified the survival marker JUN as significantly upregulated upon p53 KO (Figures 16F and 23D), emphasizing that blocking p53 expression imparts survival benefits to grafted dopamine neurons.

Questions remain regarding the source of the TNFα ligands. This dataset points out that grafted dopamine neurons show increased expression of TNFα superfamily ligands such as TNFSF12 and TNFSF10 (Figure 22E) compared to their *in vitro* counterpart.

Although the presently disclosed subject matter and its advantages have been described in detail, it should be understood that various changes, substitutions and alterations can be made herein without departing from the spirit and scope of the present disclosure. Moreover, the scope of the present application is not intended to be limited to the particular embodiments of the process, machine, manufacture, and composition of matter, means, methods and steps described in the specification. As one of ordinary skill in the art will readily appreciate from the present disclosure of the presently disclosed subject matter, processes, machines, manufacture, compositions of matter, means, methods, or steps, presently existing or later to be developed that perform substantially the same function or achieve substantially the same result as the corresponding embodiments described herein may be utilized according to the presently disclosed subject matter. Accordingly, the appended claims are intended to include within their scope such processes, machines, manufacture, compositions of matter, means, methods, or steps.

Various patents, patent applications, publications, product descriptions, protocols, and sequence accession numbers are cited throughout this application, the present disclosures of which are incorporated herein by reference in their entireties for all purposes.

WHAT IS CLAIMED IS:

1. A method for treating a subject, comprising administering to the subject one or more midbrain dopamine (mDA) neurons, wherein p53-mediated apoptosis of the one or more mDA neurons is suppressed.

2. The method of claim 1, wherein the subject suffers from a neurodegenerative disorder and/or neurodegeneration of midbrain dopamine neurons.

3. The method of claim 2, wherein the neurodegenerative disorder is selected from the group consisting of Parkinson's disease, Huntington's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), frontotemporal dementia, and combinations thereof.

4. The method of any one of claims 1-3, wherein the suppression of p53-mediated apoptosis comprises a) administering to the subject at least one compound selected from the group consisting of tumor necrosis factor alpha (TNFα) inhibitors, nuclear factor kappa B (NFκB) inhibitors, p53 inhibitors, and combinations thereof; or b) contacting the one or more mDA neurons with at least one compound selected from the group consisting of TNFα inhibitors, NFκB inhibitors, p53 inhibitors, and combinations thereof.

5. The method of claim 4, wherein the suppression of p53-mediated apoptosis comprises administering to the subject a TNFα inhibitor.

6. The method of claim 4 or 5, comprising administering the one or more mDA neurons simultaneously with the administration of the at least one compound.

7. A method of improving *in vivo* survival of one or more midbrain dopamine (mDA) neurons, comprising suppressing p53-mediated apoptosis of the one or more mDA neurons.

8. The method of claim 7, wherein the suppression of p53-mediated apoptosis comprises contacting the one or more mDA neurons with a compound selected from the group consisting of TNFα inhibitors, NFκB inhibitors, p53 inhibitors, and combinations thereof.

9. The method of claim 8, wherein the suppression of p53-mediated apoptosis comprises contacting the one or more mDA neurons with a TNFα inhibitor.

10. The method of any one of claims 1-9, wherein the suppression of p53-mediated apoptosis comprises inhibition of TNFα signaling, inhibition of NFκB signaling, inhibition of p53 signaling, or a combination of the foregoing.

11. The method of any one of claims 4, 5, 8, and 9, wherein the TNFα inhibitor is selected from the group consisting of anti-TNFα antibodies, TNFα decoy receptors, chemical compounds, nucleic acid inhibitors, small molecule inhibitors, receptor biologic inhibitors, inactive TNF fragments, TNFα circulating receptor fusion protein, xanthine derivatives, 5-HT_{2A} agonist, and combinations thereof.

12. The method of claim 11, wherein the TNFα inhibitor is an anti-TNFα antibody.

13. The method of claim 12, wherein the anti-TNFα antibody is selected from the group consisting of adalimumab, adalimumab-adbm, adalimumab-adaz, adalimumab-atto, certolizumab pegol, golimumab, infliximab, infliximab-abda, infliximab-dyyb, remtolumab, afelimomab, nerelimomab, ozoralizumab, placulumab, and combinations thereof.

14. The method of claim 13, wherein the anti-TNFα antibody is adalimumab.

15. The method of claim 4 or 8, wherein the NFκB inhibitor is selected from the group consisting of upstream inhibitors of NFκB, inhibitors of IKK activity, inhibitors of IκB phosphorylation, inhibitors of IκB degradation, proteasome inhibitors, protease inhibitors, inhibitors of NFκB nuclear translocation and expression, NFκB DNA-binding inhibitors, and NFκB transactivation inhibitors, inhibitors of NFκB directed gene transactivation, antioxidants, and combinations thereof.

16. The method of claim 4 or 8, wherein the p53 inhibitor is selected from the group consisting of JNK inhibitors, p38 MAPK inhibitors, caspase inhibitors, puma/BBC3 inhibitors, BAX inhibitors, CDK inhibitors, MDM2 and MDMX activators, and combinations thereof.

17. The method of any one of claims 1-16, wherein the suppression of p53-mediated apoptosis comprises knocking out or knocking down *TP53* gene in the one or more mDA.

18. The method of claim 17, wherein the *TP53* gene is knocked out or knocked down by a gene-engineering system.

19. The method of claim 18, wherein the gene-engineering system is a CRISPR-Cas system.

20. The method of any one of claims 1-19, wherein the one or more mDA neurons express a marker selected from the group consisting of EN1, OTX2, TH, NURR1, FOXA2, LMX1A, PITX3, LMO3, SNCA, ADCAP1, CHRNA4, ALDH1A1, SOX6, WNT1, DAT, VMAT2, GIRK2, SATB1, CALB1, CALB2, SNCG, PBX1, and combinations thereof.

21. The method of any one of claims 1-20, wherein the one or more mDA neurons are post-mitotic mDA neurons.

22. The method of any one of claims 1-21, wherein the one or more mDA neurons are *in vitro* differentiated from one or more stem cells.

23. The method of claim 22, wherein the one or more stem cells are selected from the group consisting of human stem cells, nonhuman primate stem cells, rodent nonembryonic stem cells, human embryonic stem cells, nonhuman primate embryonic stem cells, rodent embryonic stem cells, human induced pluripotent stem cells, nonhuman primate induced pluripotent stem cells, rodent induced pluripotent stem cells, and human recombinant pluripotent cells, nonhuman primate recombinant pluripotent cells, and rodent recombinant pluripotent cells.

24. The method of claim 22 or 23, wherein the one or more stem cells are human stem cells.

25. The method of any one of claims 22-24, wherein the one or more stem cells are one or more pluripotent stem cells or multipotent stem cell.

26. The method of any one of claims 22-25, wherein the one or more stem cells are selected from the group consisting of embryonic stem cells, induced pluripotent stem cells, and combinations thereof.

27. The method of claim 26, wherein the one or more stem cells are one or more induced pluripotent stem cells.

28. The method of claims 22-27, wherein the *in vitro* differentiation comprises contacting the one or more stem cells with at least one inhibitor of Small Mothers Against Decapentaplegic (SMAD) signaling, at least one activator of Sonic hedgehog (SHH) signaling, and at least one activator of wingless (Wnt) signaling.

29. The method of claim 28, wherein the concentration of the at least one activator of Wnt signaling that is contacted with the cells is increased between about 2 days and about 6 days from the initial contact of the cells with the at least one activator of Wnt signaling.

30. The method of claim 28 or 29, wherein the concentration of the at least one activator of Wnt signaling that is contacted with the cells is increased by between about 250% and about 1800% of the initial concentration of the at least one activator of Wnt signaling contacted with the cells.

31. The method of any one of claims 28-30, wherein the at least one activator of Wnt signaling comprises an inhibitor of glycogen synthase kinase 3β (GSK3β) signaling.

32. The method of any one of claims 28-31, wherein the at least one activator of Wnt signaling is selected from the group consisting of CHIR99021, CHIR98014, AMBMP hydrochloride, LP 922056, Lithium, deoxycholic acid, BIO, SB-216763, Wnt3A, Wnt1, Wnt5a, derivatives thereof, and combinations thereof.

33. The method of claim 32, wherein the at least one activator of Wnt signaling comprises CHIR99021.

34. The method of any one of claims 28-33, wherein the at least one inhibitor of SMAD signaling comprises an inhibitor of TGFβ/Activin-Nodal signaling, an inhibitor of bone morphogenetic protein (BMP) signaling, or a combination of the foregoing.

35. The method of claim 34, wherein the at least one inhibitor of TGFβ/Activin-Nodal signaling is selected from the group consisting of SB431542, derivatives of SB431542, and combinations thereof.

36. The method of claim 35, wherein the derivative of SB431542 comprises A83-01.

37. The method of any one of claims 34-36, wherein the at least one inhibitor of TGFβ/Activin-Nodal signaling comprises SB431542.

38. The method of claim 34, wherein the at least one inhibitor of BMP signaling is selected from the group consisting of LDN193189, Noggin, dorsomorphin, derivatives of LDN193189, derivatives of Noggin, derivatives of dorsomorphin, and combinations thereof.

39. The method of claim 38, wherein the at least one inhibitor of BMP comprises LDN-193189.

40. The method of any one of claims 28-39, wherein the at least one activator of SHH signaling is selected from the group consisting of SHH proteins, Smoothened agonists (SAG), and combinations thereof.

41. The method of claim 40, wherein the SHH protein is selected from the group consisting of recombinant SHHs, modified N-terminal SHHs, and combinations thereof.

42. The method of claim 41, wherein the modified N-terminal SHH comprises two isoleucines at the N-terminus.

43. The method of claim 41 or 42, wherein the modified N-terminal SHH has at least about 90% sequence identity to an un-modified N-terminal SHH.

44. The method of claim 43, wherein the un-modified N-terminal SHH is an un-modified mouse N-terminal SHH or an un-modified human N-terminal SHH.

45. The method of any one of claims 41-43, wherein the modified N-terminal SHH comprises SHH C25II.

46. The method of claim 40, wherein the SAG comprises purmorphamine.

47. The method of claim any one of claims 28-46, wherein the *in vitro* differentiation further comprises contacting the one or more stem cells with at least one activator of fibroblast growth factor (FGF) signaling.

48. The method of claim 47, wherein the at least one activator of FGF signaling is selected from the group consisting of FGF18, FGF17, FGF8a, FGF8b, FGF4, FGF2, and combination thereof.

49. The method of claim 47 or 48, wherein the at least one activator of FGF signaling comprises FGF18 or FGF8.

50. The method of claim any one of claims 28-49, wherein the *in vitro* differentiation further comprises contacting the one or more stem cells with at least one inhibitor of Wnt signaling.

51. The method of claim 50, wherein the at least one inhibitor of Wnt signaling is selected from the group consisting of IWP2, IWR1-endo, XAV939, IWP-O1, Wnt-C59, IWP-L6, and ICG-001, and combinations thereof.

52. The method of claim 50 or 51, wherein the at least one inhibitor of Wnt signaling comprises IWP2.

53. The method of any one of claims 1-52, wherein the one or more mDA neurons express a detectable level of CD184 and do not express a detectable level of CD49e.

54. A composition comprising:

(a) one or more midbrain dopamine (mDA) neurons; and

(b) at least one compound selected from the group consisting of TNFα inhibitors, NFκB inhibitors, p53 inhibitors, and combinations thereof.

55. The composition of claim 54, wherein the composition is a pharmaceutical composition further comprising a pharmaceutically acceptable carrier.

56. The composition of claim 54 or 55, wherein the composition is for treating or ameliorating a neurodegenerative disorder and/or neurodegeneration of midbrain dopamine neurons.

57. The composition of claim 56, wherein the neurodegenerative disorder is selected from the group consisting of Parkinson's disease, Huntington's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), frontotemporal dementia, and combinations thereof.

58. The composition of any one of claims 54-57, wherein the TNFα inhibitor is selected from the group consisting of anti-TNFα antibodies, TNFα decoy receptors, chemical compounds, nucleic acid inhibitors, small molecule inhibitors, receptor biologic inhibitors, inactive TNF fragments, TNFα circulating receptor fusion protein, xanthine derivatives, 5-HT_{2A} agonist, and combinations thereof.

59. The composition of claim 58, wherein the TNFα inhibitor is an anti-TNFα antibody.

60. The composition of claim 59, wherein the anti-TNFα antibody is selected from the group consisting of adalimumab, adalimumab-adbm, adalimumab-adaz, adalimumab-atto, certolizumab pegol, golimumab, infliximab, infliximab-abda, infliximab-dyyb, remtolumab, afelimomab, nerelimomab, ozoralizumab, placulumab, and combinations thereof.

61. The composition of claim 59, wherein the anti-TNFα antibody is adalimumab.

62. The composition of any one of claims 54-61, wherein the NFκB inhibitor is selected from the group consisting of upstream inhibitors of NFκB, inhibitors of IKK activity, inhibitors of IκB phosphorylation, inhibitors of IκB degradation, proteasome inhibitors, protease inhibitors, inhibitors of NFκB nuclear translocation and expression, NFκB DNA-binding inhibitors, and NFκB transactivation inhibitors, inhibitors of NFκB directed gene transactivation, antioxidants, and combinations thereof.

63. The composition of any one of claims 54-62, wherein the p53 inhibitor is selected from the group consisting of JNK inhibitors, p38 MAPK inhibitors, caspase inhibitors, puma/BBC3 inhibitors, BAX inhibitors, CDK inhibitors, MDM2 and MDMX activators, and combinations thereof.

64. The composition of any one of claims 54-63, wherein the one or more mDA neurons express a marker selected from the group consisting of EN1, OTX2, TH,

NURR1, FOXA2, LMX1A, PITX3, LMO3, SNCA, ADCAP1, CHRNA4, ALDH1A1, SOX6, WNT1, DAT, VMAT2, GIRK2, SATB1, CALB1, CALB2, SNCG, PBX1, and combinations thereof.

65. The composition of any one of claims 54-64, wherein the one or more mDA neurons are post-mitotic mDA neurons.

66. The composition of any one of claims 54-65, wherein the one or more mDA neurons are *in vitro* differentiated from one or more stem cells.

67. The composition of claim 66, wherein the one or more stem cells are selected from the group consisting of human stem cells, nonhuman primate stem cells, rodent nonembryonic stem cells, human embryonic stem cells, nonhuman primate embryonic stem cells, rodent embryonic stem cells, human induced pluripotent stem cells, nonhuman primate induced pluripotent stem cells, rodent induced pluripotent stem cells, and human recombinant pluripotent cells, nonhuman primate recombinant pluripotent cells, and rodent recombinant pluripotent cells.

68. The composition of claim 66 or 67, wherein the one or more stem cells are human stem cells.

69. The composition of any one of claims 66-68, wherein the one or more stem cells are one or more pluripotent stem cells or multipotent stem cell.

70. The composition of any one of claims 66-69, wherein the one or more stem cells are selected from the group consisting of embryonic stem cells, induced pluripotent stem cells, and combinations thereof.

71. The composition of claim 70, wherein the one or more stem cells are one or more induced pluripotent stem cells.

72. The composition of claims 66-71, wherein the *in vitro* differentiation comprises contacting the one or more stem cells with at least one inhibitor of Small Mothers Against Decapentaplegic (SMAD) signaling, at least one activator of Sonic hedgehog (SHH) signaling, and at least one activator of wingless (Wnt) signaling.

73. The composition of claim 72, wherein the concentration of the at least one activator of Wnt signaling that is contacted with the cells is increased between about 2 days and about 6 days from the initial contact of the cells with the at least one activator of Wnt signaling.

74. The composition of claim 72 or 73, wherein the concentration of the at least one activator of Wnt signaling that is contacted with the cells is increased by between about 250% and about 1800% of the initial concentration of the at least one activator of Wnt signaling contacted with the cells.

75. The composition of any one of claims 72-74, wherein the at least one activator of Wnt signaling comprises an inhibitor of glycogen synthase kinase 3β (GSK3β) signaling.

76. The composition of any one of claims 72-75, wherein the at least one activator of Wnt signaling is selected from the group consisting of CHIR99021, CHIR98014, AMBMP hydrochloride, LP 922056, Lithium, deoxycholic acid, BIO, SB-216763, Wnt3A, Wnt1, Wnt5a, derivatives thereof, and combinations thereof.

77. The composition of claim 76, wherein the at least one activator of Wnt signaling comprises CHIR99021.

78. The composition of any one of claims 72-77, wherein the at least one inhibitor of SMAD signaling comprises an inhibitor of TGFβ/Activin-Nodal signaling, an inhibitor of bone morphogenetic protein (BMP) signaling, or a combination of the foregoing.

79. The composition of claim 78, wherein the at least one inhibitor of TGFβ/Activin-Nodal signaling is selected from the group consisting of SB431542, derivatives of SB431542, and combinations thereof.

80. The composition of claim 79, wherein the derivative of SB431542 comprises A83-01.

81. The composition of claim 79, wherein the at least one inhibitor of TGFβ/Activin-Nodal signaling comprises SB431542.

82. The composition of any one of claims 78-81, wherein the at least one inhibitor of BMP signaling is selected from the group consisting of LDN193189, Noggin,

dorsomorphin, derivatives of LDN193189, derivatives of Noggin, derivatives of dorsomorphin, and combinations thereof.

83. The composition of claim 82, wherein the at least one inhibitor of BMP comprises LDN-193189.

84. The composition of any one of claims 72-83, wherein the at least one activator of SHH signaling is selected from the group consisting of SHH proteins, Smoothened agonists (SAG), and combinations thereof.

85. The composition of claim 84, wherein the SHH protein is selected from the group consisting of recombinant SHHs, modified N-terminal SHHs, and combinations thereof.

86. The composition of claim 85, wherein the modified N-terminal SHH comprises two isoleucines at the N-terminus.

87. The composition of claim 85 or 86, wherein the modified N-terminal SHH has at least about 90% sequence identity to an un-modified N-terminal SHH.

88. The composition of claim 87, wherein the un-modified N-terminal SHH is an un-modified mouse N-terminal SHH or an un-modified human N-terminal SHH.

89. The composition of any one of claims 85-88, wherein the modified N-terminal SHH comprises SHH C25II.

90. The composition of claim 84, wherein the SAG comprises purmorphamine.

91. The composition of claim any one of claims 72-90, wherein the *in vitro* differentiation further comprises contacting the one or more stem cells with at least one activator of fibroblast growth factor (FGF) signaling.

92. The composition of claim 91, wherein the at least one activator of FGF signaling is selected from the group consisting of FGF18, FGF17, FGF8a, FGF8b, FGF4, FGF2, and combination thereof.

93. The composition of claim 91 or 92, wherein the at least one activator of FGF signaling comprises FGF18 or FGF8.

94. The composition of any one of claims 72-93, wherein the *in vitro* differentiation further comprises contacting the one or more stem cells with at least one inhibitor of Wnt signaling.

95. The composition of claim 94, wherein the at least one inhibitor of Wnt signaling is selected from the group consisting of IWP2, IWR1-endo, XAV939, IWP-O1, Wnt-C59, IWP-L6, and ICG-001, and combinations thereof.

96. The composition of claim 94 or 95, wherein the at least one inhibitor of Wnt signaling comprises IWP2.

97. The composition of any one of claims 54-96, wherein the one or more mDA neurons express a detectable level of CD184 and do not express a detectable level of CD49e.

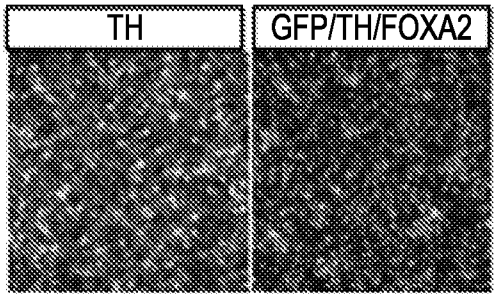


FIGURE 1A

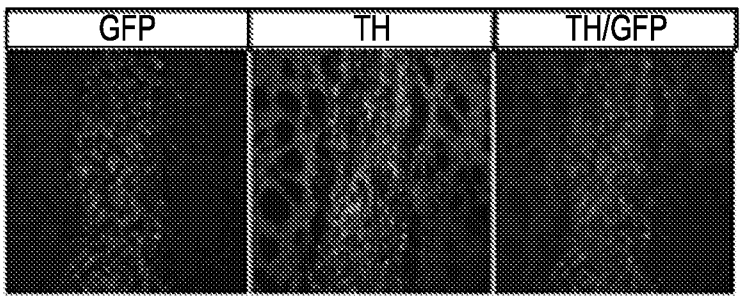


FIGURE 1B

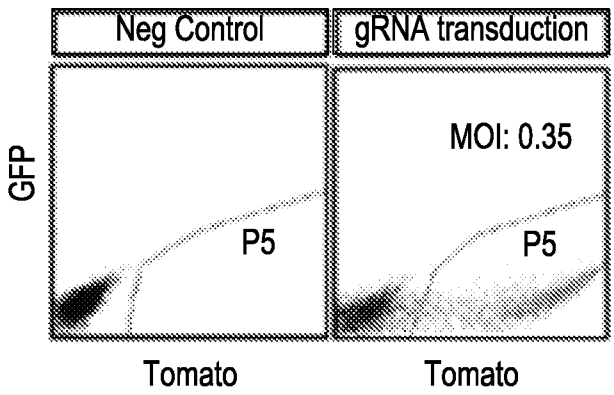


FIGURE 1C

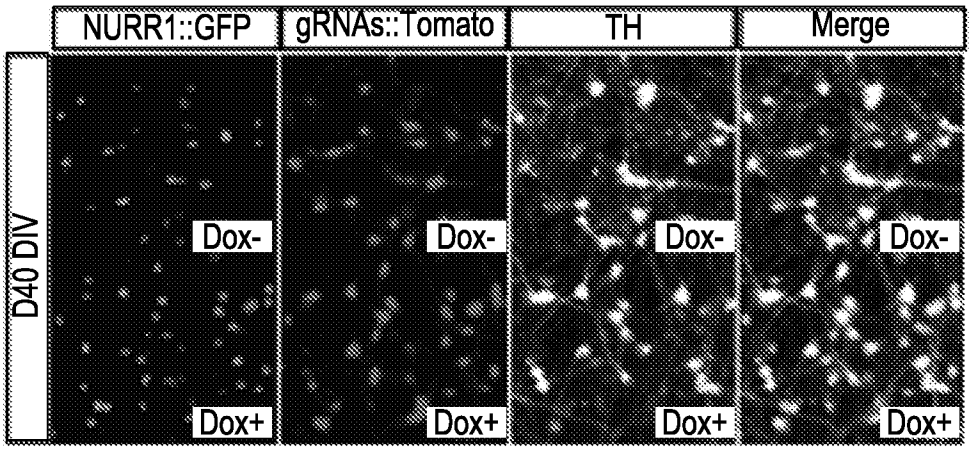


FIGURE 1D

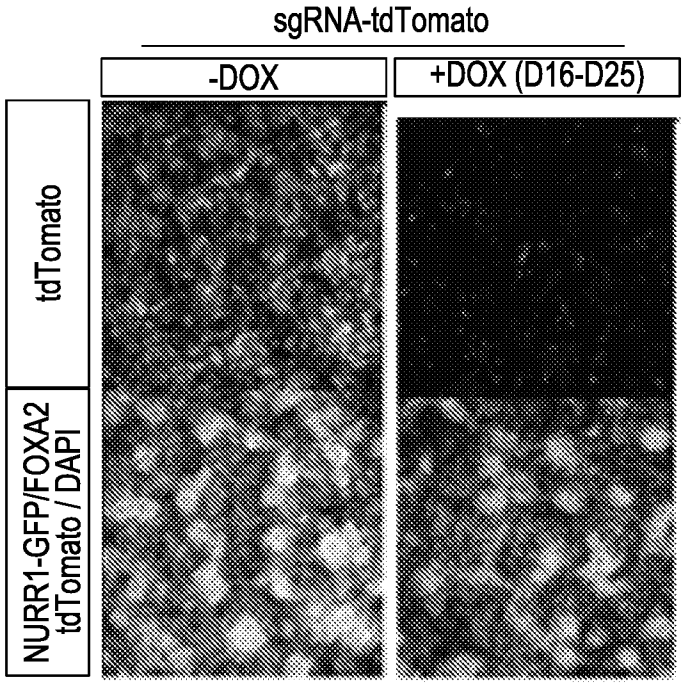


FIGURE 1E

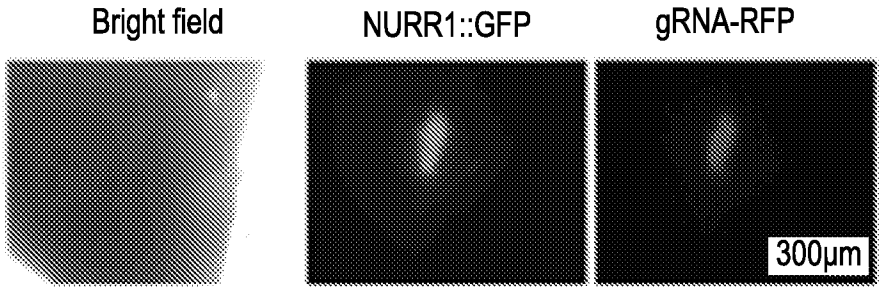


FIGURE 1F

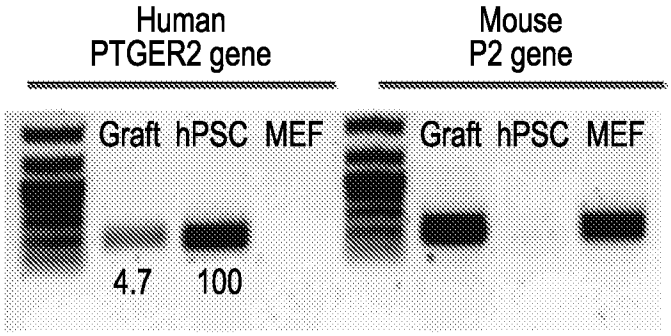


FIGURE 1G

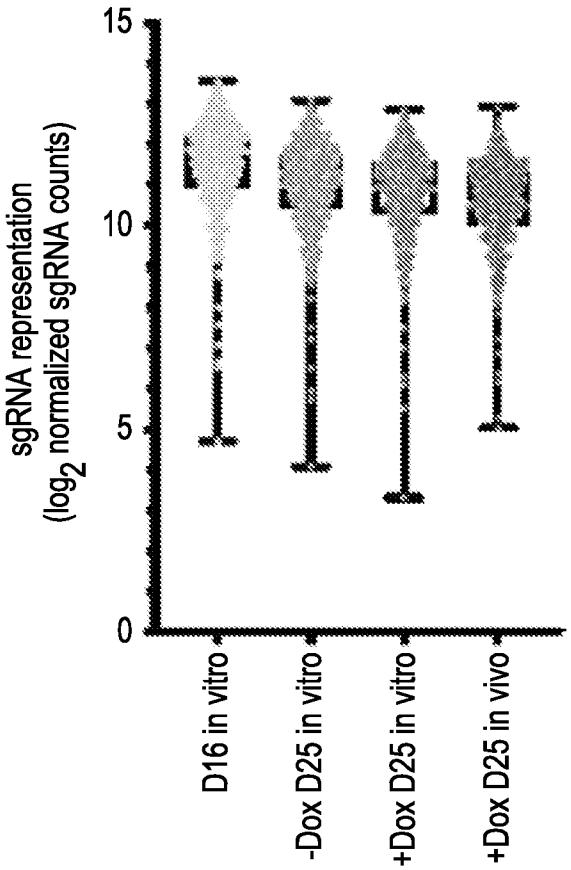


FIGURE 1H

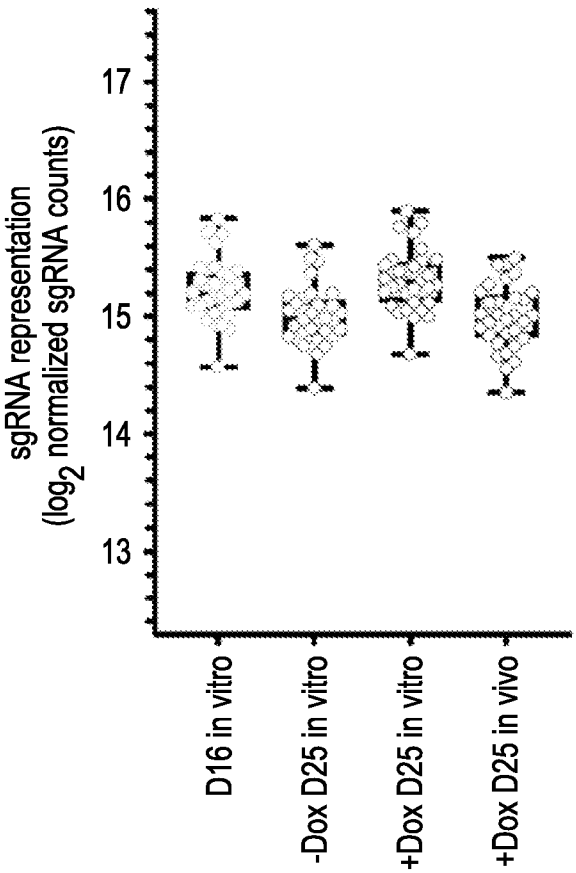

sgRNA representation
(log2 normalized sgRNA counts)
17
16
15
14
13
D16 in vitro
-Dox D25 in vitro
+Dox D25 in vitro
+Dox D25 in vivo

FIGURE 1I

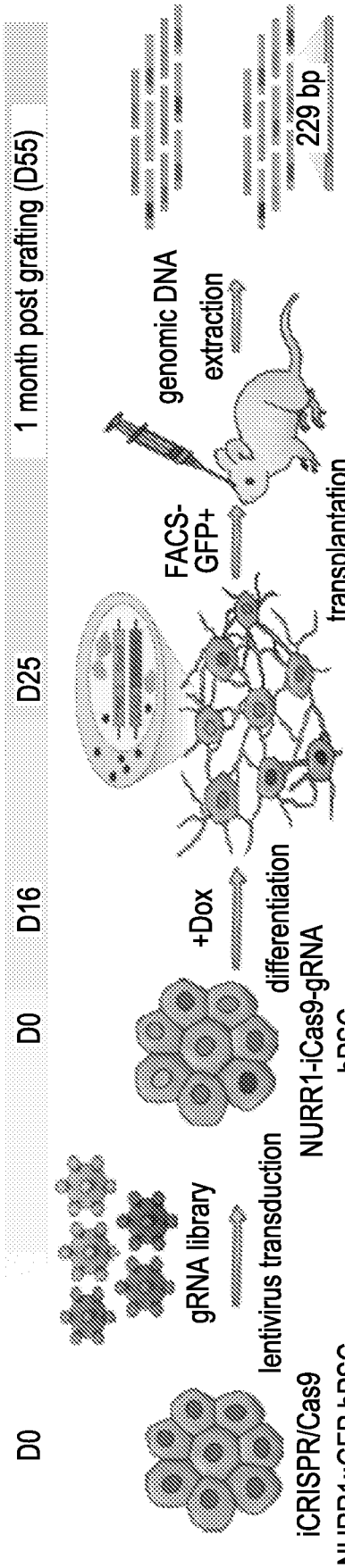

D0
D0
D16
D25
1 month post grafting (D55)
iCRISPR/Cas9
NURR1::GFP hPSC
lentivirus transduction
gRNA library
NURR1-iCas9-gRNA
hPSC
+Dox
differentiation
FACS-
GFP+
transplantation
genomic DNA
extraction
229 bp

FIGURE 2A

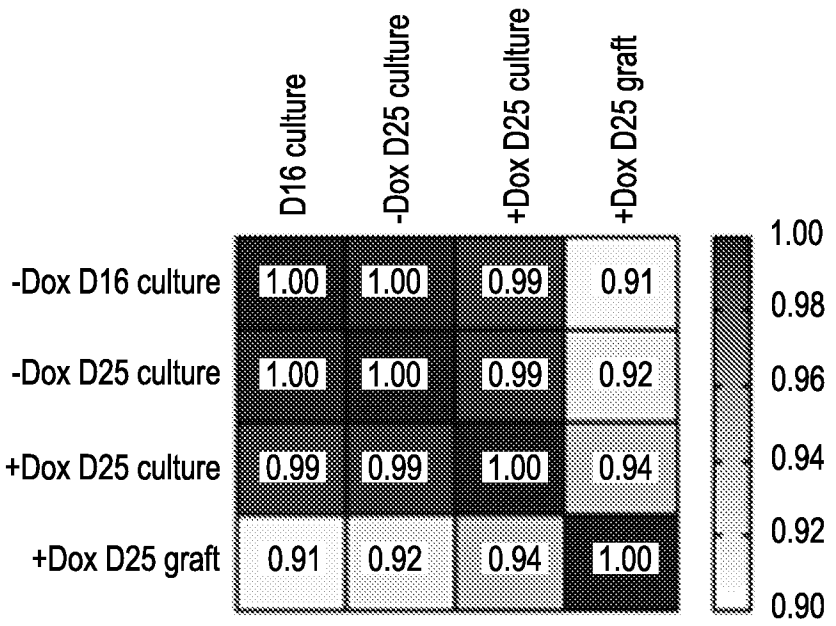


FIGURE 2B

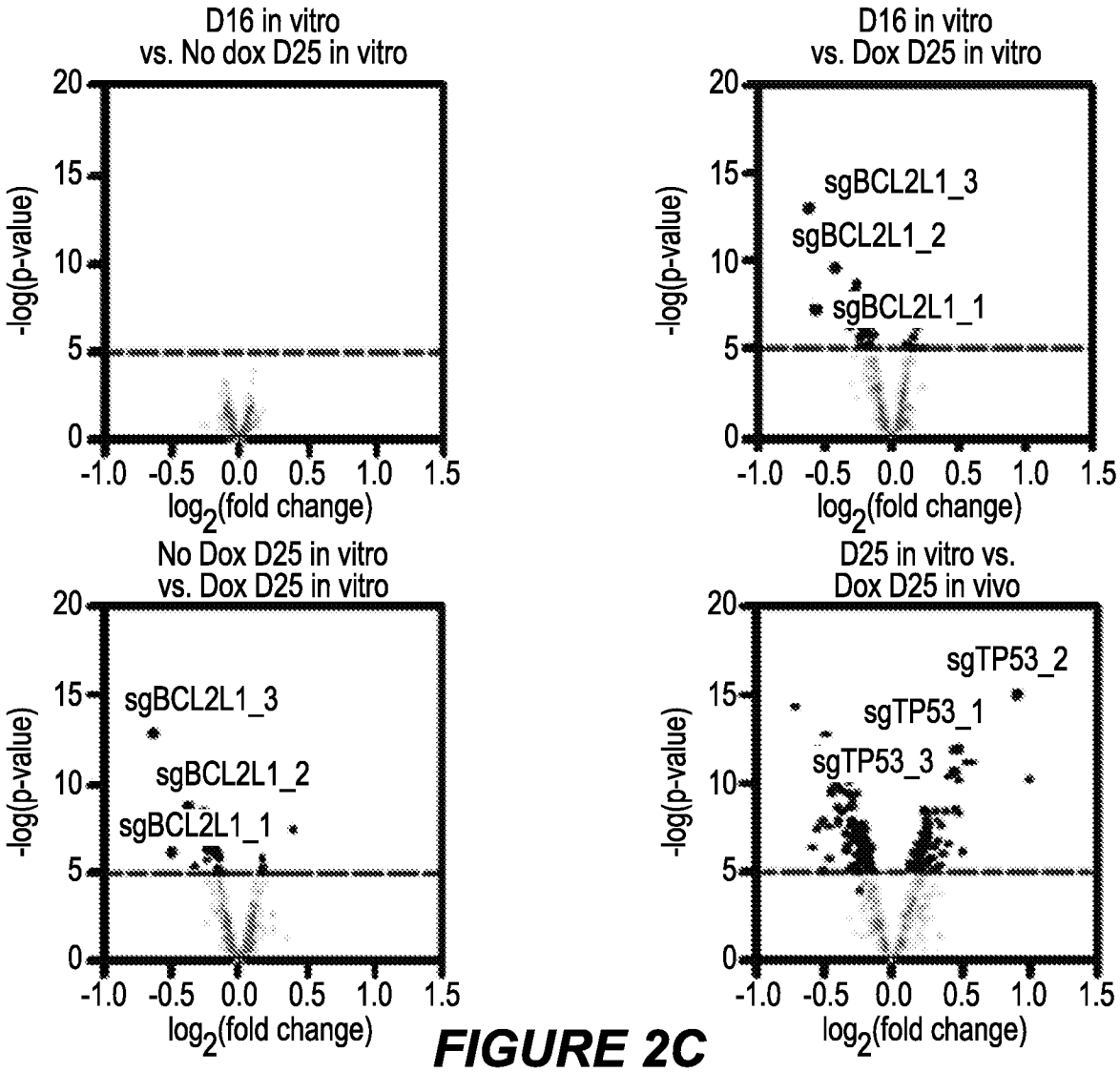


FIGURE 2C

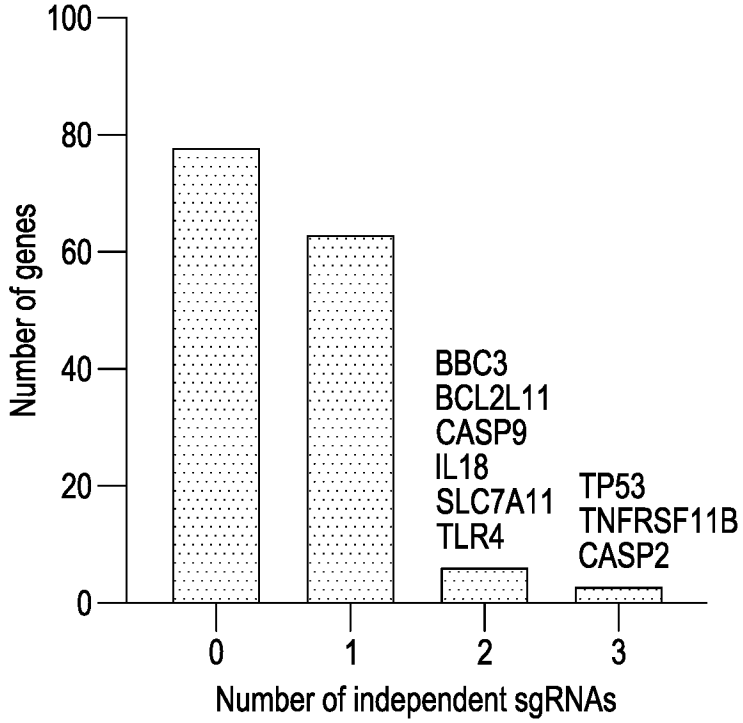


FIGURE 2D

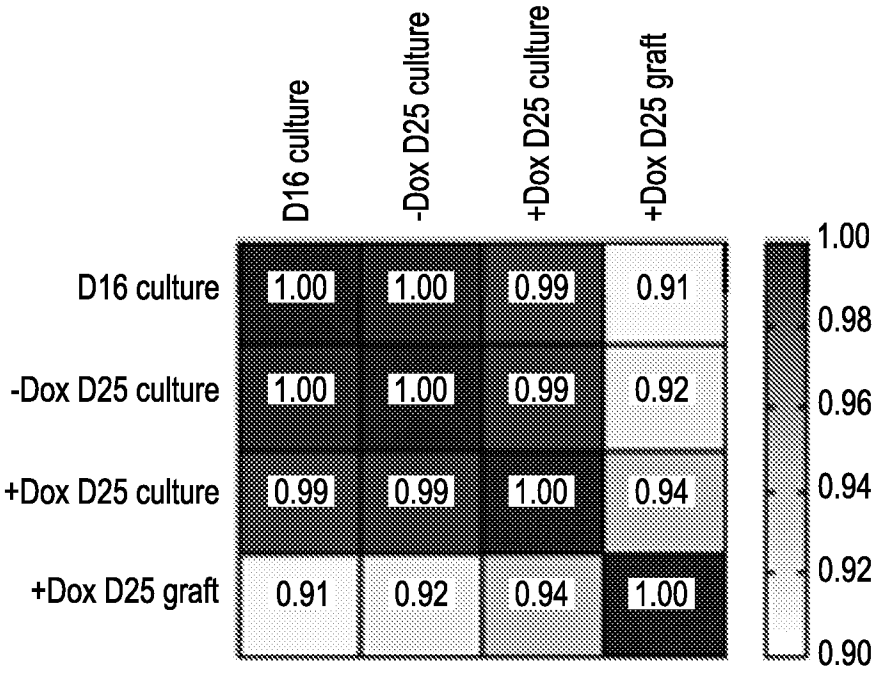


FIGURE 2E

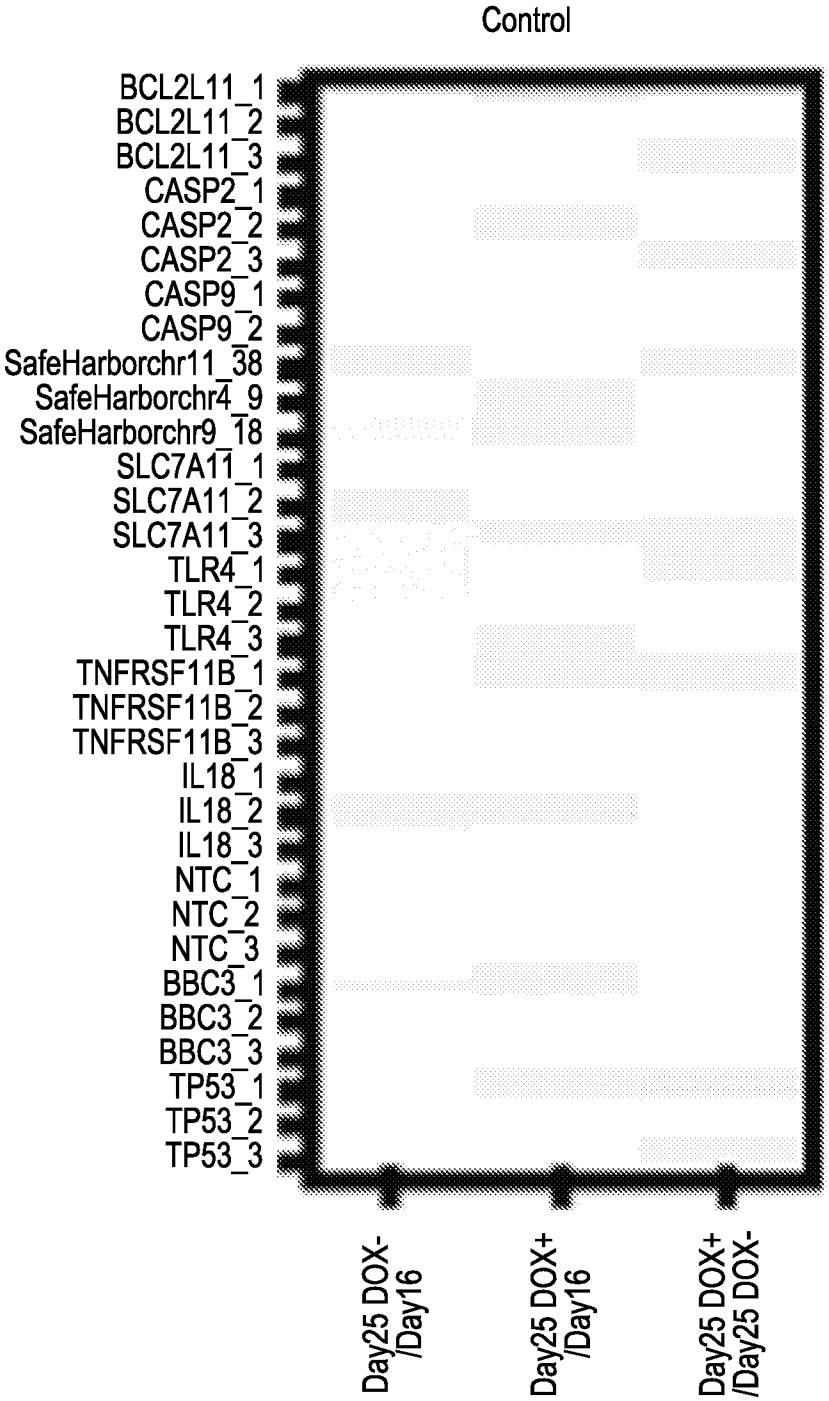

Control
BCL2L11_1
BCL2L11_2
BCL2L11_3
CASP2_1
CASP2_2
CASP2_3
CASP9_1
CASP9_2
SafeHarborchr11_38
SafeHarborchr4_9
SafeHarborchr9_18
SLC7A11_1
SLC7A11_2
SLC7A11_3
TLR4_1
TLR4_2
TLR4_3
TNFRSF11B_1
TNFRSF11B_2
TNFRSF11B_3
IL18_1
IL18_2
IL18_3
NTC_1
NTC_2
NTC_3
BBC3_1
BBC3_2
BBC3_3
TP53_1
TP53_2
TP53_3
Day25 DOX-/Day16
Day25 DOX+/Day16
Day25 DOX+/Day25 DOX-

