

Title: Doublecortin like kinase 1 regulates α -Synuclein levels and toxicity

Abbreviated title: DCLK1 regulates α -Syn levels

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Number of Pages: 45

Number of Figures: (11 main, 0 extended data)

Number of Tables: (0 main, 0 extended data)

Number of words for Abstract: 175

Number of words for Introduction: 645

Number of words for Discussion: 1261

Conflict of Interest: None declared

Acknowledgements:

The authors thank Vitaliy Bondar, Won-Seok Lee, James Orengo, Joseph McInnes and all members of the Zoghbi lab for important discussions and critical feedback on the manuscript. We also thank Marie-Françoise Chesselet (UCLA) for the gift of the Thy1- α -Syn-“Line 61” mice, as well as the NINDS human and cell data repository for patient iPSCs. We also used the WA09 human ESC line (H9 ESC) derived by Dr. James Thomson and distributed by WiCell Research Institute under SLA agreement.

This study was funded in part by grants from the Huffington Foundation (H.Y.Z.), and the Howard Hughes Medical Institute (H.Y.Z.) H.Y.Z. also received support for this study from UCB Pharma and The Hamill Foundation. M.W.C.R. received funding from the Parkinson's Foundation Stanley Fahn Junior Faculty Award (Grant No. PF-JFA-1762). S.M.H.-A. and M.J.A.W. were funded by Cure Parkinson's Trust and John Fell OUP Research Fund. We thank the Gordon and Mary Cain Pediatric Neurology

Research Foundation Laboratories for allowing us access to their stereological microscope. The IDDRC Microscopy Core (NIH U54 HD083092 from the Eunice Kennedy Shriver National Institute of Child Health and Human Development), The RNA In Situ Hybridization core at BCM (with expert assistance of Cecilia Ljungberg and funding from: NIH S100D016167 and NIH U54 HD083092), and Baylor College of Medicine Gene Vector Core were used for this project. The content is solely the responsibility of the authors.

Abstract

α -Synuclein (α -Syn) accumulation is a pathological hallmark of Parkinson's disease. Duplications and triplications of *SNCA*, the gene coding for α -Syn, cause genetic forms of the disease, which suggests that increased α -Syn dosage can drive PD. To identify the proteins that regulate α -Syn we previously performed a screen of potentially druggable genes that led to the identification of 60 modifiers. Among them, Doublecortin like kinase 1 (DCLK1), a microtubule binding serine threonine kinase, emerged as a promising target due to its potent effect on α -Syn and potential druggability as a neuron-expressed kinase. In this study, we explore the relationship between DCLK1 and α -Syn in human cellular and mouse models of PD. First, we show that DCLK1 regulates α -Syn levels post-transcriptionally. Second, we demonstrate that knockdown of *Dclk1* reduces phosphorylated species of α -Syn and α -Syn-induced neurotoxicity in the substantia nigra in two distinct mouse models of synucleinopathy. Lastly, silencing *DCLK1* in human neurons derived from individuals with *SNCA* triplications reduces phosphorylated and total α -Syn, thereby highlighting DCLK1 as a potential therapeutic target to reduce pathological α -Syn in disease.

Significance Statement

DCLK1 regulates α -Syn protein levels and *Dclk1* knockdown rescues α -Syn toxicity in mice. This study provides evidence for a novel function for DCLK1 in the mature brain, and for its potential as a new therapeutic target for synucleinopathies.

Introduction

Parkinson's disease (PD) is a common neurodegenerative condition affecting approximately 6.1 million people worldwide (Dorsey et al., 2018). PD is characterized by the death of the dopaminergic neurons of the Substantia Nigra *pars compacta* (SNc) and the accumulation of the presynaptic protein α -Synuclein (α -Syn) (Spillantini et al., 1998; Burré et al. 2016). Although the pathogenic mechanism(s) of PD remain(s) elusive, there is evidence that suggests that increased α -Syn levels can drive PD. First, humans with duplications and triplications of the *SNCA* locus (encoding α -Syn) develop autosomal dominant PD (Singleton et al., 2003; Chartier-Harlin et al., 2004; Ibáñez et al., 2004). Importantly, α -Syn levels correlate with the severity of symptoms (Devine et al., 2011). Second, some single nucleotide polymorphisms in regulatory regions of *SNCA* increase PD risk and *SNCA* transcript levels (Soldner et al., 2016). Third, the effect of drugs on *SNCA* transcript load directly correlates with PD risk at the epidemiological level (Mittal et al., 2017). Furthermore, haploinsufficiency in *GBA1* increases PD risk due to defects in the lysosomal clearance of α -Syn (Mazzulli et al., 2011). Despite this, only a few regulators of α -Syn levels have been identified previously. These include transcriptional regulators (ZSCAN21, ZSCAN219 GATA-1 and GATA-2) (Clough et al., 2009; Scherzer et al., 2008; Dermetzaki et al., 2016; Lassot et al., 2018) as well as a handful of post-translational regulators such as Polo-like kinase 2 (Oueslati et al., 2013), NEDD4 (Tofaris et al., 2011), USP9X (Rott et al., 2011), and TRIM28 (Rousseaux et al., 2016). How well most of these modulators would serve as therapeutic targets remains an open question. NEDD4 and USP9X have not been tested in vivo, and the beneficial effect of PLK2 requires increasing its kinase activity, which is pharmacologically challenging. To address the scarcity in modulators of α -Syn levels, we performed an shRNA screen on 7,787 potentially druggable genes (Rousseaux and Vázquez Vélez, et al., 2018), and identified 60 new regulators of α -Syn levels. We selected ten for further verification in human neurons and in the mouse brain. Doublecortin like kinase 1 (DCLK1) emerged as the most promising regulator because it is a neuron expressed kinase and it was also identified in a separate orthogonal siRNA screen targeting the kinome (Rousseaux et al., 2016).

DCLK1 was originally discovered based on its homology to Doublecortin (DCX) (Burgess et al., 1999; Silverman et al., 1999), a gene essential for cortical development and whose mutations lead to Subcortical band heterotopia (Des Portes et al., 1998; Gleeson et al., 1999; Horesh et al., 1999). Further studies since then have revealed that the DCLK1 protein is localized to the somato-dendritic compartment (Shin et al., 2013) and is composed of a microtubule binding segment (DCX domains) and a serine threonine kinase domain separated by a PEST rich sequence (Patel et al., 2016). The DCX domains allow DCLK1 to bind and polymerize microtubules (Lin et al., 2000), and to guide kinesin 3 coated vesicles to the dendritic compartment (Lipka et al., 2016). The function of the kinase domain is less well characterized. MAP7D1, a microtubule binding protein, DCX and DCLK1 itself are the only known phosphorylation targets of DCLK1 (Koizumi et al., 2017). Additionally, little is known about the function of DCLK1 in the adult brain.

We decided to pursue the mechanism by which DCLK1 regulates α -Syn levels and determine the effect of *Dclk1* knockdown in mouse models of synucleinopathy. We found that DCLK1 regulates α -Syn protein levels through its kinase domain independently of its catalytic activity, and that *Dclk1* knockdown reduces phosphorylated species of α -Syn (pS129) (Fujiwara et al., 2002) in the Thy1- α -Syn-“Line 61” (Rockenstein et al., 2002) model of synucleinopathy and α -Syn induced dopaminergic neuron toxicity in an AAV mediated *SNCA* overexpression mouse model. Moreover, *DCLK1* knockdown reduces α -Syn levels in *SNCA* triplication patient neurons. Thus, we reveal an important function of DCLK1 in the adult brain, and highlight its important role regulating α -Synuclein levels.

Materials and Methods

Protein extraction, SDS-PAGE and western blotting for measuring protein levels

Protein extraction

To extract protein from AAV injected brains, we dissected and flash froze (in liquid nitrogen) the posterior cortex and hippocampus from mice anesthetized at three weeks old (wild type mice) or two

months old (transgenic mice). Frozen tissue was thawed on ice and ground using a motor-powered pestle in 1X PEPI buffer (5 mM EDTA, PBS, 10 ml/g) supplemented with protease and phosphatase inhibitors (1X GenDEPOT, P3100–100, P3200–020). 125 μ L of the homogenate was saved for RNA extraction (see RNA extraction and qPCR section), the rest was mixed 1:1 with 2X RIPA (100 mM Tris, pH 7.5, 300 mM NaCl, 0.2% SDS, 1% sodium deoxycholate; 2% NP-40, 10mM EDTA, pH 8.0) buffer-containing protease and phosphatase inhibitors (1X). The lysates were then vortexed and incubated on ice for 20 minutes, before being spun down at 13,000 rpm for 20 minutes.

SDS-PAGE and western blotting

Protein samples were loaded on either 10- or 15-well Nupage 4-12% Bis-Tris gels (Invitrogen, NP0335BOX, NP0336BOX), or 17 well BOLT 4-12% Bis-Tris gels (NW04127BOX). Gels were run in MES buffer (50mM MES, 50mM Tris base, 0.1% SDS, 1mM EDTA, pH7.3) and proteins were then transferred onto Amersham™ Protran™ Premium NC Nitrocellulose membranes (0.2 μ m pore, GE, 45004004) in Tris-Glycine buffer (25mM Tris, 190mM Glycine) supplemented with 10% methanol at 0.34 amps for 1 hour.

