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2 **Unconventional secretion of α -synuclein mediated by** 3 **palmitoylated DNAJC5 oligomers**

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Abstract

Alpha-synuclein (α -syn), a major component of Lewy bodies found in Parkinson's disease (PD) patients, has been found exported outside of cells and may mediate its toxicity via cell-to-cell transmission. Here, we reconstituted soluble, monomeric α -syn secretion by the expression of DnaJ homolog subfamily C member 5 (DNAJC5) in HEK293T cells. DNAJC5 undergoes palmitoylation and anchors on the membrane. Palmitoylation is essential for DNAJC5-induced α -syn secretion, and the secretion is not limited by substrate size or unfolding. Cytosolic α -syn is actively translocated and sequestered in an endosomal membrane compartment in a DNAJC5-dependent manner. Reduction of α -syn secretion caused by a palmitoylation-deficient mutation in DNAJC5 can be reversed by a membrane-targeting peptide fusion-induced oligomerization of DNAJC5. We propose that DNAJC5 forms a palmitoylated oligomer to accommodate and export α -syn.

Introduction

Parkinson's disease, the second most common neurodegenerative disease, is characterized by the deposit of clumps of protein aggregate, lipid and damaged organelles known as Lewy bodies (LBs) (Dauer and Przedborski, 2003; Shahmoradian et al., 2019). One of the main constituents of LB is the presynaptic protein α -syn (Stefanis, 2012). α -syn is encoded by the *SNCA* gene and is highly abundant in neurons. As a small, intrinsically disordered protein containing 140 amino acids (AA), α -syn can be divided into three domains, an amphipathic N-terminal domain where most PD-related mutations are located, including A30P, E46K and A53T, a central hydrophobic region known as the non-amyloid- β component (NAC) which is essential for aggregation, and an acidic C-terminal domain (**Fig 1A**) (Alderson and Markley, 2013). α -syn can undergo a conformational change from a disordered monomer to an oligomer (Burre et al., 2014; Lashuel et al., 2002), which can further polymerize to form insoluble fibrils (Guerrero-Ferreira et al., 2019; Guerrero-Ferreira et al., 2018; Strohaker et al., 2019).

In recent years, studies have suggested that α -syn deposits are not static, but rather actively spread during disease progression. Grafted neurons in PD patients developed α -syn positive LBs years after surgery, suggesting host-to-graft pathology propagation (Kordower et al., 2008; Li et al., 2008). Based on analysis of human pathology, the Braak hypothesis posits that α -syn aggregates can spread in a stereotyped manner from the gastrointestinal tract to the brain, causing neuron loss beginning in the brainstem, extending to the midbrain, and finally to the cortex (Braak et al., 2006; Braak et al., 2003). In more recent work, Braak-like transmission of *in vitro* generated α -syn fibrils has been recapitulated in mice and

non-human primates (Chu et al., 2019; Chung et al., 2020; Kim et al., 2019; Luk et al., 2012).

Less-well understood is the molecular and cellular basis for the transfer of α -syn between cells. Several mechanisms have been proposed for α -syn export and transfer, including unconventional exocytosis (Jang et al., 2010; Lee et al., 2005), exosomes (Danzon et al., 2012; Emmanouilidou et al., 2010; Stykel et al., 2021) and membrane nanotubes (Abounit et al., 2016; Scheiblich et al., 2021). The extracellular existence of α -syn was confirmed in body fluid such as blood and cerebrospinal fluid (CSF) (Borghi et al., 2000; El-Agnaf et al., 2003). Packaging of different conformational forms of α -syn inside extracellular vesicles (EVs) has been reported but requires further vigorous scrutiny to differentiate from secreted sedimentable aggregates (Brahic et al., 2016).

DNAJC5, also known as cysteine string protein α (CSP α), is a co-chaperone of HSC70 and has been shown to control the extracellular release of many neurodegenerative disease proteins including α -syn. This process of unconventional traffic has been termed misfolding-associated protein secretion (MAPS) (Fontaine et al., 2016; Lee et al., 2016). DNAJC5 contains three domains – a common N-terminal J-domain conserved among DnaJ proteins, the cysteine-string (CS) central domain which is heavily palmitoylated and anchors the protein to late endosomes, and an overall disordered C-terminal domain (**Fig 1A**).

DNAJC5 plays a neuroprotective role in the brain (Zinsmaier, 2010). Depletion of DNAJC5 produces neurodegeneration in mice by cooperation with α -syn (Chandra et al., 2005). Interestingly, a previous study also reported that neuron-derived EVs contain DNAJC5 (Deng et al., 2017). However, the mechanism by which DNAJC5

recognizes and translocates soluble α -syn into a membrane compartment for secretion remains elusive.

In this study, we characterized the mechanism of DNAJC5-induced α -syn secretion in a cell-based secretion assay. Using biochemical characterization and imaging of internalized α -syn in enlarged endosomes as a secretory intermediate, we found previously underappreciated roles of palmitoylation and oligomerization of DNAJC5 in the regulation of α -syn secretion.

Results

Reconstitution of DNAJC5-induced α -syn secretion

Previous studies have shown that α -syn secretion can be stimulated by over-expressing DNAJC5 (Fontaine et al., 2016). The CS domain of DNAJC5 plays a role in promoting stable membrane attachment based on its overall hydrophobicity and by enabling post-translational palmitoylation catalyzed by membrane-bound Asp-His-His-Cys (DHHC) family palmitoyltransferases (Greaves and Chamberlain, 2006). Using a common human cell line, HEK293T, we first tested the expression and subcellular localization of DNAJC5 (**Fig 1B**). Further subcellular enrichment of DNAJC5 was characterized using a C-terminal FLAG tag. Coomassie blue staining revealed two bands of high and low mobility on SDS-PAGE in the membrane and cytosolic fractions, respectively (**Fig 1C**). Similar migration profiles of palmitoylated (P-) and non-palmitoylated (NP-) DNAJC5 have been reported (Greaves et al., 2012). Thus, we confirm that membrane anchoring of DNAJC5 requires palmitoylation in our assay.

We next co-expressed DNAJC5 together with α -syn to examine their secretion over time. At 16 h after transfection, we detected similar basal-level secretion of α -syn in both DNAJC5-negative and -positive conditions. Two bands of DNAJC5 corresponding to P-DNAJC5 and NP-DNAJC5 were seen in the lysate, but only the P-DNAJC5 was secreted into the medium (**Fig 1D**). The stimulation of α -syn secretion by DNAJC5 became obvious at a longer incubation time (36 h), and at this time point NP-DNAJC5 was also enriched in the medium (**Fig 1E**). In addition to wild-type (WT) α -syn, secretion of several PD-causing α -syn mutant proteins (A30P, E46K and A53T) were also induced by expressing DNAJC5 (**Fig 1 supplement 1**).

In addition to the stimulated secretion of α -syn produced by the expression of exogenous DNAJC5, we examined the dependence of a basal secretion of α -syn on endogenous DNAJC5. We fused α -syn with an N-terminal nanoluciferase (Nluc) (England et al., 2016) for sensitive, quantitative detection (**Fig 1 supplement 2A**). Stimulated secretion of Nluc- α -syn by DNAJC5 was confirmed by immunoblot, indicating that Nluc-fusion did not impede α -syn secretion (**Fig 1 supplement 2B**). Without over-expression of DNAJC5, we observed accumulation of Nluc- α -syn signal in the medium over time (**Fig 1 supplement 2C**). Quercetin is a plant-derived flavonoid that has previously been shown to inhibit DNAJC5-mediated trafficking of a bacterial toxin (**Fig 1 supplement 2D**) (Deruelle et al., 2021). Likewise, we found that quercetin also inhibited Nluc- α -syn secretion in a dose-dependent manner (**Fig 1 supplement 2E**), implying a role for endogenous DNAJC5 in α -syn secretion. Direct depletion of endogenous DNAJC5 by siRNA interference (**Fig 1 supplement**

2F) also decreased basal α -syn secretion (**Fig 1 supplement 2G**). Taken together, we established a cell-based α -syn secretion assay that is regulated by DNAJC5.

