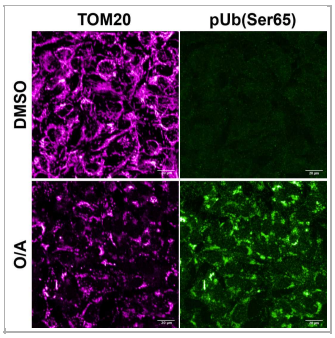

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Regulation of mitophagy by the NSL complex underlies genetic risk for Parkinson's disease: Cell-Based *in vitro* Assays

Marc P.M. Soutar[1], Daniela Melandri[1], Benjamin O'Callaghan[1], Emily Annuario[2], Amy E. Monaghan[3,4], Paul J. Whiting[3,4], Helene Plun-Favreau[5,6]

[1]Department of Neurodegenerative Diseases, UCL Queen Square Institute of Neurology, London, UK;
[2]Kings College, London, UK;
[3]UCL Alzheimer's Research UK, Drug Discovery Institute, London, UK;
[4]UCL Dementia Research Institute, London, UK;
[5]UCL Queen Square Institute of Neurology, London, UK;
[6]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20815.

Benjamin O'Callaghan

ABSTRACT

Impaired mitophagy is a key causative pathway in familial Parkinson's disease, but its relevance to idiopathic Parkinson's disease is unclear. We used a mitophagy screening assay to evaluate the functional significance of risk genes identified through genome-wide association studies and describe the cell-based *in vitro* assays used.

MATERIALS TEXT

Mitochondrial Toxins
Oligomycin (mitochondrial complex V inhibitor) was purchased from Cayman Chemicals (11341) and from Sigma-Aldrich (O4876)
Antimycin A (mitochondrial complex III inhibitor) were purchased from Sigma-Aldrich (A8674).

All siRNAs were purchased as pre-designed siGENOME SMARTpools from Dharmacon:
non-targeting (D-001206-13)
PINK1 (M-004030-02), PLK1 (L-003290-00)
KIF-11 (L-003317-00), KAT8 (M-014800-00)
KANSL1 (M-031748-00)
KANSL2 (M-020816-01)
KANSL3 (M-016928-01), HCFC1 (M-019953-01)
MCRS1 (M-018557-00)
OGT (M-019111-00)
PHF20 (M-015234-02)
WDR5 (M-013383-01).

The following antibodies were used for immunocytochemistry:
mouse anti TOM20 (Santa Cruz, sc-17764, 1:1000),
rabbit anti phospho-ubiquitin (Ser65) (Cell Signaling, 37642, 1:1000),
rabbit anti phospho-Parkin (Ser65) (Abcam/Michael J. Fox Foundation, MJF17, 1:250)
rabbit anti FLAG (Sigma-Aldrich, F7425, 1:500),
AlexaFluor 488 goat anti rabbit (Invitrogen, A11008, 1:2000),
AlexaFluor 568 goat anti mouse (Invitrogen, A11004, 1:2000).

The following antibodies were used for immunoblotting:

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Keywords: Parkinson's disease, Genetic Risk, Mitophagy, NSL Complex, GWAS, KANSL1, KAT8, mitophagy, MAPT locus, H1 Haplotype, HCS, ASAPCRN

mouse anti TIM23 (BD Biosciences, 611223, 1:1000),
rabbit anti TOM20 (Santa Cruz, sc-11415, 1:1000),
rabbit anti phospho-ubiquitin (Ser65) (Merck Millipore, ABS1513-I, 1:1000, and Cell Signaling, 37642, 1:1000),
mouse anti GAPDH (Abcam, ab110305, 1:10000),
rabbit anti KAT8 (Abcam, ab200600, 1:1000),
rabbit anti total-Tau (DAKO, 1:10000),
mouse anti V5 tag (Invitrogen, R960-25, 1:1000),
rabbit anti KANSL1 (Sigma-Aldrich, HPA006874, 1:500),
rabbit anti pParkin(Ser65) (Abcam/Michael J. Fox Foundation, MJF17, 1:1000),
mouse anti FLAG M2 (Sigma-Aldrich, F3165, 1:1000),
sheep anti pRab8A(Ser111) (MRC Protein Phosphorylation and Ubiquitylation Unit, University of Dundee, 1ug/ml preblocked with 10ug/ml non-phosphorylated peptide),
rabbit anti total Rab8 (Cell Signaling, 6975, 1:1000),
IRDye 680LT donkey anti mouse (LI-COR Biosciences, 925-68022, 1:20000),
IRDye 800CW donkey anti rabbit (LI-COR Biosciences, 925-32213, 1:20000),
IRDye 800CW Donkey anti-Goat (LI-COR Biosciences, 926-32214, 1:20000),
IRDye® 680RD Goat anti-Rabbit (LI-COR Biosciences, 926-68071, 1:20000).

