α-Synuclein fibrils subvert lysosome structure and function for the propagation of protein misfolding between cells through tunneling nanotubes

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1 **α-Synuclein fibrils subvert lysosome structure and function for the**

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Abstract

The accumulation of α-synuclein aggregates in specific brain regions is a hallmark of synucleinopathies including Parkinson's disease. α-Synuclein aggregates propagate in a "prion-like" manner and can be transferred inside lysosomes to recipient cells through tunneling nanotubes (TNTs). However, how lysosomes participate in the spreading of α-synuclein aggregates is unclear. Here, by using super-resolution and electron microscopy, we find that α-synuclein fibrils affect the morphology of lysosomes and impair their function in neuronal cells. In addition, we demonstrate that α-synuclein fibrils induce peripheral redistribution of lysosomes, likely mediated by TFEB, increasing the efficiency of α-synuclein fibrils' transfer to neighboring cells. We also show that lysosomal membrane permeabilization allows the seeding of soluble α-synuclein in cells that have taken up α-synuclein fibrils from the culture medium and, more importantly, in healthy cells in co-culture, following lysosome-mediated transfer of the fibrils. Moreover, we demonstrate that seeding occurs mainly at lysosomes in both donor and acceptor cells, after uptake of α-syn fibrils from the medium and following their transfer, respectively. Finally, by using a heterotypic co-culture system, we determine the origin and nature of the lysosomes transferred between cells and we show that donor cells bearing α-synuclein fibrils transfer damaged lysosomes to acceptor cells, while also receiving healthy lysosomes from them. These findings thus contribute to the elucidation of the mechanism by which α-synuclein fibrils spread through TNTs, while also revealing the crucial role of lysosomes, working as a Trojan horse for both seeding and propagation of disease pathology.

Introduction

The accumulation of amyloidogenic proteins in different regions of the brain is a hallmark of neurodegenerative diseases (NDs) leading to cellular dysfunction, loss of synaptic communication, and deficits in specific brain functions [1,2]. The misfolded α-synuclein (α-syn) protein accumulates in intraneuronal inclusions known as Lewy bodies and Lewy neurites in NDs known as synucleinopathies, including the second most common ND, Parkinson's Disease (PD) [3–5]. These inclusions are associated with loss of neuronal and glial cells in specific brain regions, leading to both motor (e.g., bradykinesia, tremor, rigidity) and non-motor (e.g., cognitive impairment, depression, anxiety) symptoms [6,7].

α-Syn is a small (140 amino acids) cytoplasmic protein enriched in the brain and localized to presynaptic terminals [8]. Although the precise function of α-syn remains poorly understood, it has been shown to promote membrane curvature, thus contributing to synaptic trafficking and vesicle budding [9–12], to modulate dopamine release through its association with presynaptic terminal SNARE complexes [13], and to be involved in synaptic attenuation [14]. A recent study has characterized *in vitro* a mechanism by which α-syn stabilizes, in a concentration-dependent manner, the docking of synaptic vesicles on the plasma membrane by establishing a dynamic link between the two membranes [15]. In PD brains, the spread of the pathology correlates with the presence of α-syn inclusions and follows a specific and predictable route through interconnected brain regions, mirroring the different stages of the disease [5,16]. These findings supported the hypothesis, later confirmed experimentally, that α-syn aggregates can be transferred from one cell to another both *in vitro* [17–22], and *in vivo* [23–27]. Furthermore, mounting evidence suggests that α-syn aggregates spread in a "prion-like" manner, as they are able to self-propagate by acting as "seeds" and inducing the misfolding of soluble native α-syn [19,27–35]. Thus, understanding how α-syn aggregates spread and how/where in the cell they induce misfolding of the soluble protein is crucial: i) to elucidate the pathogenesis of the disease, and ii) to devise a therapy to inhibit the progression of the pathology. Different mechanisms depending on either cell-to-cell contact or secretion have been proposed for the spread of α-syn fibrils [35]. We and others have found that, in *in vitro* and *ex vivo* cultures, tunneling nanotubes (TNTs) play a major role in this process [19–21,36].

TNTs are actin-based, thin cellular protrusions that connect remote cells [37]. They have been identified in various cell types *in vitro* [38–41] and *in vivo* [42–47]. TNTs are open-ended structures [48,49] that allow the exchange of various cargos, including entire organelles, such as mitochondria and lysosomes, between cells [19,37–40]. In addition to α-syn fibrils, other

74 amyloidogenic proteins such as prion protein [50–52], huntingtin [53], and tau [52,54,55] use

75 TNTs as highways for transfer to naïve cells, suggesting that TNTs are common routes for the

76 spread of pathogenic proteins between cells. Of particular interest, we showed that α-syn fibrils

77 can be efficiently transferred between neuronal CAD cells, primary neurons, primary

78 astrocytes, and human neural progenitor cells, and between organotypic hippocampal slices and

79 astrocytes. We found that α-syn fibrils are mostly localized inside lysosomes in all of these cell

80 types [19,21,56] and could be transferred inside lysosomes through TNTs from "infected"

81 donor cells to naïve acceptor cells in co-cultures. Importantly, once arrived in the acceptor cells,

82 α-syn fibrils were able to induce the aggregation of soluble α-syn [19]. How this occurs,

83 however, is not clear.

84 Lysosomes are cytoplasmic, membrane-enclosed, acidic organelles that have long been

85 considered as the "dustbin" and "recycling center" of the cells, as they are responsible for the

86 degradation of both exogenous and intracellular cargos destined to lysosomes via endocytic,

87 phagocytic [57,58], and autophagic pathways [59,60]. However, in recent years, mounting

88 evidence revealed that lysosomes play a more complex role in the regulation of cellular

89 homeostasis, as they are involved in crucial processes such as plasma membrane repair,

90 transcriptional control, nutrient sensing, signaling, and energy metabolism [61–65]. It is

91 therefore not surprising that lysosomal dysfunction is involved in a plethora of pathological

92 conditions, including NDs [66]. Notably, the levels of lysosome-associated membrane proteins

93 1 and 2 (LAMP1 and LAMP2) and lysosomal enzymes Cathepsin B and D were found to be

94 significantly reduced in PD patient brains, mouse and cell models [67–72]. Moreover,

95 lysosomal membrane permeabilization (LMP) has been reported in cells treated with protein

96 aggregates including α-syn [73,74].

97 In the present study, we focused on the role of lysosomes in the intercellular spread of α-syn

98 fibrils in neuronal cells. Particularly, we studied how α-syn fibrils affect lysosomes and how

99 these organelles contribute to the spread and propagation of α-syn aggregates through TNTs.

100 We found that α-syn fibrils alter the morphology of lysosomes and compromise lysosomal

101 function. We demonstrated that α-syn fibrils induce the peripheral distribution of the lysosomes

102 exhibiting functional impairment, likely mediated by TFEB, and showed that this peripheral

103 positioning enhances the efficiency of α-syn fibrils' transfer in a cell-to-cell contact-dependent

104 manner. In addition, we demonstrated that TFEB translocated to the nucleus in the presence of

105 α-syn fibrils, probably contributing to the peripheral distribution of lysosomes. We also found

106 that LMP, which is well described in cells following the uptake of α-syn fibrils from the

107 medium, also occurs in acceptor cells following the transfer of lysosomes bearing α-syn fibrils

4

108   from neighboring donor cells. Thus, by facilitating the escape of α-syn fibrils from lysosomes

109   after their transfer, LMP appears to be a relevant mechanism for the seeding of soluble α-syn

110   and propagation of the pathology in neighboring cells. Importantly, our super-resolution

111   microscopy data suggest that lysosomes create an optimal environment for seeding and are a

112   hub for the conversion of soluble α-syn into aggregates. Finally, by using a heterotypic cell co-

113   culture, we were able to track the origin and nature of the lysosomes transferred between cells.

114   We found that cells overwhelmed by the presence of α-syn fibrils impairing lysosomal function

115   transfer more damaged lysosomes to recipient cells and, in return, they receive healthy

116   lysosomes from neighboring cells devoid of α-syn, suggesting a potential rescue mechanism in

117   α-syn pathology.

Results

α-Syn fibrils affect the morphology and function of lysosomes.

We have previously reported that the majority of exogenous α-syn fibrils internalized by neuronal catecholaminergic cell line CAD localize to lysosomes [19]. We confirmed these findings by performing object-based 3D colocalization analysis (*https://imaris.oxinst.com*) in CAD cells challenged with α-syn fibrils for 18 h prior to labelling for the lysosomal marker LAMP1. We detected 74 ± 1% of α-syn fibrils colocalizing with lysosomes (LAMP1+ organelles), confirming that the majority of α-syn fibrils localize to lysosomes following their uptake (Fig 1A). In addition, we found 60 ± 1% of lysosomes colocalizing with α-syn fibrils (Fig 1A). To further investigate the lysosomal localization of α-syn fibrils and the possible morphological consequences of this localization, we performed structured illumination microscopy (SIM). We found that α-syn fibrils localized either inside lysosomes (Fig 1B, magenta arrow and enlarged images on the right) or at the lysosomal membrane (Fig 1B, green arrow and enlarged images on the right). We then measured the diameter of lysosomes in control cells and in α-syn fibril-treated cells (where we distinguished between lysosomes containing or not containing α-syn fibrils). The average diameter of lysosomes in control cells and of lysosomes not containing α-syn fibrils in treated cells was almost identical (0.43 ± 0.01 μm) whereas lysosomes containing α-syn fibrils increased their diameter by 50% (0.63 ± 0.01 μm; Fig 1B, graph), demonstrating that the enlargement induced by α-syn fibrils is restricted to the lysosomes containing fibrils.