Characterization of extracellular DNAJC5 and α -syn

In our established assay, DNAJC5 and α -syn co-secrete into the medium (**Fig 1E**). Secreted α -syn has been reported to be encapsulated inside EVs (Danzer et al., 2012). We sought to assess the EV association of secreted α -syn using a medium fractionation protocol based on EV preparations developed by our lab (**Fig 2A**) (Shurtleff et al., 2016). After serial differential centrifugation, α -syn and NP-DNAJC5 remained soluble. In comparison, P-DNAJC5 co-sedimented with other EV markers after 100k xg centrifugation (**Fig 2B**). In addition to WT α -syn, we also conducted medium fractionation with several PD-causing α -syn mutants and found they all remained soluble in culture supernatant fractions (**Fig 2 supplement 1A**). To test the EV association of P-DNAJC5, we performed a further sucrose step gradient flotation (**Fig 2 supplement 2A**) with the 100k high-speed pellet fraction (**Fig 2 supplement 2B**). P-DNAJC5 equilibrated with other EV markers to the 10%/40% interface expected for buoyant EVs (**Fig 2 supplement 2B**). We conclude that the secreted α -syn induced by DNAJC5 is neither membrane bound nor in an insoluble fibrillar form.

Next we sought to characterize the conformation of secreted soluble α -syn. The medium containing secreted α -syn was pooled, concentrated and applied to a gel filtration column. Extracellular α -syn was eluted from the column at around 60% of the column volume (**Fig 2C**), similar to the elution volume of purified monomeric α -syn (**Fig 2D and Fig 2 supplement 3E&F**). By an orthogonal assay, we examined

the interaction between tagged and untagged forms of secreted α -syn as an indicator of oligomerization. In this assay, equal amounts of plasmids expressing FLAG-tagged α -syn and non-tagged α -syn were co-transfected in HEK293T cells and the medium was collected and incubated with anti-FLAG M2 beads for to detect co-immunoprecipitation of the two forms (IP) (**Fig 2E**). FLAG-tagged α -syn migrated more slowly than non-tagged α -syn as detected in samples of the culture medium (**Fig 2F**). We found that only the FLAG-tagged α -syn was immuno-precipitated (**Fig 2F**), suggesting no stable interaction between the two species. The gel filtration and IP assays reinforced our conclusion that α -syn is secreted as a monomer.

Secretion of α -syn requires palmitoylation of DNAJC5

Membrane targeting of DNAJC5 is dependent upon palmitoylation (Greaves et al., 2008). Two specific mutations, L115R and L116 Δ in the CS domains, cause adult-onset neuronal ceroid lipofuscinosis (NCL), a type of neurodegenerative disorder (Benitez et al., 2011). NCL mutations reduce the level of DNAJC5 palmitoylation and promote aggregation of the protein (Diez-Ardanuy et al., 2017). We perturbed DNAJC5 palmitoylation by either introducing the palmitoylation-deficient mutation L115R or treating cells with the competitive palmitoyl transferase inhibitor, 2-bromopalmitic acid (2-BA) (Resh, 2006), and subsequently examined the influence on DNAJC5 membrane association. DNAJC5 palmitoylation largely decreased in the L115R mutant or upon 2-BA treatment. Correspondingly, NP-DNAJC5 accumulated in the cytosol (**Fig 3A**).

Having confirmed palmitoylation inhibition by 2-BA and the L115R mutation, we next examined their effect on α -syn secretion. Upon 10 μ M 2-BA treatment, α -syn and DNAJC5 secretion was totally abolished (**Fig 3B**). The efficacy of the inhibitor

was validated by the disappearance of the low-mobility band corresponding to P-DNAJC5 in the cell lysate (**Fig 3C**). Furthermore, 2-BA inhibited α -syn secretion in a concentration-dependent manner (**Fig 3 supplement 1B&C**). Likewise, α -syn secretion was reduced by the palmitoylation-deficient DNAJC5 (L115R) compared with DNAJC5 (WT) (**Fig 3D & 3E**). DNAJC5 has been proposed to function downstream of deubiquitinase USP19 in the MAPS pathway (Xu et al., 2018). In agreement with the model, DNAJC5 carrying either NCL mutation L115R or L116 Δ had no palmitoylated band detected in the lysate (**Fig 3 supplement 2B**) and blocked USP19-stimulated α -syn secretion (**Fig 3 supplement 2A**). These results establish that palmitoylation is essential for DNAJC5 membrane association and function in stimulating α -syn secretion.

DNAJC5-dependent internalization of α -syn into enlarged endosomes

DNAJC5 has been reported to be associated with late endosomes (Lee et al., 2018). Using immunofluorescence (IF) imaging, we found colocalization between endogenous DNAJC5 and the late-endosomal marker CD63 in certain vesicular structures (**Fig 4 supplement 1A**). To visualize the topological localization of DNAJC5 and α -syn inside or outside endosomes, we turned to a U2OS cell line expressing a fluorescent protein-fused, constitutively active form of Rab5 (Rab5^{Q79L}) (Bohdanowicz et al., 2012). As a positive control, CD63 was inside the lumen of enlarged endosomes labeled by mCherry-Rab5^{Q79L} (**Fig 4 supplement 1B**). We labeled DNAJC5 with the self-labeling HaloTag for multiple choices of color in live cell imaging (Los et al., 2008). In the lumen of enlarged endosomes, both diffuse and punctate DNAJC5 were found (**Fig 4A**). Unlike WT DNAJC5, the DNAJC5 L115R mutant became disperse in the cytosol, rather than being internalized into

enlarged endosomes (**Fig 4 supplement 2A**). In contrast, mNeonGreen (mNG)-fused α -syn showed diffuse localization in both the cytosol and nucleus but was completely excluded from enlarged endosomes in L115R mutant cells (**Fig 4B**). Notably, we observed the entry of α -syn into enlarged endosomes containing internalized DNAJC5, implying the translocation of α -syn into the membrane compartment required DNAJC5 (**Fig 4C**). With no luminal localization inside enlarged endosomes, the DNAJC5 L115R mutants also failed to induce entry of α -syn into the same compartments (**Fig 4 supplement 2B**). Consistent with these imaging results, we applied cell fractionation to separate membranes of DNAJC5- and α -syn-expressing cells (**Fig 4D**). A protease protection assay indicated that α -syn and DNAJC5 were partially resistant to digestion by proteinase K in the absence but not in the presence of Triton X-100 consistent with the conclusion that a fraction of the proteins were sequestered within membrane compartments (**Fig 4E**). Our visual inspection suggests a membrane translocation role for DNAJC5 prior to α -syn secretion.

Size and unfolding are dispensable for α -syn secretion

In our medium fractionation assay, secreted α -syn in the extracellular space was characterized as a soluble monomer (**Fig 2**). We generated a series of tandem repeats of α -syn to mimic its oligomeric states (**Fig 5A**) (Dong et al., 2018). On SDS-PAGE, α -syn tandem repeats showed a larger apparent size than their predicted molecular weights, possibly caused by their extended conformation as intrinsically disordered proteins (**Fig 2 supplement 2**). We first determined that these α -syn tandem repeats could also be secreted upon expression of DNAJC5 (**Fig 5B**), indicating that

DNAJC5 can accommodate α -syn of different sizes. Fractionation of the growth medium showed that secreted tandem repeats were also soluble (**Fig 5 supplement**).

In conventional and many unconventional secretion processes, the secreted substrates undergo unfolding prior to translocation through a narrow channel across the hydrophobic membrane barrier (Rapoport et al., 2017). We devised an approach to test the possibility of unfolding by fusion of a hyperstable three-helix bundle (3H) to either the N- or C-terminus of α -syn to impede the unfolding process (**Fig 5D**). This 3H protein was previously designed in the lowest-energy arrangements and displayed extreme thermodynamic stability and remained folded even in non-physiological denaturing conditions (Huang et al., 2014). The α -syn fusion proteins were expressed successfully in cells and co-secreted with DNAJC5 normally into the medium (**Fig 5F**). These data suggest that protein unfolding is dispensable for α -syn secretion.

