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Supplemental Information

Mitochondrial Superoxide Dismutase Specifies Early Neural Commitment by Modulating Mitochondrial Dynamics

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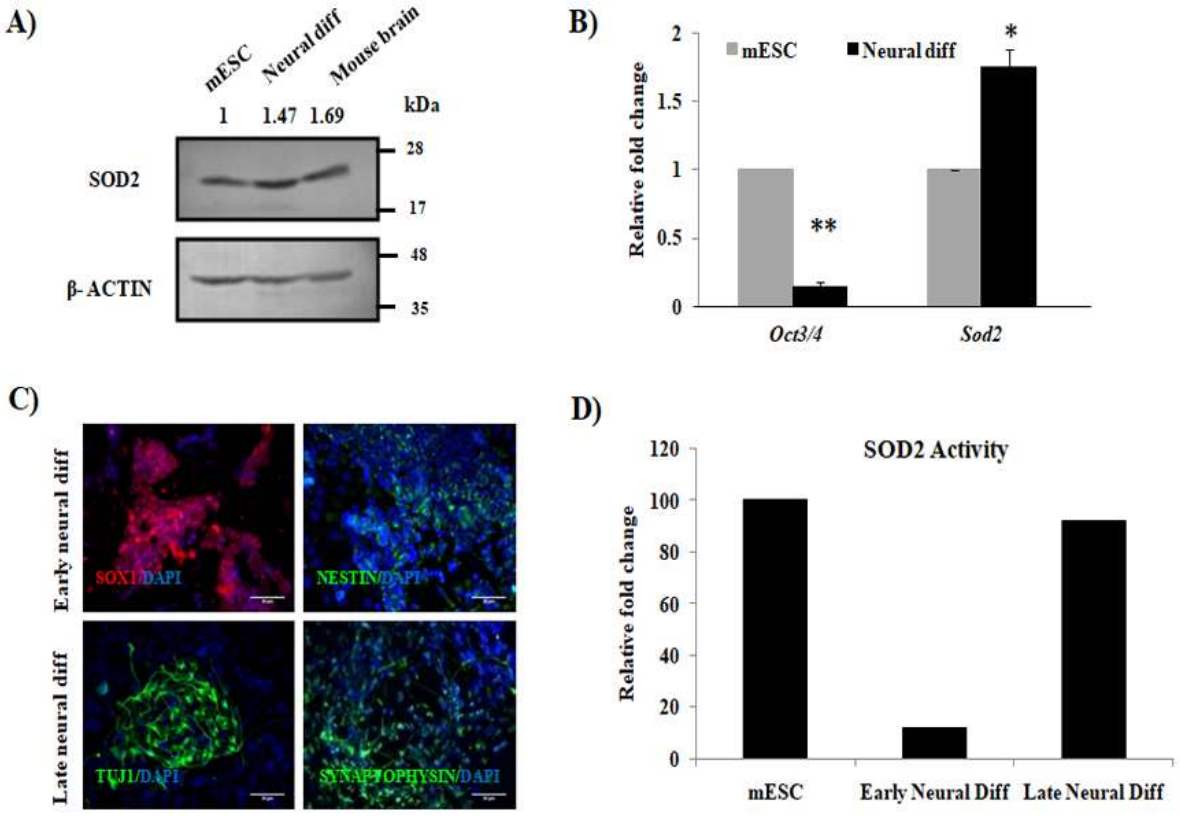

Figure S1, related to figure 1: Expression and enzymatic activity analysis of SOD2 during neural differentiation of mESCs.

(A) Protein levels of SOD2 in mESCs, neural differentiation and adult brain tissue.

(B) mRNA levels of *Oct3/4* and *Sod2* in mESCs and its neural differentiation counterpart.

(C) Representative immunofluorescence images to authenticate the early (SOX1, NESTIN) and late (TUJ1, SYNAPTOPHYSIN) neural differentiation stages of cells differentiated from mESCs.

(D) Fold change in SOD2 enzyme activity in early neural differentiation and late neural differentiation in comparison with mESCs. Mean ±SE, n= 3 independent experiments, * $p< 0.05$, ** $p < 0.01$.

Un-cropped full western blot images are available in data S1.

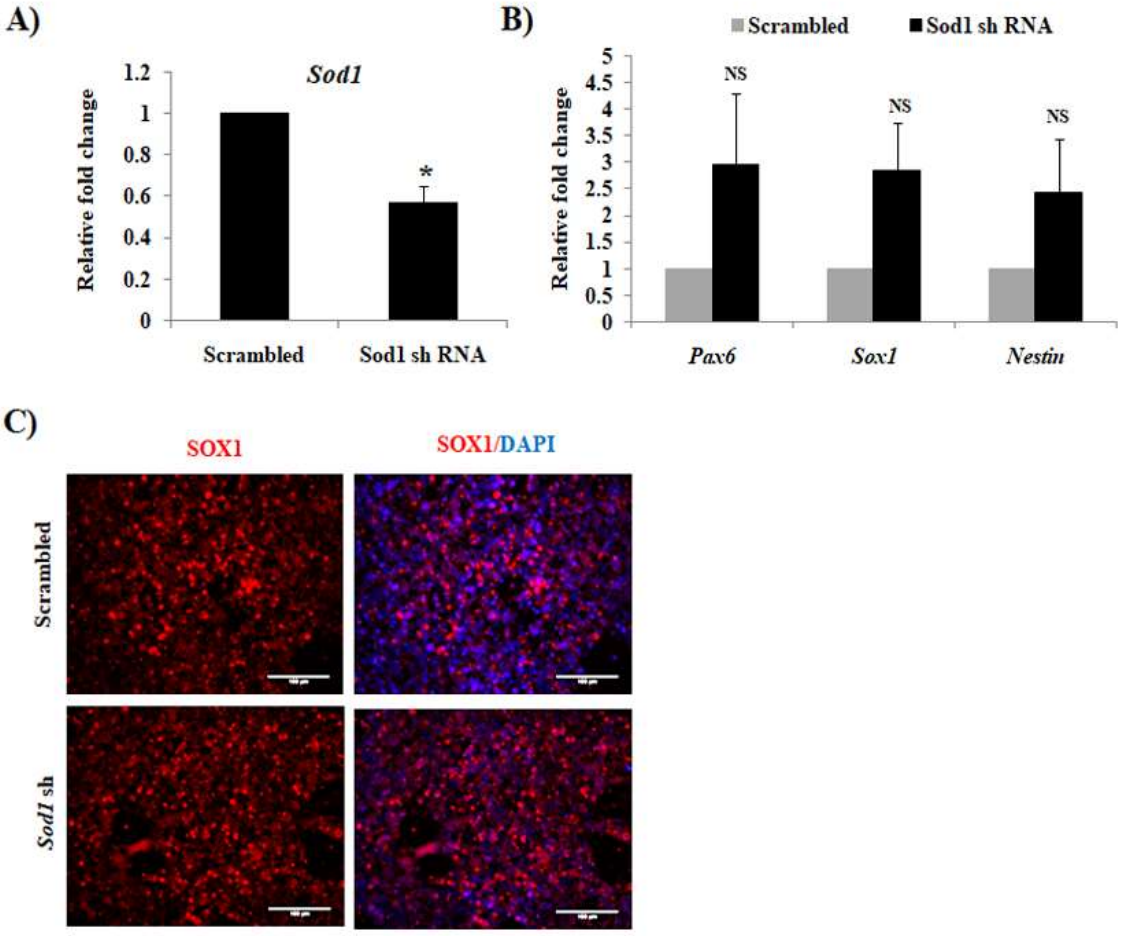

Figure S2, related to figure 1: Effect of Sod1 knockdown on neural differentiation of mESCs.