To further investigate the morphology of compartments containing α-syn fibrils, we used a correlative light-electron microscopy (CLEM) approach to identify α-syn-positive and -negative lysosomes by fluorescence microscopy (FM) and study their corresponding morphology by electron microscopy (EM). First, we cultured CAD cells transiently transfected with a plasmid encoding LAMP1-GFP and loaded with Alexa 568-tagged α-syn fibrils on gridded coverslips, and imaged α-syn fibril-treated cells in FM while marking coordinates of α-syn positive (Alexa568+/GFP+) and negative (Alexa568-/GFP+) LAMP1-GFP lysosomes. Following FM imaging, the CAD cells were prepared for EM by keeping their orientation, and serial sections were collected from the region containing the previously marked coordinates (see also Material and Methods) [75,76]. Then, the FM signal was correlated to EM images. Using this approach, we overcame the lack of suitable tools to identify α-syn fibrils directly by EM and were able to compare the structural properties of lysosomes containing or not containing α-syn fibrils. LAMP1-GFP-positive organelles colocalizing with α-syn fibrils in FM

151 were structurally identified as lysosomes by the presence of electron-dense heterogenous

152 luminal content, degraded membranes, and occasional intraluminal vesicles as morphological

153 criteria [77]. Lysosomes not containing α-syn fibrils presented a healthy lysosome morphology

154 with spherical shape and smooth membranes [77,78] (Fig 1C, green square, number 1).

155 Interestingly, most lysosomes containing α-syn fibrils were found to be quite heterogeneous in

156 structure (Fig 1C, red squares numbers 2, 3, and 4). Some had an unusual luminal content and

157 displayed curvy limiting membrane, suggesting that the presence of the fibrils could deform the

158 organelles (Fig 1C, red squares numbers 3, and 4). The aberrant, curvy organelles were

159 completely absent in control cells not treated with α-syn fibrils (S1A Fig). A subset of

160 lysosomes containing α-syn fibrils, as defined by FM, had a healthy morphology (Fig 1C, red

161 square, number 2), which could represent an early stage of fibrillar accumulation.

162 Next, we used another CLEM approach to analyse the morphology and size of α-syn-positive

163 lysosomes with high-resolution. CAD cells loaded with Alexa 568-tagged α-syn fibrils were

164 prepared for EM following the flat embedding Tokuyasu technique. This technique preserves

165 the fluorescence signal of α-syn fibrils in ultrathin sections prepared for EM, and in addition

166 allows to perform immunolabelling (both gold and fluorescent) of endogenous LAMP1 [79]

167 (Fig 1D). Following a recently developed high-throughput on-section CLEM approach [80],

168 we analysed 50 LAMP1-immunolabelled lysosomes in control cells (S1B Fig), 50 α-syn-

169 positive, and 50 α-syn-negative lysosomes in treated cells. For each lysosome, we correlated

170 EM ultrastructural details (surface area and perimeter) and the presence of LAMP1 to the FM

171 images showing the presence or absence of α-syn fibrils. Using this approach, we confirmed

172 that the lysosomes bearing α-syn fibrils were significantly larger (perimeter: 2.24 ± 0.14 μm

173 and area: 0.16 ± 0.01 μm^2) compared to lysosomes devoid of α-syn fibrils (perimeter: $1.16 \pm$

174 0.06 μm and area: 0.08 ± 0.01 μm^2) and control lysosomes (perimeter: 1.16 ± 0.04 μm and area:

175 0.08 ± 0.01 μm^2; Fig 1D, graphs).

176

177 **Fig 1. α-Syn fibrils affect the morphology of lysosomes. (A)** Representative confocal images

178 of control and Alexa 568-tagged α-syn fibril-treated (18 h) CAD cells, immunolabelled for

179 LAMP1 (green). Colocalization between LAMP1+ puncta and α-syn fibrils is indicated by

180 arrows in the inset of a selected region delimited by a square. % of α-syn fibrils colocalizing

181 with LAMP1+ puncta ($74 \pm 1\%$) and % of LAMP1+ puncta colocalizing with α-syn fibrils (60

182 $\pm 1\ \%$) performed by object-based 3D colocalization method (Imaris software) is presented.

183 Mean $\pm$ S.E.M, n=3 (30 cells per condition). Scale bar: 10 μm (for inset: 2 μm). **(B)** Super-

184   resolution (SR) images of control (left panel) and Alexa 568-tagged α-syn fibril-treated CAD

185   cells for 18 h (right panel), immunolabelled for LAMP1 (far-red) antibody (pseudo colored in

186   grey). Merge images of each condition are presented with additional HCS CellMask[TM] Blue

187   staining. Magenta and green arrows indicate the selected lysosomes having α-syn fibrils inside

188   the lysosomal lumen and on the lysosomal membrane, respectively, where higher

189   magnifications of these lysosomes and 3D reconstructions are represented in magenta and green

190   squares, respectively. Scale bar: 10 μm (for insets: 2 μm, for magenta and green insets: 0.5 μm).

191   Average diameter (μm) of lysosomes in control (0.43 ± 0.01) and in α-syn fibril-treated cells,

192   the latter sub-grouped as: lysosomes without α-syn fibrils (0.43 ± 0.01) and lysosomes with α-

193   syn fibrils (0.63 ± 0.01), is presented. Mean ± S.E.M, lysosomes' diameters were measured in

194   SR images of eight α-syn fibril-treated cells and seven control cells (155 lysosomes per

195   condition). Images were acquired by spinning-disk microscopy with SR module. ns=not

196   significant, ****P<0.0001 by Kruskal-Wallis nonparametric ANOVA test followed by Dunn's

197   multiple comparison tests. **(C)** FM image of LAMP1-GFP transfected and α-syn fibril-treated

198   CAD cells for 18 h (left panel). Enlarged merged images of correlative resin EM and FM images

199   of four selected lysosomes not containing (number 1, indicated by green square) and containing

200   α-syn fibrils (numbers 2, 3, 4, indicated by red squares) are presented. Lysosomes number 1

201   and 2 are further magnified (blue squares) and presented within the same image. Lyso:

202   lysosome, PM: plasma membrane, LD: lipid droplets. Scale bar: 10 μm (for red and green

203   insets: 0.5 μm, for blue insets: 0.2 μm). **(D)** Overlay of on-section correlative EM images

204   immuno-gold labelled for LAMP1[10] and FM images of LAMP1-GFP transfected and α-syn

205   fibril-treated CAD cells (18 h). Insets of three selected regions (indicated by red, purple, and

206   black squares) are presented. Average perimeter (μm) of lysosomes in control (1.16 ± 0.04) and

207   in α-syn fibril-treated cells: lysosomes without α-syn fibrils (1.16 ± 0.06) and lysosomes with

208   α-syn fibrils (2.24 ± 0.14; upper graph) is presented. ns=not significant, ****P<0.0001 by one-

209   way ANOVA followed by Tukey's multiple comparison tests. Average area (μm^2) of

210   lysosomes in control (0.08 ± 0.01) and in α-syn fibril-treated cells: lysosomes without α-syn

211   fibrils (0.08 ± 0.01) and lysosomes with α-syn fibrils (0.16 ± 0.01; lower graph) is presented.

212   Mean ± S.E.M. (50 cells per condition). ns=not significant, ****P<0.0001 by Kruskal-Wallis

213   nonparametric ANOVA test followed by Dunn's multiple comparison tests. Scale bar: 10 μm

214   (for insets: 0.5 μm). The data underlying this Figure may be found in S1 Data.

215

216   In spite of these changes in lysosome morphology, the presence of α-syn fibrils had no effect

217   on the abundance of lysosomes. Indeed, the average number of LAMP1-positive lysosomes per

218   cell (control cells: 222 ± 6; α-syn fibril-treated cells: 218 ± 7; Fig 2A, left panel) and the level

219   of LAMP1 (control: 0.9 ± 0.2 AU; α-syn: 0.7 ± 0.1 AU; Fig 2A, right panel) did not show any

220   significant difference in the presence or absence of α-syn fibrils. Overall, these results indicate

221   that, while the overall amount of lysosomes is not affected by α-syn fibrils, lysosomes

222   containing α-syn exhibit changes in size and morphology.

