**H63D variant of the homeostatic iron regulator (*HFE)* gene alters α-synuclein expression, aggregation, and toxicity**

Yunsung Kim1, Mark C. Stahl1, #, Xuemei Huang1,2,3, James R. Connor2, 3, \*

1Department of Neurology, Penn State College of Medicine, Hershey, PA, USA

2Department of Neurosurgery, Penn State College of Medicine, Hershey, PA, USA

3Translational Brain Research Center, Penn State College of Medicine, Hershey, PA, USA

#Present address: Neurocrine Biosciences, San Diego, CA, USA

\*Corresponding author: 500 University Drive, Hershey, PA; jconnor@pennstatehealth.psu.edu

**Abstract**

Pathological features of Parkinson’s disease include the formation of Lewy bodies containing α-synuclein and the accumulation of iron in the substantia nigra. Previous studies have suggested that iron accumulation contributes to the Parkinson’s disease pathology through reactive oxygen species production and accelerated α-synuclein aggregation. The current study examines the effects of commonly occurring H63D variant of the homeostatic iron regulatory (*HFE)* gene on α-synuclein pathology in cell culture and animal models. H63D *HFE* expression in SH-SY5Y cells lowered endogenous α-synuclein levels and significantly decreased pre-formed fibril induced α-synuclein aggregation. H63D *HFE* cells were also protected from pre-formed fibril induced apoptosis. Autophagic flux, a major pathway for α-synuclein clearance, was increased in H63D *HFE* cells. Expression of REDD1 was elevated and rapamycin treatment was unable to further induce autophagy, indicating mTORC1 inhibition as the main mechanism of autophagy induction. Moreover, siRNA knockdown of REDD1 in H63D *HFE* cells decreased autophagic flux and increased the sensitivity to PFF mediated toxicity. While iron chelator (deferiprone) treatment rescued WT *HFE* cells from pre-formed fibril toxicity, it exacerbated or was unable to rescue H63D *HFE* cells. In the *in vivo* pre-formed fibril intracranial injection model, H67D *Hfe* (mouse homolog of the human H63D *HFE* variant) C57BL/6J × 129 mice showed less α-synuclein aggregation and less decline in motor function compared to WT *Hfe*. Collectively, this study suggests that H63D *HFE* variantmodifies α-synuclein pathology through the induction of autophagy and has the potential to impact the pathogenesis and treatment response in Parkinson’s disease.

**Keywords**

Parkinson’s disease, α-synuclein, iron, HFE, deferiprone

**Abbreviations**

CTF = corrected total fluorescence, DFP = deferiprone, IRE = iron response element, IRP = IRE binding protein, PFF = pre-formed fibril, RRID = Research Resource Identifier (see scicrunch.org), ThS = Thioflavin S, ThT = Thioflavin T

**Introduction**

Iron accumulation is a consistent feature of Parkinson’s disease pathology. Multiple post-mortem and MRI based studies have shown that compared to healthy controls, patients with Parkinson’s disease have higher iron in the basal ganglia (Dexter *et al.* 1983; Wang *et al.* 2016; Dexter *et al.* 1991; Chen *et al.* 1993). Iron is an important cofactor for many cellular processes and its intracellular concentration is tightly regulated due to potential production of reactive oxygen species (ROS) through the Fenton reaction (Winterbourn 1995; Beard *et al.* 1996). In Parkinson’s disease, excess iron is thought to contribute to increased expression and aggregation of α-synuclein, the main protein component of Lewy bodies (Xiao *et al.* 2018; Abeyawardhane *et al.* 2018; Wang *et al.* 2019). The role of iron in Parkinson’s disease progression is under active clinical investigation in ongoing studies of an iron chelator, deferiprone, as a potential therapeutic option for Parkinson’s disease (Devos *et al.* 2014; Martin-Bastida *et al.* 2017).

α-Synuclein oligomers and fibrils are widely considered to be the main toxic components of Parkinson’s disease pathology (Winner *et al.* 2011; Rockenstein *et al.* 2014). Neurons containing aggregated α-synuclein demonstrate loss of normal neurotransmission and disruption of multiple cellular processes including protein degradation pathways and mitochondrial function (Wong and Krainc 2017). Various models of α-synuclein aggregation have been developed to study its toxicity. α-Synuclein pre-formed fibrils (PFFs) are derived from aggregated recombinant α-synuclein monomers. Injection of PFFs into non-transgenic mice have been observed to induce prion-like spread of α-synuclein aggregates by ‘seeding’ recruitment of endogenous soluble α-synuclein (Volpicelli-Daley *et al.* 2011; Luk *et al.* 2009; Volpicelli-Daley *et al.* 2014).

Autophagy is one of the main pathways for clearance of aggregated α-synuclein and has been shown to be a vital homeostatic pathway in terminally differentiated cells such as neurons (Vogiatzi *et al.* 2008; Hara *et al.* 2006; Komatsu *et al.* 2006). Macroautophagy is the ‘classical’ autophagy pathway involving autophagosome formation followed by fusion with the lysosome. This study focuses on macroautophagy as the pathway of α-synuclein degradation. Nutrient-sensitive mTORC1 is the main negative regulator of autophagy through its interaction with autophagy initiating ULK1 complex (Jung *et al.* 2009; Heras-Sandoval *et al.* 2014). REDD1 inhibits mTORC1 and induction of REDD1 expression has been associated with hypoxia, ER stress, and iron chelation (Ohyashiki *et al.* 2009; Dennis *et al.* 2013; Whitney *et al.* 2009). Autophagy has been a focal point of investigations in the context of Parkinson’s disease pathogenesis in part due to the high prevalence of glucocerebrosidase (*GBA*)mutations in the Parkinson’s disease population. *GBA* mutation is the most common genetic risk factor for Parkinson’s disease (Gan-Or *et al.* 2015; O’Regan *et al.* 2017). Dysfunction of glucocerebrosidase causes lysosomal storage disease (Gaucher’s) and has been shown to promote α-synuclein accumulation through autophagic inhibition (Du *et al.* 2015).

The direct effects of iron on α-synuclein homeostasis has been postulated through the identification of a putative iron response element at the 5’-UTR of α-synuclein mRNA along with metal binding sites located on the protein (Friedlich *et al.* 2007; Febbraro *et al.* 2012). However, the impact of genes involved in iron regulation on Parkinson’s disease risk and molecular pathology is poorly understood. Polymorphisms in the *HFE* gene which encodes for the homeostatic iron regulator protein, HFE, have been identified to be a disease modifier in multiple neurodegenerative diseases including amyotrophic lateral sclerosis and Alzheimer’s disease (Nandar *et al.* 2014; Su *et al.* 2013; Wang *et al.* 2004; Ali-Rahmani *et al.* 2014b). HFE limits iron uptake into cells through its interaction with the transferrin receptor (Barton *et al.* 2015). *HFE* variants were first identified to be associated with hereditary hemochromatosis (HH), which is an iron overloading disease that affects the liver, heart, pancreas, and the skin (Simon *et al.* 1976). C282Y is the most common variant associated with HH, but it has a low prevalence in the general population ranging from 0 to 9.2% (Hanson *et al.* 2001). The highly prevalent H63D *HFE* variant, which is estimated to be present in 13.5% of the US population and 5-22% across the world, is rarely associated with HH but has been shown to alter brain iron metabolism (Steinberg *et al.* 2001; Nandar *et al.* 2013; Hanson *et al.* 2001). Furthermore, H67D *Hfe* (homologous to human H63D *HFE*) expression in mice reportedly protects dopaminergic neurons from paraquat toxicity, indicating a potentially protective role of this *HFE* variant in Parkinson’s disease (Nixon *et al.* 2018).

In this study, we investigated the effects of the H63D *HFE* variant on α-synuclein metabolism *in vitro* and *in vivo* using pre-formed fibrils as a model of Parkinson’s disease. We also tested the effects of an iron chelator, deferiprone, on the toxicity of α-synuclein PFFs in cells expressing WT and H63D *HFE*.

**Materials and Methods**

**Cell culture**

This study was not pre-registered. SH-SY5Y neuroblastoma cells transfected to stably express WT or H63D *HFE* were used for all experiments (Lee *et al.* 2007). Dr. Sang Lee, Ph.D. (Penn State College of Medicine) kindly provided the cells. SH-SY5Y are not listed as a commonly misidentified cell line by the ICLAC. The parental SH-SY5Y cells were last authenticated on 1/18/19 through Genetica. *HFE* gene transfection and expression were confirmed using PCR and western blot using FLAG/HFE antibodies. Western blots of whole cell homogenates were also used to identify any differences in protein expression. Cells were cultured in accordance with guidelines from American Type Culture Collection (ATCC). Briefly, cells were cultured in DMEM/F12 medium (Invitrogen; Cat no: 11330032) containing 10% fetal bovine serum (HyClone; Cat no: SH30071.03IH25-40), 100 U/mL penicillin-streptomycin (Gibco; Cat no: 15140122), MEM non-essential amino acids (Gibco; Cat no: 11140050), and 250ug/mL Geneticin (Gibco; Cat no: 10131027). Cells were incubated in 5% CO2 at 37oC and media was changed every 3-5 days. Cells were passaged for a maximum of ten passages.