A) Knock-down efficiency of *Sod1* shRNA in mESCs.

B) Transcript analysis showing effect of *Sod1* knockdown on neural differentiation of mESCs.

C) Immunofluorescence for SOX1 in neural differentiation of mESCs upon *Sod1*inhibition. Data represented as mean ±SE, n= 3 independent experiment, *p< 0.05. Scale bar= 100 µm.

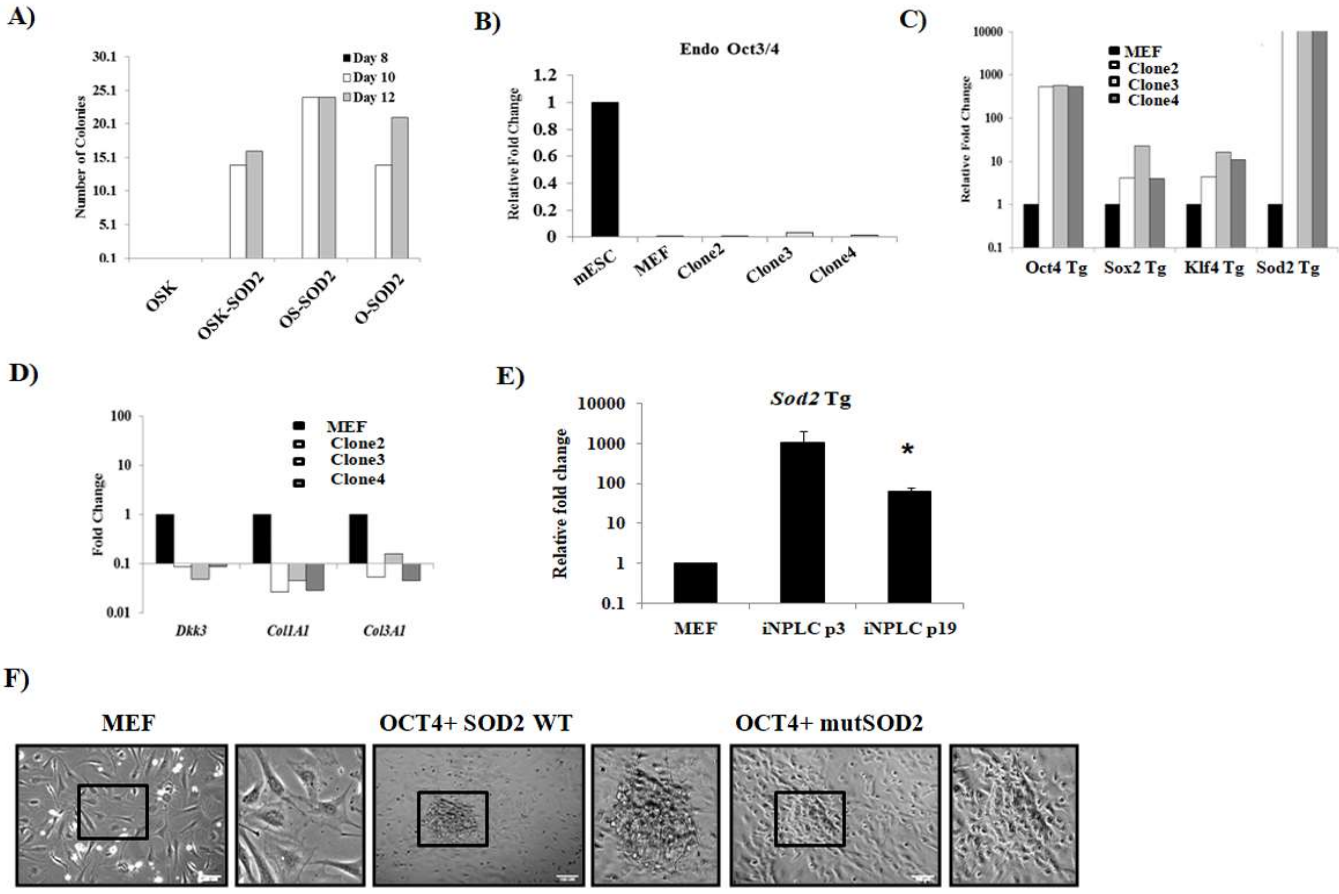

Figure S3, related to figure 3: SOD2 and mutSOD2 mediated transdifferentiation of MEFs to induced neural progenitor like cells (iNPLCs).

(A) Number of colonies developed at day 8, 10 and 12 upon transduction of MEFs with various combinations of reprogramming factors and SOD2.

(B) Transcript analysis of endogenous OCT4 in MEFs and i-NPLC colonies in comparison to mESCs.

(C) Expression of different transgenes in O-SOD2 colonies obtained from MEFs.

(D) mRNA levels of fibroblast markers *Dkk3*, *Col1A1* and *Col3A1* in O-SOD2 clones with respect to starting cell material MEFs.

(E) SOD2 transgene (Tg) expression in MEFs and i-NPLC colonies at passage number 3 and 19.

(F) Phase contrast images of untransduced MEFs and those transduced withOCT4 and SOD2 WT; OCT4 and mutSOD2, cultured in ESC medium. Scale bar represents 100 µm. Data is representative of three individual biological samples. * $p < 0.05$.