223   α-Syn fibrils have been shown to impair lysosomal function [67–72]. Thus, next we addressed

224   their effect on lysosomes in our model system. We first assessed the acidity of the lysosomes

225   in cells treated or not with α-syn fibrils by using a fluorogenic acidotropic dye, LysoTracker

226   Deep Red (LysoTracker DR). The fluorescence intensity of LysoTracker DR was significantly

227   decreased (~41 %) in α-syn fibril-treated cells compared to untreated control cells (Fig 2B),

228   suggesting alkalinization of lysosomes in the presence of α-syn fibrils. Second, we used a DQ-

229   BSA assay in which the fluorescent signal is detected only when the fluorogenic substrate is

230   hydrolyzed by lysosomal proteases. We observed that the DQ-BSA fluorescence intensity was

231   significantly decreased (~45 %) in the presence of α-syn fibrils compared to the control (Fig

232   2C), suggesting that the activity of the lysosomal proteases was compromised. Finally, we used

233   a more specific approach to evaluate lysosomal function by quantifying the activity of the

234   lysosomal enzyme Cathepsin B (CathB). The intracellular activity of CathB was detected as

235   fluorescent puncta produced by the hydrolysis of the fluorogenic substrate Magic Red in live

236   cells. We observed a significant decrease (~42 %) in CathB enzyme activity in cells challenged

237   with α-syn fibrils compared to control cells (Fig 2D). Since the lysosomal pool was similar in

238   control and α-syn fibril-treated cells, these data indicate that the decrease in the fluorescent

239   signal of LysoTracker DR, DQ-BSA, and Magic Red is likely due to the presence of α-syn

240   fibrils.

241   Altogether, our data indicate that the majority of the α-syn fibrils localize to the lysosomes,

242   where they induce lysosomal enlargement and structural changes, and impair lysosome

243   function.

244

245   **Fig 2. α-Syn fibrils affect the function of lysosomes. (A)** Average number of lysosomes

246   (LAMP1+ puncta) per cell in control (222 ± 6) and in α-syn fibril-treated CAD cells for 18 h

247   (218 ± 7) is presented. Mean ± S.E.M, *n*=5 (50 cells per condition). ns=not significant by

248   Whitney-Mann *U*- test (left panel). Western blot showing LAMP1 and α-tubulin expression in

control and α-syn fibril-treated cells. Integrated Density of LAMP1 protein expression levels normalized to α-tubulin in control (0.9 ± 0.2) and α-syn fibril-treated cells (0.7 ± 0.1) are presented in Arbitrary Units (AU). Mean ± S.E.M, n=3. ns=not significant by paired t-test. **(B)** Representative confocal images of control and Alexa 568-tagged α-syn fibrils challenged CAD cells (18 h), labelled with LysoTracker DR (20 nM for 20 min; LysoTracker DR is pseudo colored in grey). Dashed lines represent the cell contour. Average Integrated Density of control cells ($1.4 \times 10^7 \pm 9 \times 10^5$) and α-syn fibril-treated cells ($8.3 \times 10^6 \pm 5 \times 10^5$) is presented. Mean ± S.E.M, n=3 (40 cells per condition). ****P<0.0001 by Mann-Whitney U-test. Scale bar: 10 μm. **(C)** Representative confocal images of control and Alexa 568-tagged α-syn fibrils (red) challenged CAD cells for 18 h, treated with DQ-BSA (green) for 90 min. Dashed lines represent the cell contour. Average Integrated Density measured for control cells ($3.8 \times 10^6 \pm 1 \times 10^5$) and α-syn fibril-treated cells ($1.7 \times 10^6 \pm 6 \times 10^4$) is presented. Mean ± S.E.M, n=3 (25 cells per condition). ****P<0.0001 by Mann-Whitney U-test. Scale bar: 10 μm. **(D)** Representative confocal images of control and Alexa 488-tagged α-syn fibrils (green) challenged CAD cells for 18 h treated with 1X Magic Red Cathepsin B (red). Dashed lines represent the cell contour. Average Integrated Density measured for control cells ($1.3 \times 10^6 \pm 6 \times 10^4$) and α-syn fibril-treated cells ($7.5 \times 10^5 \pm 5 \times 10^4$) is presented. Mean ± S.E.M, n=5 (100 cells per condition). ****P<0.0001 by Mann-Whitney U-test. Scale bar: 10 μm. The data underlying this Figure may be found in S1 Data.

Lysosomes containing α-syn fibrils undergo Lysosomal Membrane Permeabilization (LMP).

α-Syn [73,74], as well as other amyloidogenic aggregates such as tau [81,82] and amyloid beta [83], induce LMP following their uptake from the culture medium in different cell types. This allows escape of α-syn aggregates from the lysosome to act on the soluble α-syn in the cytosol. However, the fate of these lysosomes has not been assessed. Furthermore, we previously demonstrated that α-syn spread between cells occurs mainly by direct cell-to-cell transfer of lysosomes bearing aggregates through tunneling nanotubes (TNTs), cytoplasmic protrusions extending from the cell periphery and connecting distant cells [21,35,52] (S2 Fig). Therefore, the question arises as to whether LMP also occurs after lysosomes bearing α-syn have been transferred to healthy neighboring cells. To address this question, we assessed first whether α-syn fibrils also induced LMP in our neuronal CAD cells. We used the Galectin-3 (Gal3) puncta

assay, based on the localization of Gal3 to damaged lysosomes. CAD cells were transiently transfected with Gal3-GFP and challenged or not with α-syn fibrils for 18 h, prior to labelling for LAMP1 (Figs 3A and S3A number 1). After image acquisition by confocal microscopy, we quantified Gal3-GFP puncta formation in α-syn fibril-treated and non-treated cells. We detected a significantly higher percentage of cells exhibiting Gal3-GFP puncta in the presence of α-syn fibrils ($46 \pm 2\%$) compared to the control condition ($7 \pm 1\%$) (Fig 3B, left graph), in which most cells displayed cytosolic Gal3-GFP [73,74]. However, in cells exhibiting Gal3-GFP puncta, there was no significant difference in the average number of puncta between control and α-syn fibril-treated cells (8 ± 1 and 12 ± 1 respectively; Fig 3B, right graph). In addition, we performed object-based 3D colocalization analysis to evaluate the association of Gal3-GFP with both α-syn fibrils and LAMP1+ lysosomes. We detected $38 \pm 4\%$ of Gal3-GFP puncta colocalizing with α-syn fibrils, $47 \pm 3\%$ of Gal3-GFP puncta colocalizing with LAMP1+ lysosomes, and $35 \pm 3\%$ of Gal3-GFP puncta colocalizing with both α-syn fibrils and lysosomes (Fig 3A, magenta arrowheads, and C). These findings demonstrate that more than one third of Gal3-GFP puncta are recruited to the ruptured lysosomes containing α-syn fibrils. Furthermore, by using SIM, we could clearly observe the recruitment of Gal3-GFP to the damaged lysosomes positive for α-syn fibrils, which appeared to be either inside their lumen (Fig 3D) or at their membrane (Fig 3E).

Since lysosomes under LMP can be targeted to the autophagy pathway in order to be degraded (a process called lysophagy) [84], next we investigated this possible scenario in CAD cells treated with α-syn fibrils in comparison with control cells. As a positive control we treated CAD cells with L-leucyl-L-leucine methyl ester (LLOMe), which is a well-known inducer of LMP. Cells were then labeled for ubiquitin, LC3, and LAMP1, and colocalization of these markers were analysed. As expected, in CAD cells treated with LLOMe, we could detect good colocalization between LC3, ubiquitin and LAMP1, whereas in control cells we could only detect a few LC3 and ubiquitin puncta and no colocalization between these puncta and lysosomes (S4 Fig). In three independent experiments, we could only detect one lysosome that colocalized with LC3 and ubiquitin in α-syn fibril-treated cells (S4 Fig); thus, lysophagy was an extremely rare event under these conditions and was not statistically different from the control condition.

Fig 3. α-Syn fibrils induce LMP. (A) Representative confocal images of Gal3-GFP transfected control (upper panel) and Alexa 568-tagged α-syn fibril-treated CAD cells for 18 h (lower

panel), labelled with LAMP1-Alexa 647 antibody (pseudo colored in grey). Higher magnifications of the selected region in α-syn fibril-treated cells (white squares) are presented on the upper-left part of each image. Arrowheads indicate a lysosome having α-syn fibrils colocalizing with Gal3 puncta. Scale bar: 10 μm (for insets: 5 μm). **(B)** % of control (7 ± 1%) and α-syn fibril-treated CAD cells for 18 h (46 ± 2%) under LMP (left graph), and average number of Gal3-GFP puncta for control (8 ± 1) and α-syn fibril-treated cells (12 ± 1) under LMP (right graph). Mean ± S.E.M, n=3 (50 cells per condition). ****P<0.0001 by Mann-Whitney U-test. **(C)** % of Gal3-GFP puncta colocalizing with α-syn fibrils (38 ± 4%), with LAMP1 (47 ± 3%), and with both α-syn fibrils and LAMP1 (35 ± 3%) calculated by object-based 3D colocalization (Imaris software). Mean ± S.E.M, n=3 (30 cells per condition). **(D)** SR image showing the recruitment of Gal3-GFP to a lysosome containing α-syn fibrils puncta in its lumen. **(E)** SR image showing the recruitment of Gal3-GFP to a lysosome having α-syn fibrils at its membrane. In D and E, insets of two selected regions (white squares) from different Z-planes of the same Gal3-GFP transfected and α-syn fibril-treated CAD cell, labelled for HCS CellMask™ Blue, DAPI, and LAMP1 (pseudo colored in grey) are presented. Images were acquired with SR SIM (Zeiss, LSM 780-Elyra PS.1). Scale bars: 10 μm (for insets: 1 μm). The data underlying this Figure may be found in S1 Data.

α-Syn fibrils seed *de novo* formation of aggregates at the lysosomes.

