# Unfolding the role of Ire1 and DNAJB1 in Parkinson's disease

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**Abstract**

Parkinson's disease (PD) is a movement disorder of the brain which is hallmarked by the presence of large protein-rich cytoplasmic aggregates, called Lewy bodies (LBs), in the neurons. The protein α-synuclein (α-syn) is abundant in these aggregates, and can itself form aggregates. Several mutations linked to α-syn are also linked to an increased risk of contracting PD. In healthy neurons, the chaperone heat shock protein 70 (Hsp70) is able to refold misfolded proteins such as aggregated α-syn, with the help of its co-chaperones, the DNAJ proteins. In addition, toxic protein aggregation is normally stopped by the unfolded protein response (UPR) in the endoplasmic reticulum, including the protein Ire1 which indirectly activates genes involved in protein folding. Here, it is shown that knocking out the DNAJ protein DNAJB1 in HEK293 cells overexpressing α-syn, by use of CRISPR/Cas9 technology, leads to a significant increase of α-syn aggregates. In addition, reintroducing the DNAJB1 gene into these knockout cells decreases the aggregate percentage to a level comparable with control cells. Furthermore, introducing a H32Q mutant version of DNAJB1, which cannot interact with HSP70, into the knockout cells, does not decrease the level of α-syn aggregation. This suggests that the DNAJB1 protein may have an important function in suppressing the formation of the toxic α-syn aggregates in cells and could be involved in a potential new PD therapeutic. Furthermore, the effect of conditionally knocking out the UPR protein Ire1 in the dopaminergic neurons of a Parkinsonian mouse model expressing human α-syn-GFP has been investigated. After 6 months, the lack of the protein does not seem to result in a behavioral difference in the mice compared to control, and thereby does not share the therapeutic potential of DNAJB1.

## Introduction

Neurodegenerative diseases are defined as chronic disorders caused by progressive loss of neurons in the central nervous system [1]. Depending on the disease, this can have several different detrimental effects for the patient. One of the most common neurodegenerative diseases is Parkinson's disease (PD), whose cardinal symptoms are tremors, rigidity, and postural imbalance, and is characterized by the degeneration of dopaminergic neurons in the *substantia nigra pars compacta* of the brain [2].

A hallmark of PD is the formation of large protein-rich cytoplasmic aggregates called Lewy bodies (LBs). The most abundant protein in these aggregates is a small 140-amino acid protein named α-synuclein (α-syn), which is mainly found in the central nervous system and is widely expressed in the neurons of the brain. The role of α-syn in the cell has not been completely deciphered, but studies have suggested that it has an important role in the presynaptic terminal, where it is believed to associate with SNARE proteins and thereby aid in the vesicle release during synapse transmission. Furthermore, missense mutations and multiplications of the *SNCA* gene, which encodes for α-syn, have been linked to PD [3].

The aggregation of α-syn to form fibrils, which partake in LB formation, is not exclusive only to PD. Indeed, the several diseases that involve similar α-syn inclusions are categorized under the umbrella term synucleinopathies [3]. In fact, accumulation of misfolded proteins is a common denominator of not only synucleinopathies, but of several neurodegenerative diseases, explaining why the cell has numerous mechanisms with the purpose of maintaining correct protein folding and preventing toxic aggregation. One of these mechanisms involves the molecular chaperone family of 70-kDa heat shock proteins (Hsp70s), which are involved in several folding processes including folding of newly synthesized proteins as well as refolding of misfolded and aggregated proteins. Indeed, Hsp70 has been shown to prevent the dopaminergic neuronal loss associated with PD [4]. Hsp70 binds to hydrophobic protein regions and utilizes ATP hydrolysis to energize the refolding of misfolded proteins [5], but cannot accomplish this without a family of co-chaperones, the DNAJ proteins, which supply the misfolded protein substrate and stimulate ATP hydrolysis.

The human genome encodes over 40 different DNAJ proteins, all of which contain the J domain that is used to bind Hsp70 [6]. Among these is the cytosolic protein DNAJB1, which is constitutively expressed in neuronal tissue [7] and is upregulated during heat shock stress. Overexpression of DNAJB1 in polyglutamine disease mouse models using viral vectors has

been shown to suppress polyglutamine inclusion formation. In addition, stress-responding DNAJB1 has been suggested to be released into the extracellular space through an exosomal pathway, and subsequently to be internalized into neighboring cells to promote their proteostasis [8]. Furthermore, *in vitro* studies combining the Hsp70 family member Hsc70 with an Hsp110 family nucleotide exchange factor and DNAJB1 have shown the ability to disaggregate α-syn fibrils [9].

In addition to DNAJB1, another DNAJ protein that has been studied in the context of aggregated proteins is DNAJB6. DNAJB6 is present in neurons and has two isoforms; the longer DNAJB6a which is predominantly localized in the nucleus, and the shorter DNAJB6b which is more common cytoplasmically[10]. DNAJB6 has indeed been shown to be present in LBs in PD patients[11]. Furthermore, the protein has been shown to reduce the aggregation of polyglutamine *in vivo*[12]. Moreover, Hansen et al. (unpublished data, 2017) have shown that knocking out the DNAJB6 gene in α-syn-dsRed-overexpressing cells leads to a significant increase in α-syn aggregates, an effect that is reversed by the reintroduction of DNAJB6.

The work of chaperones such as Hsp70 and co-chaperones like DNAJB1 and DNAJB6 play an important role against upheaval of the cell's protein homeostasis. However, should this defense fail, there are numerous further protective mechanisms to ensure proteostasis. Firstly, misfolded proteins that are unable to be refolded become targeted for endoplasmic reticulum-associated degradation (ERAD). If this contribution still does not restore the protein balance of the cell, the ER will induce the unfolded protein response (UPR) [13].

There are three main pathways that are activated in the UPR: the Activating Transcription Factor 6 (ATF6) pathway, the PKR-like endoplasmic reticulum kinase (PERK) pathway, and the Inositol Requiring Enzyme 1 (Ire1) pathway. Normally, all these pathways are inhibited by binding to the endoplasmic reticulum (ER) chaperone protein BiP. When misfolded proteins accumulate, however, BiP dissociates from the receptors to bind to the exposed hydrophobic regions of misfolded proteins. This dissociation enables each pathway to become activated and induce downstream effects. Under these conditions, ATF6 gets exported from the ER and activated in the Golgi apparatus, leading to its transport to the nucleus, where it activates transcription of genes involved in protein folding and ERAD. PERK, in turn, gets trans-autophosphorylated and can thereby phosphorylate the translation initiation factor eIF2α, resulting in decrease of global protein synthesis and thereby a reduction of ER protein load. Finally, as BiP dissociates from the N terminus of the Ire1 protein, Ire1 oligomerizes

and autophosphorylates. This activates the ER lumen-exposed endoribonuclease domain of the protein, which subsequently cleaves an intron from the mRNA of the transcription factor XBP1, resulting in a more potent frameshift variant. XBP1, in turn, enables activation of genes involved in ERAD, protein folding, lipid synthesis, and RNA degradation. In addition to the action of this ER lumen-exposed domain of Ire1, the cytosolic kinase domain of the protein can promote a pro-apoptotic signal should the load of misfolded proteins increase to unsustainable levels. There are two different homologs of Ire1 in the body, Ire1α and Ire1β. Ire1β is only found in the intestine while the homolog that will be discussed in this report, Ire1α, is expressed throughout the body including the brain. Altogether, these pathways guide a protective response that includes both decrease of protein translation and upregulation of a specific subset of chaperones to increase the folding capacity of the cell. [14]. The UPR has been suspected to play a part in neurodegenerative diseases involving aggregated proteins. Indeed, upregulation of XBP1 in a Parkinsonian mouse model has been shown to suppress the degeneration of dopaminergic neurons [15]. In addition, overexpression of BiP reduced α-syn toxicity in human α-syn-expressing rats [16].