FIGURE 2F

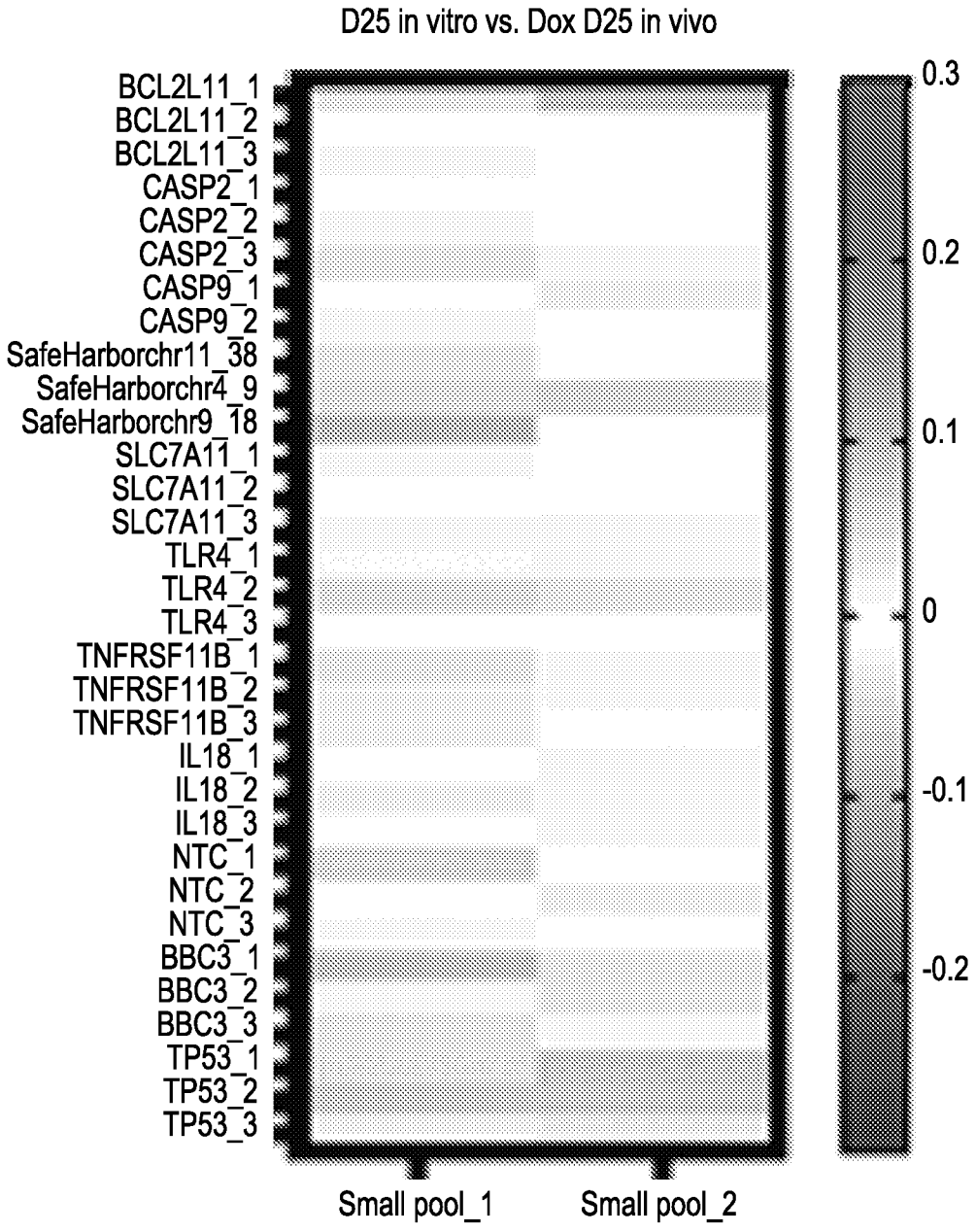


FIGURE 2F (Continued)

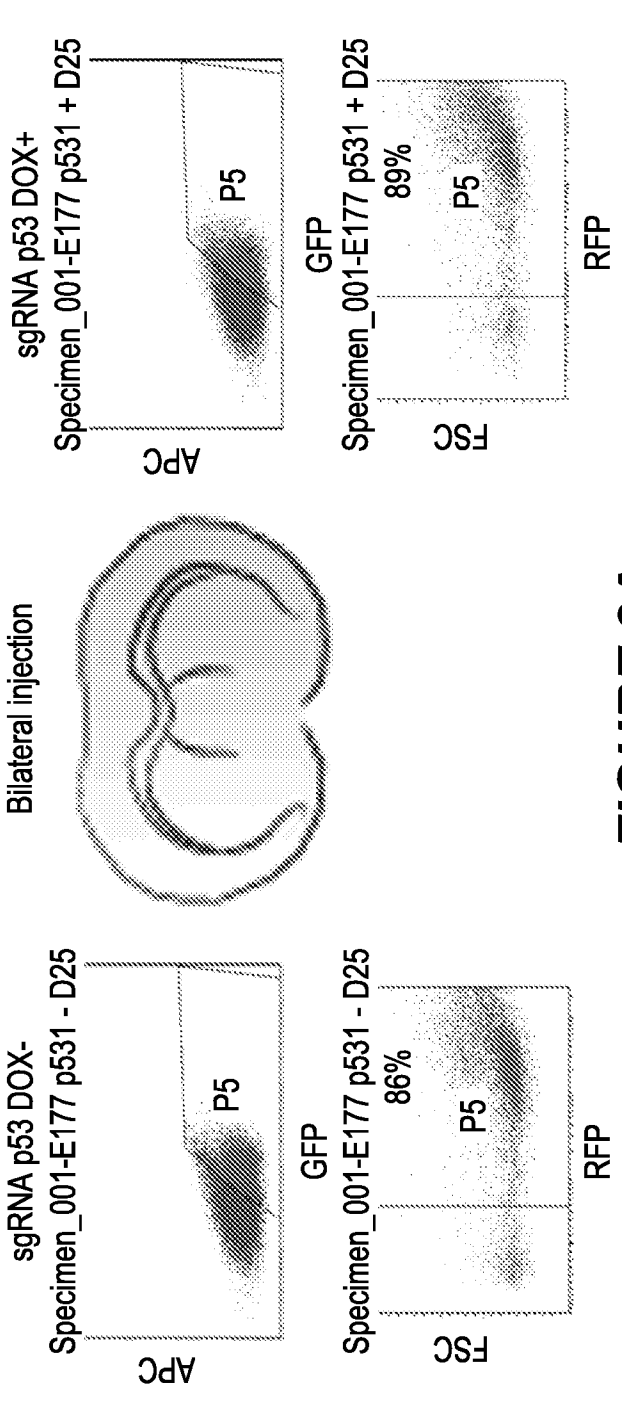


FIGURE 3A

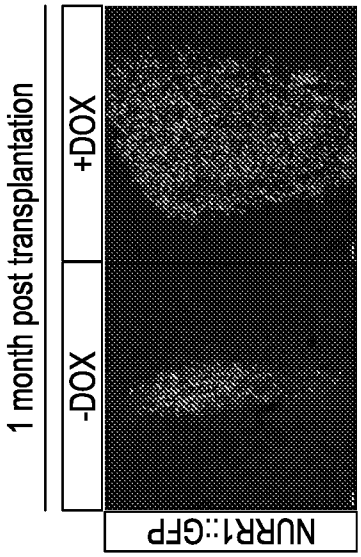


FIGURE 3C

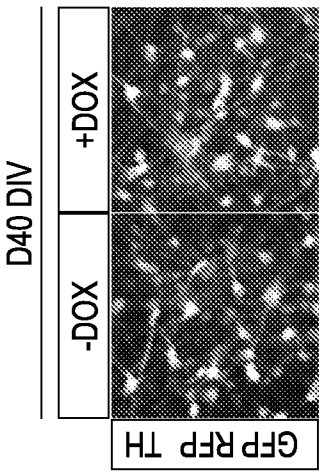


FIGURE 3B

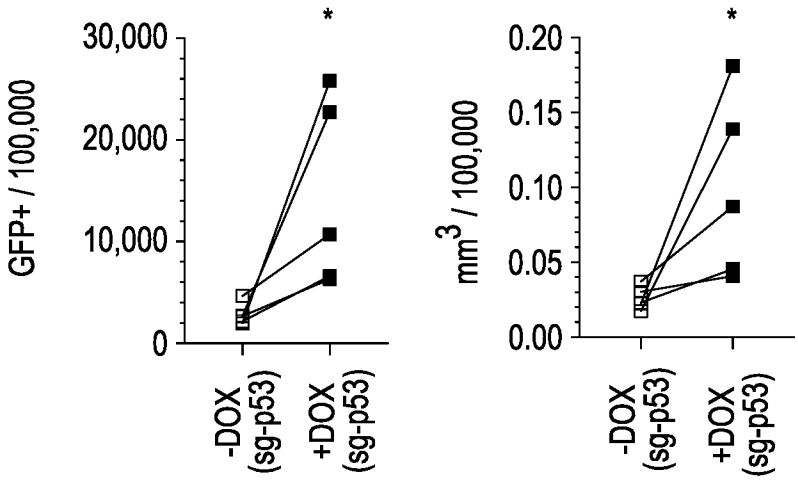


FIGURE 3D

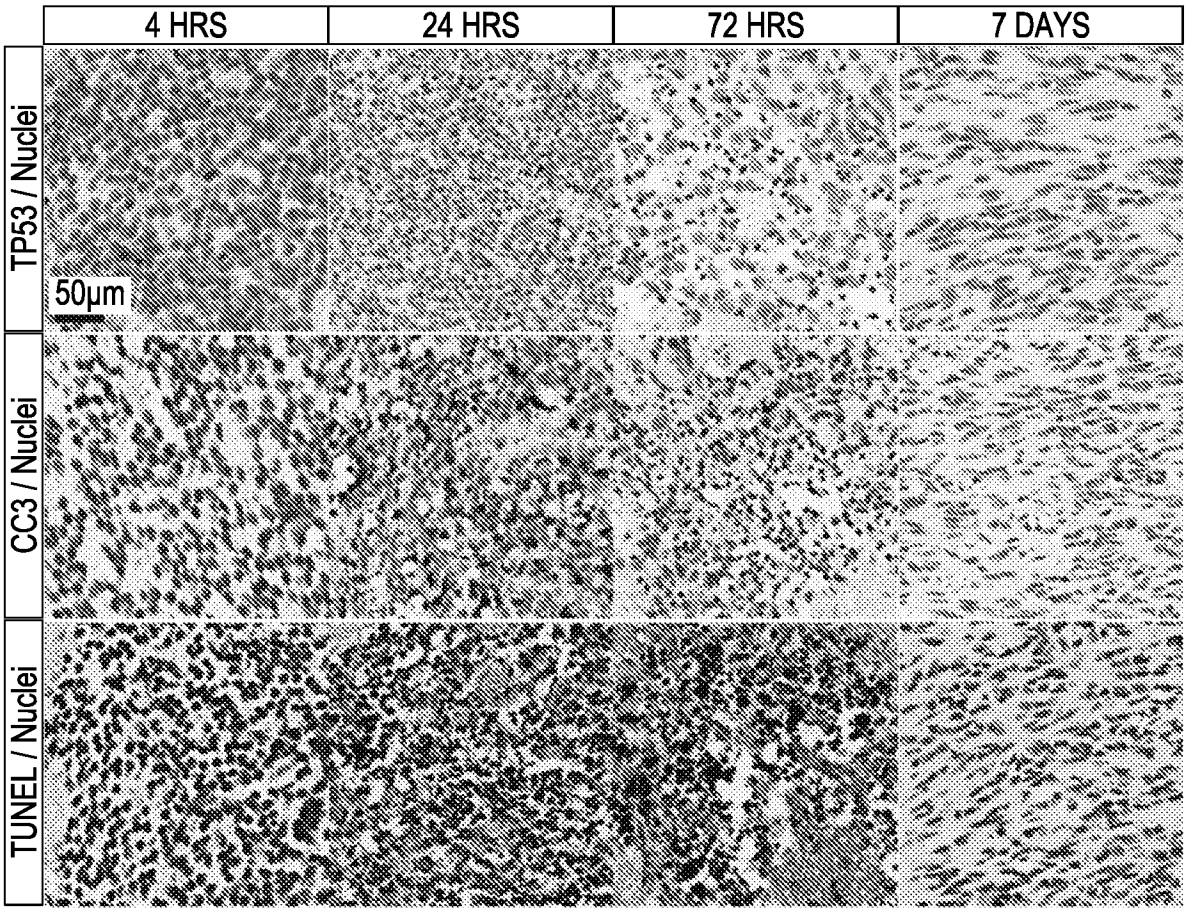


FIGURE 3E

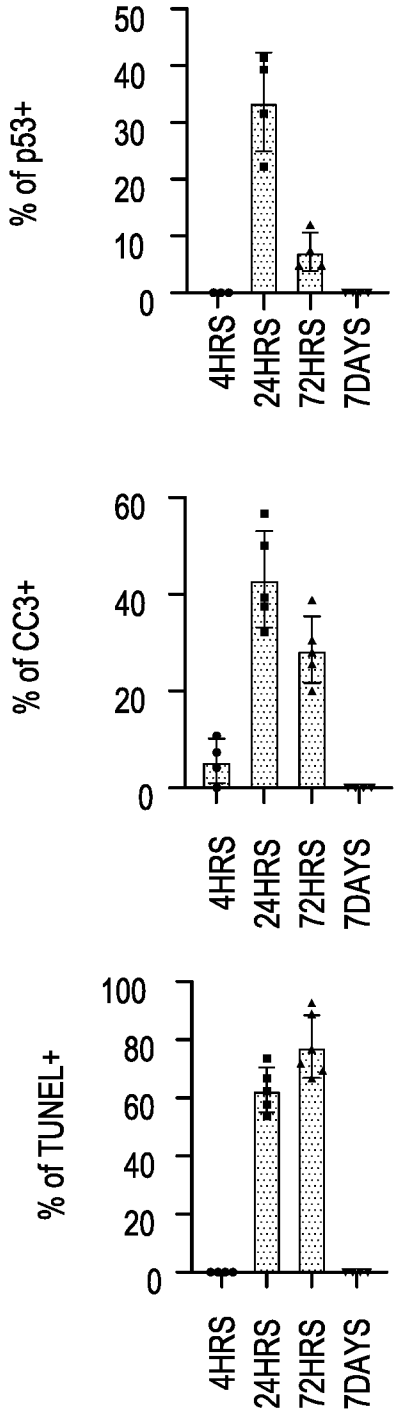

% of p53+
50
40
30
20
10
0
4HRS
24HRS
72HRS
7DAYS
% of CC3+
60
40
20
0
4HRS
24HRS
72HRS
7DAYS
% of TUNEL+
100
80
60
40
20
0
4HRS
24HRS
72HRS
7DAYS

FIGURE 3F

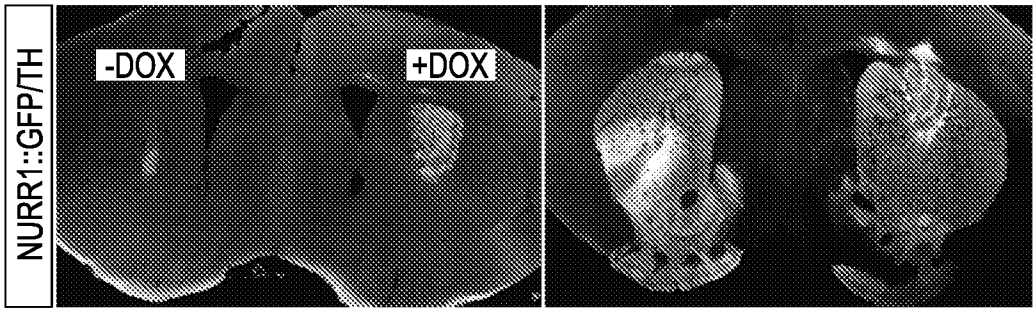


FIGURE 4A

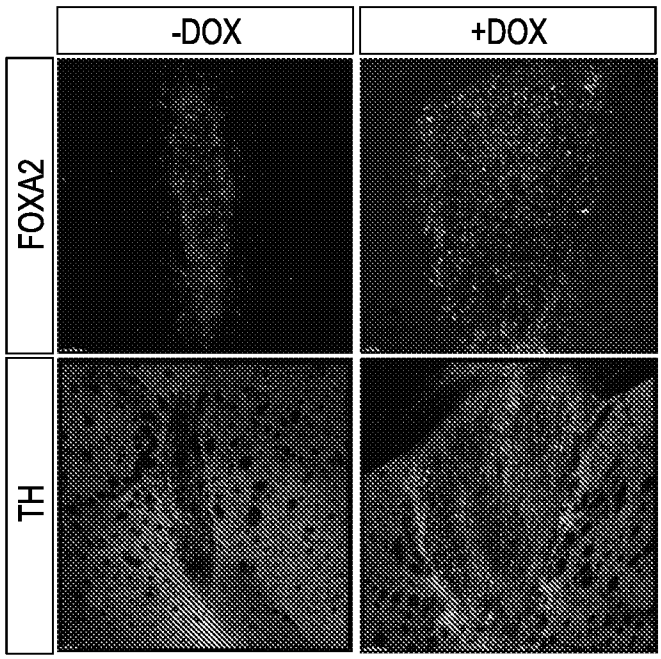


FIGURE 4B

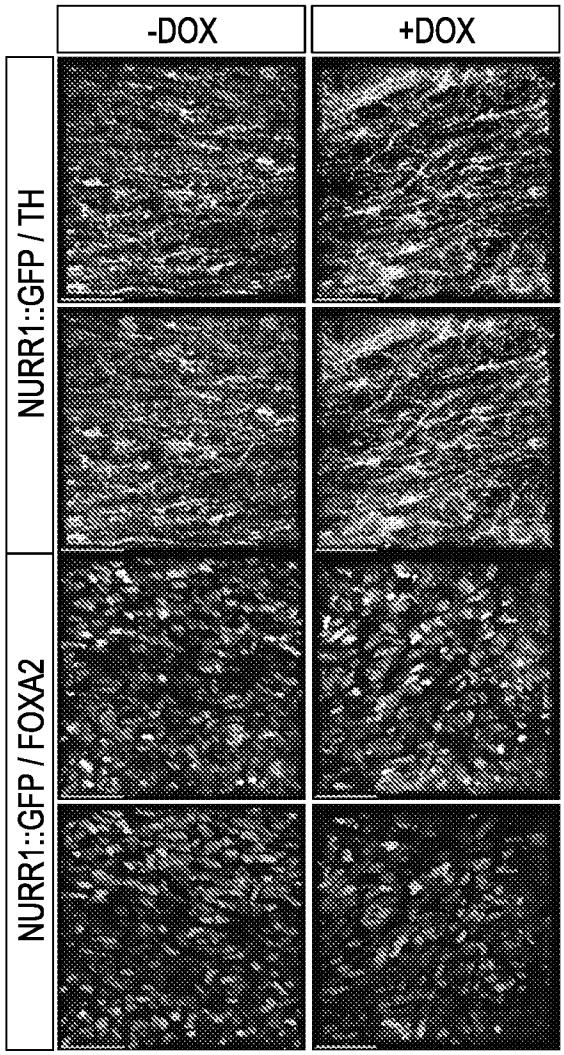


FIGURE 4C

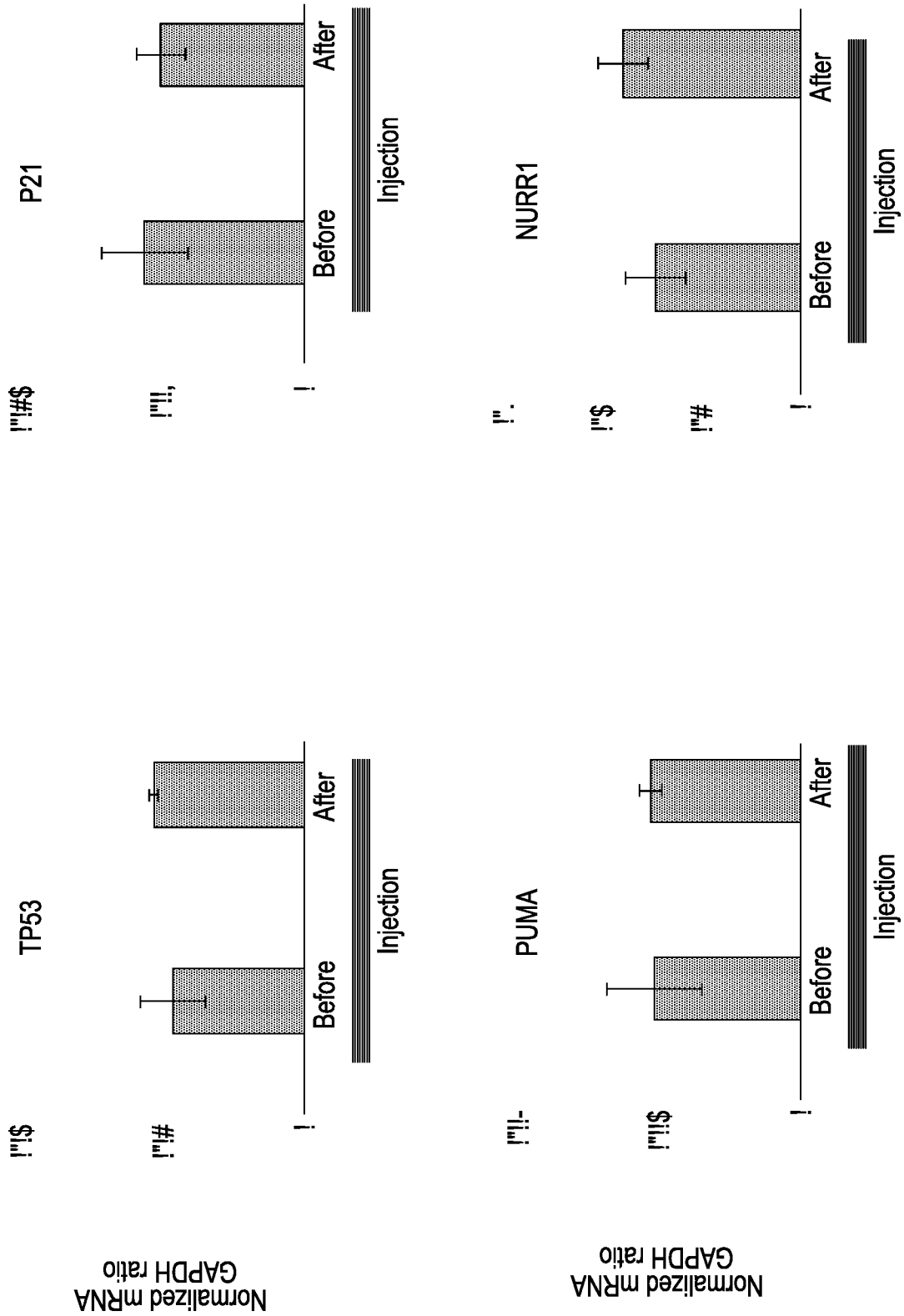

P21
NURR1
TP53
PUMA
Before
After
Injection
Normalized mRNA
GAPDH ratio

FIGURE 4D

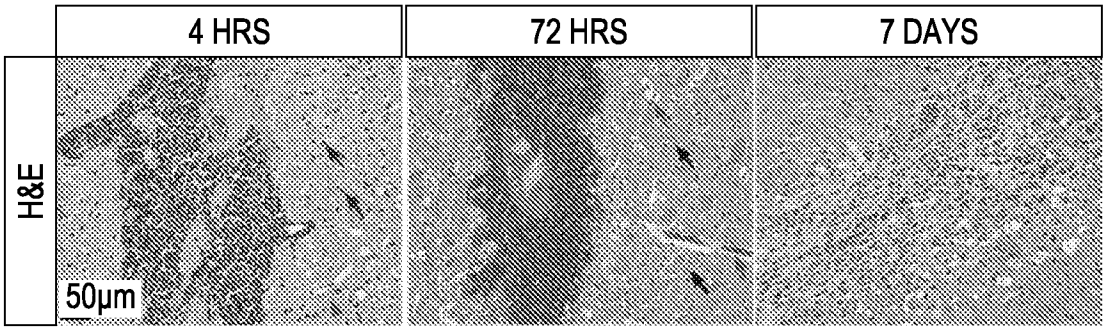


FIGURE 5A

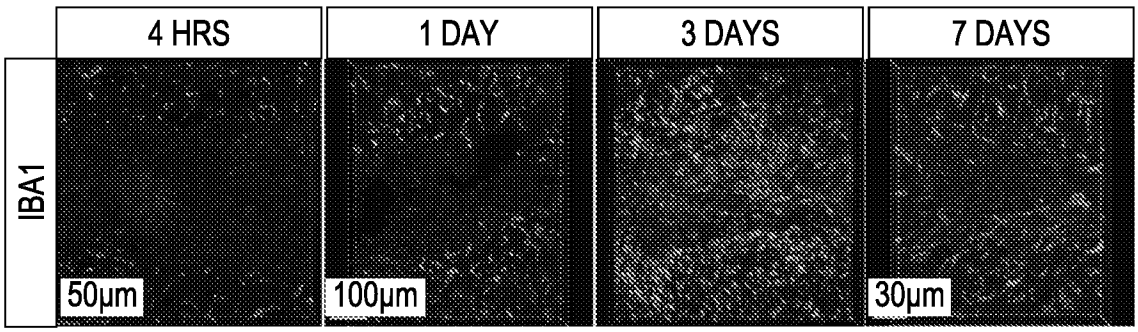


FIGURE 5B

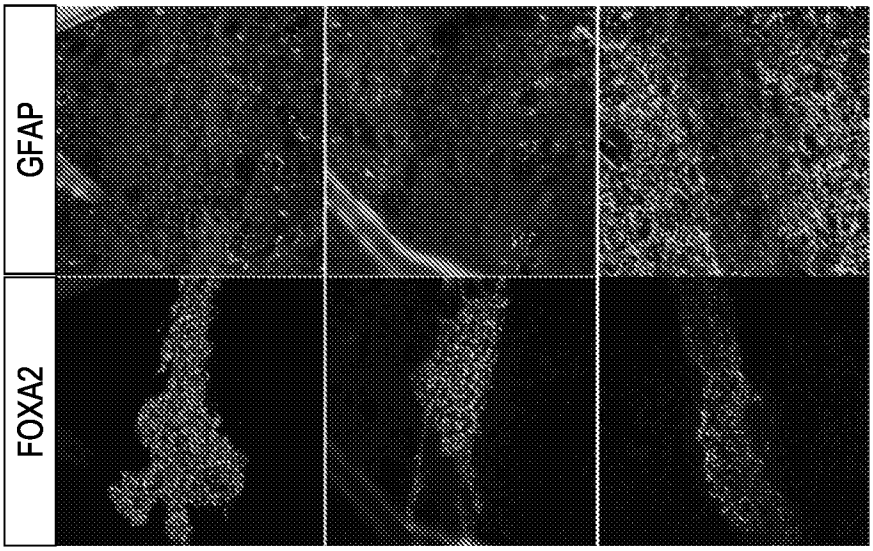


FIGURE 5C

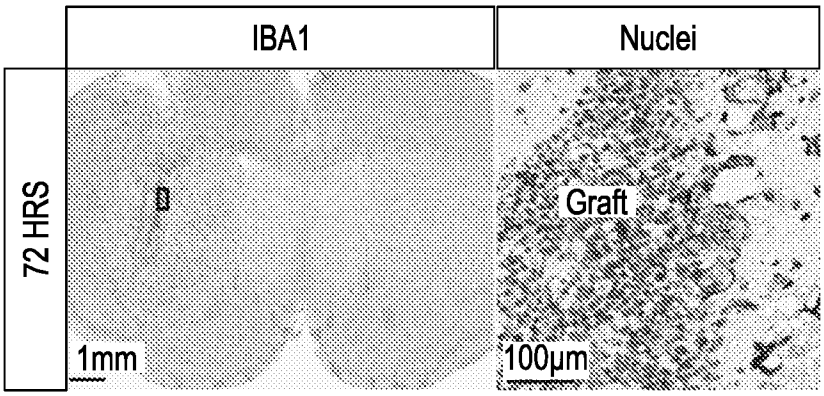


FIGURE 5D

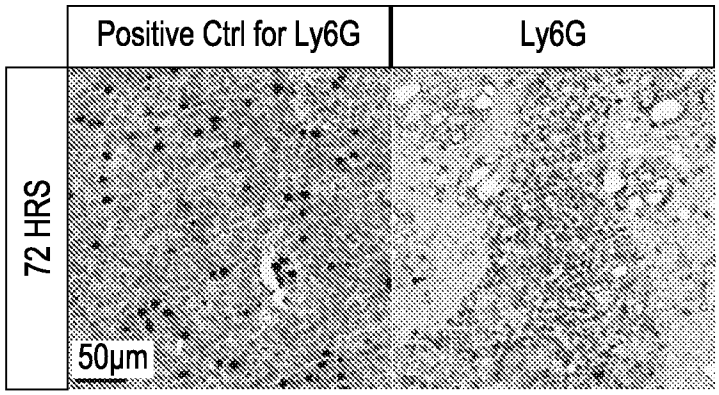


FIGURE 5E

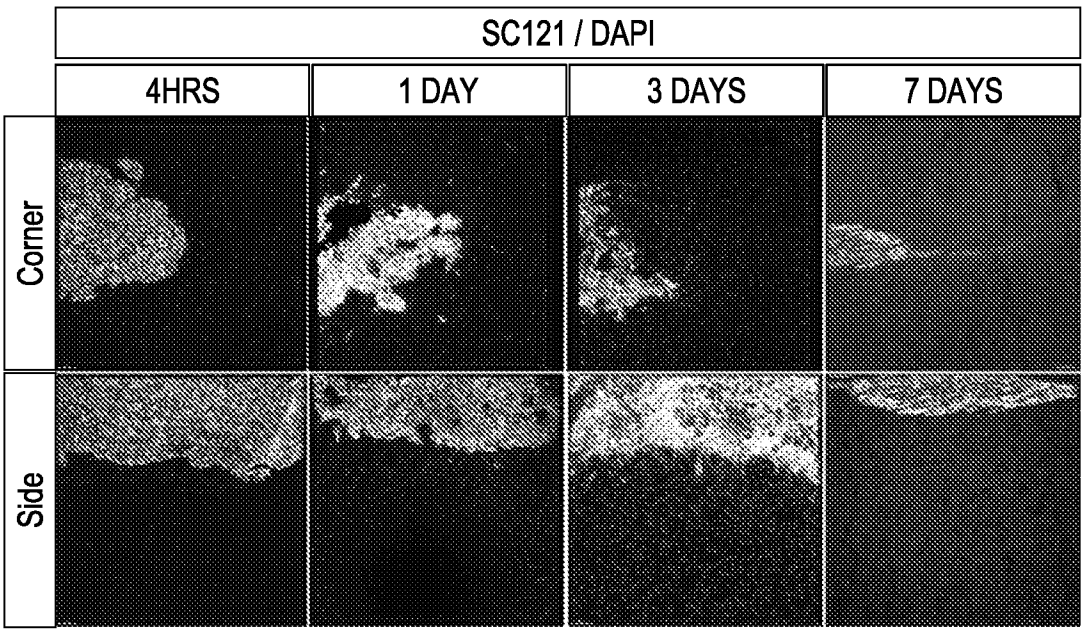


FIGURE 6

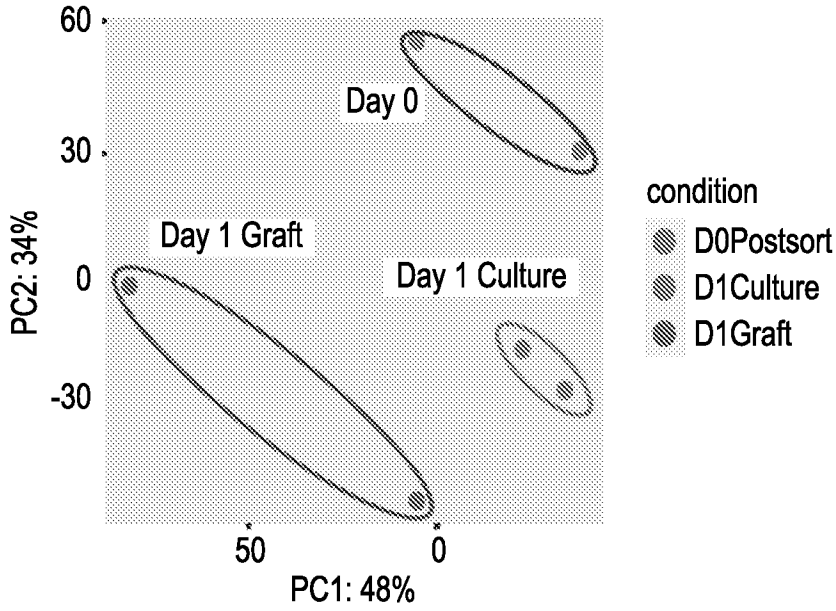


FIGURE 7A

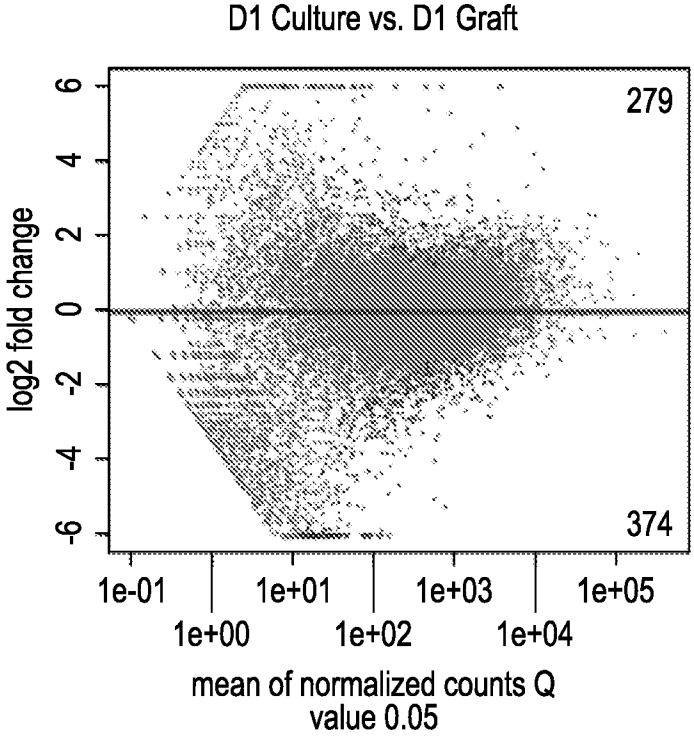


FIGURE 7B

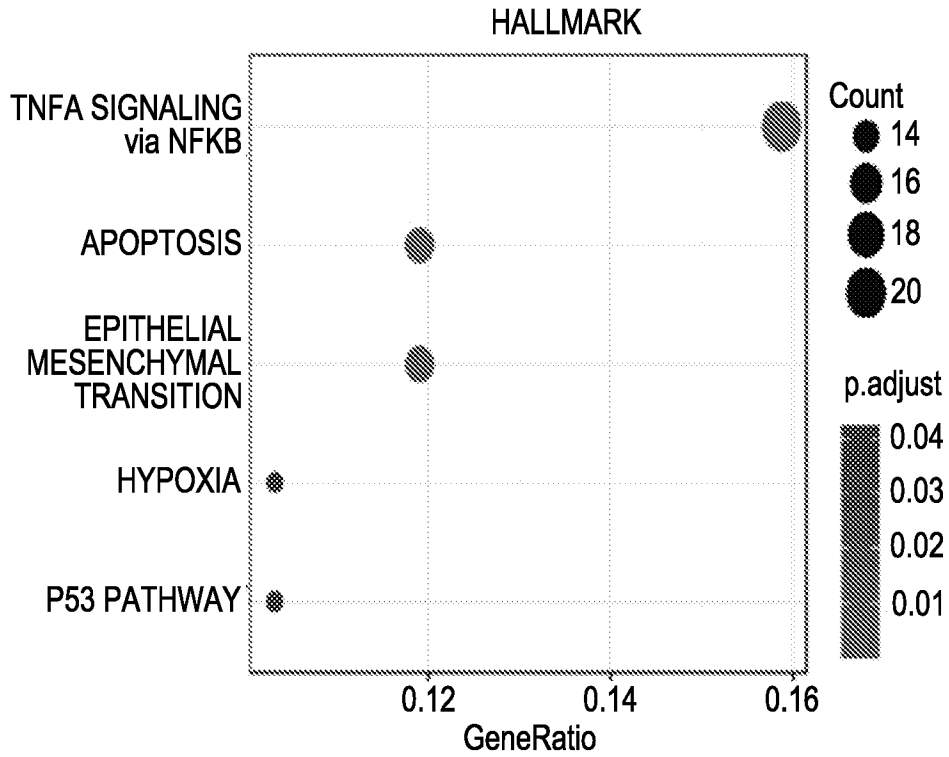


FIGURE 7C

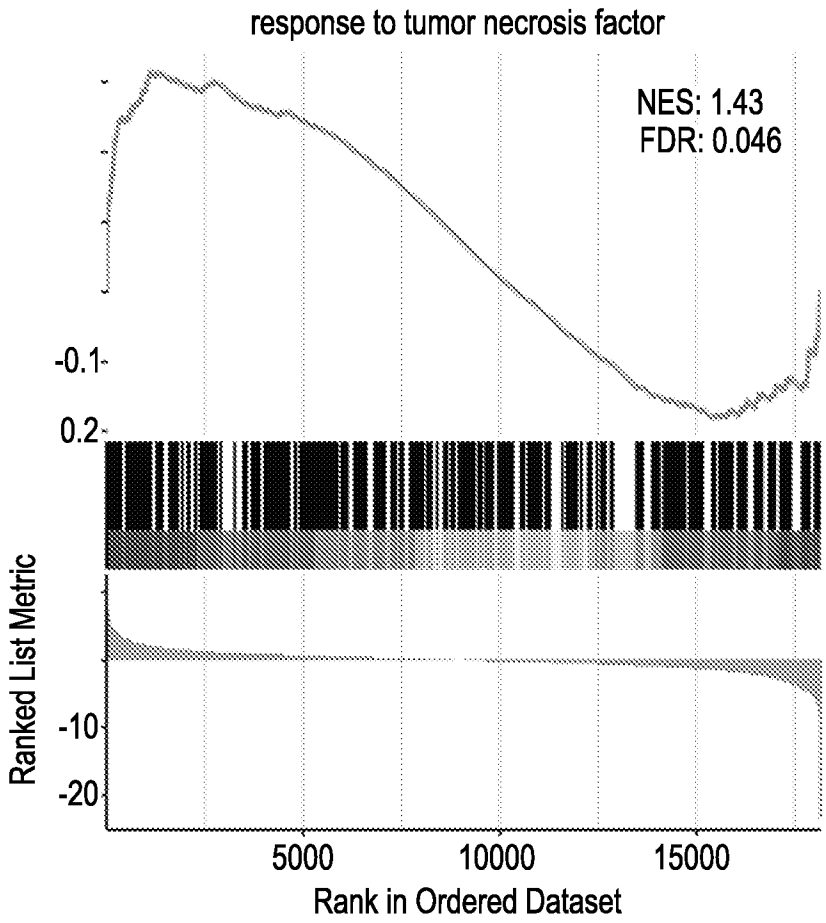


FIGURE 7D

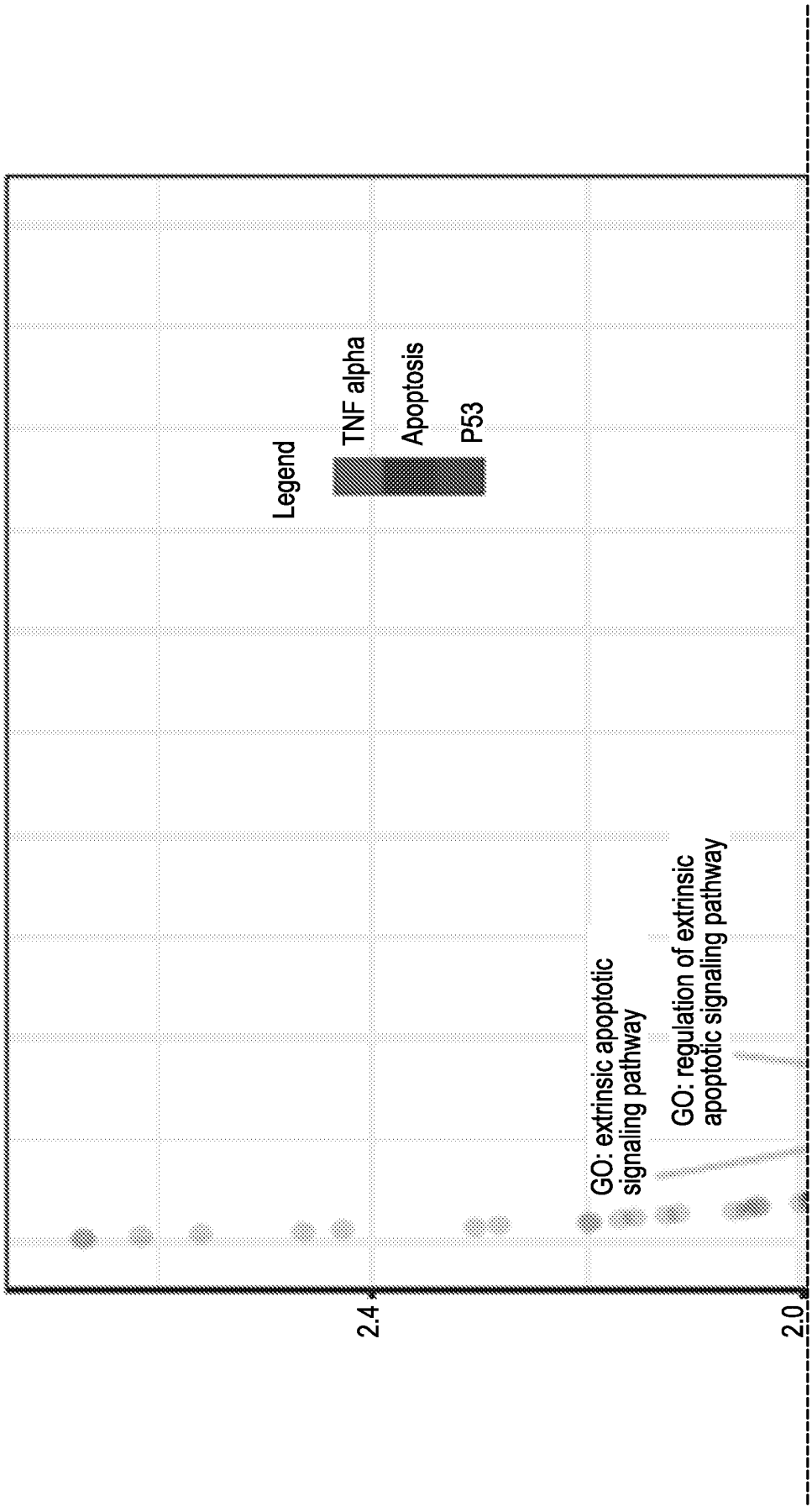

Legend
TNF alpha
Apoptosis
P53
GO: extrinsic apoptotic signaling pathway
GO: regulation of extrinsic apoptotic signaling pathway
2.4
2.0

FIGURE 7E

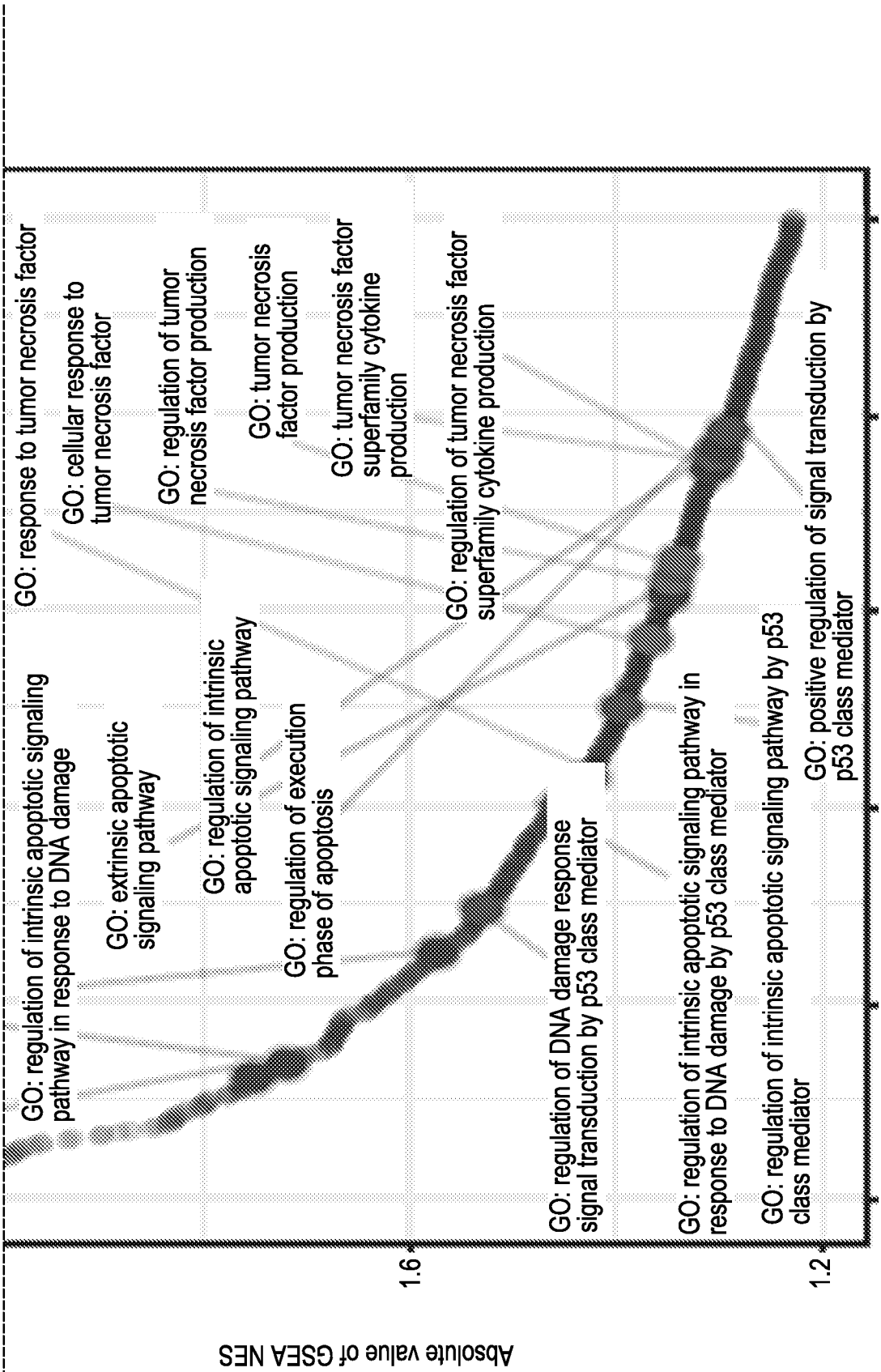


FIGURE 7E (Continued)