LMP has been proposed to be a relevant mechanism for α-syn fibrils to escape from lysosomes and interact with soluble α-syn to induce seeding [19,85]. Therefore, we investigated the seeding activity of exogenous α-syn fibrils in CAD cells. To this aim, CAD cells were transiently transfected with a plasmid encoding α-syn-GFP, a soluble fluorescent reporter of α-syn, and the cells were challenged or not with Alexa 568-tagged α-syn fibrils for 18 h. The formation of α-syn-GFP aggregates detected as green puncta was then quantified (Figs 4A and S3A number 2). We found that 76 ± 5% of cells treated with α-syn fibrils produced newly formed α-syn-GFP aggregates, whereas this percentage was significantly lower (13 ± 2%) in control cells transfected with α-syn-GFP only (Fig 4B, left graph). In addition, the average number of newly formed α-syn-GFP puncta detected in the cells treated with the fibrils (27 ± 3) was significantly higher than the α-syn-GFP puncta detected in the control condition (12 ± 1; Fig 4B, right graph), suggesting that the exogenous α-syn fibrils induce efficient seeding of the soluble protein. These data were in line with previous findings [86–89]; however, the

346 question remains as to whether lysosomes participate to this seeding event and how/where in

347 the cell it occurs. To investigate whether seeding is associated with the lysosomes, we

348 performed super-resolution microscopy in CAD cells transfected with α-syn-GFP, loaded with

349 α-syn fibrils and labelled for LAMP1. We observed three different scenarios: i) lysosomes with

350 both α-syn fibrils and α-syn-GFP aggregates colocalizing at the lysosomal membrane (Fig 4C),

351 ii) lysosomes with α-syn fibrils and α-syn-GFP aggregates colocalizing inside their lumen (Fig

352 4D), and iii) lysosomes containing both α-syn fibrils and α-syn-GFP aggregates inside their

353 lumen, but not necessarily co-localizing (Fig 4E). In addition, we performed object-based 3D

354 colocalization analysis between newly formed α-syn aggregates (α-syn-GFP puncta), α-syn

355 fibrils and lysosomes. We observed that 69 ± 6 % of the newly formed aggregates colocalized

356 with LAMP1+ lysosomes, 74 ± 7% colocalized with α-syn fibrils, and 63 ± 6 % of them were

357 positive for both α-syn fibrils and LAMP1+ organelles (Fig 4F), indicating that in CAD cells

358 the majority of the seeding takes place at lysosomal compartments following α-syn fibrils

359 uptake.

360 To understand whether the localization of α-syn-GFP aggregates to lysosomes was consistent

361 with the beginning of the seeding event and not a post seeding delivery of newly formed fibrils,

362 we monitored the occurrence of seeding by live imaging microscopy. We first transfected CAD

363 cells with soluble α-syn-GFP and then immediately after the addition of α-syn fibrils we started

364 to monitor the seeding event by detecting the formation of green puncta (α-syn-GFP newly

365 formed aggregates) in these cells compared to control cells by live spinning-disk microscopy.

366 We found that the seeding first became apparent about 2 h from the addition of the α-syn fibrils

367 and increased with time (S1 Movie and S5A Fig). We did not observe any newly formed α-syn-

368 GFP puncta in untreated control cells (S2 Movie and S5B Fig). Next, we performed super-

369 resolution microscopy in order to investigate whether these newly formed aggregates were

370 associated with the lysosomes by immunolabelling LAMP1 at different time points (1h, 2h, and

371 4h). Interestingly, at all these time points (even in the rare events found at 1 hour), we found

372 that newly formed aggregates colocalized with α-syn fibrils and LAMP1 (Fig 4G-I). Therefore,

373 newly formed α-syn-GFP aggregates following α-syn fibrils addition are associated with

374 lysosomes at the very beginning of the seeding event, supporting our hypothesis that lysosomes

375 function as hubs for the seeding of newly formed α-syn aggregates.

376 Overall, these results indicate that exogenous α-syn fibrils induce *de novo* aggregation of

377 soluble α-syn in association with lysosomes.

378

Fig 4. α-Syn fibrils seed *de novo* formation of aggregates associated with lysosomes. (A) Representative confocal images of α-syn-GFP transfected control or Alexa 568-tagged α-syn fibril-treated CAD cells (18 h). Scale bar: 10 μm (for insets: 5 μm). **(B)** % of control (13 ± 2%) and α-syn fibril-treated CAD cells for 18 h (76 ± 5%) that formed α-syn-GFP aggregates (left graph). Mean ± S.E.M, *n*=3 (45 cells per condition). ****P<0.0001 by two tailed *t*-test. Average number of α-syn-GFP puncta per cell in control (12 ± 1) and α-syn fibril-treated cells (27 ± 3) (right graph). Mean ± S.E.M, *n*=3 (45 cells per condition). **P=0.005 by Mann-Whitney *U*-test. **(C)** SR image showing α-syn fibrils puncta and α-syn-GFP+ aggregate colocalizing on the lysosomal membrane in CAD cells treated with α-syn fibrils for 18 h. **(D)** SR image showing colocalization between α-syn fibrils puncta and α-syn-GFP+ aggregate inside the lysosomal lumen, and α-syn fibrils on the lysosomal membrane in CAD cells treated with α-syn fibrils for 18 h. **(E)** SR image showing α-syn fibrils puncta and α-syn-GFP+ aggregate inside the lysosomal lumen in CAD cells treated with α-syn fibrils for 18 h. **(F)** Object-based 3D colocalization analysis (Imaris software) between α-syn-GFP aggregates and α-syn fibrils (74 ± 7%), α-syn-GFP aggregates and LAMP1+ lysosomes (69 ± 6 %), and α-syn-GFP aggregates with both α-syn fibrils and LAMP1+ lysosomes (63 ± 6 %). Mean ± S.E.M, *n*=2 (16 super-resolution images, ~2000 lysosomes were analysed). **(G)** SR image showing α-syn fibrils puncta and α-syn-GFP+ aggregate colocalizing with LAMP1 on the lysosomal membrane in CAD cells treated with α-syn fibrils for 1 h. **(H)** SR image demonstrating α-syn fibrils puncta and α-syn-GFP+ aggregate colocalizing with LAMP1 both on the lysosomal membrane and inside the lysosomal lumen in CAD cells treated with α-syn fibrils for 2 h. **(I)** SR image showing α-syn fibrils puncta and α-syn-GFP+ aggregate colocalizing with LAMP1 on the lysosomal membrane in CAD cells treated with α-syn fibrils for 4 h. In C, D, E, G, H, I insets of selected regions (white squares) from different α-syn-GFP transfected and α-syn fibril-treated CAD cells, labelled for DAPI and LAMP1 (pseudo colored in grey) are presented. Images were acquired with a spinning-disk microscope with SR module. Scale bars: 10 μm (for insets: 1 μm; 0.5 μm for inset in G). The data underlying this Figure may be found in S1 Data.

α-Syn-loaded lysosomes localize to the cell periphery and are less functional.

The results presented above highlight the role of lysosomes in the propagation of α-syn misfolding after uptake of the fibrils from the culture medium. However, they do not address i) how propagation of misfolding occurs following the transfer of lysosomes containing α-syn aggregates to healthy neighboring cells [19,21], and ii) whether/how lysosome damage induced

14

412 by α-syn aggregates favors the spread of pathology [19,32]. Lysosomes are dynamic organelles

413 that move bidirectionally between the perinuclear and peripheral regions of the cell [90–92].

414 As our results indicate that the α-syn fibrils interfere with lysosome morphology and function,

415 we hypothesized that they could also have an impact on lysosome positioning. Therefore, we

416 evaluated the distribution of lysosomes in CAD cells challenged with α-syn fibrils compared to

417 control cells. Cells were labelled for LAMP1, HCS CellMask™ Blue (cell outline stain), and

418 DAPI (nuclear stain), and the number of lysosomes in the perinuclear and peripheral regions in

419 each condition was quantified (see Materials and Methods; Fig 5A). In agreement with previous

420 findings that lysosomes are mainly located in the perinuclear region in healthy non-polarized

421 cells [93], we detected 57 ± 1% of the lysosomes at the perinuclear region in control CAD cells

422 (Fig 5A, white arrows, and B). In contrast, in cells treated with α-syn fibrils, the situation was

423 reversed, with the majority of lysosomes (56 ± 1%) found in the peripheral region (Fig 5A,

424 green, red, yellow arrows, and B).

425 Since our results indicate that α-syn fibrils affect lysosomal pH and function (Fig 2B-D), to

426 understand whether there is a correlation between lysosome distribution and function, we next

427 evaluated the distribution of lysosomes containing α-syn fibrils and quantified the perinuclear

428 and peripheral distribution of only functional lysosomes positive for LysoTracker DR and

429 Magic Red CathB. We found that α-syn fibril-containing LysoTracker DR positive and Magic

430 Red CathB positive lysosomes were more perinuclear (54 ± 2% and 56 ± 1%, respectively; Fig

431 5C), suggesting that peripheral lysosomes containing α-syn fibrils are less functional.

432 These results thus show that α-syn fibrils promote redistribution of lysosomes towards the cell

433 periphery. Furthermore, this pool of peripheral lysosomes is less acidic and has less degradative

434 capacity.