**Calcein assay**

Calcein-AM (Sigma; Cat no: 17783) was used to measure the labile iron pool in SH-SY5Y cells. Cells were seeded in 6 well plates and treated with 0.15uM Ca-AM in assay buffer (PBS, 1mg/mL bovine serum albumin, 20mM HEPES, pH 7.3) for 10 min in 37oC. Cells were trypsinized, washed, and resuspended with 1mL of assay buffer. 100uL of cell suspension was added per well to a 96 well plate and fluorescence was measured (SpectraMax Gemini EM; excitation 488nm, emission 518 nm). Remaining cell suspension was spun down at 8000 rcf for 3 min and cell pellet was lysed with RIPA lysis buffer (150mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 7.4) plus Roche Complete Protease Inhibitor tablet (Cat no: 11697498001). Total protein concentrations of the lysates were measured using the Qubit Protein Assay Kit (Invitrogen; Cat no: Q33212).

**ELISA and Western blots**

α-Synuclein ELISA was performed using Invitrogen Human α-synuclein ELISA kit (Cat no: KHB0061). Cells were lysed in Cell Extraction Buffer (150mM NaCl, 1.0% Triton x-100, 50mM Tris, pH 7.4, 1mM PMSF, Roche Complete Protease Inhibitor tablet) by incubation on ice for 30 min with occasional vortexing. Lysates were centrifuged at 13,000xg, 4oC, for 10 minutes. Total protein concentration was quantified using the Qubit Protein Assay Kit. Protocol from the ELISA kit was followed and 96 well plate was read with SpectraMax 340pc. For western blots, cells were washed twice with cold PBS and collected using cell scrapers. RIPA buffer with Roche protease inhibitor (Cat no: 11697498001) and phosphatase inhibitor (Roche; Cat no: 4906845001) was used for lysis. Samples were incubated on ice for 30 min, sonicated, and centrifuged at 16,000 xg for 20 min. Total protein concentrations of the lysates were measured using the Qubit Protein Assay Kit. Loading dye and reducing agent from SDS-Sample Prep Kit (Thermo; Cat no: 8988) were added and the lysates were boiled at 70oC for 10 min. For PFF treated cell lysates, reducing agent was omitted in sample preparation. Samples were separated on 4-12% Bis-Tris gel (Life Technologies; Cat no: NP0321BOX) in NuPAGE MES SDS Running Buffer (Invitrogen; Cat no: NP0002) and transferred to PVDF membrane using the iBlot2 transfer system (Invitrogen; Cat no: IB24001) at P3 setting. PageRuler Plus Prestained Protein Ladder (ThermoFisher; Cat no: 26620) was loaded along with the samples. For α-synuclein blots, membrane was then incubated in 0.4% paraformaldehyde for 30 min to increase the signal as previously published (Lee and Kamitani 2011). Membranes were block in 5% milk or 5% BSA in TBST (25mM Tris, 150mM NaCl, 0.1% Tween 20, pH 7.5) for 1 hour. Primary antibody (Novus α-Synuclein 2A7, BD Biosciences α-Synuclein Syn1, Cell Signaling H-ferritin, Cell Signaling LC3B D11, ProteinTech REDD1, ProteinTech β-actin) was then added for overnight incubation at 4oC, followed by washes with TBST. Appropriate secondary antibody (Goat anti-mouse IgG HRP conjugated secondary, Goat anti-rabbit IgG HRP conjugated secondary) was added for 1.5 hour at room temperature. Detailed list of antibodies can be found in **Table 1**. Pierce ECL western blot substrate (Thermo; Cat no: 32209) was used for detection. Images were acquired with GE Amersham Imager 600. Band densities were quantified using ImageJ version 1.52a.

**Table 1. List of antibodies**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Name** | **Host** | **Type** | **Dilution** | **Citation** |
| α-Synuclein [2A7] | Mouse | Monoclonal | WB – 1:1000  ICC – 1:1000 | RRID: AB\_1555287  Novus Cat# NBP1-05194 |
| α-Synuclein [Syn1] | Mouse | Monoclonal | WB – 1:500 | RRID: AB\_398107  BD Biosciences Cat# 610786 |
| α-Synuclein [EPR20535] | Rabbit | Monoclonal | IHC – 1:5000 | Abcam Cat# ab212184 |
| Phospho Ser129 α-Synuclein | Rabbit | Monoclonal | IHC – 1:500 | RRID: AB\_869973  Abcam Cat# ab51253 |
| H-ferritin [D1D4] | Rabbit | Monoclonal | WB – 1:1000 | RRID: AB\_11217441  Cell Signaling Technology Cat# 4393 |
| LC3B [D11] | Rabbit | Monoclonal | WB – 1:1000 | RRID: AB\_2137707  Cell Signaling Technology Cat# 3868 |
| LC3B | Rabbit | Polyclonal | ICC – 1:1000 | RRID: AB\_10003146  Novus Cat# NB100-2220 |
| REDD1 | Rabbit | Polyclonal | WB – 1:500 | RRID: AB\_2245711  Proteintech Cat# 10638-1-AP |
| ULK1 [D8H5] | Rabbit | Monoclonal | WB – 1:1000 | RRID: AB\_11178668  Cell Signaling Technology Cat# 8054 |
| Phospho Ser757 ULK1 | Rabbit | Polyclonal | WB – 1:500 | RRID: AB\_10829226  Cell Signaling Technology Cat# 6888 |
| β-actin | Mouse | Monoclonal | WB - 1:10,000 | RRID: AB\_2289225  Proteintech Cat# 60008-1-Ig |
| Goat Ant-Mouse IgG - H&L (HRP) | Goat | Polyclonal | WB – 1:2500 | RRID: AB\_955439  Abcam Cat# ab6789 |
| Goat Anti-Rabbit IgG-H&L (HRP) | Goat | Polyclonal | WB – 1:2500 | RRID: AB\_524669  Novus Cat# NB 7160 |
| Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) | Goat | Polyclonal | ICC – 1:2000  IHC – 1:1000 | RRID: AB\_2630356  Abcam Cat# ab150077 |
| Goat Anti-Mouse IgG H&L (Alexa Fluor® 568) | Goat | Polyclonal | ICC – 1:2000 | Abcam Cat# ab175473 |
| Biotinylated Anti-Rabbit IgG | Goat | Polyclonal | IHC – 1:200 | RRID: AB\_2336820  Vector Laboratories Cat# PK-6101 |

**α-Synuclein pre-formed fibrils**

Purified α-synuclein monomers for PFF fibrilization was purchased from Proteos, Inc (Cat no: RP-003) and PFFs were formed according to the provided protocol. α-Synuclein protein products from Proteos, Inc. are made in partnership with the Michael J. Fox Foundation and protein quality is monitored by SDS-PAGE, HPLC-SEC, and LAL assays. Briefly, monomer was diluted to 5mg/ml with final buffer composition of 100mM NaCl, 10mM Phosphate, pH 7.2-7.6 and constantly agitated at 600rpm, 37oC for 7 days. Fibril formation was confirmed using Thioflavin T (Sigma; Cat no: T3516) fluorescence assay and sedimentation followed by gel electrophoresis (**Supplemental Fig. 1A, Supplemental Fig. 1B**). Thioflavin T (final concentration 0.25uM dissolved in PBS) was added to α-synuclein monomer, α-synuclein PFF, and PBS only and incubated at room temperature for 15 minutes in a 96 well plate. Fluorescence was measured using SpectraMax Gemini EM plate reader (excitation 440nm, emission 482nm). Sedimentation was performed by centrifugation at 100,000xg for 30 min. Samples were loaded on 4-12% Bis-Tris gel (Life Technologies; Cat no: NP0321BOX) and ran at 200V for 25min. Gels were stained with coomassie blue (BioRad; Cat no: 161-0436). α-Synuclein PFFs were aliquoted and stored in -80oC until use. For SH-SY5Y cell treatment with PFFs, cells were seeded in 6 well plates (~1x106 cells/ well). Cells were seeded on coverslips for immunocytochemistry. α-Synuclein PFFs were thawed at room temperature, diluted to 0.1mg/mL with sterile DPBS, and sonicated (1s pulses at 10% power for 5 minutes). Sonicated PFFs were diluted with cell culture media then added to 6 well or 96 well plates. The sonication step is expected to produce a heterogeneous population of fibrils ranging in length from 20 to 100nm (Patterson *et al.* 2019).