After being transferred, membranes were blocked in 5% milk in TBS-T for 1 hour, and probed with one of the following primary antibodies in 5% milk overnight: anti α -Syn (C20, Santa Cruz Biotechnology, sc-7011-R, RRID:AB_2192953) 1/500, anti-human α -Syn (MJFR1, Abcam, ab13850, RRID:AB_25372171) 1/1000, anti- α Syn (Clone 42, BD Biosciences ,610787, RRID:AB_398108) 1/2000, anti-pS129 α -Syn (Abcam, ab51253, RRID:AB_869973) 1/500, anti-Delk1 (Abcam, ab31704, RRID:AB_873537) 1/500, anti- β III Tubulin (Sigma-Aldrich, T8578, RRID:AB_1841228) 1/10000, anti-Vinculin (Sigma-Aldrich, V9131, RRID:AB_477629) 1/10000, anti-Flag (M2, Sigma-Aldrich, F1804, RRID:AB_262044) 1/1000 , anti-APP (22C11, Millipore, MAB348, RRID:AB_94882) 1/2000, and anti-Tau (Dako, A0024, RRID:AB_10013724) 1/5000. The next day, membranes were probed with secondary antibodies for 1 hour. For chemiluminescent western

blotting donkey anti-mouse HRP conjugated antibody (Jackson Immnunoresearch, 715-035-150,RRID:AB_2340770) or goat anti-rabbit HRP conjugated antibody (BIO-RAD, 170-5046, RRID:AB_11125757) were used 1/10000 in 5% milk. For LiCor fluorescent western blotting IRDye® 680RD Goat anti-Rabbit IgG (LICOR, 926-6807, RRID:AB_10956166) or IRDye 800CW Goat anti-Mouse IgG (LICOR, 926-32210,RRID:AB_621842) were diluted 1/10,000 in LI-COR Odyssey TBS blocking buffer (927–50003). The membranes were imaged on an Amersham Imager 600 (GE,29083461) using Amersham ECL Prime Reagent (GE,45010090) for chemiluminescence or on a LICOR Odyssey imager for fluorescent blots. All western blot data was quantified using ImageStudioLite (LICOR).

RNA extraction and cDNA production

We used PEPI buffer to homogenize the tissue (see protein extraction section). RNA from mouse brain was then extracted using TRIzol (Invitrogen, Cat# 15596-026) and total RNA were purified using the miRNeasy micro kit (Qiagen, 217004) according to the manufacturer's instructions. cDNA was generated using the QuantiTect Reverse Transcription Kit (Qiagen, 205313) according to the manufacturer instructions.

qPCR

qPCR was performed with PowerUp SYBR Master Mix (Fisher Scientific, A25777) on a Bio-Rad CFX96 instrument. *Dcl1* primers specific for exon junctions of either the full length, kinase domain or total transcripts (see below). 10 ng of cDNA was used in technical triplicate for each primer pair. The average dCt was calculated from triplicate values and the relative abundance of the measured transcripts was determined using the ddCT method (Pfaffl, 2002). For all experiments in mice *Ppia* (see below) was used to normalize ddCT values (Kim et al.,2014). For all experiments in human neurons *TUBB3* (a well-known marker of neurons) (Latremoliere, et al., 2018) was used to normalize ddCT values.

Mouse Primers:

174 *Dclk1* All transcripts FW: 5'-CTGGGTTAATGATGATGGTCTCC-3'

175 *Dclk1* All transcripts RV: 5'-ACAGAAACTCCTGCTGCAGT-3'

176 *Dclk1* Full length FW: 5'-TCCTTCGAGCAGGTTCTCAC-3'

177 *Dclk1* Full length RV: 5'-GAAGGCACATCACCTGCTTC-3'

178 *Dclk1* Kinase domain FW: 5'-GAAGTTAATGGAACCCCTGGTAG-3'

179 *Dclk1* Kinase domain RV: 5'-GAGAGATCCTCTGCTTCCGC-3'

180 *Snca* FW: 5'-GTGACAACAGTGGCTGAGAAGAC-3'

181 *Snca* RV: 5'-GGTACCCCTCCTCACCCCTTG-3'

182 *Ppia* FW: 5'-GCATACAGGTCCTGGCATCT-3'

183 *Ppia* RV: 5'-CCATCCAGCCATTCAGTCTT-3'

184

185 Human Primers:

186 *SNCA* FW: 5'-TGTTCTCTATGTAGGCTCCA-3'

187 *SNCA* RV: 5'-ACTTGCTCTTTGGTCTTCTC-3'

188 *TUBB3* FW: 5'-ATCAGTGATGAGCATGGCA-3'

189 *TUBB3* RV: 5'-ACTTGTGAGAAGAGGCCTC-3'

190

191 **Immunoprecipitation**

192 *In vivo* IPs

193 For in vivo IPs, mice of both sexes were anesthetized with isoflurane. After anesthesia, cervical

194 dislocation was performed and the brain was dissected out of the skull. The brain was then cut in half and

195 flash frozen in liquid nitrogen. Each brain half was lysed in NETT (NaCl 170 mM, EDTA 1 mM, Tris 50

196 mM pH 7.4, Triton X-100 0.5%) buffer with protease and phosphatase inhibitors using dounce

197 homogenizers. The lysates were then incubated on ice for 20 minutes and spun down at 13,000 rpm for 20

198 minutes at 4°C. 50 µL of cleared lysate was kept for input. 400 µL of each lysate was conjugated with

either: Normal Rabbit IgG (Millipore, 12-370, RRID:AB_145841) or DCLK1 antibody (Abcam, ab31704, RRID:AB_873537) for 2 hours. Meanwhile, 30 μ L of Protein A Dynabeads (Invitrogen, 100-02D) were washed 3 times in 500 μ L of NETT buffer. Each lysate/antibody complex was then added to the beads and incubated for 30 minutes at 4°C. The beads were then washed 5 times with 1 ml of NETT buffer and the samples were resuspended and eluted in the same NETN:Laemmli buffer mixture as described above. The samples were loaded onto an SDS-PAGE gel as described above.

IPs in cells

For experiments in cells, HEK293T cells (ATCC®, CRL-3216™) were seeded on 6 well dishes at 500,000 cells/well (Corning, 08-772-1B). The next day, the cells were transfected with 250 ng of either empty vector or DCLK1-3XFlag using MIRUS TransIT-293 reagent (MIR 2706) according to manufacturer's instructions. 48 hours post transfection, 10 μ L of Protein A Dynabeads (Invitrogen, 100-02D) per sample were washed 3 times in NETT buffer supplemented with 1X protease and phosphatase inhibitor cocktails (Genedepot, P3100-100, P3200-020). They were then incubated with 1 μ g of either anti- α -Syn rabbit polyclonal (C-20, Santa Cruz Biotechnology, sc-7011-R, RRID:AB_2192953) or normal rabbit IgG (Millipore, 12-370, RRID:AB_145841) for 2 h at 4°C. The beads were then washed 3 times. During the antibody-bead conjugation, cells were resuspended in PBS and spun down at 5,000 rpm for 5 minutes. The cell pellet was gently resuspended in NETT buffer supplemented with protease and phosphatase inhibitors and incubated on ice for 20 minutes. Meanwhile, the beads were washed 3 times in NETT buffer. The protein lysates were then spun down at 13,000 rpm for 20 minutes at 4°C. 50 μ L (5%) of cell lysate were kept for input and each cell lysate was then added to 10 μ L of conjugated beads. The samples were then incubated for 30 minutes at 4°C. The beads were washed 5 times in 1 mL of NETT buffer and the washed samples were eluted in a 1:1 mix of NETT buffer, and 2X Laemmli sample buffer at 85 °C for 10 minutes, spun down for 30 seconds at 13,000 rpm, and loaded onto an SDS-PAGE gel as described above.

In vitro kinase assay

This assay was performed in a similar way to what was described in (Bondar et al., 2018). Full length human *SNCA* DNA was cloned into a pDEST15 vector (GST-tagged, Invitrogen, 11802014), and transformed into BL21AI (Invitrogen, C607003). GST- α -Syn were purified through a GST column. Briefly, 5 μ g of recombinant α -Syn was incubated with 250 ng of active DCLK1 (SignalChem, D14-10G-10) for 1 hour at 30°C in kinase buffer (50 mM PO₄ pH 7.4, 150 mM NaCl, 20 mM MgCl₂, 0.1 mg/ml BSA, 1 mM dithiothreitol) with phosphatase inhibitor (Roche, 04906837001), 20 mM cold adenosine triphosphate (ATP) (Invitrogen, PV3227) and 1.2 ml of 0.01 mCi/ml ³²P ATP (PerkinElmer, BLU502A250UC). The reactions were terminated by adding sample buffer and boiling the sample at 95°C for 10 minutes. Samples were then run an SDS-PAGE gel as described in the SDS-PAGE section of these methods. The gel was then Coomassie stained (InstantBlue, VWR, 95045-070) for 20 minutes, and exposed to an X-Ray film (GE, 28906845) for 1 hour.

Immunofluorescence and immunohistochemistry

Tissue preparation

For immunohistochemical experiments, mice were transcardially perfused with PBS followed by 4 % Paraformaldehyde (PFA). Brains were dissected and fixed in 4 % PFA for 2 days, dehydrated for 24 hours in 15% Sucrose (w/v, in PBS) followed by a 2-day incubation in 30% Sucrose solution (in PBS), all at 4°C. The brains were then frozen on dry ice in OCT compound (VWR, 25608-930) and sectioned on a cryostat (Leica CM 3050S). Sections containing the striatum were sectioned at 20 μ m and sections containing the SNc were sectioned at 40 μ m. Sections were kept in 1X PBS with 0.02% NaN₃ until use.

For immunofluorescence, tissue was drop fixed in 4% PFA and dehydrated in sucrose solutions in the same way as what was described for IHC sections. All sections for IF were sectioned at 20 μ m.

Staining.