435

Peripheral α-syn-loaded lysosomes are preferentially transferred to neighboring cells.

437 Our next question was to understand whether the effect of α-syn on lysosome positioning could

438 be relevant for the propagation of the pathology and in which way. Peripheral lysosomes could

439 be more prone to exocytosis and therefore could increase the secretion of α-syn fibrils to the

440 extracellular environment. Alternatively, peripheral lysosomes could be more apt for transfer

441 to healthy neighboring cells through TNTs. To distinguish between these alternative scenarios,

442 we first examined lysosomal exocytosis by comparing the amount of CathB released into the

443 culture medium of control and α-syn fibril-treated CAD cells using an ELISA method. These

444 experiments showed no significant difference in the amount of CathB released by control (70

15

445   ± 1 ng/mL) and α-syn fibril-treated cells (75 ± 7 ng/mL; Fig 5D). As an alternative approach,

446   we measured the integrated fluorescence density of LAMP1 at the plasma membrane (PM) in

447   cells treated or not treated with α-syn fibrils. To this end, we used an antibody recognizing a

448   luminal epitope of LAMP1, which is displayed to the extracellular milieu after fusion of

449   lysosomes with the PM in non-permeabilized cells (Fig 5E). We did not observe a difference

450   in the average integrated density of PM-associated LAMP1 between control ($9x10^4 ± 2x10^4$

451   AU) and α-syn fibril-treated cells ($9x10^4 ± 3x10^4$ AU; Fig 5E, graph). These results indicate

452   that, despite their different distributions, lysosomes are exocytosed at a similar rate in α-syn

453   fibril-treated and control cells.

454   Next, to understand whether the peripheral positioning of lysosomes could affect their transfer

455   to neighboring cells, we compared the efficiency of lysosome transfer by using a co-culture

456   assay where donor cells were transiently transfected with LAMP1-GFP and either treated or

457   not with α-syn fibrils for 18 h (α-syn co-culture and control co-culture, respectively), and

458   acceptor cells were transiently transfected with H2B-mCherry (Figs 5F and S3B). We observed

459   that the percentage of acceptor cells that received LAMP1-GFP puncta (lysosomes) from donor

460   cells was significantly higher in the α-syn co-culture compared to the control co-culture (29 ±

461   3% and 18 ± 2%, respectively) (Fig 5G, left graph). Lysosome transfer through secretion was

462   evaluated by a "secretion test" (S3I Fig) whereby acceptor cells treated with the conditioned

463   medium of control or α-syn fibril-loaded donor cells were quantified for the presence of

464   LAMP1-GFP puncta. We did not observe a significant difference between the two secretion

465   tests (9 ± 2% and 7 ± 1% in control and α-syn secretion, respectively; Fig 5G, left graph). In

466   addition, we did not observe any difference in the average number of LAMP1-GFP puncta

467   detected per acceptor cell either in the co-culture conditions (2 ± 0.2 for both co-culture

468   conditions) or in the secretion test conditions (1 ± 0.1 for both secretion conditions; Fig 5G,

469   right graph). These results confirm our previous data that lysosome transfer from donor to

470   acceptor cells is predominantly mediated by cell-to-cell contact, likely through TNTs rather

471   than secretion [19,21,22]. Importantly, we showed that donor cells challenged with α-syn fibrils

472   transfer lysosomes more efficiently to the acceptor cells compared to control donor cells.

473   Moreover, we performed object-based 2D colocalization analysis in the acceptor cells that

474   received both LAMP1-GFP+ lysosomes and α-syn fibrils in order to evaluate the number of

475   lysosomes containing α-syn fibrils transferred to the acceptor cells. We found 51 ± 6% of α-

476   syn fibrils colocalizing with LAMP1-GFP+ lysosomes and 51 ± 6% of lysosomes colocalizing

477   with α-syn fibrils, suggesting that half of the lysosomes transferred from donor to acceptor cells

478   carried α-syn fibrils (Fig 5H).

479   Taken together, our data indicate that lysosomes that are more peripherally located in the

480   presence of α-syn fibrils are not secreted more compared to control cells, but instead are more

481   efficiently transferred to acceptor cells by cell-to-cell contact.

482

483   **Fig 5. α-Syn-loaded lysosomes localize to the cell periphery, are less functional, and are**

484   **preferentially transferred to neighboring cells. (A)** Representative confocal images of

485   control and Alexa 568-tagged α-syn fibril-treated CAD cells (18 h) labelled for LAMP1-Alexa

486   488 antibody, HCS CellMask^{TM} Blue, and DAPI. White arrows indicate the perinuclear

487   lysosome clusters in control cells; green, red, and yellow arrows indicate peripheral lysosomes,

488   α-syn fibrils, and lysosomes colocalizing with α-syn fibrils, respectively. Scale bar: 10 μm. **(B)**

489   % of LAMP1+ lysosomes in perinuclear (purple dots) and peripheral regions (black dots) of

490   the control cells and cells challenged with α-syn fibrils is presented. In control cells: % of

491   perinuclear lysosomes: $57 \pm 1\%$, and peripheral lysosomes: $43 \pm 1\%$; in α-syn fibril-treated

492   cells: % of perinuclear lysosomes: $44 \pm 1\%$, and peripheral lysosomes: $56 \pm 1\%$. Mean $\pm$ S.E.M,

493   *n*=5 (40 cells per condition). ns=not significant, ****P<0.0001 by one-way ANOVA followed

494   by Tukey's multiple comparison tests. **(C)** Graph representing the distribution of LysoTracker

495   DR+ and Magic Red CathB+ lysosomes colocalizing with the α-syn fibrils in perinuclear

496   (purple dots) and peripheral region (black dots). % of LysoTracker DR+ lysosomes colocalizing

497   with α-syn fibrils in perinuclear region: $54 \pm 2\%$, and in peripheral region: $46 \pm 2\%$. % of Magic

498   Red CathB+ lysosomes colocalizing with α-syn fibrils in perinuclear region: $56 \pm 1\%$, and in

499   peripheral region: $44 \pm 1\%$. Mean $\pm$ S.E.M, *n*=4 (40 cells per condition). ns=not significant by

500   one-way ANOVA followed by Tukey's multiple comparison tests. **(D)** Quantity of secreted

501   CathB (ELISA) in the media of control (70 ± 1 ng/mL) and α-syn fibril-treated CAD cells (75

502   ± 7 ng/mL). Mean $\pm$ S.E.M, *n*=3. ns=not significant by Mann-Whitney *U*-test. **(E)**

503   Representative confocal images of control and Alexa 568-tagged α-syn fibril-treated CAD cells

504   for 18 h, immuno-labelled for LAMP1-Alexa 488 antibody in non-permeabilized conditions.

505   Scale bar: 10 μm. Average Integrated Density of LAMP1 immunofluorescence at the cell

506   surface in control ($9x10^4 \pm 2x10^4$) and in α-syn fibril-treated cells ($9x10^4 \pm 3x10^4$) is presented.

507   Mean $\pm$ S.E.M, *n*=3 (20 cells per condition). ns=not significant by paired *t*-test. Scale bar: 10

508   μm. **(F)** Representative confocal images of donor and acceptor cells after 24 h of co-culture.

509   Donor cells were transfected with LAMP1-GFP and treated with Alexa 568-tagged α-syn fibrils

or not (referred to as "α-syn co-culture" and "control co-culture", respectively). Acceptor cells were transfected with H2B-mCherry. Scale bar: 20 μm. **(G)** % of acceptor cells that received LAMP1+ puncta (lysosomes) from donor cells in control co-culture (18 ± 2%), in α-syn co-culture (29 ± 3%), in control secretion test (9 ± 2%), and in α-syn secretion test (7 ± 1%) (left graph). Mean ± S.E.M, *n*=4 (150 acceptor cells per condition). ns=not significant, ***P=0.0004 by one-way ANOVA followed by Tukey's multiple comparison tests. The average number of LAMP1-GFP+ puncta detected per acceptor cell in control co-culture (2 ± 0.2), in α-syn co-culture (2 ± 0.2), in control secretion (1 ± 0.1), and in α-syn secretion (1 ± 0.1) is presented (right graph). Mean ± S.E.M, *n*=4 (150 acceptor cells per condition). ns=not significant, ***P=0.0004 by Kruskal-Wallis nonparametric ANOVA test followed by Dunn's multiple comparison tests. **(H)** % of α-syn fibril puncta colocalizing with LAMP1-GFP+ puncta (51 ± 6%) and % of LAMP1-GFP+ puncta colocalizing with α-syn fibrils puncta (51 ± 6%) is presented. Mean ± S.E.M, *n*=4 (20 acceptor cells that received both α-syn fibrils and LAMP1-GFP puncta were analysed). The data underlying this Figure may be found in S1 Data.

TFEB as a player in lysosome redistribution upon α-syn loading.

The Transcription Factor EB (TFEB) regulates lysosome biogenesis and function by coordinating the expression of lysosomal components [94]. Furthermore, TFEB has also been shown to influence lysosome positioning [95–97]. To investigate a possible role of TFEB in the peripheral redistribution of lysosomes upon α-syn loading, we first asked whether the lysosomal loading of α-syn fibrils triggers TFEB nuclear translocation. To address this question, we expressed TFEB-WT-GFP in CAD cells challenged or not with α-syn fibrils for 18 h (S3A Fig number 3). We found a significant increase in the percentage of CAD cells having nuclear TFEB upon α-syn loading compared to control cells (control: 18 ± 4%; α-syn: 57 ± 6%, Fig 6A).