**Immunocytochemistry and image analysis**

Cells were grown on coverslips and fixed with 4% paraformaldehyde (in PBS) for 10 min at room temperature. Fixed cells were washed 3 times with cold PBS then permeabilized with 0.1% Triton X-100 in PBS for 20 min. Coverslips were washed 3 times with PBST (PBS with 0.1% Tween 20) and blocked in 5% BSA in PBST for 1 hour at room temperature. Primary antibody (Novus α-Synuclein 2A7, Novus LC3B) incubation was overnight at 4oC (**Table 1**). Thioflavin S (Sigma; Cat no: T1892) was dissolved in 1:1 ethanol/PBS solution. Coverslips were incubated in 0.025% ThS for 8 min and washed 3 times with 80% ethanol/PBS solution. Appropriate secondary antibodies (Goat anti-rabbit IgG Alexa Fluor 488 secondary, Goat anti-mouse IgG Alexa Fluor 568 secondary) were added for 1 hour at room temperature, then finally washed before being mounted on microscope slides with Vectashield antifade mounting medium with DAPI (Cat no: H-1200). LC3 images were taken with confocal microscope (Nikon Eclipse FN-1/C2+). Slides were labeled by numeric code for staining, image acquisition, and data analysis to blind the experimenter. α-Synuclein and ThS fluorescence images were taken with Echo Revolve hybrid upright-inverted microscope. LC3-positive puncta/cell and fluorescence intensities were quantified using ImageJ version 1.52a (Kimura *et al.* 2009). Corrected total fluorescence (CTF) was calculated by using the formula: [integrated density – (selected area \* mean fluorescence of background)]/ number of cells in the selected area.

**Autophagy, Cell viability, and apoptosis assays**

Autophagic flux was measured using LC3 western blot and immunofluorescent staining. Cells were treated with 100nM Bafilomycin A1 (Sigma; B1703) for 4hr or 50uM Chloroquine (Sigma; Cat no: C6628) for 2.5 hrs prior to lysis. Cell viability was measured using ATP-based CellTiter-glo assay (Promega; Cat no: G7570) and 96 well plate was read with SpectraMax Gemini EM plate reader. Caspase 3/7 activity was measured with Caspase-glo assay (Promega; Cat no: G8090). For Annexin V-FITC/ 7-AAD flow cytometry experiments, cells were treated seeded in 6 well plates and treated with 1.5uM α-synuclein monomer or 1.5uM monomer + 5uM PFF for 7 days. Cells were trypsinized, washed with PBS, and resuspended with Annexin binding buffer (BioLegend; Cat no: 422201). Samples were incubated with Annexin V-FITC stain (Biolegend; Cat no: 640905) for 15 min at room temperature. 5ul of 7-AAD (BioLegend; Cat no: 420403) was added for each 500uL sample. 10-color BD FACSCanto (BD Biosciences) in Penn State College of Medicine’s Flow Cytometry Core was used to detect Annexin V-positive cells.

**esiRNA knockdown**

WT and H63D *HFE* SH-SY5Y cells were transfected with REDD1 esiRNA (Sigma; Cat no: EHU009051) or control EGFP esiRNA (Sigma, Cat no: EHUEGFP) using Lipofectamine RNAiMAX (ThermoFisher, Cat no: 13778100). esiRNAs are a heterogenous mixture of siRNAs that target the same cDNA sequence (**Table 2**). siRNAs and lipofectamine were diluted in Opti-MEM (ThermoFisher, Cat no: 31985062). 250uL of 100nM esiRNA in Opti-MEM + Lipofectamine solution was added to each well of a 6 well plate. 48 hours after treatment, cells were trypsinized and seeded for further experiments. To assess autophagic flux, cells were treated with Bafilomycin A1 as described above and harvested for lysis. For PFF toxicity experiments, esiRNA treated cells were exposed to 1.5uM α-synuclein monomer or 1.5uM monomer + 5uM PFF for 5 days. Cell viability was measured using an ATP-based CellTiter-glo assay (Promega; Cat no: G7570).

**Table 2. esiRNA target sequences**

|  |  |
| --- | --- |
| **esiRNA** | **Target Sequence** |
| **EGFP** | GTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAA  CGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGT  TCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGC  AGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCT  ACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCG  AGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTG  GGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGG  CATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACC  AGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCG  CCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGA  TCACTCTCGGCATGGACGAGCTGTA |
| **REDD1** | ACTACTGCGCCTGGCCTACAGCGAGCCGTGCGGCCTGCGGGGGGCGCTGCTGGACGTCTGCGTGGAGCA  GGGCAAGAGCTGCCACAGCGTGGGCCAGCTGGCACTCGACCCCAGCCTGGTGCCCACCTTCCAGCTGAC  CCTCGTGCTGCGCCTGGACTCACGACTCTGGCCCAAGATCCAGGGGCTGTTTAGCTCCGCCAACTCTCCC  TTCCTCCCTGGCTTCAGCCAGTCCCTGACGCTGAGCACTGGCTTCCGAGTCATCAAGAAGAAGCTGTACA  GCTCGGAACAGCTGCTCATTGAGGAGTGTTGAACTTCAACCTGAGGGGGCCGACAGTGCCCTCCAAGAC  AGAGACGACTGAACTTTTGGGGTGGAGACTAGAGGCAGGAGCTGAGGGACTGATTCCTGTGGTTGGAAA  ACTGAGGCAGCCACCTAA |

**Mouse α-synuclein PFF intracranial injections**

Mouse PFFs were prepared using the same protocol as the human PFFs described above. After dilution (to 2.5mg/ml) and sonication of the fibrils, 3-month-old C57Bl/6J x 129 male mice expressing wild-type or H67D *Hfe* (RRID:IMSR\_JAX:023025) were stereotactically injected with the 5ug PFF (Nandar *et al.* 2013; Luk *et al.* 2009). H67D *Hfe* is the mouse homolog to the human H63D *HFE* variant. H67D is a 199C to G point mutation in exon 2 of mouse *Hfe* and mice expressing H67D *Hfe* have been shown to have increased hepatic iron loading and altered brain iron profiles (Nandar *et al.* 2013; Tomatsu *et al.* 2003). No randomization method was used for allocation of mice because all animals received the PFF injection. Mice were anesthetized using 5% isoflurane mixture and maintained under anesthesia using a 1-2% mixture during the entirety of the procedure. After shaving the head, povidone-iodine was used to disinfect the area. Mice were mounted on the stereotaxic frame and corneal lubrication was applied. Total of 14 mice were injected (WT *Hfe*: 4 males, 3 females; H67D *Hfe*: 3 males, 4 females) with PFF in the right striatum and PBS in the left striatum (+0.2 mm relative to Bregma, +2.0 mm lateral to midline, +2.6 mm beneath the dura) (**Fig. 1**). Injection was performed using a 22-gauge needle and syringe (Hamilton; Cat no: 80285) at an approximate rate of 0.1uL per min for total of 2.5uL per injection site. The needle was in place for >5 min at each target. Bone wax was applied to prevent any leakage and the skin was sutured. Mice were allowed to recover in a 37oC environment before transportation. Subcutaneous buprenorphine (0.05mg/kg) was administered for analgesia. Mice were monitored daily for 7 days post-surgery to assess for acute distress. From then on, mouse weights were checked once per month until the endpoint of 3 months post-injection. There were no predetermined exclusion criteria (no exclusions were made) and all 14 mice what were initially injected survived until the endpoint. The mice were individually housed in standard mouse cages at the in-house animal facility of Penn State College of Medicine. Mice had access to food and water *ad libitum*. Penn State College of Medicine Institutional Animal Care and Use Committee approved all procedures (PRAMS200747464).

**Rotor rod motor behavior assessment**

At 1 week (baseline), 1 month, 2 months, and 3 months post-injection, all mice underwent rotor rod (Columbus Instruments, Columbus, OH, USA) testing to assess their motor function (**Fig. 1**). Behavioral tests were conducted in the morning (9-11am). To blind the person conducting the behavioral test to the mouse genotype, each mouse was assigned a number and marked by ear notching prior to the first rotor rod session. The corresponding genotype for each mouse was unmasked for data analysis at endpoint. Mouse weights were recorded prior to each rotor rod session. No significant difference in weight between genotypes were observed (data not shown). Before each set of mice, rotor rod apparatus was cleaned with 70% ethanol to minimize odor cues. Total session lasted for 250s and speed was ramped up from 4rpm to 35rpm uniformly. Time to first fall and total number of falls during each session was recorded. Training sessions were completed 4 days and 2 days prior to the actual recorded rotor rod session using the same settings as described.

**Immunohistochemistry**

At the endpoint (3 months post-injection), mice were injected with ketamine (100mg/kg)/xylazine (10mg/kg) cocktail and perfused with Ringer’s solution. Brains were removed and preserved with 4% paraformaldehyde in 0.1M PBS. Paraformaldehyde was replaced with 70% ethanol, then tissue was paraffin-embedded and sectioned into 5um slices. Slides were coded using the mouse number as described above to blind the experimenter during staining, image acquisition, and data analysis. Slides were deparaffinized, rehydrated, and incubated in sodium citrate buffer for antigen retrieval. Sections were blocked with 2% milk in PBS for 1hr at room temperature. Primary antibody incubation was performed overnight at 4°C. Alexa488 conjugated anti-rabbit secondary antibody or biotinylated anti-rabbit secondary antibody (**Table 1**) was added and immunoreactivity was detected with 3,3’-diaminobenzidine Peroxidase (HRP) substrate kit (Vector Laboratories; Cat no: SK-4100). Slides were imaged with Echo Revolve hybrid upright-inverted microscope. Corrected total fluorescence (CTF) was calculated by using the formula: [integrated density – (selected area \* mean fluorescence of background)]/ corrected DAPI intensity in selected area.