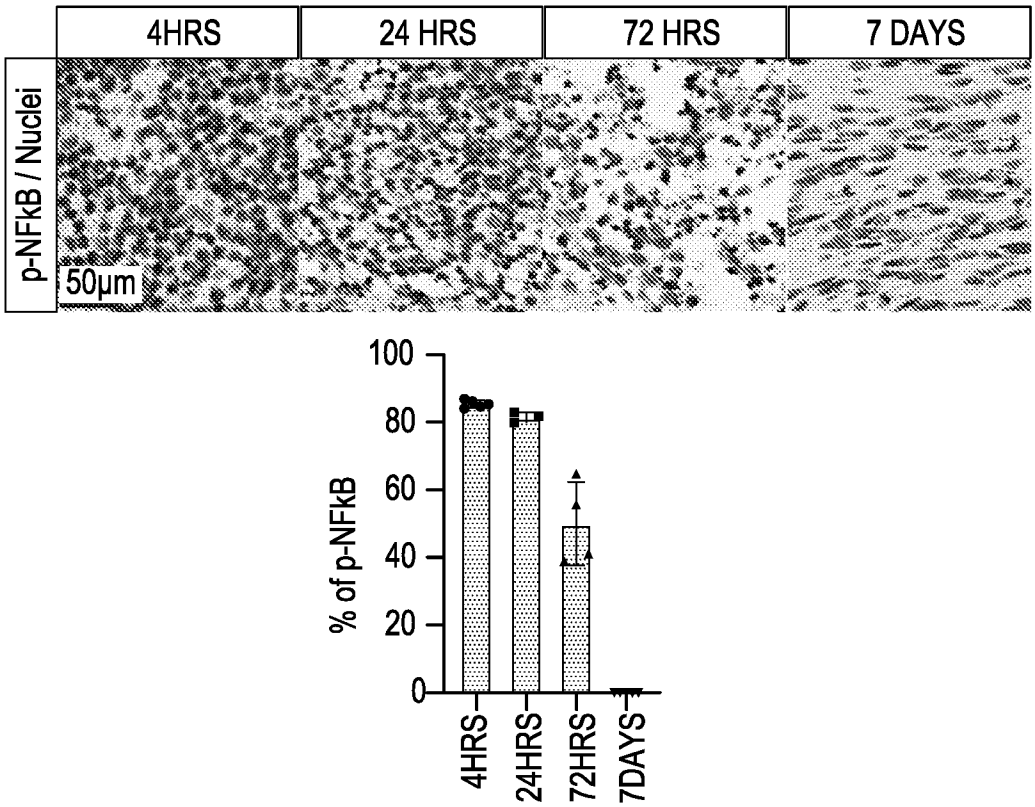


FIGURE 7F

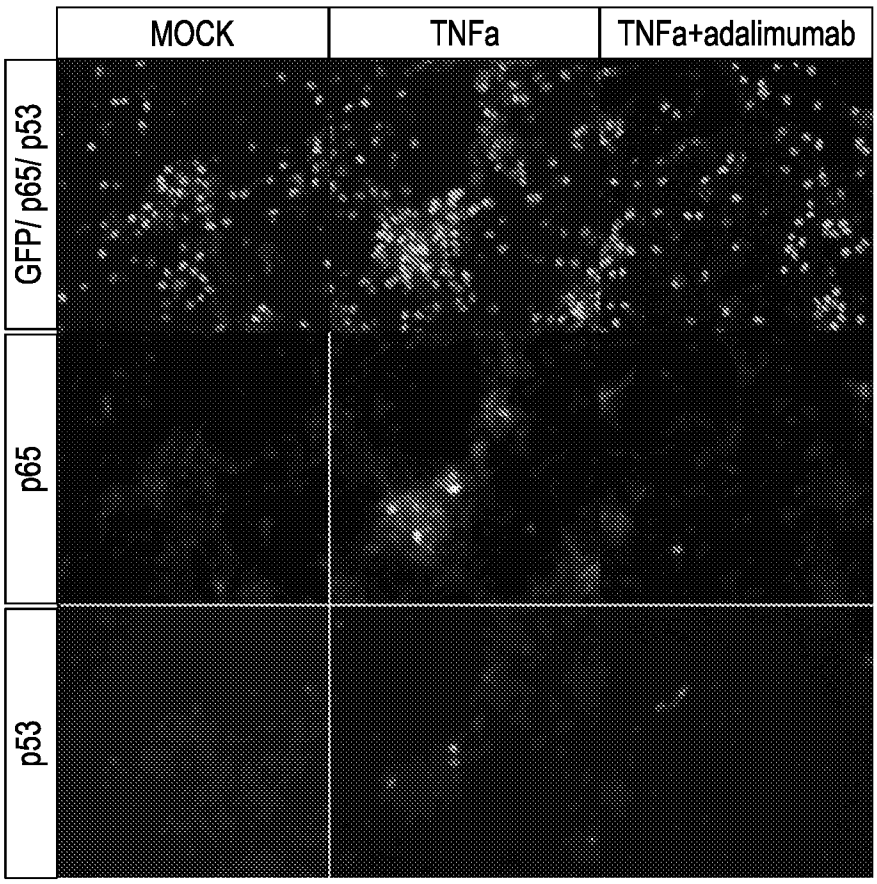


FIGURE 7G

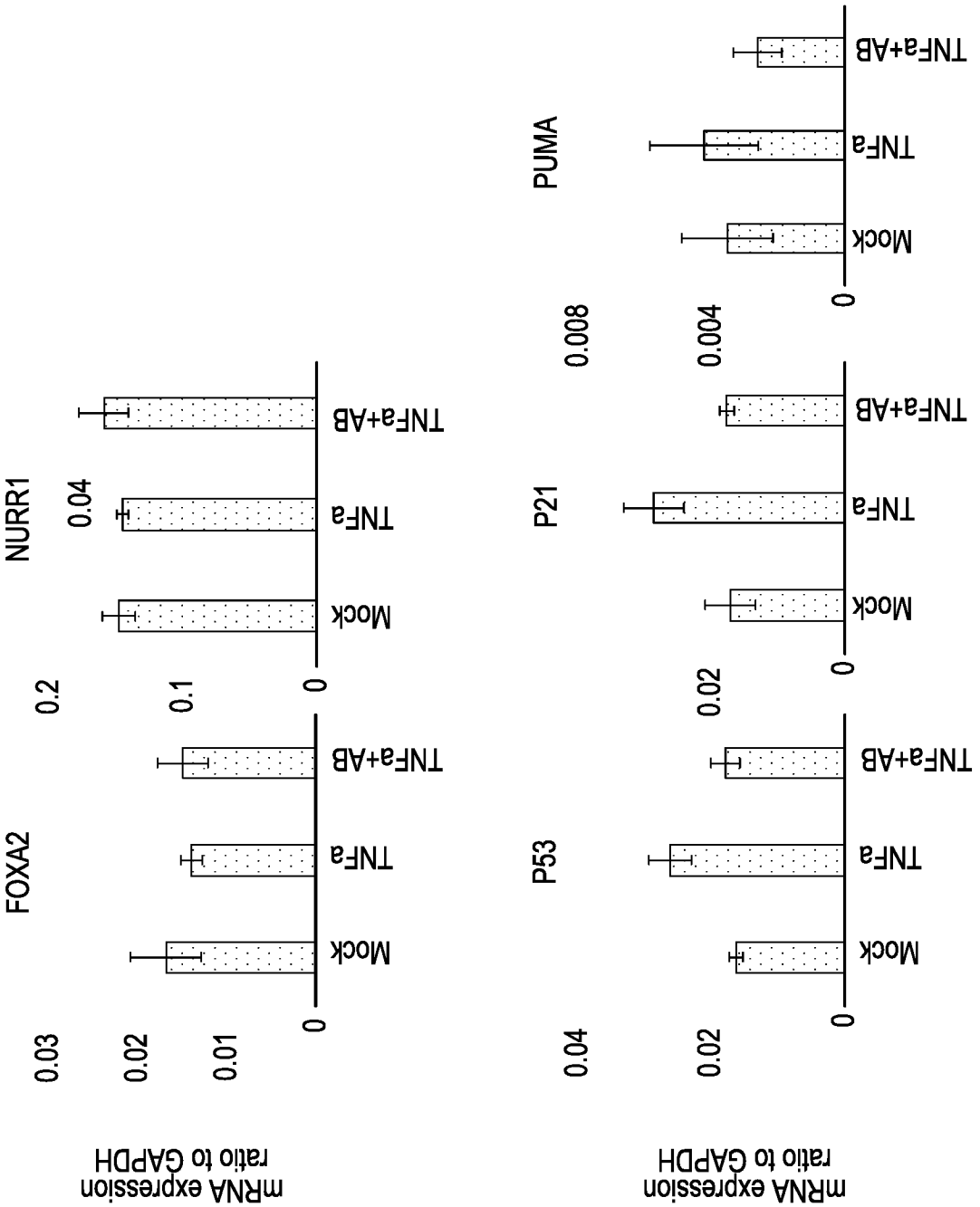

mRNA expression ratio to GAPDH
FOXA2
0.03
0.02
0.01
0
Mock
TNFa
TNFa+AB
NURR1
0.2
0.1
0
Mock
TNFa
TNFa+AB
P53
0.04
0.02
0
Mock
TNFa
TNFa+AB
P21
0.02
0
Mock
TNFa
TNFa+AB
PUMA
0.008
0.004
0
Mock
TNFa
TNFa+AB

FIGURE 7H

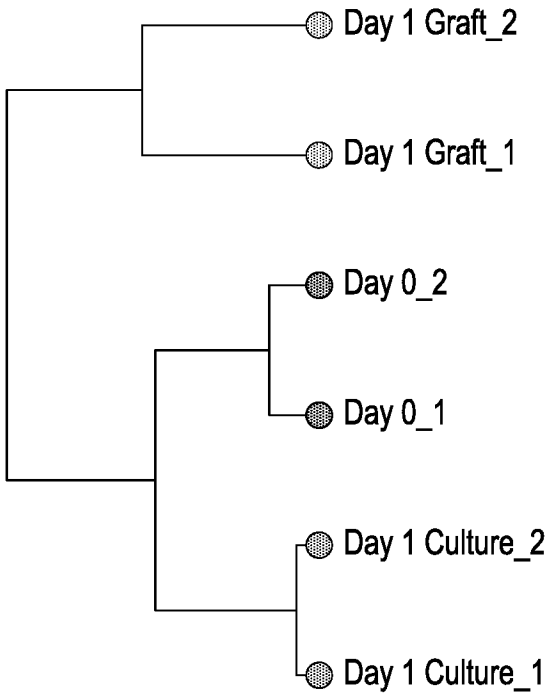

Day 1 Graft_2
Day 1 Graft_1
Day 0_2
Day 0_1
Day 1 Culture_2
Day 1 Culture_1

FIGURE 8A

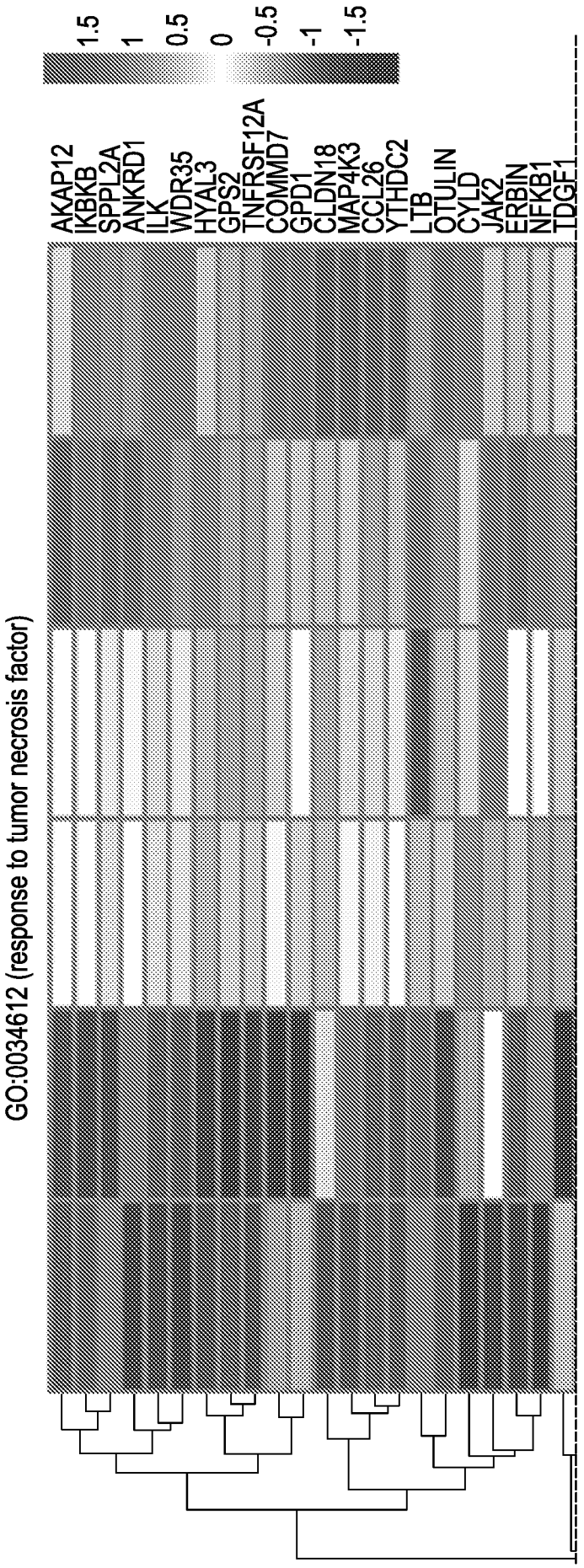

1.5
1
0.5
0
-0.5
-1
-1.5
AKAP12
IKBKB
SPPL2A
ANKRD1
ILK
WDR35
HYAL3
GPS2
TNFRSF12A
COMMD7
GPD1
CLDN18
MAP4K3
CCL26
YTHDC2
LTB
OTULIN
CYLD
JAK2
ERBIN
NFKB1
TDGF1
GO:0034612 (response to tumor necrosis factor)

FIGURE 8B

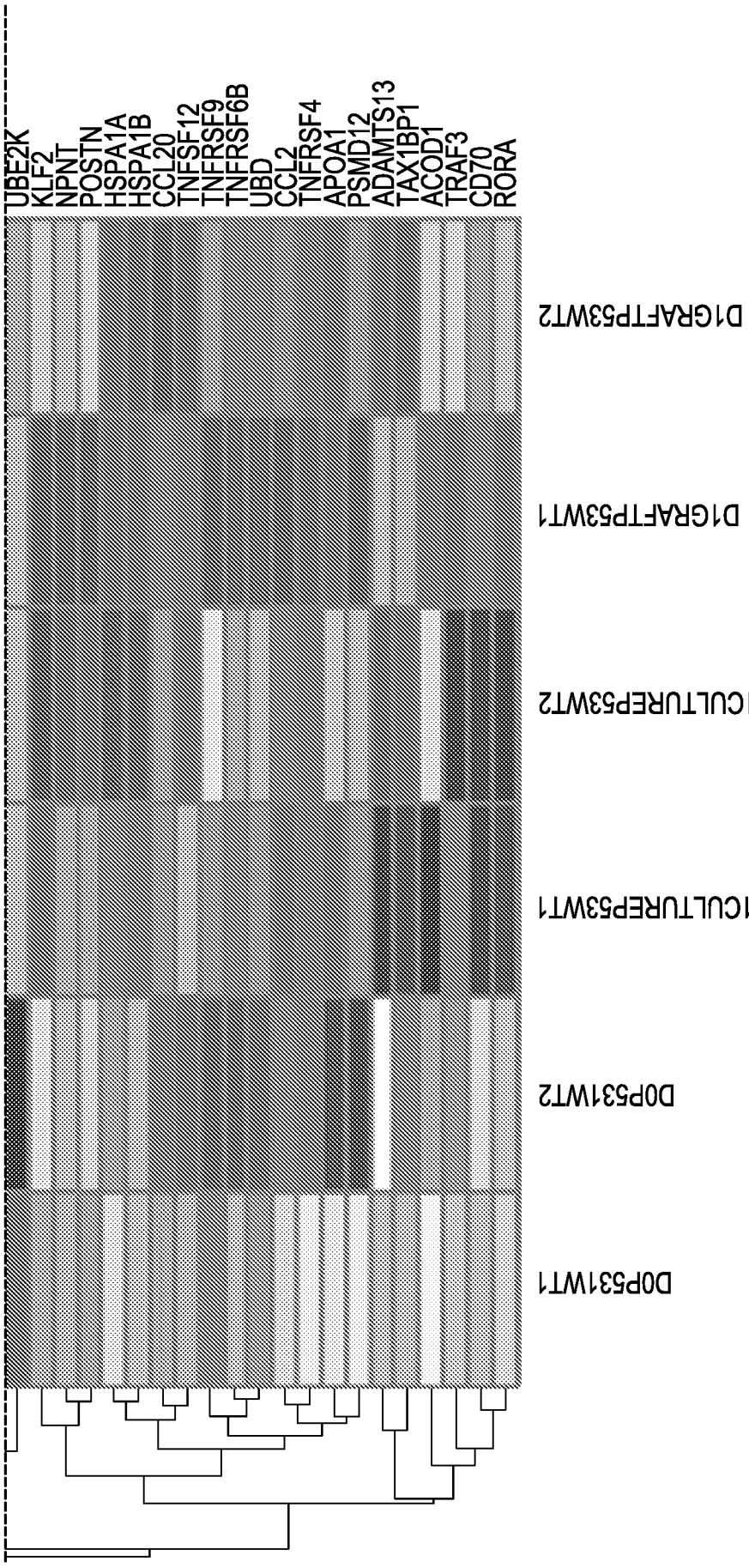


FIGURE 8B (Continued)

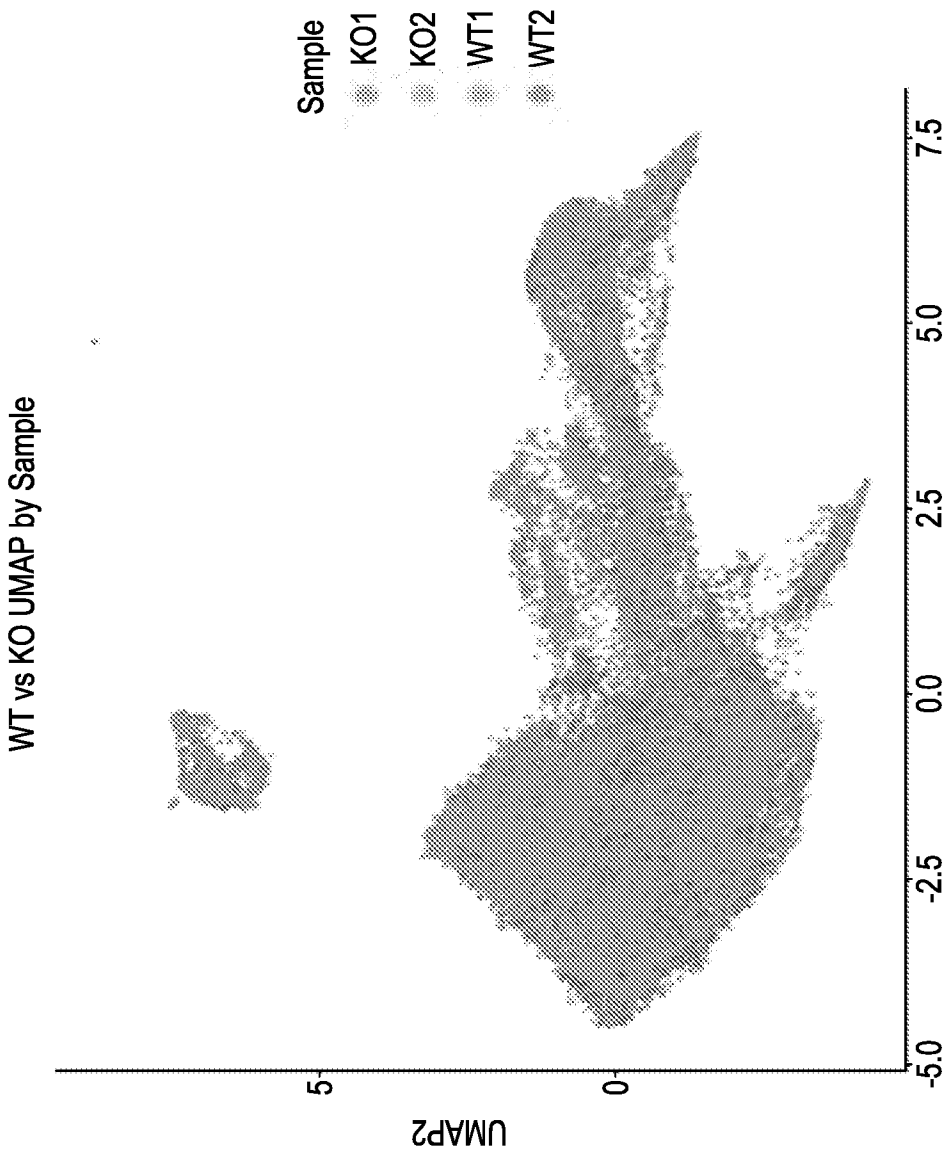

WT vs KO UMAP by Sample
Sample
KO1
KO2
WT1
WT2
UMAP2
5
0
-5.0
-2.5
0.0
2.5
5.0
7.5
UMAP 1

FIGURE 8C

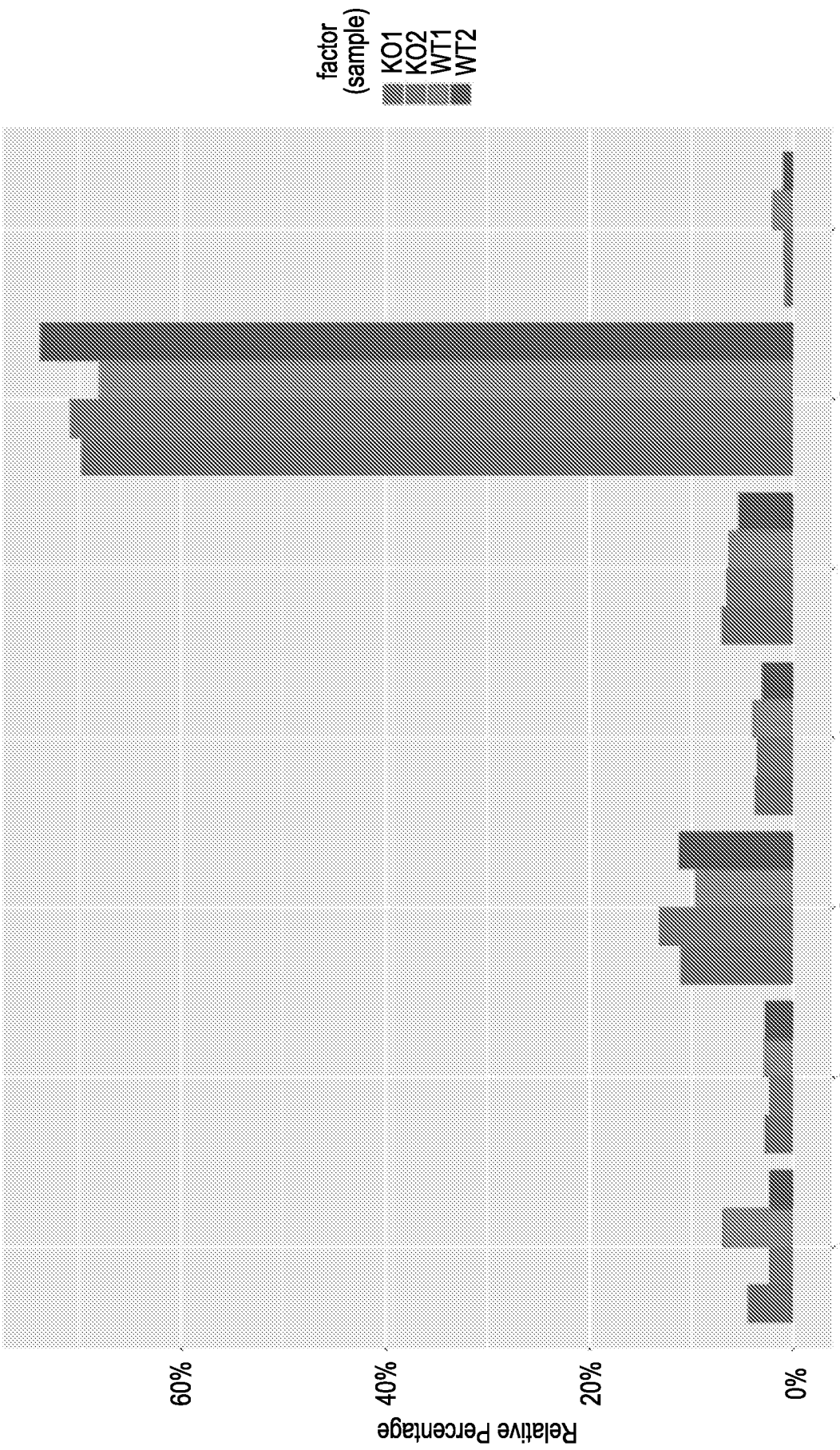

factor
(sample)
KO1
KO2
WT1
WT2
Relative Percentage
0%
20%
40%
60%
1
2
3
4
5
6
7
cluster

FIGURE 8D

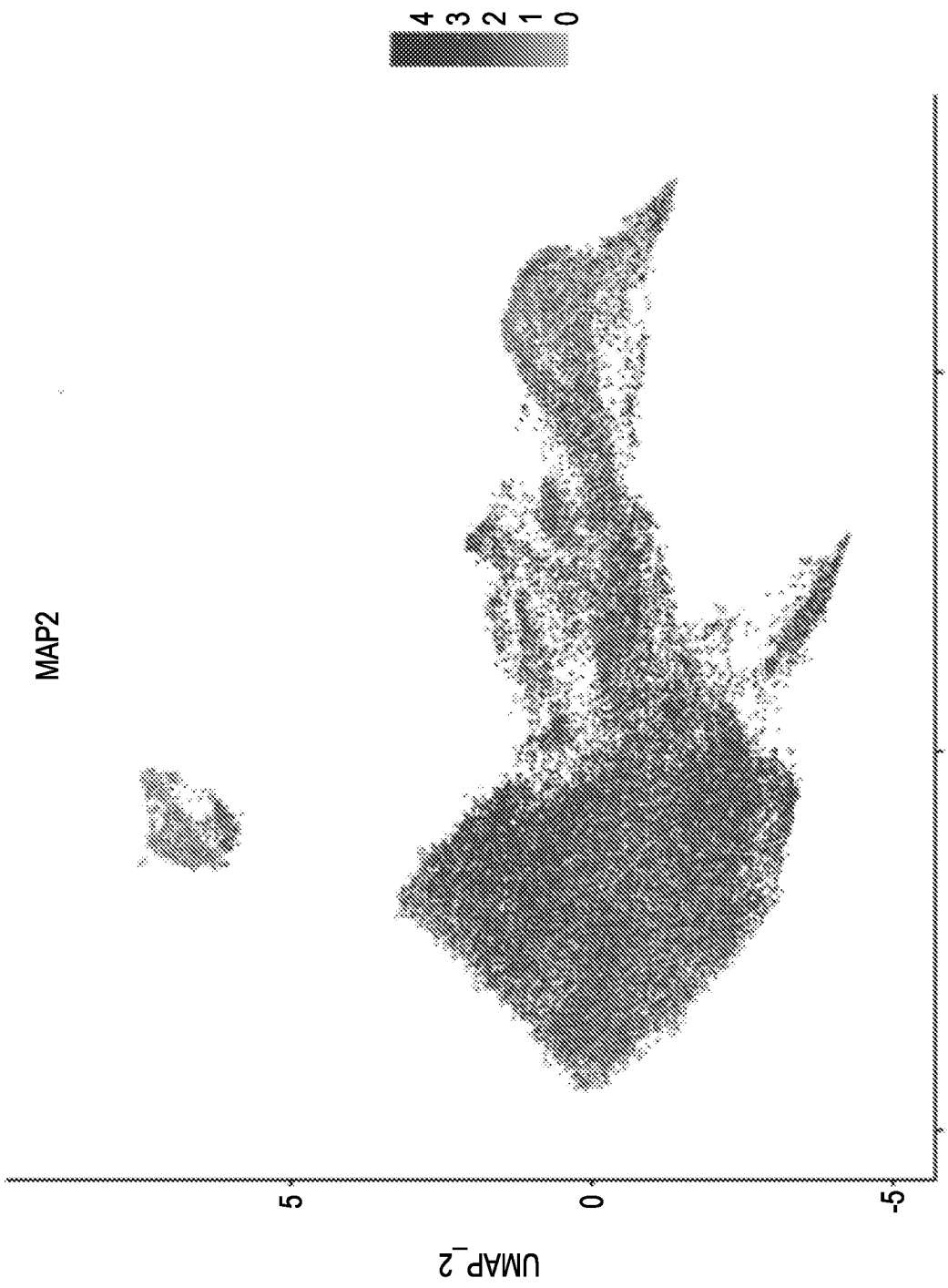
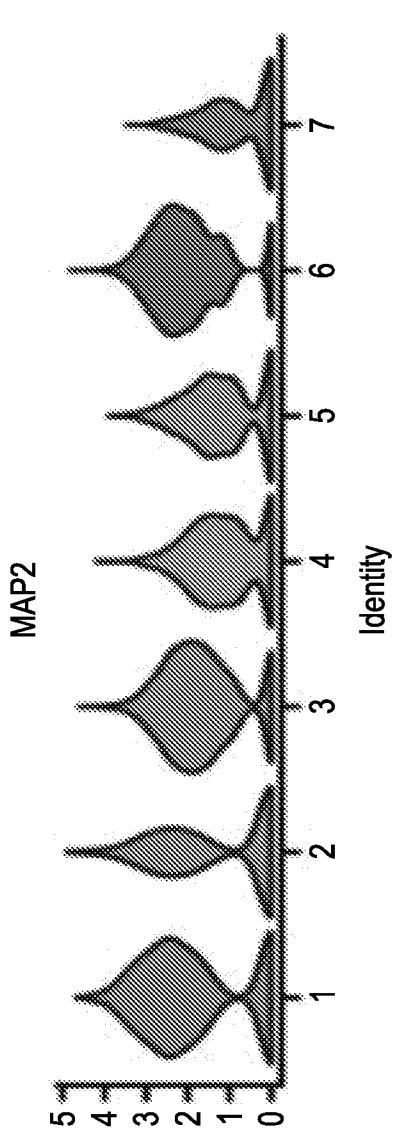


FIGURE 8E



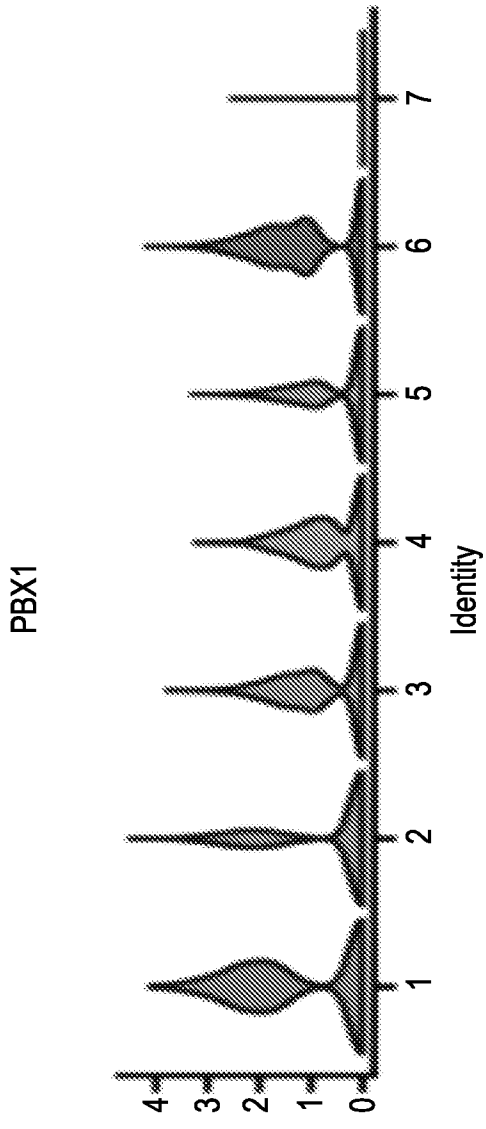


FIGURE 8F

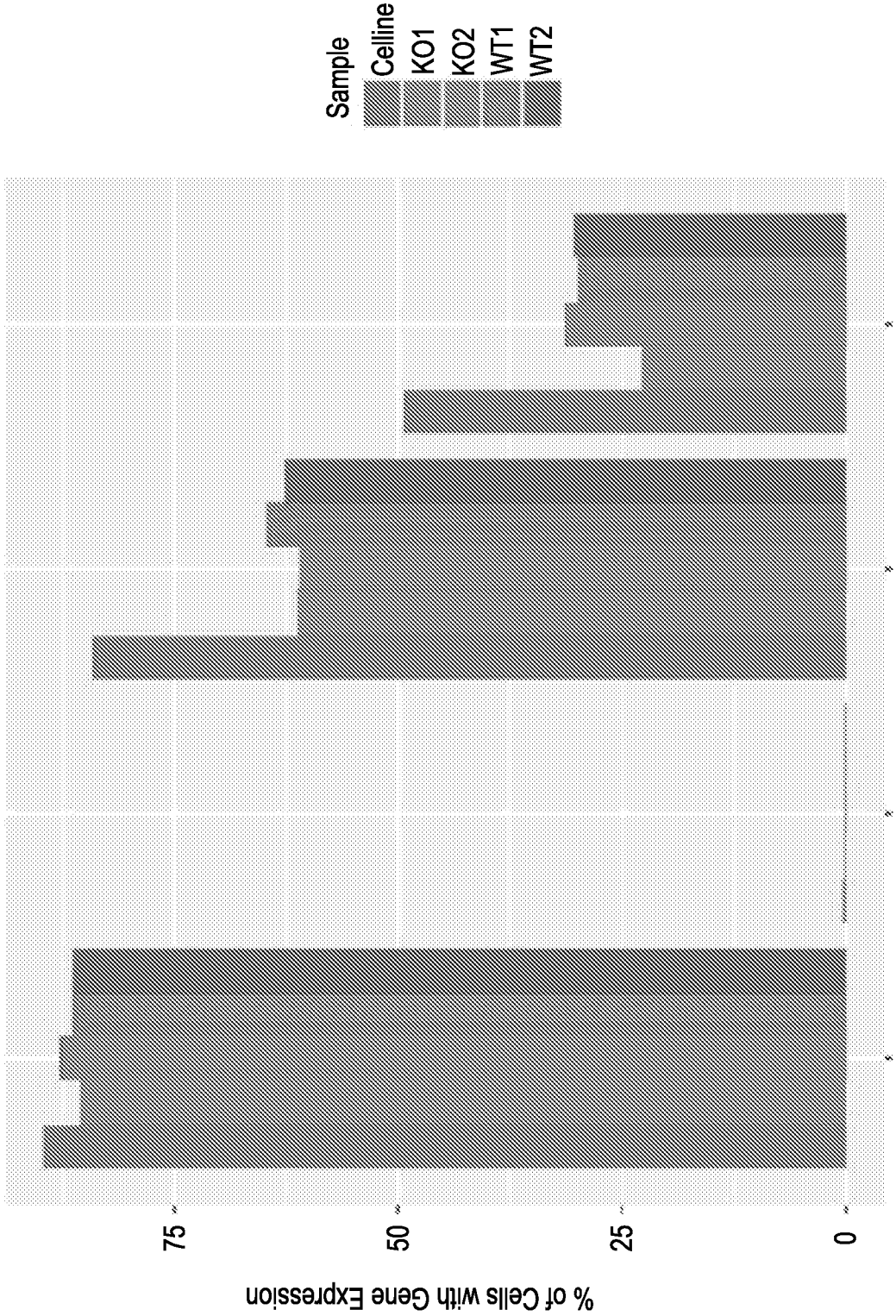


FIGURE 8F (Continued)

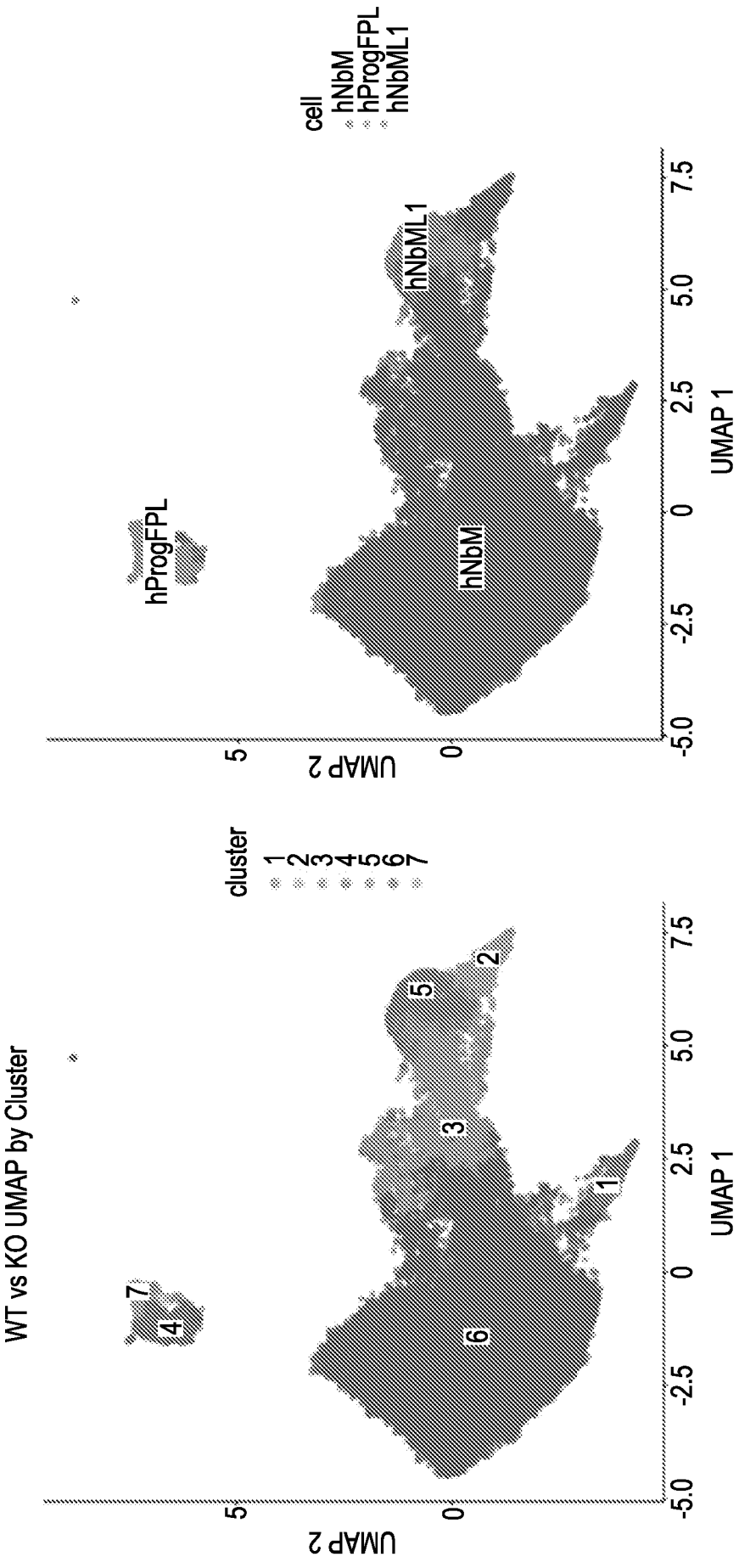

WT vs KO UMAP by Cluster
cluster
1
2
3
4
5
6
7
UMAP 2
UMAP 1
5
0
-5.0
-2.5
0
2.5
5.0
7.5
cell
hNbM
hProgFPL
hNbML1
hProgFPL
hNbML1
hNbM

FIGURE 9A

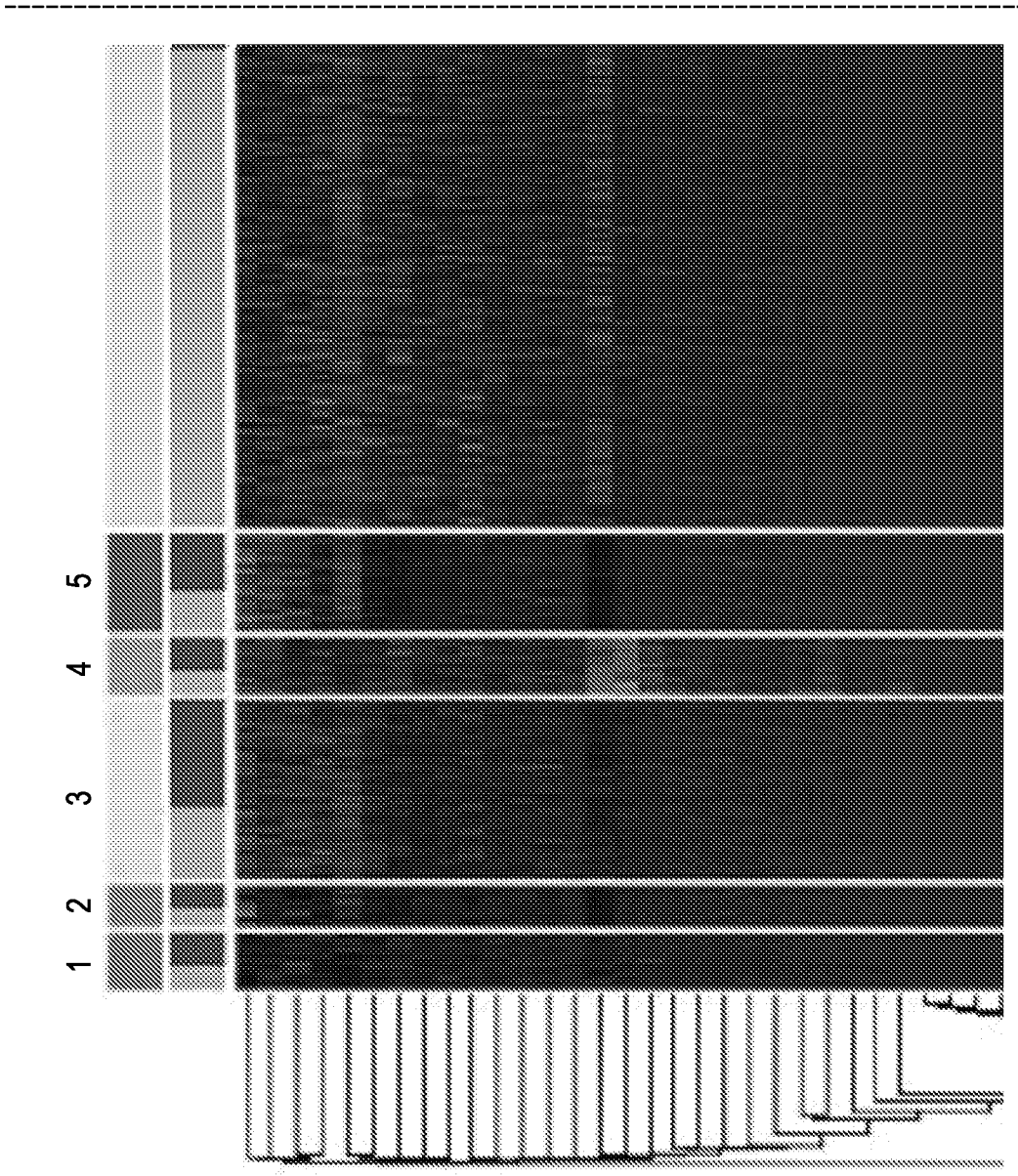


FIGURE 9B

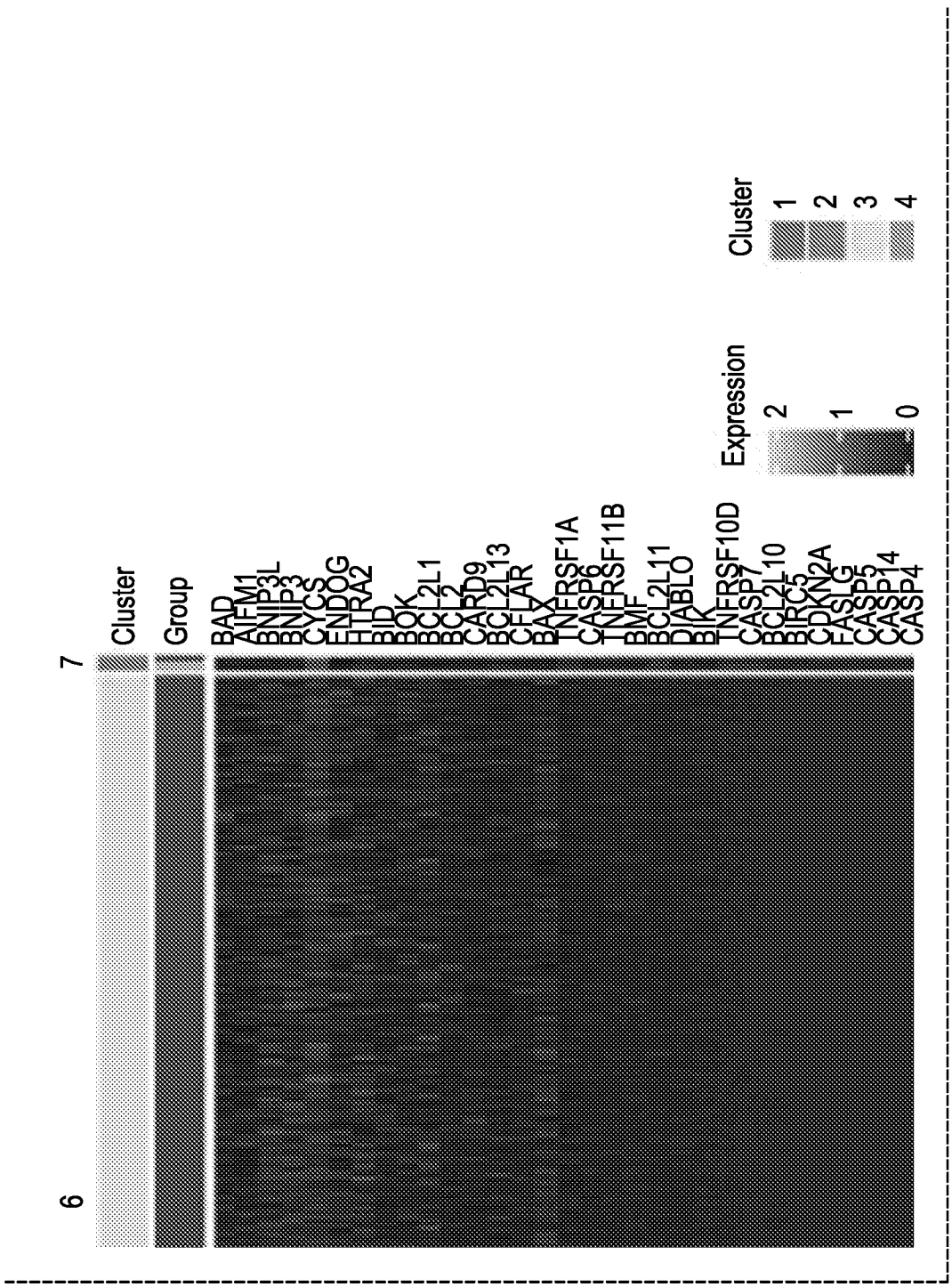


FIGURE 9B (Continued)