Next, we assessed the effect of TFEB overexpression on lysosome distribution in CAD cells. We transiently transfected CAD cells with plasmids encoding wild-type TFEB (TFEB-WT-GFP) or constitutively active mutant TFEB (TFEB-mut-GFP) (i.e., nuclear TFEB), or with empty plasmid CMV-GFP (control), and quantified the number of lysosomes in the perinuclear and peripheral regions. We found that cells overexpressing the constitutively active form of TFEB showed more peripheral lysosomes compared to both control cells and cells overexpressing TFEB-WT-GFP (TFEB-mut-GFP: 59 ± 1%; TFEB-WT-GFP: 54 ± 2%; CMV-

542   GFP: 52 ± 1%; Fig 6B). These results indicate that TFEB promotes the peripheral distribution

543   of lysosomes in CAD cells.

544   To determine whether TFEB participates in peripheral redistribution of lysosomes upon α-syn

545   loading, we silenced the expression of TFEB in CAD cells by RNA interference (Fig 6C). CAD

546   cells transfected with scrambled siRNA (control) or siTFEB were challenged with α-syn fibrils

547   for 18 h. Then, we quantified the positioning of lysosomes containing α-syn fibrils (Fig 6D).

548   As expected, control cells showed more peripheral α-syn-loaded lysosomes (52.1 ± 1%). In

549   contrast, in siTFEB-treated CAD cells α-syn-loaded lysosomes were slightly but significantly

550   more perinuclear (50.7 ± 1%). These findings indicate that silencing of endogenous TFEB

551   reduced the peripheral redistribution of lysosomes upon α-syn loading, suggesting the

552   involvement of TFEB in this event.

553

554   **Fig 6. The role of TFEB in lysosome redistribution upon α-syn loading. (A)** % of cells

555   overexpressing TFEB-WT-GFP having nuclear TFEB in control CAD cells (18 ± 4%) and α-

556   syn fibril-treated CAD cells for 18 h (57 ± 6%). Mean ± S.E.M, *n*=3 (50 cells per condition).

557   ****P<0.0001 by Unpaired t test. **(B)** % of LAMP1+ lysosome distribution in perinuclear

558   (purple dots) and peripheral regions (black dots) of cells overexpressing CMV-GFP (control),

559   TFEB-WT-GFP, or TFEB-mut-GFP. In control CMV-GFP cells: % of perinuclear lysosomes:

560   48 ± 1%, and peripheral lysosomes: 52 ± 1%; in TFEB-WT-GFP cells: % of perinuclear

561   lysosomes: 46 ± 2%, and peripheral lysosomes: 54 ± 2%; in TFEB-mut-GFP cells: % of

562   perinuclear lysosomes: 41 ± 1%, and peripheral lysosomes: 59 ± 1%. Mean ± S.E.M, *n*=3 (30

563   cells per condition). ***P<0.001 by one-way ANOVA followed by Tukey's multiple

564   comparison tests. **(C)** Western blot prepared from cell extracts collected from control,

565   scrambled siRNA (sicontrol)-, and siTFEB-pretreated CAD cells for 60 h, showing TFEB and

566   GAPDH (loading control) expression. **(D)** Graph representing the distribution of lysosomes

567   containing α-syn fibrils in perinuclear (purple dots) and peripheral region (black dots) of α-syn

568   fibril-treated sicontrol and siTFEB cells. % of lysosomes containing α-syn fibrils of sicontrol

569   cells in perinuclear region: 49 ± 1%, and in peripheral region: 52 ± 1%. % of lysosomes

570   containing α-syn fibrils of siTFEB cells in perinuclear region: 51 ± 1%, and in peripheral

571   region: 49 ± 1%. Mean ± S.E.M, *n*=4 (30 cells per condition). ns=not significant, *P=0.0288

572   by one-way ANOVA followed by Tukey's multiple comparison tests. The data underlying this

573   Figure may be found in S1 Data.

574

Lysosome positioning affects the efficiency of α-syn fibrils' transfer.

To further investigate the effect of lysosome positioning on α-syn fibrils transfer, we set-up different co-culture assays where we monitored the amount of α-syn transfer using donor cells with more peripheral or more perinuclear lysosomes. To this end, we respectively overexpressed or silenced the Arf-like small GTPase Arl8b, which controls the movement of lysosomes towards the cell periphery [98,99]. To examine the effect of peripheral lysosome redistribution, Arl8b-GFP transfected or control donor CAD cells were loaded with α-syn fibrils and co-cultured with acceptor CAD cells transiently transfected with H2B-GFP to distinguish them from the donors (referred to as Arl8b-GFP co-culture and control co-culture, respectively; Figs 7A and S3C). We then quantified the presence of α-syn fibrils in the acceptor cells in each co-culture condition. We detected a significant increase in the percentage of acceptor cells that received α-syn fibrils in the Arl8b-GFP co-culture ($65 \pm 4\%$) compared to control co-culture ($49 \pm 4\%$; Fig 7B, left graph). As a control, we monitored the transfer of α-syn fibrils through the culture supernatant, thus checking a possible effect on transfer through a secretion mechanism. As previously found [19], the amount of α-syn transfer by secretion was very low compared with the transfer in co-culture, and did not significantly change in the two co-culture conditions ($8 \pm 2\%$ and $10 \pm 2\%$, in control and Arl8b-GFP secretion conditions, respectively; Fig 7B, left graph). Despite changes in the percentage of cells that took up α-syn fibrils in co-cultures, we did not find a significant difference in the average number of α-syn puncta per acceptor cell in any of these conditions (in average: 3 ± 0.4, 3.4 ± 0.4, 1.3 ± 0.4, 2 ± 0.3 α-syn fibrils puncta per acceptor cell in control co-culture, Arl8b-GFP co-culture, control secretion, and Arl8b-GFP secretion, respectively; Fig 7B, right graph).

To corroborate these observations in a different cell type, we performed the same experiment in HeLa cells (Figs S3D and S6) in which the effect of Arl8b on lysosome positioning was extensively characterized [90,91]. We observed that the percentage of acceptor cells that received α-syn fibrils was $28 \pm 3\%$ and $60 \pm 5\%$ in control and Arl8b-GFP co-cultures, respectively (S6A and B Fig, left graph). Again, we did not observe any significant increase in the transfer of α-syn fibrils through secretion from the donors overexpressing Arl8b-GFP in comparison to control donors ($8 \pm 3\%$ and $1 \pm 1\%$ respectively; S6B Fig, left graph). As in CAD cells, the average number of α-syn puncta per acceptor cell was not significantly different in HeLa co-cultures and secretion conditions (3 ± 1 and 5 ± 1 in control and Arl8b-GFP co-cultures, respectively; 1 ± 0.0 and 2 ± 0.2 in control and Arl8b-GFP secretion tests, respectively; S6B Fig, right graph).

608 Furthermore, we silenced the expression of Arl8b in CAD cells by RNA interference (Fig 7C).

609 Briefly, we pretreated donor cells with siRNA targeting Arl8b (siArl8b) and scrambled siRNA

610 (sicontrol) for 60 h prior to loading with α-syn fibrils for 18 h; in parallel, untreated control

611 cells were challenged with α-syn fibrils. These donors were then co-cultured with acceptor cells

612 transiently transfected with H2B-GFP (Figs 7D and S3E). In control co-culture and in sicontrol

613 co-culture, the percentage of acceptor cells that received α-syn fibrils was 55 ± 4% and 60 ±

614 3%, respectively, whereas in siArl8b co-culture we observed a significant lower percentage of

615 28 ± 3% (Fig 7E, left graph). We did not detect any significant difference in α-syn transfer

616 through secretion (6 ± 1%, 9 ± 1%, and 6 ± 1% in control, sicontrol, and siArl8b secretion

617 conditions, respectively; Fig 7E, left graph). The average number of α-syn puncta detected in

618 acceptor cells in co-cultures and secretion conditions was not significantly changed (1-2 puncta

619 per cell in all the conditions; Fig 7E, right graph).

620 To further test the importance of lysosome positioning in α-syn fibrils transfer, we performed

621 similar experiments in HeLa cells with knock out (KO) of components of the lysosome-

622 positioning machinery. These components included the myrlysin (also known as BORCS5)

623 subunit of BORC, which promotes recruitment of Arl8b to membranes [100], and the kinesins

624 KIF1B and KIF5B [101–105], which drive lysosome movement towards the cell periphery [90].

625 KO of these proteins impairs lysosome movement towards the cell periphery [90,100]. These

626 KO donor cells, in parallel to control donors (WT HeLa) were loaded with α-syn fibrils and co-

627 cultured with WT HeLa cells expressing H2B-GFP (Figs S3F and S6C). Acceptor cells were

628 then analysed for the presence of α-syn fibrils. The percentage of acceptor cells that received

629 α-syn fibrils in control co-culture was 29 ± 2%, whereas in myrlysin-KO and KIF1B-KIF5B-

630 double-KO (KIF1B-5B dKO) co-cultures this percentage was significantly decreased to 16 ±

631 2% and 12 ± 2%, respectively (S6D Fig, left graph). α-Syn fibrils' transfer through secretion

632 was similar in all the conditions (5 ± 2%, 6 ± 1%, 6 ± 1% in control, myrlysin-KO, and KIF1B-

633 5B dKO secretion conditions, respectively; S6D Fig, left graph). We did not observe a

634 significant difference in the average number of transferred α-syn puncta in any of the co-

635 cultures/secretion conditions (1-2 puncta per cell in all the conditions; S6D Fig, right graph).