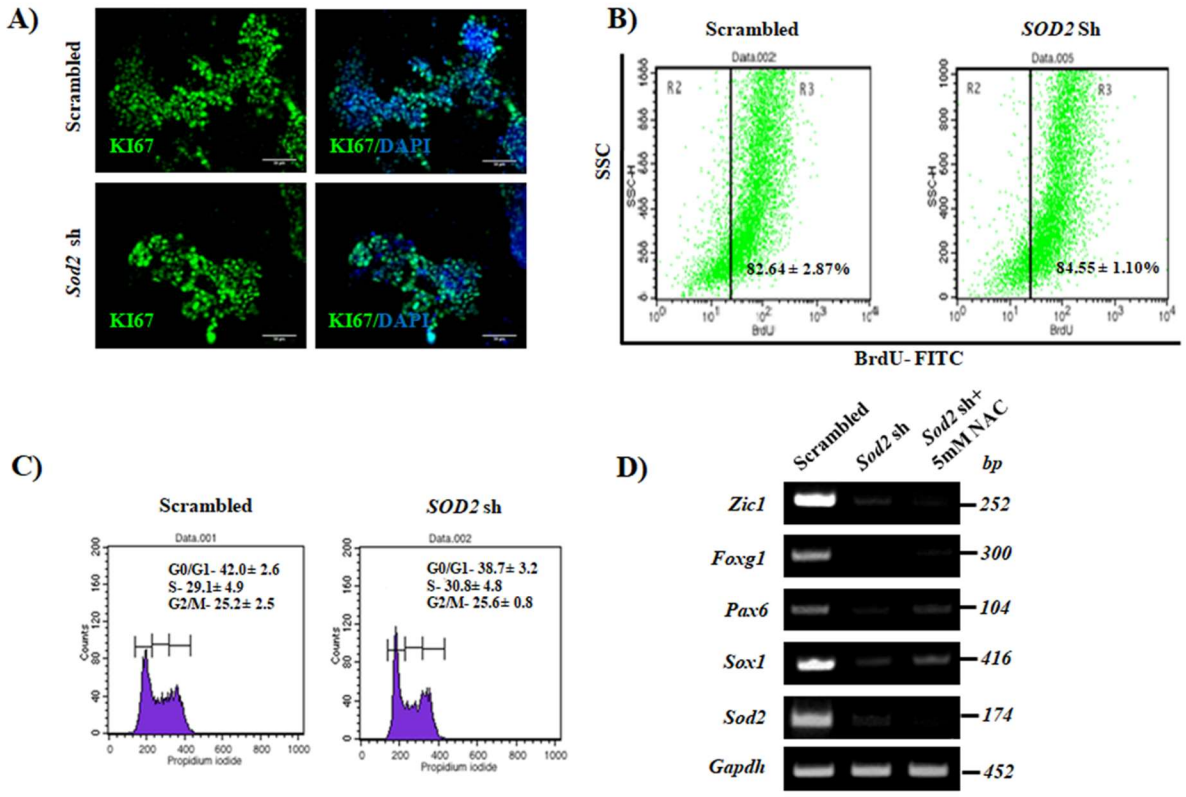

Figure S4, related to figure 4: SOD2 mediated neurogenesis is not due to modulation in cell proliferation or ROS.

(A) Representative immunofluorescence images of Ki67 staining in early neural differentiation of mESCs upon *SOD2* knockdown.

(B) Flow cytometric analysis of BrdU incorporation in early neural differentiation of mESCs upon *SOD2* knockdown.

(C) Cell cycle analysis of early neural differentiation of mESCs upon *SOD2* knockdown.

(D) mRNA levels of early neural markers in neural differentiation with *SOD2* knockdown, and N-acetylcysteine (NAC) treatment with *SOD2* knockdown. Mean ±SE, n= 3 independent experiments.

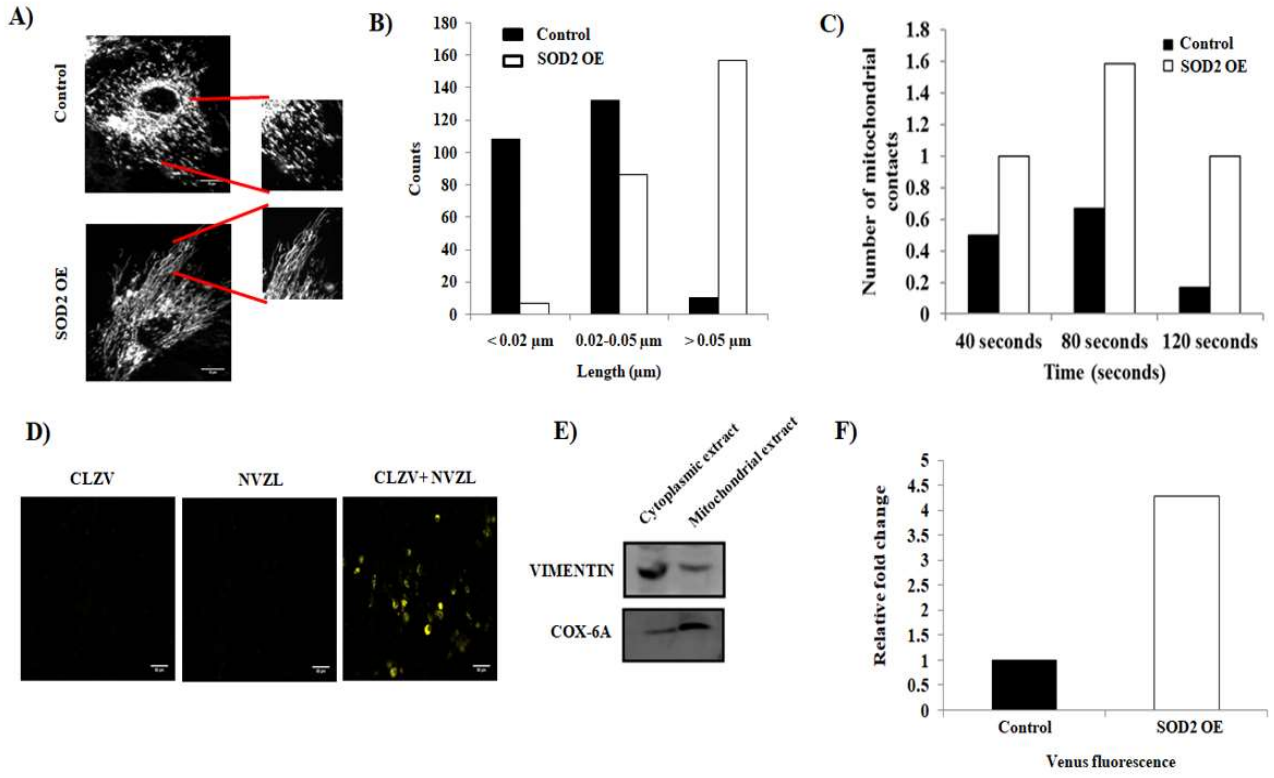

Figure S5, related to figure 5: Over-expression of SOD2 enhances mitochondrial fusion in MEFs.

(A) Representative images of mitochondria in MEFs where SOD2 over-expressed cells show enhanced mitochondrial fusion

(B) Quantification of mitochondrial length upon SOD2 Over-expression in MEFs

(C) Number of mitochondrial contacts at different time intervals in mitochondria of MEFs where SOD2 is over-expressed

(D) Representative image of the MEFs transfected with individual constructs (CLZV and NVZL) or with both the constructs (CLZV+NVZL). Cells transfected with both constructs exhibit Venus fluorescence confirming the bio-complementation process.

(E) Purity of mitochondrial isolation shown by enrichment of mitochondrial specific marker COX-6A in isolated mitochondria

(F) Increase in venus fluorescence indicating mitochondrial fusion upon SOD2 over-expression in MEFs.

Un-cropped full western blot images are available in data S1.

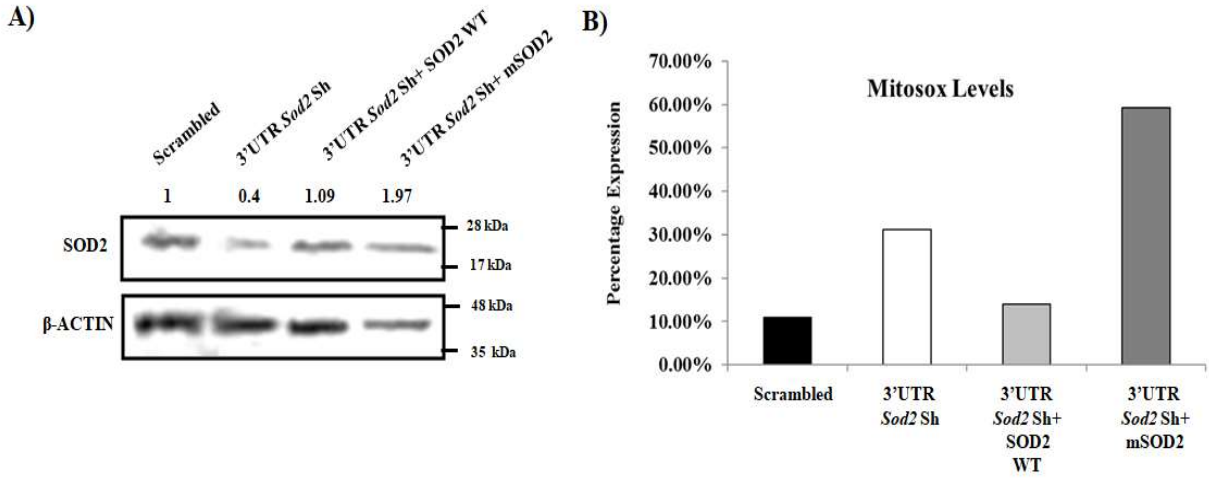

Figure S6, related to figure 5: SOD2 antioxidant mutant hampers the superoxide dismutation by SOD2.