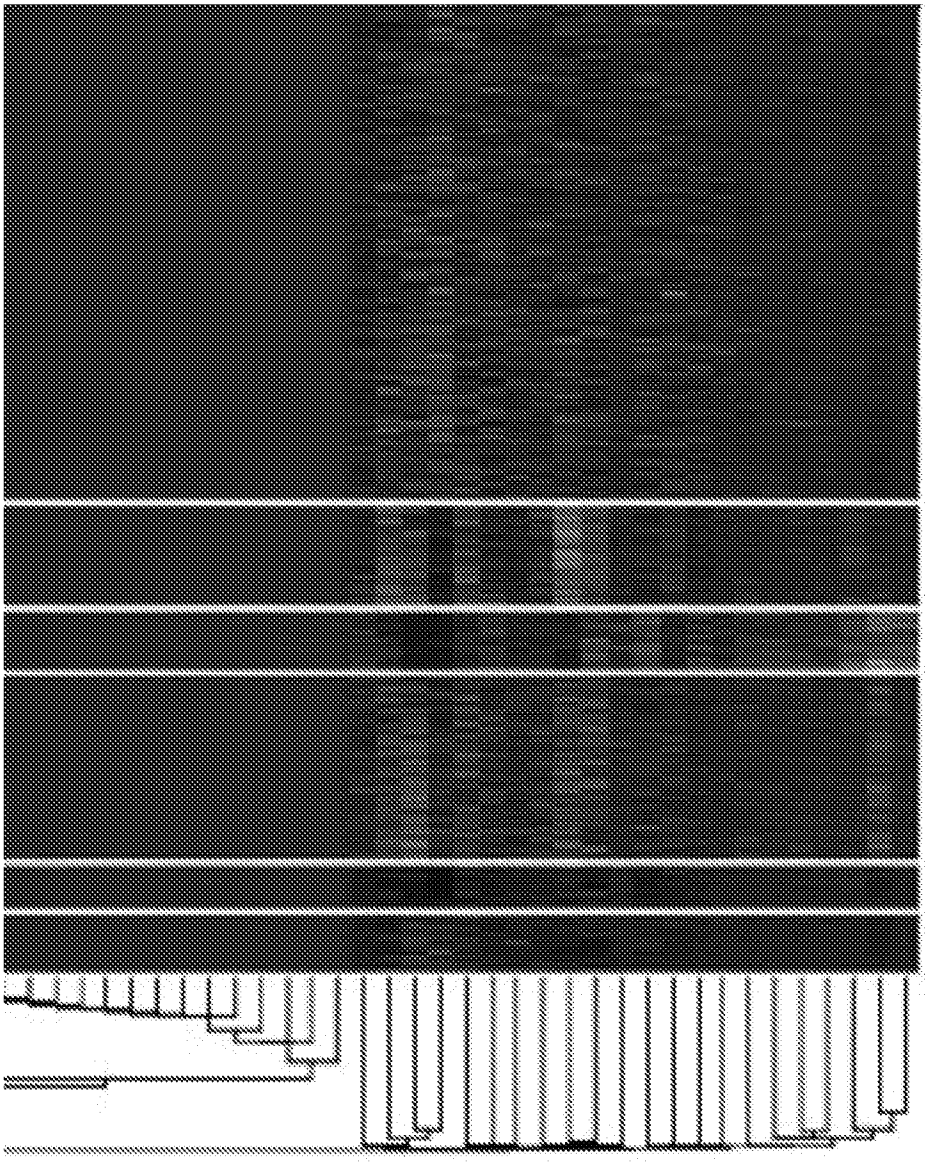


FIGURE 9B (Continued)

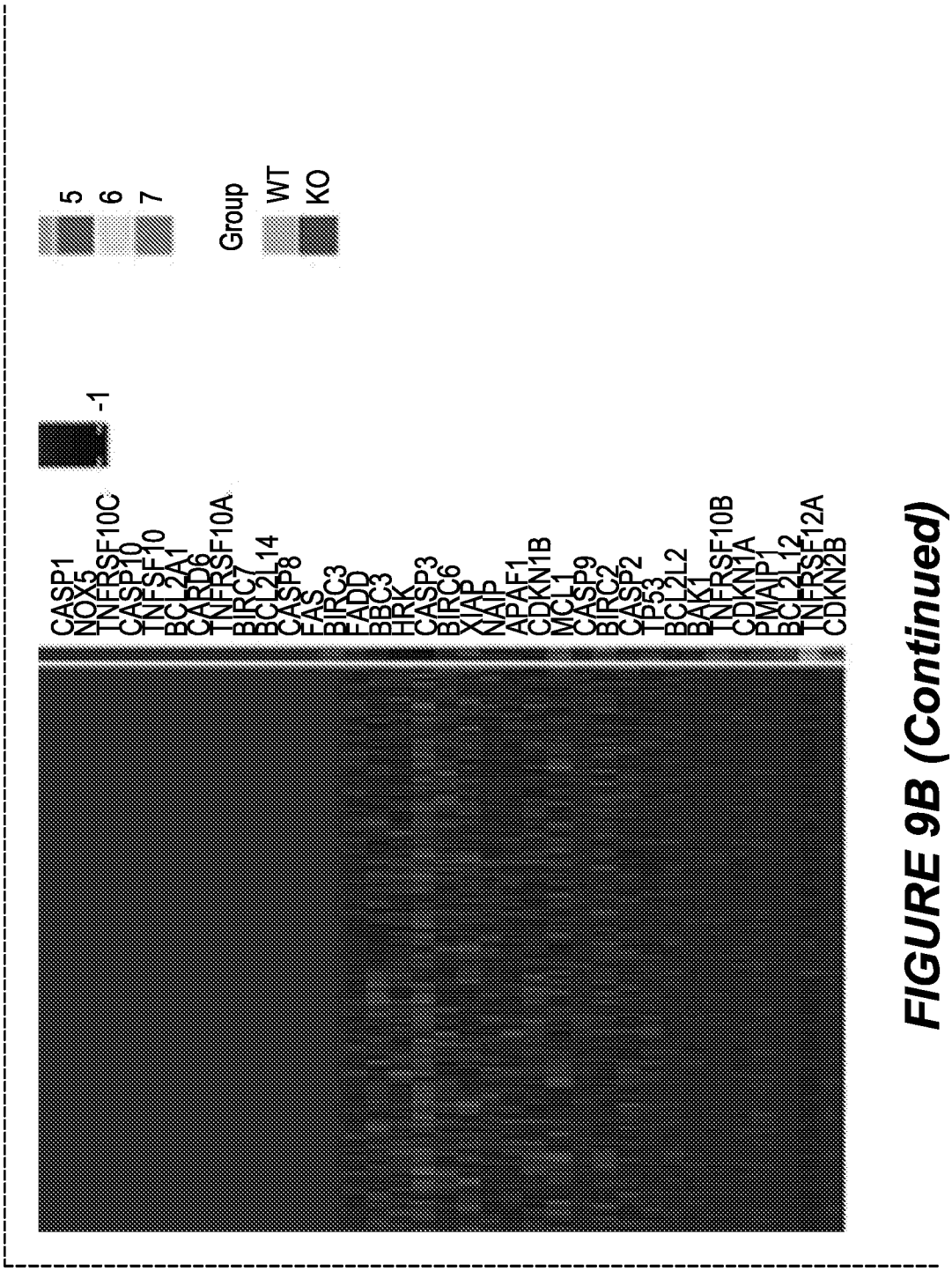


FIGURE 9B (Continued)

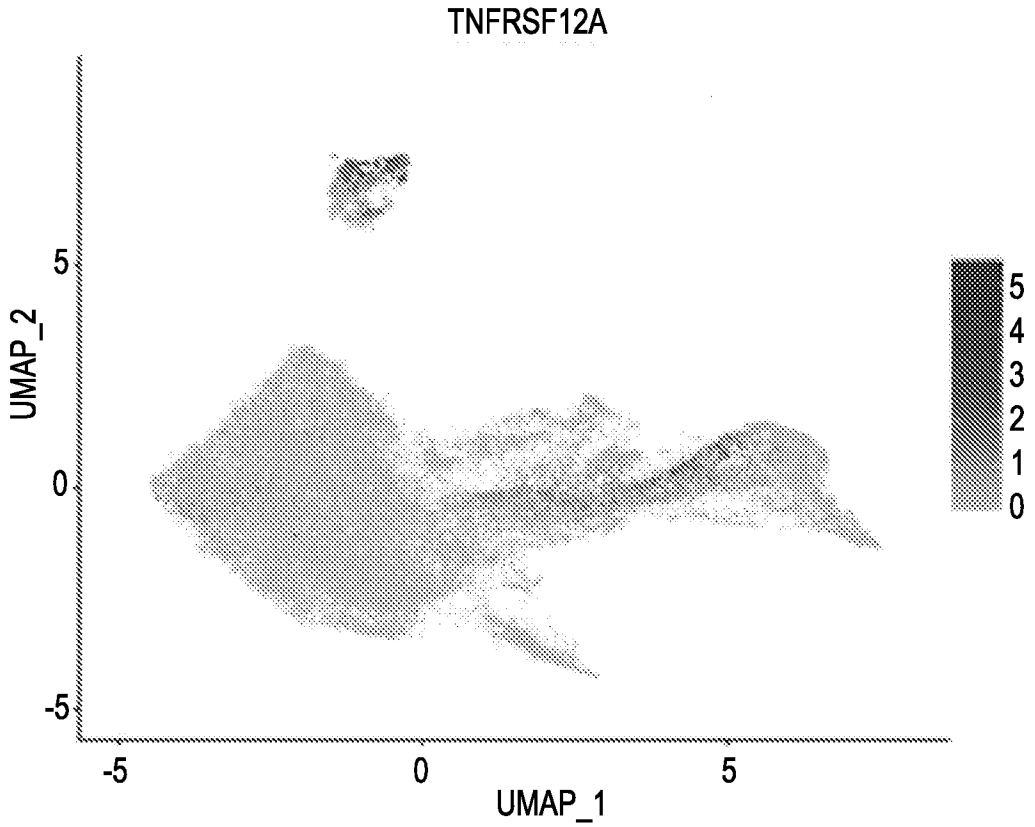


FIGURE 9C

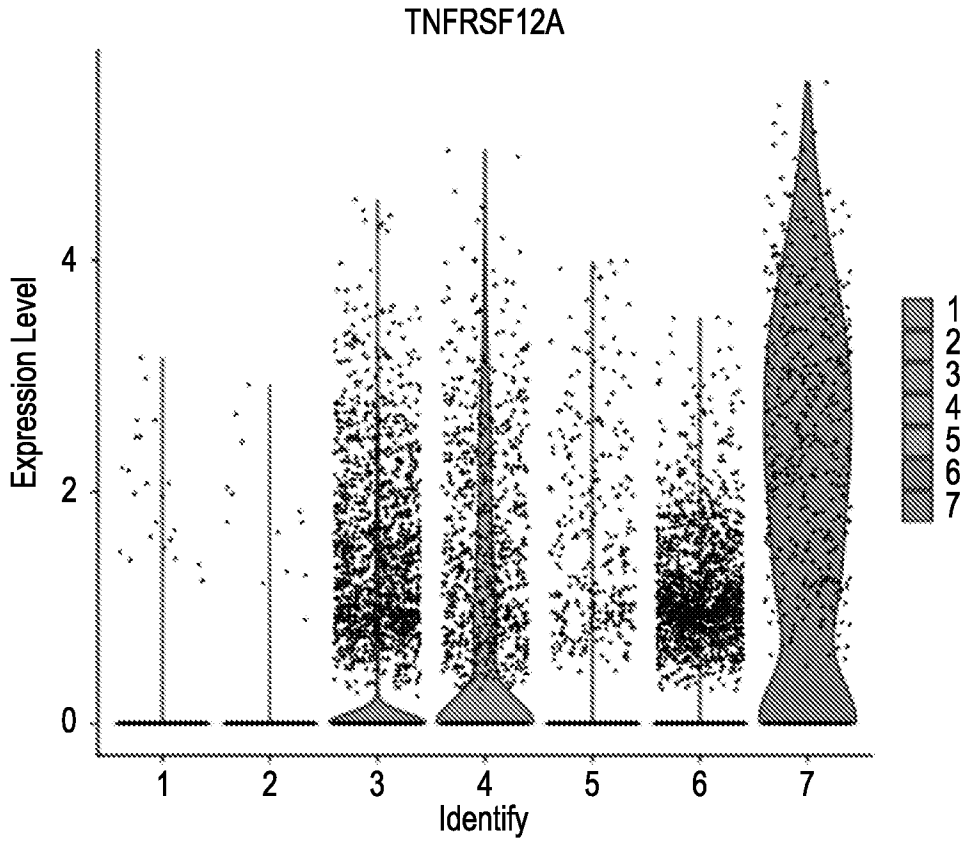


FIGURE 9D

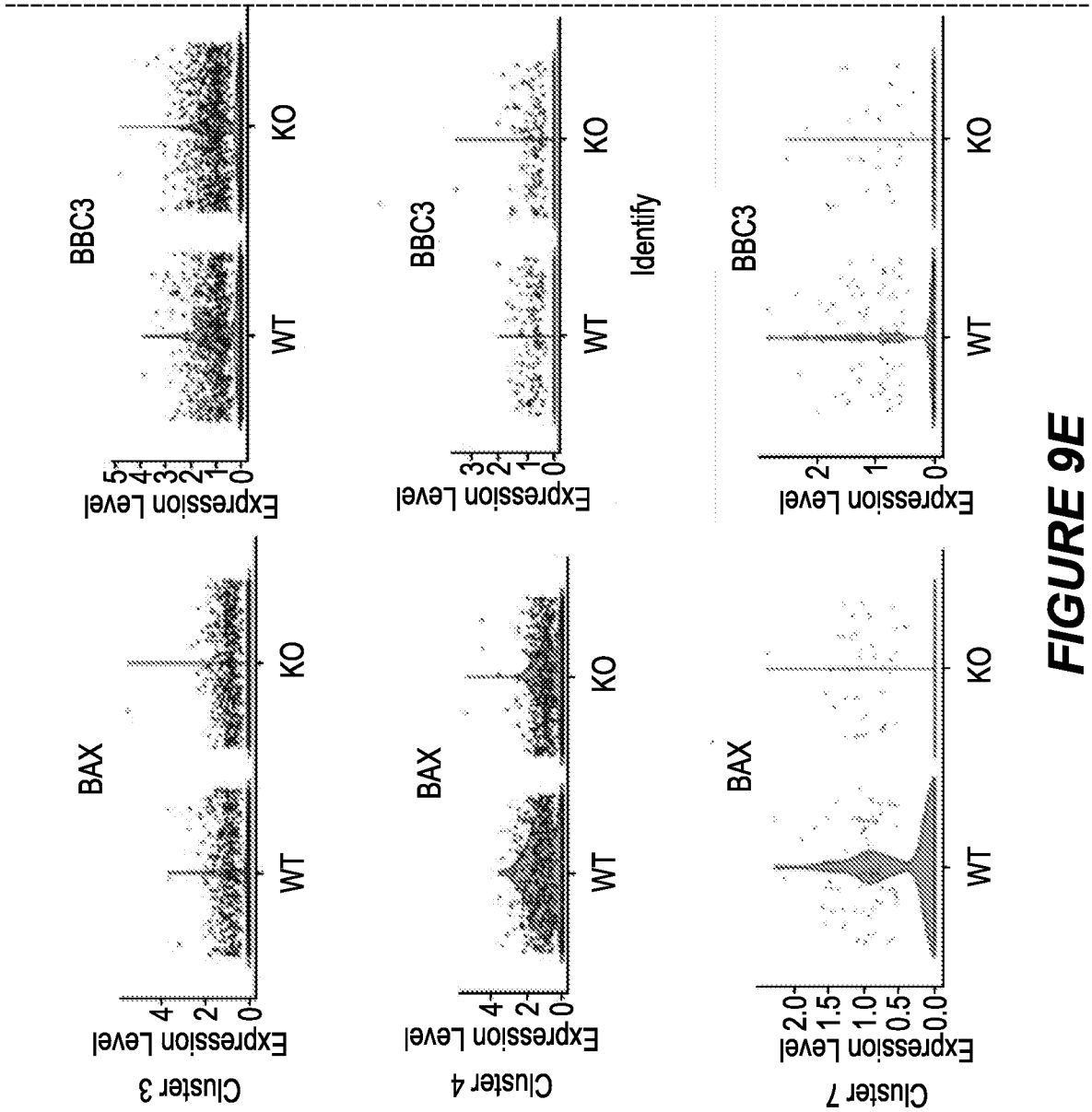

Cluster 3
Expression Level
BAX
WT
KO
BBC3
Cluster 4
Identify
Cluster 7

FIGURE 9E

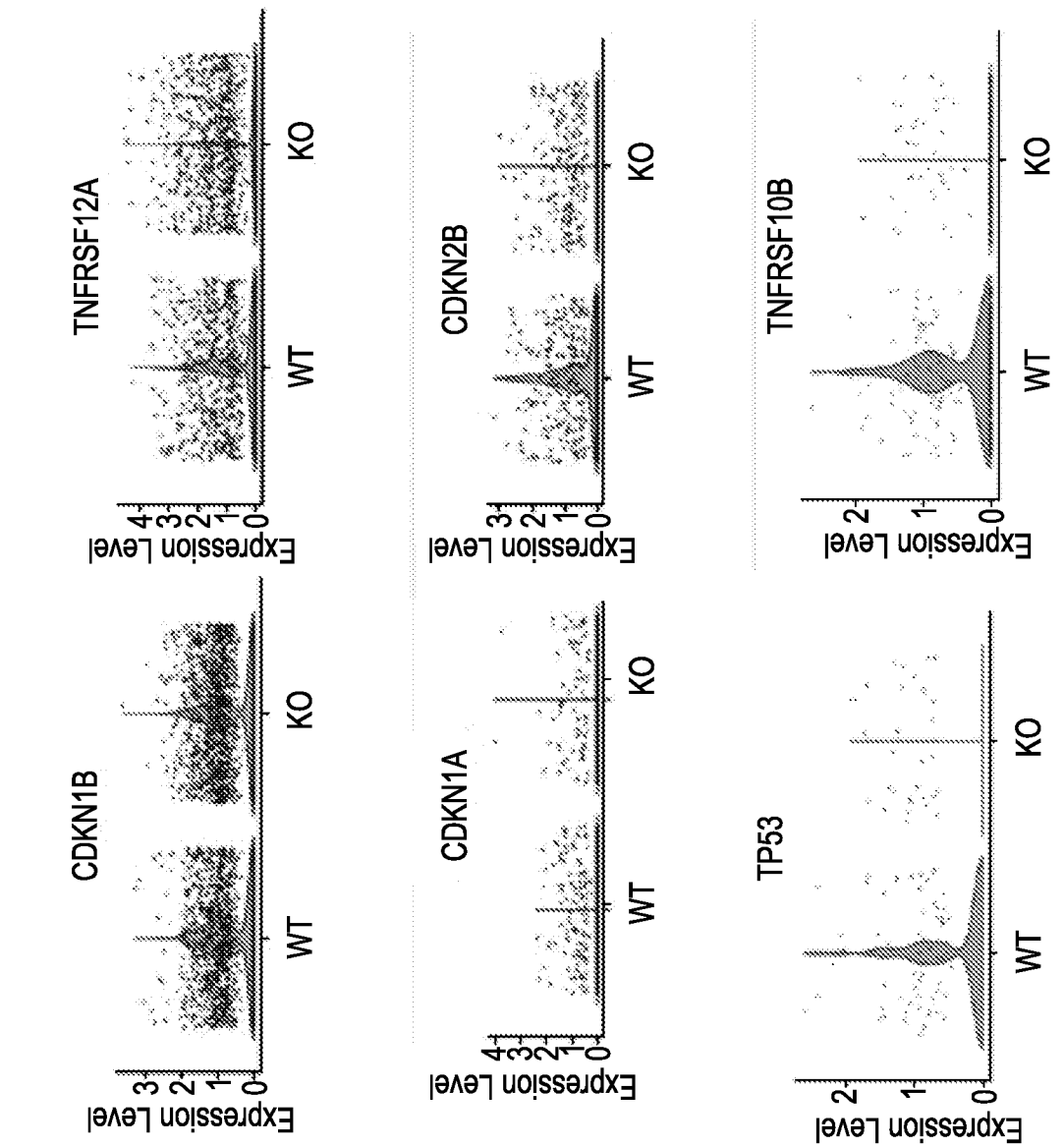


FIGURE 9E (Continued)

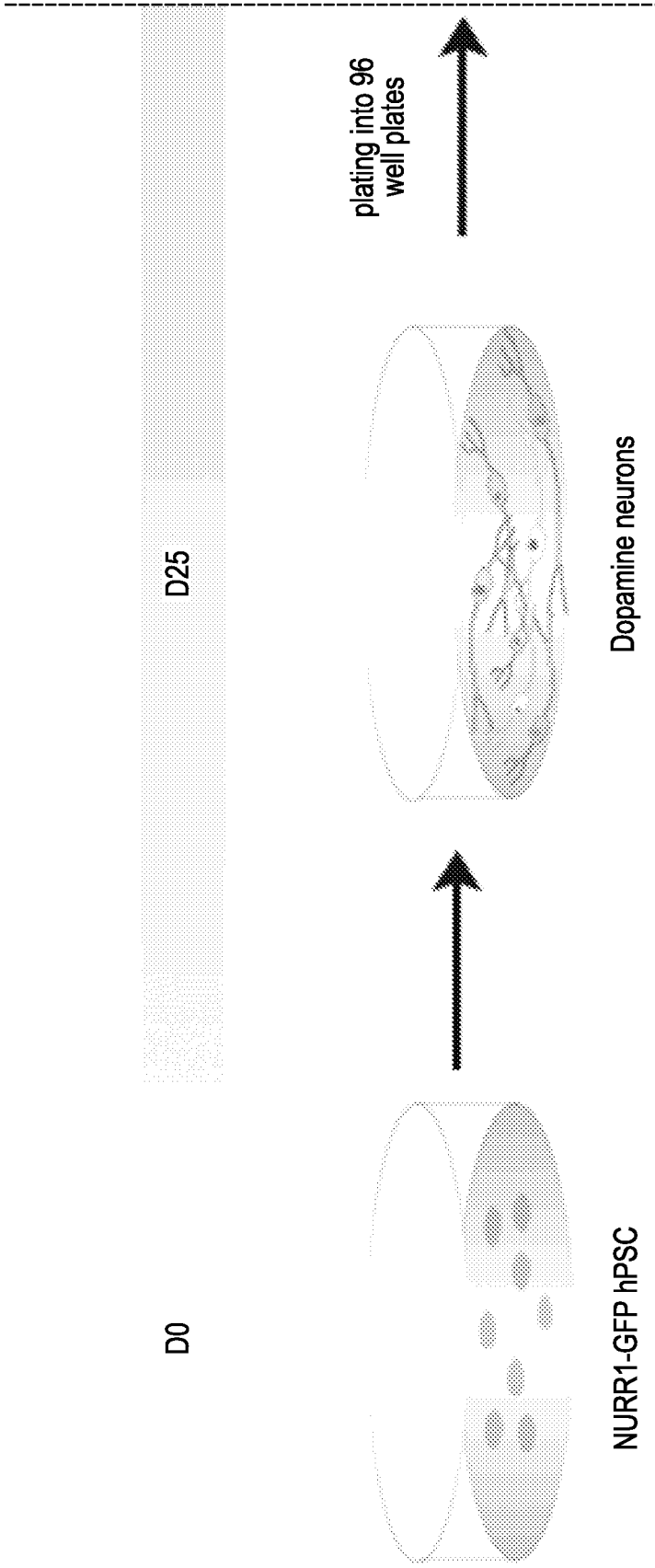

plating into 96 well plates
D25
Dopamine neurons
D0
NURR1-GFP hPSC

FIGURE 10A

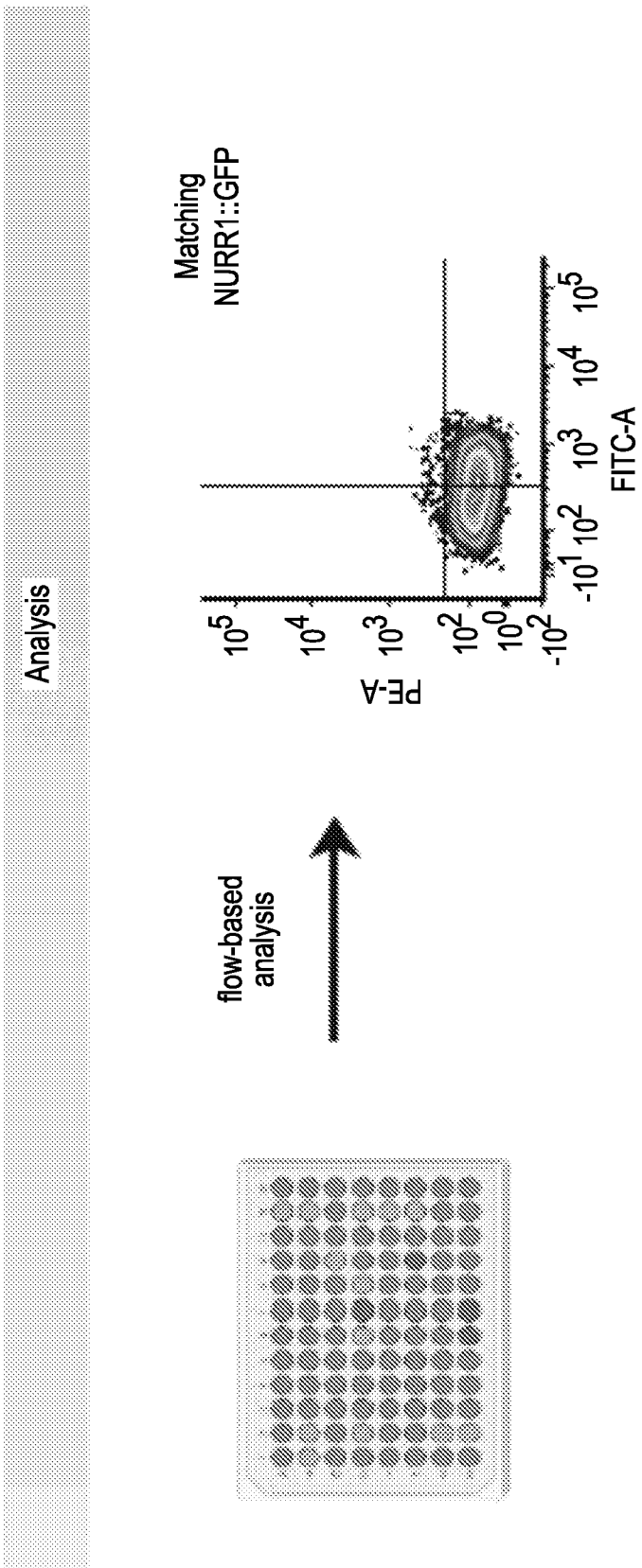


FIGURE 10A (Continued)

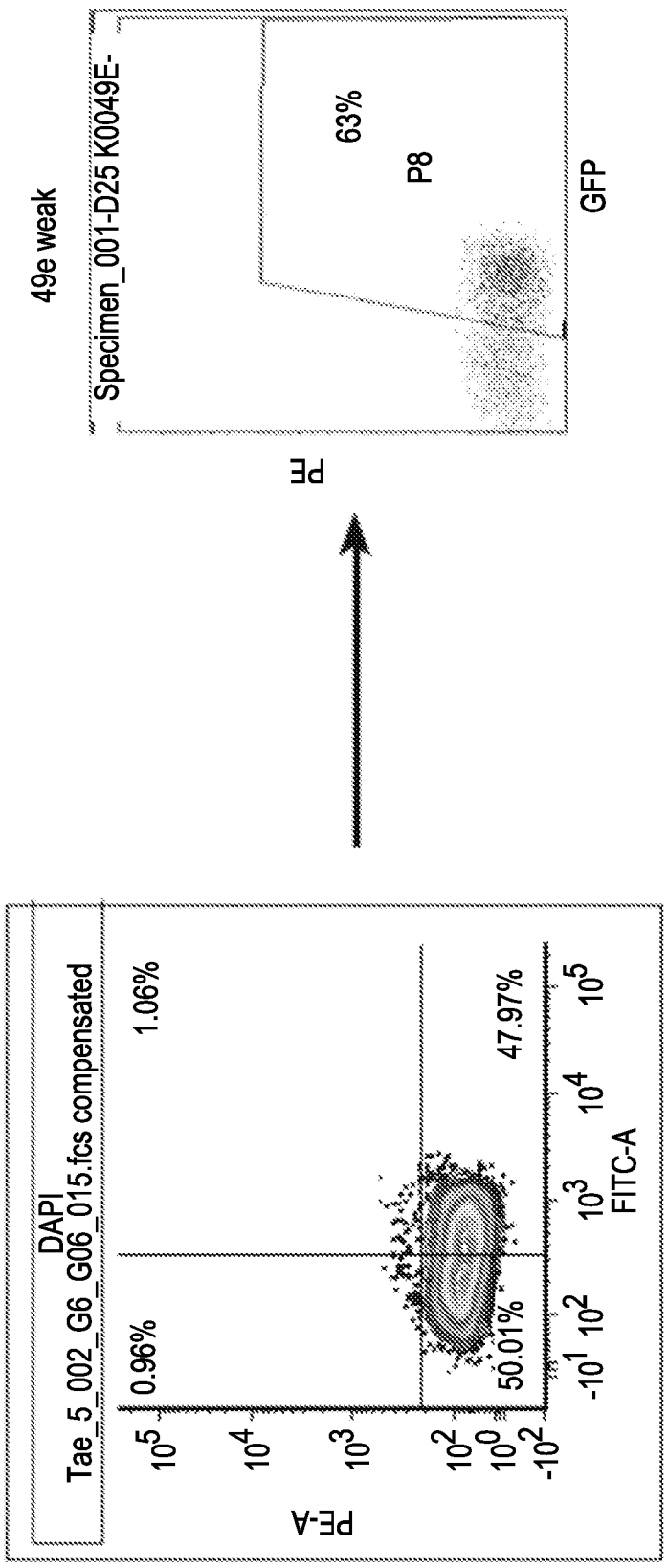

49e weak
Specimen_001-D25 K0049E-
63%
P8
GFP
PE
DAPI
Tae_5_002_G6_G06_015.fcs compensated
1.06%
0.96%
50.01%
47.97%
PE-A
FITC-A

FIGURE 10B

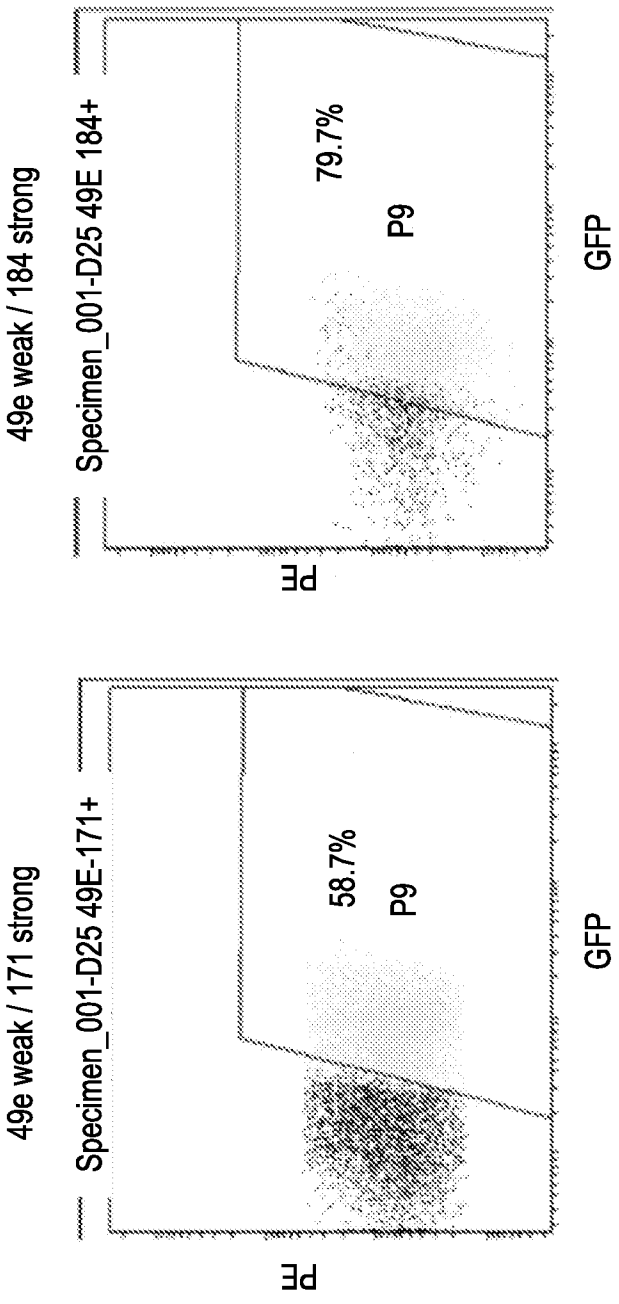


FIGURE 10B (Continued)

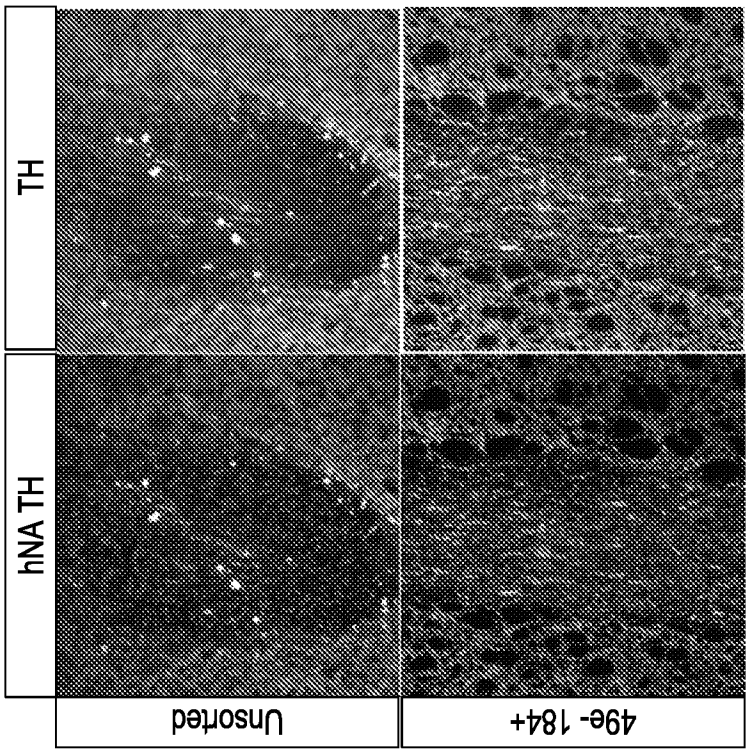


FIGURE 10E

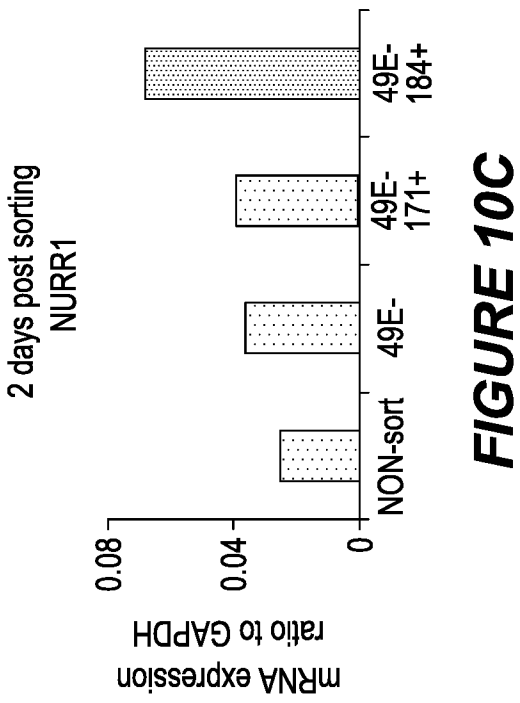


FIGURE 10C

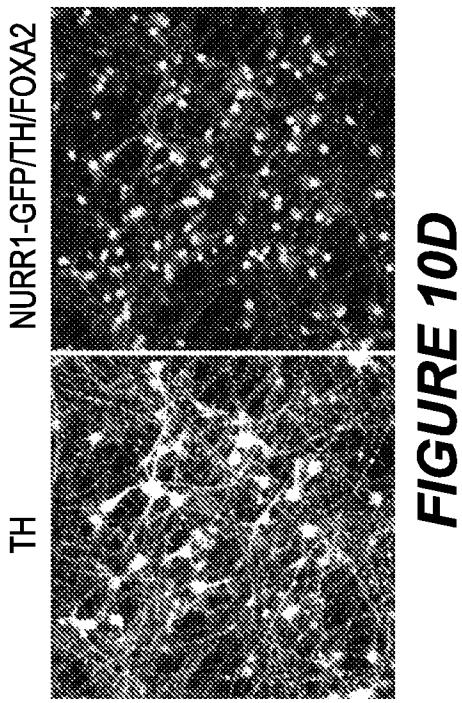


FIGURE 10D

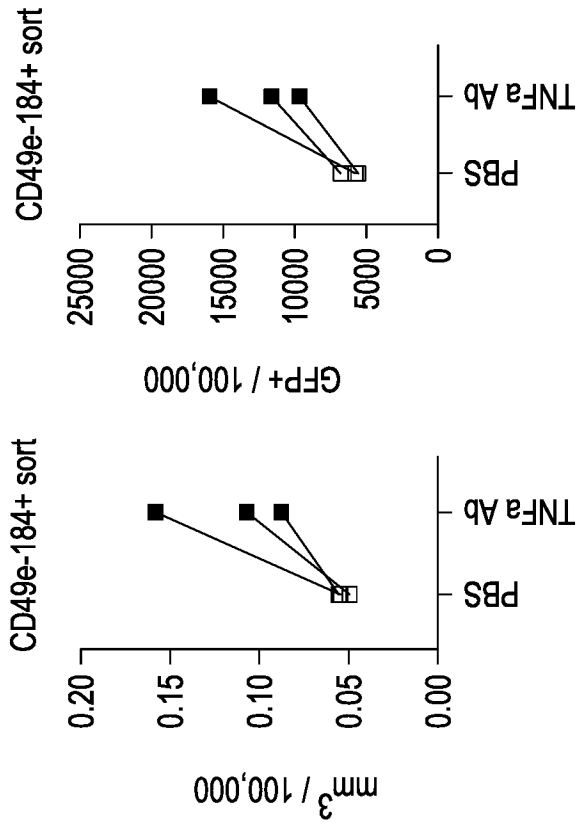


FIGURE 10G

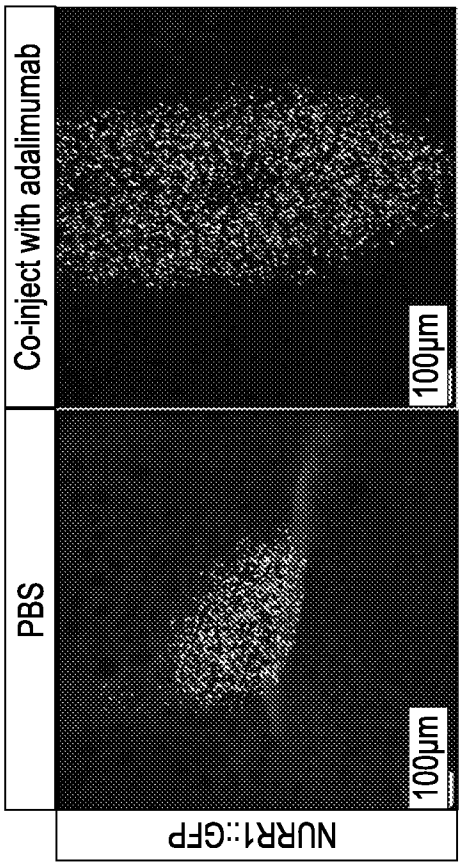


FIGURE 10F

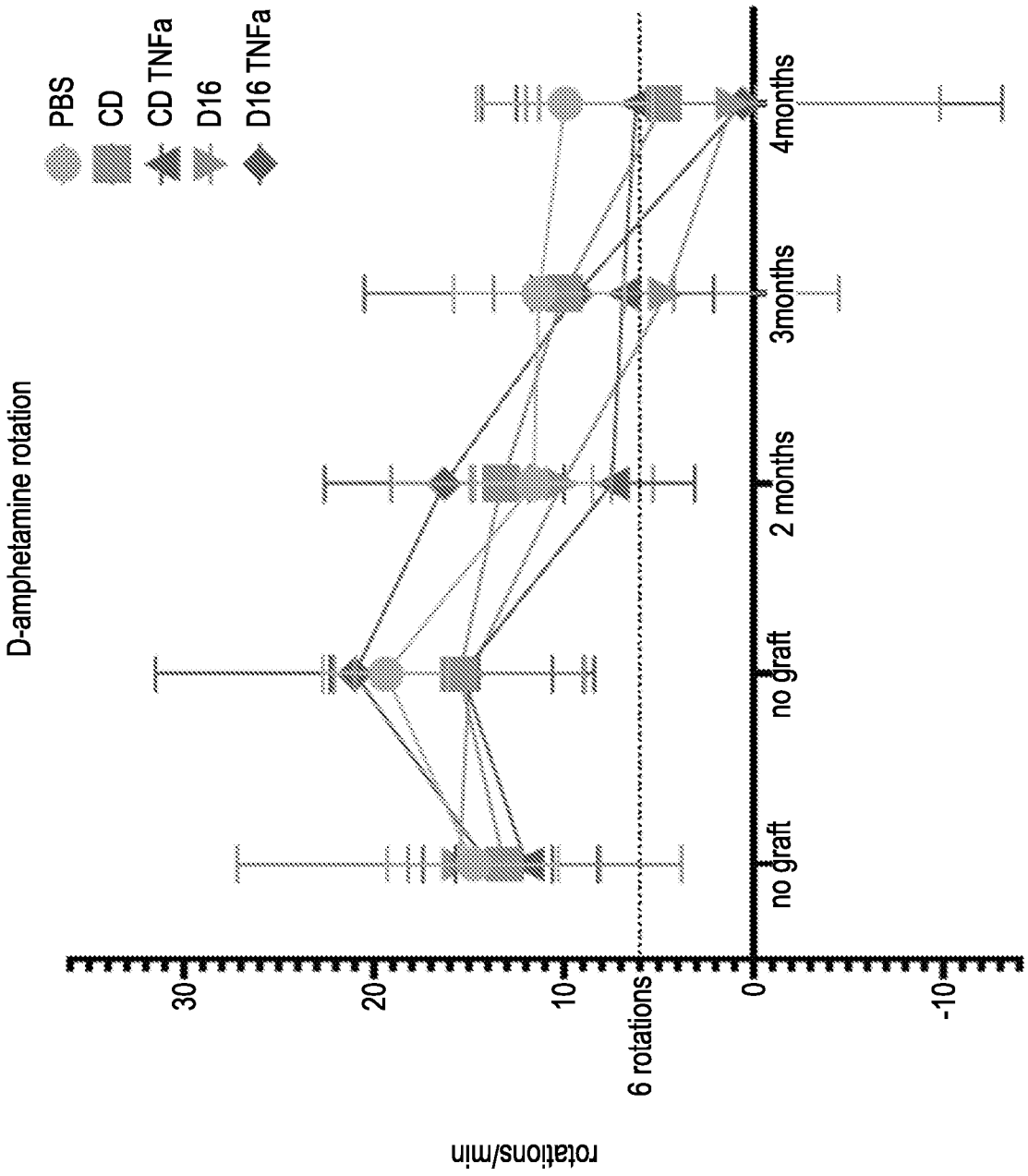

D-amphetamine rotation
PBS
CD
CD TNFa
D16
D16 TNFa
30
20
10
6 rotations
0
-10
rotations/min
no graft
no graft
2 months
3months
4months

FIGURE 10H

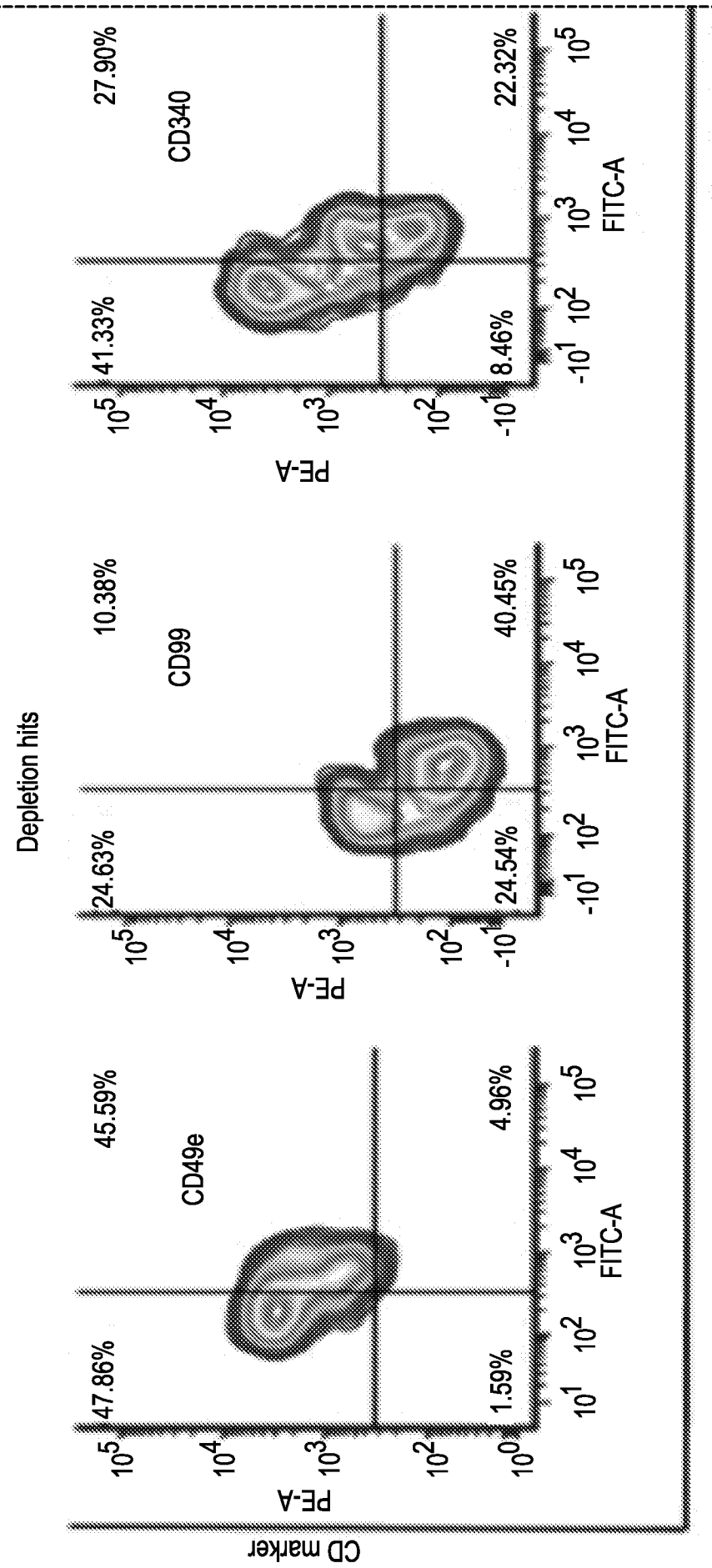

Depletion hits
CD marker
CD49e
CD99
CD340
PE-A
FITC-A
NURR1-GFP
47.86%
1.59%
45.59%
4.96%
24.63%
24.54%
10.38%
40.45%
41.33%
8.46%
27.90%
22.32%