While there are numerous additional mechanisms in the cell that assist in the clearance of misfolded proteins, the two that will mainly be investigated in this study are the Ire1 pathway and the DNAJB1/Hsp70 pathway. In this master thesis I demonstrate that knocking out DNAJB1 in HEK293 cells causes increased α-syn aggregation, which is dependent on the J-domain catalytic activity of the protein. In addition, I present data from mouse models studies addressing whether Ire1α could play a role in PD progression.

## Materials and Methods

### DNAJB1

#### *Establishing DNAJB1 knockouts*

HEK293 cells overexpressing α-syn attached to a red fluorescent protein (dsRed) were used to induce and visualize α-syn aggregation. While HEK293 cells originate from human embryonic kidney, the cells show several neuronal phenotype characteristics and are therefore widely used in neuroscience research [17]. The DNAJB1 gene was knocked out in these cells by CRISPR/Cas9 plasmid transfection. The CRISPR/Cas9 system originates from the adaptive immune response of bacteria and archaea, and utilizes a pre-designed guide RNA sequence that leads the protein Cas9 to the gene of interest. Cas9 then creates a double-stranded DNA

break at the specified location, which the cell then tries to repair, resulting in a mutation that in this case created a knockout of the DNAJB1 gene [18]. For this system, DNAJB1-specific guide sequences were created and cloned into a Cas9-Green fluorescent protein (GFP) construct (Addgene cat# 48138).

The insert of the guides into the plasmid was accomplished by mixing the guide RNAs with T4 polynucleotide kinase and T4 ligation buffer in order to promote phosphorylation and annealing of the oligonucleotides. A thermocycler was used with the following temperature differences: 37°C x 30 min, 95°C x 5 min, ramp down to 25°C at 5°C per min. The phosphorylated and annealed oligonucleotides were set up in a combined digestion and ligation reaction with the Cas9-Green fluorescent protein construct and incubated in a thermocycler (6 cycles of 37°C x 5 min and 21°C x 5 min). The resulting ampicillin-resistant plasmids with incorporated guides were transformed into competent bacteria by heat shock and were plated onto ampicillin-containing dishes. After overnight incubation, colonies were selected for Miniprep plasmid isolation (Zyppy Plasmid Miniprep Kit). The plasmids were sequenced to ensure correct oligo incorporation. The guide sequences used were 5'CACCGTCTCCTCGTCCGACGCGCCG3', 5'AAACCGGCGCGTCGGACGAGGA GA3', 5'CACCGCGGGTCGCTGAGCACGTCGT3', 5'AAACACGACGTGCTCAGC GACCCGC3'.

In order to transfect the construct into the cells, plasmids and Lipofectamine LTX (Thermo Fisher Scientific) were separately incubated with Opti-MEM medium (Thermo Fisher Scientific) for 5 minutes, after which they were combined and incubated for an additional 20 minutes. Subsequently, the solution was added to the cells dropwise. After overnight incubation, medium was changed, and 36 hours after transfection, GFP-positive cells were single sorted using Fluorescence Activated Cell Sorting (FACS). In this method, single cells are passed in front of a laser, which detects the fluorescence of the cell, and which subsequently sorts the cells emitting the fluorescence of interest into individual 96 wells [19]. Following this, the cells were grown in DMEM (Sigma-Aldrich) plus 10% FBS (Sigma-Aldrich) and penicillin/streptomycin (Gibco Life Technologies), and surviving colonies were grown in 96 well plates until they were subconfluent, and then transferred to 12 well plates.

*Identifying DNAJB1 knockouts*

Subconfluent cells from 12 well plates were detached by trypsinization and split into two fractions: half of them were frozen using freezing media (60% FBS, 30% medium, 10%

DMSO) for further investigating, and the other half was transferred into an Eppendorf tube after which the cells were lysed using lysis buffer (0.5% Triton X-100, 50 mM Tris/HCl pH 7.4, 175 mM NaCl, 5 mM EDTA pH 8.0, 1% protease inhibitor mixture). The protein concentration was determined using the Bradford assay, in which the dye Coomassie Brilliant Blue G-250 is converted from its red to its blue form when bound to protein [20]. Samples were compared to a standard with BSA concentrations ranging from 0 to 2000 µg/ml using cuvette spectrophotometry. Protein concentrations were used to calculate appropriate volumes of lysates for the western blot which was then performed. The proteins were separated in 8 % polyacrylamide gels (resolving gel: 2% Glycerol, 0.5M Tris pH 8.8, 0.1% SDS, Acrylamide 8%/Bis 0.9%, 1% TEMED, 0.06% APS, stacking gel: 0.06 M Tris pH 6.8, 0.05% SDS, Acrylamide 2%/Bis 0.2%, 0.05% TEMED, 0.03% APS) and were then transferred onto a PVDF membrane, blocked with 5% (w/v) dried skimmed milk in PBS-T (8% NaCl, 0.2% KCl, 1.44% $Na_2HPO_4$, $KH_2PO_4$, 0.05% Tween) and stained with a primary anti-DNAJB1 antibody (Enzo Life Sciences, Cat# ADI-SPA-400-D) in 2% (w/v) dried skimmed milk in PBS-T, and a secondary HRP-conjugated goat anti-rabbit antibody (Dako) in 2% (w/v) dried skimmed milk in PBS-T, in order to identify knockouts. Three knockouts of DNAJB1 were investigated. Further lysates were made and western blot was performed a second time to assure that the knockouts were identified correctly. The blots were also stained with a primary anti-α-syn (BD, Cat# 610787) antibody to visualize the total amount of α-syn in the lysates, as well as with anti-phospho-α-syn (Abcam, Cat# ab59264) antibody, corresponding to the phosphorylated Ser129 amino acid in aggregated α-syn. Secondary antibodies used were HRP-conjugated goat anti-rabbit and goat anti-mouse from Dako.

*Quantifying α-syn aggregates*

50 000 cells, counted using a hemocytometer, were transferred to a 24 well plate with coverslips and left overnight to grow. The cells were washed with PBS (Thermo Fisher Scientific), fixed with 4% PFA for 20 minutes, re-washed with PBS and then mounted on a microscope slide using Vectashield mounting medium (H-1200, Vector) containing the DNA-specific stain DAPI [21]. Using a fluorescent microscope, at least 200 cells were photographed and their α-syn aggregates were counted. This was repeated three times for each sample. For all experiments, the knockouts were compared to a wildtype control.