636 Overall, these data indicate that the contact-dependent transfer of α-syn is affected by the

637 position of lysosomes, with more transfer when lysosomes are peripheral and less transfer when

638 lysosomes are perinuclear.

639 As we have shown that lysosomes containing α-syn are transferred from acceptor cells inside

640 TNTs [52] (S2 Fig), we wondered whether TNT formation was affected by the manipulations

21

that alter lysosome positioning. Thus, we performed two sets of TNT counting experiments in control, Arl8b-GFP-overexpressing or siArl8b/sicontrol-treated CAD cells in the presence or absence of α-syn fibrils. These cells were labelled with WGA and the percentage of TNT-connected cells was quantified for each condition (see Materials and Methods). We did not observe a significant difference in the percentage of TNT-connected cells between control (45 ± 3%) and Arl8b-GFP-overexpressing cells (46 ± 2%; Fig 7F, left graph). However, in the presence of α-syn fibrils the percentage of TNT-connected cells was significantly increased to 60 ± 5% in control and 64 ± 5% in Arl8b-GFP-overexpressing cells (Fig 7F, left graph). Similarly, the percentage of TNT-connected cells remained unchanged between control (44 ± 4%), sicontrol (45 ± 4%), and siArl8b (43 ± 4%) pretreated cells, whereas in the presence of α-syn fibrils the percentage of TNT-connected cells significantly increased to 62 ± 4% in control, 62 ± 5% in sicontrol, and 66 ± 3% in siArl8b-treated cells (Fig 7F, right graph). These data indicate that lysosome positioning has no effect on TNT formation per se, but it is the presence of α-syn fibrils that increases the number of TNT-connected cells, in line with previous findings [19].

Taken together, these results indicate that lysosome positioning affects the efficiency of α-syn fibrils' transfer. Moreover, α-syn fibrils increase the number of TNT-connected cells independent of lysosome positioning.

Fig 7. Lysosome positioning affects the efficiency of α-syn fibrils' transfer. (A) Representative confocal images of donor and acceptor cells after 24 h of co-culture. Donor cells were either loaded with Alexa 568-tagged α-syn fibrils only (control co-culture) or transfected with Arl8b-GFP prior to be treated with α-syn fibrils (Arl8b-GFP co-culture). Acceptor cells were transfected with H2B-GFP. Co-cultures were labelled with HCS CellMask^{TM} Blue and DAPI. Arrows indicate the α-syn fibrils received by acceptor cells and asterisks indicate the cells having α-syn fibrils in each co-culture condition. Scale bar: 10 μm. **(B)** % of acceptor cells received α-syn fibrils in control co-culture (49 ± 4%), in Arl8b-GFP co-culture (65 ± 4%), in control secretion (8 ± 2%), and in Arl8b-GFP secretion (10 ± 2%) (left graph). Mean ± S.E.M, *n*=3 (90 acceptor cells per condition). ns=not significant, **P=0.0038 by Kruskal-Wallis nonparametric ANOVA test followed by Dunn's multiple comparison tests. Average number of α-syn fibrils puncta in control co-culture (3 ± 0.4), in Arl8b-GFP co-culture (3.4 ± 0.4), in control secretion (1.3 ± 0.4), and in Arl8b-GFP secretion (2 ± 0.3) (right graph). Mean ± S.E.M, *n*=3 (90 acceptor cells per condition). ns=not significant, by one-way ANOVA followed by

674   Tukey's multiple comparison tests. **(C)** Western blot prepared from cell extracts collected from

675   control, scrambled siRNA (sicontrol)-, and siArl8b-pretreated CAD cells for 60 h, showing

676   Arl8b and α-tubulin (loading control) expression. Integrated Density of Arl8b protein

677   expression levels normalized to α-tubulin in control (0.80 ± 0.03), sicontrol- (0.82 ± 0.02), and

678   siArl8b-pretreated cells (0.19 ± 0.09) are presented in Arbitrary Units (AU). Mean ± S.E.M,

679   *n*=3. ns= not significant, ***P=0.0004 by one-way ANOVA followed by Tukey's multiple

680   comparison tests. **(D)** Representative confocal images of donor and acceptor cells after 24 h of

681   co-culture. Donor CAD cells were pretreated with sicontrol or siArl8b for 60 h or left untreated

682   prior to be challenged with Alexa 568-tagged α-syn fibrils (referred to as control, sicontrol, and

683   siArl8b co-cultures, respectively). Acceptor cells were transfected with H2B-GFP. Co-cultures

684   were labelled with HCS CellMask^{TM} Blue and DAPI. Arrows indicate the α-syn fibrils received

685   by acceptor cells and asterisks indicate the cells having α-syn fibrils in each co-culture

686   condition. Scale bar: 10 μm. **(E)** % of acceptor cells that received α-syn fibrils in control co-

687   culture (55 ± 4%), sicontrol co-culture (60 ± 3%), siArl8b co-culture (28 ± 3%), control

688   secretion (6 ± 1%), sicontrol secretion (9 ± 1%), and siArl8b secretion (6 ± 1%) (left graph).

689   Mean ± S.E.M, *n*=4 (80 acceptor cells per condition). ns= not significant, ****P<0.0001 by

690   one-way ANOVA followed by Tukey's multiple comparison tests. Average number of α-syn

691   puncta per acceptor cell in control co-culture (2 ± 0.2), sicontrol co-culture (2 ± 0.1), siArl8b

692   co-culture (2 ± 0.3), control secretion (1 ± 0.2), sicontrol secretion (2 ± 0.2), and siArl8b

693   secretion (2 ± 0.3) (right graph). Mean ± S.E.M, *n*=4 (80 acceptor cells per condition). ns= not

694   significant by Kruskal-Wallis nonparametric ANOVA test followed by Dunn's multiple

695   comparison tests. **(F)** Control, Arl8b-GFP transfected, and sicontrol- or siArl8b-pretreated

696   CAD cells for 60 h were challenged or not with α-syn fibrils (18 h) and labelled with WGA for

697   TNT counting experiments. % of TNT-connected cells in control (45 ± 3%), Arl8b-GFP (46 ±

698   2%), control-α-syn (60 ± 5%), and Arl8b-GFP-α-syn (64 ± 5%) conditions. Mean ± S.E.M, *n*=4

699   (115 cells per condition). ns= not significant (left graph). % of TNT-connected cells in control

700   (44 ± 4%), sicontrol (45 ± 4%), siArl8b (43 ± 4%), control-α-syn (62 ± 4%), sicontrol-α-syn

701   (62 ± 5%), and siArl8b-α-syn (66 ± 3%) is presented (right graph). Mean ± S.E.M, *n*=3 (90

702   cells per condition). ns= not significant, *P<0.05, **P<0.01 by one-way ANOVA followed by

703   Tukey's multiple comparison tests. The data underlying this Figure may be found in S1 Data.

704

705   **α-Syn fibrils induce LMP upon arrival in the acceptor cells.**

706   Our data show that more than 50% of α-syn fibrils in acceptor cells colocalize with LAMP1,
707   indicating that they are still associated with lysosomes after the transfer (Fig 5H). Moreover,
708   we have previously reported that α-syn fibrils can induce seeding of soluble α-syn once they
709   are transferred to acceptor cells [19]. Based on the results presented here one possible
710   explanation for this observation would be that α-syn fibrils also trigger LMP in acceptor cells
711   following their transfer, similar to what we have observed in donor cells (Fig 3A and B). To
712   assess this hypothesis, we performed co-culture experiments where donor cells were either
713   transiently transfected with H2B-mCherry (control co-culture) or treated with α-syn fibrils for
714   18 h (α-syn co-culture), and co-cultured with acceptor cells transiently transfected with Gal3-
715   GFP (Figs 8A and S3G number 1). Acceptor cells were then evaluated for the presence of Gal3-
716   GFP puncta. The percentage of acceptor cells exhibiting LMP (Fig 8A, white arrows) was
717   significantly higher in the α-syn co-culture ($33 \pm 5\%$) compared to control co-culture ($6 \pm 2\%$)
718   (Fig 8B, upper graph). We also quantified the average number of Gal3-GFP puncta per acceptor
719   cell and we found a significant increase in α-syn co-culture (14 ± 1) compared to control co-
720   culture where some LMP occurs spontaneously (8 ± 1; Fig 8B, lower graph). We also performed
721   super-resolution microscopy in α-syn co-culture labelled for lysosomes with antibody to
722   LAMP1 and observed Gal3-GFP and α-syn fibrils puncta both in close proximity inside the
723   lysosomal lumen (Fig 8C) and colocalizing on the lysosomal membrane (Fig 8D). These results
724   thus show that, following their transfer, α-syn-containing lysosomes undergo LMP in acceptor
725   cells.
726
727   **Fig 8. α-Syn fibrils induce LMP upon arrival in the acceptor cells. (A)** Representative
728   confocal images of donor and acceptor cells after 24 h of co-culture. Donor cells were
729   transfected with H2B-mCherry (control co-culture) or treated with Alexa 568-tagged α-syn
730   fibrils (α-syn co-culture). Acceptor cells were transfected with Gal3-GFP. Co-cultures were
731   labelled with HCS CellMask™ Blue. Red arrows indicate the α-syn fibrils received by acceptor
732   cells, white arrows indicate the Gal3-GFP puncta formation (LMP) in the acceptor cells that
733   received α-syn fibrils. Scale bar: 10 μm. **(B)** % of acceptor cells under LMP in control ($6 \pm 2\%$)
734   and α-syn ($33 \pm 5\%$) co-cultures (upper graph). Average number of Gal3-GFP puncta formation
735   in acceptor cells under LMP in control (8 ± 1) and α-syn (14 ± 1) co-cultures (lower graph).
736   Mean $\pm$ S.E.M, n=5 (50 acceptor cells per condition). *P=0.04, ****P<0.0001 by Mann-
737   Whitney U- test. **(C)** SR image of a co-culture where donor cells were treated with α-syn fibrils
738   for 18 h and acceptor cells were transfected with Gal3-GFP, labelled for LAMP1-Alexa 647