**Statistical analysis**

Sample size for the animal study was calculated using data from Luk, et al. 2009, which showed about 15% difference in time to fall between control and 3 months post PFF injection. Alpha of 0.05 and beta of 0.2 was used to arrive at the calculated sample size of 7 per group (Unless otherwise noted, values presented in figures are means with standard errors of the mean. GraphPad Prism version 8.2.1 was used for statistical analyses. Student’s t-test or ANOVA with *post hoc* multiple comparisons test were used. No test for normality or outliers was performed because these tests lack power with low sample sizes. Statistical analysis method used for each figure is noted in the legend.

**Data availability**

Data that support the findings in this study are available upon request.

**Results**

**H63D HFE cells have increased iron and decreased α-synuclein expression**

The intracellular labile iron pool (LIP) was increased in SH-SY5Y neuroblastoma cells expressing H63D *HFE* compared to wildtype, as shown by the decrease in calcein fluorescence (**Fig. 2A**). Calcein fluorescence is quenched by iron and therefore is inversely related to LIP. H-ferritin expression, which is controlled by an iron response element and iron regulatory protein interaction at the 5’-UTR, was increased in H63D *HFE* cells (**Fig. 2B**). Collectively, these results confirm that intracellular iron is increased in the H63D *HFE* SH-SY5Y cells. The consequence of H63D *HFE* expression on α-synuclein expression was investigated by western blot (**Fig. 2C**) and ELISA (**Fig. 2D**), both of which demonstrated significantly lower α-synuclein level in H63D *HFE* cells compared to WT cells.

**H63D HFE decreases PFF induced α-synuclein aggregation and cell death**

α-Synuclein preformed-fibrils were used to induce α-synuclein aggregation in WT and H63D *HFE* expressing SH-SY5Y cells. PFF formation was confirmed by sedimentation and Thioflavin T fluorescence (**Supplemental Fig. 1A, Supplemental Fig. 1B**). Expression of putative receptors for α-synuclein PFFs in WT and H63D HFE cells were confirmed using western blot (**Supplemental Fig. 1C, Supplemental Fig. 1D**). Cells were treated with 1.5uM α-synuclein monomer or PFF for 7 days, then stained with Thioflavin S (ThS), anti-α-synuclein antibody, and 4′,6-diamidino-2-phenylindole (DAPI) stain as the nuclear stain (**Fig. 3A**). Fold change (PFF/monomer) in corrected total fluorescence (CTF) for ThS and α-synuclein was decreased for H63D *HFE* cells, indicating lower level of α-synuclein aggregation (**Fig. 3B**). CTF was calculated by subtracting background intensity from integrated fluorescence density. This was confirmed with time course experiments in which Thioflavin T fluorescence was measured from cell culture media collected 3, 5, and 7 days post-PFF treatment (**Fig. 3C**). By day 7 post-treatment, ThT fluorescence was significantly higher in media collected from WT *HFE* cells. Similarly, α-synuclein western blots were performed using whole-cell lysates prepared 3, 5, and 7 days post-PFF treatment (**Fig. 3D**). At all time-points, the total α-synuclein level was higher in WT *HFE* cells than in H63D *HFE* cells. PFF-induced cell death was also measured. Previous work (Mahul-Mellier *et al.* 2015) demonstrated that treatment of SH-SY5Y cells with α-synuclein PFFs results in measurable toxicity only in the presence of additional α-synuclein monomers; therefore, cells were treated with combinations of 1.5uM monomer and increasing concentrations of PFFs (1.5 – 10uM) for 7 days. A PFF dose-dependent decrease in cell viability was seen with both WT and H63D *HFE* cells (**Fig. 4A**). Viability was normalized to the group treated with α-synuclein monomer to account for potential differences in cell number. Notably, H63D *HFE* cells showed greater cell viability compared to wildtype. The lower levels of apoptotic cell death in H63D *HFE* cells occurred after 1.5uM monomer + 5uM PFF treatment was confirmed with caspase 3/7 activity assay (**Fig. 4B**) and annexin V-FITC/ 7-AAD flow cytometry (**Fig. 4C**). Fold change in caspase 3/7 activity and percent annexin V-positive cells after PFF treatment were significantly lower for H63D *HFE* cells than WT *HFE*.

**H63D HFE increases autophagy through REDD1 inhibition of mTORC1**

Autophagy is the main pathway for clearance of aggregated α-synuclein. Baseline autophagic flux in WT and H63D *HFE* SH-SY5Y cells was measured using autophagy inhibitors bafilomycin A1 (BafA1) and chloroquine (CQ). Cells were treated with 100nM BafA1 for 4hr or 50uM CQ for 2.5 hrs. Lysates were used for western blot against LC3B. LC3B-II band densities were quantified and autophagic flux was calculated by the difference in LC3B-II band density between control and autophagy inhibitor treated groups normalized to β-actin. Autophagic flux was significantly increased in H63D *HFE* cells (**Figs. 5A, 5B**). As expected, immunofluorescence revealed build-up of LC3-positive puncta with BafA1 treatment. Greater increase in LC3-positive puncta/cell was observed for H63D *HFE* cells compared to wildtype (**Fig. 5C**), confirming the LC3B-II western analysis. A potential mechanism for increased baseline autophagy is through the inhibition of mTORC1. mTORC1 is a negative regulator of autophagy and its activity can be inhibited by TSC1/TSC2 complex and drugs like rapamycin (Heras-Sandoval *et al.* 2014), resulting in a net increase in autophagic flux. When WT and H63D *HFE* cells were treated with rapamycin, autophagy induction was only detected in WT *HFE* cells (**Fig. 6A**). The lack of further autophagy induction by rapamycin in the H63D *HFE* cells suggested the possibility that mTORC1 pathway was maximally inhibited. To evaluate this, we compared the levels of phosphorylated ULK1 in WT and H63D *HFE* cells. ULK1 is phosphorylated by mTORC1 and is involved in autophagy initiation. ULK1 phosphorylation at Ser757 was decreased in H63D *HFE* cells (**Fig. 6B**). Furthermore, REDD1, which is known to inhibit mTORC1, was found be to elevated (**Fig. 6C**). REDD1 knockdown by siRNA resulted in decreased autophagic flux in H63D *HFE* cells confirming the REDD1-mTORC1 mediated autophagy induction (**Fig. 6D**). Overall, these results indicate that H63D *HFE* cells have increased levels of autophagic flux via elevated REDD1 expression and subsequent mTORC1 inhibition. Finally, REDD1 knockdown diminished the viability of H63D *HFE* cells with PFF treatment while having minimal effects in WT *HFE* cells (**Fig. 6E**), establishing a direct mechanistic connection between autophagy induction and protection from PFF toxicity.

**H63D HFE alters response to DFP treatment**

We investigated the effects of H63D *HFE* gene on the neuroprotective properties of the iron chelator deferiprone (DFP) in the pre-formed fibril model. Iron chelation by DFP treatment was confirmed with calcein fluorescence and H-ferritin western blots (**Supplemental Fig. 2A, Supplemental Fig. 2B**). Cells were treated with 1.5uM PFF for 5 days, then 1, 10, 30uM of deferiprone was added to the media for 2 additional days. Iron chelation has been previously reported to repress mTOR activity through the induction of REDD1 expression (Ohyashiki *et al.* 2009). Changes in autophagic flux with DFP treatment was assessed using LC3 western blot (**Fig. 7A)**, which showed autophagy induction in WT *HFE* cells but decreased flux at lower DFP concentration or return to baseline at higher DFP concentration in H63D *HFE* cells. Consistent with this result, co-treatment with DFP and α-synuclein PFFs resulted in a decrease in total α-synuclein level in WT cells that was significant at the highest DFP dose. In contrast, H63D *HFE* cells had significantly elevated levels of α-synuclein at the low DFP concentration of 1uM while at high concentrations, α-synuclein levels were essentially unaffected (**Fig. 7B)**. Similar response patterns were observed for cell viability after 1.5uM monomer + 5uM PFF treatment (**Fig. 7C**).

**H67D *Hfe* mice are relatively resistant to PFF mediated loss of motor function and α-synuclein aggregation**

To investigate the impact of H63D *HFE* in an *in vivo* model of Parkinson’s disease, WT and H67D *Hfe* expressing mice were injected with α-synuclein PFFs. Previous studies with H67D *Hfe* (mouse homolog of the human H63D *HFE* variant) mice showed altered brain iron regulation (Nandar *et al.* 2013) and protection from paraquat induced toxicity (Nixon *et al.* 2018). Male and female C57BL/6J × 129 WT and H67D *Hfe* mice were stereotactically injected with 5ug of PFF in the right striatum and PBS in the left striatum. Motor function was assessed using a rotor rod test. WT *Hfe* mice had a significant increase in the total number of falls by 3 months post-injection (**Fig. 8A**) and a significant decrease in time to first fall at 2 months and 3 months post-injection (**Fig. 8B**). H67D *Hfe* mice did not show a decline in motor function by the end point of the study (3 months). Immunohistochemistry was used to detect the level of pS129 α-synuclein at 3 months post-injection. Stronger pS129 α-synuclein staining was seen in the cortex of WT *Hfe* mice ipsilateral to the PFF injection compared to H67D *Hfe* mice (**Fig. 8C**). Fluorescent secondary antibody was used to quantify the pS129 α-synuclein staining intensity. WT *Hfe* mice had significantly higher pS129 α-synuclein staining compared to H67D *Hfe* mice (**Fig. 8D**).