(A) Western blot showing SOD2 expression in cells transduced with scrambled control, 3' UTR *Sod2* sh RNA with and without over-expression of SOD2 WT and mutSOD2. Un-cropped full western blot images are available in data S1.

(B) Mitochondrial superoxide levels measured in mESCs expressing scrambled control, 3' UTR *Sod2* sh RNA with and without over-expression of SOD2 WT and mutSOD2.

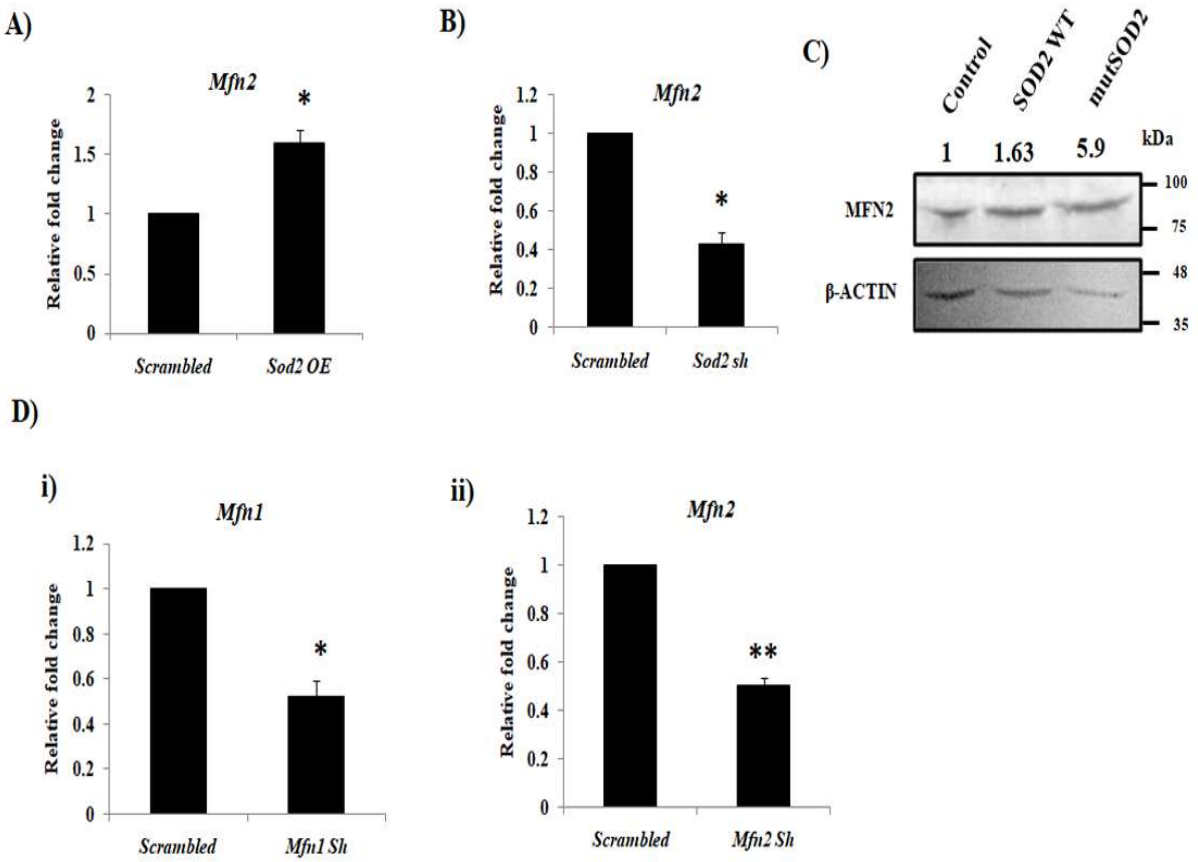

Figure S7, related to figure 6: SOD2 antioxidant mutant has similar effects on MFN2 expression as that of SOD2 WT.

(A and B) Expression analysis of *Mfn2* transcripts upon SOD2 over-expression (A) and knockdown (B).

(C) Western blot showing the effect of SOD2 WT and mutSOD2 over-expression on MFN2 levels.

(D) Knockdown efficiency of *Mfn1* shRNA and *Mfn2* shRNA shown by qPCR. Data is representative of Mean ±SE, n= 3 independent experiments, * $p< 0.05$, ** $p < 0.01$.

Transparent Methods

Ethics Statement

All experiments were approved and performed in compliance with the regulations of the Manipal University Animal ethical Committee and in accordance with the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care.

Cell lines and differentiation

mESCs: mouse embryonic stem cells, R1 (a kind gift from Catherine Verfaille, KU Leuven), were cultured on mitotically inactivated mouse embryonic fibroblast (MEF) feeder layer in DMEM high glucose (Gibco) supplemented with 8% FBS (HiMedia), 1X NEAA (Gibco), 1mM GlutaMax (Gibco), 1X Penstrep (Gibco), 1X sodium pyruvate (Gibco) and 100 μM β-Mercaptoethanol (Sigma). To maintain pluripotency, 1000U/mL of LIF (Millipore) was added to the medium. Prior to differentiation, mESCs were cultured on 0.1% gelatin coated dishes for 48 hours.

P40H1 is a hippocampal cell line derived from post natal day 40 mouse. P40H1 cells were cultured in DMEM high glucose supplemented with 5% FBS, 1X NEAA, 1mM GlutaMax and 1X Penstrep.

MEF and HEK293T cells were cultured in medium containing DMEM high glucose with 10% FBS, 1XNEAA, 1mM GlutaMax and 1X Penstrep. The list and specification of the reagents used are provided in Table S2.

Endodermal differentiation

Approximately 2×10^4 cells were plated onto a 12 well plate coated with matrigel in differentiation medium supplemented with 100 ng/mL Activin (Peprotech# 10429- HNAH), 50 ng/mL Wnt3A and 2% FBS. After three days of differentiation, primitive endoderm specific cues were provided. Briefly, 100 ng/mL Activin and 1.5% FBS were supplemented with fresh differentiation medium. Differentiation was terminated on day 6 and the cells were harvested for transcript and protein analyses.