Immunohistochemistry was performed as previously described (Rousseaux et al., 2016). Briefly, floating sections were washed 3 times in PBS, and then incubated with rabbit anti-Tyrosine Hydroxylase antibody (EMD Millipore, AB152, RRID:AB_390204) diluted 1/10,000 in PBS supplemented with 5 % FBS, 0.3 % Triton X-100 overnight at 4°C. Afterwards, the sections were washed 3 more times and stained with the VECTASTAIN® Elite® ABC HRP Kit (Peroxidase, Rabbit IgG) (Vector laboratories, PK-6101) according to manufacturer's instructions. We then used the DAB Peroxidase (HRP) Substrate Kit (with Nickel), 3,3'-diaminobenzidine (Vector laboratories,SK-4100) to develop the sections. Sections were mounted on superfrost plus slides (Fisher Scientific, 22-037-246) and dried at room temperature. Slides were then dehydrated by incubating them in the following series of solutions: PBS, H₂O, 70% Ethanol, 95% Ethanol, 100% Ethanol, and Xylene, before mounting coverslips using Richard-Allan Scientific™ Cytoseal™ XYL (ThermoFisher, 8312-4).

Immunofluorescence was performed as previous described (Rousseaux et al., 2016). Briefly, floating sections were washed 3 times in PBS, and then incubated with one or two of the following antibodies: mouse anti- α -Syn clone 42 (BD, 610787, RRID:AB_398108) 1/500 (recognizes human and mouse α -Syn), rat anti-human α -Syn 15G7 (Enzo, ALX-804-258-L001,RRID:AB_2050691) 1/250, Phospho- α -Syn (Ser129) (D1R1R) Rabbit mAb (CST, 23706S, RRID:AB_2798868) 1/500, Rabbit polyclonal anti-Dcl1 1/500 (Abcam, ab31704), mouse anti-Neurofilament-M (RMO 14.9, 1/1000) (CST, 2838, RRID:AB_561191), mouse anti-Synaptophysin (7H12, 1/1000) (CST, 9020, RRID:AB_2631095) or mouse anti-Tyrosine Hydroxylase 1/10,000 (Immunostar, 22941, RRID:AB_572268) diluted in PBS supplemented with 5% FBS, 0.3% Triton X-100 overnight at 4°C. The next day, sections were washed 3 times in PBS before being incubated with one of the following antibodies diluted in the same solution used for the primary antibody: Invitrogen Goat anti-Mouse IgG (H+L) Alexa Fluor 488 (A-11001, RRID:AB_2534069), Goat anti-Rabbit IgG (H+L) Alexa Fluor 555 (A-21428, RRID:AB_2535849), or Goat anti-Rat IgG (H+L), Alexa Fluor 555 (A-21434, RRID:AB_2535855) , Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 555 (A32727, RRID: AB_2633276), and

Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 647 (A32728, RRID: AB_2633277). Sections were then washed 3 more times in PBS and mounted using ProLong™ Gold Antifade Mountant (Invitrogen, P36930).

Human neuron preparation.

Immunofluorescence of human neurons was performed similarly to what was described previously (Jiang and Chen et al., 2017). Briefly, human neurons were cultured for three weeks. Cells were washed in PBS to remove the media. They were then subjected to a 20 minute fixation in 4% PFA and then washed three times with PBS. Finally, we performed a 10 minute permeabilization in 0.1% Triton-PBS and 30 minute blocking with 5% donkey serum before applying primary antibody. The following primary antibodies were used in human neuron studies: Rabbit Anti-GABA (Millipore Sigma, A2052-25UL, RRID: AB_477652), Mouse anti-VGLUT1 (Millipore Sigma, AMAb91041-100UL, RRID:AB_2665777), Rabbit Anti-MAP2 (EMD Millipore, AB5622, RRID:AB_91939), and Mouse Anti-Tubulin (Beta III): (EMD Millipore, MAB1637, RRID: AB_2210524). All secondary antibodies were used 1/1000. The rest of the procedure is similar to what is described for IF in mouse brain.

Immunofluorescent imaging

All imaging was performed on a confocal microscope (Leica STED TCS SP8), using LAS X software (Leica) to select optimal settings for image capture. All confocal IF images presented in this study were taken with the 63X objective. All YFP images represent fluorescence coming directly from this protein, no anti-YFP immunostaining was performed. Epi-fluorescent IF images were taken at 20X.

Immunofluorescent quantification

All quantifications were performed using Fiji software (Schindelin et al., 2012). Briefly, Z-stacks were loaded onto the software. For quantification of α -Syn fluorescence in the soma, the experimenter selected the brightest YFP positive cells (marking the cell as infected with AAV) and quantified the mean

fluorescent signal of pS129 α -Syn or human α -Syn (555 or 568) and Neurofilament-M (647). from each YFP positive soma in the YFP (488) channel as well as the 568 channel (pS129 α -Syn or human α -Syn). The mean fluorescence values of the fluorophore of interest were normalized to the Neurofilament-M signal before comparison. For quantification of α -Syn fluorescence outside of the soma, the experimenter quantified the raw integrated density of fluorescence inside and outside cells in an individual plane of a z stack for the previously mentioned α -Syn species as well as Synaptophysin (647). Then experimenter subtracted the signal emanating from cells, and normalized the α -Syn fluorescence values to those of Synaptophysin prior to comparison between experimental and control conditions. Notably, raw integrated density was selected as this represents the sum of every pixel, and avoids falsely low values attributed to punctate nature of pre-synaptic protein staining.

To estimate the proportion of cells expressing both α -Syn and Dcl1, the experimenter manually counted cells in sections from 3 animals. The number of cells expressing both markers was divided by the total number of visualized cells. To analyze the co-localization between different α -Syn species and Neurofilament-M, YFP and Synaptophysin, the experiment utilized the co-localization test function of Fiji software. The observed R^2 were then plotted, and compared between controls and experimental conditions. The same method was used to calculate the colocalization between Dcl1 and α -Syn in the wild type mouse cortex and substantia nigra, as well as for Dcl1 and TH in the substantia nigra.

Stereotaxic injections, stereological counting of dopaminergic neurons, and densitometric measurements of Striatal fiber density

Stereotaxic injections

Stereotaxic injections were performed as previously described (Rousseaux et al., 2016) with some small modifications to the protocol. Briefly, 2-month-old male C57B6/J mice were anesthetized, cleaned with antiseptic and a midline incision was made to expose lambda and bregma. Subsequently, a Hamilton needle containing 2 μ L of virus was used to deliver virus cocktail to the SNc (coordinates from bregma:

AP -2.9; ML 1.3; DV -4.2) at 0.2 μ L/s rate over 10 minutes. The virus cocktail included either shLuciferase + SNCA-Flag or shDcl1 + SNCA-Flag (3×10^9 gc, 1×10^9 gc) (see Production of AAV8 section below). After the injection was finished, the needle was removed, and the incision was sutured and glued. Animals were housed for 2 months post injections before being sacrificed. All surgeries carried out in mice were approved by the Institutional Animal Care and Use Committee for Baylor College of Medicine and Affiliates and in accordance with institutional animal protocols.

Stereological counts of dopaminergic neurons

Stereological counts were performed as previously described (Rousseaux et al., 2016). Briefly, sections from -2.54 to -4 from Bregma were selected for staining in intervals of six. IHC for TH was performed as described above. Sections were then coded so that the experimenter was blinded. All IHC for stereology was imaged using at 1000x (the representative images were taken using a 20X objective) on a Zeiss AXIO observer microscope equipped with a motorized stage (MBF). Optical fractionation was then used to estimate total dopaminergic cell counts in the SNc of each group (Stereo Investigator, MBF). To conduct this method, the experimenter outlined the ipsilateral (injected) and contralateral (not injected) SNc for each section using the software. Then the software automatically generated random windows that sampled each section, and the experimenter counted TH+ cell bodies. The software then estimated the total number of cells using the measured tissue thickness.

Quantification of Striatal TH density

Quantification of striatal TH optical density was performed as previously described (Rousseaux et al., 2016). Briefly, images of the striatum corresponding to the ipsilateral and contralateral sides were captured using the 20X objective (Zeiss, Axio Scan.Z1). Images were then converted to gray scale using an in-house automated Adobe Photoshop CS5 workflow (Automator, Mac OSX). Five regions across each striatal section were sampled and normalized using the negative staining from the corpus callosum. Normalized values across three sections were used to compare groups.

Cell line culture and transfection

Cell culture

HEK293T cells (ATCC®, CRL-3216™) were cultured on DMEM (Corning, MT10013CV) supplemented with 10% fetal bovine serum (Atlanta Biological, S11150), and 1X antibiotic/antimycotic (Gibco, 15240062).

Transfection

Transfection experiments for measurement of protein levels were carried out in 24 well plates (Corning, CLS3527-100EA). HEK293T cells were seeded at 50,000 cells/well. They were then transfected with 1µg of DNA using MIRUS TransIT-293 reagent (MIR 2706) according to the manufacturer's instructions. For IP transfections see immunoprecipitation section of methods section.

Viral transduction.

HEK293T cells were seeded in 96 well plates (5,000 cells/well) and infected with excess of virus (10 µL of concentrated virus). 24 hours later, cells were selected with puromycin (1µg/µl) for an additional 72 hours. The cells were then cultured for at least 10 days in Complete DMEM before experimental use.

siRNA mediated knockdown

Transient knockdown of Delk1 was accomplished by transfecting Silencer siRNAs (32nM) from Thermofisher (catalog #:AM51331, siRNA IDs: 574, and 575) using 0.5µL of Dharmafect (Dharmacon, T-2001-01) per well in HEK293T cells plated in 24 wells (50,000 cells/well). The cells were cultured in antibiotic free media for 72 hours prior to lysis.