**Discussion**

Although the H63D *HFE* polymorphism is expected to result in an increased amount of intracellular iron that could theoretically exacerbate Parkinson’s-related pathology such as oxidative stress or α-synuclein aggregation, no association between this allele frequency and Parkinson’s disease risk has been identified (Borie *et al.* 2002; Dekker *et al.* 2003; Akbas *et al.* 2006; Aamodt *et al.* 2007; Rhodes *et al.* 2014; Buchanan *et al.* 2002; Guerreiro *et al.* 2006; Halling *et al.* 2008; Biasiotto *et al.* 2008; Greco *et al.* 2011; Mariani *et al.* 2013; Mariani *et al.* 2016). However, many of these studies have small patient cohorts (n < 500); therefore, it is unclear whether the impact of H63D *HFE* on Parkinson’s disease has adequately been assessed. Furthermore, in a mendelian randomization modeling study where the effect of multiple iron related genes on Parkinson’s disease was investigated, increased iron levels as determined by genotype of iron related genes was found to decrease the risk for Parkinson’s disease (Pichler *et al.* 2013). In this study, we found that the presence of H63D *HFE* gene variant altered the metabolism of α-synuclein in cell culture and in animal model, consistent with increased autophagic flux. We also showed that H63D *HFE* expressing SH-SY5Y cells were resistant to the neuroprotective effects of an iron chelator (deferiprone). SH-SY5Y is an immortalized cell line that is derived from a neuroblastoma and therefore possess characteristics of cancer cells that differ from dopaminergic neurons. To validate the *in vitro* findings which demonstrated decreased α-synuclein aggregation and PFF mediated toxicity, intracranial PFF injection mouse model was used to show the impact of the *HFE* variant on α-synuclein *in vivo*. Overall, our data support the idea that H63D *HFE* gene variant modulates sensitivity to α-synuclein toxicity via its effects on protein degradation, which may lead to altered Parkinson’s disease pathology.

The PFF model of Parkinson’s disease is well established in cells and animals for inducing α-synuclein ‘seeding’ and spread, which are key components of Parkinson’s disease α-synucleinopathy (Volpicelli-Daley *et al.* 2011; Luk *et al.* 2009). H63D *HFE* cells showed decreased α-synuclein aggregation and increased cell viability with PFF treatment. Furthermore, we show that with PFF injection, H67D *Hfe* (homologous to human H63D *HFE*) mice are better able to maintain their motor function and have decreased α-synuclein aggregation, supporting the concept that H63D *HFE* variant may be protective against Parkinson’s disease. Additionally, mice that carry H67D *Hfe* have been reported to have decreased sensitivity to paraquat treatment, a mitochondrial neurotoxin model of Parkinson’s disease (Nixon *et al.* 2018). NRF2, a regulator of oxidative damage response, has also been shown to be elevated in H67D *Hfe* mice (Nandar *et al.* 2013), indicating an elevated anti-oxidant response with the *HFE* variant expression. The cellular pathophysiology of Parkinson’s disease involves intertwined pathways of mitochondrial oxidative damage and aggregated protein clearance. This current study adds to the growing literature showing the impact of *HFE* variant on multiple aspects of Parkinson’s disease pathology.

The relationship of iron and α-synuclein expression has been postulated as a direct molecular effect through a putative iron responsive element (IRE) in the 5’-UTR of α-synuclein mRNA that has sequence homology to that found in the mRNA for H- and L-ferritin (Febbraro *et al.* 2012; Friedlich *et al.* 2007). The release of IRE binding protein (IRP) from IRE in iron-replete conditions allows for increased ribosome binding of the mRNA and translation (Pantopoulos 2004). The putative IRE has been used to explain why multiple studies have observed increased α-synuclein levels with exogenous iron treatment (Xiao *et al.* 2018; Bartels *et al.* 2019), but studies using the yeast three hybrid system and IRP pull down have not detected binding of IRPs to the α-synuclein IRE (Chen and Olsthoorn 2019; Koukouraki and Doxakis 2016). Herein we report that H63D *HFE* expressing cells in which the mutation is associated with increased labile iron and a functioning IRE-IRP system, as indicated by the induction of H-ferritin expression, α-synuclein expression level is decreased. A functional IRE/IRP in the 5’ UTR would have been expected to be associated with an increase expression of α-synuclein. Thus, our study is consistent with the data that the IRE-like sequence of α-synuclein is nonfunctional, although we cannot rule out that the impact of the H63D *HFE* variant, which significantly alters other aspects of cellular metabolism such as autophagy (shown herein) and cholesterol synthesis (Ali-Rahmani *et al.* 2014a), may indirectly alter the expression of α-synuclein.

We tested the hypothesis that the mechanism by which the H63D *HFE* variant altered α-synuclein level was through altered autophagy. Although autophagy was first discovered as a starvation response mechanism, many studies have now shown that constitutive autophagy is an essential homeostatic process, especially for neurons. Knockout mice lacking key proteins for autophagy (ATG8, ATG5) show signs of neurodegeneration, motor deficiencies, and accumulation of protein aggregates (Komatsu *et al.* 2006). Hara, et al. 2006 further showed that baseline autophagy in neurons is responsible for turnover of diffuse cytosolic proteins. Dysregulation of autophagy has been shown in multiple models of Parkinson’s disease; therefore, we investigated autophagy as a potential protective mechanism in H63D *HFE* cells. Compared to wildtype, H63D *HFE* cells had increased baseline autophagic flux. H63D *HFE* cells and also failed to induce autophagy with mTORC1 inhibitor, rapamycin, treatment indicating that mTORC1 activity is already inhibited. This is consistent with the increased expression of REDD1, a known mTORC1 inhibitor. Phosphorylation of ULK1 at Ser757 was also decreased in H63D *HFE* cells. ULK1 complex initiates autophagy and Ser757 phosphorylation by mTORC1 has been shown to inhibit its activity (Kim *et al.* 2011). REDD1 expression has been shown to be affected by various cellular stresses including hypoxia and ER stress (Whitney *et al.* 2009; Dennis *et al.* 2013; Ohyashiki *et al.* 2009). The H63D HFE protein has been shown to induce prolonged ER stress independent of cellular iron status (Liu *et al.* 2011). Furthermore, we showed that REDD1 knockdown decreases the autophagic flux and increases α-synuclein PFF toxicity for the H63D *HFE* cells. Taken together, our data show that elevated autophagy through REDD1 inhibition of mTORC1 in H63D *HFE* cells is directly responsible for the reduction in α-synuclein PFF toxicity.

Because the H63D *HFE* variant alters iron status and α-synuclein metabolism, we hypothesized that iron chelation therapy would be impacted by the presence of this genotype. Deferiprone (DFP) is an iron chelator that is under active investigation as a disease modifying therapy for Parkinson’s disease (Clinicaltrials.gov Identifier: NCT02655315). DFP treatment has been shown to reduce brain iron levels by MTI (Devos *et al.* 2014). Preclinical models using 6-OHDA and MPTP have shown that DFP is neuroprotective (Devos *et al.* 2014; Dexter *et al.* 2011). While those neurotoxins cause dopaminergic neuronal cell death, they fail to model α-synuclein aggregation. Using PFFs, we showed that DFP is effective in reducing α-synuclein aggregation and protecting from cell death in WT *HFE* expressing cells but had mixed effects in the H63D *HFE* cells which mirrored changes in autophagy. In H63D *HFE* cells, the decrease in autophagy with low dose of DFP treatment could be due to the loss of increase in intracellular iron from the *HFE* variant. With higher dose of DFP, cellular iron deficiency can be achieved leading to autophagy induction. The pilot clinical trials of DFP for Parkinson’s disease have shown some efficacy. DFP treatment was shown to decrease brain iron content by MRI and improvement Unified Parkinson’s Disease Rating Scale part III scores have been observed (Martin-Bastida *et al.* 2017; Devos *et al.* 2014). Our data suggest that stratifying the patients according to their *HFE* genotype could further amplify the effects of the DFP treatment, and likely other therapeutic strategies, in clinical trials and lead to personalized treatment strategies for Parkinson’s disease patients.