Mesodermal differentiation

mESCs were seeded at a density of 1.5×10^5 cells/sq.cm and maintained in differentiation medium overnight. The medium was then supplemented with10 ng/mL each of EGF (Peprotech# 10605-HNAE), bFGF (Peprotech# 10014- HNAE) and PDGF (R & D systems 220-BB). Differentiation was carried out for 6 days post which the cytokines were withdrawn. The cells were cultured in the medium without cytokines for 4 days to achieve terminal differentiation.

Neural differentiation

mESCs were cultured in N2B27 medium which is a 1:1 mixture of DMEM-F12 with 1X N2 plus supplement (Thermofisher Scientific- #**17502001**)and Neurobasal medium (Gibco) with 1X B27 (Thermofisher Scientific- #12587001) supplemented with 1X Glutamax, 1X Penstrep, 100 µM β-Mercaptoethanol and 10^{-8} M Retinoic acid. The differentiation protocol was staged as early (end of 3 days of differentiation) and late (end of 7 days of differentiation) neural differentiation. The stages were characterized for their efficiency by the respective marker profiles.

Oligodendrocyte differentiation

Cells were seeded at a density of $5*10^4$ cells/cm^2 on ultra-low attachment plates and cultured for 4 days. On day 4 of differentiation, the medium was supplemented with 0.2M retinoic acid (RA). On the next two days, 0.2M RA and 1µM Purmorphamine were added to the differentiation medium. The spheres formed under low attachment were disaggregated and plated on 0.01% polyornithine (Sigma #P4957) coated dishes in differentiation medium DMEM F12, 1X N2 plus supplement, 1mM sodium pyruvate , 1% NEAA, 100 µM β- Mercaptoethanol along with 20 ng/mL bFGF.

Animals

We used 3-4 week old male Swiss Albino mice for mRNA expression analysis of *Sod2* in the whole brain lysate. We used p2 mice to inject iNPLCs labeled with GFP to one of the cortical hemispheres to test their ability to differentiate *in vivo*. The mice post injections were allowed to grow till 4 weeks. Post this, the animals were sacrificed and the brains were harvested for immuno-histochemical analysis.

Western blotting

Cells were lysed with RIPA buffer containing 1X protease inhibitors (Sigma #S8830). The suspension was rocked gently on ice for 20 min and centrifuged at 12000rpm for 15 min. and the clear lysates were stored at -80°C till further use. The samples were prepared by heating with Laemmli buffer at 95°C. Proteins were resolved on a 12% SDS-Polyacrylamide gel and then transferred onto an activated PVDF membrane using a semi-dry blotting apparatus. The non-specific binding on the membrane was blocked with 3% BSA or 3% skimmed milk in 1X TBST. The blots were then incubated with indicated primary antibodies at a concentration of 1:1000 overnight on a rocker at 4^0C. The blots were then washed with 1X TBST thrice and appropriate HRP-conjugated secondary antibodies were added at a concentration of 1:1000. The blots were incubated for 1 hour at room temperature on a rocker and then washed thrice with 1X TBST. The blots were developed using WesternBright ECL HRP susbtrate (Advansta #K-12045-D20) on LI-COR C digit blot scanner. The list and dilutions of primary and secondary antibodies used in this study are provided in Table S2.

qRT-PCR analysis

Cells were lysed with RNAiso Plus (Takara Bioscience) and total RNA was isolated according to the manufacturer's protocol. RNA was quantified by Nanodrop (ThermoScientific Corporation) and 1µg of RNA was converted to cDNA using RevertAid First Strand cDNA synthesis Kit (ThermoScientific Corporation). For transcript analysis, PCR amplification was performed using 2X EmeraldAmp GT PCR Master mix (Takara Biosciences) and the amplified products were visualized on a 2% agarose gel. qPCR analysis was performed using 2X SYBR Green kit (Takara Biosciences) and gene specific primers (Key resource table 1) on a 7500 Real time PCR machine (Applied Biosystems). The list of primers used in this study is provided in Table S1.

Immunofluorescence

Cells were fixed with 4% PFA for 20 min, permeabilized using 0.5% TritonX-100 in PBS for 30 min, and blocked with 20% FBS in PBS for 20 min, at room temperature. Three PBS washes were given after each step. Primary antibodies were added at appropriate concentrations in PBS and incubated at 4^0C overnight. The cells were washed thrice with 1X PBS and incubated with secondary antibodies conjugated with fluorescence tags at a concentration of 1:1000 in room

temperature for 1 hour. List of primary and secondary antibodies used in the study have been provide in Key resource table 2. The cells were counterstained with 1:10000 DAPI for 2 min. After washing with 1X PBS, the cells were visualized under Olympus X73 inverted microscope and images were captured at 20X. Images were pseudo-colored and analyzed using ImageJ software.

Immunohistochemistry:

To track S2 cells injected into infant mouse cortex, we performed IHCs on 4 week old mouse brains. The tissues were fixed with 4% PFA at 4˚C for 72 hours. Cryo-sectioning was performed to obtain 10 µm thick brain sections. The sections were washed once with 1X PBS and incubated with 1:100 primary antibody overnight at 4˚C. Washes were performed with 1X PBS. Secondary antibody was diluted in PBS to a final concentration of 1:600 and the sections were incubated with it for 4 hours at room temperature. The sections were washed thrice with 1X PBS and counter-stained with 1:10000 DAPI. The stained neurons were imaged under 20X objective in Nikon TE 2000 inverted epi-fluorescence microscope.

BrdU incorporation assay

To determine the proliferative status of cells during early neural differentiation, the cells were treated with a final concentration of 10µM BrdU after 18 hours of plating and were differentiated till day 3. After this, the cells were fixed with cold methanol for 15 min at room temperature and then washed thrice with 1X PBS containing 0.1% tween-20 (Sigma) (PBST). Hydrochloric acid, at a working concentration of 2M was added and the samples were incubated at room temperature for 30 minutes. Cells were then permeabilized with 1X BD PermWash for 20 min, blocked with 20% FBS for 30 min at room temperature and incubated with anti-BrdU antibody at a concentration of 1:400 on a rocker at 4^0C overnight. Post washes, the cells were incubated with secondary antibody at a concentration of 1:1000 for 2 hours at room temperature and were analyzed on Flow cytometer for BrdU expression.

Flow cytometry

Cells were trypsinized, washed with 1X Ca^{2+}/Mg^{2+} free PBS, and fixed with 0.1% PFA for 1 hour at room temperature. Post washes with 1X PBS, the cells were permeabilized using BD 1X PermWash and blocked with 20% FBS. Primary antibody was added at a concentration of 1:400

and the cells were incubated on a rocking platform at 4^0C overnight. After three washes with 1X PBS, the cells were incubated with appropriate secondary antibody at a dilution of 1:1000 for one hour at room temperature. The cells were washed thrice with 1X PBS and the events were acquired on BD FACSCalibur analyzer.

DCFHDA staining

Cells were trypsinized and resuspended in serum-free basal medium. DCFHDA (Thermofisher Scientific #D399) was added at a final concentration of 20µM and incubated at 37^0C for 20 min in dark. Events were acquired on BD FACSCalibur to understand ROS levels in mESCs, early neural differentiation and late neural differentiation.

Cell cycle analysis

Cells were trypsinzed and washed once with 1X Ca^{2+}/Mg^{2+} free PBS. The pellet was resuspended in 1mL of hypotonic propidium iodide (PI) solution containing 0.1% sodium citrate, 40µg/mL RNase, 25µg/mL PI and 0.03% NP40 in nuclease free water. The samples were incubated for 10 min on ice in dark and the cell cycle profile was analyzed on BD FACSCalibur.