XPACK fusion rescues DNAJC5 L115R secretion deficiency by induced oligomerization

DNAJC5 has been reported to have an intrinsic propensity to form SDS-resistant oligomers (Zhang and Chandra, 2014). In the whole membrane immunoblot of extracellular DNAJC5, we noticed many diffuse, ladder-like bands above the two corresponding to P-DNAJC5 and NP-DNAJC5 (**Fig 6A**), possibly higher molecular weight (HMW) oligomers. This apparent oligomerization of DNAJC5 became more obvious when the J domain was deleted (**Fig 6 supplement 1A**). The formation of HMW-DNAJC5 was not due to inter-molecular disulfide bonds because the diffuse bands persisted in the presence of a reducing agent (**Fig 6 supplement 1B**). To assess the size of these HMW species of DNAJC5, we evaluated a cell lysate by gel

filtration chromatography. HMW-DNAJC5 fractionated according to its apparent size, forming a stair-like pattern on the DNAJC5 immunoblot (**Fig 6B**). HMW-DNAJC5 appeared not to be in protein aggregates based chromatography within the gel filtration column volume (**Fig 6B**). These results suggest the presence of higher-ordered, SDS-resistant DNAJC5 oligomers both in intracellular and extracellular fractions.

XPACK (XP) is a membrane-targeting peptide sequence used widely in studies of cargo loading into exosomes and delivery to target cells of choice (Yim et al., 2016). The exosome loading process by XPACK is also dependent on two lipidation reactions – myristoylation on the first glycine and palmitoylation on the second cysteine (**Fig 6 supplement 1C**) (Zacharias et al., 2002). Given the similarity of membrane localization and lipidation between XPACK and the CS domain of DNAJC5, we tested the effect of XPACK fusion with DNAJC5 on the latter's ability to induce α -syn secretion. Upon fusion with XPACK to the N-terminus, only one band with same mobility as P-DNAJC5 appeared in the WT DNAJC5 XPACK fusion protein (XP-WT) sample, in contrast to the two bands of both P- and NP-DNAJC5 in the WT DNAJC5 sample (**Fig 6 supplement 1D**). Furthermore, XP-DNAJC5 was tightly associated with membranes (**Fig 6 supplement 1E**). This suggested that XPACK lipidation is non-reversible, resulting in complete lipidation of DNAJC5. Both the slow electrophoretic mobility and membrane association of XP-DNAJC5 were reversed by 2-BA treatment, indicating XPACK lipidation included palmitoylation (**Fig 6 supplement 1E**). We also examined the effect of XPACK fusion on the palmitoylation-deficient mutant of DNAJC5 (L115R). In this case, XPACK fusion did not change the mobility of DNAJC5 mutant L115R from NP-DNAJC5 to P-DNAJC5 but resulted in several weak protein bands between the

two (**Fig 6 supplement 1D**). We introduced a point mutation of serine to leucine into the XPACK sequence to block its normal lipidation, naming it Dead XPACK (DXP) (**Fig 6 supplement 1C**). The DXP-DNAJC5 L115R species had the same mobility as NP-DNAJC5 (**Fig 6 supplement 1D**).

We conducted the same cellular fractionation with cells expressing DNAJC5 XP-L115R. In this case, XP-L115R migrated slowly and remained near the top of the gels, suggesting XPACK induces high-order oligomerization or aggregation of DNAJC5 L115R (**Fig 6C**). XP-L115R was also highly enriched in the membrane fraction through XPACK-mediated lipidation (**Fig 6C**). In spite of this apparent difference between DNAJC5 XP-L115R and WT DNAJC5, α -syn secretion was stimulated by the expression of both species (**Fig 6D**). Treatment with the palmitoylation inhibitor 2-BA restored mobility of XP-L115R to a position similar to that of monomeric DNAJC5 (**Fig 6E**). Correspondingly, secretion of α -syn was no longer stimulated by the palmitoylation deficient DNAJC5 XP-L115R monomer (**Fig 6D**).

The data from the cellular fractionation and secretion assays showed similar effects for WT DNAJC5 and DNAJC5 XP-L115R. We employed imaging methods to examine the subcellular localization of DNAJC5 XP-L115R fused with a C-terminal HaloTag. In contrast to the diffuse distribution of the DNAJC5 L115R mutant, which was excluded from the interior of enlarged endosomes (**Fig 4 supplement 2A**), punctate DNAJC5 XP-L115R were widely associated with enlarged endosomes (**Fig 6 supplement 2A**). Internalization events were found in several enlarged endosomes (**Fig 6 supplement 2A, magnified insets**). mNG- α -syn was also incorporated into endosomal compartments when co-expressed with DNAJC5 XP-L115R (**Fig 6**

supplement 2B). Our imaging data corroborate the biochemical similarity between WT DNAJC5 and DNAJC5 XP-L115R.

The J domain and C-terminal tail (C tail) of DNAJC5 are dispensable for α -syn secretion

The secretion deficiency caused by the L115R mutation highlights the importance of the CS domain of DNAJC5 in regulating α -syn secretion. The structure of the J domain of DNAJC5 has been solved by nuclear magnetic resonance (NMR) (Patel et al., 2016). Recent progress in deep learning algorithms, exemplified by AlphaFold, enables atomic accuracy in protein structure prediction (Jumper et al., 2021). We searched the public AlphaFold database to examine the predicted structure of full-length DNAJC5. In the predicted structure, the J domain shows a conserved overall globular J protein fold on the N-terminus, linked by the helical CS domain to the flexible C-terminal tail. Only a short helix is predicted to reside within the C-tail (**Fig 7A**). We refined the boundary of each domain in DNAJC5 based on the predicted structure.

Using this information, we showed that oligomerization of DNAJC5 increased when the J domain was deleted (**Fig 6 supplement 1A and Fig 7C**). Deletion of the J domain did not alter the level of α -syn secretion induced by DNAJC5, indicating that this domain was dispensable for α -syn secretion (**Fig 7B**). Next, we examined the function of the C-tail by truncating about ten amino acids at a time, resulting in a series of C-terminal truncated DNAJC5 constructs, i.e., DNAJC5 Δ C10, Δ C20, Δ C30, Δ C40. All four DNAJC5 C-terminal truncations were able expressed and formed oligomers in cells (**Fig 7E**). C-terminal truncated oligomers were co-secreted

with α -syn into the medium (**Fig 7D**). This result demonstrates that neither the J domain nor the C-tail is required for DNAJC5 to induce α -syn secretion.

Discussion

Transmission of protein aggregates and subsequent self-amplification is emerging as a common theme across various neurodegenerative diseases. DNAJC5 has been shown to control the release of neurodegenerative disease proteins but the detailed molecular mechanism remains elusive. In this study, we first reconstituted DNAJC5-regulated α -syn secretion in cultured HEK293T cells. By combining this assay with medium and cellular fractionation, we demonstrated that membrane-anchoring of DNAJC5 through palmitoylation is crucial for its secretion and the secreted α -syn as a soluble monomer. In addition, we observed the topological locations of DNAJC5 and α -syn inside enlarged endosomes, presumably at an intermediate stage prior to secretion. Furthermore, artificially-devised lipidation (XPACK) induced DNAJC5 oligomers and restored α -syn secretion. Finally, we provide evidence that both palmitoylation and oligomerization are solely dependent on the CS domain, which is required for α -syn secretion. Based on our biochemical assays and imaging observations, we propose that palmitoylated DNAJC5 oligomers function at a step involving membrane translocation of cytosolic α -syn, enabling it to become competent for secretion (**Fig 7F**).