In conclusion, the findings presented in this study demonstrate that H63D *HFE* variant has a significant impact on α-synuclein homeostasis of the cell through its modulation of autophagy, leading to protection in *in vitro* PFF model and *in vivo* PFF intracranial injection models of Parkinson’s disease. Deferiprone treatment, as expected, was found to be effective in reducing α-synuclein aggregation and increasing cell viability but only with WT *HFE* expression. Given the high prevalence of H63D *HFE* variant in the general population, our study establishes *HFE* genotype as a potential disease modifier of Parkinson’s disease. Furthermore, our findings argue for the importance of stratification by *HFE* genotype for evaluation of therapeutic trials with iron chelator therapy.

**Acknowledgements**

We thank the Penn State Flow Cytometry Core for their technical support, Sang Lee, Ph.D. for providing *HFE* transfected SH-SY5Y cells, and Salvatore Stella, Jr., Ph.D. for his confocal imaging support. We would also like to acknowledge Elizabeth Neely and Becky Webb for their technical expertise on mouse handling and stereotactic intracranial injections.

**Funding**

This study was partially supported by funds from the G.M Leader Family and the Soter S. and Carolyn C. Harbolis Alzheimer’s Research Endowment.

**Competing interests**

Dr. James Connor, Ph.D. is an editor for the Journal of Neurochemistry

**References**

Aamodt A. H., Stovner L. J., Thorstensen K., Lydersen S., White L. R., Aasly J. O. (2007) Prevalence of haemochromatosis gene mutations in Parkinson’s disease. *J. Neurol. Neurosurg. Psychiatry* **78**, 315–7.

Abeyawardhane D. L., Fernández R. D., Murgas C. J., Heitger D. R., Forney A. K., Crozier M. K., Lucas H. R. (2018) Iron Redox Chemistry Promotes Antiparallel Oligomerization of α-Synuclein. *J. Am. Chem. Soc.* **140**, 5028–5032.

Akbas N., Hochstrasser H., Deplazes J., Tomiuk J., Bauer P., Walter U., Behnke S., Riess O., Berg D. (2006) Screening for mutations of the HFE gene in Parkinson’s disease patients with hyperechogenicity of the substantia nigra. *Neurosci. Lett.* **407**, 16–19.

Ali-Rahmani F., Grigson P. S., Lee S., Neely E., Connor J. R., Schengrund C.-L. (2014a) H63D mutation in hemochromatosis alters cholesterol metabolism and induces memory impairment. *Neurobiol. Aging* **35**, 1511.e1–12.

Ali-Rahmani F., Schengrund C.-L., Connor J. R. (2014b) HFE gene variants, iron, and lipids: a novel connection in Alzheimer’s disease. *Front. Pharmacol.* **5**, 165.

Bartels M., Weckbecker D., Kuhn P. H., Ryazanov S., Leonov A., Griesinger C., Lichtenthaler S. F., Bötzel K., Giese A. (2019) Iron-mediated aggregation and toxicity in a novel neuronal cell culture model with inducible alpha-synuclein expression. *Sci. Rep.* **9**.

Barton J. C., Edwards C. Q., Acton R. T. (2015) HFE gene: Structure, function, mutations, and associated iron abnormalities. *Gene* **574**, 179–192.

Beard J. L., Dawson H., Piñero D. J. (1996) Iron metabolism: a comprehensive review. *Nutr. Rev.* **54**, 295–317.

Biasiotto G., Goldwurm S., Finazzi D., Tunesi S., Zecchinelli A., Sironi F., Pezzoli G., Arosio P. (2008) HFE gene mutations in a population of Italian Parkinson’s disease patients. *Park. Relat. Disord.* **14**, 426–430.

Borie C., Gasparini F., Verpillat P., Bonnet A. M., Agid Y., Hetet G., Brice A., Dürr A., Grandchamp B. (2002) Association study between iron-related genes polymorphisms and Parkinson’s disease. *J. Neurol.* **249**, 801–804.

Buchanan D. ., Silburn P. ., Chalk J. ., Couteur D. . Le, Mellick G. . (2002) The Cys282Tyr polymorphism in the HFE gene in Australian Parkinson’s disease patients. *Neurosci. Lett.* **327**, 91–94.

Chen J. C., Hardy P. A., Kucharczyk W., Clauberg M., Joshi J. G., Vourlas A., Dhar M., Henkelman R. M. (1993) MR of human postmortem brain tissue: correlative study between T2 and assays of iron and ferritin in Parkinson and Huntington disease. *AJNR. Am. J. Neuroradiol.* **14**, 275–81.

Chen S.-C., Olsthoorn R. C. L. (2019) Relevance of the iron-responsive element (IRE) pseudotriloop structure for IRP1/2 binding and validation of IRE-like structures using the yeast three-hybrid system. *Gene* **710**, 399–405.

Dekker M. C. J., Giesbergen P. C., Njajou O. T., Swieten J. C. van, Hofman A., Breteler M. M. B., Duijn C. M. Van (2003) Mutations in the hemochromatosis gene (HFE), Parkinson’s disease and parkinsonism. *Neurosci. Lett.* **348**, 117–119.

Dennis M. D., McGhee N. K., Jefferson L. S., Kimball S. R. (2013) Regulated in DNA damage and development 1 (REDD1) promotes cell survival during serum deprivation by sustaining repression of signaling through the mechanistic target of rapamycin in complex 1 (mTORC1). *Cell. Signal.* **25**, 2709–2716.

Devos D., Moreau C., Devedjian J. C., Kluza J., Petrault M., Laloux C., Jonneaux A., et al. (2014) Targeting chelatable iron as a therapeutic modality in Parkinson’s disease. *Antioxid. Redox Signal.* **21**, 195–210.

Dexter D. T., Carayon A., Javoy-Agid F., Agid Y., Wells F. R., Daniel S. E., Lees A. J., Jenner P., Marsden C. D. (1991) Alterations in the levels of iron, ferritin and other trace metals in Parkinson’s disease and other neurodegenerative diseases affecting the basal ganglia. *Brain* **114**, 1953–1975.

Dexter D. T., Statton S. A., Whitmore C., Freinbichler W., Weinberger P., Tipton K. F., Corte L. Della, Ward R. J., Crichton R. R. (2011) Clinically available iron chelators induce neuroprotection in the 6-OHDA model of Parkinson’s disease after peripheral administration. *J. Neural Transm.* **118**, 223–31.

Dexter D. T., Wells F. R., Agid F., Agid Y., Lees A. J., Jenner P., Marsden C. D. (1983) Increased nigral iron content in postmortem Parkinsonian brain. *Lancet* **ii**, 1099–102.

Du T. T., Wang L., Duan C. L., Lu L. L., Zhang J. L., Gao G., Qiu X. B., Wang X. M., Yang H. (2015) GBA deficiency promotes SNCA/α-synuclein accumulation through autophagic inhibition by inactivated PPP2A. *Autophagy* **11**, 1803–1820.

Febbraro F., Giorgi M., Caldarola S., Loreni F., Romero-Ramos M. (2012) α-Synuclein expression is modulated at the translational level by iron. *Neuroreport* **23**, 576–580.

Friedlich A. L., Tanzi R. E., Rogers J. T. (2007) The 5’-untranslated region of Parkinson’s disease alpha-synuclein messengerRNA contains a predicted iron responsive element. *Mol. Psychiatry* **12**, 222–223.

Gan-Or Z., Dion P. A., Rouleau G. A. (2015) Genetic perspective on the role of the autophagy-lysosome pathway in Parkinson disease. *Autophagy* **11**, 1443–1457.

Greco V., Marco E. V. De, Rocca F. E., Annesi F., Civitelli D., Provenzano G., Tarantino P., et al. (2011) Association study between four polymorphisms in the HFE, TF and TFR genes and Parkinson’s disease in Southern Italy. *Neurol. Sci.* **32**, 525–527.

Guerreiro R. J., Bras J. M., Santana I., Januario C., Santiago B., Morgadinho A. S., Ribeiro M. H., Hardy J., Singleton A., Oliveira C. (2006) Association of HFE common mutations with Parkinson’s disease, Alzheimer’s disease and mild cognitive impairment in a Portuguese cohort. *BMC Neurol.* **6**, 24.

Halling J., Petersen M. S., Grandjean P., Weihe P., Brosen K. (2008) Genetic predisposition to Parkinson’s disease: CYP2D6 and HFE in the Faroe Islands. *Pharmacogenet. Genomics* **18**, 209–212.

Hanson E. H., Imperatore G., Burke W. (2001) HFE Gene and Hereditary Hemochromatosis: A HuGE Review. *Am. J. Epidemiol.* **154**, 193–206.

Hara T., Nakamura K., Matsui M., Yamamoto A., Nakahara Y., Suzuki-Migishima R., Yokoyama M., et al. (2006) Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* **441**, 885–889.

Heras-Sandoval D., Pérez-Rojas J. M., Hernández-Damián J., Pedraza-Chaverri J. (2014) *The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration*. Pergamon.

Jung C. H., Jun C. B., Ro S. H., Kim Y. M., Otto N. M., Cao J., Kundu M., Kim D. H. (2009) ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol. Biol. Cell* **20**, 1992–2003.

Kim J., Kundu M., Viollet B., Guan K. L. (2011) AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat. Cell Biol.* **13**, 132–141.