FIGURE 11A

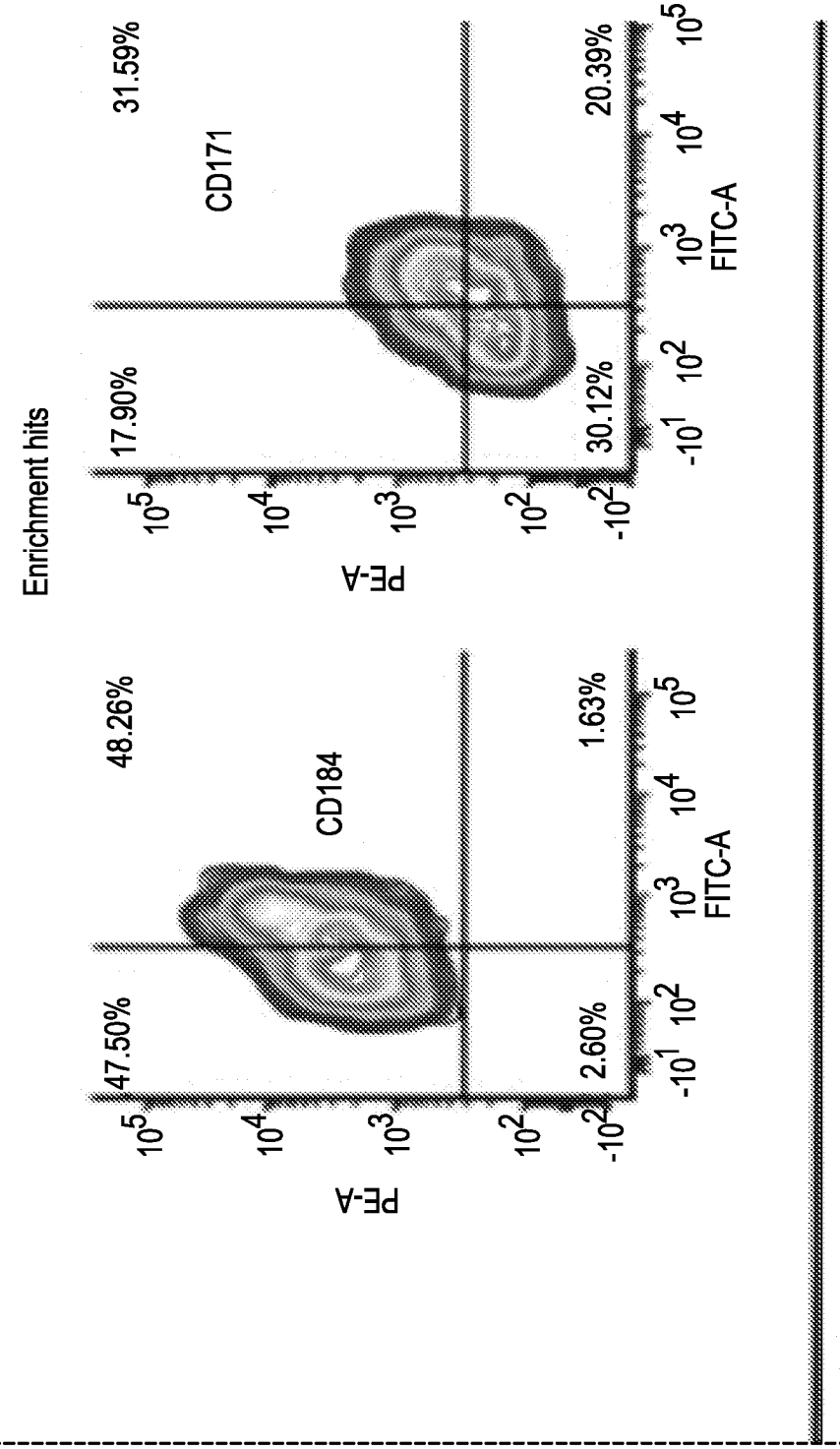


FIGURE 11A (Continued)

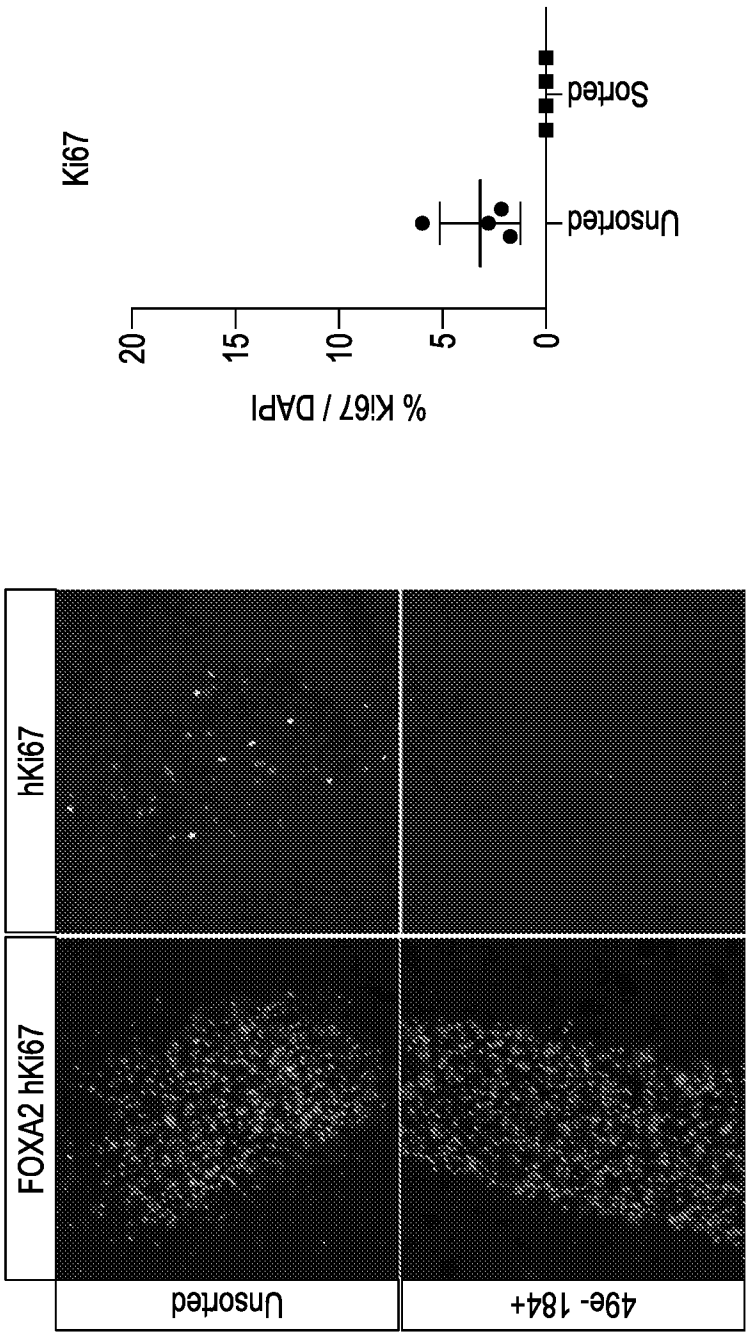

Ki67
Sorted
Unsorted
20
15
10
5
0
% Ki67 / DAPI
hKi67
FOXA2 hKi67
Unsorted
49e- 184+

FIGURE 11B

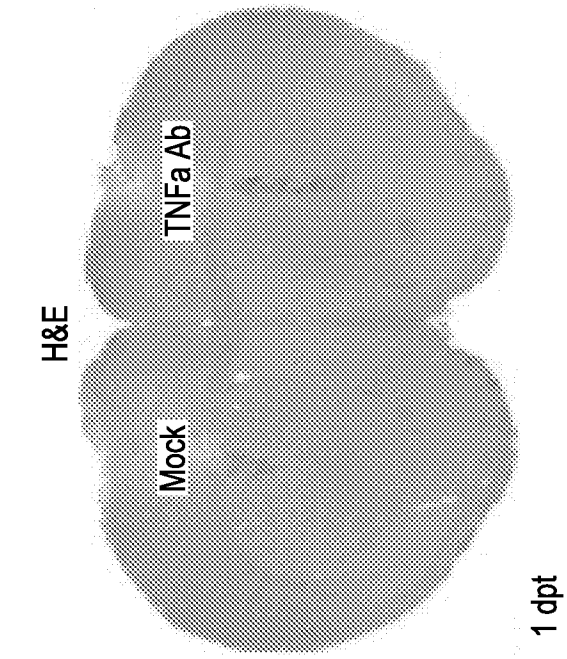


FIGURE 11D

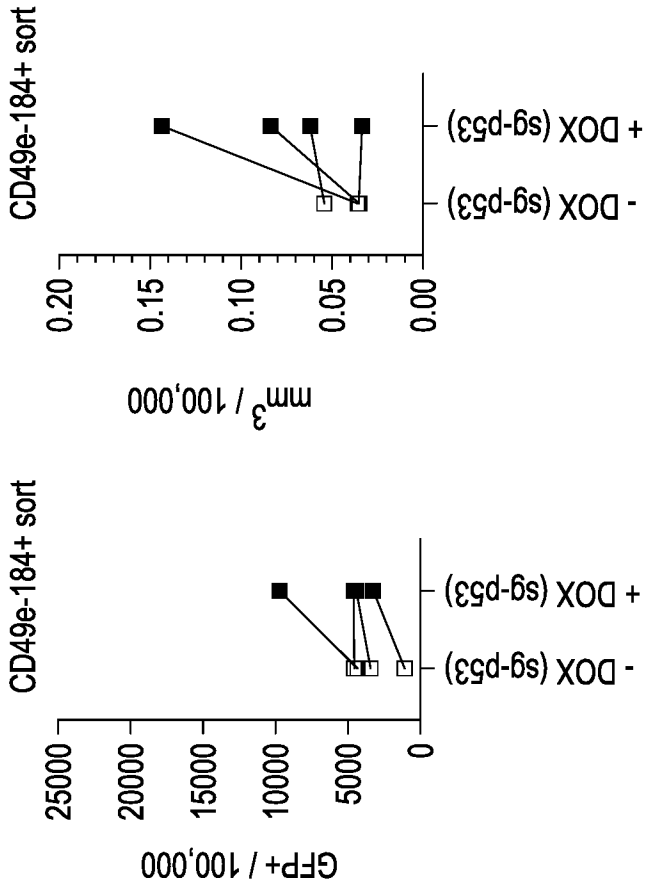


FIGURE 11C

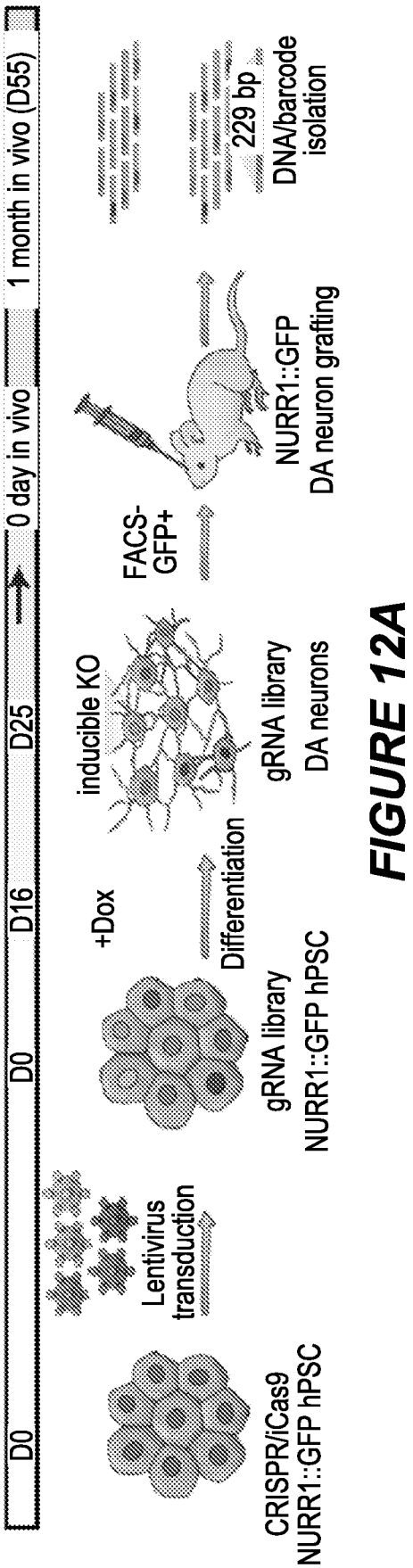


FIGURE 12A

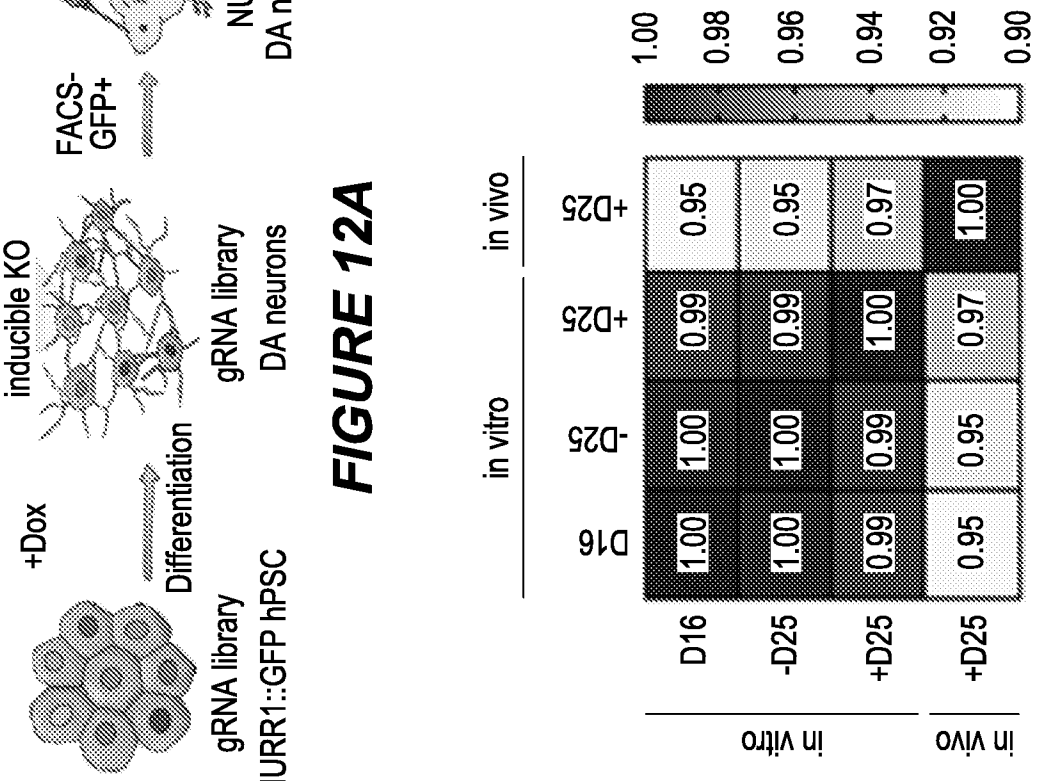


FIGURE 12B

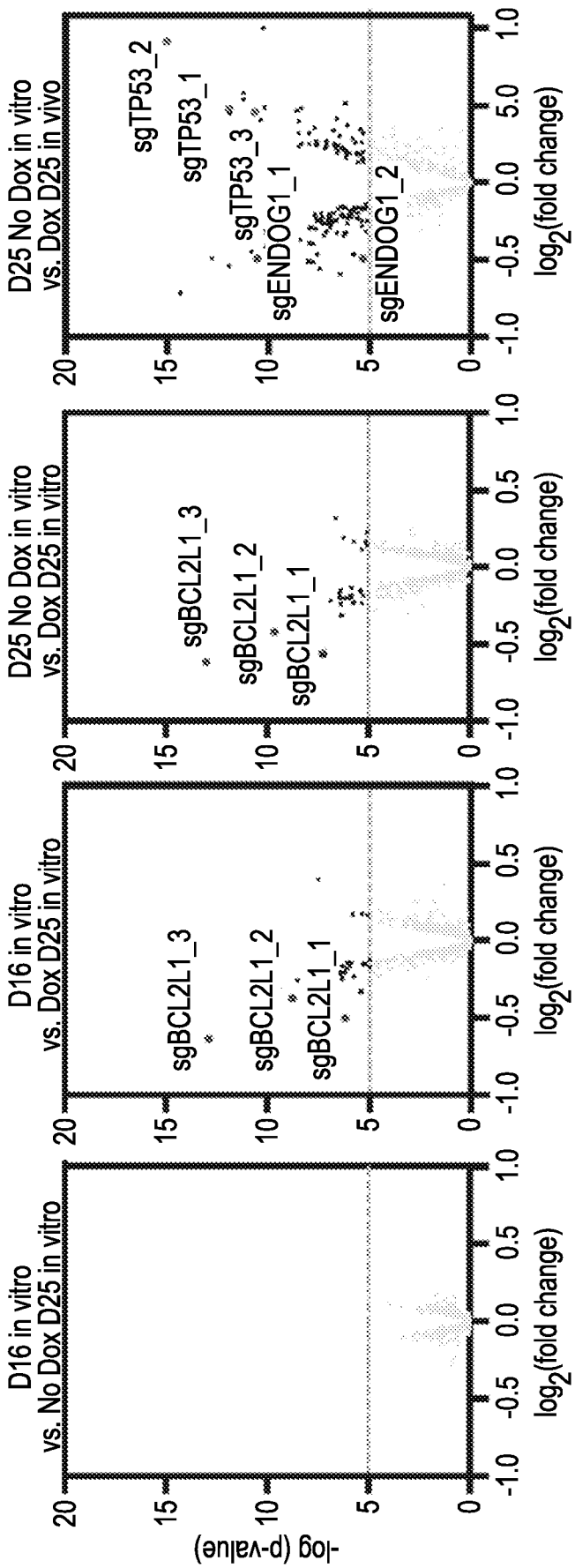

D16 in vitro
vs. No Dox D25 in vitro
D16 in vitro
vs. Dox D25 in vitro
D25 No Dox in vitro
vs. Dox D25 in vitro
D25 No Dox in vitro
vs. Dox D25 in vivo
-log (p-value)
log2(fold change)
sgBCL2L1_1
sgBCL2L1_2
sgBCL2L1_3
sgENDOG1_1
sgENDOG1_2
sgTP53_1
sgTP53_2
sgTP53_3

FIGURE 12C

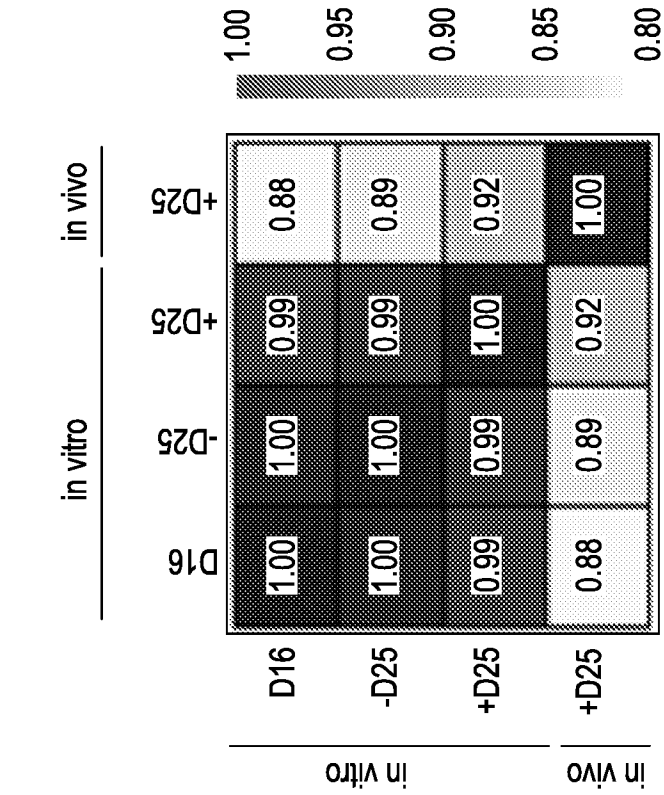


FIGURE 12E

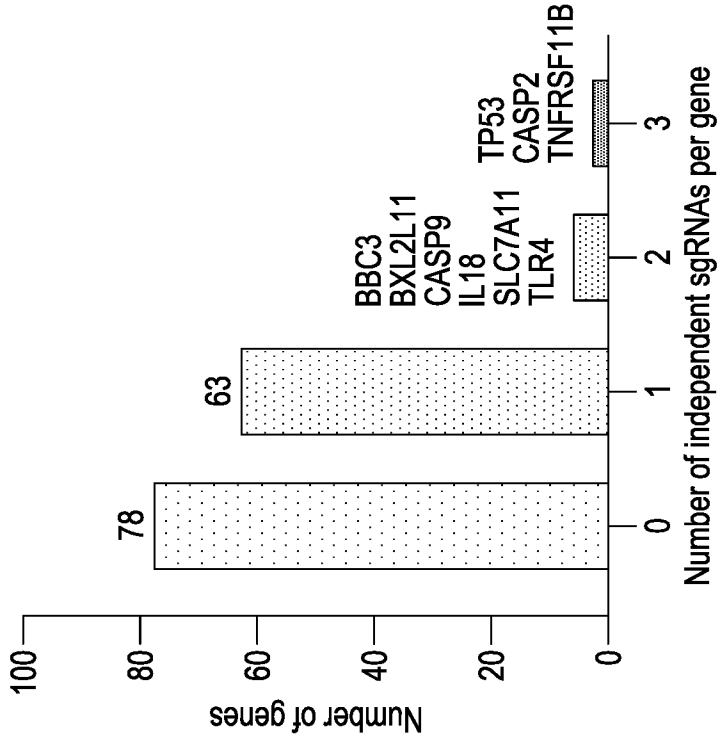


FIGURE 12D

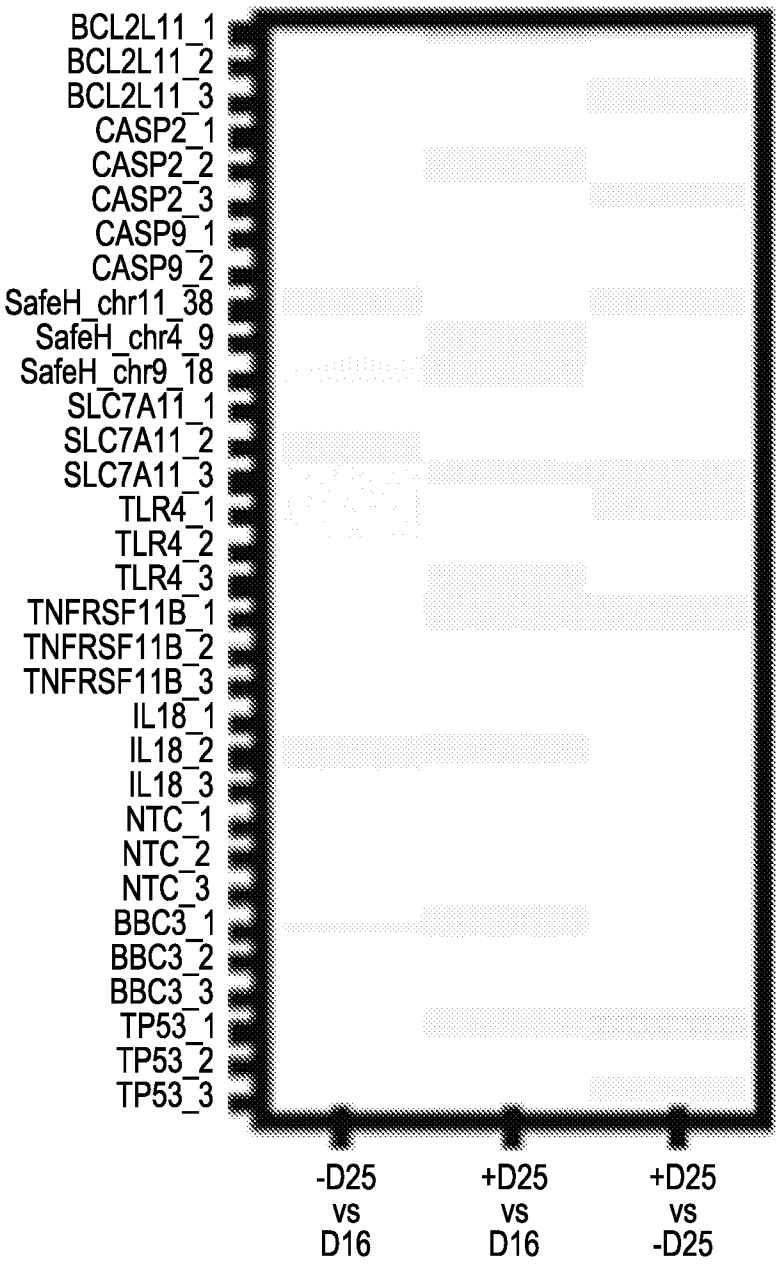

BCL2L11_1
BCL2L11_2
BCL2L11_3
CASP2_1
CASP2_2
CASP2_3
CASP9_1
CASP9_2
SafeH_chr11_38
SafeH_chr4_9
SafeH_chr9_18
SLC7A11_1
SLC7A11_2
SLC7A11_3
TLR4_1
TLR4_2
TLR4_3
TNFRSF11B_1
TNFRSF11B_2
TNFRSF11B_3
IL18_1
IL18_2
IL18_3
NTC_1
NTC_2
NTC_3
BBC3_1
BBC3_2
BBC3_3
TP53_1
TP53_2
TP53_3
-D25 vs D16
+D25 vs D16
+D25 vs -D25

FIGURE 12F

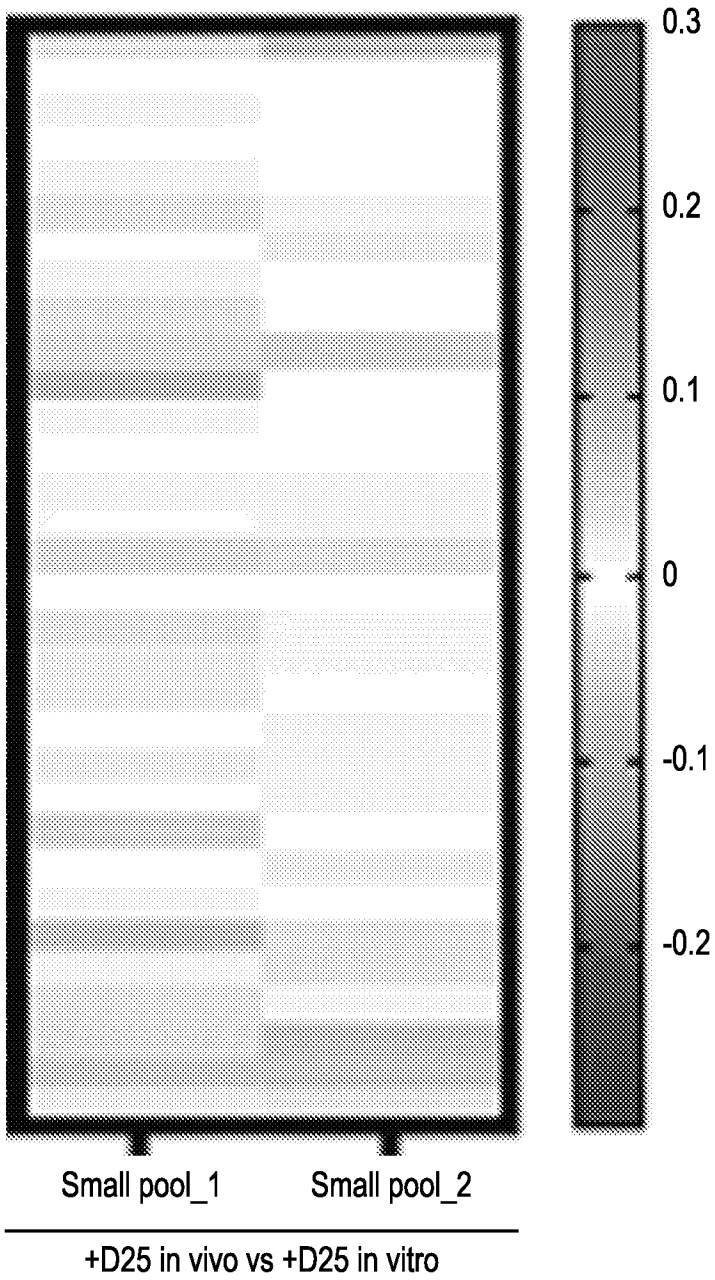


FIGURE 12F (Continued)

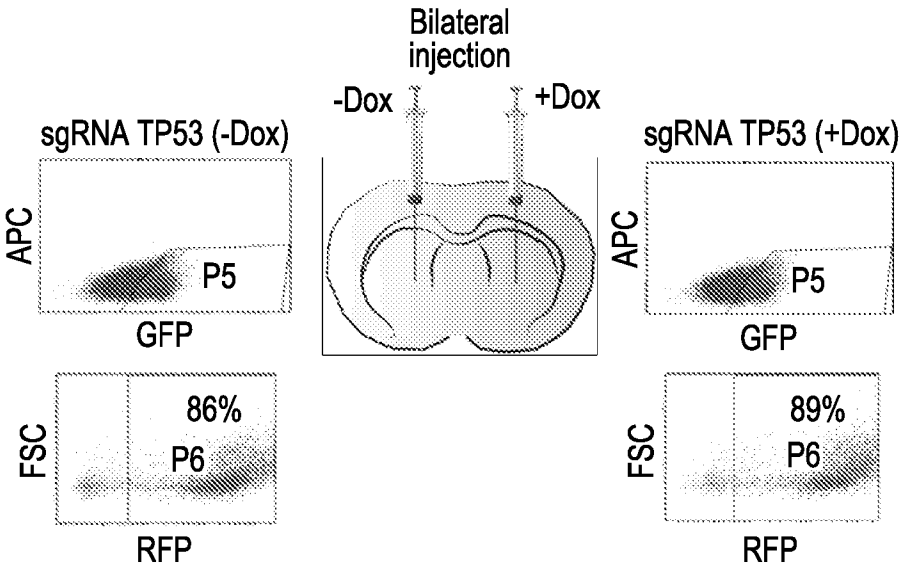


FIGURE 13A

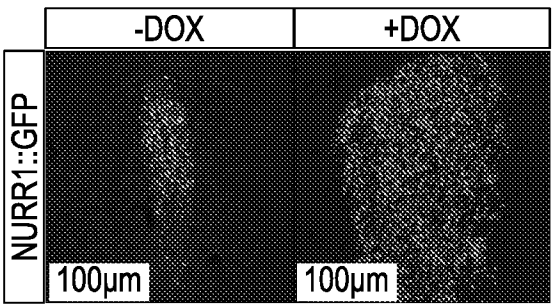


FIGURE 13B

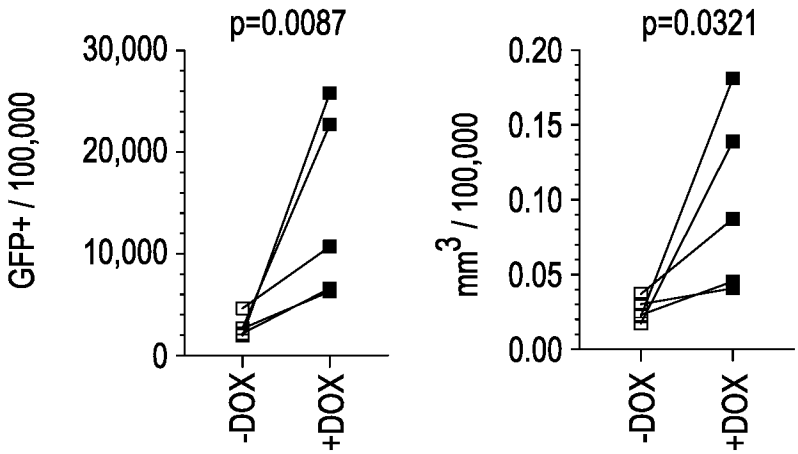


FIGURE 13C

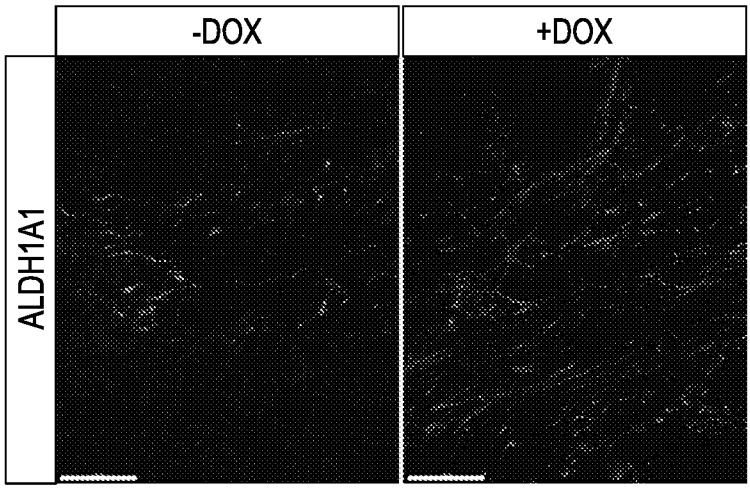


FIGURE 13D

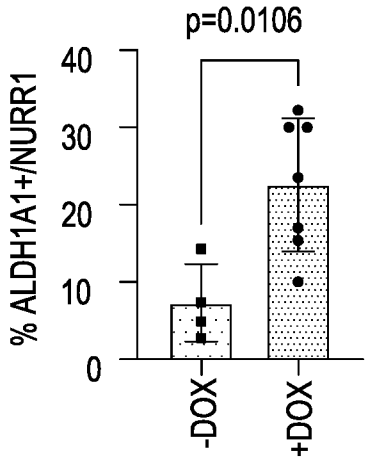


FIGURE 13E

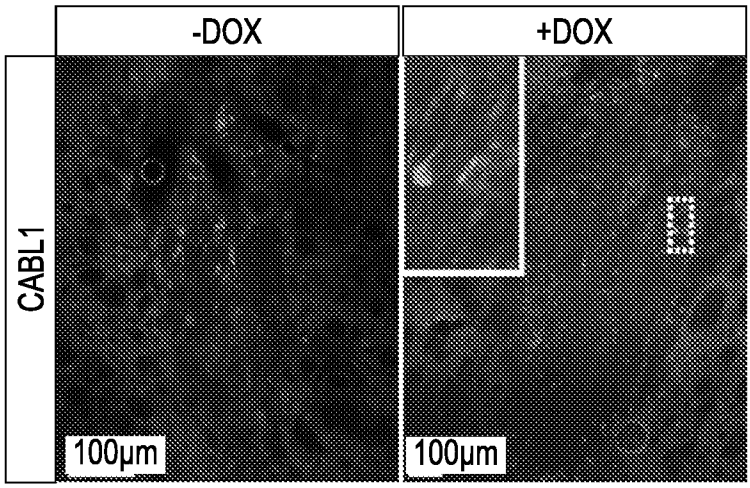


FIGURE 13F

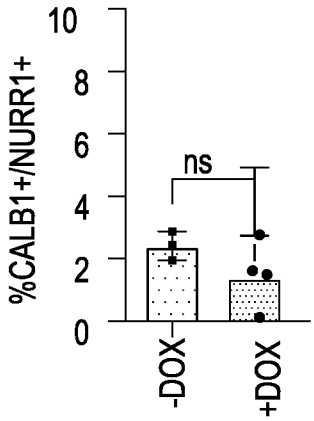
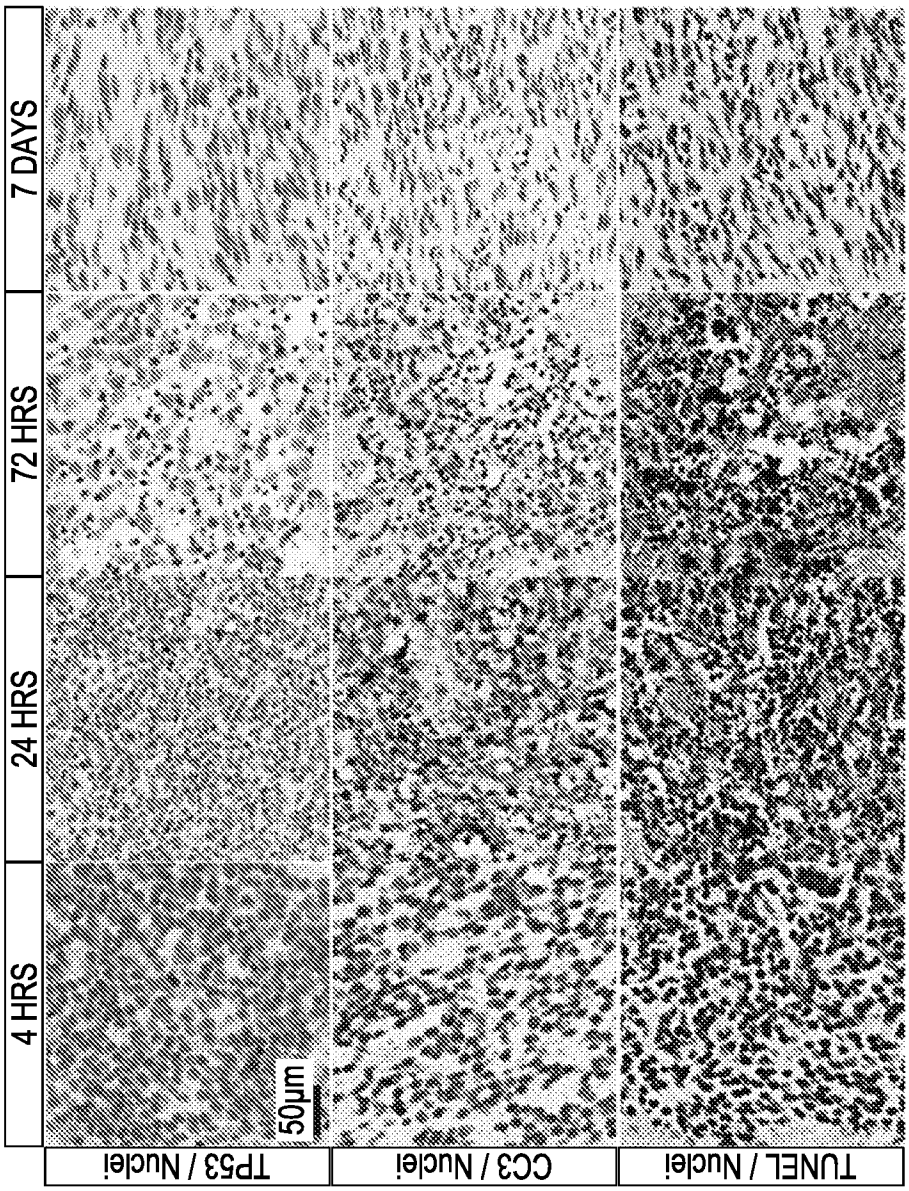


FIGURE 13G

FIGURE 14A

FIGURE 14B

FIGURE 14C



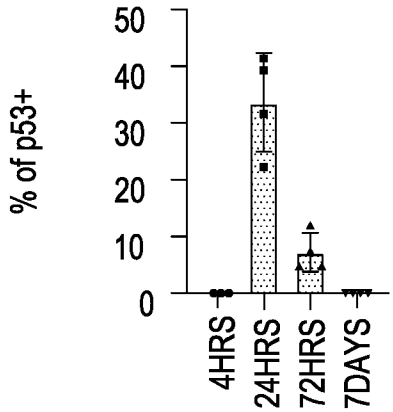


FIGURE 14D

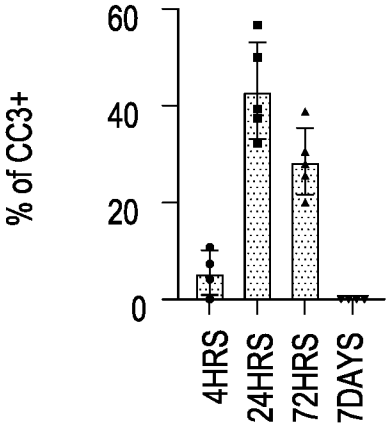


FIGURE 14E

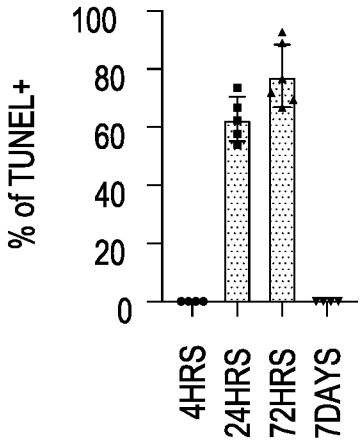


FIGURE 14F

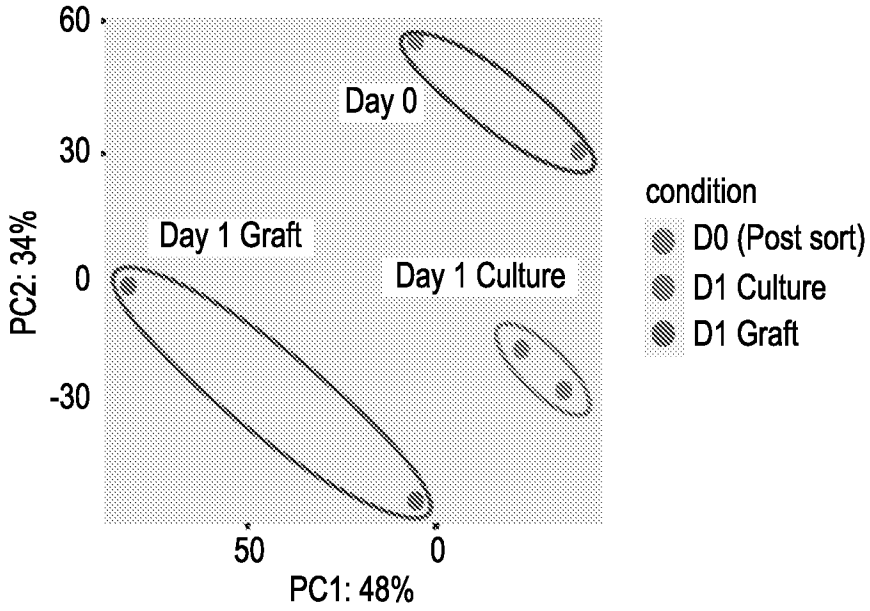


FIGURE 15A

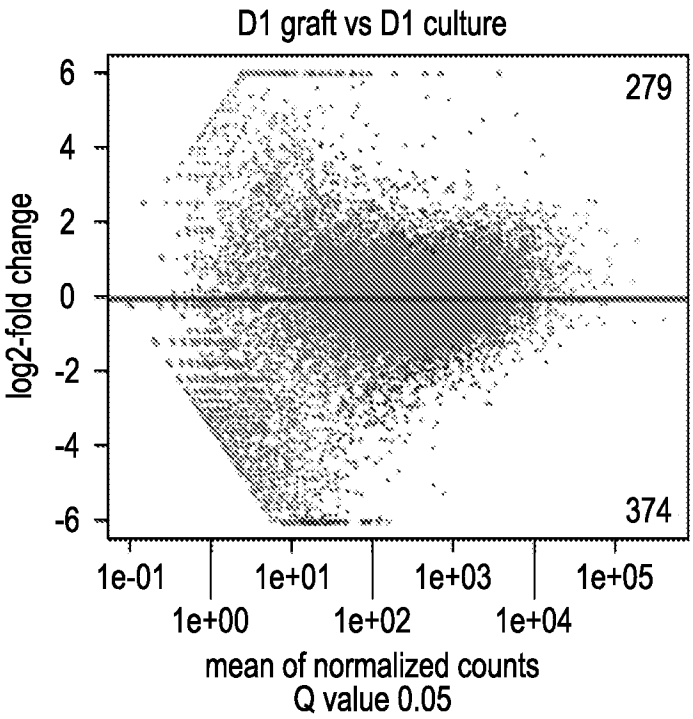


FIGURE 15B

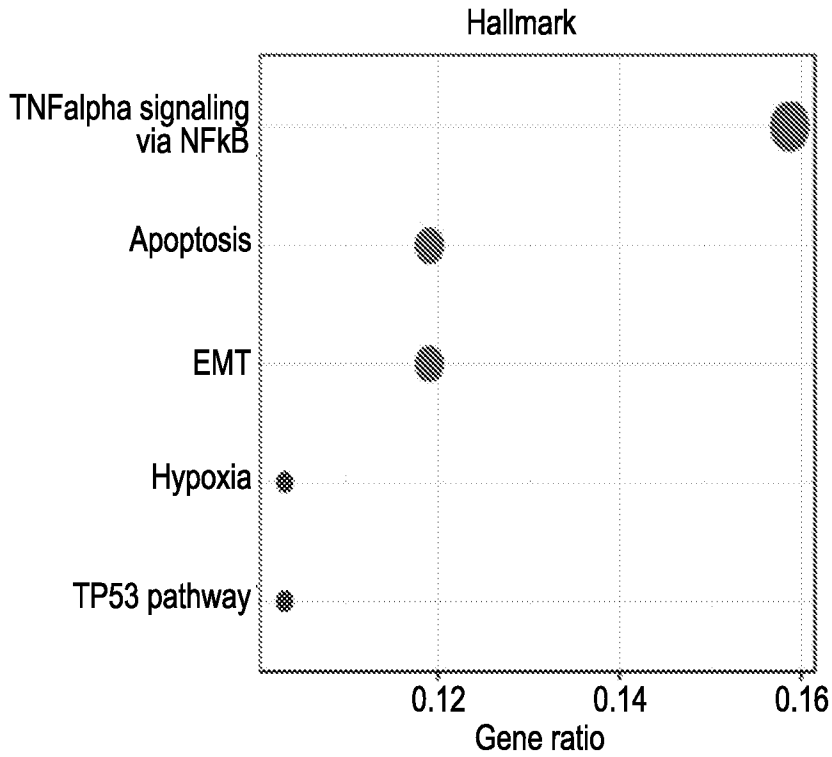


FIGURE 15C

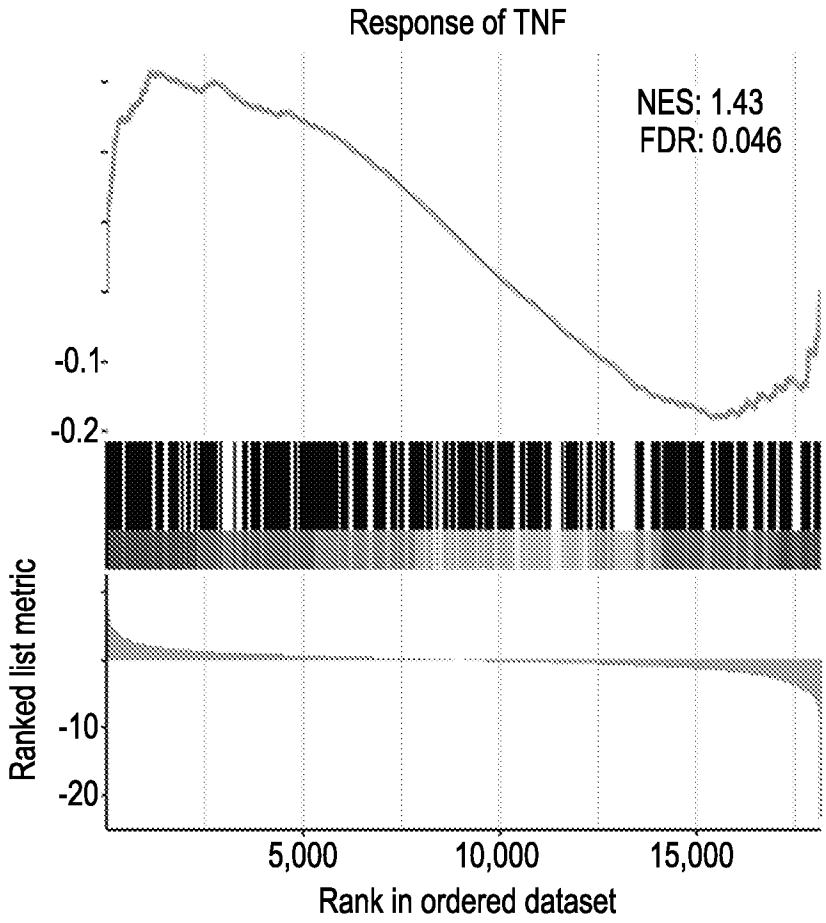


FIGURE 15D

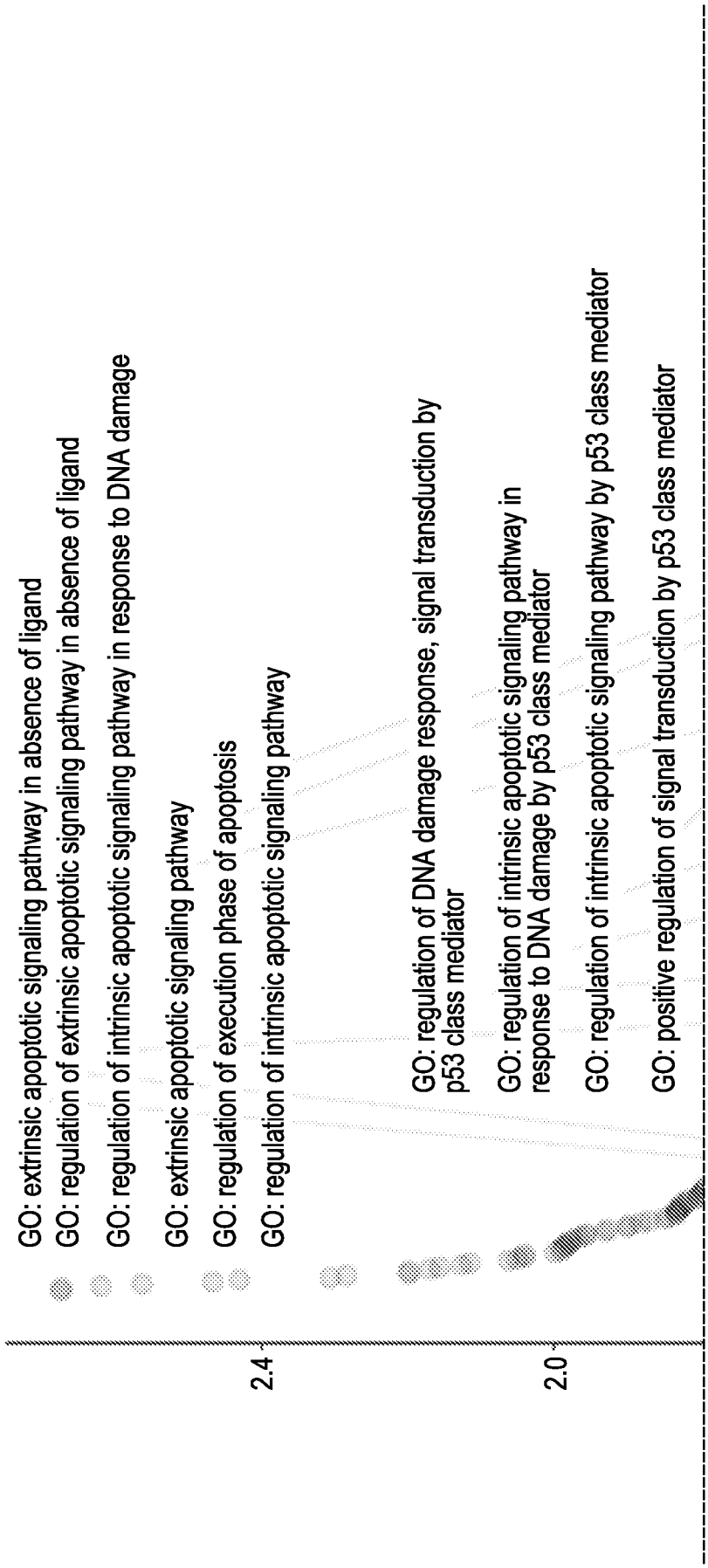

GO: extrinsic apoptotic signaling pathway in absence of ligand
GO: regulation of extrinsic apoptotic signaling pathway in absence of ligand
GO: regulation of intrinsic apoptotic signaling pathway in response to DNA damage
GO: extrinsic apoptotic signaling pathway
GO: regulation of execution phase of apoptosis
GO: regulation of intrinsic apoptotic signaling pathway
GO: regulation of DNA damage response, signal transduction by p53 class mediator
GO: regulation of intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator
GO: regulation of intrinsic apoptotic signaling pathway by p53 class mediator
GO: positive regulation of signal transduction by p53 class mediator
2.4
2.0