Using the criteria of differential sedimentation and gel filtration chromatography, we conclude that α -syn is secreted in cultures cells as a soluble monomeric species not enclosed within extracellular vesicles, regardless of mutations modeled on PD (**Fig 2 supplement 1A**) or as expressed in tandem arrays or in gene fusions to

tightly folded proteins (**Fig 5 supplement**). Consistent with our results, other MAPS substrates were also reported to be soluble (Lee et al., 2016). Although α -syn oligomers have also been found in EVs (Danzer et al., 2012; Emmanouilidou et al., 2010; Guo et al., 2020) we see no evidence for this in our immunoblot experiments (**Fig 2B**). With a more sensitive and quantitative Nluc assay, 85% of secreted α -syn was found to be soluble (**Fig 2 supplement 1B**). Given that DNAJC5 can be found in membrane buoyant EVs (**Fig 2 supplement 2B**) and α -syn is known to destabilize lipid membrane (Fusco et al., 2017), we sought to detect soluble EV luminal proteins as an indicator that α -syn may have escaped by rupture of the EV membrane. However, we failed to see any increase in EV luminal markers in the high-speed supernatant fraction from cells overexpressing α -syn (**data not shown**). How α -syn passes across a membrane *en route* to secretion and whether insoluble α -syn aggregates can be secreted through the same pathway, remain to be addressed.

Numerous neuronal proteins are palmitoylated, including synaptic scaffolding proteins, signaling proteins and synaptic vesicle proteins (Linder and Deschenes, 2007). Protein palmitoylation has been implicated in the pathogenesis of neurodegenerative diseases (Cho and Park, 2016). In PD particularly, a recent study reported that upregulation of cellular palmitoylation decreased α -syn cytoplasmic inclusions (Ho et al., 2021). The neuropathology and behavior deficiency of Huntington disease (HD) mice can be reversed by boosting brain palmitoylation (Virlogeux et al., 2021). In the case of DNAJC5, L115R and L116 Δ , the two adjacent mutations causing decreased palmitoylation of DNAJC5 monomers, lead to a familial form of NCL (Benitez et al., 2011; Diez-Ardanuy et al., 2017). Our results suggested that the secretion of neurodegenerative disease

proteins is also dependent on palmitoylation, possibly alleviating the cellular burden of protein aggregate accumulation. Notably, the general inhibition of cellular palmitoylation by 2-BA led to a complete block of α -syn secretion, whereas the specific palmitoylation deficient DNAJC5 mutant L115R only partially decreased α -syn secretion (**Fig 3B-E**). The difference implies the existence of palmitoylation-dependent factors other than DNAJC5.

Although DNAJC5 co-expressed with α -syn and palmitoylation are required for secretion, EV-associated P-DNAJC5 clearly separated from soluble α -syn in the culture medium (**Fig 2B and Fig 2 supplement 2B**). At which step do the two separate? In our live cell imaging experiments, the internalized DNAJC5 inside enlarged endosomes had both punctate and diffuse distributions (**Fig 4A**). This may represent the soluble NP-DNAJC5 and membrane-attached P-DNAJC5, respectively. In the time-lapse imaging of internalized α -syn induced by DNAJC5, both DNAJC5 and α -syn moved dynamically inside the compartment, without significant co-localization (**Fig4 supplementary video**). This observation suggests that the separation of DNAJC5 and α -syn may occur prior to their secretion when the late endosome and plasma membrane fuse.

Zhang et al. (2020) have reported a novel membrane channel, TMED10, for the unconventional secretion of IL-1 β . These authors speculate an activation-on-demand oligomerization of TMED10 membrane subunits to form a conducting channel for substrate translocation, a process they refer to as THU (Zhang et al., 2020). α -syn was reported to not depend on TMED10 for unconventional secretion (Zhang et al., 2020). Similarly, in chaperone-mediated autophagy (CMA), cytosolic substrates are proposed to be translocated into lysosomes through a

channel formed by the oligomerization of a single-transmembrane protein, LAMP2A (Bandyopadhyay et al., 2008). The secretion of α -syn has been shown to be independent of CMA (Lee et al., 2018). Without a transmembrane domain, membrane-tethered P-DNAJC5 oligomer is unlikely to be a channel for translocation. A recent preprint has identified CD98hc, an amino acid transporter subunit, to be a DNAJC5 interactor that is required for α -syn secretion (Lee et al., 2021 preprint). It remains to be determined whether CD98hc or other as yet uncharacterized membrane proteins are directly involved in α -syn membrane translocation.

In SEC61-mediated co-translational translocation, substrates enter the SEC61 translocon in an unfolded state (Rapoport et al., 2017). In THU and CMA, substrate unfolding is also required for translocation across the membrane (Kaushik and Cuervo, 2018; Zhang et al., 2020). In striking contrast, unfolding and size is not a limiting factor for α -syn secretion (**Fig 5**). As a precedent of translocation without unfolding, studies have shown the import of folded proteins into the matrix of peroxisomes and obviously through the nuclear pore (Kim and Hettema, 2015; Lin and Hoelz, 2019). DNAJC5 forms a series of extremely stable oligomers, which may provide versatile adaptors to accommodate diverse misfolded or folded substrates with different dimensions. The structure of DNAJC5 oligomers may shed light on the principle of this folding-independent translocation pathway.

Materials and methods

408 Key Resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti- α -synuclein	BD Biosciences	Cat# 610787
Rabbit polyclonal anti-DNAJC5	RayBiotech	Cat# 144-10489-200
Mouse monoclonal anti-alpha Tubulin	Abcam	Cat# ab7291
Mouse monoclonal anti-Alix	Santa Cruz Biotechnology	Cat# Sc-53540
Rabbit monoclonal anti-CD9	Cell Signaling Technology	Cat# 13174S
Mouse monoclonal anti-PDI	Enzo Life Sciences	Cat# ADI-SPA-891-D
Mouse monoclonal anti-CD63	Fisher Scientific	Cat# BDB556019
Mouse monoclonal anti-Flotillin-2	BD Biosciences	Cat# 610383
Mouse monoclonal anti-Transferrin Receptor	Thermo Fisher SCIENTIFIC	Cat# 13-6800
Mouse monoclonal anti-FLAG	Sigma-Aldrich	Cat# F9291
Bacterial and virus strains		
XL1-Blue competent cells	MacroLab Berkeley	N/A
Rossetta (DE3) pLysS competent cells	MacroLab Berkeley	N/A
Chemicals, peptides, and recombinant proteins		
Anti-FLAG M2 Affinity Gel	Sigma-Aldrich	Cat# A2220-5ML
Dimethyl sulfoxide (DMSO)	Fisher Scientific	Cat# BP231-100
Quercetin	Sigma-Aldrich	Cat# Q4951-10G

2-Bromopalmitic acid	Millipore Sigma	Cat# 21604-1G
HaloTag® Oregon Green® Ligand	Promega	Cat# G2802
HaloTag® TMR® Ligand	Promega	Cat# G8251
Prolong Gold with DAPI	Fisher Scientific	Cat# P36931
α -syn tandem repeats protein	This paper	N/A
Critical Commercial Assays		
Nano-Glo® Luciferase Assay System	Promega	Cat# N1150
Experimental Models: Cell Lines		
HEK293T Cells	Cell culture facility, UC Berkeley	N/A
U2OS Cells	Cell culture facility, UC Berkeley	N/A
Oligonucleotides		
FlexiTube GeneSolution GS80331for DNAJC5	Qiagen	1027416
Recombinant DNA plasmids		
mCherry-Rab5CA(Q79L)	Addgene	Cat# 35138
BFP-Rab5CA(Q79L)	This study	N/A
SNCA (Myc-DDK-tagged)-Human synuclein, apha	OriGene Technoogy	Cat# RC221446
CSP (DNAJC5) (NM_025219) Human Tagged ORF Clone	OriGene Technology	Cat# RC208826
TCH1003-MGC premier cDNA clone for USP19	transOMIC	Cat# TCH1003