*Creating DNAJB1 and DNAJB1 H32Q plasmids*

In order to reintroduce the DNAJB1 gene into the knockouts, human DNAJB1 cDNA and H32Q mutant human DNAJB1 cDNA were amplified using primers containing XhoI (5'TCGACTCGAGCTATGGGTAAAGACTACTACCAGACGTTGGG3') and BamHI (5'TCGAGGATCCCTATATTGGAAGAACCTGCTCAAGTACGGTTC3') restriction site overhangs. cDNA was isolated by size through gel electrophoresis, as was Kanamycin-resistant plasmid vector pAcGFP-1-C1 (Clontech). Subsequently, the isolated DNA was purified from the gel using Zymoclean Gel DNA Recovery Kit. All DNA was then digested by restriction nucleases *Bam*HI and *Xho*I, and the plasmid vector was ligated to both variants separately. Afterwards, the solutions were added to competent bacteria (XL1-Blue), which were heat shocked to enable transformation and incubated with SOC medium (Thermo Fisher Scientific) for 2 hours on a shaker at 37°*C*. Next, the bacteria were plated onto agar plates containing Kanamycin and were incubated overnight. Four colonies of each sample were then isolated and incubated overnight in a shaker in 5 ml LB medium containing Kanamycin. The following day, a MiniPrep (Zyppy Plasmid Miniprep Kit) was performed to isolate the plasmids. To make sure that the DNAJB1 gene had successfully integrated in the plasmid, 1 µg of the purified plasmid DNA was digested using restriction nucleases *Bam*HI and *Xho*I. The resulting DNA was separated by size to enable identification of the DNAJB1 gene. The plasmid was sequenced to ensure correct integration.

*Transfection of plasmids and quantification of α-syn aggregates*

To introduce the gene into the knockout cells, the plasmids and Lipofectamine LTX (Thermo Fisher Scientific) were independently incubated with Opti-MEM (Thermo Fisher Scientific) for 5 minutes at room temperature, and subsequently combined for an additional 20 minute incubation. After adding the solution dropwise to 30 000 cells grown in a 24 well plate with coverslips overnight, the cells were incubated 24 hours. Medium was changed, and the following day the cells were washed with PBS, fixed with 4% PFA for 20 minutes and then mounted onto microscope slides using Vectashield mounting medium containing DAPI after an additional PBS washing. At least 200 GFP and dsRed positive cells were counted and their aggregates quantified. The experiment was repeated 3 times for each sample.

*DNAJB6a knockout*

As earlier results by Hansen et al., (unpublished data, 2017) have shown that DNAJB6 is able to significantly suppress α-syn aggregation in HEK293 cells overexpressing α-syn-dsRed, the effect of knocking out DNAJB6a was investigated. The knockout of DNAJB6a was created using the same CRISPR/Cas9 method as the DNAJB1 knockouts, described above. The sequences for the guides used were 5'CACCGTCCTACAGAATTGTCGAGAA3' and 5'AAACTTCTCGACAATTCTGTAGGAC3'. The primary antibodies used to identify the knockout by western blot were anti-α-syn (BD, Cat# 610787), anti-phospho-α-syn (Abcam, Cat# ab59264), and anti-DNAJB6 (Abcam, Cat# ab198995). Secondary antibodies used were HRP-conjugated goat anti-rabbit and goat anti-mouse from Dako. The knockout cells were grown on coverslips, mounted onto microscope slides, and quantified in the same manner as the DNAJB1 knockouts described above.

*DNAJB6 and DNAJB1 double knockout*

Since both DNAJB6 and DNAJB1 show a significant increase in α-syn aggregation when knocked out, a double knockout of both proteins was created in α-syn-dsRed-expressing HEK293 cells. The knockout was created using the same method as for DNAJB1 described above. The sequences used for the DNAJB1 knockout were 5'CACCGTCTCCTCGTCCG ACGCGCCG3', 5'AAACCGGCGCGTCGGACGAGGAGA3', 5'CACCGCGGGTCGCT GAGCACGTCGT3', and 5'AAACACGACGTGCTCAGCGACCCGC3'. The sequences used for DNAJB6 knockout were 5'CACCGGGAGGCATATGAAGTGCTGT3' and 5'AAACAGATATCGGAAACTGGCACTC3'. The antibodies used for western blot were anti-DNAJB1 antibody (Enzo Life Sciences, Cat# ADI-SPA-400-D) and anti-DNAJB6 (Abcam, Cat# ab198995). The secondary antibody used was HRP-conjugated goat anti-rabbit from Dako.

<u>Ire1 knockout mice</u>

*Generation of transgenic mice*

Hansen et al., 2013 have previously established a mouse model of Parkinson's disease, where human α-syn attached to GFP is expressed under control of the endogenous mouse α-syn promoter through pronuclear injection of a bacterial artificial chromosome [22]. This model mouse was used in the following experiments to investigate the effect of knocking out the Ire1α gene in wildtype and Parkinson-model mice. All mice used were female; as Hansen et

al., 2013, showed that the Parkinsonian symptoms were more abundant in female α-syn-GFP expressing mice than in males.

In order to create mice with a conditional dopaminergic knockout of Ire1, the Cre-Lox method was used. Mice with Cre recombinase expressed on their dopaminergic neurons (The Jackson Laboratory), courtesy of the endogenous dopamine transporter promoter, were bred with mice that have *loxP* flanking their Ire1α gene. By *loxP*-specific recombination, Cre recombinase deleted the Ire1α gene [23]. Breeding the α-syn-GFP-expressing mice with the conditional dopaminergic Ire1 knockout mice resulted in the main mouse model investigated in the study: a Parkinson model mouse with a conditional knockout of the Ire1α gene. In addition to this model, three control groups were tested, resulting in the following four groups being studied;

1. Wildtype Ire1α and no expression of human α-syn-GFP
2. Conditional Ire1α knockout and no expression of human α-syn-GFP
3. Wildtype Ire1α and expression of human α-syn-GFP
4. Conditional Ire1α knockout and expression of human α-syn-GFP

*Genotyping*

Mouse ear biopsies were analyzed using genotyping lysis buffer (50 mM Tris pH 8.0, 5 mM EDTA, 100 mM NaCl, 0.5% SDS) in combination with Proteinase K at 56ºC overnight. Subsequently, DNA was precipitated by addition, incubation, centrifugation and removal of saturated NaCl followed by addition, centrifugation and removal of 99% ethanol. 70% ethanol was used to wash the DNA pellet before resuspension in 10 mM Tris pH 7.4, 0.1 mM EDTA. Using α-syn-GFP and dat-cre primers, specific for the α-syn-GFP gene and the Cre recombinase gene, respectively, PCR was performed. The presence of the amplified genes was analyzed by size through agarose gel electrophoresis.

*Behavioral tests*

Behavioral test were performed on all animals at around 3 and 6 months of age. The two tests that were used are the rotarod assay and the open field assay. Before all experiments the mice were allowed to familiarize themselves with the test room for one hour. The number of mice used for the tests were as follows (Ire1α-/αsyn-, Ire1α+/αsyn-, Ire1α-/αsyn+, Ire1α+/αsyn+ respectively): 3 months of age (10, 7, 8, 11), 6 months of age: (4, 7, 1, 9).

*Rotarod*

The rotarod assay allows the animals to walk on an accelerating rod in order to assess the motor abilities and balance of the animals (Rotamax 4/8, Columbus Instruments, USA, 3.8 cm rod diameter, 4.5 cm wide) [24]. Prior to testing, the mice were pre-trained by walking for 20 seconds at a rod rotating at a speed of 5 rounds per minute (rpm). Subsequently, the mice were placed on the rod once more as the speed increased gradually from 5 to 40 rpm over 5 minutes. When a mouse fell down from the rod the time was stopped and noted. 4 trials (two each day, with at least an hour between each trial) were averaged. If the mouse held on to the rod or by other means stayed on the rod without running or walking, the trial ended there, for the purpose of measuring the same kind of motor abilities in all mice. The rotarod machine was cleaned with 30 % ethanol between each animal.