Transfection and transduction

One day prior to transfection, HEK 293T cells were seeded at a density of $0.2*10^5/cm^2$. The transfection reagent –X-tremeGENE (Merck #6366236001) – was added to OptiMEM (Gibco) at a concentration of 1:4 of the plasmid amount in a sterile polystyrene tube. The tube was thoroughly vortexed for 15 seconds and incubated at room temperature for 10 min. The plasmids of interest, with their respective packaging vectors, were added at recommended concentrations and the tube was vortexed for 15 seconds. The mixture was incubated at room temperature for 20-30 min. and the transfection mixture was added carefully to the cells, which were then incubated at 37^0C with 5% CO_2. The supernatants containing viral particles were collected 48 hours and 72 hours post transfection. Viral particles were concentrated by incubating the supernatants with a final concentration of 50% sterile PEG solution on a rocking platform at 4^0C overnight and then centrifuging at 1600 rpm for 1 hour at 4^0C. The viral pellet was resuspended in minimal volume of basal medium and added to the recipient cells. Cells were provided normal growth medium 24 hours post transduction and selected with 1µg/mL Puromycin wherever applicable. Transduced

cells were further trypsinized and used for different experiments. The list of plasmids used in this study is provided in Table S2.

Derivation and maintenance of O-SOD2 WT and O-mutSOD2 clones

MEFs were transduced with various combinations of retroviral constructs of mOCT4, mSOX2, mKLF4 (kind gift from Dr. Shinya Yamanaka; Addgene #13366, #13367 and #13370 respectively) and pMIG-SOD2 WT. In an independent experiment, MEFs were transduced with mOCT4 and pMIG-mutSOD2. The cells were cultured in mESC medium supplemented with LIF from 48 hours post transduction. After five days of transduction, the cells were trypsinized using 0.25% Trypsin-EDTA Thermofisher Scientific #25200056) and plated on inactivated MEF feeder layer (iMEF) at a density of $0.15*10^6$cells.The medium was changed every alternate day. The colonies were manually picked and then expanded on iMEF feeders.

Differentiation of OS2 cells into mature neurons and glial cells

OS2 cells were cultured in mES-LIF medium (DMEM + 16.6% FBS + 2mM L-Glutamine + 1% Sodium Pyruvate + 0.1M β-mercaptoethanol) on gelatin coated dishes for upto 20 passages, post which they were cultured on matrigel coated plates with Neural Stem Cell medium (DMEM/F12 + 1X N2 + 1X B27 + 80mg Glucose + 0.22% BSA fraction V + 10ng Epidermal Growth Factor + 8ng/mL Fibroblast Growth Factor + 100ng/mL Insulin) before inducing them for neuronal and glial cell differentiation. Media change was given every 2- 3 days and cells post P30 were considered for differentiation.

For neuronal differentiation, 30,000 cells/well were plated onto matrigel coated 12 well plates. After 24 hours of plating, cells were fed with Neural differentiation medium-1 [DMEM/F12 + 1X B27 + 2mM L-Glut + 0.22% BSA fraction V+ 2nM Retinoic Acid + 10uM AraC (Cytosine-B-D-arabinofuranoside hydrochloride) + 10uM Valproic Acid]. Post 72 hrs, the media was changed to Neural differentiation medium-2 (DMEM/F12 + 1X B27 + 2mM L-Glut + 0.22% BSA fraction V + 2nM Retinoic Acid + 5uM AraC + 10uM Valproic Acid) for 3 days. Post 3 days, the cells were cultured in Neural differentiation medium-3 (Electrophys medium, StemCell Technologies with 20ng/mL of BDNF, 20ng/mL GDNF and 10uM Valproic Acid) for 7 days with a media change every 3 days.

For glial differentiation, 5,000 cells/well were plated in Matrigel coated 12 well plates. Post 24 hours of plating, astrocyte differentiation medium was added to the cells (DMEM/F12 + 1X B27 + 2mM L-Glut + 1% FBS). The cells were maintained in the medium for 5 days with media change every 72 hrs before harvesting them for gene and protein expression analysis.

Microarray

The samples for gene expression were labeled using Agilent Quick-Amp labeling Kit (p/n5190-0442). About 500ng each of total RNA were reverse transcribed at 40°C using oligodT primer tagged to a T7 polymerase promoter and converted to double stranded cDNA (ds cDNA). Synthesized ds cDNA were used as template for cRNA generation by *in vitro* transcription and the dye Cy3 CTP (Agilent) was incorporated during this step. Labeled cRNA was cleaned up using QiagenRNeasy columns (Qiagen, #74106) and quality was assessed for yields and specific activity using the Nanodrop ND-1000.

600ng of labeled cRNA sample was fragmented at 60ºC and hybridized on to Agilent's Mouse_GXP_8x60K (AMADID: 28005). Fragmentation of labeled cRNA and hybridization were done using the Gene Expression Hybridization kit (Agilent Technologies, *In situ* Hybridization kit, Part Number 5190-0404). Hybridization was carried out in Agilent's Surehyb Chambers at 65º C for 16 hours. The hybridized slides were washed using Agilent Gene Expression wash buffers (Agilent Technologies, Part Number 5188-5327) and scanned using the Agilent Microarray Scanner (AgilentTechnologies, Part Number G2600D).

Data extraction from Images was done using Feature Extraction software Version 11.5.1.1 of Agilent. Images were quantified using Feature Extraction Software (Version-11.5 Agilent). Feature extracted raw data was analyzed using GeneSpring GX software from Agilent. Normalization of the data was done in GeneSpring GX using the 75th percentile shift and fold expression values were obtained with respect to Specific control Samples. Differential expression patterns were identified among the samples. Significant genes up regulated fold> 1.0 (logbase2) and down regulated <-1.0 (logbase2) in the test samples with respect to control sample were identified. Statistical student T-test was performed and p-value among the replicates was calculated based on volcano plot algorithm. Differentially regulated genes were clustered using hierarchical clustering based on Pearson coefficient correlation algorithm to identify significant

gene expression patterns. Biological analysis was performed for the differentiallyexpressed genes based on their functional category and pathways using Biological Analysis tool DAVID (http://david.abcc.ncifcrf.gov/).

SOD2 activity assay

Cells were harvested and the protein concentrations were determined using BCA estimation kit (Novagen, USA) with BSA as the standard. Equal amounts of protein from each sample were taken to assess SOD2 activity using a colorimetric method, according to manufacturer's instructions (Biovision #K335). Briefly, the samples were incubated with WST solution followed by the addition of dilution buffer. Release of superoxide was initiated by addition of enzyme working solution and the samples were incubated for 20 minutes at 37^0C. Absorbance was recorded at 450nm using Ensightmultiwell plate reader and SOD2 activity was calculated.

Mitochondrial superoxide staining

Cells were trypsinized, washed once with 1X PBS to remove traces of FBS and resuspended in serum free basal medium. Mitosox red (Thermofisher Scientific #M36008) was added to the suspension at a final concentration of 20 μM. The samples were incubated at 37^0C in dark for 20 min. The cells were washed once with basal medium and the events were acquired on BD FACS Aria II.