FIGURE 15E

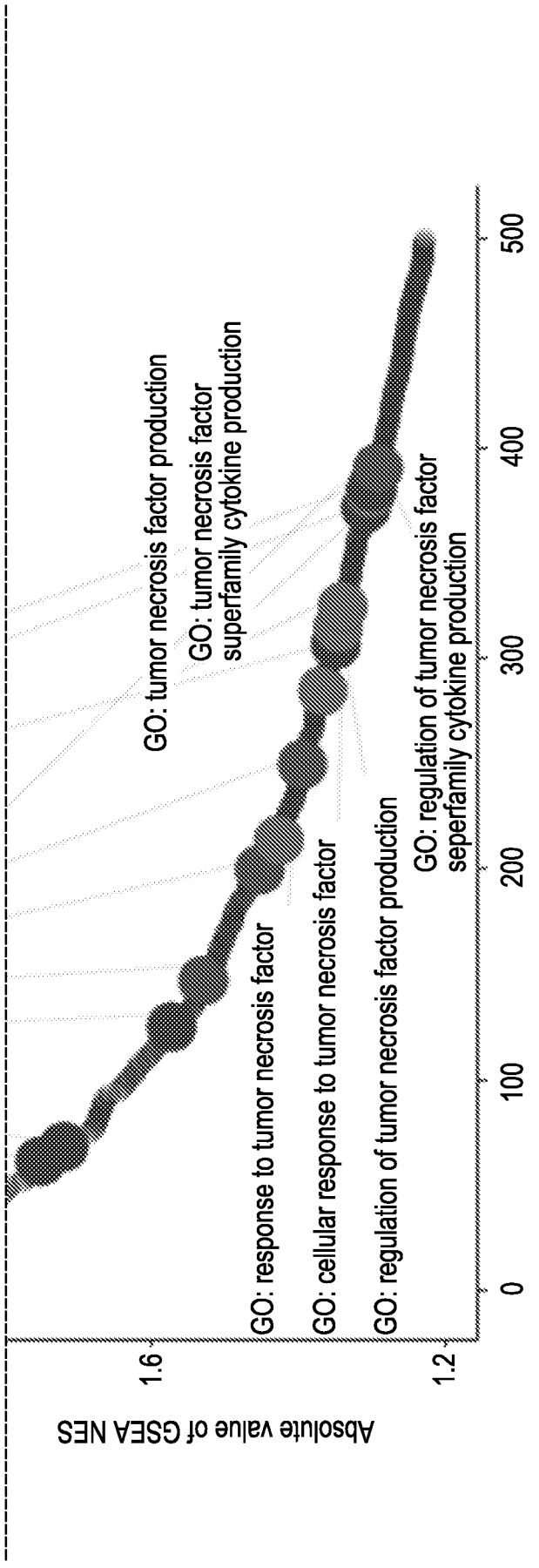


FIGURE 15E (Continued)

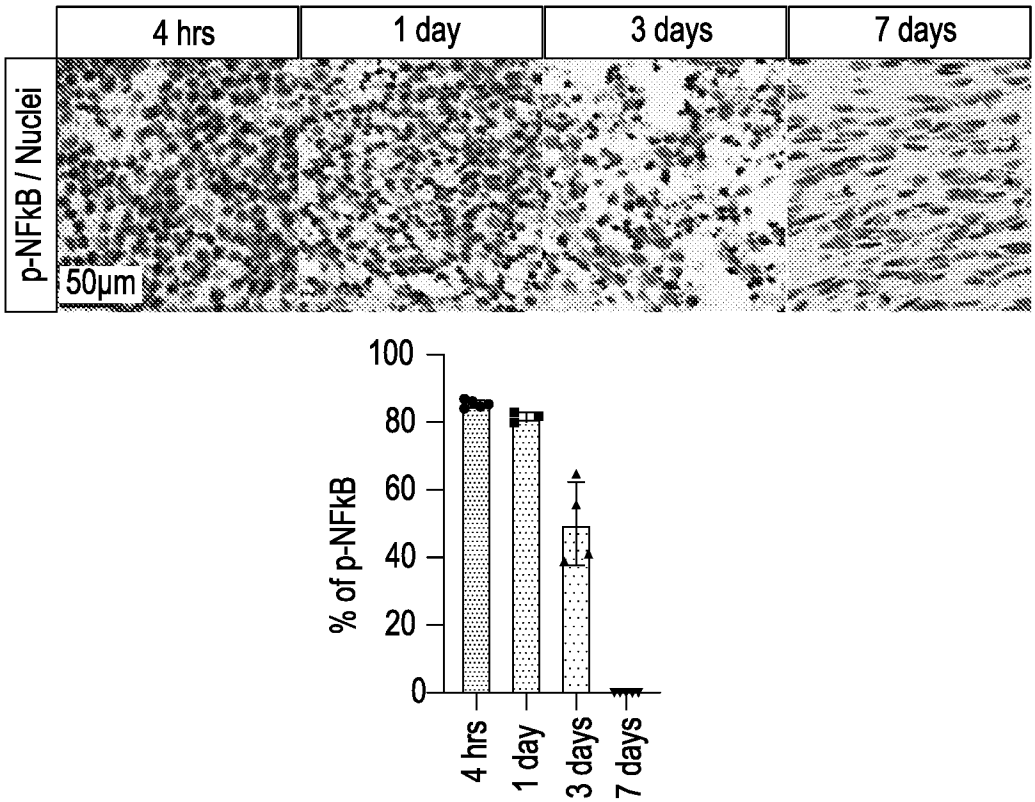


FIGURE 15F

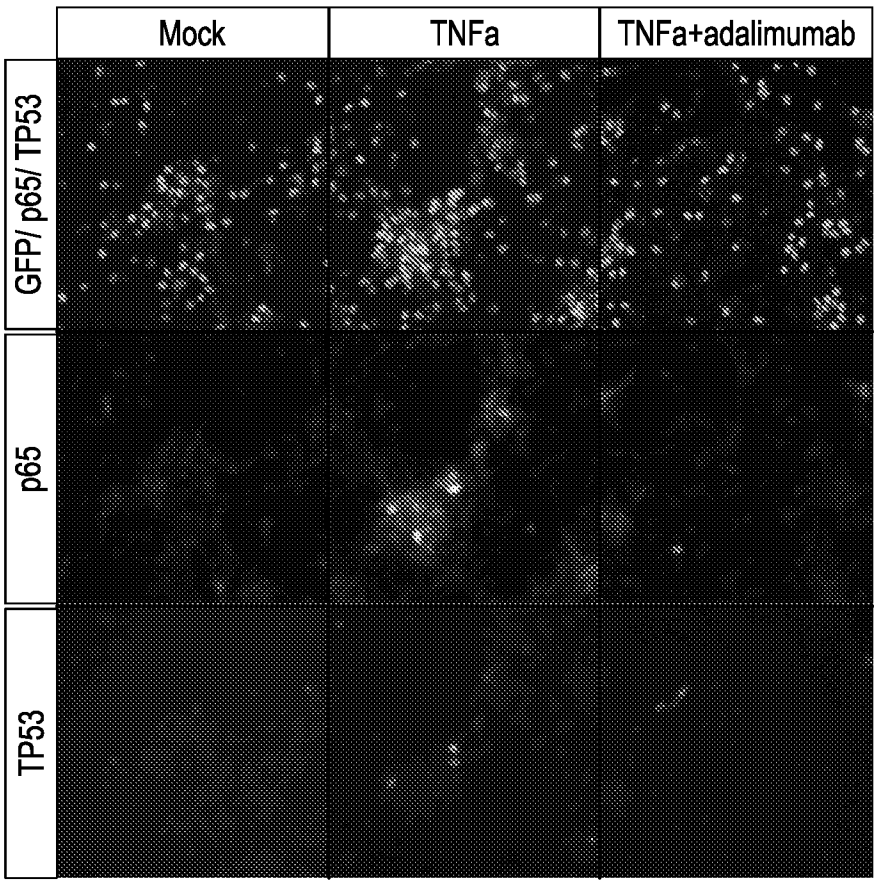


FIGURE 15G

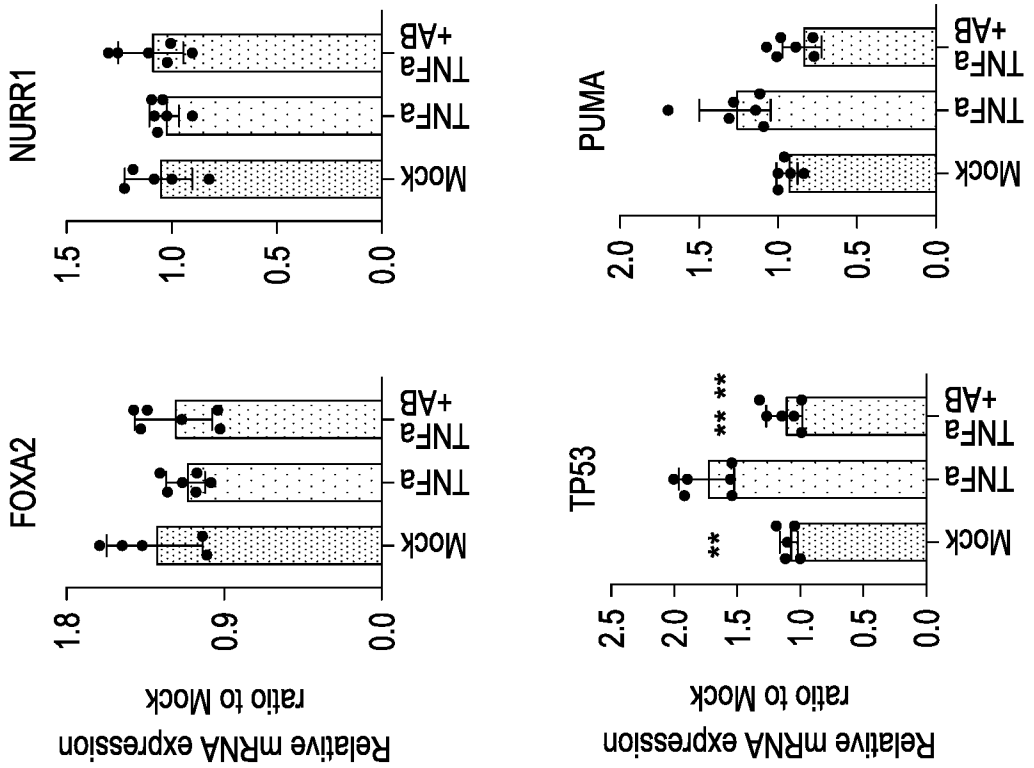


FIGURE 15I

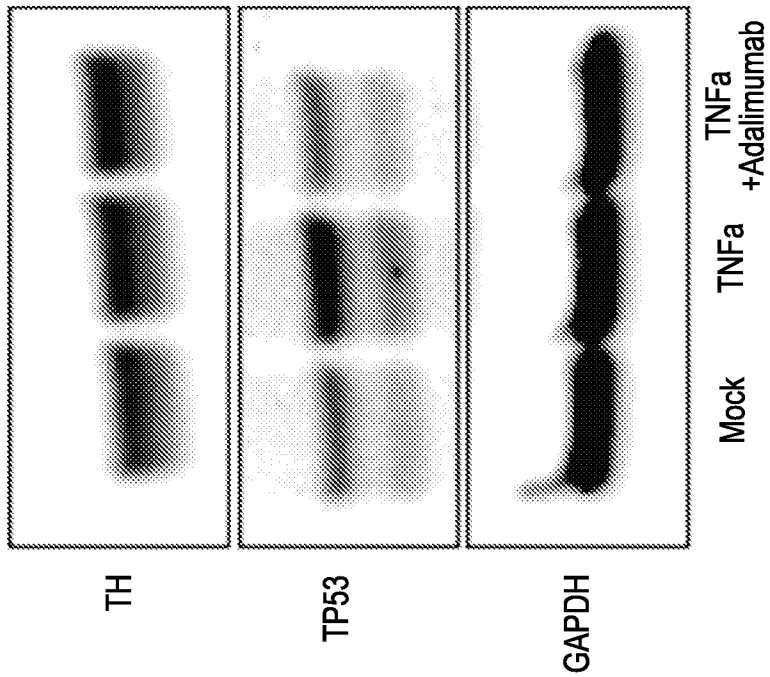


FIGURE 15H

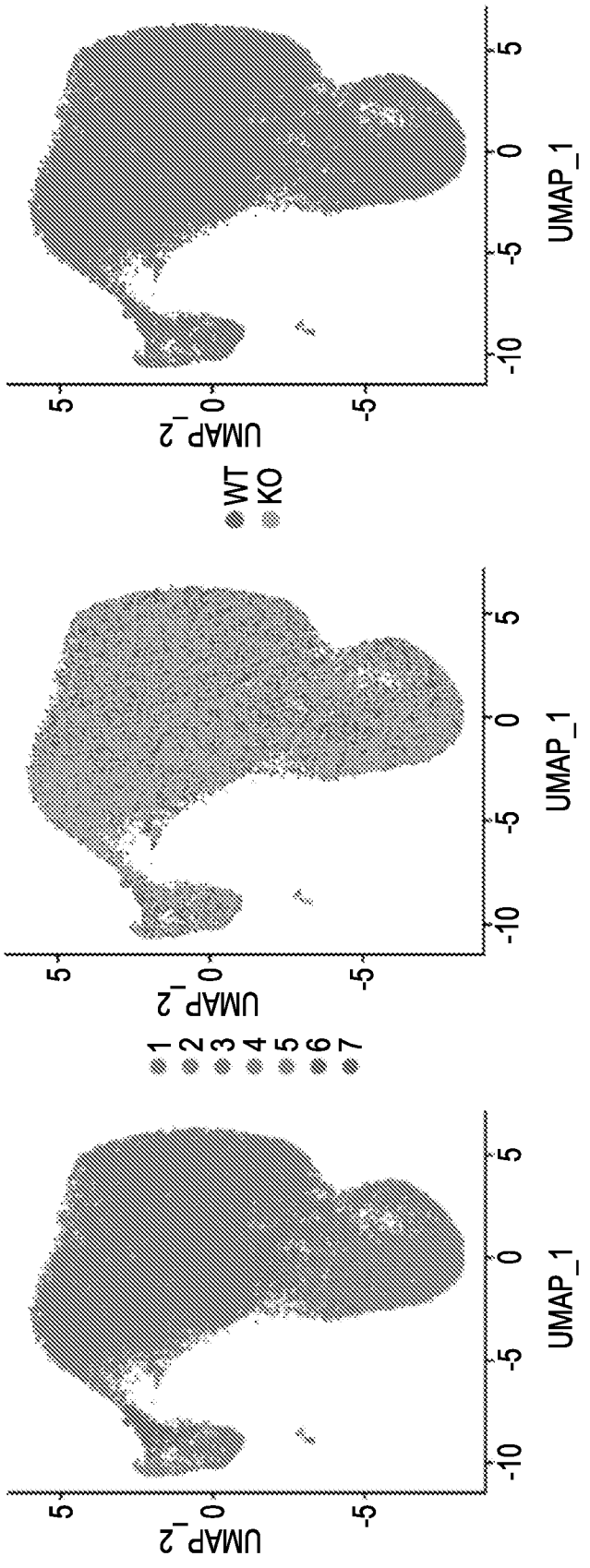


FIGURE 16A

FIGURE 16B

FIGURE 16C

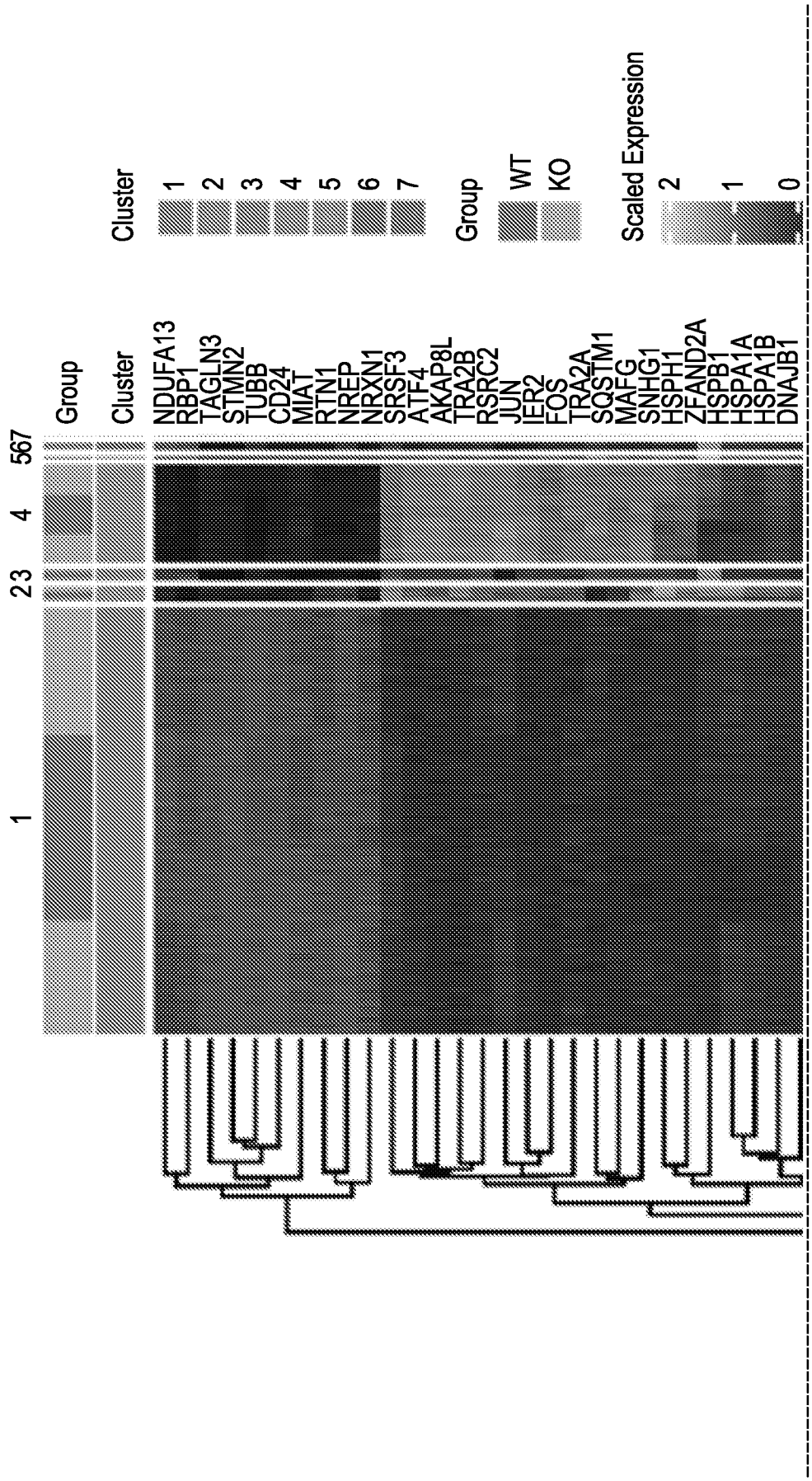

Cluster
1
2
3
4
5
6
7
Group
WT
KO
Scaled Expression
2
1
0
Group
Cluster
NDUFA13
RBP1
TAGLN3
STMN2
TUBB
CD24
MIAT
RTN1
NREP
NRXN1
SRSF3
ATF4
AKAP8L
TRA2B
RSRC2
JUN
IER2
FOS
TRA2A
SQSTM1
MAFG
SNHG1
HSPH1
ZFAND2A
HSPB1
HSPA1A
HSPA1B
DNAJB1
1
23
4
567

FIGURE 16D

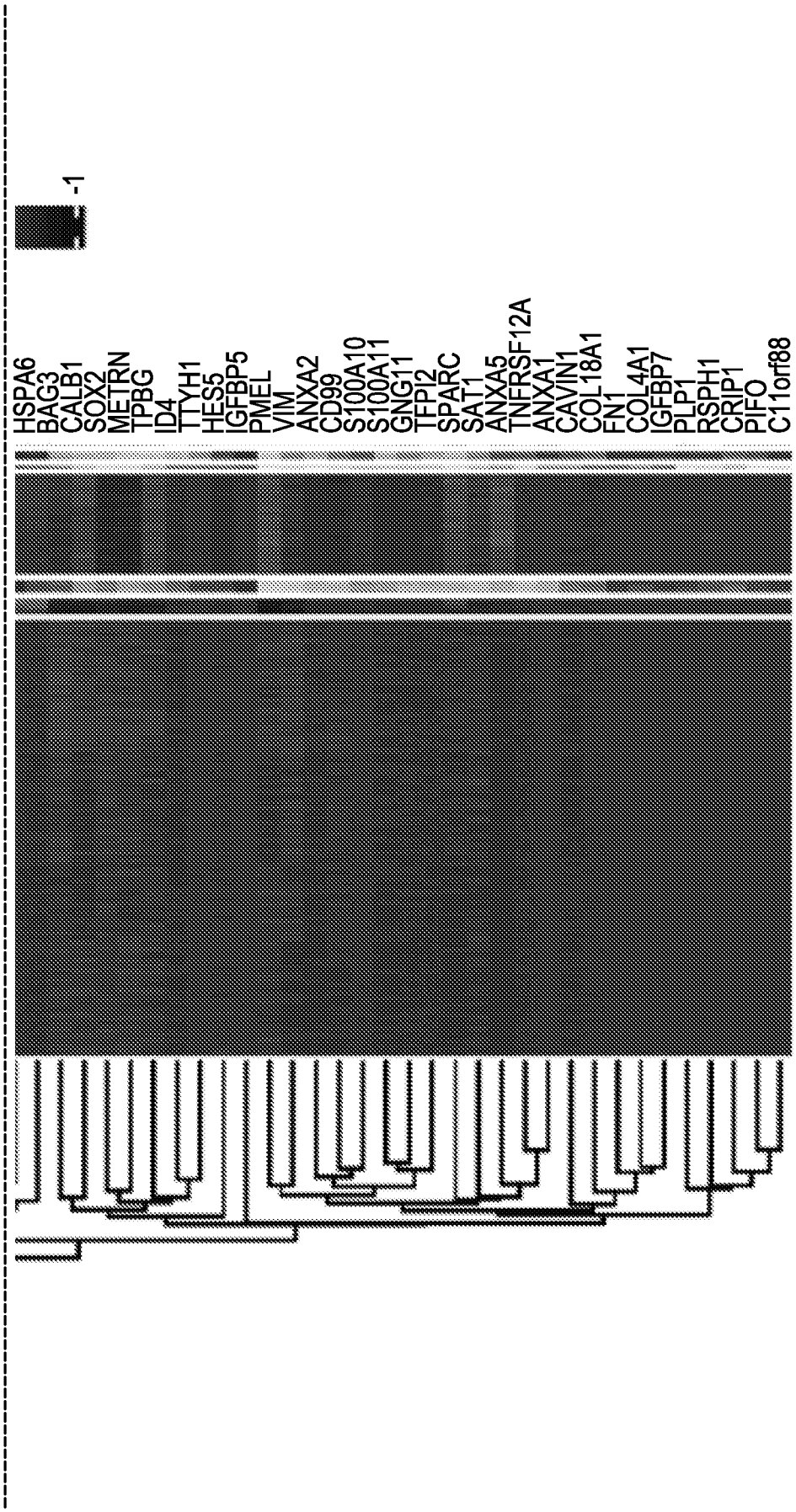


FIGURE 16D (Continued)

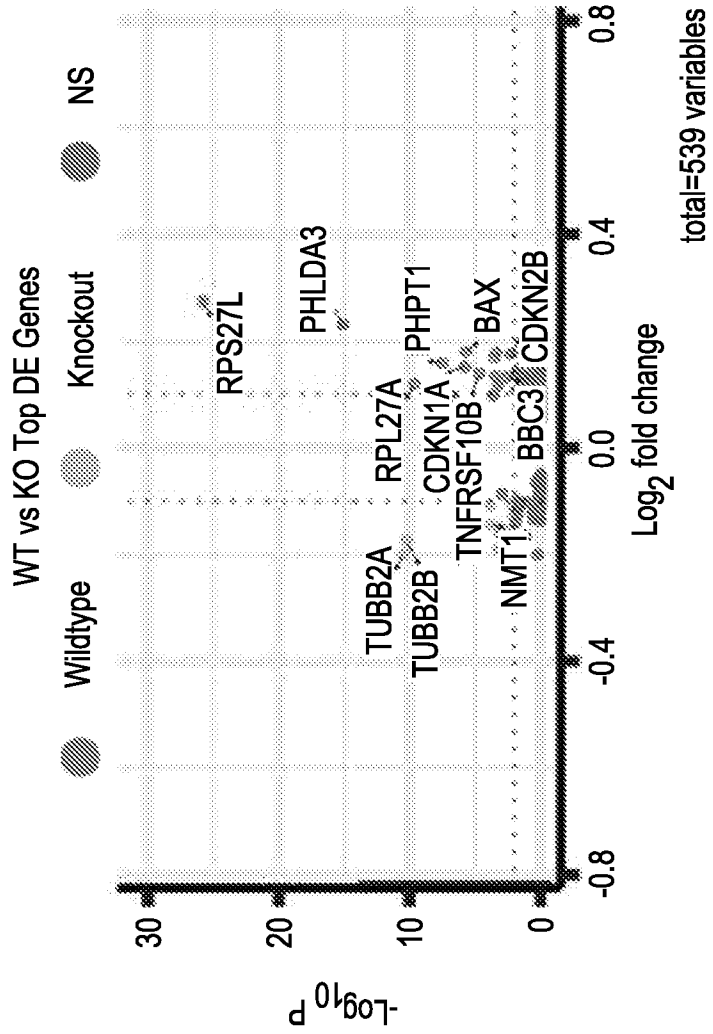

WT vs KO Top DE Genes
Wildtype
Knockout
NS
-Log10 P
30
20
10
0
-0.8
-0.4
0.0
0.4
0.8
Log2 fold change
total=539 variables
RPS27L
PHLDA3
TUBB2A
RPL27A
PHPT1
TUBB2B
CDKN1A
BAX
TNFRSF10B
BBC3
CDKN2B
NMT1

FIGURE 16E

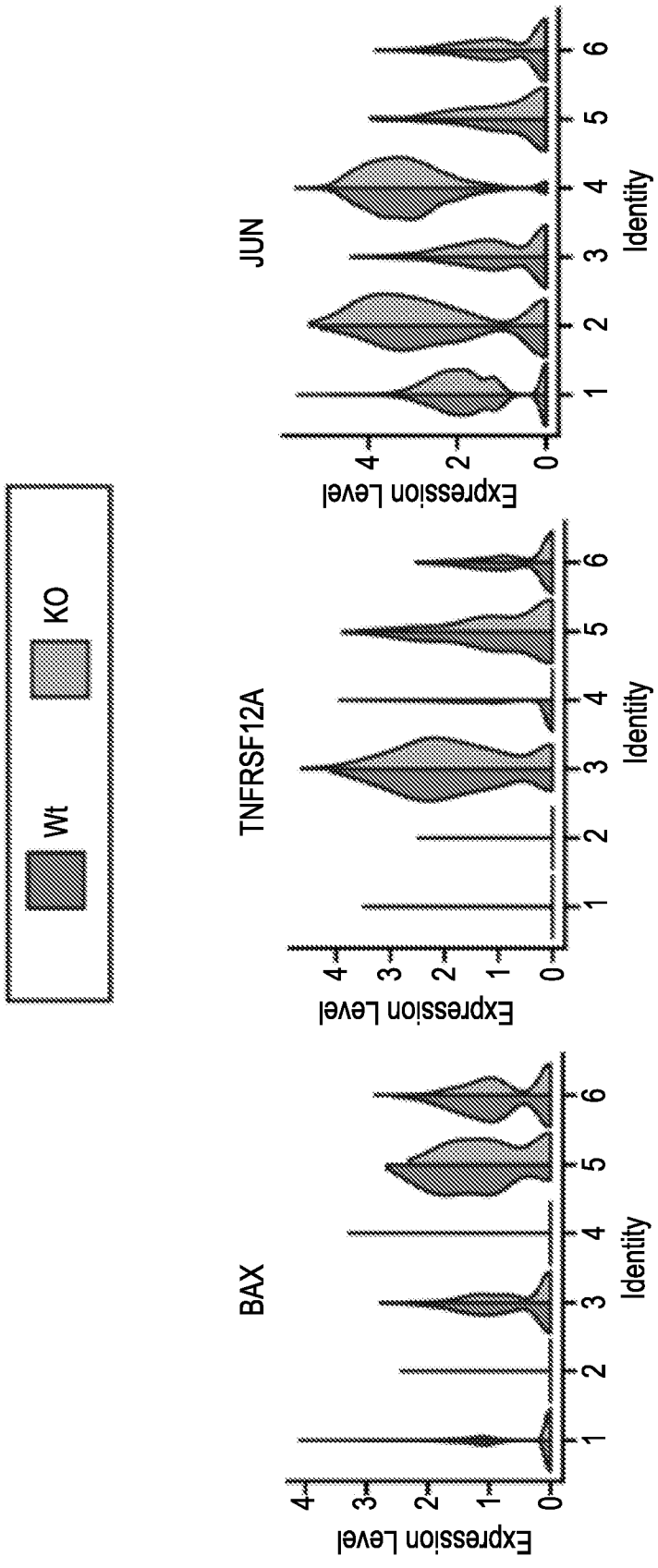


FIGURE 16F

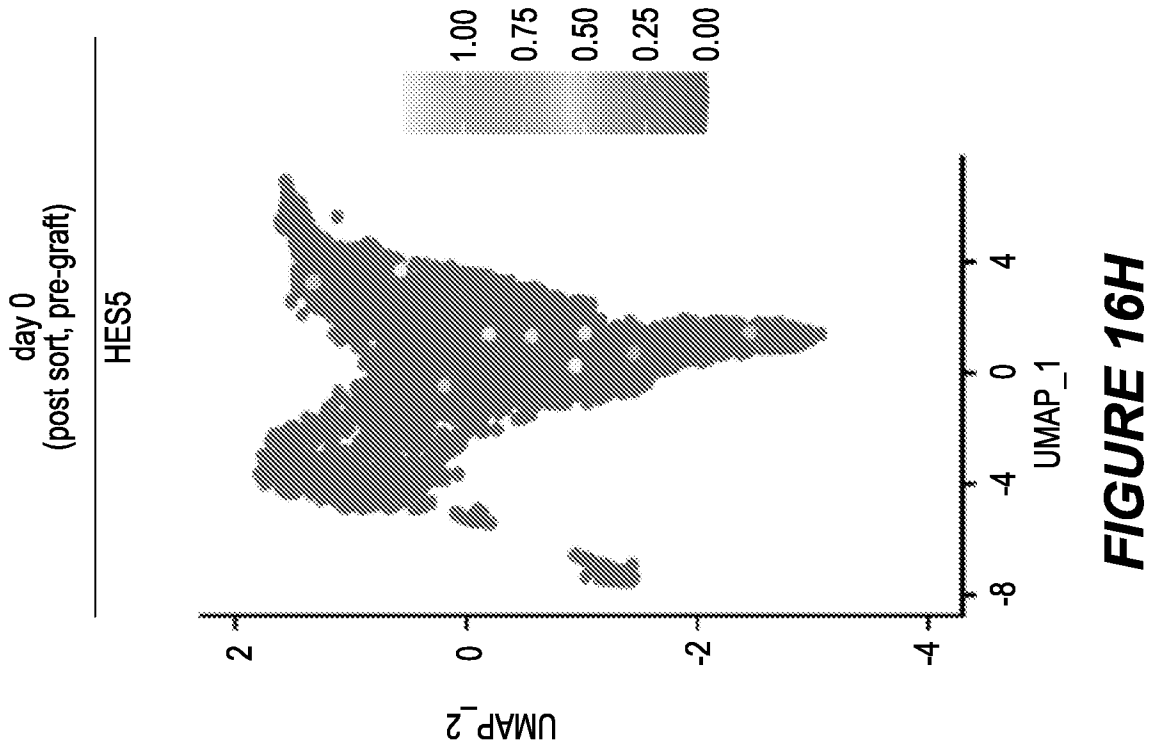


FIGURE 16H

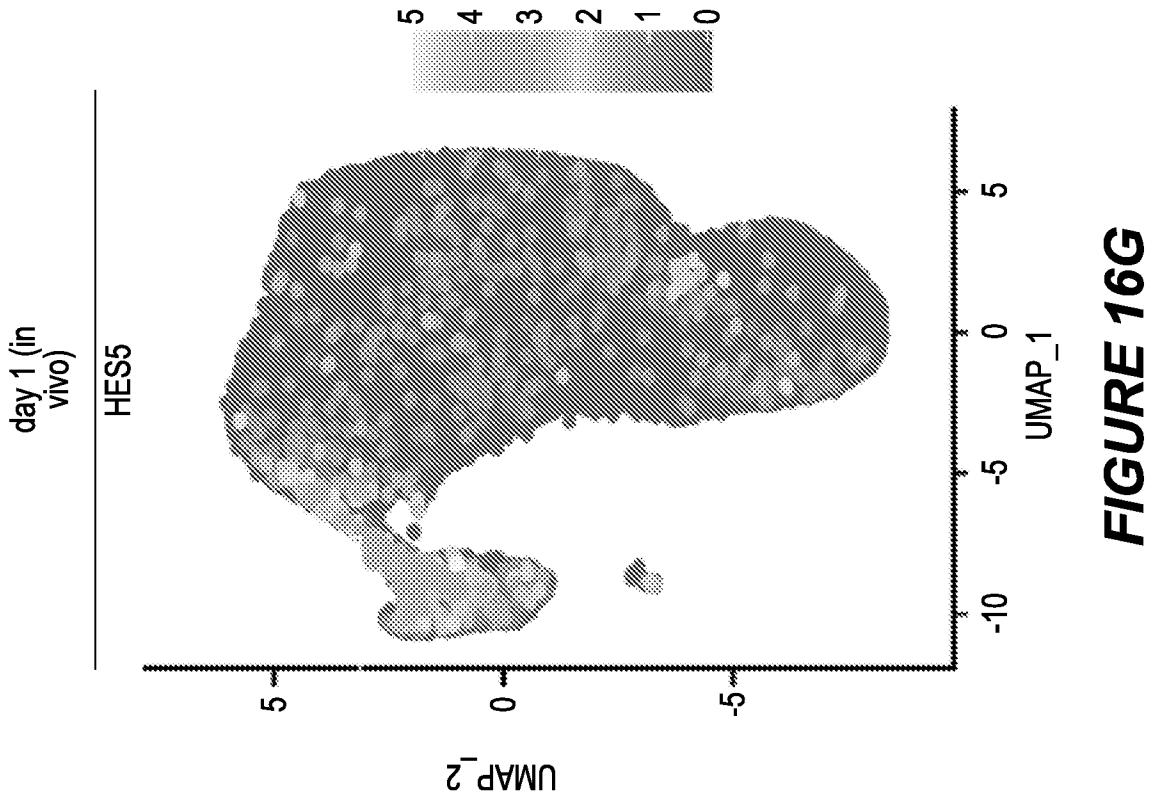


FIGURE 16G

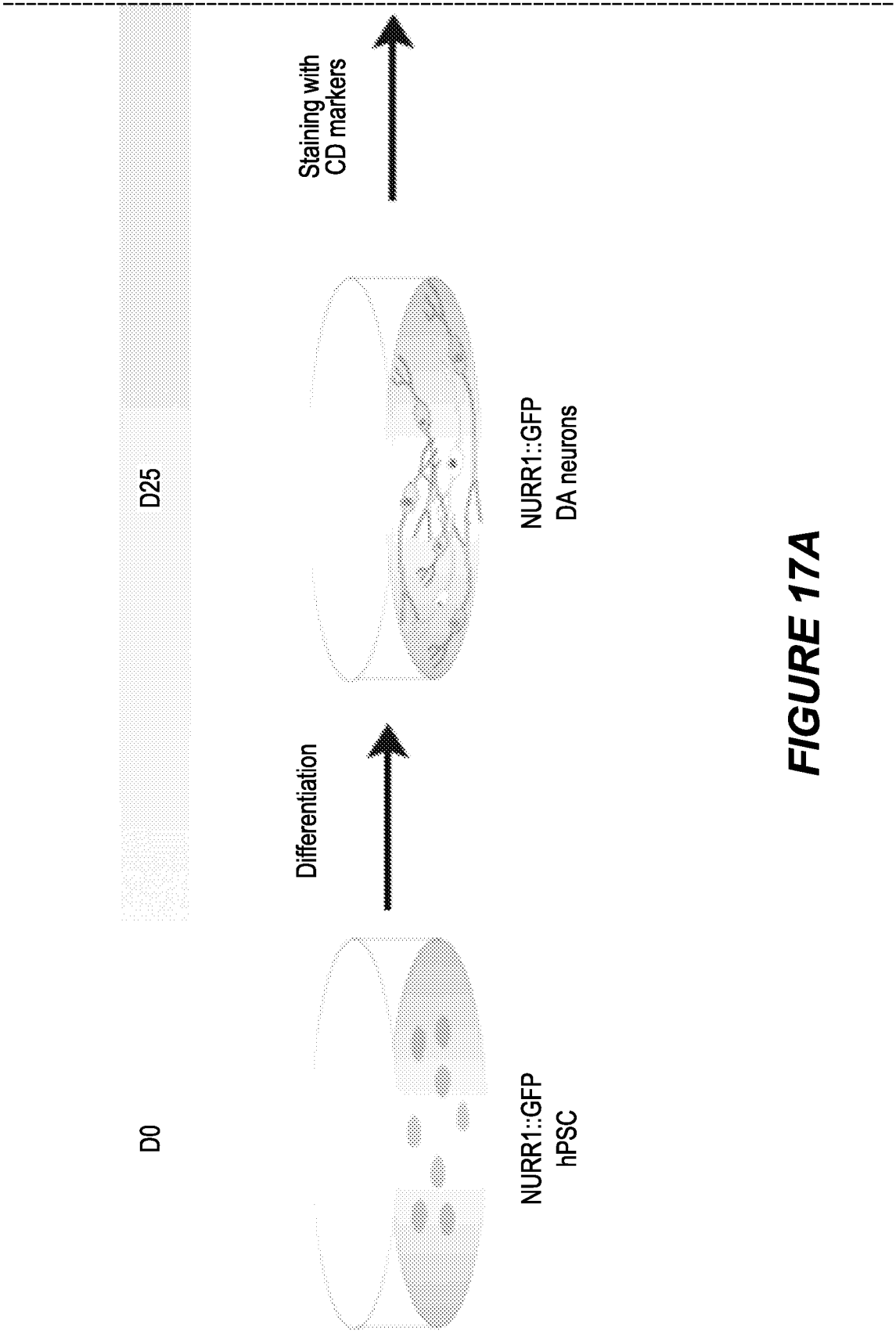

D0
NURR1::GFP
hPSC
Differentiation
D25
NURR1::GFP
DA neurons
Staining with
CD markers