Lysosomal and proteosomal inhibition

To inhibit the lysosomal function in HEK293T cells, cells were treated 18 hours prior to lysis with 100nM of Bafilomycin A1 (Millipore-Sigma, 196000). To inhibit proteosomal function, cells were treated 12 hours prior to lysis with 1000nM of MG132 (Millipore-Sigma, 474790). The efficacy of

lysosomal inhibition was assessed by western blotting with rabbit anti-LC3 (Novus biologicals, NB100-2220, RRID: AB_10003146). The efficacy of proteosomal inhibition was determined by immunoblotting for poly-ubiquitinated proteins using mouse anti-ubiquitin (P4D1) antibody (CST, 3936, RRID: AB_331292)

Human neuron culture

Generation of human neurons by differentiation of iPSCs

The derivation and culture of human neurons for shRNA knockdown experiments was performed as described previously (Rousseaux, and Vázquez-Vélez et al., 2018). Briefly, we generated and expanded neuronal progenitors from the ND50042 line (*SNCA* triplication female patient), and from WA09 (H9) embryonic stem cells (ESCs) (WiCell technologies, WAe009-A). We obtained NN0000049 iPSCs from the NINDS Human Cell and Data repository (Patient #:NDS00201,iPSC line ID: ND50040; RUID=NN0000049). We obtained H9 ESCs from Dr. Jean Kim at Baylor College of Medicine under agreement with WiCell Technology. NPCs were cultured for at least one week after thawing (with media changes every two to three days). Accutase was then used to passage the NPCs at a density of 15,000 cells per cm² in 24-well plates coated with Cultrex (Stem Cell Qualified, Reduced Growth Factor BME, Trevigen, # 3434-010-02). Three weeks later, the neurons were infected with pGIPZ lentiviruses carrying shRNA (Dharmacon) at an MOI of 20. Six days post infection, the neurons were selected with 1 mg/ml puromycin for 5-6 days. At 14 days post infection, protein was extracted as described in the protein extraction methods section.

Direct conversion of induced pluripotent stem cells (iPSCs) into human neurons

SNCA triplication (Patient #:NDS00201,iPSC line ID: ND34391; RUID= NN0003871) and isogenic control (*SNCA*^{WT}) induced pluripotent stem cells (iPSCs) were generated as previously described (Heman-Ackah et al., 2017). To confirm successful differentiation of each line, a modified version of the direct differentiation protocol described previously by others was used (Thoma et al., 2012; Zhang et al.,

2013; Ho et al., 2016). Briefly, 2×10^6 iPSCs suspended as single cells in mTeSR1 basal media (STEMCELL Technologies, 85850) with $10 \mu\text{M}$ of Y-27632 (Tocris, TB1254-GMP) were infected with a modified version of hNGN2-eGFP-Puro plasmid (RRID:Addgene_79823). In this plasmid which was a gift from Dr. Kristen Brennand (Ho et al., 2016) the puromycin selection cassette was replaced with blasticidin selection, and the GFP fluorescent marker with mCherry. The iPSCs were incubated in suspension overnight and moved to Matrigel (Corning, 477343-706) coated plates on the following day. After several days of expansion and passaging with Accutase (A6964, Millipore Sigma), the iPSCs were plated (0.125×10^6 cells/cm²) in 12-well tissue culture plates coated Cultrex (Stem Cell Qualified, Reduced Growth Factor BME, Trevigen, # 3434-010-02). They were then induced using Neuronal induction media (NIM – DMEM/F-12:Neurobasal (1:1) with 1X Penicillin-Streptomycin, 2% B-27 without vitamin A, 1% N-2, 2 mM Glutamax, all from ThermoFisher, Cat#A1647801, 21103049, 10378016, 12587010, 17502001, and 35050061 respectively) supplemented with doxycycline ($2 \mu\text{g/ml}$) (STEMCELL Technologies, 72742), and SMAD inhibitors, $1 \mu\text{M}$ dorsomorphin (sc-361173, Santa Cruz Biotechnology) and $10 \mu\text{M}$ SB431542 (S1067, Selleck Chemicals). Two days later, the media was changed to Neural Differentiation media (NDM - Neurobasal medium with 1X Penicillin-Streptomycin, 2% B-27 without vitamin A, 2 mM Glutamax) supplemented with 20 ng/ml BDNF, 10 ng/ml GDNF, 10 ng/ml NT-3 (all three were human animal free peptides from Peprotech, AF-450-02, AF-450-10, and AF-450-03 respectively), $100 \mu\text{M}$ db-cAMP (sc-201567B, Santa Cruz Biotechnology), and $200 \mu\text{M}$ ascorbic acid (A8960, Millipore Sigma) supplemented with doxycycline, and $20 \mu\text{g/ml}$ of blasticidin (ant-bl-05, Invivogen) for selection. Blasticidin selection was performed for six days (from day two to day eight). The cultures were supplemented with $1 \mu\text{g/ml}$ laminin (3400-010-02, Travigen) once every 4 days and with matrigel once every 8 days to prevent detachment. The cells were kept in culture for a total of 30 days. At the end of this period, protein was extracted as described in the protein extraction section of these methods.

Production of vectors for transfection experiments

To express different functional domains of DCLK1 in HEK293T cells, we produced a series of new vectors. All of these vectors are based on a modified pcDNA3 that is compatible with Gateway cloning and encodes a 3' 3X-Flag tag (Klisch et al., 2017). We obtained human *DCLK1* cDNA (RC217050, OriGene), and PCR amplified the full length DCLK1 (1-2188bp), PEST-Kinase domain (862-1941bp), and Doublecortin domains (169-807bp) from the original vector. We then used the Gateway™ BP Clonase™ II Enzyme mix (11789020, Thermofisher Scientific) to transfer these sequences unto the Gateway™ pDONR™221 Vector (12536017, Thermofisher Scientific). Finally, each insert was transferred unto our customized pcDNA3-3X-Flag vector using Gateway™ LR Clonase™ II Enzyme mix (11791020, Thermofisher Scientific). All cloning reactions were performed according to the manufacturer's instructions.

To generate kinase dead forms of full length, and PEST-Kinase domain constructs, we used QuikChange II XL Site-Directed Mutagenesis Kit (200521, Agilent) to perform an alanine substitution on the catalytic domain of DCLK1 (D511A) (Patel et al., 2016) according to the manufacturer's instructions. To generate kinase domain constructs that lack the PEST sequence (862-1020bp), we also used site directed mutagenesis to eliminate this sequence from our PEST-Kinase domain constructs.

Production and titering of lentiviruses

Production

Lentiviruses were made according to what was described previously (Rousseaux, and Vázquez-Vélez et al., 2018). HEK293T cells were seeded so that they were 90% confluency on the day of transfection in 150 mm dishes. They were transfected using MIRUS-TRANS-IT 293 reagent with pGIPz, psPAX2, pMD2.G (at a 4:3:1 ratio, 45 µg total for 150 mm dish). Media was collected 48 and 72 hours later. It was spun down for 10 minutes at 4,000 rpm at 4C. The viral media was then filtered using 45 µm PES filters to remove cellular debris before being concentrated 100X overnight using Lenti-X concentrator (Clontech, 631231), according to manufacturer instructions.

Titerting

Titerting was performed as previously described (Rousseaux, and Vázquez-Vélez et al., 2018). Briefly, HEK293T cells were seeded at 50,000 cells/well in a 24 well plate. The next day, a serial dilution of the virus was made and cells were infected with each dilution. After four days of culture, GFP positive colonies were counted and the viral titers were estimated.

Mouse breeding and p0 intraventricular injections

CFW of both sexes (Charles Rivers, 024), C57B6/J (Jackson laboratory), and male Thy1- α -Syn (“Line 61”) (Rockenstein et al., 2002) mice were used for p0 intraventricular injections. We will refer to these mice as Thy1- α -Syn here after. We had previously obtained the Thy1- α -Syn, and had back crossed into the C57B6/J background (Rousseaux et al., 2016). In all experiments, mice were allowed to breed for 3 days before the male was removed. In the case of experiments using C57B6/J mice, FVB foster mothers were used to house the pups after manipulation to avoid maternal cannibalization. In experiments using the Thy1- α -Syn, transgenic females were bred to wildtype males. Because the transgene is on the X chromosome, only the male offspring can be used as females have a larger degree of variability because of X-inactivation. In the case of wildtype CFW mice, a foster mother was not necessary, and the mice were kept with their mothers after injections. Both sexes were used in this case. All procedures carried out in mice were approved by the Institutional Animal Care and Use Committee for Baylor College of Medicine and Affiliates.

Production of AAV8

We used previously generated AAV8 vectors containing either an shRNA against Luciferase (negative control) or against *Dclk1* (Rousseaux, and Vázquez-Vélez et al., 2018). Viruses were produced in collaboration with the Baylor College of Medicine Viral Vector core.

P0 AAV Injections

478 Injections were performed as previously described (Kim et al., 2013; Rousseaux, and Vázquez-
479 Vélez et al., 2018). Briefly, shRNA sequences were obtained using the SplashRNA algorithm and cloned
480 into YFP and miR-E containing AAV8 vectors (Muhar et al., 2013; Fellmann et al., 2017).

482 **Statistical analyses**

483 For all experiments comparisons of two groups were performed using Student's t-test, and
484 comparisons of three or more groups were performed using one-way ANOVA followed by Dunnett's
485 multiple comparison test. All analyses were conducted using Prism 6 software (GraphPad).