α -synuclein-A30P	Gift of Dr. Thomas Südhof lab	N/A
α -synuclein-E46K	Gift of Dr. Thomas Südhof lab	N/A
α -synuclein-A53T	Gift of Dr. Thomas Südhof lab	N/A
α S-2 (α -syn- α -syn) tandem dimer	Gift of Michael Woodside lab	N/A
α S-4 (α -syn- α -syn- α -syn- α -syn) tandem Tetramer	Gift of Michael Woodside lab	N/A
DNAJC5-L115R	This study	N/A
DNAJC5-L116 Δ	This study	N/A
DNAJC5 (WT)-HaloTag	This study	N/A
DNAJC5 (L115R)-HaloTag	This study	N/A
XPACK-DNAJC5 (L115R)-HaloTag	This study	N/A
mNeonGreen- α -synuclein	This study	N/A
³ H- α -synuclein	This study	N/A
α -synuclein- ³ H	This study	N/A
XPACK-DNAJC5 (WT)	This study	N/A
XPACK-DNAJC5 (L115R)	This study	N/A
DEAD XPACK-DNAJC5 (L115R)	This study	N/A
DNAJC5- Δ J (Δ 14-82)	This study	N/A
DNAJC5- Δ C10 (D189-198)	This study	N/A

DNAJC5-ΔC20 (D179-198)	This study	N/A
DNAJC5-ΔC30 (D169-198)	This study	N/A
DNAJC5-ΔC40 (D151-198)	This study	N/A
Software and algorithms		
Fiji (ImageJ)	NIH	https://imagej.nih.gov/ij/
PyMOL	Schrödinger	https://pymol.org/2/
Prism 8	Graphpad	https://www.graphpad.com/scientific-software/prism/
AlphaFold Protein Structure Database	DeepMind	https://alphafold.ebi.ac.uk/

Cell culture and transfection

Cells were grown at 37°C in 5% CO₂ and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). For secretion assays, the FBS concentration was reduced to 1% for up to 36 h. The growth rate of cells slowed down but the cells remained viable. For EV preparation and medium fractionation, cells were grown in DMEM supplemented with exosome-depleted FBS. Exosome-depleted FBS was prepared by overnight centrifugation of 30% diluted FBS in DMEM at 100,000xg. Transfection of plasmids into cells was performed using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocols. SiRNA knockdown was performed using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocols.

Reconstitution of α-syn secretion in HEK293T cells

HEK293T cells (cultured in 6-well plates) were cultured to 60% confluence and co-transfected with plasmids encoding different constructs of α-syn and DNAJC5.

At 4 h after transfection, cell culture medium was replaced with DMEM supplemented with 1% FBS containing indicated drugs for treatment. At indicated time points, media fractions were collected and centrifuged at 1,000xg for 10 min to remove floating cells and cell debris. The media were mixed with methanol/chloroform to precipitate proteins which were collected by centrifugation (10,000 g x 10 min) and resuspended in SDS-PAGE sample loading buffer to achieve concentration (20-fold). Cells were lysed in lysis buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1% Triton X-100). Both the concentrated media and cell lysate fractions were analyzed by immunoblot.

For a nanoluciferase-based assay, the media fractions were collected and centrifuged at 1,000xg for 10 min. The supernatant fractions were harvested and further diluted with PBS buffer (1000-fold). The nanoluciferase activity was assayed using a Nano-Glo[®] Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's protocol.

Membrane and Cytosol fractionation

Cells (one 10cm dish) were cultured to 70% confluence and transfected with different constructs of DNAJC5. One day after transfection, the transfected cells were harvested by scraping in 1 ml B88 (20 mM HEPES-KOH, pH 7.2, 250 mM sorbitol, 150 mM potassium acetate and 5 mM magnesium acetate) plus a cocktail of protease inhibitors (Sigma-Aldrich, St. Louis, MO) and were further homogenized by passing through a 22G needle 10 times. Cell homogenates were centrifuged at 500xg for 10 min. The recovered supernatant fractions, post-nuclear supernatant (PNS), were centrifuged at 100,000xg for 1.5 h. The supernatant was collected and the centrifugation was repeated to achieve a clarified cytosol fraction. The pellet fraction was washed and resuspended in the same volume of

B88. The resuspended material was also centrifuged again to collect the washed membrane. Membranes were lysed in lysis buffer.

For membrane fractionation, the PNS was subjected to differential centrifugation at 3,000xg (10 min), 25,000xg (20 min) and 100,000xg (30min). Membrane fractions were normalized to phosphatidylcholine content and analyzed by immunoblot (Ge et al., 2013).

For proteinase K protection assays, the 25,000xg membrane fraction was aliquoted into three tubes: one without proteinase K, one with proteinase K (10 µg/ml), and one with proteinase K plus TritonX-100 (0.5%). The reaction was conducted on ice for 20 min and stopped by sequential addition of PMSF (1mM) and sample buffer, which were then heated on metal block at 95°C for 5 min and samples were analyzed by SDS-PAGE and immunoblot.

Medium fractionation and extracellular vesicle preparation

Conditioned medium was harvested and centrifuged first at 1,500xg for 20 min followed by 10,000xg for 30 min and 100,000xg for 1.5 h. The supernatant fractions at each step were collected and treated with methanol/chloroform to precipitate proteins which were then collected by centrifugation and pellet fractions were and resuspended in sample buffer for concentration (20-fold). The sedimented fractions at each step were also collected and resuspended in sample buffer. All the fractions were analyzed by immunoblot.

EVs were further floated by a sucrose step gradient centrifugation. The pellet fraction from a 100,000xg centrifugation was resuspended in PBS and mixed with 60% sucrose buffer (10mM Tris-HCl pH 7.4, 100 mM NaCl) to achieve a final sucrose concentration >50% as measured with a refractometer. Aliquots of 40% (5

ml) and 10% (2 ml) sucrose buffer were sequentially overlaid above the sample. The tubes were then centrifuged at 150,000xg for 16 h in an SW41 Ti swinging-bucket rotor (Beckman Coulter). After centrifugation, fractions (0.5 ml for each) were collected from top to bottom and samples analyzed by SDS PAGE and immunoblot.

Co-immunoprecipitation

Media fractions were collected and centrifuged at 1,000xg for 10min. The supernatant fractions were collected and concentrated (20-fold) using a 10 kDa Amicon filter (Millipore, Billerica, MA). Concentrated media (1 ml) were incubated with 20 μ l of anti-FLAG M2 affinity gel (Sigma-Aldrich, St. Louis, MO) for 1 h at 4°C. After washing 5x with lysis buffer, SDS-PAGE sample loading buffer was added to the beads and samples were processed for SDS PAGE and immunoblot.

Protein purification

The purification of different α -syn tandem-oligomer constructs was performed as previously described (Dong et al., 2018). Briefly, an osmotic shock protocol was adapted to enrich proteins released from the periplasm of transfected *E. coli*. The supernatant fraction containing released proteins was subjected to ammonium sulfate (AS) precipitation, with 50%, 45% and 40% saturated concentration of AS for monomer, dimer and tetramer, respectively. After overnight precipitation, the precipitated proteins were collected by centrifugation at 100,000xg for 30 min. The pellet fractions were dissolved in Buffer A (20mM Tris-HCl pH 8.0) and clarified by repeated centrifugation at 100,000xg for 30 min.

Clarified supernatants were applied to an equilibrated HiPrep Q Fast Flow 16/10 column (GE Healthcare, Chicago, IL). Eluted proteins were collected, concentrated by 10 kDa Amicon filter (Millipore, Billerica, MA) and further purified by gel filtration (Superdex-200, GE Healthcare). PBS was used as gel filtration buffer. Purified proteins were assessed by SDS-PAGE followed by coomassie-blue staining.