Mitochondrial length and contact measurement

Mitochondria were labeled by transiently transfecting P40H1 cells with pLVmitoDsred construct (addgene 44386) and imaged using Olympus FV3000 inverted microscope at 63X magnification or Olympus X73 inverted microscope at 20X magnification. Images were processed using ImageJ software. The length of mitochondria was measured using line tool in ImageJ and tabulated. The number of mitochondrial kiss and run events were counted across different time frames using the live videos captured with DsRed labeled mitochondria.

shRNA cloning

Oligonucleotides (Supplementary table 1) of mouse *Sod2*, *Mfn1*and *Mfn2* were procured and allowed to anneal with respective reverse complimentary sequences a 95^0C for 5 min. The reaction mixture was slowly cooled down to room temperature and then transformed to DH5α competent cells along with the pLKO 1.puro lentiviral vector backbone digested with AgeI and EcoRI. Plasmid was isolated from the colonies obtained and sequenced for the selection of positive clones.

For *Sod2* inducible shRNA cloning, the annealed oligonucleotides were cloned into Tet- pLKO-puro vector.

Cloning SOD2 over-expression construct

SOD2 CDS was amplified from mESCcDNA using PhusionTaq polymerase enzyme and cloned into pMIG MCS IRES GFP backbone. The clones obtained were confirmed by DNA sequencing.

Generation of SOD2 antioxidant mutant clone

Point mutations were introduced in SOD2 sequence so that the protein formed is incapable of quenching mitochondrial superoxide. Two such mutations were performed to convert D-183 to N-183 and W-185 to F-185. The template selected was pMIG-Sod2-IRES-GFP. In order to generate site specific mutagenesis, this WT plasmid was amplified using sense and antisense oligonucleotides containing mutations and a *MluI* restriction site for the ease of screening positive clones. The oligonucleotides were 5' – GCTGGGGATTAACGTGTTTGAGCACGCGTACTACC-3' and 5' – GGTAGTACGCGTGCTCAAACACGTTAATCCCCAGC – 3'. The clones obtained were digested with methylation dependent restriction enzyme *DpnI* for the removal of WT SOD2 template and transformed using DH5-α. The colonies were screened for the positive clone by restriction digestion of the plasmid with *MluI* and were confirmed further by DNA sequencing.

Isolation of mitochondria

Cells were washed once with ice-cold 1X PBS, scrapped on ice, transferred to a chilled microcentrifuge tube and spun at 800g for 5 min at 4^0C. The cell pellet was resuspended in 3mL of ice cold Isolation Buffer c (IBc-10mM Tris-base, 1mM EGTA< 0.2M Sucrose at pH 7.4) and

homogenized using a pre-chilled Teflon dounce homogenizer. The homogenate was spun at 600g for 10 min at 4^0C. The supernatant was collected and centrifuged at 7000g for 10 min at 4^0C. The supernatant was discarded and the pellet was washed with 200µL of ice cold IBc and spun at 7000g for 10 min at 4^0C. The mitochondrial pellet was resuspended in minimal volume of IBc and further used for mitochondrial fusion assay.

Mitochondrial fusion assay

Mitochondrial fusion assay was performed as previously published (Schauss*et.al.,* 2010) with some modifications. Briefly, the mitochondria were isolated from two different populations of cells each harboring one half of venus and luciferase construct that is targeted to mitochondria. The mitochondrial pellets were resuspended in a reaction buffer containing 10 mM HEPES (pH 7.4), 110 mMMannitol, 68 mM Sucrose, 80 mMKCl, 0.5 mM EGTA, 2 mM Mg $(CH_3COO)_2$, 0.5 mM sodium succinate and 1 mM DTT. The mitochondria were then concentrated by centrifuging at 9000g for 1 min. The samples were incubated on ice for 30 min, then resuspended in the reaction buffer, and further incubated on ice for 30 min. Mitochondria were resuspended and warmed to 37^0C for 10 min, resuspended in minimal volume of the reaction buffer, and venus fluorescence was recorded at 515 nm $_{(Exc)}$ and 530 nm $_{(Emi)}$.

Quantification and Statistical Analysis

Image analysis and quantification of mitochondrial length and contacts was performed on ImageJ software.

Adobe photoshop was used to prepare figures with 300 dpi resolution.

Statistical analysis was performed using a two-tailed Student t-test. P values of $p \leq 0.05$ (*) were considered significant; $p \leq 0.005$ (**) and $p \leq 0.001$ (***) were considered highly significant.

Table S1: Primer sequences used for transcript analysis related to Figure 1, Figure 2, Figure 3, Figure 4, Figure 6, Figure S1, Figure S2, Figure S3, Figure S4, Figure S5, Figure S6 and Figure S7.

Primer Name	Sequence	Amplicon size (bp)
Gapdh	Forward primer: ACCACAGTCCATGCCATCAC Reverse primer: TCCACCACCCTGTTGCTGTA	452
Oct4	Forward primer: GAGGAGTCCAGGACATGAA Reverse primer: AGATGGTGGTCTGGCTGAAC	153
Map2	Forward primer: TCAGGAGACAGGGAGGAGAA Reverse primer: GTGTGGAGGTGCCACTTTTT	112
Sod2	Forward primer: CCGAGGAGAAGTACCACGAG Reverse primer: GCTTGATAGCCTCCAGCAAC	174
Sod1	Forward primer:CGGTGAACCAGTTGTGTTGT Reverse primer: AGTCACATTGCCCAGGTCTC	190
Pax6	Forward primer: TCCCAGGGATCTGAGAATTG Reverse primer: CACAACGGTTTGAAATGACG	104
Mog	Forward primer: ACCAAGAAGAGGCAGCAATG Reverse primer: GGTCCAAGAACAGGCACAAT	259
Olig2	Forward primer: CAGCGAGCACCTCAAATCTA Reverse primer: CACAGTCCCTCCTGTGAAGC	199
Foxg1	Forward primer: ACCTGTCCCTCAACAAGTGC Reverse primer: ACGTGGTCCCGTTGTAACTC	300
Pdgfr-a	Forward primer: CACACCGGATGGTACACTTG Reverse primer: GGCAGAGTCATCCTCTTCCA	159
Sox1	Forward primer: CTGCTCAAGAAGGACAAGTA Reverse primer: CTCATGTAGCCCTGAGAGT	416
Zic1	Forward primer: GCCCTTCAAAGCCAAATACA Reverse primer: TTGCAAAGGTAGGGCTTGTC	252
Tuj1	Forward primer: TAGACCCCAGCGGCAACTAT	127