The generation of rabbit monoclonal anti PINK1 antibody has been described in the following manuscript:
Sci Rep. 2015. DOI: https://doi.org/10.1038/s41598-018-26949-6

The generation and use of sheep anti pRab8A(Ser111) antibody has been described in the following manuscript:
EMBO J. 2015. DOI: https://doi.org/10.15252/embj.201591593

Cell Culture and siRNA Transfection

1

Culture cells in Dulbecco's Modified Eagle Medium (DMEM, Gibco, 11995-0 65) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Gibco) in a humidified chamber at 37 °C with 5% CO_2.

Note

Parkin over-expressing (POE) SHSY5Y cells are a kind gift from H. Ardley and have been described in the following manuscript.
Mol Biol Cell. 2003. DOI: https://doi.org/10.1091/mbc.e03-02-0078

Mt-Keima POE SHSY5Y cells were a kind gift of C. Luft and have been described in the following manuscript.
Autophagy. 2019. DOI: https://doi.org/10.1080/15548627.2019.1603549

PINK1-HA overexpressing SHSY5Y cells were a kind gift from E. Deas and have been described in the following manuscript.
Hum Mol Genet. 2011. DOI: https://doi.org/10.1093/hmg/ddq526

2 For siRNA transfection, transfect cells using DharmaFECT1 transfection reagent (Dharmacon, T-2001-03) according to the manufacturer's instructions.

High Content siRNA Screen

3 **Cell Plating and siRNA Transfection**
Dispense siRNA into Geltrex-coated 96-well CellCarrier Ultra plates (Perkin Elmer) at a final concentration of [M] 30 nanomolar (nM) using the Echo 555 acoustic liquid handler (Labcyte).

4 30m
Add 25 µL of DMEM containing 0.48% of DharmaFECT1 transfection reagent to each well and incubate for 00:30:00.

5 Seed POE SHSY5Y cells using the CyBio SELMA (Analytik Jena) at 15,000 cells per well, 100 µl per well in DMEM + [M] 10 % (v/v) FBS.

6 3d
Incubate cells for 72:00:00 in a humidified chamber at 37 °C with 5% CO_2.

7 3h
Treat cells with [M] 10 micromolar (µM) oligomycin/ [M] 10 micromolar (µM) antimycin for 03:00:00 to induce mitophagy.

8 15m
Immunoflourescence and Image Capture Analysis
Fix cells with [M] 4 % (w/v) PFA (Sigma-Aldrich,F8775), 00:15:00, Room temperature.

9 Remove PFA solution and wash cells 3x with PBS.

10 Block and permeabilise cells with [M] 10 % (v/v) FBS, [M] 0.25 Mass / % volume Triton X-100 in PBS, 1 h, RT.

2h

11 Immunostain cells with rabbit anti pUb(Ser65) (Cell Signaling, 37642, 1:1000) and mouse anti TOM20 (Santa Cruz, sc-17764, 1:1000) primary antibodies in [M] 10 % (v/v) FBS/PBS, 02:00:00, Room temperature.

1h

12 After 3x PBS washes, add AlexaFluor 568 anti-mouse and 488 anti-rabbit secondary antibodies and Hoechst 33342 (Thermo Scientific, 62249) in [M] 10 % (v/v) FBS/PBS, (1:2000 dilution for all) and incubate for 01:00:00, Room temperature.

13 Remove secondary antibodies and wash cells 3x with PBS.

14 Image plates using the Opera Phenix (Perkin Elmer).
Acquire 5x fields of view and 4x 1 µm Z-planes per well, using the 40X water objective, NA1.1.