Immunofluorescence and live-cell Imaging

For immunofluorescence, U2OS cells were washed once with PBS and immediately fixed by 4% EM-grade paraformaldehyde (Electron Microscopy Science, Hatfield, PA) for 10 min at room temperature. Cells were washed three times with PBS and blocked and permeabilized for 30 min in permeabilization buffer (5% FBS and 0.1% saponin in PBS). Cells were then incubated with 1:100 dilution of primary antibodies overnight at 4°C. After three washes with PBS, cells were incubated with 1:500 dilution of fluorophore-conjugated secondary antibodies for 30 min at room temperature. Prolong Gold with DAPI (Thermo Fisher) was used as mounting solution. Images were acquired with a Zeiss LSM900 confocal microscope and analyzed with Fiji/ImageJ software (<https://imagej.nih.gov/ij/>).

For live-cell imaging, cells were cultured in 35mm glass bottom dishes (MatTek). The addition of HaloTag fluorescent ligands were added according to the manufacturer's protocol (Promega). After incubation, the medium was replaced with Opti-MEM supplemented with 10% FBS. Imaging was performed using a Zeiss LSM900 confocal microscope in a temperature-controlled (37°C and 5% CO₂) environment.

521

522 **Additional information**

523 **Competing Interests**

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Figure legends

Figure 1. Reconstitution of α -syn secretion regulated by palmitoylated DNAJC5 in HEK293T cells.

(A) Schematic diagrams of α -syn and DNAJC5. Domains are highlighted in different colors. Red arrows indicate known disease-causing mutations on each protein.

(B) Membrane and cytosol fractionation scheme. Briefly, homogenized HEK293T cells were centrifuged at low speed to prepare a post-nuclear supernatant (PNS). High-speed centrifugation was then performed to separate the insoluble membrane (M) from cytosol (C).

(C) Partition of palmitoylated DNAJC5 (P-DNAJC5) and non-palmitoylated DNAJC5 (NP-DNAJC5) between the membrane (M) and cytosol (C) fractions. DNAJC5 was immunoprecipitated from cytosol and membrane with anti-FLAG resin and evaluated by Coomassie-blue stained SDS-PAGE.

(D) α -syn secretion 16 h after transfection. The secretion of P-DNAJC5 in the medium was detected.

(E) α -syn secretion 36 h after transfection. NP-DNAJC5 was also secreted in the medium together with α -syn.

Figure 1 – figure supplement 1. Secretion of α -syn variants. HEK293T cells were co-transfected with Parkinson's disease (PD)-causing α -syn mutant (A30P, E46K, A53T) and DNAJC5.

(A) Secretion of α -syn variants into conditioned medium. Medium was collected, concentrated and evaluated by immunoblot.

(B) Expression of α -syn variants in HEK293T cells.

Figure 1 – figure supplement 2. Basal secretion of α -syn is partially dependent on endogenous DNAJC5.

(A) Schematic diagram of Nanoluciferase (Nluc)-fused α -syn.

(B) Nluc- α -syn secretion was stimulated by DNAJC5 expression. Plasmids encoding Nluc- α -syn and DNAJC5 were co-transfected into HEK293T cells. Expression and secretion of proteins were detected with immunoblot 36 h after transfection.

(C) Time-dependent of accumulation of Nluc- α -syn in the medium without DNAJC5 overexpression. After transfecting HEK293T cells with Nluc- α -syn alone, fractions of medium were collected at indicated time points.

(D) Chemical structure of Quercetin (QLT), a reported DNAJC5 inhibitor.

(E) QLT inhibited endogenous Nluc- α -syn secretion in a concentration-dependent manner. AU, arbitrary unit. Secretion assay similar to (B) was performed with treatment of indicated concentration of QLT. Amounts of secreted proteins were quantified with nanoluciferase assay 36hr after transfection.

(F) HEK293T cells were co-transfected with Nluc- α -syn and siRNA targeting DNAJC5. At 36 h post transfection, cells were harvested for testing knockdown (KD) efficiency by immunoblot.

(G) Nluc- α -syn secretion decreased in DNAJC5 KD cells compared with control.

Figure 2. Characterization of secreted α -syn.

(A) Medium fractionation scheme.

(B) Secreted α -syn was soluble. Differential centrifugation was performed with conditioned medium from HEK293T cells transfected with DNAJC5 and α -syn. Alix and CD9, exosome markers. PDI, an endoplasmic reticulum (ER) marker, was used as exosome-negative marker.

(C) Gel filtration fractionation of medium. Conditioned medium was concentrated and subjected to gel filtration fractionation. Fractions were evaluated by anti- α -syn immunoblot.

(D) Chromatograms of tandem α -syn monomer (blue curve), dimer (green curve) and tetramer (red curve) were overlaid. In comparison, the relative intensity of secreted α -syn in each fraction was plotted as blue bars.

(E) Schematic diagram of co-immunoprecipitation (co-IP) of secreted α -syn and FLAG- α -syn. Shown here is possible interaction between α -syn (blue circle) and FLAG- α -syn (yellow circle) in a representative tetrameric conformation.

(F) Anti-FLAG Immunoprecipitation (FLAG-IP) of media from cells transfected with indicated plasmids. Both the medium input and FLAG-IP samples were evaluated with anti- α -syn immuno(western)blot (anti- α -syn WB).

Figure 2 – figure supplement 1. Solubility of secreted α -syn variants.

(A) Medium fractionation of secreted α -syn PD mutants (A30P, E46K and A53T). After differential centrifugation of medium, supernatant (10ks and 100ks) and pellet fractions (100kp) were evaluated by immunoblot.

(B) Medium fractionation of basal secreted Nluc- α -syn without DNAJC5 overexpression. Similar fractionation assay was performed in (A) with medium from HEK293T cells transfected with Nluc- α -syn alone. Secreted Nluc- α -syn was quantified with nanoluciferase assay. AU, arbitrary unit.

Figure 2- figure supplement 2. DNAJC5 enriched in buoyant EV fraction.

(A) Schematic diagram of EV flotation protocol. Briefly, high-speed pellet fractions of growth medium were resuspended in 60% sucrose buffer and overlaid sequentially with 40% and 10% sucrose buffer. The tubes were centrifuged at 150,000 (150k)xg at 4°C for overnight. Buoyant EVs floated at the 10%/40% sucrose interface, separated from other insoluble materials.

(B) Immunoblots across the sucrose step gradient. DNAJC5 as well as other classical exosome markers (Flot-2, CD63 and CD9) were highly enriched in fractions 4 to 6 at the 10%/40% interface.

Figure 2 – figure supplement 3. Assessment of tandem α -syn oligomers by gel filtration chromatography.

(A) Chromatogram of purified tetrameric α -syn tandem oligomer (α -syn- α -syn- α -syn- α -syn).

(B) Coomassie-blue stained SDS-PAGE of fractions from gel filtration of tetrameric α -syn tandem oligomer (α -syn- α -syn- α -syn- α -syn).

(C) Chromatogram of purified dimeric α -syn tandem oligomer (α -syn- α -syn).

(D) Coomassie-blue stained SDS-PAGE of fractions from gel filtration of dimeric α -syn tandem oligomer (α -syn- α -syn).

(E) Chromatogram of purified WT α -syn (α -syn).

(F) Coomassie-blue stained SDS-PAGE of fractions from gel filtration of WT α -syn (α -syn).

Figure 3. Disruption of palmitoylation of DNAJC5 inhibited α -syn secretion.

(A) Inhibition of DNAJC5 palmitoylation by 2-bromopalmitic acid (2-BA) or introduced mutation L115R. Cellular fractionation was performed with HEK293T cells transfected with WT DNAJC5 and treated with 10 μ m 2-BA, or transfected with DNAJC5 L115R mutant. TFR, transferrin receptor. PNS, post-nuclear supernatant. C, cytosol. M, membrane.

(B) α -syn secretion was blocked with 2-BA treatment. HEK293T cells transfected with indicated plasmids were treated with DMSO or 10 μ m 2-BA. Media were collected and secretion was evaluated by SDS PAGE and immunoblot.

(C) Palmitoylation of DNAJC5 was blocked in HEK293T cells treated with 2-BA.