*Open field assay*

The open field assay placed the animals in 40.6×40.6×38.1 cm (d×w×h) plexiglass chambers cleaned with water and 30 % ethanol. The mice were allowed to explore the cage freely for one hour while their movements were recorded using an infrared photobeam system, where beam-breaks were recorded [25]. The purpose of this test was to record the total distance the animals moved during the hour, to assess locomotor activity. After resting for at least an hour, animals were intraperitoneally injected with 5 mg/kg amphetamine in saline solution and the test was immediately repeated. Amphetamine causes an inhibition of the dopamine transporter (DAT) leading to increase of dopamine in the synaptic cleft of dopaminergic neurons, causing an activation of these synapses [26]. Hence, the injection of amphetamine activates the neurons of the *substantia nigra pars compacta* that should be relevant in Parkinson's disease. Between all tests the cages were cleaned. Results were analyzed using the ANY-maze software.

*Statistics*

The significance of the results was analyzed using student's t test, and variance was investigated by standard deviation, for all experiments.

**Results**

DNAJB1

To investigate whether DNAJB1 may play a role in α-syn aggregation, we decided to knock out DNAJB1 with the help of specific DNAJB1 gene targeting RNA guides by the use of the

CRISPR/Cas9 system. The cell line of choice for the study was HEK293 cells overexpressing a transgene of dsRed fused to the C-terminus of α-syn. Cells transiently transfected with guide RNA and GFP-Cas9 were sorted by FACS 36 hours after the transfection and surviving potential knockout colonies were grown in 96 well plates. Subsequently, cell lysates were analyzed by western blot, leading to the discovery of three independent complete DNAJB1 knockout cell lines (Figure 1 A). Furthermore, these cell lines were analyzed using antibodies reflecting total α-syn level, phospho-Ser129-α-syn level, as well as actin loading control. The clones were shown to have a similar total α-syn level, while the phospho-Ser129-α-syn level, which is a known marker of α-syn aggregation [27], was higher in knockout 3 than in wildtype and both the other knockouts (Figure 1 A).

When visualizing the knockout and wildtype cells using fluorescence microscopy, significantly more red puncta (interpreted as α-syn aggregates) were seen in the knockouts than in the control (Figure 1 C). Around 8.4% of the knockout cells had red puncta compared to 3.4% cells of the wildtype (Figure 1 B). To confirm that the increase in red puncta in the knockouts was indeed due to lack of DNAJB1, a GFP-tagged version of DNAJB1 was reintroduced into the cells by transfection (Figure 2 B). The reintroduction of DNAJB1 resulted in a significant decrease of α-syn aggregates to around 4.4% in knockout 1 (Figure 2 A). Similar significances were seen in all three knockouts.

In order to verify that the decrease of aggregates after DNAJB1 reintroduction was due to DNAJB1 interacting with Hsp70 through its J domain, a control experiment was set up. By using a mutated version of DNAJB1 H32Q, which cannot interact with Hsp70, it was possible to confirm the importance of the J domain for the interaction between chaperone and co-chaperone [28]. Indeed, transfection of H32Q DNAJB1 into DNAJB1 knockout cells did not decrease the amount of red puncta in the cells significantly, and the aggregate percentage was quantified to 8.3% in knockout 1 (Figure 2 A, Figure 2 C), with similar results in the other knockouts. This suggests that the J domain, and thereby the interaction with Hsp70, is vital to the α-syn-disaggregating properties of DNAJB1.

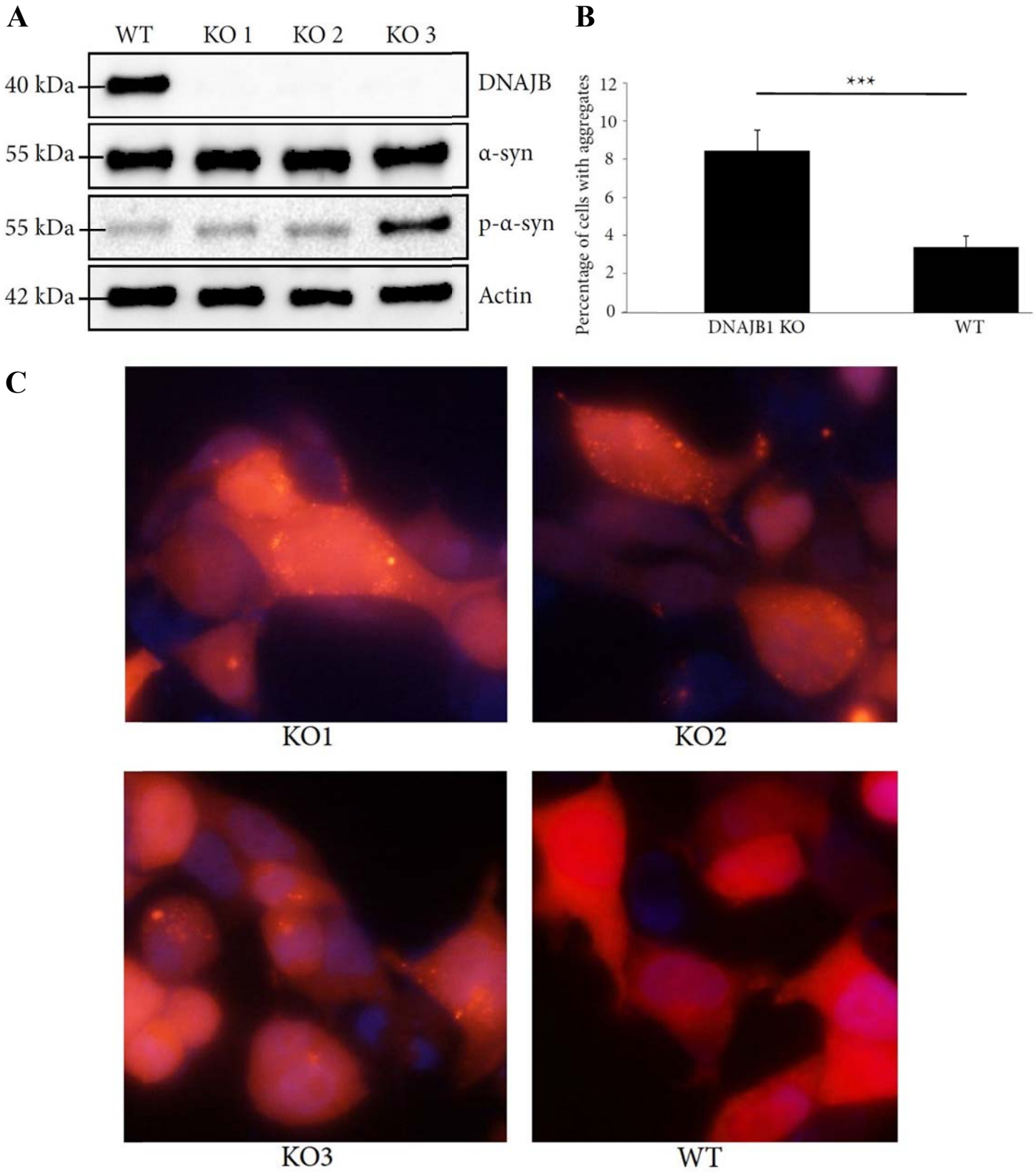


**Figure 1. Knockout of DNAJB1 in HEK293-α-syn-dsRed cells causes increased α-syn aggregation.** **(A)** Western blot depicting DNAJB1 protein levels, phospho-Ser129-α-syn levels, and total α-syn-dsRed levels in HEK293-α-syn-dsRed cells, for wildtype control and three DNAJB1 knockouts. The detection of actin was used as loading control. **(B)** The percentage of cells with α-syn aggregates (red puncta) in knockout compared to control. The results of the three knockouts were averaged. Statistical analysis performed by student's t test. *:P<0.05, **:P<0.01, ***:P<0.001. Standard deviation displayed by error bars. **(C)** Fluorescence microscopy of each DNAJB1 knockout as well as wildtype control in HEK293-α-syn-dsRed cells. The aggregates are visualized as red puncta due to expression of α-synuclein with dsRed. The cell nuclei are stained with DAPI. Pictures were taken at 60X magnification.