Kimura S., Fujita N., Noda T., Yoshimori T. (2009) Monitoring autophagy in mammalian cultured cells through the dynamics of LC3, in *Methods Enzymol.*, Vol. 452, pp. 1–26. Academic Press.

Komatsu M., Waguri S., Chiba T., Murata S., Iwata J. I., Tanida I., Ueno T., et al. (2006) Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* **441**, 880–884.

Koukouraki P., Doxakis E. (2016) Constitutive translation of human α-synuclein is mediated by the 5’-untranslated region. *Open Biol.*

Lee B. R., Kamitani T. (2011) Improved immunodetection of endogenous α-synuclein. *PLoS One* **6**, e23939.

Lee S. Y., Patton S. M., Henderson R. J., Connor J. R. (2007) Consequences of expressing mutants of the hemochromatosis gene (HFE) into a human neuronal cell line lacking endogenous HFE. *FASEB J.* **21**, 564–576.

Liu Y., Lee S. Y., Neely E., Nandar W., Moyo M., Simmons Z., Connor J. R. (2011) Mutant HFE H63D Protein Is Associated with Prolonged Endoplasmic Reticulum Stress and Increased Neuronal Vulnerability. *J. Biol. Chem.* **286**, 13161–13170.

Luk K. C., Song C., O’Brien P., Stieber A., Branch J. R., Brunden K. R., Trojanowski J. Q., Lee V. M.-Y. (2009) Exogenous α-synuclein fibrils seed the formation of Lewy body-like intracellular inclusions in cultured cells. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 20051–20056.

Mahul-Mellier A. L., Vercruysse F., MacO B., Ait-Bouziad N., Roo M. De, Muller D., Lashuel H. A. (2015) Fibril growth and seeding capacity play key roles in α-synuclein-mediated apoptotic cell death. *Cell Death Differ.* **22**, 2107–2122.

Mariani S., Ventriglia M., Simonelli I., Bucossi S., Siotto M., Donno S., Vernieri F., Squitti R. (2016) Association between sex, systemic iron variation and probability of Parkinson’s disease. *Int. J. Neurosci.* **126**, 354–360.

Mariani S., Ventriglia M., Simonelli I., Spalletta G., Bucossi S., Siotto M., Assogna F., Melgari J. M., Vernieri F., Squitti R. (2013) Effects of hemochromatosis and transferrin gene mutations on peripheral iron dyshomeostasis in mild cognitive impairment and Alzheimer’s and Parkinson’s diseases. *Front. Aging Neurosci.* **5**.

Martin-Bastida A., Ward R. J., Newbould R., Piccini P., Sharp D., Kabba C., Patel M. C., et al. (2017) Brain iron chelation by deferiprone in a phase 2 randomised double-blinded placebo controlled clinical trial in Parkinson’s disease. *Sci. Rep.* **7**, 1398.

Nandar W., Neely E. B., Simmons Z., Connor J. R. (2014) H63D HFE genotype accelerates disease progression in animal models of amyotrophic lateral sclerosis. *Biochim. Biophys. Acta - Mol. Basis Dis.* **1842**, 2413–2426.

Nandar W., Neely E. B., Unger E., Connor J. R. (2013) A mutation in the HFE gene is associated with altered brain iron profiles and increased oxidative stress in mice. *Biochim. Biophys. Acta - Mol. Basis Dis.* **1832**, 729–741.

Nixon A. M., Meadowcroft M. D., Neely E. B., Snyder A. M., Purnell C. J., Wright J., Lamendella R., Nandar W., Huang X., Connor J. R. (2018) HFE Genotype Restricts the Response to Paraquat in a Mouse Model of Neurotoxicity. *J. Neurochem.* **145**, 299–311.

O’Regan G., Desouza R. M., Balestrino R., Schapira A. H. (2017) *Glucocerebrosidase Mutations in Parkinson Disease*.

Ohyashiki J. H., Kobayashi C., Hamamura R., Okabe S., Tauchi T., Ohyashiki K. (2009) The oral iron chelator deferasirox represses signaling through the mTOR in myeloid leukemia cells by enhancing expression of REDD1. *Cancer Sci.* **100**, 970–977.

Pantopoulos K. (2004) *Iron metabolism and the IRE/IRP regulatory system: An update*. New York Academy of Sciences.

Patterson J. R., Polinski N. K., Duffy M. F., Kemp C. J., Luk K. C., Volpicelli-Daley L. A., Kanaan N. M., Sortwell C. E. (2019) Generation of Alpha-Synuclein Preformed Fibrils from Monomers and Use In Vivo. *J. Vis. Exp.* 148.

Pichler I., Greco M. F. Del, Gögele M., Lill C. M., Bertram L., Do C. B., Eriksson N., et al. (2013) Serum Iron Levels and the Risk of Parkinson Disease: A Mendelian Randomization Study. *PLoS Med.* **10**.

Rhodes S. L., Buchanan D. D., Ahmed I., Taylor K. D., Loriot M. A., Sinsheimer J. S., Bronstein J. M., et al. (2014) Pooled analysis of iron-related genes in Parkinson’s disease: Association with transferrin. *Neurobiol. Dis.* **62**, 172–178.

Rockenstein E., Nuber S., Overk C. R., Ubhi K., Mante M., Patrick C., Adame A., et al. (2014) Accumulation of oligomer-prone α-synuclein exacerbates synaptic and neuronal degeneration in vivo. *Brain* **137**, 1496–1513.

Simon M., Bourel M., Fauchet R., Genetet B. (1976) Association of HLA A3 and HLA B14 antigens with idiopathic haemochromatosis. *Gut* **17**, 332–334.

Steinberg K. K., Cogswell M. E., Chang J. C., Caudill S. P., McQuillan G. M., Bowman B. A., Grummer-Strawn L. M., Sampson E. J., Khoury M. J., Gallagher M. L. (2001) Prevalence of C282Y and H63D Mutations in the Hemochromatosis (HFE) Gene in the United States. *JAMA* **285**, 2216.

Su X. W., Lee S. Y., Mitchell R. M., Stephens H. E., Simmons Z., Connor J. R. (2013) H63D HFE polymorphisms are associated with increased disease duration and decreased muscle superoxide dismutase-1 expression in amyotrophic lateral sclerosis patients. *Muscle and Nerve* **48**, 242–246.

Tomatsu S., Orii K. O., Fleming R. E., Holden C. C., Waheed A., Britton R. S., Gutierrez M. A., Velez-Castrillon S., Bacon B. R., Sly W. S. (2003) Contribution of the H63D mutation in HFE to murine hereditary hemochromatosis. *Proc. Natl. Acad. Sci.* **100**, 15788–15793.

Vogiatzi T., Xilouri M., Vekrellis K., Stefanis L. (2008) Wild type α-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells. *J. Biol. Chem.* **283**, 23542–23556.

Volpicelli-Daley L. A., Luk K. C., Lee V. M.-Y. (2014) Addition of exogenous α-synuclein preformed fibrils to primary neuronal cultures to seed recruitment of endogenous α-synuclein to Lewy body and Lewy neurite–like aggregates. *Nat. Protoc.* **9**, 2135–2146.

Volpicelli-Daley L. A., Luk K. C., Patel T. P., Tanik S. A., Riddle D. M., Stieber A., Meaney D. F., Trojanowski J. Q., Lee V. M. Y. (2011) Exogenous Alpha-Synuclein Fibrils Induce Lewy Body Pathology Leading to Synaptic Dysfunction and Neuron Death. *Neuron* **72**, 57–71.

Wang J.-Y., Zhuang Q.-Q., Zhu L.-B., Zhu H., Li T., Li R., Chen S.-F., et al. (2016) Meta-analysis of brain iron levels of Parkinson’s disease patients determined by postmortem and MRI measurements. *Sci. Rep.* **6**, 36669.

Wang R., Wang Y., Qu L., Chen B., Jiang H., Song N., Xie J. (2019) Iron-induced oxidative stress contributes to α-synuclein phosphorylation and up-regulation via polo-like kinase 2 and casein kinase 2. *Neurochem. Int.* **125**, 127–135.

Wang X. S., Lee S., Simmons Z., Boyer P., Scott K., Liu W., Connor J. (2004) Increased incidence of the Hfe mutation in amyotrophic lateral sclerosis and related cellular consequences. *J. Neurol. Sci.* **227**, 27–33.

Whitney M. L., Jefferson L. S., Kimball S. R. (2009) ATF4 is necessary and sufficient for ER stress-induced upregulation of REDD1 expression. *Biochem. Biophys. Res. Commun.* **379**, 451–455.

Winner B., Jappelli R., Maji S. K., Desplats P. A., Boyer L., Aigner S., Hetzer C., et al. (2011) In vivo demonstration that α-synuclein oligomers are toxic. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 4194–4199.

Winterbourn C. C. (1995) Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicol. Lett.* **82**–**83**, 969–974.

Wong Y. C., Krainc D. (2017) α-Synuclein toxicity in neurodegeneration: mechanism and therapeutic strategies. *Nat. Med.* **23**, 1–13.