FIGURE 17A

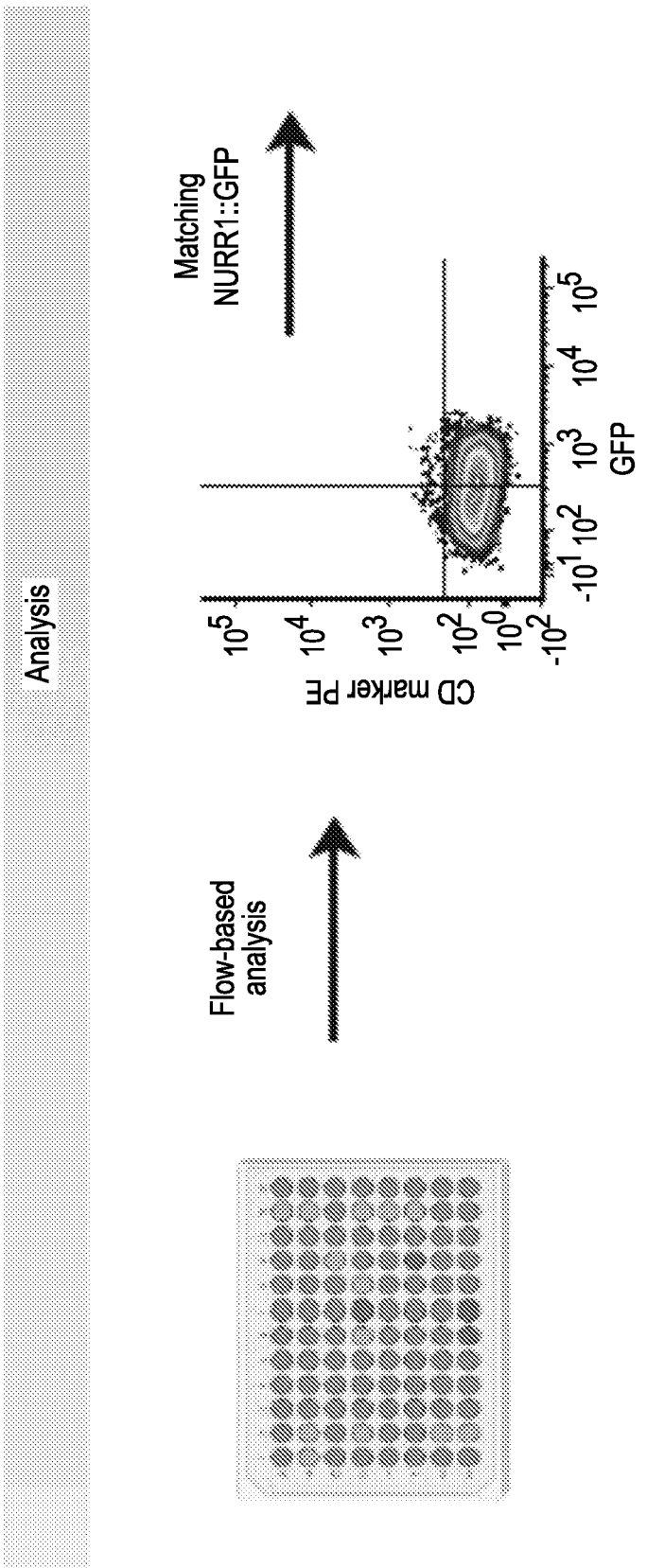


FIGURE 17A (Continued)

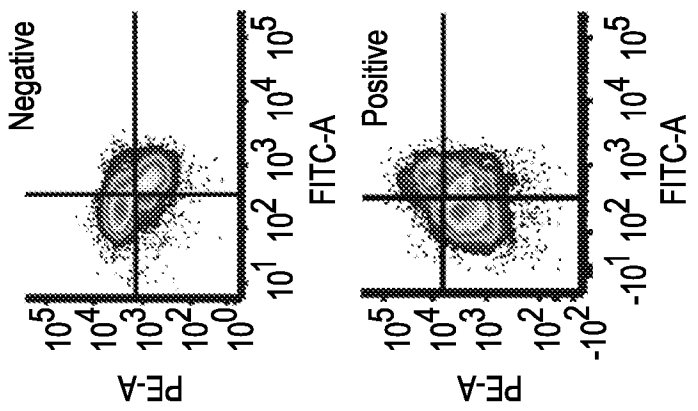


FIGURE 17A (Continued)

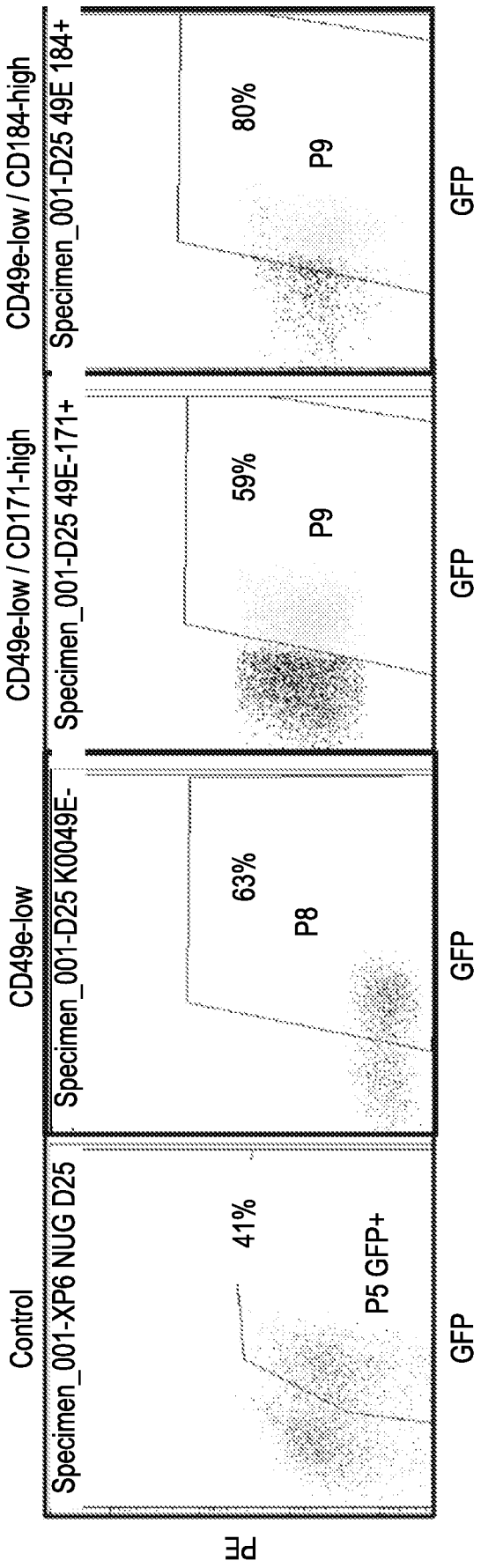

Control
Specimen_001-XP6 NUG D25
41%
P5 GFP+
CD49e-low
Specimen_001-D25 K0049E-
63%
P8
CD49e-low / CD171-high
Specimen_001-D25 49E-171+
59%
P9
CD49e-low / CD184-high
Specimen_001-D25 49E 184+
80%
P9
PE
GFP
GFP
GFP
GFP

FIGURE 17B

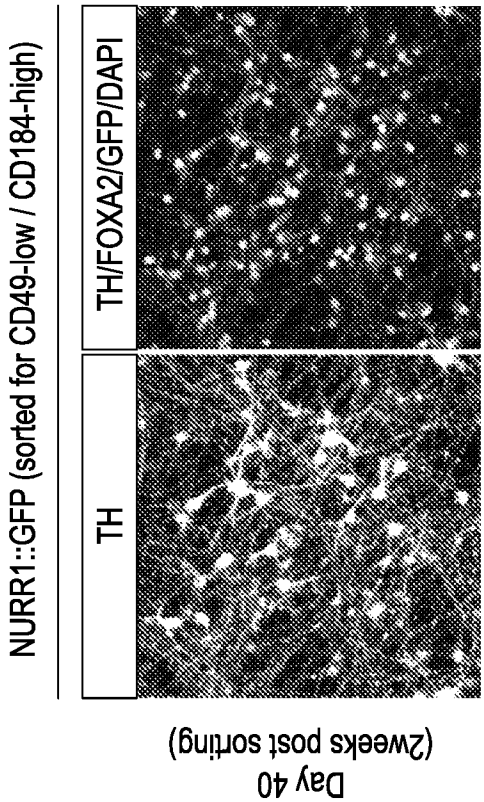


FIGURE 17D

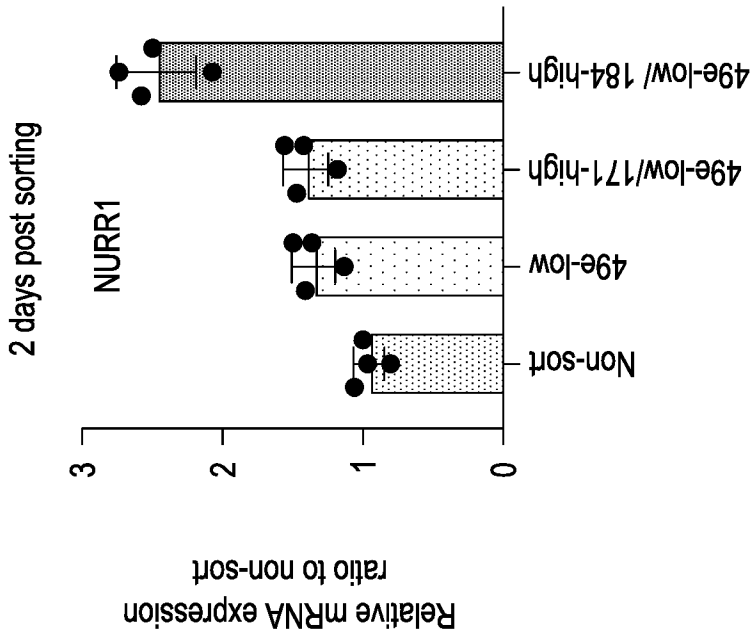


FIGURE 17C

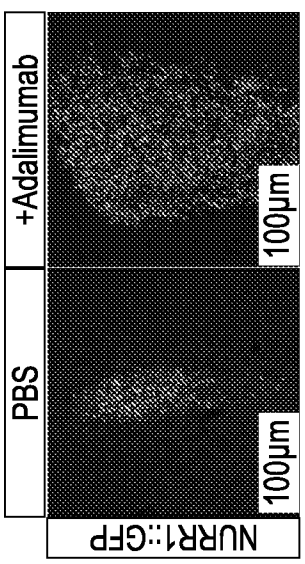


FIGURE 18A

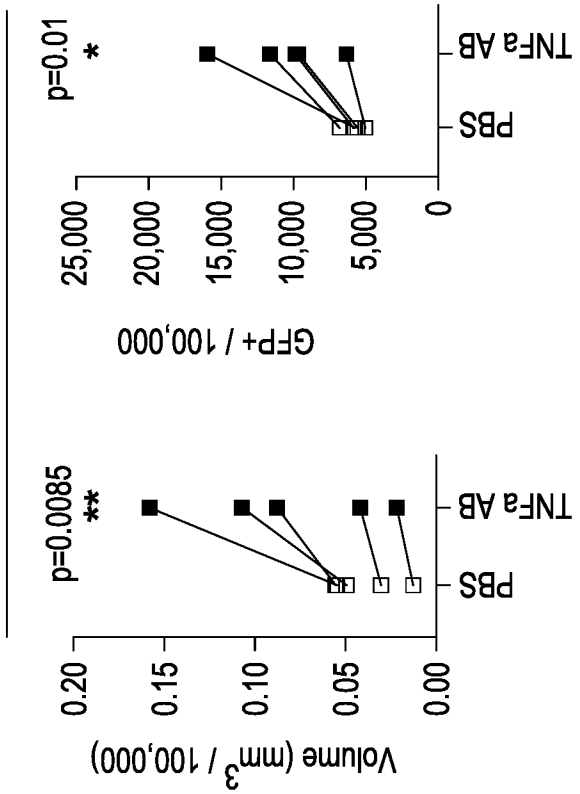


FIGURE 18B

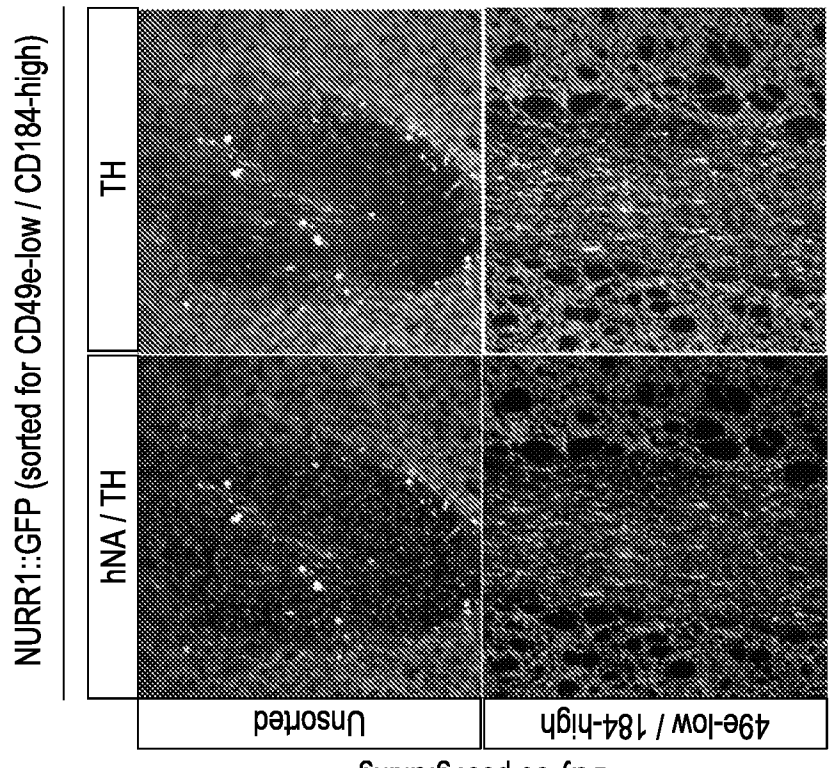


FIGURE 17E

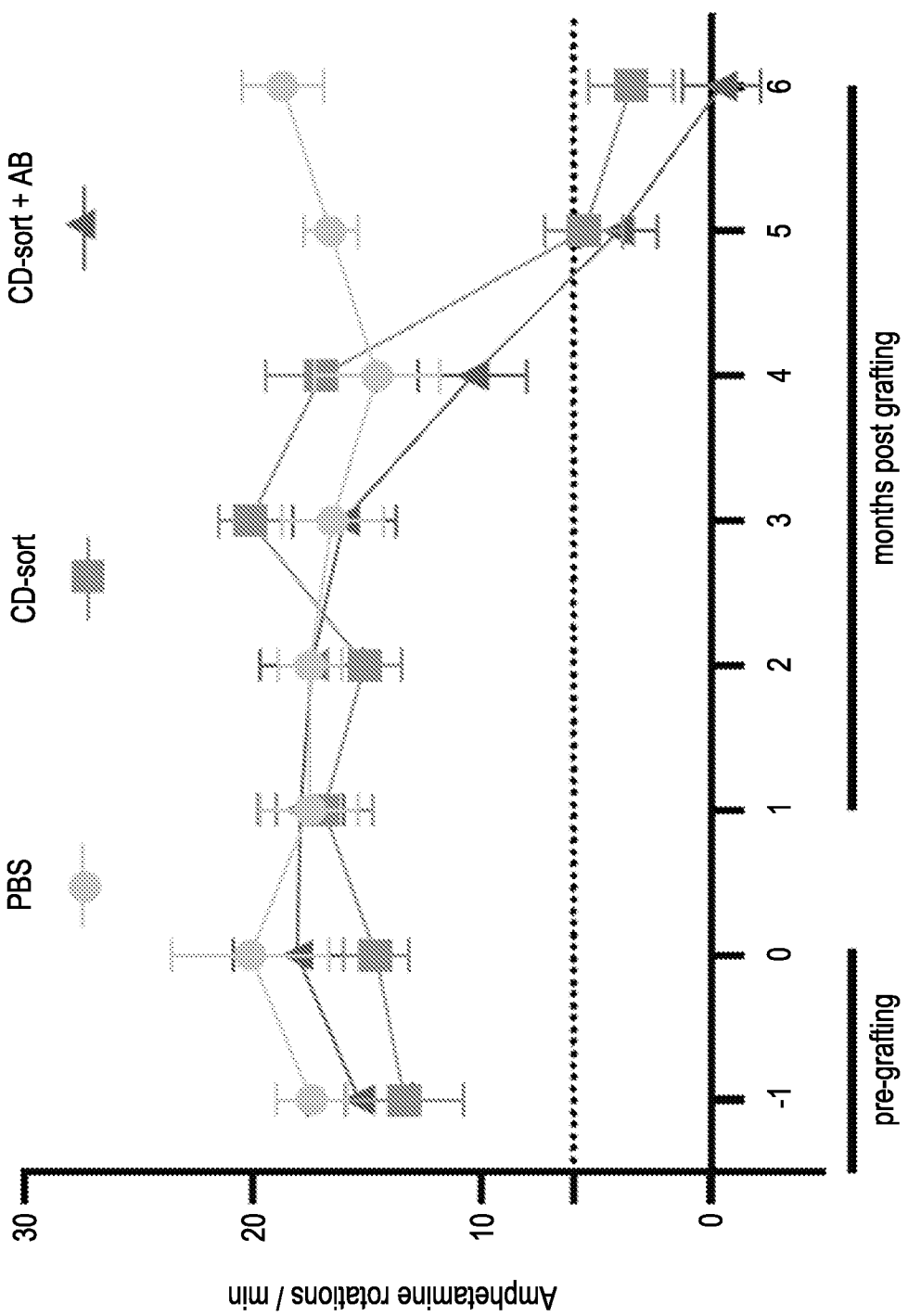

PBS
CD-sort
CD-sort + AB
Amphetamine rotations / min
30
20
10
0
-1
0
1
2
3
4
5
6
pre-grafting
months post grafting

FIGURE 18C

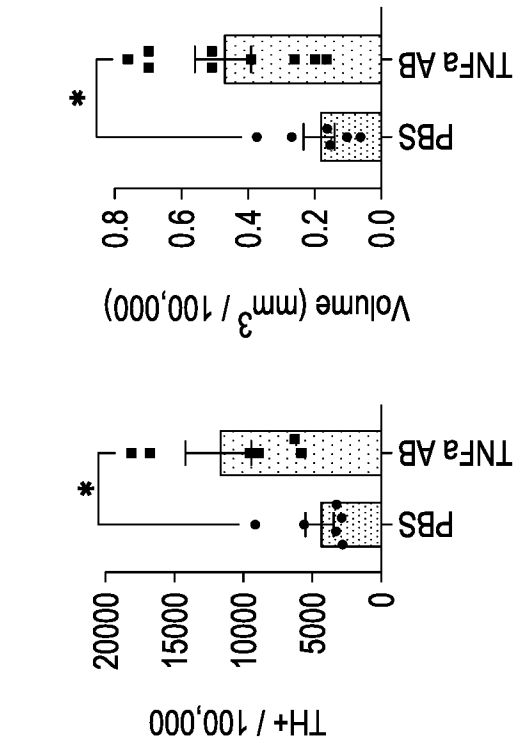


FIGURE 18E

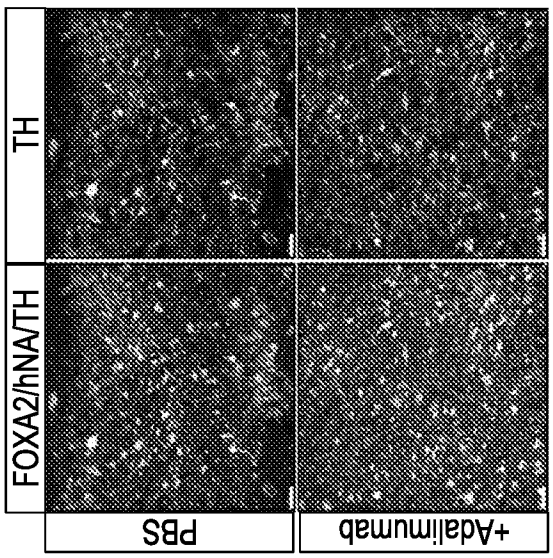
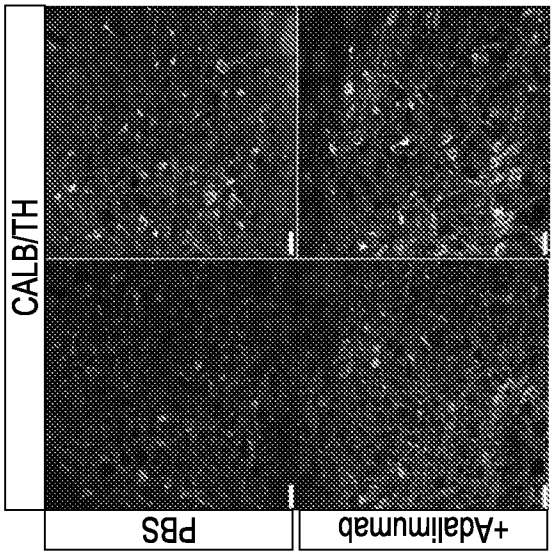


FIGURE 18D



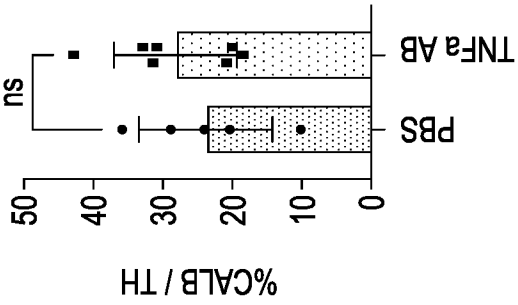
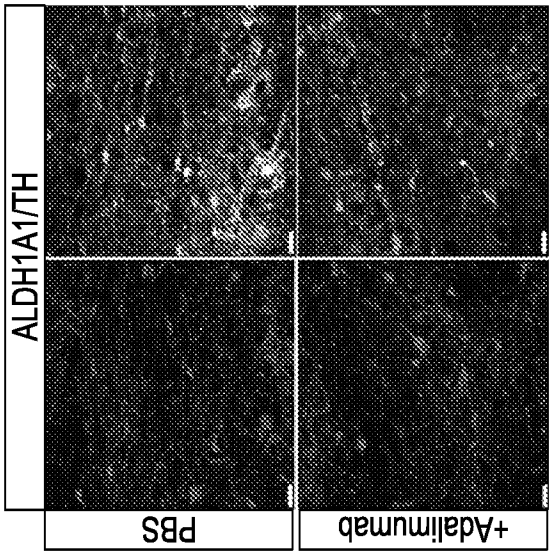


FIGURE 18G



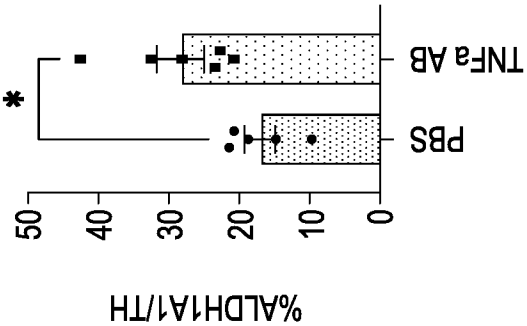


FIGURE 18F

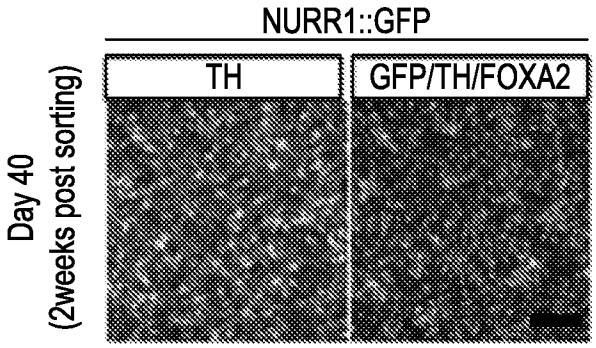


FIGURE 19A

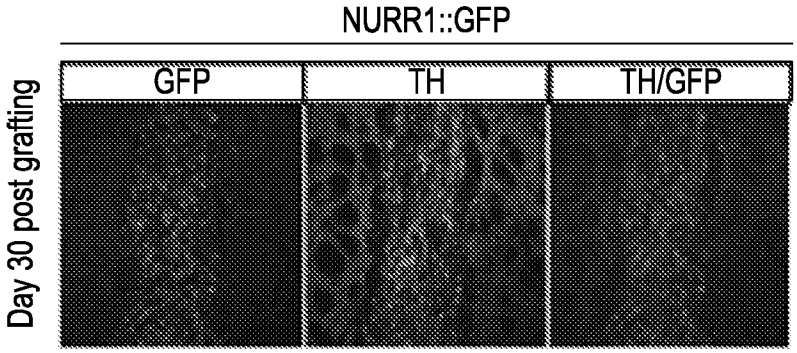


FIGURE 19B

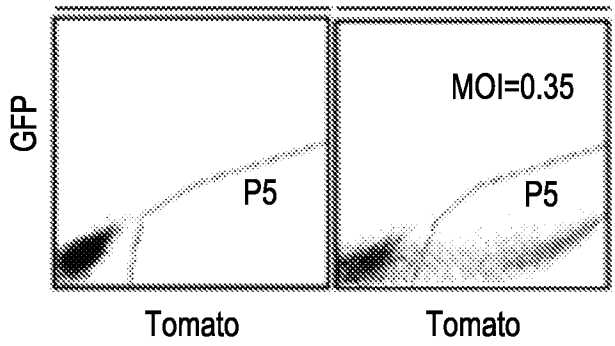


FIGURE 19C

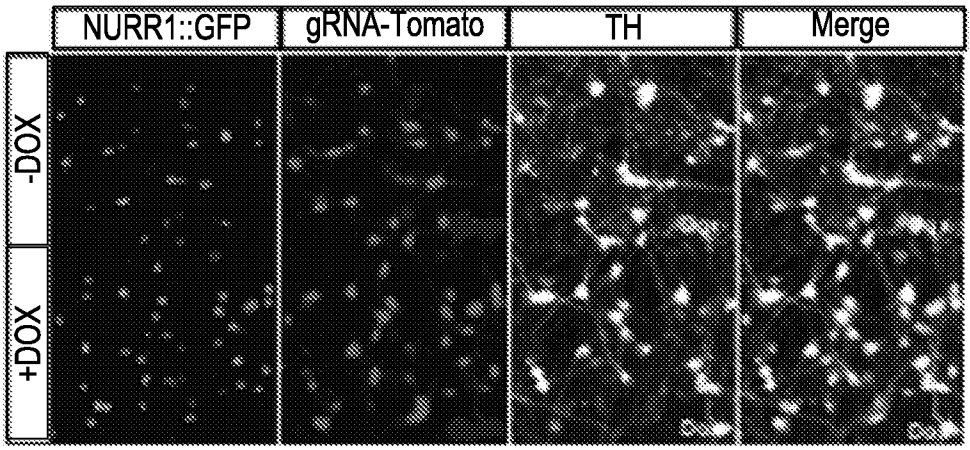


FIGURE 19D

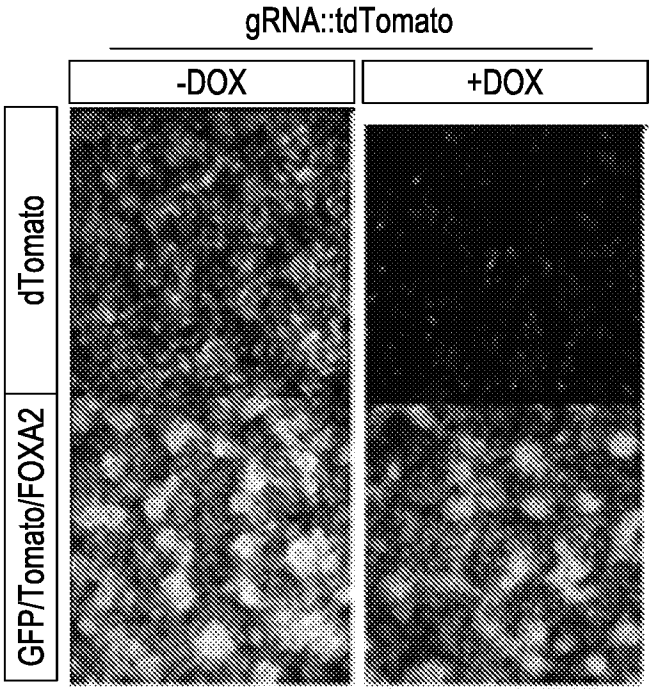


FIGURE 19E

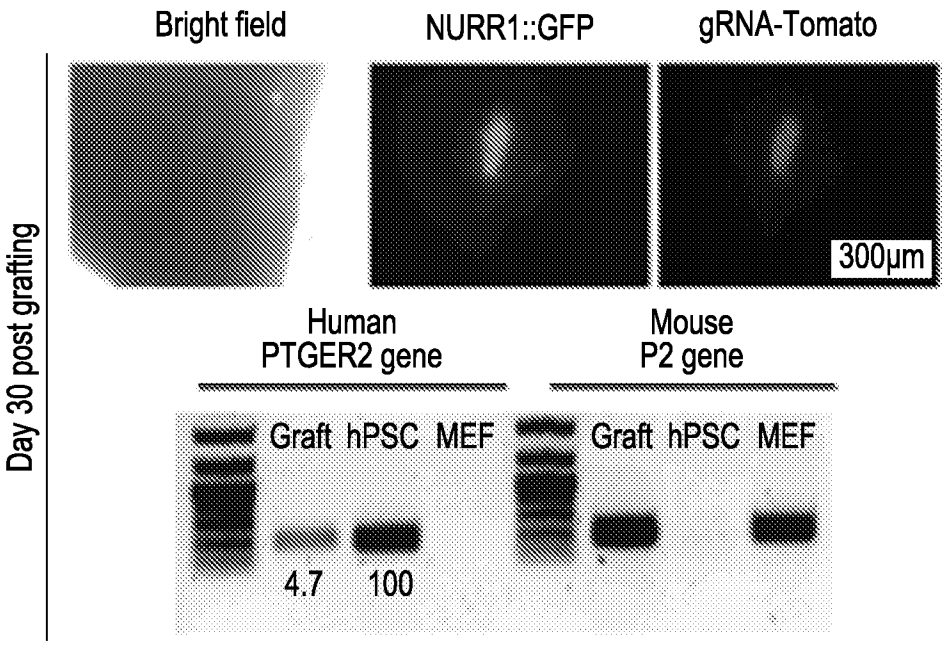


FIGURE 19F

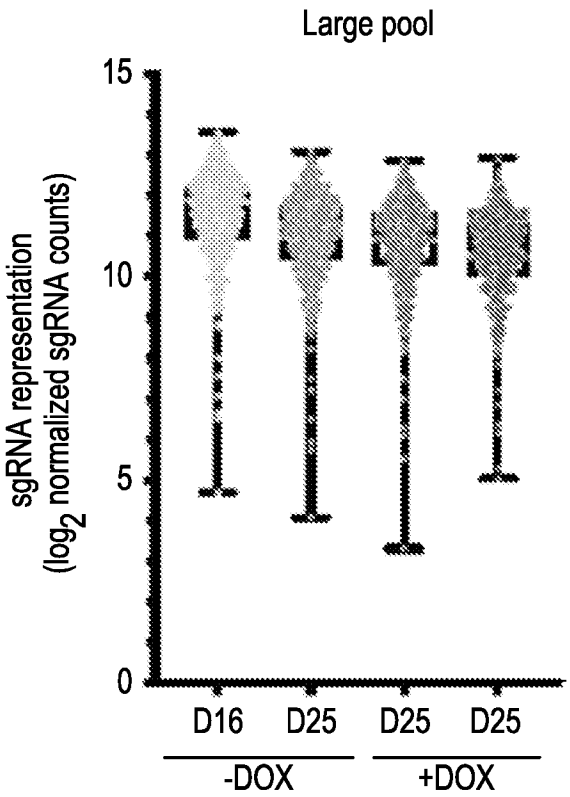


FIGURE 19G

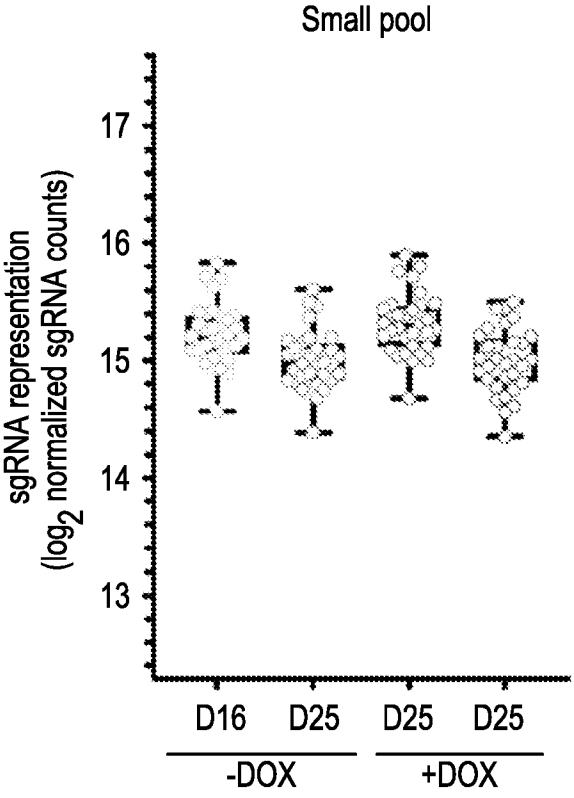

Small pool
sgRNA representation
(log2 normalized sgRNA counts)
17
16
15
14
13
D16
D25
D25
D25
-DOX
+DOX