Earlier work by Hansen et al., (unpublished data, 2017) has shown that knocking out the DNAJ protein DNAJB6 in α-syn-dsRed-expressing HEK293 cells leads to a significant increase in α-syn aggregates. In aforementioned study, both isoforms of DNAJB6, DNAJB6a and DNAJB6b, were knocked out. In continuation with this work, a knockout of only the DNAJB6a isoform was investigated, in order to examine the individual abilities of the isoform. A knockout was accomplished and confirmed by western blot, after which levels of total α-syn as well as phospho-α-syn were investigated (Figure 3A). The levels of both the aggregation-dependent phospho-α-syn as well as the total α-syn levels were similar in both knockout and wildtype. Subsequently, the percentage of red puncta was quantified as described earlier, and there was shown not to be any significant difference between knockout and wildtype (Figure 3B). While the DNAJB6a isoform alone does not seem to have an effect on the aggregation of α-syn, the complete knockout of DNAJB6 still gives a pronounced effect, similar to the clear effect of a DNAJB1 knockout. Therefore, a double knockout of the two proteins was made (Figure 3C), to enable further investigation of their effect on α-syn aggregation.

Altogether, these results suggest that DNAJB1 has a disaggregating effect on α-syn that leads to an increase in aggregates when the protein is knocked out. The α-syn aggregate suppressing characteristics of DNAJB1 are dependent on its interaction with Hsp70 through the J domain. On the other hand, the α-syn aggregate suppressing features of the DNAJB6 protein do not seem to be due to the DNAJB6a isoform, but more likely because of the DNAJB6b isoform. By producing a combined knockout of both DNAJB1 and DNAJB6, further studies can be made on the effects of these proteins on α-syn aggregation.

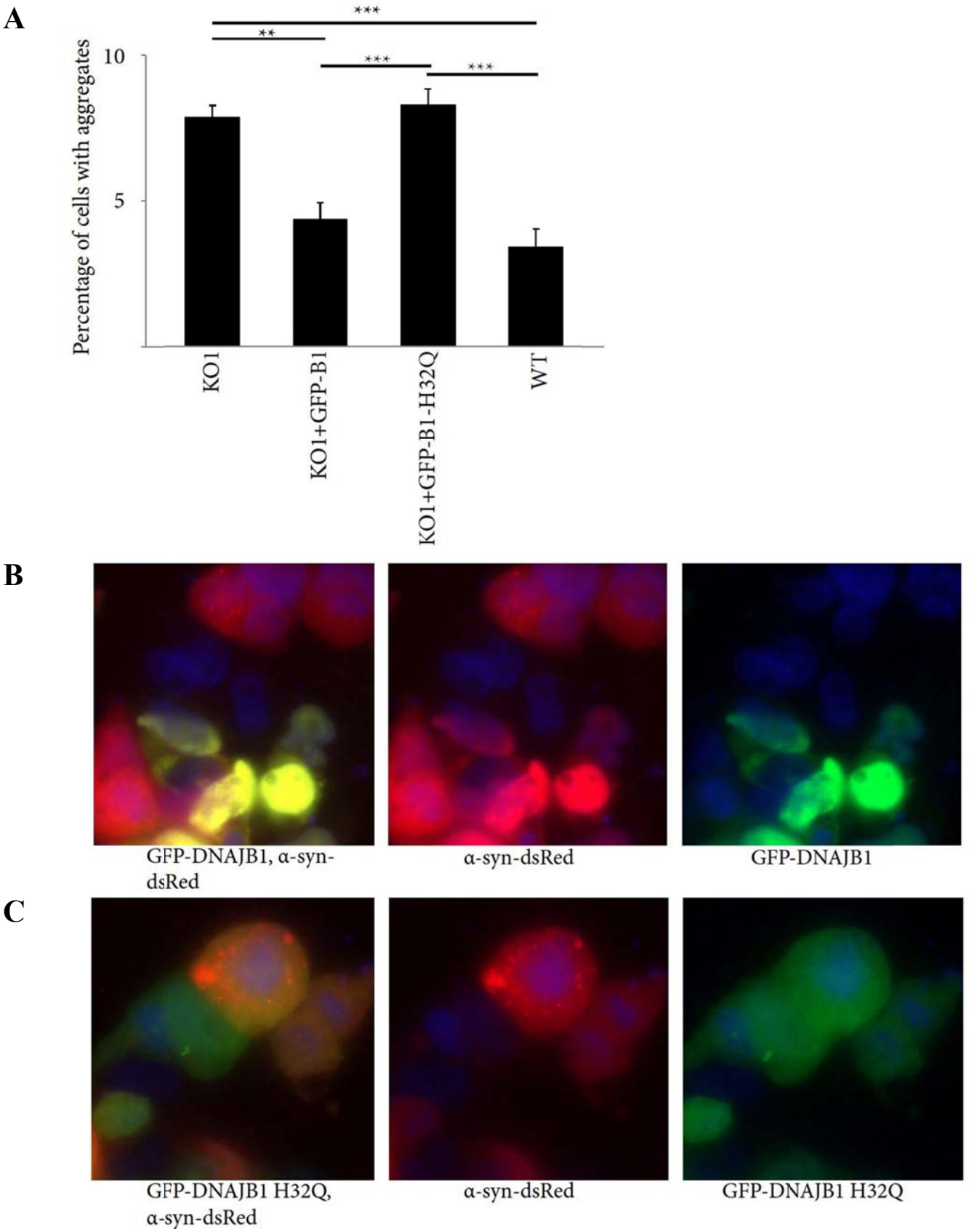


**Figure 2. DNAJB1 knockout aggregation increase is dependent on DNAJB1 J domain. (A)** Displays the percentage of cells with α-syn aggregates for DNAJB1 KO 1, for KO1 transfected with the DNAJB1-GFP expression plasmid, for KO1 transfected with the DNAJB1 H32Q mutant expression plasmid, and for the wildtype control, in HEK293-α-syn-dsRed cells. Statistical analysis performed by student's t test. *:P<0.05, **:P<0.01, ***:P<0.001. **(B)** Fluorescence microscopy of KO1 transfected with GFP-DNAJB1. The cell nuclei are stained with DAPI. Pictures were taken at 60X magnification. **(C)** Fluorescence microscopy of KO1 transfected with GFP-DNAJB1 H32Q. The cell nuclei are stained with DAPI. Pictures were taken at 60X magnification.

Ire1

Parkinson-model mice expressing α-syn-GFP under the control of the endogenous mouse α-syn promoter were mated with dopaminergic conditional Ire1 knockout mice to create four experimental groups: (wt Ire/wt α-syn-GFP), (tg Ire/wt α-syn-GFP), (wt Ire/tg α-syn-GFP), (tg Ire/tg α-syn-GFP). In order to investigate the behavioral symptoms of these animals, the mice were studied using the rotarod test and the open field assay, at 3 and 6 months of age. The rotarod assay measures the animals' gross motor skills by testing their ability to run on a rod of increasing speed without falling. At 3 months, the experiment showed no significant difference in motor skill between any of the groups (Figure 4 A). Similarly, after 6 months, there still was no significant variation between the groups (Figure 4 B).