15 Analyse images in an automated way using the Columbus 2.8 analysis system (Perkin Elmer) to measure the integrated intensity of pUb(Ser65) within the whole cell.

16 First of all, load the image as a maximum projection, before segmenting to identify the nuclei using the Hoechst 33342 channel (method B).

17 To identify the cytoplasm, use the “Find Cytoplasm” building block (method B) on the sum of the Hoechst and Alexa 568 channels.

18 Identify pUb(Ser65) as spots (method B) on the Alexa 488 channel, before measuring their integrated intensity.

19 **Screen Quality Control, Data Processing and Candidate Selection**
Quality control screen plates based on the efficacy of the PINK1 siRNA control and O/A treatment window (minimum 3-fold).
Check data for edge effects using Dotmatics Vortex visualization software.

20 Quality control raw data using robust Z prime > 0.5.

Process data using Python for Z score calculation before visualization in Dotmatics Vortex.
Candidates should be considered a hit where Z score is ≥ 2 or ≤ -2, and where replication of efficacy is seen both within and across plates.

21 **siRNA Libraries**

Resuspend siRNAs in RNase-free water for a final concentration of [M] 20 micromolar (µM) .

Note

siRNA libraries were purchased from Dharmacon as an ON-TARGETplus SMARTpool Cherry-pick siRNA library, 0.25 nmol in a 384-well plate.

22 Add SCR, PINK1 and PLK1 or KIF11 controls to the 384-well plate at a concentration of [M] 20 micromolar (µM) before dispensing into barcoded assay-ready plates.

KANSL1 iNeuron Culture and Differentiation

23

Culture human induced pluripotent stem cells (hIPSCs) on Geltrex (Thermofisher) coated culture dishes in mTeSR1 (StemCell Technologies) and maintained in a humidified 37 °C incubator, 5% CO2.

Note

Isogenic hIPSC lines with/without a heterozygous loss of function (LoF) frameshift mutation in Exon2 of the KANSL1 gene (c.531insT), which have also being stable transduced with transgenes permitting doxycycline-inducible overexpression of murine Ngn2 were a kind gift from the lab of N. Nadif Kasri and have been described in the following manuscript
Autophagy. 2022. DOI:https://doi.org/10.1080/15548627.2021.1936777

24 Differentiate isogenic KANSL1$^{+/+}$ and KANSL1$^{+/-}$ hiPSCs into excitatory cortical neurons by doxycycline induced overexpression of murine Ngn2.

Note

iNeuron differentiation protocol adapted from that described in the following manuscripts.
Autophagy. 2022. DOI: https://doi.org/10.1080/15548627.2021.1936777
J Vis Exp. 2017. DOI: https://doi.org/10.3791/54900

25 On d0 dissociate hiPSCs into a single cell suspension using accutase (Sigma).

26 Plate cells onto geltrex coated dishes in induction medium consisting of DMEM/F12 supplemented with 1x Glutamax, 1x non-essential amino acids (NEAAs), 1x N2-supplement (all Thermofisher) and [M] 4 µg/ml doxycycline (Sigma).
Supplement induction media with [M] 10 micromolar (µM) Y-27632 Rho Kinase inhibitor (ROCKi, Peprotech) during initial seeding.
Seed 7.5x10^5 cells per 6-well.

2d

27 24:00:00 later (d1) and 48:00:00 later (d2) perform a full medium change with freshly prepared induction media without Y-27632 ROCKi.

1d

28 On d3 perform a full medium change with freshly prepared N2-B27 media consisting of a 1:1 mixture of DMEM/F12:Neurobasal supplemented with 0.5x N2-supplement, 0.5x B27 supplement, 0.5x NEAAs, 0.5x Glutamax, [M] 45 micromolar (µM) 2-Mercaptoethanol (all Thermofisher), [M] 2.7 µg/ml insulin (Sigma).
Supplement N2-B27 media with [M] 2 micromolar (µM) Cytosine β-D-arabinofuranoside (ARA-C) (Sigma) on d3.