FIGURE 19H

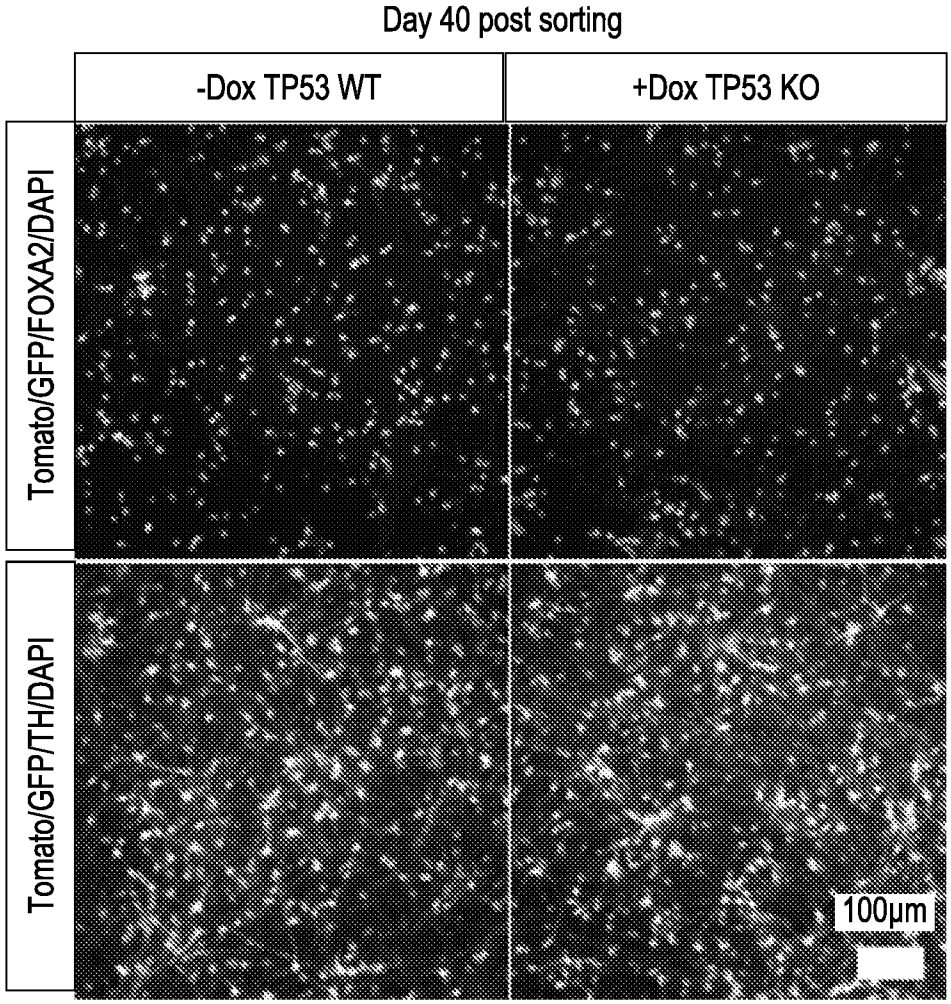


FIGURE 20A

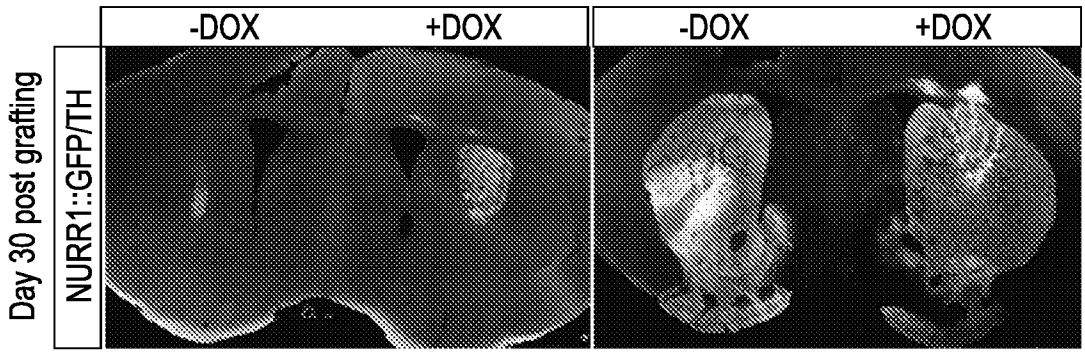


FIGURE 20B

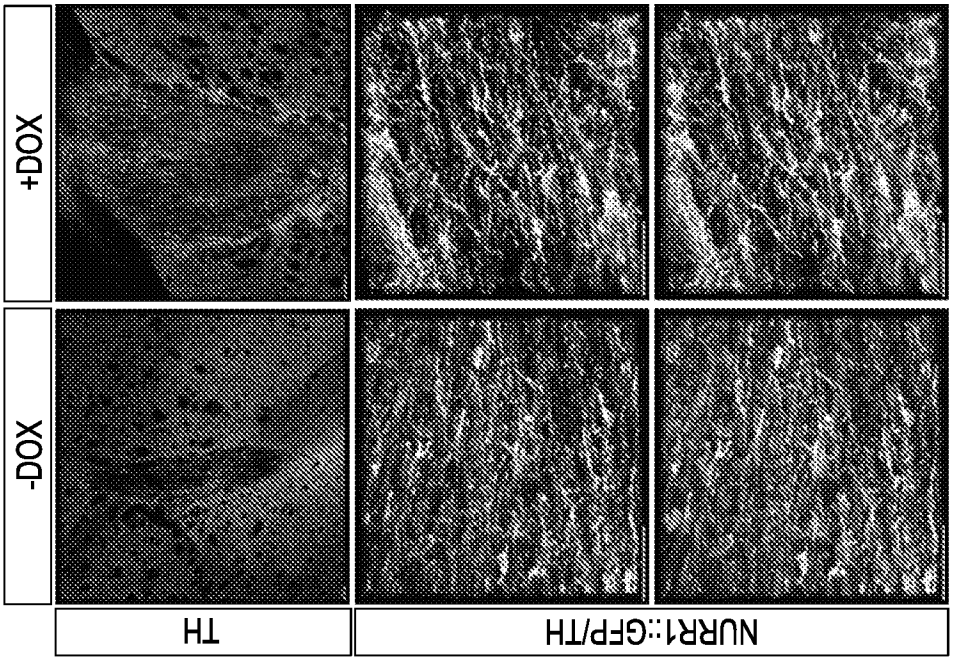


FIGURE 20D

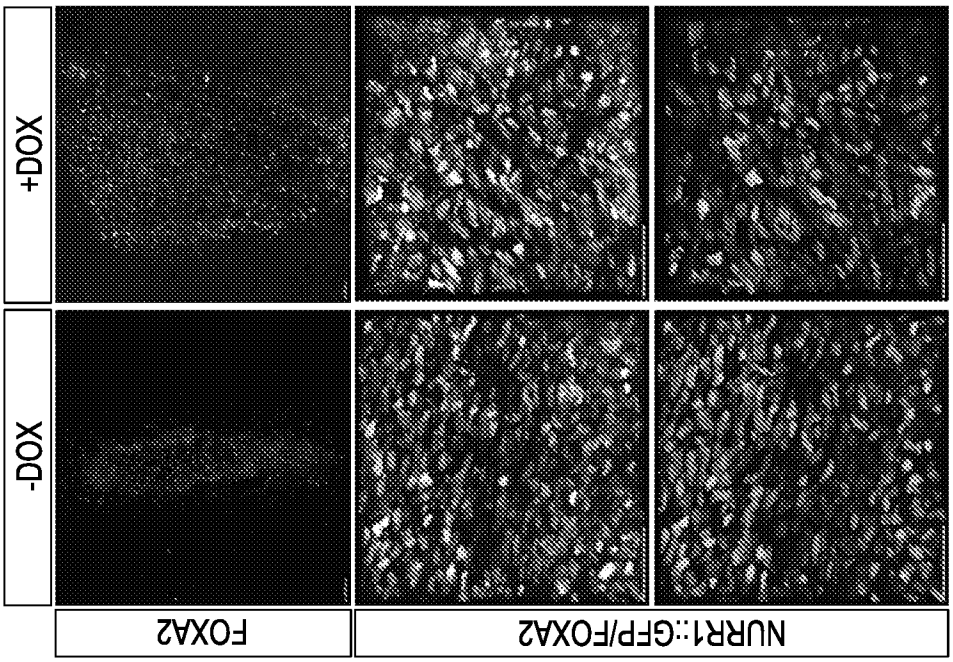


FIGURE 20C

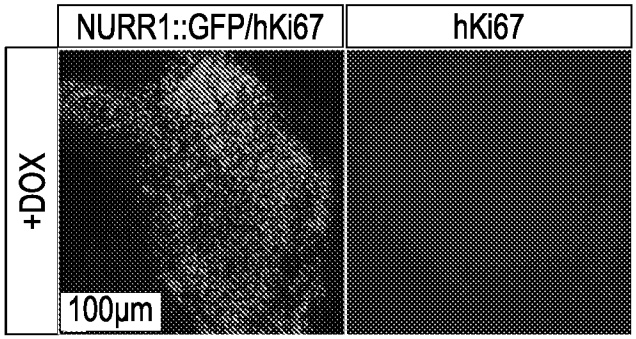


FIGURE 20E

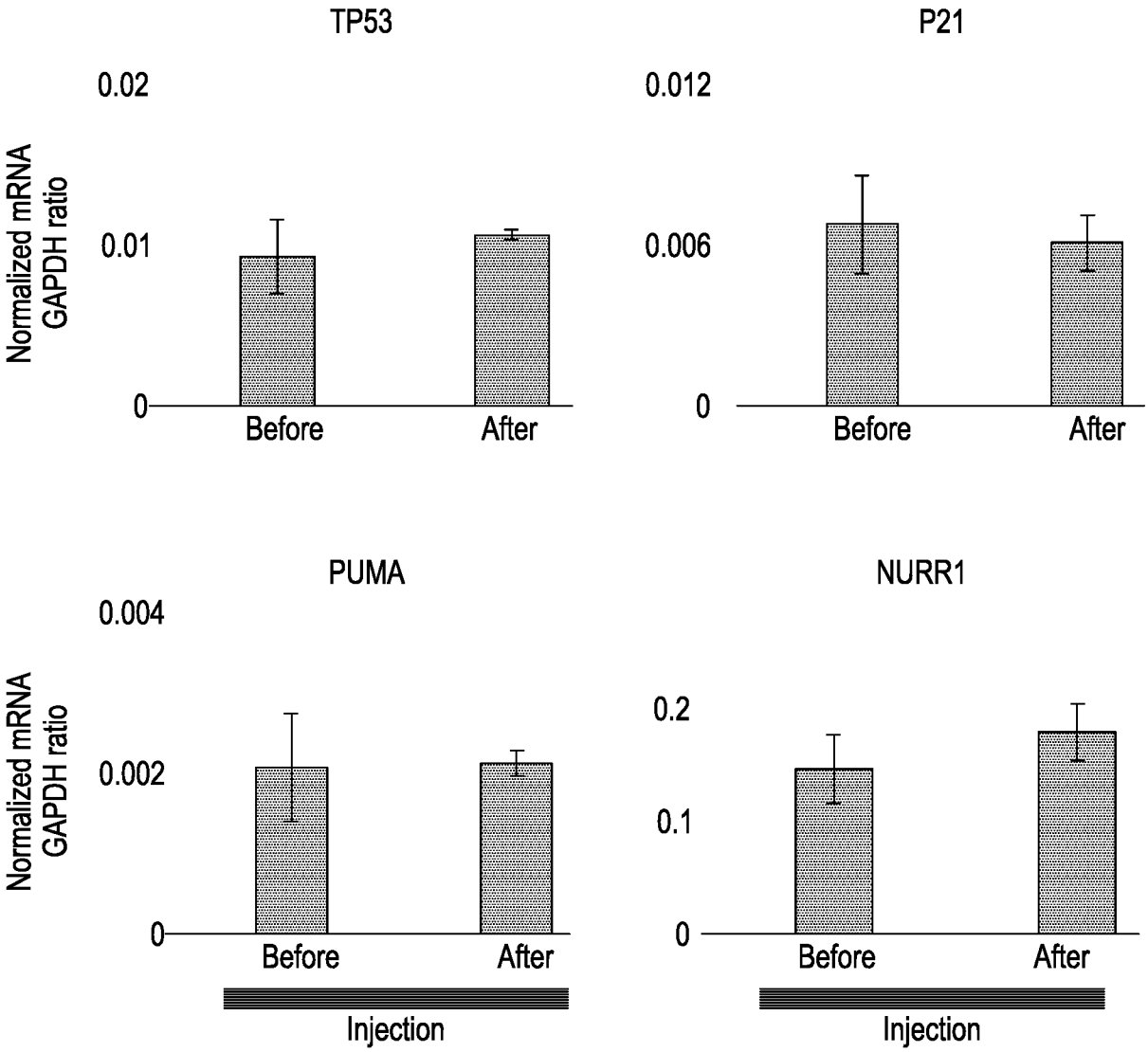


FIGURE 20F

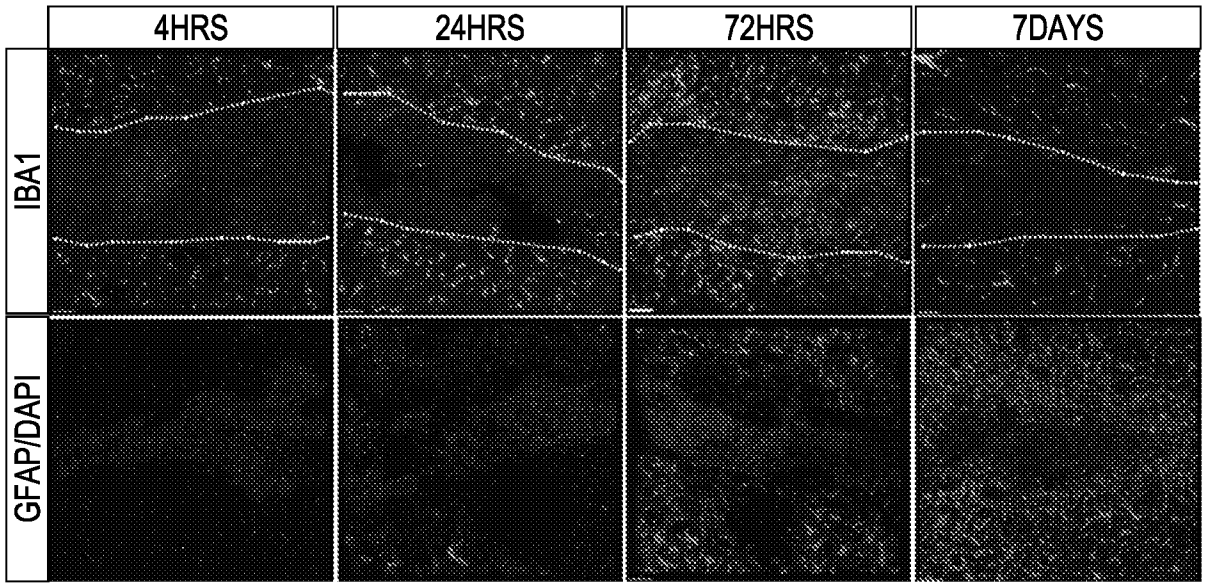


FIGURE 21A

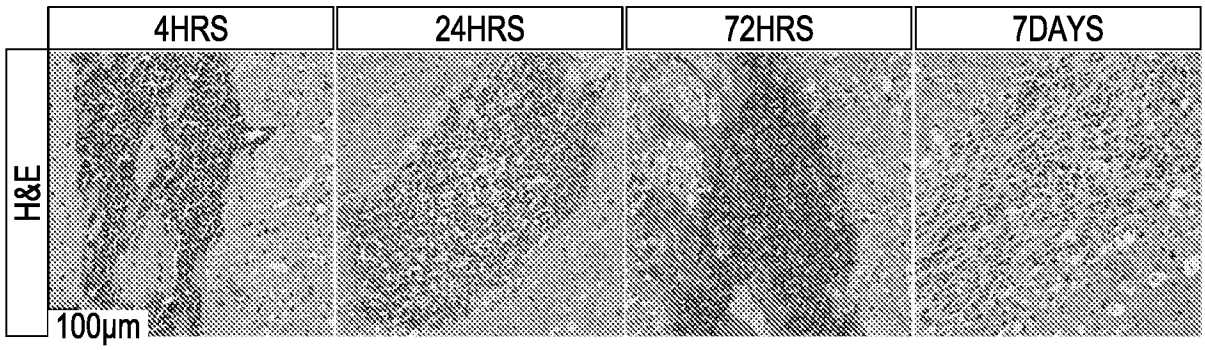


FIGURE 21B

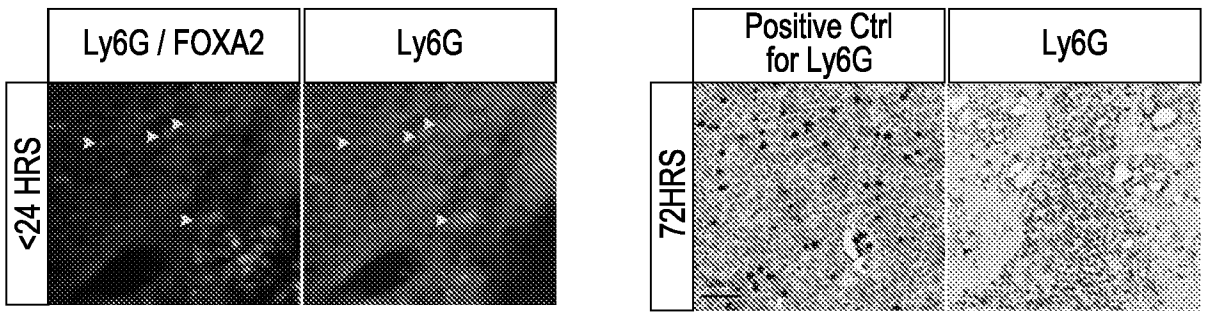


FIGURE 21C

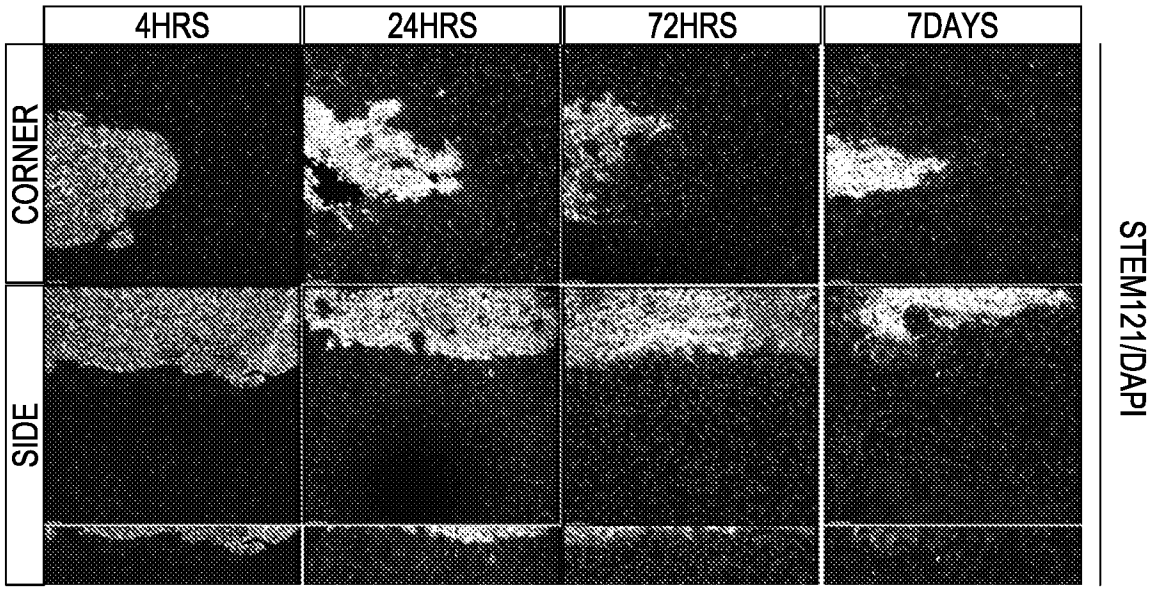


FIGURE 21D

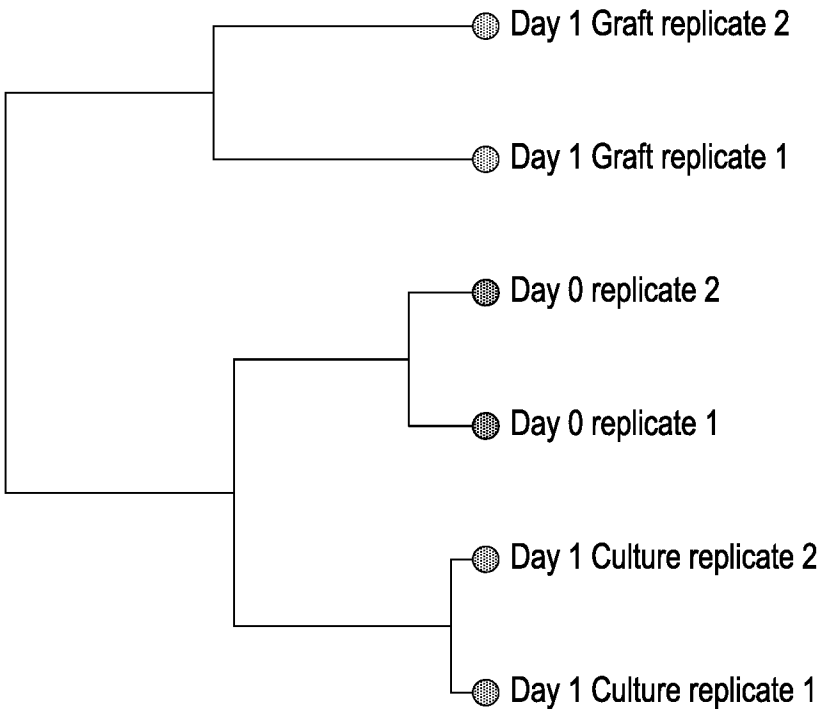


FIGURE 22A

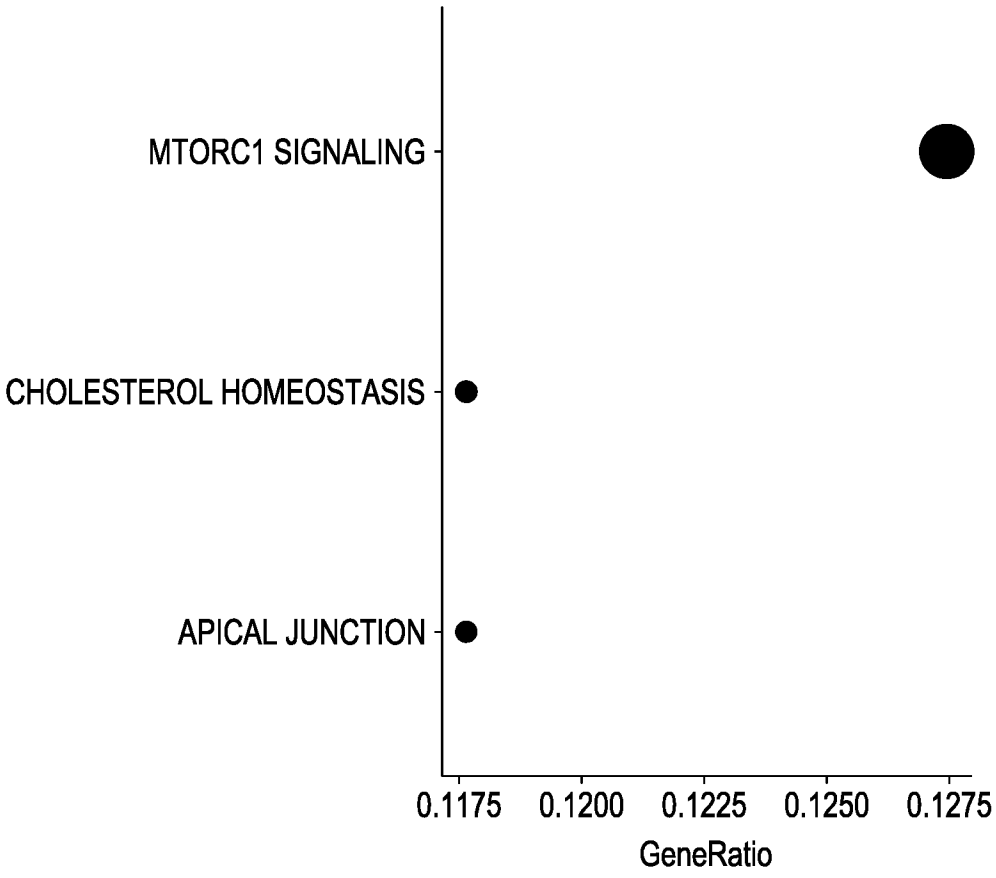

MTORC1 SIGNALING
CHOLESTEROL HOMEOSTASIS
APICAL JUNCTION
0.1175
0.1200
0.1225
0.1250
0.1275
GeneRatio

FIGURE 22B

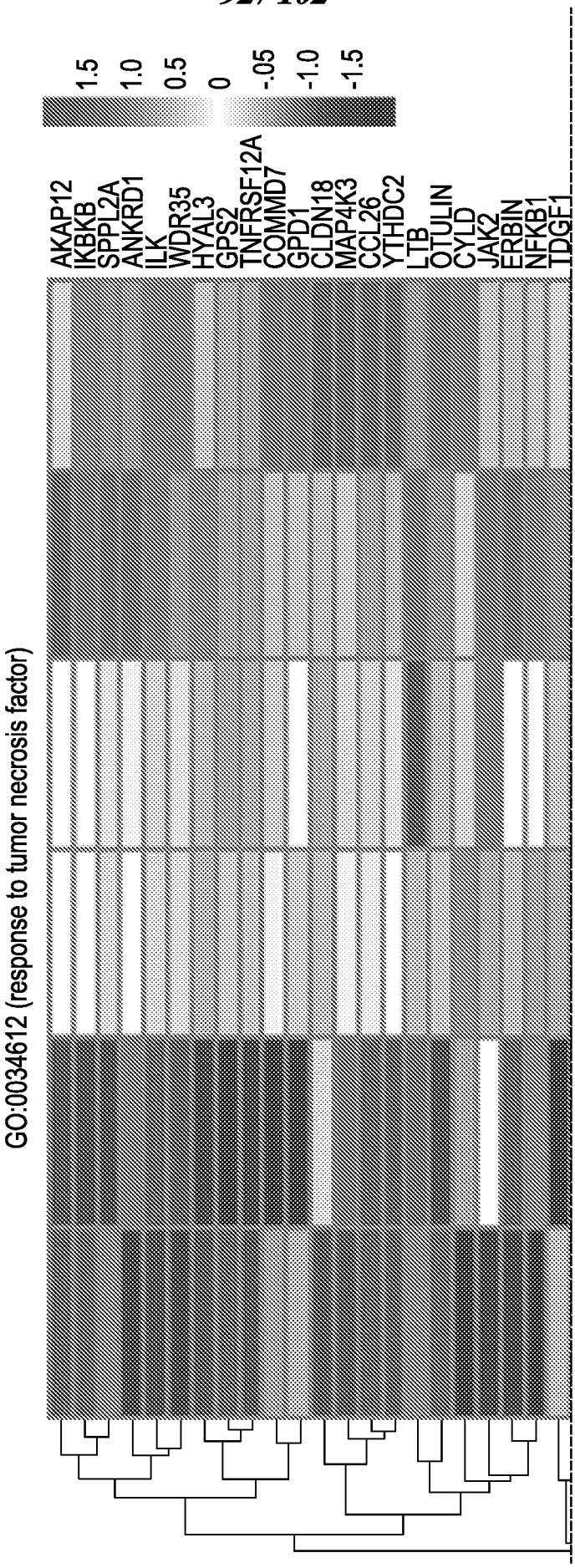

1.5
1.0
0.5
0
-.05
-1.0
-1.5
AKAP12
IKBKB
SPPL2A
ANKRD1
ILK
WDR35
HYAL3
GPS2
TNFRSF12A
COMMD7
GPD1
CLDN18
MAP4K3
CCL26
YTHDC2
LTB
OTULIN
CYLD
JAK2
ERBIN
NFKB1
TDGF1
GO:0034612 (response to tumor necrosis factor)

FIGURE 22C

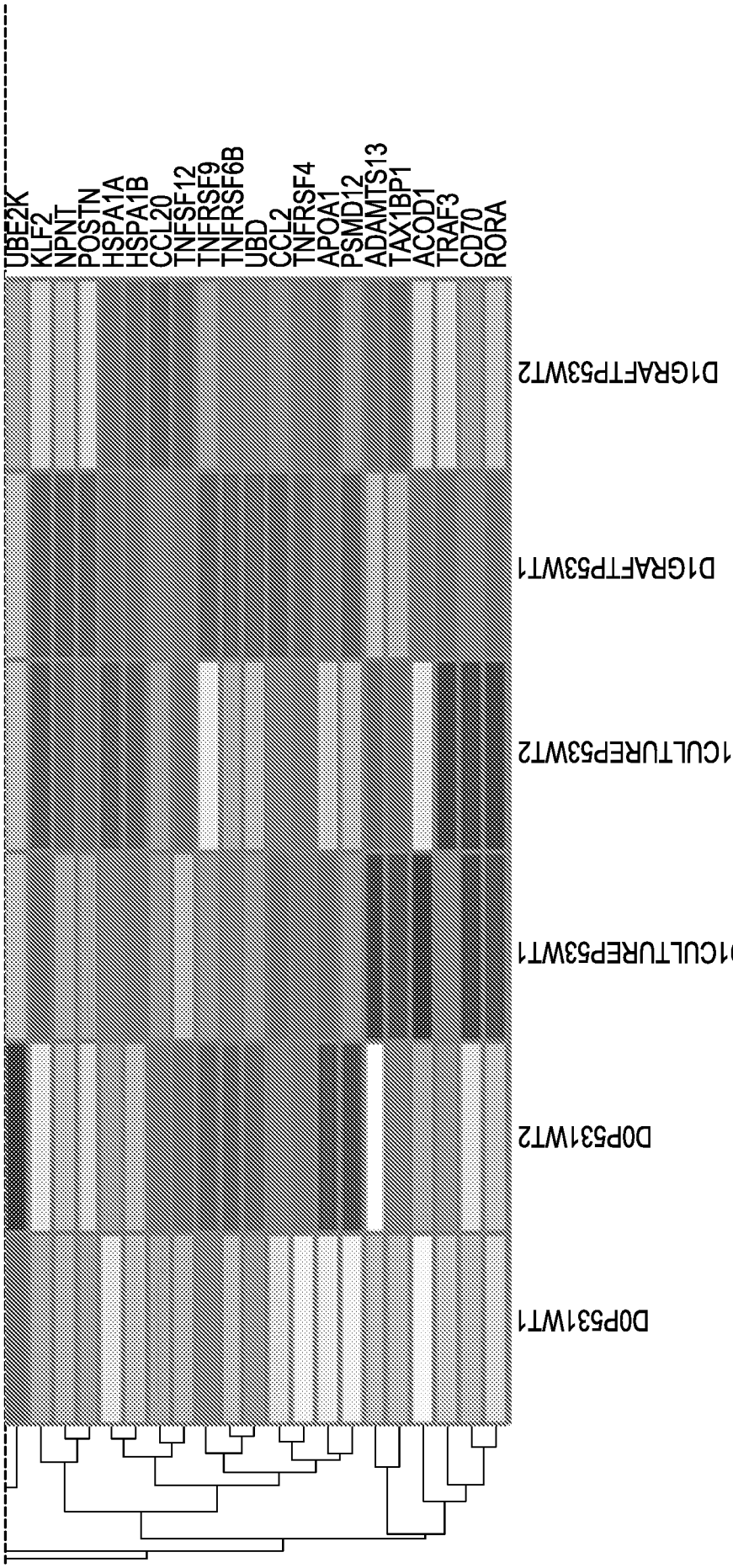


FIGURE 22C (Continued)

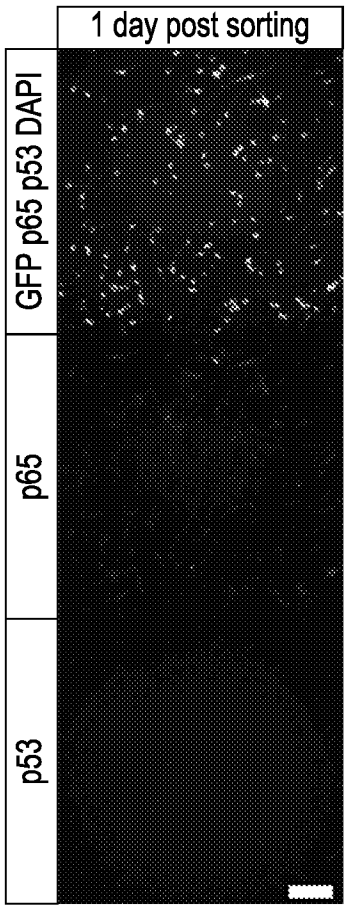


FIGURE 22D

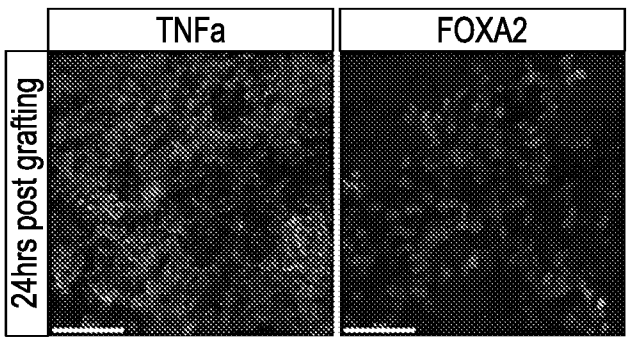


FIGURE 22E

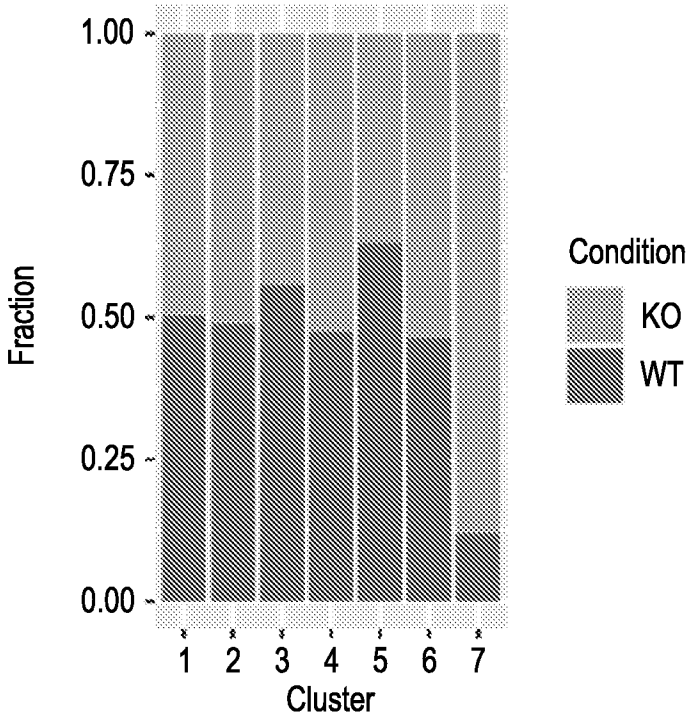


FIGURE 23A

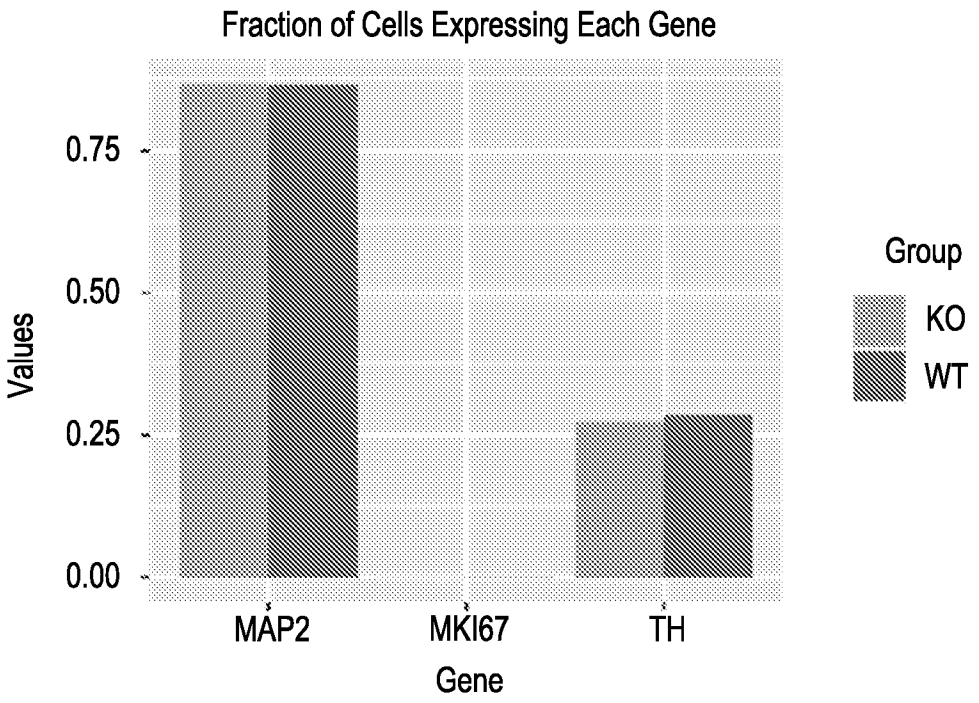


FIGURE 23B

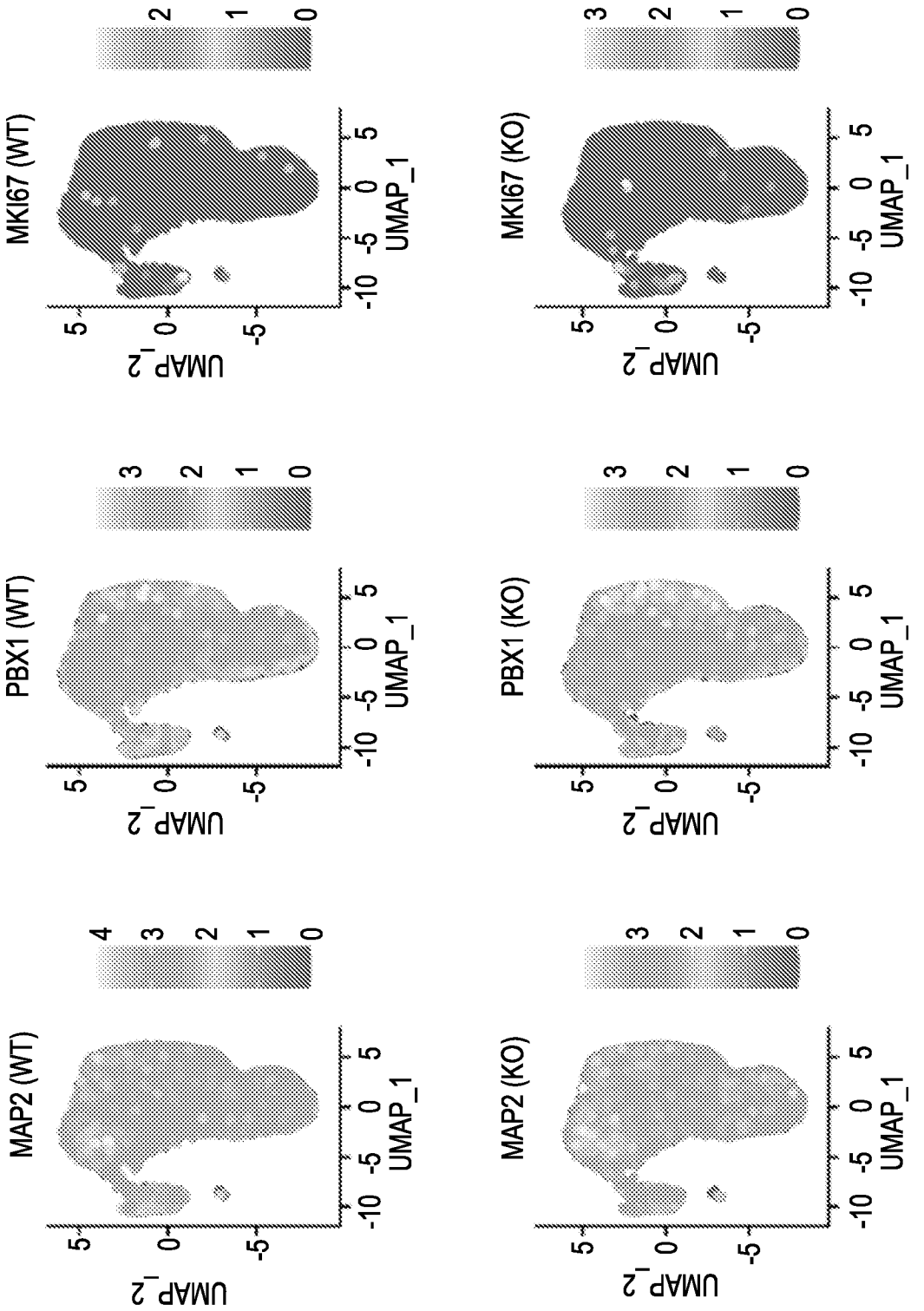


FIGURE 23C

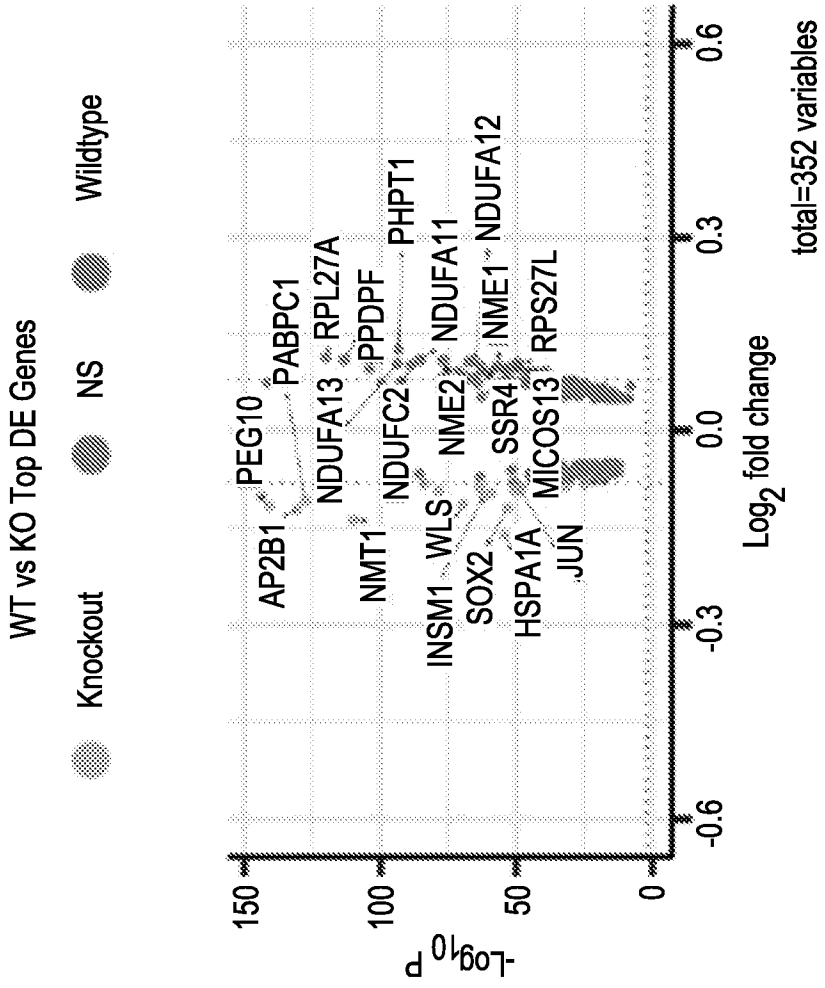

WT vs KO Top DE Genes
Knockout
NS
Wildtype
PEG10
PABPC1
AP2B1
RPL27A
NDUFA13
PPDPF
PHPT1
NMT1
NDUFC2
NDUFA11
INSM1
WLS
NME2
NME1
NDUFA12
SOX2
SSR4
RPS27L
HSPA1A
MICOS13
JUN
-Log10 P
150
100
50
0
-0.6
-0.3
0.0
0.3
0.6
Log2 fold change
total=352 variables

FIGURE 23D

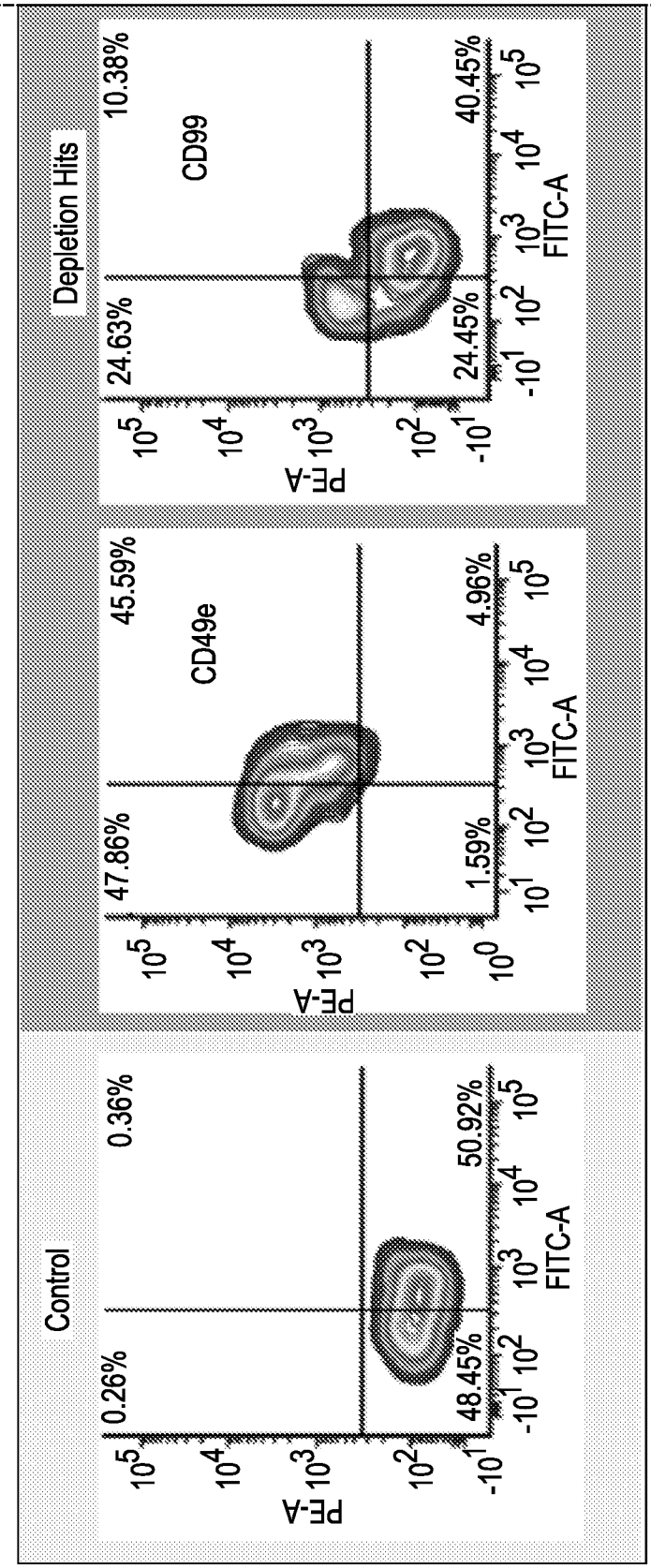

Control
0.26%
0.36%
48.45%
50.92%
PE-A
FITC-A
Depletion Hits
CD49e
47.86%
45.59%
1.59%
4.96%
CD99
24.63%
10.38%
24.45%
40.45%

FIGURE 24A

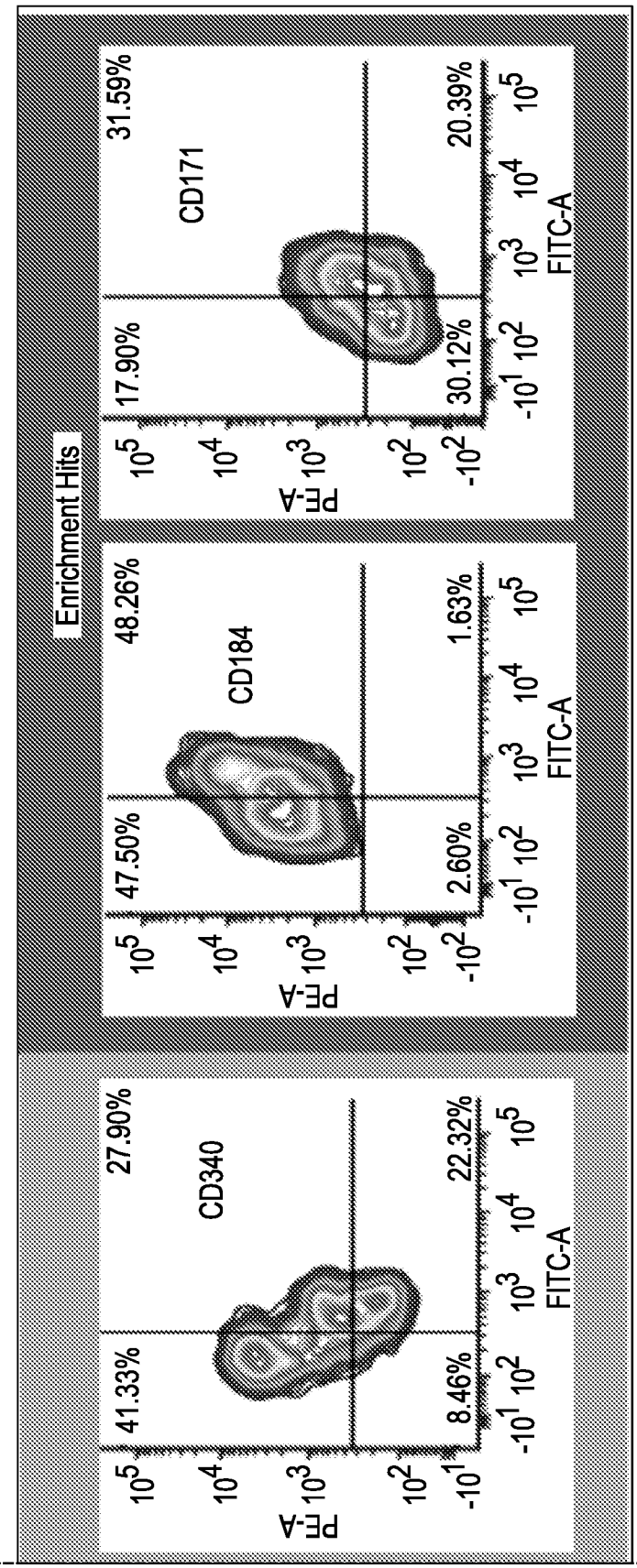


FIGURE 24A (Continued)

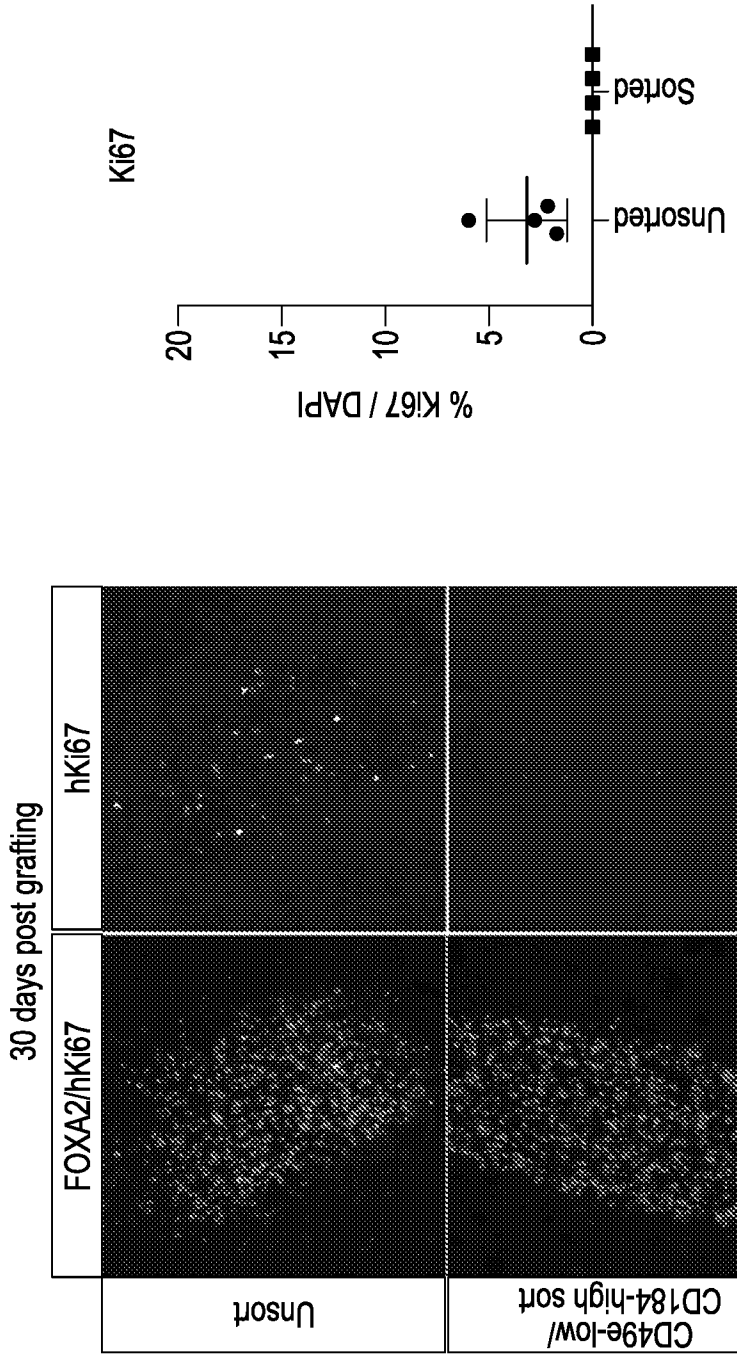

30 days post grafting
FOXA2/hKi67
hKi67
Unsort
CD49e-low/
CD184-high sort
Ki67
% Ki67 / DAPI
0
5
10
15
20
Unsorted
Sorted

FIGURE 24B

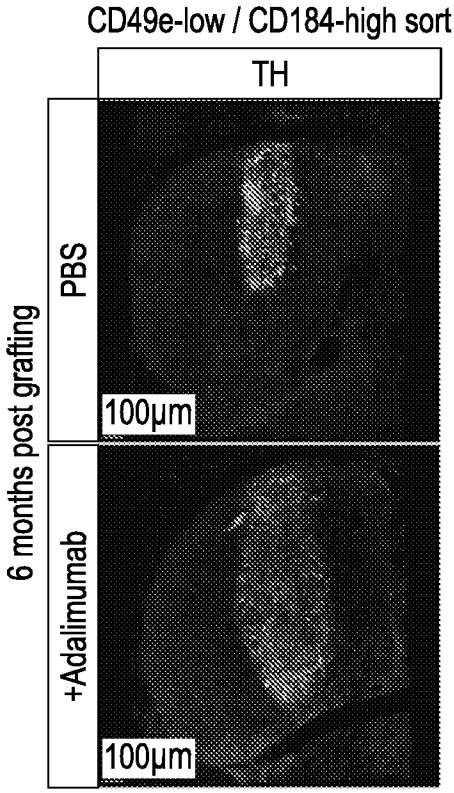


FIGURE 25A

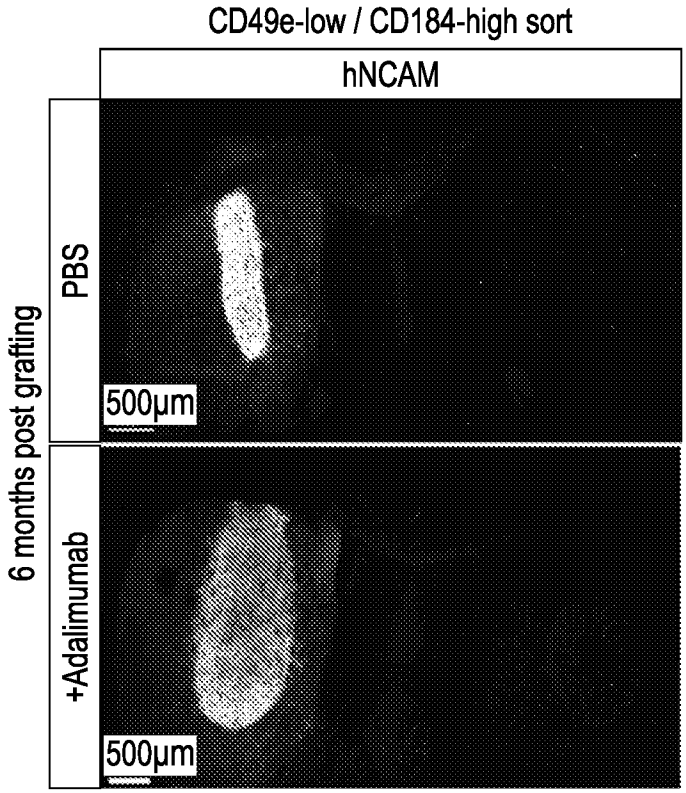


FIGURE 25B

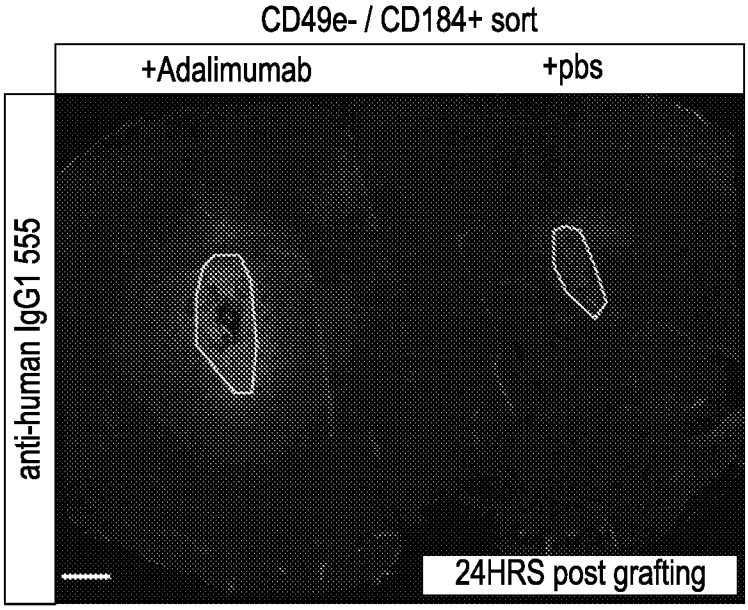

CD49e- / CD184+ sort
+Adalimumab
+pbs
anti-human IgG1 555
24HRS post grafting

FIGURE 25C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2023/015644

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - INV. - A61P 25/28 (2023.01)
ADD. - A61K 31/7088; A61K 39/395; A61P 25/00; A61P 25/16 (2023.01)

CPC - INV. - A61P 25/28; C07K 14/4746; C07K 14/4747 (2023.05)
ADD. - G01N 2510/00 (2023.05)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic database consulted during the international search (name of database and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2020/097380 A1 (BERG LLC) 14 May 2020 (14.05.2020) entire document	7, 8, 54-57
---		---
Y		1-5, 9
Y	US 2015/0010514 A1 (MEMORIAL SLOAN-KETTERING CANCER CENTER) 08 January 2015 (08.01.2015) entire document	1-5
Y	SHAO et al. " TNF-α-induced p53 activation induces apoptosis in neurological injury," J Cell Mol Med, 28 April 2020 (28.04.2020), Vol. 24, Pgs. 6796-6803. entire document	5, 9
A	NAIR " Activation of p53 signaling initiates apoptotic death in a cellular model of Parkinson's disease," Apoptosis, 14 March 2006 (14.03.2016), Vol.11, Pgs. 955-66. entire document	1-5, 7-9, 54-57
E, X	KIM et al. " TNF-NFkB-p53 axis restricts in vivo survival of hPSC-derived dopamine," bioRxiv, 31 March 2023 (31.03.2023), Pgs. 1-50. Retrieved from the Internet: <https://www.biorxiv.org/content/biorxiv/early/2023/03/31/2023.03.29.534819.full.pdf> on 30 May 2023 (30.05.2023). entire document	1-5, 7-9, 54-57

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 May 2023	JUN 22 2023
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Taina Matos Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2023/015644

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed.

   b. ☒ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)),

      ☒ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

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Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6, 10-53, 58-97
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.