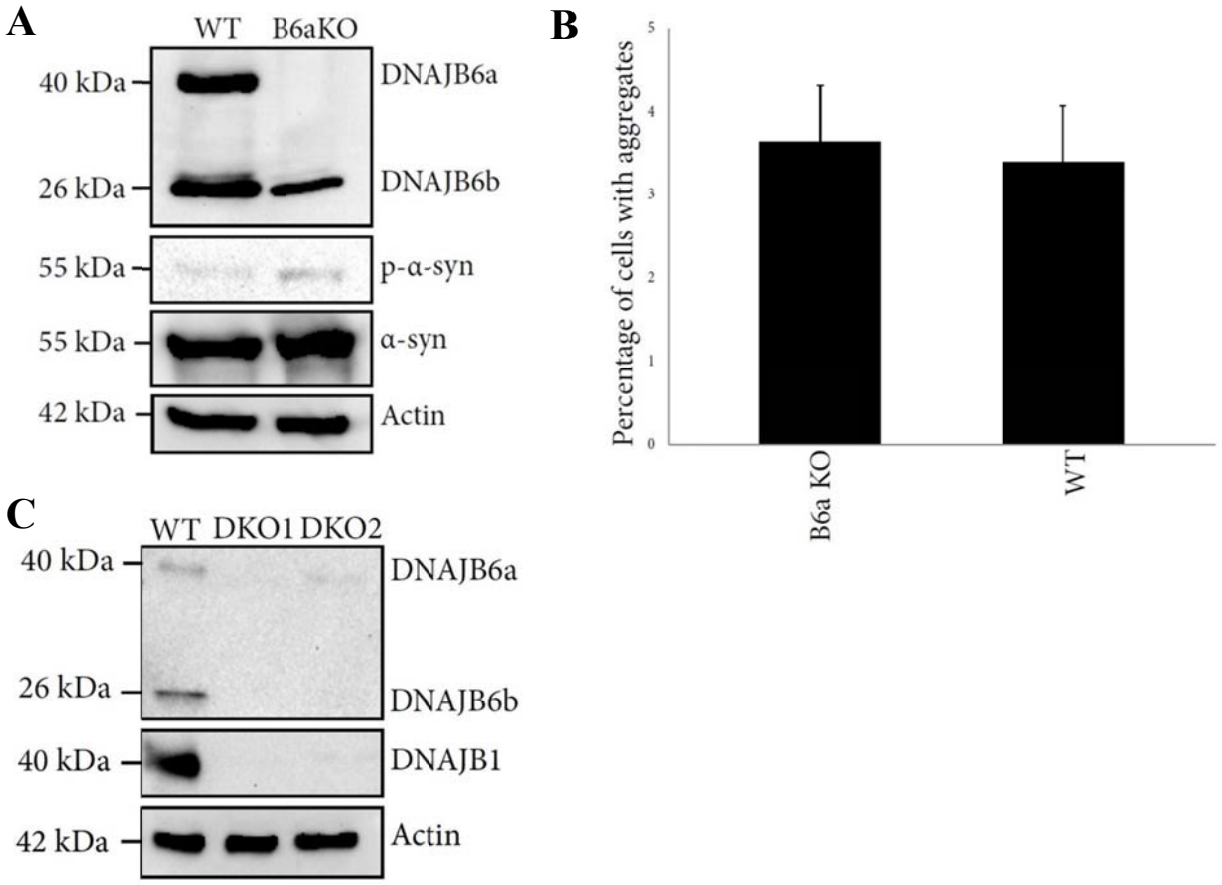


**Figure 3. Knockout of DNAJB6a and double knockout of DNAJB6b and DNAJB1 in HEK293-α-syn-dsRed cells. (A)** Western blot depicting DNAJB6a protein levels, phospho-Ser129-α-syn levels, and total α-syn-dsRed levels in HEK293-α-syn-dsRed cells, for wildtype control and DNAJB6a knockout. The detection of actin was used as loading control. **(B)** The percentage of cells with α-syn aggregates (red puncta) in DNAJB6a knockout compared to control. Standard deviation displayed by error bars. **(C)** Western blot depicting a DNAJB6 and DNAJB1 protein levels, in HEK293-α-syn-dsRed cells, for wildtype control and two double DNAJB6b and DNAJB1 knockout. The detection of actin was used as loading control.

The open field assay measures the locomotor activity of the mice during a one hour long exploration of a plexiglass chamber. The mice were first tested without any treatment, and then tested again after an amphetamine injection (5 mg/kg), to cause increase of extracellular dopamine and activation of dopaminergic synapses. At 3 months, no treatment, there is no significant difference in locomotor activity between the four groups (Figure 5 A). When the mice are injected with amphetamine, however, a significant difference emerges between (tg Ire/wt α-syn-GFP) and (wt Ire/tg α-syn-GFP) groups, where the (tg Ire/wt α-syn-GFP) has a lower distance travelled than the (wt Ire/tg α-syn-GFP) (Figure 5 B). After 6 months, no treatment, there still is no significant difference between any of the groups (Figure 5 C). However, there is only one value measured from the (wt Ire/tg α-syn-GFP) group and therefore significance calculations involving this group cannot be performed. In addition, it is discouraged to draw conclusions from this value in the amphetamine treatment (Figure 5 D), as it may look much lower than the rest but is still the result of only one mouse which may or may not be an outlier. The other groups do not show any significant differences under amphetamine treatment.

Altogether, the gross motor skill measured by the rotarod test appears unaffected by the genetic alterations after 3 and 6 months. Similarly, the locomotor activity of the mice without treatment is seemingly comparable between groups. While the amphetamine-treated 3 month old mice seem to exhibit a significance between two groups, the change cannot be tracked at 6 months due to lack of mice.

**Discussion**

DNAJB1

Using DNAJB1 knockout HEK293 cells overexpressing α-syn-dsRed have shown the ability of DNAJB1 to suppress α-syn aggregate formation by exposing a significant increase of α-syn aggregates when the gene is knocked out (Figure 1 C). In addition, this upsurge is diminished when DNAJB1 is reintroduced into the cell by transfection (Figure 2 A). However, the introduction of a DNAJB1 H32Q mutant, which cannot interact with Hsp70 due to a substitution in its J domain, cannot decrease the amount of α-syn aggregates in the cells