487 **Results**

488 *Dclk1 regulates α -Syn levels post transcriptionally*

489 We previously used AAV8 mediated intraventricular delivery of shRNAs to knockdown *Dclk1* in
490 the postnatal brain (posterior cortex and hippocampus) (**Figure 1A**), and found that this reduces α -Syn
491 protein (Rousseaux, and Vázquez-Vélez et al., 2018, **Figure 1B**). We used the posterior cortex and
492 hippocampus for these experiments, because this region has the highest infection rate using this method
493 (Kim et al., 2013). We hypothesized that *Dclk1* regulates the levels of α -Syn post transcriptionally
494 because the two screens that identified *DCLK1* were performed on transgenic cell lines which lack the
495 regulatory elements that control *SNCA* transcription (Rousseaux et al., 2016, Rousseaux, and Vázquez-
496 Vélez et al., 2018). To test this, we performed qPCR to detect the relative abundance of *Snca* transcript
497 after *Dclk1* knockdown. We did not find any significant difference in *Snca* transcript after *Dclk1*
498 knockdown in the mouse brain (**Figure 1B**), confirming that *Dclk1* regulates α -Syn protein levels post-
499 transcriptionally. To test whether this modulatory effect is specific to α -Syn, we measured the levels of
500 two other neurodegenerative disease-driving proteins: tau and App. In both cases, *Dclk1* knockdown did
501 not alter their protein levels (**Figure 1C**). The *Dclk1* gene has multiple transcripts that include alternative
502 start sites (Burgess and Reiner, 2002; Deuel et al., 2006; Koizumi et al., 2006). In the adult brain, these

transcripts code for either the full-length protein or a shortened form that does not include the DCX domains, herein referred to as the “kinase domain” (**Figure 1D**). To determine which forms of *Dclk1* are affected by our shRNA we measured the transcript and protein levels. We found that our shRNA could knockdown both the full length *Dclk1* as well as the kinase domain transcripts (**Figure 1E**).

The Dclk1 kinase domain, but not its catalytic activity, is required for regulation α -Syn levels

To gain insight into the mechanism by which Dclk1 regulates α -Syn levels, we first asked whether these two proteins reside in similar neuronal compartments. To answer this question, we labeled sections of mouse cortex and SNc with anti-Dclk1 and anti- α -Syn antibodies to visualize neuronal soma (**Figure 2A**). We also labeled the substantia nigra pars reticulata (SNr), striatum and hippocampus to visualize neuronal fibers (dendrites and pre-synaptic terminals) (**Figure 2B**). Finally, we also stained nigral sections with anti-TH antibodies and anti-Dclk1 antibodies to determine directly if dopaminergic neurons express Dclk1 (**Figure 2C**). We found that the majority of neurons (approximately 80%) in the cortex and the SNc express both α -Syn and Dclk1 (**Figure 2A,D**). Moreover, the co-localization of α -Syn and Dclk1 was significantly higher in the soma of cortical neurons than in their corresponding fibers (average fiber $R^2=0.513$ vs average soma $R^2=0.701$). The difference was also found in the substantia nigra (average SNr fiber $R^2=0.152$ vs average SNc soma $R^2=0.403$) (**Figure 2A-B,E**). It should be noted that the R^2 values vary greatly between brain region which is possibly due to differences in tissue architecture. Notably, Dclk1 and α -Syn fibers in other regions (striatum, hippocampus) were visibly opposed with little overlap. This pattern of co-localization is not surprising given that outside of the soma, Dclk1 has been reported to be localized to the dendritic compartment (Shin et al., 2013), and α -Syn has been reported to be localized to the pre-synaptic compartment (Withers et al., 1997; Kahle et al., 2000; Unni et al., 2010; Burré et al., 2016). Additionally, Dclk1 staining co-localized more strongly with TH staining in dopaminergic neuronal soma (SNc) than in their fibers (SNr) (**Figure 2C,E**).

Given that the two proteins exist in the same subcellular location, we next asked if they could interact. We performed immunoprecipitation (IP) of endogenous α -Syn in HEK293T cells transfected with either empty vector or flag tagged DCLK1 and found that overexpressed DCLK1 immunoprecipitates with endogenous α -Syn in cells. (**Figure 3A**). Next, we tested if this interaction could be detected in vivo. To do so we immunoprecipitated endogenous Dclk1, and probed for endogenous α -Syn in the brains of wild type mice. We found that α -Syn could be detected at long exposures (**Figure 3B**). It is worth noting that while we did detect an endogenous interaction between α -Syn and Dclk1, the amount of endogenous α -Syn that interacted with Dclk1 was very small. These data suggests that the two proteins may interact.

Since Dclk1 and α -Syn can interact (albeit weakly) and co-exist in the soma of neurons, and Dclk1 is a protein kinase, we next asked whether DCLK1 can directly phosphorylate α -Syn. To test this, we performed an in vitro kinase assay. We found that active DCLK1 is capable of auto-phosphorylation but it does not phosphorylate recombinant GST- α -Syn. In contrast, c-Abl, a known α -Syn kinase (Mahul-Mellier et al., 2014; Brahmachari et al., 2016) phosphorylates α -Syn (**Figure 3C**). This result suggests that Dclk1 may not directly phosphorylate α -Syn. This is perhaps not surprising as α -Syn lacks the proposed consensus motif for Dclk1 (R-R-X-X-S*/T*) (Shang et al., 2003; Koizumi et al., 2017).

We next determined if the kinase activity of DCLK1 is required for its effect on α -Syn levels. To answer this question, we performed shRNA-mediated knocked down of endogenous *DCLK1* in HEK293T cells and tested whether overexpression of shRNA-resistant flag-tagged constructs containing wildtype DCLK1 or catalytically dead (DCLK1-D511A) (**Figure 4A**) could rescue the effect on α -Syn levels. We found that both forms of DCLK1 were able to rescue the decrease in α -Syn levels seen upon *DCLK1* knockdown (**Figure 4B**). Additionally, both wild type and catalytically dead kinase domain had the same effect on α -Syn levels, regardless of the presence or absence of the PEST sequence (**Figure 4C-D**). Conversely, the DCX domains alone failed to change α -Syn levels (**Figure 4E**). Taken together these

data suggest that DCLK1 regulates α -Syn levels via its kinase domain, but that this effect is not dependent on its kinase activity.

Knockdown of DCLK1 reduces α -Synuclein levels via lysosomal degradation in cells

To elucidate how the binding of DCLK1 affects α -Syn protein levels, we asked if DCLK1 protects α -Syn protein from lysosomal or proteasomal degradation. To test this, we transiently knocked down *DCLK1* in HEK293T cells with siRNAs and added either inhibitors of lysosomal function (Bafilomycin A1) or proteasomal function (MG132; **Figures 5A-C**). We found that only exposure to Bafilomycin A1 selectively mitigated the effect of siDCLK1 on α -Syn protein levels (**Figure 5B**), whereas proteasomal inhibition had no effect (**Figure 5C**). This is consistent with reports indicating that the primary mode of α -Syn degradation is lysosomal (Cuervo, 2004; Lee, 2004; Vogiatzi et al., 2008; Oueslati et al., 2013; Tofaris et al., 2011).

Dclk1 knockdown preferentially affects the levels of pS129- α -Syn in transgenic mice and ameliorates toxicity in an AAV mediated mouse model

After initially focusing on the mechanism of regulation of endogenous α -Syn by Dclk1 in the wild type context, we next tested whether this translates to the context of disease, in which pathogenic species α -Syn are increased and drive toxicity. To capture the full spectrum of synucleinopathy phenotypes, we used two models to test whether Dclk1 inhibition would prove beneficial. First, to test the effect on the levels of pS129- α -Syn we used the Thy1- α -Syn “Line 61” (Rockenstein et al., 2002) mouse model of PD, henceforth referred to as Thy1- α -Syn. This line overexpresses wild type human *SNCA* at tenfold normal levels throughout the brain (Rockenstein et al., 2002; Chesselet et al., 2012). Importantly, pS129 α -Syn is thought to be associated with pathology in humans, because it is drastically elevated in the brains of PD patients and in Lewy bodies (Fujiwara et al., 2002; Anderson et al., 2006). To test the effect of Dclk1 loss on α -Syn-induced pathology in mice, we used neonatal intraventricular injection of

AAV8 carrying a *Dcl1* shRNA for widespread delivery. Because the *SNCA* transgene is on the X chromosome, we crossed Thy1- α -Syn female mice with wild type males, and injected their litters (**Figure 6A**). We analyzed male mice due to the mosaicism in females arising from X chromosome inactivation (Disteche and Berletch, 2015). Two months after the injection, we sacrificed the animals, dissected the posterior cortex and hippocampus and analyzed the different α -Syn species biochemically and histologically. We first performed immunofluorescence to determine the proportion of cortical neurons infected with our virus. We found that approximately 60% of Neurofilament-M (NF-M) positive soma were also positive for YFP (indicating viral infection) (**Figure 6B**). Next, we confirmed *Dcl1* knockdown in the posterior cortex and hippocampus via western blot and immunofluorescence (**Figures 6C-D**). We found that *Dcl1* knockdown robustly reduces the amount pS129- α -Syn by western blot (by 33%) (**Figure 6E**). However, in the context of 10X overexpression of α -Syn, we did not detect significant changes in human or in total α -Syn by western blot after *Dcl1* knockdown (**Figure 6F**). To determine if this was due to a lack of interaction between mouse *Dcl1* and the overexpressed human α -Syn, we pulled down endogenous *Dcl1* and blotted for all three α -Syn species. We found that pS129, human, and total α -Syn were all able to interact with *Dcl1* (**Figure 6G**).