1w 5d

29 Every 3-4 days thereafter, perform a half media change with N2-B27 lacking ARA-C.

1d

30 Perform a half media change with N2-B27 on d16 and collect cells for experimental assays 24 h later on d17.

CRISPRi-i3N iNeuron Culture and Differentiation

31 hiPSCs are cultured as outlined above for KANSL1 hiPSCs in Step 23 go to step #23

Note

A hiPSC line stably transduced with transgenes permitting doxycycline-inducible overexpression of murine Ngn2 at the AAVS1 safe-harbor locus, and stably transduced with constitutively expressed enzymatically dead Cas9 (dCas9)-KRAB transcriptional repressor fusion protein at the CLYBL promoter safe-harbor locus (CRISPRi-i3N hiPSCs) was a kind gift from the labs of M. E. Ward and M. Kampmann, and have been described in the following manuscripts.
Curr Protoc cell Biol. 2018. DOI: https://doi.org/10.1002/cpcb.51
Neuron. 2019. DOI: https://doi.org/10.1016/j.neuron.2019.07.014

32 First transduce CRISPRi-i3N hiPSCs with lentiviral particles encoding a mCherry-reporter and sgRNA sequences targeting the promoter regions of *KANSL1*, *KAT8* or *PINK1,* or a non-targeting sgRNA control.
Then differentiated transduced cells into excitatory cortical neurons by doxycycline induced overexpression of murine Ngn2.

> Note
>
> CRISPRi-i3N-iNeuron differentiation protocol adapted from the following manuscripts.
> *Curr Protoc cell Biol*. 2018. DOI: https://doi.org/10.1002/cpcb.51
> *Neuron*. 2019. DOI: https://doi.org/10.1016/j.neuron.2019.07.014

> Note
>
> See Steps 39-41 for lentiviral particle generation and encoded sgRNA sequences.

33 1d
On d-1 dissociate CRISPRi-i3N hiPSCs into a single cell suspension using accutase and reverse transduce with sgRNA lentiviral supernatant in mTeSR1 supplemented with 5ug/ml polybrene and [M] 10 micromolar (µM) Y-27632 ROCKi.
Seed 4.5x10^5 cells onto Geltrex-coated 6-well plates.

34 1d
24:00:00 later (d0) change media to induction media (composition outlined above in Step 26 go to step #26 for KANSL1 iNeurons).

35 3d
24:00:00 later (d1) and 48:00:00 later (d2) perform a full medium change with freshly prepared induction media.

36 1d
On d3, dissociate differentiating iNeurons into a single cell suspension using accutase (Sigma) and seed in N2-B27 media (composition outlined above in Step 28 go to step #28 for KANSL1 iNeurons) into Geltrex-coated 96-well CellCarrier Ultra plates for immunofluorescence (IF) (3x10^4 cells per well) and 12-well plates (5x10^5 cells per well) for biochemistry purposes.

37 1w 5d
Perform a half media change with N2-B27 the following day (d4) and every 3-4 days thereafter.

1d

38 Perform a half media change with N2-B27 on d16 and collect cells for experimental assays 24 h later on d17.

Lentiviral Particle Generation

1d

39

Transfect 70-90% confluent Lenti-X human embryonic kidney 293T cells (HEK293T) cultured in DMEM 10% FBS media with pMD2.G and pCMVR8.74 alongside appropriate delivery plasmids: pLV[Exp]-U6>sgRNA-hPGK>mApple (Vectorbuilder) plasmids (for sgRNA), empty pLVX-EF1α-IRES-Puro (Clontech, Takara Bio), V5-KANSL1 pLVX-EF1α-IRES-Puro, or V5-KAT8 pLV[Exp]-EF1α-IRES-Puro at a 1:1:2 molar mass ratio using Lipofectamine 3000 (Invitrogen).