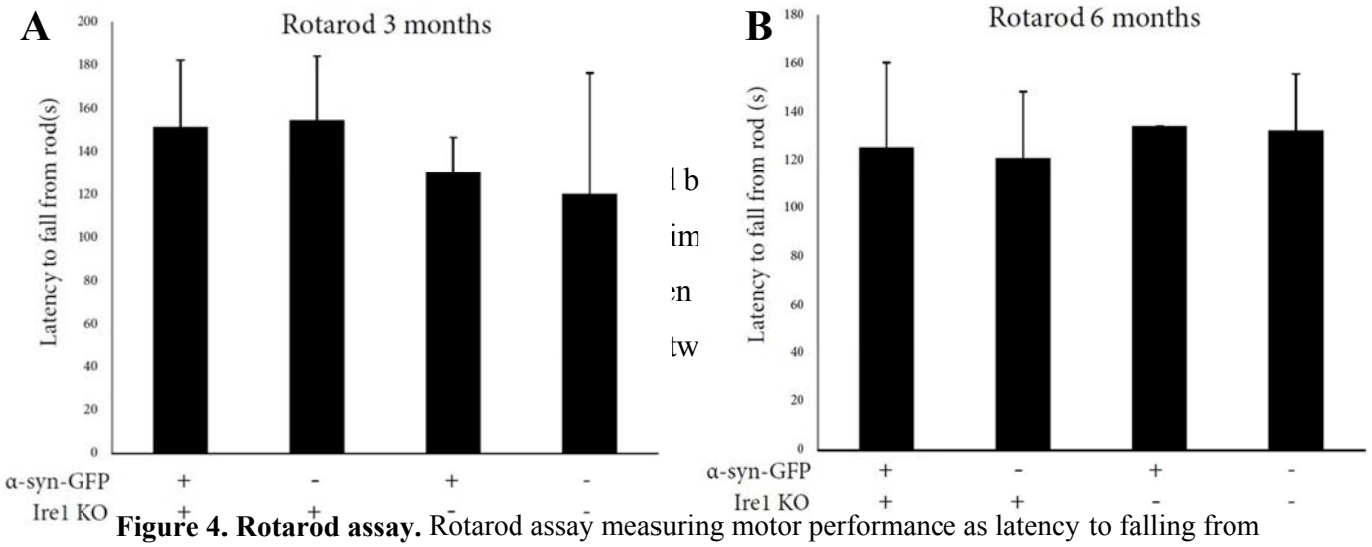


**Figure 4. Rotarod assay.** Rotarod assay measuring motor performance as latency to falling from accelerating rod (s) for **(A)**3 month old mice, **(B)** 6 month old mice. Mice investigated are (tg Ire1[+]/tg α-synuclein-GFP[+]), (tg Ire1[+]/wt α-synuclein-GFP[-]), (wt Ire1[-]/tg α-synuclein-GFP[+]), (wt Ire1[-]/wt α-synuclein-GFP[-]).

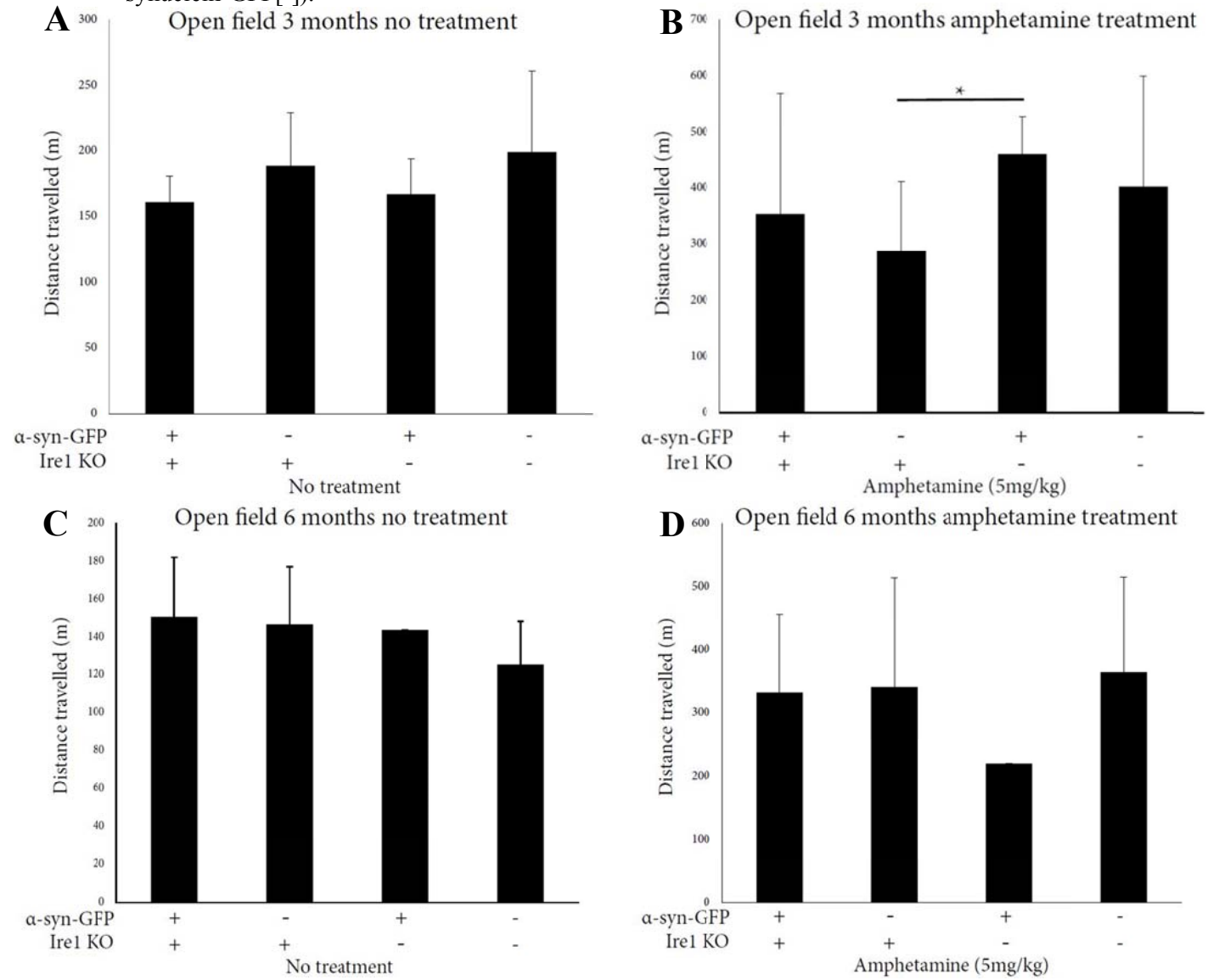


**Figure 5. Open field assay.** Open field assay measuring locomotor activity as distance travelled (m) for **(A)** 3 month old mice without treatment, **(B)**3 month old mice treated intraperitoneally with amphetamine (5 mg/kg), **(C)** 6 month old mice without treatment, **(D)** 6 month old mice treated intraperitoneally with amphetamine (5 mg/kg). Mice investigated are (tg Ire1[+]/tg α-syn-GFP[+]), (tg Ire1[+]/wt α-syn-GFP[-]), (wt Ire1[-]/tg α-syn-GFP[+]), (wt Ire1[-]/wt α-syn-GFP[-]). Statistical analysis performed by student's t test. *:P<0.05, **:P<0.01, ***:P<0.001.

(Figure 2 A). This suggests that it is, in fact, the interaction with Hsp70 through the J domain that is the main pathway of α-syn disaggregation for DNAJB1. Similarly, previous studies have shown that poly-Q inclusion formation is decreased by the presence of DNAJB1, but only when its J domain is non-mutated [28].

In addition, western blot staining (Figure 1 A) showed that while the total α-syn aggregation levels remain similar between knockout and wildtype, the amount of phosphorylated α-syn seems to be increased in one knockout clone only. Since only three clones are investigated, it is difficult to state whether the effect is individual to this clone only or if the phosphorylated α-syn level is heightened in a larger percentage of the DNAJB1 knockouts. Since phosphorylation of α-syn is a post-translational modification that is enhanced in Lewy Bodies and correlated to increased aggregation of the protein, these results would suggest that the third knockout has a higher level of aggregation than control and both of the two other knockouts [22]. The fact that the total α-syn level remains steady between clones suggests that the third clone does not have increased α-syn. In addition, when the fluorescence microscopy quantification is compared between clones, the third knockout does not have a significantly larger percentage of aggregates than the other knockout clones (Figure 2 A). Therefore, the reason for this difference is not easily explainable, but could be attained to an increased amount of post-translational phosphorylation, or to the aggregates themselves having more phosphorylated α-synuclein exposed to the reach of antibodies.