We hypothesized that this preferential effect may be due to the abundant overexpressed α -Syn found at the pre-synaptic compartment that does not co-localize with *Dcl1* (**Figure 2**). To test this hypothesis, we first compared the co-localization of pS129 and human α -Syn to the soma and neuronal fibers (see extended **Figure 7-1 for negative controls**). Using Synaptophysin as a marker, we found that human α -Syn colocalizes more strongly with neuronal fibers than pS129 α -Syn. In contrast to this result, we found that pS129- α -Syn colocalized more strongly with markers of neuronal soma (NF-M and YFP) than human α -Syn. (**Figure 7A**). Building on this finding, we measured somatic pS129 and human α -Syn mean fluorescence intensity (normalized to NF-M) in mice injected with either shLuciferase or sh*Dcl1*. We found that *Dcl1* knockdown reduced both forms of α -Syn in neuronal soma (**Figures 7B-C**). Importantly the observed reduction of pS129 α -Syn was more dramatic than the observed reduction of

human α -Syn. Conversely, neither species of α -Syn in neuronal fibers were affected by *Dclk1* knockdown (normalized to Synaptophysin) (**Figures 7D-E**). Taken together these results suggest that in this context, *Dclk1* knockdown preferentially reduces somatic species of α -Syn.

We next tested if *Dclk1* knockdown can affect α -Syn induced neurotoxicity. Since *Dclk1* is expressed in nigral neurons (**Figure 2C**), and because the Thy1- α -Syn model does not recapitulate the dopaminergic cell loss observed in PD, we utilized a viral model of α -Syn-induced Parkinsonism (Kirik et al., 2002; Ip et al., 2017). We first verified appropriate viral targeting of the SNc by IF (**Figure 8A**), as well as *Dclk1* knockdown (**Figure 8B**). We also ensured that human α -Syn overexpression caused significant loss of Tyrosine hydroxylase (TH) positive dopaminergic neurons in the SNc two months post-injection (**Figure 8C-D**).

Next, we performed unilateral stereotaxic injections into the SNc of wild type male mice with a Flag-tagged *SNCA* cDNA or empty vector together with an shRNA targeting *Dclk1* (shRNA) or *Luciferase* (shLuciferase) control (sh*Dclk1*+empty vector vs shLuciferase+empty vector vs sh*Dclk1*+*SNCA* vs shLuciferase+*SNCA*, respectively). (**Figures 9A-D**). As has been previously shown (Kirik et al., 2002; Ip et al., 2017), viral overexpression of *SNCA* resulted in significant loss of TH positive dopaminergic neurons in the substantia nigra as determined by unbiased stereology (**Figure 9B,D**), as well as dopaminergic denervation in the striatum (**Figure 9C,E**) when compared to the uninjected contralateral side or animals injected with the shRNAs and an empty vector. Importantly, we found that co-injection of sh*Dclk1* with *SNCA*, was able to rescue these phenotypes, but co-injection with shLuciferase was not (**Figures 9B-E**). Taken together, these results suggest that *Dclk1* knockdown reduces phosphorylated species of α -Syn and rescues α -Syn induced dopaminergic neurotoxicity in the SNc in mice.

DCLK1 regulates α -Syn levels in neurons derived from a PD patient

Having shown that Dclk1 interacts with and modulates α -Syn levels, we next asked whether this effect is conserved in a human neuronal model derived from an individual with α -Syn-driven PD. We derived cortical neurons from induced pluripotent stem cells (iPSCs) obtained from two clones from a patient with *SNCA* triplication (Meunter et al., 1998; Singleton et al., 2003), an isogenic control corrected for the mutation, and the H9 line of embryonic stem cells. Using the dual-SMAD inhibition protocol, we first differentiated one of the iPSC clones (RUID:NN0000049, clone 1) as well as H9 ESCs into neural progenitor cells and then to cortical neurons. For the second *SNCA* triplication clone (RUID: NN0003871) (Heman-Ackah, et al., 2017) as well as its isogenic control, we utilized *NGN2* mediated direct neuronal conversion method (Thoma et al., 2012; Zhang et al., 2013; Ho et al., 2016). We first confirmed differentiation by staining for neuronal markers (β III-Tubulin and MAP2), as well as markers of glutamatergic (Vglut1) and GABAergic (GABA) neurons (**Figures 10A-B**). Next, we confirmed that neurons from both of these *SNCA* triplication clones, regardless of the derivation method, carry a twofold increase in *SNCA* RNA when compared to their respective controls (**Figure 10C-D**), confirming that these lines are reproducing what would be expected from the genomic duplication. We also observed that both clones contain high levels of pathologically associated pS129 α -Syn species. Surprisingly, we found that total α -Syn protein levels are increased approximately five-fold when compared to controls in both differentiation paradigms and using both controls (**Figure 10C-D**). Finally, to test the effect of *DCLK1* in the context of patient neurons we performed shRNA-mediated knockdown of *DCLK1* in *SNCA* triplication neurons derived from clone 1 and confirmed that this intervention potently reduces both pS129 α -Syn and total α -Syn. (**Figure 10E**). Taken together these data suggest that *DCLK1* is a strong regulator of α -Syn levels in patient neurons.

Discussion

The role of α -Syn in Parkinson's disease and other synucleinopathies has been under intense study since its discovery over thirty years ago (Maroteaux et al., 1988; Uéda et al., 1993; Spillantini et al.,

1998). Although much has been learned about how α -Syn oligomerizes (Pieri et al., 2016; Bengoa-Vergniory et al., 2017), aggregates (Spillantini et al., 1998), spreads (Luk et al., 2012; Mao et al., 2016; Wong and Krainc, 2017), and causes toxicity (Martin, 2006; Devi et al., 2008; Mazzulli et al., 2016), relatively little is known about the molecules that control its protein levels. The fact that duplications and triplications in *SNCA* cause genetic forms of PD (Singleton et al., 2003; Chartier-Harlin et al., 2004; Ibáñez et al., 2004) further underscores the importance of understanding this regulation. With that in mind, recent efforts by our lab have set out to find regulators of α -Syn levels (Rousseaux et al., 2016, Rousseaux, and Vázquez-Vélez et al., 2018). DCLK1 emerged as one of the most promising targets for downstream validation both due to its strong and consistent modulation of endogenous α -Syn levels in two independent screens, and because of the potential of its druggability as a brain-resident kinase. In this study, we sought to gain insight into the mechanism of how DCLK1 regulates α -Syn levels. Additionally, we wanted to determine if the knockdown of *DCLK1* would be beneficial in mouse and human genetic models of synucleinopathy.

DCLK1 has been almost exclusively studied in the context of neurodevelopment. In the past, much of the research has focused on the microtubule polymerizing function (Lin et al., 2000) of the doublecortin domains that the protein possesses, how this is necessary for neuronal migration, formation of the corpus callosum (Deuel et al., 2006; Koizumi et al., 2006), and regulation of dendrite maturation (Shin et al., 2013). Apart from phosphorylation of MAP7D1, DCX and itself (Koizumi et al., 2017), no other function of the kinase domain of this protein has been reported. Moreover, there is currently no evidence that DCLK1 regulates the levels of any of these targets. Our data suggest that DCLK1 regulates α -Syn post transcriptionally via its kinase domain, independent of its catalytic activity, PEST sequence, and the DCX domains. Additionally, this effect appears to be dependent on lysosomal function. Thus, when *DCLK1* is knocked down, α -Syn protein levels decrease due to lysosomal degradation (**Figure 11**). It should be noted that a wide variety of non-catalytic functions for kinases have been found previously. They include allosteric regulation of other kinases, and scaffolding for protein complexes (Kung and Jura,

2016). Despite this, how DCLK1 regulates α -Syn levels remains elusive. Importantly, we found that endogenous α -Syn can interact with overexpressed DCLK1. However, the interaction that was observed between endogenous proteins in vivo is very weak. It will be important in the future to further dissect how DCLK1 regulates α -Syn levels. It is possible that DCLK1 acts as a scaffold for another protein that promotes α -Syn stability or that DCLK1 functionally inhibits proteins that target α -Syn to the lysosome.

It is also worth mentioning that despite equivalent amounts of transfection, the kinase dead versions of DCLK1 seem to be more stable than their wildtype counterparts. This observation is perhaps, not surprising given that mutations in enzymes that reduce activity are often accompanied by an increase in protein stability (Beadle and Shoichet, 2002)

To study the relationship between *Dclk1* and α -Syn in the disease context we turned to animal and cellular models. The first, the Thy1- α -Syn model overexpresses wildtype human α -Syn tenfold throughout the brain. When we knocked down *Dclk1* in these mice, we were surprised to find the dramatic reduction only in the levels of the pS129 α -Syn, a species of α -Syn associated with pathology in humans with PD. (Fujiwara et al., 2002; Arawaka, 2006). We found that this is due to the localization of the different species of α -Syn. The transgenic α -Syn in these mice is mostly pre-synaptic. In contrast pS129 α -Syn is mostly localized to the soma, which explains why in this model of synucleinopathy, pS129 α -Syn is drastically reduced with *Dclk1* knockdown despite no apparent change in total α -Syn. Interestingly, we did observe a reduction in total α -Syn after *Dclk1* knockdown in the context of the wildtype mouse brain. Given all of our data, we propose that *Dclk1* knockdown reduces the somatic α -Syn in this context as well, which explains why only 18% of α -Syn is affected.

This preferential effect on somatic α -Syn could be an attractive feature of this modulator for therapeutic purposes, as inhibiting the interaction between *Dclk1* and α -Syn could preferentially affect the α -Syn that is mislocalized and driving toxicity, while leaving the normal function of α -Syn unaffected. DCLK1 is also an example of how the regulation of protein levels not only depends on the cellular context, but on the context of the subcellular organelle. It should be noted however, that we did

observe a significant decrease in α -Syn levels after *DCLK1* knockdown in *SNCA* triplication patient neurons which suggests that DCLK1 can regulate α -Syn levels in a disease relevant context, albeit not in the context of massive (tenfold) α -Syn overexpression such as is the case for the Thy1- α -Syn mice.