Note

pMD2.G (Addgene plasmid #12259) and pCMVR8.74 (Addgene plasmid # 12259) were gifts from Didier Trono. KANSL1 cDNA (ENST00000432791.7) with N-terminal V5 tag was cloned into the pLVX-EF1α-IRES-Puro plasmid using SpeI and NotI restriction sites. See Table below for sgRNA plasmids and V5-KAT8 pLV[Exp]-EF1α-IRES-Puro plasmids (Vectorbuilder)

A	B	C	D
Gene Target	**sgRNA Sequence (5'-3')**	**Plasmid**	**Plasmid Map**
Non-Targeting	gctgcatggggcgcgaatca	pLV[Exp]-U6>NT-Seq1-hPGK>mApple	https://en.vectorbuilder.com/vector/VB210129-1165psy.html
KANSL1	gccgccgcggcgagacgagt	pLV[Exp]-U6>KANSL1_P1_Seq1-hPGK>mApple	https://en.vectorbuilder.com/vector/VB210126-1227jjz.html
KAT8	ggttgcggcggggacttcag	pLV[Exp]-U6>KAT8_P1_Seq1-hPGK>mApple	https://en.vectorbuilder.com/vector/VB210129-1139enp.html
PINK1	ggcctgtcgcaccgccatgg	pLV[Exp]-U6>PINK1_P1_Seq1-hPGK>mApple	https://en.vectorbuilder.com/vector/VB210129-1160qka.html
N/A	N/A	pLV[Exp]-EF1A>V5/hKAT8[NM_032188.3]:IRES:Puro	https://en.vectorbuilder.com/vector/VB220307-1149nfg.html

sgRNA sequences and encoding plasmids, and V5-KAT8 encoding plasmid.

1d

40 The next day, perform a full media change with mTeSR1 (for sgRNA lentivirus) or DMEM [M] 10 % (v/v) FBS media and culture cells for 24:00:00.

41 Collect the lentivirus containing mTeSR1 / DMEM [M] 10 % (v/v) FBS and dilute 1:2 with fresh mTeSR1 or [M] 10 % (v/v) FBS before filtering through 0.44 µm PES filters.

Mitochondrial Enrichment and Western Blotting

3d

42 

Transfect POE SHSY5Y and H4 cells with [M] 100 nanomolar (nM) siRNA and incubate for 72:00:00.

43 Culture KANSL1 iNeurons as detailed above in Steps 23-30 go to step #23.

44 Use whole cell lysates from POE SHSY5Y cells, H4 cells, and KANSL1 iNeurons.
For some experiments, POE SHSY5Y lysates you can first fractionate into cytoplasmic and mitochondria-enriched preparations to facilitate detection of mitochondrial localised proteins of interest.

Note

Mitochondrial enrichment and Western blotting protocols have been described previously in the following manuscript.
Sci Rep. 2018. DOI: https://doi.org/10.1038/s41598-018-26949-6

KANSL1 and KAT8 siRNA KD Rescue

45 

Transfect POE SHSY5Ys with 25 nM siRNA (d0) and incubate for 48 h.

46 Transduce siRNA KD cells with lentivirus in the presence of 10 µg/ml polybrene (d2).

47 Perform a full media change the following day (d3) and collect 4 days post siRNA transfection (and 2 days post lentivirus transduction) (d4).

pRab8A(Ser111) Measurements

48

Transfect SHSY5Y cells stably overexpressing PINK1-HA with 100 nM siRNA and incubate for 72 h.

49 Immunoprecipitate 200ug of protein (whole cell lysate) with Protein A Dynabeads™ (Invitrogen) prebound with 0.5ug of rabbit anti total-Rab8 antibody (Cell Signaling, 6975) at 4 °C overnight.

50 Elute samples from the beads by heating at 95 °C in 2x LDS supplemented with 50mM DTT for 5min.

Immunoflourescence

51

Reverse transfect SHSY5Y cells with 50 nM siRNA in 96-well CellCarrier Ultra plates according to the manufacturer's instructions and incubate for 72 h.

52 Culture CRISPRi-i3N iNeurons as described above in Steps 31-38 go to step #31 .

53 Treat, fix and stain cells as per the screening protocol detailed above in Steps 8-13 go to step #8 .

54 For visualisation purposes, select brightness and contrast settings on the SCR (siRNA KD SHSY5Y) or no transduction (No TD) (CRISPRi-i3N iNeurons) controls and apply the same settings to all other conditions. Present representative images as maximum projections of the channels for one field of view.

55

Extract total RNA from cells using the Monarch Total RNA Miniprep Kit (New England Bioscience) with inclusion of the optional on-column DNAse treatment.