While the disaggregation results are promising, there are several interesting experiments that could be done in this area in the future. Firstly, it would be interesting to further investigate the interaction between Hsp70 and DNAJB1 by for example an Hsp70 inhibitor. By inhibiting Hsp70, one could investigate if DNAJB1 has further beneficial effects in the cell independently of Hsp70 when its J domain is not mutated. In addition, the results could be cemented by further experiments measuring the difference of α-syn aggregate levels between knockout and control. LBs and other large pathological α-syn aggregates in PD have been shown to be insoluble, a fact that could be used to separate from the soluble fraction and quantify them[29]. If, however, the red puncta viewed in the fluorescence microscope are not in fact insoluble, native gels could be used to visualize the aggregates, since native gels allow complexes of protein to remain folded as they were in the cell[30].

Furthermore, there is a third partner in the disaggregating activity that could be investigated: the nucleotide exchange factors (NEFs). Their role in the activity of DNAJ and Hsp70 is to

escalate replacement of ADP with ATP, and thereby to increase the rate of disaggregation [31]. There is a lot of diversity in the family of NEFs in human cells, and it has been found that different NEFs can have very different levels of disaggregation enhancement. Apg2 and Hsp105α of the Hsp110 NEF family, for example, have been shown to increase disaggregation efficiency in combination with Hsp70 and DNAJB1, while NEF Bag-1 does not [9]. Indeed, the ineffectiveness of Bag-1 together with DNAJB1 and Hsp70 has been shown in Poly-Q inclusions, as well [28]. Therefore, in the future it might be interesting to further investigate the particular NEFs that work best together with DNAJB1.

PD is known to cause degeneration of dopaminergic cells, and therefore an additional relevant experiment would be to investigate the effect knocking out DNAJB1 has on cell death. In addition, the cells we use in this study are originally human embryonic kidney cells. While HEK293 cells have been shown to possess several neuronal phenotype characteristics[17], it is important to continue these studies in an actual neuronal cell line, such as the neuroblastoma cell line Neuro-2A[32]. Indeed, as the project progresses, it would be interesting to move the search into a primary neuronal cell line, where there is potential to dissect out dopaminergic neurons exclusively. Furthermore, it would give the possibility to study typically neuronal characteristics, such as axonal function, more closely. In fact, α-syn has been shown to accumulate in axons in primary neurons that have been seeded with preformed α-syn fibrils[29].

Subsequently, there are several possible *in vivo* models that could be relevant. Firstly, a knockout model of DNAJB1 is yet to be produced, but by using the Cre-LoxP method similarly to the way the Ire1 knockout mice were created in this report, a line of knockout mice could be made. This line could then be mated with PD model mice, producing a DNAJB1 knockout PD mouse that could be used for both behavioral test and brain studies. Secondly, it has been shown that the use of recombinant adeno-associated viral vectors (rAAV) that express human α-syn leads to progressive loss of dopaminergic neurons in *substantia nigra pars compacta*. Therefore, rAAVs expressing α-syn can readily be used as PD models[33]. In addition, these viruses can be used to overexpress promising therapeutic proteins such as DNAJB1 in the brain, to analyze the effects on behavior. Furthermore, rAAV could be used to combine overexpression of DNAJB1 with overexpression of a NEF deemed appropriate through cell studies, to increase efficiency of the chaperone system. Another alternative is to induce PD-like symptoms in DNAJB1 knockout animals using the rAAV virus.

While there are indeed multiple possible experiments concerning DNAJB1, there is also the possibility to further explore the double knockout of DNAJB1 and DNAJB6. Since these proteins both have similar effects on the aggregation of α-syn, reintroduction tests may shed a light on whether the mechanisms of these proteins in relation to Hsp70 differ completely or are functionally redundant.

In order to further understand the future results of analyzing the double knockouts, it is important to analyze the results of the DNAJB6a knockout. Since the percentages of red puncta in wildtype control and DNAJB6a are not significantly different (Figure 3B), the main disaggregating effect of DNAJB6 on α-syn aggregates must stem from the DNAJB6b isoform or the combination of both DNAJB6a and DNAJB6b. α-syn has been shown to be expressed in both nucleus and cytoplasm, while DNAJB6b is mostly cytosolic and DNAJB6a is mostly nucleic[10]. A possible explanation for the indifferent results of the DNAJB6a knockout and control could be that the majority of the aggregates of the α-syn-dsRed model used in these experiments are localized to the cytoplasm, even though α-syn is expressed in both nucleus and cytoplasm. That would result in a decreased effect of DNAJB6a, as it is located to the nucleus. Therefore, it may be beneficial to further analyze the model used, and where the majority of the α-syn-dsRed aggregates reside. To sufficiently rule out any potential benefits of DNAJb6a, it could also be useful to overexpress it in a DNAJB6 knockout cell line, to see if it could rescue the induced aggregation alone.

Ire1

The role of one of the main three UPR pathways, the Ire1 pathway, was investigated in this study by knocking out the Ire1α gene in mice with overexpression of α-syn-GFP. Since the UPR has an important role in maintaining cellular protein homeostasis, this genetic change was hypothesized to result in an increase of aggregated α-syn in neurons, leading to death of dopaminergic neurons and thereby a decrease in the motor control of the mice. This was tested through behavioral tests. However, at 3 months old, there was no significant difference between groups in either of the tests. The reason for this could be that the mice are young, and that the aggregation of α-syn has yet to give an observable effect in behavior, which would be consistent with the fact that age is a risk factor for PD. Indeed, the α-syn-GFP-expressing mouse model used to represent PD did not show any significant behavioral differences at three months of age, which could explain the results. However, the mouse model did show a significant decrease in locomotor activity in the open field assay when administered with

amphetamine at 7 months [22], a difference that was not seen in this study at 6 months. The only significance seen at 6 months was that between (tg Ire/wt α-syn-GFP) and (wt Ire/tg α-syn-GFP). However, since these two groups show no significant difference to any of the two other controls, and since they share no variable, it is difficult to draw any sustainable conclusion from this result.

While the results seem to be similar between all groups at 6 months, it is still interesting to continue the study up to 9 and 12 months, since PD is an age-related disease. In addition, the mouse model used showed no significant differences between groups in the rotarod assay until 12 months of age was reached. Therefore, the effect of Ire1 might be present but strongly age-related. Furthermore, it could be worth-while to sacrifice some 12 month old mice, to investigate the effect of the knockout on the neuronal tissue. This might clear up if the α-syn aggregation has increased in the knockout mice, compared to control, and if it has had an effect on the amount of dopaminergic neurons.

## Conclusion

In conclusion, knocking out DNAJB1 in α-syn-dsRed overexpressing HEK293 cells leads to an increase in red puncta, which we interpret as α-syn aggregates. Reintroducing DNAJB1 to the cells through plasmid transfection leads to an upsurge in disaggregation, resulting in an α-syn aggregate level comparable to control, an effect which seems dependent on the function of the J domain. Knocking out Ire1 in α-syn-GFP-expressing mice, on the other hand, does not seem to have an effect on the motor behavior of the mice. However, considering that age is a common risk factor for PD, the effect of the knockout may become more visible as the mice grow older.

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