It should also be noted that we also discovered that despite having the expected two-fold increase in *SNCA* transcript dosage, our *SNCA* triplication patient lines had approximately a five-fold increase in α -Syn protein relative to controls. This finding warrants further study and could indicate that the triplication of *SNCA* overwhelms the machinery controlling α -Syn protein levels.

To study the functional effects of *Dclk1* knockdown in the context of α -Syn induced neurotoxicity we used a mouse viral overexpression model in which *SNCA* is overexpressed in the SNc. We found that *Dclk1* knockdown rescues dopaminergic neuron death in this model (**Figure 9**). These results highlight the therapeutic potential of targeting this interaction as modulating DCLK1 levels not only changes α -Syn levels but shows a clear functional benefit. Another important question is if modulating Dclk1 levels in the adult brain is safe. This is especially important because Dclk1 regulates microtubular stability (Lin et al., 2000). Previous attempts to generate *Dclk1* knockout mice have failed to remove the truncated transcripts coding for the kinase domain because of the large number of unique *Dclk1* transcripts in the mouse brain (Deuel et al., 2006; Koizumi et al., 2006). One advantage of our approach, is that our shRNA targets all forms of *Dclk1* and it circumvents any developmental roles of the gene because the shRNA is delivered postnatally. Importantly, although overexpression of *DCLK1* results in microtubule polymerization (Lin et al., 2000), the knockdown or knockout of full length *DCLK1* has been shown to affect microtubular stability only in conjunction with knockdown of other members of its protein family (*DCX* or *DCLK2*) (Koizumi et al., 2006; Deuel et al., 2006; Shin et al., 2013), which suggests that partial inhibition of Dclk1 may be safe. However, the next logical step is to conditionally knock out all transcripts of *Dclk1* in the adult brain to test the safety of removing it in the mature brain in the same way we recently did for *Trim28* (Rousseaux et al., 2018).

In this study we identified a new function in the mature brain for DCLK1. Moreover, we established the feasibility of targeting DCLK1 as a regulator of α -Syn levels, and reveal that its reduction had the most dramatic effect on the phosphorylated and most toxic form of α -Syn. Because there is ample evidence showing that α -Syn levels can drive disease, lowering DCLK1 levels, in combination with other modulators of α -Syn, is a viable disease modifying strategy for PD.

Author Contributions:

G.E.V.V., M.W.C.R., and H.Y.Z. conceived and designed the study. G.E.V.V., and K.G. carried out all experiments except the stereotaxic injections, in vitro kinase assay and qPCR experiments. R.R. and C.A. performed the in vitro kinase assay. J.P.R. carried out the qPCR experiments. M.W.C.R. and G.E.V.V. carried out the adult stereotaxic injections. F.A-N, A.B., E.C. and G.E.V.V. performed human neuron experiments. S.M.H-A, and M.J.A.W. contributed reagents and scientific insight. M.W.C.R. contributed scientific insight and edited the paper. G.E.V.V. and H.Y.Z. wrote the paper.

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Figure Legends:

Figure 1: Doublecortin like kinase 1 regulates α -Syn levels post-transcriptionally. **A)** Diagram of the experimental strategy. CFW wild type females were bred to male wild type mice. Neonatal intraventricular injections of AAVs carrying shRNA were carried out on their pups and the injected mice were sacrificed at 3 weeks of age. The posterior cortex and associated hippocampus were dissected for western blotting and qPCR. **B)** Western blot ($t= 3.376$, $P= 0.0045$) and (RNA) ($t= 0.9981$, $P= 0.3418$) for α -Syn protein (C-20) and transcript levels after Dclk1 knockdown in the mouse brain. Each data point represents individual animals. **C)** Tau ($t= 1.457$, $P= 0.1713$) and App ($t= 0.05485$, $P= 0.1713$) protein levels measured by western blot after Dclk1 knockdown in the brain. Each data point represents individual animals. **D)** Diagram of Dclk1 transcripts and protein products expressed in the mouse brain. The site targeted by the shRNA (exon 6 in full length transcript, exon 2 in shortened products) is shown. **E)** Dclk1 protein and transcript levels in mouse brains after *Dclk1* knockdown measured by western blotting and qPCR respectively. (Protein full length, $t= 9.706$, $P=0.0002$; protein kinase domain, $t=4.042$, $P=0.0099$; RNA all, $t=7.726$, $p<0.0001$; RNA full length, $t= 4.798$, $P=0.0001$). Error bars represent SEM.

Student's t-Test, NS \geq 0.05, *, **, ***, **** denote $P\leq$ 0.05, $P\leq$ 0.01, $P\leq$ 0.001, and $P\leq$ 0.0001, respectively.

Figure 2: Doublecortin like kinase 1 and α -Synuclein are both present in neuronal soma. A)

Representative immunofluorescence (IF) images of neuronal soma stained with anti-Dclk1 and anti- α -Syn (BD42) in the cortex and substantia nigra pars compacta (SNc). Yellow arrows indicate neuronal soma that express both proteins. **B)** Representative images of immunofluorescence (IF) of anti-Dclk1 and anti- α -Syn (BD42) positive neuronal fibers in the substantia nigra pars reticulata (SNr), striatum, and hippocampus. Dclk1 positive fibers appear to be opposed to α -Syn positive fibers in all three regions. **C)** Representative images of immunofluorescence (IF) using anti-Dclk1 and anti-TH antibodies in the substantia nigra pars compacta (SNc) and substantia nigra pars reticulata (SNr). Neuronal soma that are positive for TH and Dclk1 are indicated with yellow arrows. TH positive fibers and Dclk1 positive fibers in the SNr appear to be opposed. **D)** The proportion of neurons in either the cortex or the SNc that are Dclk1 and α -Syn positive was quantified and is shown as a bar graph. Each data point represents one animal. Error bar represents SEM. **E)** Co-localization analysis of cortical and nigral sections. The extent of co-localization was quantified as the degree of positional correlation between anti-Dclk1 and anti- α -Syn pixels (blue bars) (cortex $t=14.52$, $P=0.0001$; SNc/SNr, $t=6.593$, $P=0.0027$). The same analysis was done for anti-Dclk1 and anti-TH staining (light purple bars) ($t=9.380$, $p=0.0007$). Error bars represents SEM. Student's t-Test, NS \geq 0.05, *, **, ***, **** denote $P\leq$ 0.05, $P\leq$ 0.01, $P\leq$ 0.001, and $P\leq$ 0.0001, respectively.

Figure 3: Doublecortin like kinase 1 can interact with α -Synuclein but does not phosphorylate it. A)

Representative blot of co-IP experiments performed in HEK293T cells transfected with either empty vector or DCLK1-3Xflag. Endogenous α -Syn was pulled down with anti- α -Syn antibody (C-20), and the blots were probed with anti-Flag antibody (M2, Sigma-Aldrich). **B)** Representative blots of co-

immunoprecipitation (co-IP) experiments from mouse brain using anti-Dclk1 antibodies to pull down endogenous Dclk1 and immunoblotting of α -Syn (BD42). **C)** In vitro kinase assay. Active DCLK1 was incubated with recombinant α -Syn. Active c-Abl serves as a positive control. Radioactive ATP was used to detect phosphorylation.

Figure 4: Doublecortin like kinase 1 regulates α -Synuclein levels independently of its kinase activity. **A)** Diagram of DCLK1 transcript showing shRNA targeting. **B-E)** DCLK1 flag tagged constructs were transfected in HEK293T cells after shRNA knockdown of DCLK1. α -Syn protein levels were measured by western blot to determine which constructs could rescue the effect of the shRNA. Each data point represents independent sets. (B, F= 31.6, $P<0.0001$; C,F=15.97; $P=0.004$; D,F=7.877, $P=0.005$; E,F=15.21, $P=0.0004$) Error bars represent SEM. One-way ANOVA, followed by Dunnet's multiple comparison test NS \geq 0.05, *, **, ***, **** $P\leq$ 0.05, $P\leq$ 0.01, $P\leq$ 0.001, and $P\leq$ 0.0001, respectively.

Figure 5: Doublecortin like kinase 1 knockdown reduces α -Synuclein levels via lysosomal degradation. **A)** Western blot of HEK293T samples transfected with two *DCLK1* siRNAs. siDCLK1-A was selected for further experiments. (siDCLK1-A, $t=24.97$, $P<0.0001$; siDCLK1-B, $t=7.659$, $P<0.0003$) **B)** 100nM of Bafilomycin-A1 was used to inhibit lysosomal activity for 18 hours prior to cell lysis. α -Syn levels (MJFR1) were measured by western blot 72 hours after siRNA transfection. LC3 immunoblot shows the expected increase in LC3-II after treatment with the drug. (F=6.211, $P=0.0345$) **C)** 1000nM of MG132 was used to inhibit proteasomal activity for 12 hours prior to cell lysis. α -Syn levels (MJFR1) were measured by western blot 72 hours after siRNA transfection. Immunoblot of poly-ubiquitinated proteins is shown to demonstrate the efficacy of the drug (F=14.41, $P=0.0033$). Error bars represent SEM. Student's t-test (A) and One-way ANOVA, followed by Dunnet's multiple comparison test (B-C) NS \geq 0.05, *, **, ***, **** $P\leq$ 0.05, $P\leq$ 0.01, $P\leq$ 0.001, and $P\leq$ 0.0001, respectively.