56 Reverse transcribe RNA with SuperScript™ IV reverse transcriptase and random hexamers (Invitrogen).

57 Dilute the cDNA product then subject to quantitative real-time PCR (qPCR) using Fast SYBR™ Green Master Mix (Applied Biosystems) and gene specific primer pairs (See Table Below) on a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems).

List of primer pairs used for RT-qPCR of target genes

58 Calculate relative mRNA expression levels using the $2^{-\Delta\Delta Ct}$ method and *RPL18A* (SHSY5Y) or *UBC* (iNeurons) as the house-keeping gene.

Mitophagy Measurement Using mt-Keima Reporter

3d

59

Reverse transfect stable mt-Keima expressing POE SHSY5Y cells with [M] 50 nanomolar (nM) siRNA in 96-well CellCarrier Ultra plates according to the manufacturer's instructions and incubate for 72:00:00.

60 For the assay, replace the cell medium with phenol-free DMEM + 10% FBS containing Hoechst 33342 (1:10000) and either DMSO or [M] 1 micromolar (µM) oligomycin/ [M] 1 micromolar (µM) antimycin to induce mitophagy.

61 Image cells immediately on the Opera Phenix (PerkinElmer) at 37 °C with 5% CO_2, acquiring 15x single plane fields of view, using the 63X water objective, NA1.15.

Note

The following excitation wavelengths and emission filters were used: cytoplasmic Keima: 488 nm, 650–760 nm; lysosomal Keima: 561 nm, 570–630 nm; Hoechst 33342: 375 nm, 435–480 nm.

62 Images were analysed in an automated way using the Columbus 2.8 analysis system (Perkin Elmer) to measure the mitophagy index.

63 Identify cells using the nuclear signal of the Hoechst 33342 channel, before segmenting and measuring the area of the cytoplasmic and lysosomal mt-Keima.

64 Calculate the mitophagy index as the ratio between the total area of lysosomal mitochondria and the total area of mt-Keima (sum of the cytoplasmic and lysosomal mtKeima areas) per well.

Mitochondrial Membrane Potential Measurements using TMRM

3d

65 Transfect POE SHSY5Ys with [M] 50 nanomolar (nM) siRNA in 96-well CellCarrier Ultra plates using Dharmfect1 and incubate for 72:00:00.

66 Perform Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM) measurements in redistribution mode where a decrease in TMRM signal intensity is associated with mitochondrial membrane potential (ψ_m) depolarisation (confirmed post-imaging by treatment with [M] 1 micromolar (µM) Carbonyl cyanide m-chlorophenyl hydrazone, CCCP, ψ_m depolarising agent).

40m

67 Incubate live cells in [M] 25 nanomolar (nM) TMRM (Sigma Aldrich) diluted in Hanks' Balanced Salt Solution (HBSS, Gibco) buffered with [M] 10 millimolar (mM) HEPES (Sigma Aldrich), pH 7.4 for 00:40:00 at 37 °C.

68 Image cells on the Opera Phenix (PerkinElmer) at 37 °C, acquiring confocal z-stacks for 27 fields of view across 3 individual wells per experimental condition using the 40X water objective, NA1.1.

> **Note**
>
> Excite TMRM with the 561 nm laser (50% power) and collect signal with 570-630nm emission filter.

69 Analyse images in an automated way using the Columbus 2.8 analysis system (Perkin Elmer) to measure the TMRM signal intensity of maximum intensity projections.

Statistical Analysis

70 Normalise intensity measurements from imaging experiments for each experiment.
N numbers shown in figure legends should refer to the number of independent, replicate experiments.
Within each experiment, calculate the mean values of every condition from a minimum of 3 technical replicates.

71 Normalise integrated density measurements from Western blot experiments to control conditions.
Wherever possible, avoid normalisation to conditions for statistical comparisons in order to maintain experimental error associated.

72 Subject Data to either one-way or two-way ANOVA with Dunnett's post-hoc analysis for multiple comparisons, or other appropriate statistical analysis.
Present data with error bars showing mean ± standard deviation (SD) from replicate experiments.

Note

We use GraphPad Prism 9 (La Jolla, California, USA) for statistical analyses and graph production.