	Reverse primer: GTTCCAGGTTCCAAGTCCACC	
N-Caherin	Forward primer: GATTTCAAGGTGGACGAGGA Reverse primer: CACTGTGCTTGGCAAGTTGT	223
Mixl1	Forward primer: CTACCCGAGTCCAGGATCCA Reverse primer: ACTCCCCGCCTTGAGGATAA	101
Vimentin	Forward primer: AGAGAGGAAGCCGAAAGCA Reverse primer: CTTTCATACTGCTGGCGCAC	248
Sox17	Forward primer: CACAACGCAGAGCTAAGCAA Reverse primer: TTGTAGTTGGGGTGGTCCTG	128
Cxcr4	Forward primer: GTGCAGCAGGTAGCAGTGAC Reverse primer: GGCAGGAAGATCCTGTTGAA	207
Foxa2	Forward primer: CCCGGGACTTAACTGTAACG Reverse primer: TCATGTTGCTCACGGAAGAG	152
NeuN	Forward primer: GCACAGACTCATCCTGAGCA Reverse primer: GGTGGAGTTGCTGGTTGTCT	115
Nurr1	Forward primer: AGTCTGATCAGTGCCCTCGT Reverse primer: GATCTCCATAGAGCCGGTCA	162
Tau	Forward primer: GGTCCATGTCTCCTTCTTGG Reverse primer: TCTTCTGTCCTCGCCTTCTG	132
Gata6	Forward primer: CAACACAGTCCCCGTTCTTT Reverse primer: TGGTACAGGCGTCAAGAGTG	122
Flk1	Forward primer: CCCGCATGAAATTGAGCTAT Reverse primer: AAACATCTTCGCCACAGTCC	175
Vegf	Forward primer: CTGCTCTCTTGGGTGCACTG Reverse primer: TTCACATCTGCTGTGCTGTAG	375
Otx2	Forward primer: GGAAGAGGTGGCACTGAAAA Reverse primer: ACTGGCCACTTGTTCCACTC	188
P75	Forward primer: GCTCAGGACTCGTGTTCTCC Reverse primer: TGGCTATGAGGTCTCGCTCT	285
Plzf	Forward primer: GTGCCCAGTTCTCAAAGGAG	131

	Reverse primer: CTCCATGTGCTGCTGGAGT	
Gfap	Forward primer: GGAGAGGGACAACTTTGCAC Reverse primer: TCCAGCGATTCAACCTTTCT	165
S100β	Forward primer: GGTGACAAGCACAAGCTGAA Reverse primer: GTCCAGCGTCTCCATCACTT	120
Mfn1	Forward primer: ATTGGGGAGGTGCTGTCTC Reverse primer: TCAGGAAGCAGTTGGTTGTG	142
Mfn2	Forward primer: TCCAAGGTCAGGGGTATCAG Reverse primer: CAATCCCAGATGGCAGAACT	133
Nestin	Forward primer: CTGCAGGCCACTGAAAAGTT Reverse primer: GTGTCTGCAAGCGAGAGTTC	187
Dkk3	Forward primer: GGAGGAAGCTACGCTCAATG Reverse primer: CGTGCTGGTCTCATTGTGAT	175
Col1A1	Forward primer: GCCAAGAAGACATCCCTGAA Reverse primer: TCTTCATTGCATTGCACGTC	142
Col3A1	Forward primer: GCACAGCAGTCCAACGTAGA Reverse primer: TCTCCAAATGGGATCTCTGG	185
Mfn1 sh RNA	CCGGTACGGAGCTCTGTACCTTTATCTCGAGATAAAGGTAC AGAGCTCCGTATTTTTG	
Mfn2 sh RNA	CCGGGGCAGTTTGAGGAGTGCATTTCTCGAGAAATGCACT CCTCAAACTGCCTTTTTTG	
SOD2 3'UTR sh RNA	CCGGCCCAAACCTATCGTGTCCATTCTCGAGAATGGACAC GATAGGTTTGGGTTTTTG	
SOD2 inducible sh RNA	CCGGGAGGCTATCAAGCGTGACTTTCTCGAGAAAGTCACG CTTGATAGCCTCTTTTTG	

Table S2: List of antibodies used for protein expression analysis related to figure 1, figure 2, figure 3, figure 4, figure 6, figure S1, figure S2, figure S4, figure S5, figure S6 and figure S7; chemicals used for cell culture and differentiation; plasmid constructs used for retroviral and lentiviral transductions.

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti β-ACTIN	Santa Cruz	#sc-47778
Rabbit anti NUCLEOLIN	Sigma aldrich	#N2662
Rabbit anti SOD2	EMD Millipore	# AB10346
Rabbit anti SOX1	Abcam	# ab109290
Rabbit anti FOXG1	Abcam	#ab18259
Rabbit anti MFN1	Cloudclone	PAC619Mu01
Rabbit anti MFN2	CST	mAB #9482
Mouse anti BrdU	DSHB	#G3G4
Mouse anti NESTIN	BD Pharmingen	#556309
Rabbit anti COX 6A	CusaBio	#CSB-PA637381XA01CXY
Rabbit anti FIS1	CusaBio	#CSB-PA008684LA01HU
Mouse anti PAX6	DSHB	#DSHB-S1-1281
Rabbit anti ZIC1	Abcam	#ab134951
Rabbit anti OLIG2	Abcam	#ab81093
Rabbit anti VIMENTIN	CusaBio	#CSB-MA000319
Mouse anti NF200 kDa	Abcam	#ab40796
Mouse anti SYNAPTOPHYSIN	Novus Biologicals	#NB300-653SS
Mouse anti S100β	BD Biosciences	#612376

Rabbit anti GFAP	BD Biosciences	#610565
Rabbit anti TUJ1	Abcam	#ab18207
Mouse anti KI67	BD Pharmingen	#550609
Rabbit anti-mouse FITC	SigmaAldrich	#AP160F
Goat anti Mouse AF594	ThermoFisher Scientific	#11005
Donkey anti-Rabbit AF594	ThermoFisher Scientific	#21207
Donkey anti-Rabbit AF488	ThermoFisher Scientific	#21206
Plasmid Constructs		
pMIG-SOD2-IRES-GFP WT	In-house	
pMIG-SOD2-IRES-GFP mutant	In-house	
pLKO-mSod2 shRNA	Sigma Aldrich	TRCN0000324404
pLKO-mMnSOD/Sod2 3'UTR shRNA	In-house	
Sod2 sh RNA tet inducible	In-house	
Sod2 tet inducible	In-house	
Mfn1 sh RNA	In-house	
Mfn2 sh RNA	In-house	
pQCXIP-C-Mito luciferase Zipper Venus (C-mito LZV)	Prof. Heidi McBride's lab	
pQCXIP-N-Mito Venus Zipper luciferase (N-mito VZL)	Prof. Heidi McBride's lab	
pLV mito dsRed	Addgene	#44386

Chemicals and Reagents	Source	Identifier
BMP4	ThermoFisher Scientific	PHC9533
Retinoic acid	Merck	R2625- 100MG
GDNF	R&D systems	212-GD-01M
BDNF	R&D systems	248-BDB
EGF	Peprotech	315-09
FGF-2	Peprotech	100-18B
Cytosine-B-D-arabinofuranoside hydrochloride	SigmaAldrich	C6645
Valproic acid	SigmaAldrich	P4543-10G
N-acetyl cysteine	Merck	A7250-25G
BSA Fraction V	SigmaAldrich	# 10735086001
LIF	Merck	ESG1106
DMEM F12	Gibco	# 11320-033
DMEM HighGucose	Gibco	# 11965-092
Neurobasal medium	Gibco	# 38210000
NEAA	Gibco	# 11140-050
L-GlutaMax	Gibco	# 35050-061
Anti anti	Gibco	# 15240062
Sodium Pyruvate	Gibco	# 11360070
β-Mercaptoethanol	Sigma	# M7522
N2 plus supplement	Invitrogen	# 17502048
B27 without Vitamin A	Invitrogen	# 12587-010
FBS	HiMedia	RM9951