(D) DNAJC5 L115R mutant reduced α -syn secretion compared with WT DNAJC5. Secretion assay with HEK293T cells transfected with indicated plasmids encoding DNAJC5 variant was performed similar to (B).

(E) DNAJC5 L115R was non-palmitoylated in HEK293T cells.

Figure 3 – figure supplement 1. Dose-dependent inhibition of α -syn secretion by 2-bromopalmitic acid (2-BA).

(A) Chemical structure of palmitoylation inhibitor 2-bromopalmitic acid (2-BA).

The single bromo substituent at position 2 is highlighted by a red dashed square.

(B) α -syn secretion in the medium was inhibited by 2-BA in a dose-dependent

manner. Ten μ m 2-BA was serially diluted by DMSO into 5 μ m, 2 μ m, 1 μ m

solution. HEK293T cells were first transfected with DNAJC5 and α -syn. After

medium replacement, 2-BA of indicated concentration was added to cell

culture. Media was collected after 36h and followed by sample preparation,

SDS PAGE and immunoblot.

(C) With increasing concentration of 2-BA, P-DNAJC5 decreased and NP-

DNAJC5 increased in HEK293T cells.

Figure 3 – figure supplement 2. Blockage of USP19-induced α -syn secretion by DNAJC5 L115R or L116 Δ mutant.

(A) USP19 induced α -syn secretion in the medium, which was further enhanced

by WT DNAJC5 and blocked by two DNAJC5 palmitoylation-deficient

mutants (L115R and L116 Δ).

(B) Both mutations, L115R and L115 Δ , inhibited DNAJC5 palmitoylation in

HEK293T cells.

Figure 4. Topological localization of α -syn and DNAJC5 in enlarged endosomes.

(A) DNAJC5 was internalized inside enlarged endosomes. Live U2OS cells

expressing mCherry-Rab5^{Q79L} (red) showed circular enlarged endosomes

labeled by Rab5 mutant. DNAJC5-HaloTag (green) was visualized by addition

of HaloTag[®] Oregon Green[®] Ligand. Representative enlarged endosomes show

diffuse (1) or punctate (2 and 3) internalized DNAJC5. Scale bar: 15 μ m in overviews and 1 μ m in magnified insets.

(B) α -syn was excluded from enlarged endosomes. In live U2OS cells, expression of BFP-Rab5^{Q79L} (blue) produced enlarged endosomes of similar morphology compared with mCherry-Rab5^{Q79L}. mNeonGreen- α -syn (mNG- α -syn, green) was expressed both in the nucleus and cytosol. No mNG- α -syn was found inside enlarged endosomes (1, 2, 3). Scale bar: 20 μ m in overviews and 1 μ m in magnified insets.

(C) α -syn enters into enlarged endosomes in the presence of DNAJC5. DNAJC5-HaloTag (red) and mNG- α -syn (green) were co-expressed in U2OS cells carrying BFP-Rab5^{Q79L} (blue) mutant and imaged. No mNG- α -syn was internalized in endosome without DNAJC5-HaloTag inside (1). In contrast, mNG- α -syn was found inside endosomes with DNAJC5-HaloTag inside (2 and 3). Scale bar: 10 μ m in overviews and 1 μ m in magnified insets.

(D) α -syn and DNAJC5 co-fractionated in membrane fractionation. HEK293T cell homogenate was sequentially centrifuged at increasing velocity from 3000g (3k), 25,000g (25k) and 100,000g (100k). The 25k membrane fraction had the highest amount of both α -syn and DNAJC5.

(E) Proteinase K protection assay of 25k membrane containing α -syn and DNAJC5.

Figure 4 – figure supplement 1. Immunofluorescence (IF) images of endogenous DNAJC5 and enlarged endosomes.

- (A) Co-localization between endogenous CD63 (red) and DNAJC5 (green). U2OS cells were cultured and fixed, incubated with corresponding antibodies for IF detection of endogenous CD63 and DNAJC5. Representative co-localized region is shown in magnified insets. Scale bar, 20 μ m.
- (B) CD63 (green) was inside the enlarged endosomes labeled by peripheral mCherry-Rab5^{Q79L} (red). U2OS expressing mCherry-Rab5^{Q79L} were fixed, followed by IF using anti-CD63 antibody. Scale bar, 25 μ m.

Figure 4 – figure supplement 2. Live-cell images of U2OS cells expressing DNAJC5 L115R mutant and α -syn.

- (A) Live cell imaging of U2OS cells transfected with DNAJC5 (L115R)-HaloTag and mCherry-Rab5^{Q79L}. DNAJC5 (L115R)-HaloTag (green) is diffuse in cytosol and outside of the enlarged endosomes labeled by peripheral mCherry-Rab5^{Q79L} (red). Scale bar, 10 μ m in overviews and 1 μ m in magnified insets.
- (B) Live cell imaging of U2OS cells transfected with mNeonGreen (mNG)- α -syn, DNAJC5 (L115R)-HaloTag and mCherry-Rab5^{Q79L}. In the condition of co-expression with DNAJC5 (L115R)-HaloTag (red), no mNG- α -syn (green) was found inside of enlarged endosomes labeled by peripheral BFP-Rab5^{Q79L} (blue). Scale bar, 10 μ m in overviews and 1 μ m in magnified insets.

Figure 4 - supplement video. Time lapse of movement of internalized α -syn and DNAJC5 in the enlarged endosomes. DNAJC5-HaloTag (red) and mNG- α -syn (green) were co-expressed in U2OS cells carrying BFP-Rab5^{Q79L} (blue) mutant and imaged. Scale bar: 10 μ m.

Figure 5. Secretion of tandem α -syn oligomers and α -syn fused with thermostable helix bundle protein.

(A) Schematic diagrams of tandem α -syn oligomers. α -syn protomers (yellow) were linked head to tail by flexible linker (green) to mimic increased size of α -syn oligomers.

(B) Secretion of tandem α -syn oligomers in medium. Secretion assay was performed with media from HEK293T cells culture transfected with indicated tandem α -syn oligomers. Tandem α -syn oligomers are more sensitively detected by immunoblot and were exposed for shorter time compared with WT α -syn.

(C) Expression of tandem α -syn oligomers in HEK293T cells. *a non-specific band.

(D) Schematic diagrams of N-terminal fused and C-terminal fused thermostable three helix bundle (3H-) α -syn. 3H was shown as three blue dashes, α -syn was shown as orange circle.

(E) Secretion of 3H-SYN and SYN-3H in medium. Secretion assay was performed with media from HEK293T cells culture transfected with indicated 3H-fused α -syn constructs. *a non-specific band.

(F) Expression of 3H-SYN and SYN-3H in HEK293T cells. *a non-specific band.

Figure 5 – figure supplement. Medium fractionation of secreted tandem α -syn oligomers.

Figure 6. XPACK (XP)-induced DNAJC5 L115R oligomerization rescued α -syn secretion.

- (A) Ladder pattern of higher molecular weight (HMW) DNAJC5 oligomers in the medium. Medium from HEK293T cell culture transfected with DNAJC5 was centrifuged at 1000 (1k) g, 10,000 (10k) g and 100,000 (100k) g, followed by immunoblot of supernatants (s) at each centrifugation step.
- (B) Fractionation of HMW-DNAJC5 with gel filtration. HEK293T cells transfected with DNAJC5 were lysed, clarified and subjected to gel filtration. HMW-DNAJC5 of different sizes were separated based on their corresponding molecular weight.
- (C) XP-DNAJC5 L115R mutant forms a membrane-bound oligomer. Cellular fractionation was performed with HEK293T cells transfected with indicated DNAJC5 variants. Note the substantial change of electrophoretic mobility of XP-DNAJC5 L115R on SDS-PAGE.
- (D) α -syn secretion induced by XP-DNAJC5 L115R. Secretion assay was performed with HEK293T cells transfected with indicated plasmids. Ten μ m 2-BA was used to block induced α -syn.
- (E) Expression of α -syn and DNAJC5 variants in HEK293T cells. Note the substantial change in electrophoretic mobility of 2-BA-treated XP-DNAJC5 L115R on SDS-PAGE.