Xiao Y., Chen X., Huang S., Li G., Mo M., Zhang L., Chen C., et al. (2018) Iron promotes α-synuclein aggregation and transmission by inhibiting TFEB-mediated autophagosome-lysosome fusion. *J. Neurochem.* **145**, 34–50.

**Figure legends**

**Fig. 1. Mouse PFF injection and behavioral testing timeline**. 3-month-old WT and H67D *Hfe* mice were injected with PBS and 5ug αSyn PFF. One week after the injection, mice were trained on the rotor rod and their baseline motor function (time to first fall, total number of falls) was recorded. At 1 month, 2 months, and 3 months post-injection, body weights was measured to assess overall health of the mice and rotor rod testing was performed. At 3 months post-injection, brain tissues were harvested for histological studies.

**Fig. 2**. **H63D *HFE* expressing cells have decreased α-synuclein** (A) Calcein fluorescence was measured from WT and H63D *HFE* expressing cells as a marker of intracellular labile iron. Ca fluorescence is inversely related to LIP. n = 3 independent cell culture preparations (3 technical replicates per data point), \*\*\* p < 0.001, Student’s t-test (B) H-ferritin western blot shows increased expression in H63D *HFE* cells compared to wildtype (C) Representative immunoblot showing decreased α-synuclein level in SH-SY5Y cells expressing H63D *HFE* compared to WT *HFE* expressing cells. Quantification is shown on the right. n = 3 independent cell culture preparations, \* p < 0.05, Student’s t-test (D) ELISA was performed to quantify α-synuclein content (ng/mL) normalized to total protein. n = 4 independent cell culture preparations (3 technical replicates per data point), \* p < 0.05, Student’s t-test

**Fig. 3. α-Synuclein aggregation is decreased in H63D *HFE* cells after PFF treatment.** (A) Representative images showing Thioflavin S, α-synuclein, and DAPI fluorescence staining on WT and H63D *HFE* cells after 7 days of 1.5uM α-synuclein monomer or PFF treatment. (B) Fold change in α-synuclein and ThS Corrected Total Fluorescence (CTF) with PFF treatment compared to control is shown. CTF was calculated from 3 independent cell culture preparations with at least 4 images taken per slide. \* p < 0.05, \*\* p < 0.01, Student’s t-test (C) Thioflavin T fluorescence was measured from cell culture media collected at 3, 5, 7 days post 1.5uM PFF treatment. n = 3 independent cell culture preparations (3 technical replicates per data point), \*\* p < 0.01, ANOVA with Sidak’s test for multiple comparisons. (D) Representative western blots showing α-synuclein aggregation at 3, 5, and 7 days post 1.5uM PFF treatment. Quantification is shown at right. n = 3 independent cell culture preparations, \* p < 0.05, \*\* p < 0.01, ANOVA with Sidak’s test for multiple comparisons.

**Fig. 4**. **H63D *HFE* cells are protected from PFF mediated toxicity.** (A) Cell viability was measured using CellTiter-glo and normalized to PBS treated control. Cells were treated with 1.5uM α-Syn monomer, 3.5uM PFF, or combination of 1.5uM monomer and increasing PFF concentrations for 7 days. n = 4 independent cell culture preparations (2 technical replicates per data point), \* p < 0.05, \*\* p < 0.01, ANOVA with Sidak’s test for multiple comparisons. (B) H63D *HFE* cells had decreased induction of caspase 3/7 activity after 7-day 5uM PFF + 1.5uM monomer co-treatment. Caspase activity was measured with Caspase-glo and PFF/monomer fold change was calculated. n = 4 independent cell culture preparations (3 technical replicates per data point), \*\* p < 0.01, Student’s t-test (C) Cells were treated with 5uM PFF + 1.5uM monomer for 7 days then stained with Annexin V-FITC and 7-AAD to assess for apoptosis. Representative flow cytometry output is shown. Fold change in % Annexin V positive cells with PFF treatment was quantified from 5 independent cell culture preparations. \*\*\* p < 0.001, Student’s t-test.

**Fig. 5**. **Baseline autophagic flux is increased in H63D *HFE* cells.** (A) Representative LC3 western blot with 4hr 100nM bafilomycin A1 or DMSO treatment. Autophagic flux is calculated as the difference in LC3-II band intensity between DMSO and BafA1 treatment group normalized to β-actin. n = 6 independent cell culture preparations, \*\* p < 0.01, Student’s t-test. (B) Cells were treated with 50uM chloroquine or PBS for 2.5 hrs and lysed for western blot. Representative blot shows larger increase in LC3-II band intensity with CQ treatment. n = 3 independent cell culture preparations, \* p < 0.05, Student’s t-test. (C) Top panels show increase in LC3 fluorescence signal with BafA1 treatment in WT and H63D *HFE* cells. Bottom panels show LC3 positive puncta formation (arrows) in WT and H63D *HFE* cells with BafA1 treatment. Fold change in number of LC3 positive puncta per cell after BafA1 treatment was calculated. n=3 independent cell culture preparations (100 cells counted per slide), \* p < 0.05, Student’s t-test.

**Fig. 6**. **H63D HFE increases autophagy through REDD1 inhibition of mTORC1**

(A) Cells were co-treated with 200nM Rap and 100nM BafA1 for 4hrs. Representative immunoblot for LC3 is shown. Autophagic flux was calculated from LC3-II and β-actin band intensities. n = 3 independent cell culture preparations, \* p < 0.05, Student’s t-test. (B) Western blots for ULK1 phosphorylated at Ser757, total ULK1, and β-actin loading control. Quantification was performed and ratio of p-Ser757-ULK1 to total ULK1 was calculated. n = 3 independent cell culture preparations, \* p <0.05, Student’s t-test. (C) Representative blot of REDD1 from WT and H63D *HFE* cell lysates. Band density was quantified and normalized to β-actin. n = 3 independent cell culture preparations, \* p < 0.05, Student’s t-test. (D) siRNA knockdown of REDD1 was performed with siEGFP as the control siRNA. Cells were then treated with 100nM BafA1 for 4 hrs. Representative image of LC3, REDD1, and β-actin western blots are shown. n = 4 independent cell culture preparations, \*\* p < 0.01, Student’s t-test. (E) After siRNA knockdown (REDD1 or EGFP), cells were treated with 1.5uM α-synuclein monomer or 1.5uM monomer + 5uM PFF for 5 days. Cell viability was measured using CellTiter-glo. Viability is normalized to α-synuclein monomer only treated cells. n = 3 independent cell culture preparations (siRNA knockdowns) with 2 technical replicates per data point, \*\* p < 0.01, Student’s t-test.

**Fig. 7. H63D HFE modulates response to DFP treatment.** (A) After 2 days of DFP treatment, cells were treated with 100nM BafA1 or DMSO for 4hrs. Representative LC3 western blots are shown. Autophagic flux was calculated from LC3-II band intensity normalized to β-actin. n = 3 independent cell culture preparations, \*\* p < 0.01, ## p < 0.01, ANOVA with Dunnett’s test for multiple comparisons (B) WT and H63D *HFE* cells were treated with 1.5uM PFF for 7 days. Deferiprone (0, 1, 10, 30uM) was added for the last two days. Representative western blot from cell lysates is shown. Total α-synuclein was quantified and normalized to β-actin. n = 3 independent cell culture preparations, \* p < 0.05, ## p < 0.01, ANOVA with Dunnett’s test for multiple comparisons. (C) Cell viability was measured using CellTiter-glo after 1.5uM monomer + 5uM PFF and DFP treatment. Values were normalized to DFP only control. n = 4 independent cell culture preparations (2 technical replicates per data point), \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, ## p < 0.01, ### p < 0.001, ANOVA with Dunnett’s test for multiple comparisons.

**Fig. 8 H67D *Hfe* mice are protected from PFF mediated loss of motor function and α-synuclein aggregation.** 3-month-old WT and H67D *Hfe* mice were injected with PBS in the left striatum and 5ug mouse PFF in the right striatum. Baseline motor function was assessed using rotor rod one week after the injection. Subsequent rotor rod testing was performed monthly until end point of 3 months post-injection. (A) Total number of falls during the rotor rod session (250s). n = 7 mice, \* p < 0.05, Two-way ANOVA with Dunnett’s multiple comparisons. (B) Time to first fall from the rotor rod. n = 7 mice, \* p < 0.05, Two-way ANOVA with Dunnett’s multiple comparisons. (C) Cortex near the site of injection was stained for pS129 α-synuclein to confirm the seeding of the fibrils. Uninjected mice are shown as controls. WT *Hfe* mice show stronger staining for pS129 α-synuclein on the side of PFF injection. (D) Histology sections were stained for pS129 α-synuclein with fluorescent secondary antibody and DAPI. Corrected total fluorescence (CTF) was quantified by subtracting the background signal and normalizing by DAPI intensity. Significantly higher CTF was measured from WT *Hfe* mice compared to H67D *Hfe* in the PFF injected hemisphere. n = 7 mice (3 fields of view imaged per slide), \*\*\* p < 0.001, Student’s t-test.