Figure 6: Doublecortin kinase 1 knockdown preferentially reduces pS129- α -Synuclein in a mouse model of synucleinopathy. **A)** Diagram of experimental strategy. Thy1- α -Syn females were bred to male wild type mice. Neonatal intraventricular injections of AAVs carrying shRNA were carried out on their pups and male transgenic mice were sacrificed at 2 months of age. The posterior cortex and hippocampus were dissected and used for all experiments **B)** Measurement of the proportion of AAV infected cells in the posterior cortex by immunofluorescence. The number of YFP cells was divided by the number of Neurofilament-M (NF-M) positive cells in each section ($t=0.1199$, $P=0.9058$). **C)** Western blot measurement of Dclk1 levels from Thy1- α -Syn animals after shRNA mediated *Dclk1* knockdown. (Full length, $t=9.706$, $P=0.0002$; Kinase domain, $t=4.042$, $P=0.0099$). **D)** Representative IF images from the cortex of Thy1- α -Syn animals for verification of Dclk1 knockdown. Dclk1 mean fluorescence intensity was measured in the soma of neurons and normalized to mean fluorescence intensity of NF-M for quantification. ($t=5.972$, $P<0.001$) **E)** Western blots of pS129- α -Syn protein levels ($t=3.419$, $P=0.0189$). **F)** Western blots of human α -Syn (MJFR1) and total α -Syn (BD42) protein levels (Human α -Syn, $t=0.9493$, $P=0.3791$; Total α -Syn, $t=1.347$, $P=0.2266$). **G)** Endogenous Dclk1 immunoprecipitation in the Thy1- α -Syn brain followed by immunoblot for α -Syn species. pS129- α -Syn (D1R1R), Human α -Syn (MJFR1), and total α -Syn (BD42) were detected upon Dclk1 pulldown. All IF images were taken at 63X, and data points represent the individual sections, $n=3-4$ per condition. For western blotting and qPCR each data point represents individual animals. Error bars represent SEM. Student's t-Test, NS ≥ 0.05 , *, **, ***, **** denote $P\leq 0.05$, $P\leq 0.01$, $P\leq 0.001$, and $P\leq 0.0001$, respectively.

Figure 7: Knockdown of Doublecortin like kinase 1 preferentially affects α -Synuclein species in the soma of Thy1- α -Syn mouse cortex. **A)** Colocalization analysis of pS129- α -Syn (D1R1R), and overexpressed human α -Syn (15G7) with markers for neuronal fibers (Synaptophysin) and neuronal soma

(Neurofilament-M and YFP) (neuronal fibers, $t=8.067$, $P=0.0005$; Soma NF-M, $t=6.881$, $P=0.001$; Soma YFP, $t=7.906$, $P=0.0005$). **B)** Representative images (top) and quantification (bottom) of mean fluorescence intensity of pS129- α -Syn in neuronal soma relative to NF-M ($t=5.181$, $P<0.0001$). **C)** Representative images (top) and quantification (bottom) of mean fluorescence intensity of human- α -Syn in neuronal soma relative to NF-M ($t=2.239$, $P=0.0433$). **D)** Representative images and quantification of raw integrated fluorescence intensity of pS129- α -Syn in neuronal fibers normalized to Synaptophysin. The fluorescence from cell bodies was subtracted prior to analysis ($t=0.637$, $P=0.5331$). **E)** Representative images and quantification of raw integrated fluorescence intensity of human- α -Syn in neuronal fibers normalized to Synaptophysin. The fluorescence from cell bodies was subtracted prior to analysis ($t=0.1854$, $P=0.8549$). All IF images were taken at 63X, 3-4 animals were used per condition, and data points represent the individual sections (3 sections per animal). Error bars represent SEM. Student's t-Test, NS ≥ 0.05 , *, **, ***, **** denote $P\leq 0.05$, $P\leq 0.01$, $P\leq 0.001$, and $P\leq 0.0001$, respectively.

Extended data Figure 7-1: Immunofluorescence controls for pS129 α -Syn and human α -Syn specific antibodies in the mouse brain. **A)** Only a small amount of background is visible in the cortex of wild type mice when stained with anti-pS129 α -Syn (D1R1R). **B)** Staining in the cortex of a Thy1- α -Syn transgenic animal. Note that abundant signal is detected in neuronal soma. **C)** Wild type mouse midbrain stained with anti-human α -Syn (15G7), no signal is detected. **D)** Thy1- α -Syn transgenic mouse midbrain stained with anti-human α -Syn (15G7). There is both clear punctate (synaptic compartment) and somatic signal. This somatic signal is more obvious than in the cortical sections used for shRNA knockdown experiments. All images taken with an epifluorescence microscope at 20X. A, and B have been zoomed in to emphasize neuronal soma.

Figure 8: Stereotaxic injection of shDclk1 AAVs was used to knockdown *Dclk1* and overexpress human α -Synuclein in the SNc of adult mice. **A)** Representative 20X IF images showing proper targeting of the SNc. Infected dopaminergic neurons are labeled with anti-TH antibody and YFP from the virus. **B)** Measurement of Dclk1 knockdown in the SNc. Dclk1 mean fluorescence intensity was normalized to TH signal in sections of animals infected with either shLuciferase or shDclk1 Student's t-Test, *** denotes $P \leq 0.001$ ($t=10.03$, $P=0.0006$). Images are 63X. **C)** Representative 20X scans of ipsilateral (injected) and contralateral (uninjected) midbrain after AAV-SNCA injection. The needle tract (yellow box) shows human α -Syn expression (15G7) in YFP positive cells. There is a clear reduction in TH positive cells in the injected SNc (green box) when compared to the contralateral SNc (red box). **D)** 63X IF images from the areas described in C). Error bars represent SEM.

Figure 9: Doublecortin kinase 1 knockdown rescues α -Synuclein induced dopaminergic neurotoxicity. **A)** Diagram of the experiment. Wild type mice were injected in the SNc with AAVs carrying *SNCA* or empty vector and shLuciferase or shDclk1. Two months later the tissues were harvested to measure the degeneration of dopaminergic neurons and the striatal fibers. **B)** Representative images taken at 20X Substantia nigra pars compacta (SNc) TH staining for dopaminergic neurons of ipsilateral sections injected with either shLuciferase + empty vector, shDclk1+ empty vector, shLuciferase+*SNCA* or shDclk1+*SNCA* compared to the corresponding contralateral uninjected sections. **C)** Representative images of striatal (TH positive) fibers from the same animals taken at 20X. **D)** Stereological counts of dopaminergic neurons (TH positive) in the substantia nigra parts compacta (SNc) of ipsilateral sections injected with either shLuciferase + empty vector, shDclk1+empty vector, shLuciferase+*SNCA* or shDclk1+*SNCA* compared to the corresponding contralateral uninjected sections (and each other). Each data point represents a single animal ($F= 6.584$, $P<0.0001$). **E)** Densitometric measurement of Tyrosine hydroxylase fibers in the striatum from the same animals. Each data point represents a single animal ($F=4.303$, $P=0.0013$). Error bars represent SEM. One-way ANOVA followed by Dunnett's multiple

comparisons test $NS \geq 0.05$, *, **, ***, **** denote $p \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, and $P \leq 0.0001$, respectively.

Figure 10: Doublecortin like kinase 1 knockdown reduces α -Synuclein levels in human neurons derived from a patient with *SNCA* triplication. **A)** Representative immunofluorescence images of GABA and β III-Tubulin staining in H9 neurons, clone NN0000049 (*SNCA* Triplication clone 1), clone NN0003871 (*SNCA* Triplication clone 2) and its respective isogenic control. **B)** Representative immunofluorescence images of Vglut1 and MAP2 staining in H9 neurons, clone NN0000049 (*SNCA* Triplication clone 1), clone NN0003871 (*SNCA* Triplication clone 2) and its respective isogenic control. **C)** Measurement of RNA and protein *SNCA* levels of clone 1 of *SNCA* triplication patient neurons using qPCR and western blotting after neuronal differentiation compared to H9 ESC derived neurons. Both pS129 (D1RIR) and total α -Syn were measured (BD42) (RNA, Student's t-test, $t=5.35$, $P=0.0005$; Protein pS129- α -Syn, Student's t test, $t=4.326$, $P=0.0035$; Human- α -Syn, Student's t test, $t=7.815$, $P<0.0001$). **D)** Measurement of RNA and protein *SNCA* levels of clone 2 using qPCR and western blotting in directly converted neurons compared to their isogenic corrected control. Both pS129 (D1RIR) and total α -Syn were measured (BD42). (RNA, Student's t-test, $t=3.333$, $P<0.0157$; Protein pS129- α -Syn, Student's t test, $t=6.542$, $P<0.0001$; Human- α -Syn, Student's t test, $t=8.138$, $P<0.0001$). **E)** Western blot measurement of pS129- α -Syn (D1RIR), total α -Syn (BD42) and DCLK1 levels in differentiated *SNCA* triplication patient (clone 1) neurons after shRNA mediated *DCLK1* knockdown (pS129- α -Syn, One way ANOVA, $F=73.45$, $P<0.0001$; Human- α -Syn, One way ANOVA, $F=21.68$, $P<0.0001$; Full length DCLK1, $F=7.992$, $P=0.0018$; Kinase domain, $F=53.49$, $P<0.0001$). Each data point represents independent sets of neurons. Error bars represent SEM. One-way ANOVA, followed by Dunnet's multiple comparisons test $NS \geq 0.05$, *, **, ***, **** $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, and $P \leq 0.0001$, respectively.

1129 **Figure 11: Doublecortin like kinase 1 regulates somatic α -Synuclein levels in a lysosomal dependent**
1130 **manner.** α -Syn binds vesicles in the pre-synaptic compartment where Dclk1 is not present. The proteins
1131 interact in the soma where Dclk1 stabilizes α -Syn protein levels. Dclk1 knockdown interrupts this
1132 interaction and causes a reduction in levels via lysosomal degradation.
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