Figure 6 – figure supplement 1. Characterization of HMW-DNAJC5 and XPACK fusion.

- (A) DNAJC5 (Δ J) forms a series of SDS-resistant oligomers. HEK293T cells transfected with WT DNAJC5 or DNAJC5 (Δ J) were lysed and evaluated by immunoblot using anti-DNAJC5 antibody.

- (B) HMW-DNAJC5 is not formed by non-specific disulfide bonds. HEK293T cells transfected with DNAJC5 were lysed for SDS-PAGE followed by immunoblot. The loading samples for SDS-PAGE were prepared with increasing amount of DTT up to 40 mM.
- (C) Schematic diagram of XPACK. XPACK is myristoylated on the first glycine (G) and palmitoylated on the second cysteine (C). Replacement of Serine (S) at position 5 with a hydrophobic isoleucine (I) abolishes normal lipidation of XPACK.
- (D) Assessment of different XPACK fusion constructs. Cell lysate containing different DNAJC5 constructs were evaluated by anti-DNAJC5 immunoblot.
- (E) Membrane-association of XP-DNAJC5 dependent on palmitoylation. HEK293T cells transfected with XP-DNAJC5 were treated with DMSO or 10 μ M 2-BA. After cell culture for 24 h, cellular fractionation was performed with homogenized cells. Distribution of XP-DNAJC5 in cytosol (C) and membrane (M) fractions were evaluated with immunoblot.
- Figure 6 – figure supplement 2.** Live-cell images of U2OS cells expressing XP-DNAJC5 L115R mutant and α -syn.
- (A) Internalization of punctate XP-DNAJC5 (green) into enlarged endosomes labeled by mCherry-Rab5^{Q79L} (red). U2OS cells were transfected with XP-DNAJC5 and mCherry-Rab5^{Q79L}. Live cell imaging was performed 24 h after transfection. Representative enlarged endosomes with XP-DNAJC5 are shown in magnified inset. Scale bar: 10 μ m in overviews and 1 μ m in magnified insets.
- (B) α -syn enters into enlarged endosomes in the presence of XP-DNAJC5. XP-DNAJC5-HaloTag (red) and mNG- α -syn (green) were co-expressed in U2OS

cells carrying BFP-Rab5^{Q79L} (blue) mutant and imaged. Representative enlarged endosome with both XP-DNAJC5-HaloTag and mNG- α -syn inside is shown in magnified inset. Scale bar: 10 μ m in overviews and 1 μ m in magnified insets.

Figure 7. Domain mapping of secretion-competent DNAJC5.

- (A) Predicted structure of DNAJC5 by AlphaFold. Color scheme: J domain (magenta), Cys string domain (yellow) and C-terminal helix (green).
- (B) DNAJC5 (Δ J) was competent to induce α -syn secretion into the medium. HEK293T cells were transfected with indicated plasmids. Media were collected after 36 h and evaluated with immunoblot.
- (C) DNAJC5 (Δ J) formed oligomers in HEK293T cells.
- (D) C-terminal truncated DNAJC5 constructs were competent to induce α -syn secretion in the medium. HEK293T cells were transfected with C-terminal truncated DNAJC5 and α -syn. DNAJC5 antibodies cannot recognize DNAJC5 (Δ C30) and DNAJC5 (Δ C40) because of a missing epitope in the C-terminus. Instead, DNAJC5 (Δ C30) and DNAJC5 (Δ C40) were detected by C-terminal FLAG tags. All the C-terminal truncated DNAJC5 constructs showed smear-like oligomers.
- (E) Expression of C-terminal truncated DNAJC5 constructs inside of HEK293T cells. Immunoblot of anti-FLAG antibody and anti-DNAJC5 antibody cross-validated the existence of oligomers.
- (F) A model for palmitoylated DNAJC5 oligomer-mediated α -syn secretion.

1085 **Figure 1 – source data.** Uncropped immunoblot and gel images corresponding to
1086 Figure 1.

1087 **Figure 1 – figure supplement 1 – source data.** Uncropped immunoblot images
1088 corresponding to Figure 1 supplement 1.

1089 **Figure 1 – figure supplement 2 – source data.** Uncropped immunoblot images
1090 corresponding to Figure 1 supplement 2.

1091 **Figure 2 – source data.** Uncropped immunoblot corresponding to Figure 2.

1092 **Figure 2 – figure supplement 1 – source data.** Uncropped immunoblot images
1093 corresponding to Figure 2 supplement 1.

1094 **Figure 2 – figure supplement 2 – source data.** Uncropped immunoblot images
1095 corresponding to Figure 2 supplement 2.

1096 **Figure 2 – figure supplement 3 – source data.** Uncropped gel images
1097 corresponding to Figure 2 supplement 3.

1098 **Figure 3 – source data.** Uncropped immunoblot corresponding to Figure 3.

1099 **Figure 3 – figure supplement 1 – source data.** Uncropped immunoblot
1100 corresponding to Figure 3 supplement 1.

1101 **Figure 3 – figure supplement 2 – source data.** Uncropped immunoblot
1102 corresponding to Figure 3 supplement 2.

1103 **Figure 4 – source data.** Uncropped immunoblot corresponding to Figure 4.

1104 **Figure 5 – source data.** Uncropped immunoblot corresponding to Figure 5.

Figure 5 – figure supplement – source data. Uncropped immunoblot

corresponding to Figure 5 supplement.

Figure 6 – source data. Uncropped immunoblot corresponding to Figure 6.

Figure 6 – figure supplement 1 – source data. Uncropped immunoblot

corresponding to Figure 6 supplement 1.

Figure 7 – source data. Uncropped immunoblot corresponding to Figure 7.

Figure 1. Reconstitution of α -syn secretion regulated by palmitoylated DNAJC5 in HEK293T cells.

1126 **Figure 1 – figure supplement 1. Secretion of α -syn variants.**

1127

Figure 1 – figure supplement 2. Basal secretion of α -syn is partially dependent on endogenous DNAJC5.

Figure 2. Characterization of secreted α -syn.

Figure 2 – figure supplement 1. Solubility of secreted α -syn variants.

1139 **Figure 2- figure supplement 2. DNAJC5 enriched in buoyant EV fraction.**

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Figure 2 – figure supplement 3. Assessment of tandem α -syn oligomers with gel filtration chromatography.

Figure 3. Disruption of palmitoylation of DNAJC5 inhibited α -syn secretion.

Figure 3 – figure supplement 1. Dose-dependent inhibition of α -syn secretion by 2-bromopalmitic acid (2-BA).

Figure 3 – figure supplement 2. Blockage of USP19-induced α -syn secretion by DNAJC5 L115R or L116 Δ mutant.

Figure 4. Topological localization of α -syn and DNAJC5 in enlarged endosomes.

1160 **Figure 4 – figure supplement 1.** Immunofluorescence (IF) images of endogenous
1161 DNAJC5 and enlarged endosomes.

1162

Figure 4 – figure supplement 2. Live-cell images of DNAJC5 L115R mutant and α -syn.

1167 **Figure 4 - supplement video.** Time lapse of movement of internalized α -syn and
 1168 DNAJC5 in the enlarged endosomes. Blue, Rab5^{Q79L}; Green, α -syn; Red,
 1169 DNAJC5. Scale bar: 10 μ m.

Figure 5 – figure supplement. Medium fractionation of secreted tandem α -syn oligomers. P-DNAJC5 was depleted in the supernatant after centrifugation at 100,000 (100k) xg. However, no significant decrease of tandem α -syn oligomers in the supernatant was observed.

Figure 6. XPACK (XP)-induced DNAJC5 L115R oligomerization rescued α -syn secretion.

Figure 6 – figure supplement 1. Characterization of HMW-DNAJC5 and XPACK fusion.

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