739   antibody (pseudo colored in grey). A selected region where Gal3-GFP puncta were observed

740   (indicated by a white square in the acceptor cell of the co-culture image), was further magnified

741   to observe one lysosome (indicated by the small white square) having α-syn fibrils puncta and

742   recruited Gal3-GFP. Image was acquired with a Spinning Disk microscope with SR module.

743   Scale bar: 10 μm (for the inset: 0.2 μm). **(D)** SR image of an acceptor cell demonstrating Gal3-

744   GFP puncta recruited at the lysosomal membrane where α-syn fibrils were detected in close

745   proximity. Selected lysosome is indicated by a white square. Image was acquired by SR SIM

746   (Zeiss, LSM 780-Elyra PS.1). Scale bar: 5 μm (for the inset: 0.5 μm). In C, the signal was

747   boosted to make the selected lysosome visible; in D, the acceptor cell was presented with HCS

748   CellMask™ Blue labelling as this cell was poorly transfected but accumulation of Gal3-GFP

749   puncta was clearly detectable. The data underlying this Figure may be found in S1 Data.

750

751   **α-Syn fibrils induce seeding after their transfer into acceptor cells and this event is**

752   **preferentially associated with lysosomes.**

753   To further investigate the seeding mechanism in acceptor cells that received lysosomes

754   containing α-syn fibrils, we used again our α-syn-GFP soluble fluorescent reporter expressed

755   in acceptor cells co-cultured with donor cells, either challenged with α-syn fibrils or transfected

756   with H2B-mCherry (α-syn co-culture and control co-culture respectively; Figs 9A and S3G

757   number 2). We then quantified the presence of α-syn-GFP puncta (indicating aggregation of the

758   soluble protein; Fig 9A, white arrows) in the acceptor cells that received α-syn fibrils (Fig 9A,

759   red arrows). We found that 27 ± 4 % of acceptor cells contained 7 ± 1 α-syn-GFP puncta in α-

760   syn co-culture, whereas in control co-culture only $10 \pm 4\%$ of the acceptor cells had 2 ± 0.2 α-

761   syn-GFP puncta (Fig 9B).

762   Next, to address whether the seeding in acceptor cells was associated with lysosomes, we

763   performed super-resolution microscopy. After labelling the cells in co-culture with antibody to

764   LAMP1, we examined the subcellular localization of α-syn-GFP aggregates in acceptor cells.

765   We observed four different scenarios: i) lysosomes containing α-syn fibrils and colocalizing

766   with α-syn-GFP puncta surrounding the lysosomal membrane (Fig 9C), ii) lysosomes filled

767   with α-syn fibrils with discrete α-syn-GFP deposits on their membrane (Fig 9D), iii) lysosomes

768   containing α-syn-GFP puncta with α-syn fibrils in close proximity (Fig 9E), and iv) lysosomes

769   containing both α-syn fibrils and α-syn-GFP puncta partially colocalizing in their lumen (Fig

770   9F). In addition, we performed 3D object-based colocalization analysis between α-syn-GFP

771　puncta, α-syn fibrils, and LAMP1+ lysosomes. We observed that $60 \pm 7\%$ of the newly formed

772　α-syn-GFP aggregates colocalized with LAMP1+ lysosomes (Fig 9G). Overall, these data

773　indicate that the majority of the seeding of endogenously expressed soluble α-syn takes place

774　at lysosomal compartments in acceptor cells after the transfer of α-syn fibrils.

775

776　**Fig 9. α-syn fibrils induce seeding after their transfer into acceptor cells and this event is**

777　**frequently associated with lysosomes. (A)** Representative confocal images of donor and

778　acceptor cells after 24 h of co-culture. Donor cells were transfected with H2B-mCherry (control

779　co-culture) or treated with Alexa 568-tagged α-syn fibrils for 18 h (α-syn co-culture). Acceptor

780　cells were transfected with α-syn-GFP. Inset of acceptor cells (indicated by white squares) is

781　presented at the right top corner of acceptor cells' panel. White arrows indicate α-syn-GFP

782　aggregate formation, red arrows indicate α-syn fibrils received by acceptor cells in α-syn co-

783　culture. Scale bar: 10 μm (for the inset: 5 μm). **(B)** % of acceptor cells that formed α-syn-GFP

784　aggregates in control ($10 \pm 4\%$) and in α-syn ($27 \pm 4\%$) co-cultures (left graph). Average

785　number of α-syn-GFP puncta detected in acceptor cells in control (2 ± 0.2) and in α-syn (7 ± 1)

786　co-cultures is presented (right graph). Mean $\pm$ S.E.M, *n*=3 (150 acceptor cells per condition).

787　*P=0.01 and **P=0.003 for % of aggregates and average number of aggregates in control

788　versus α-syn co-cultures, respectively, by Mann Whitney *U*-test. **(C)** SR image showing a

789　lysosome containing α-syn fibrils puncta colocalizing with α-syn-GFP+ aggregate at the

790　lysosomal membrane. **(D)** SR image showing a lysosome containing α-syn fibrils where α-syn-

791　GFP+ aggregate is located mostly in a restricted portion of the lysosomal membrane. **(E)** SR

792　image showing a lysosome having α-syn-GFP+ aggregate where α-syn fibrils puncta were

793　found to be in close proximity of the lysosomes. **(F)** SR image showing a lysosome where both

794　α-syn fibrils and α-syn-GFP aggregates are located to the lumen of the lysosome and partially

795　colocalizing. **(G)** Object-based 3D analysis (Imaris software) of colocalization between α-syn-

796　GFP and LAMP1+ lysosomes ($60 \pm 7\%$). Mean $\pm$ S.E.M, *n*=2 (15 super-resolution images,

797　~3000 lysosomes were analysed). Insets of images in C, D (acquired with Spinning-Disk

798　microscope with SR module) and E, F (acquired with SR SIM, Zeiss, LSM 780-Elyra PS.1)

799　from four selected regions (white squares) and from different acceptor cells transfected with α-

800　syn-GFP and that received α-syn fibrils, labelled for LAMP1 Alexa 647 antibody (pseudo

801　colored in grey). Scale bars: 10 μm (for the insets: 1 μm). The data underlying this Figure may

802　be found in S1 Data.

803

α-Syn-loaded cells transfer damaged lysosomes to healthy cells and receive healthy lysosomes in return.

In our homotypic co-culture system between CAD cells, we could not address the nature of the transferred lysosomes (i.e., whether the lysosomes that are transferred have LMP or not) nor the directionality of lysosome transfer (from donor to acceptor and/or from acceptor to donor). To answer these fundamental questions, we designed a heterotypic co-culture assay using mouse CAD cells as donors and human HeLa cells as acceptors. Briefly, CAD cells were transiently transfected with Gal3-GFP, loaded or not with α-syn fibrils, and used as the donor cell population; on the other hand, HeLa cells stably expressing Gal3-Turquoise were used as the acceptor cell population. Donor and acceptor cells were co-cultured for 24 h prior to be fixed and immunolabelled with either a specific anti-mouse LAMP1 antibody (to detect the lysosomes originated from donor cells) or a specific anti-human LAMP1 antibody (to detect the lysosomes originated from acceptor cells; S3H Fig).

First, we tested this heterotypic system by evaluating: i) the efficiency of α-syn fibrils' transfer from donor CAD cells to acceptor HeLa cells, and ii) the occurrence of seeding in the acceptor cells. The percentage of acceptor HeLa cells that received α-syn fibrils from donors was $65 \pm 3\%$ with an average of 4 ± 0.2 α-syn puncta per acceptor cell (S7A-B Fig). As expected, also in this case, both the percentage of α-syn fibrils ($7 \pm 2\%$) and the average number of α-syn fibrils puncta per acceptor cell (2 ± 0.2) was significantly lower in the secretion condition (S7B Fig). When donor CAD cells loaded with α-syn fibrils were co-cultured with HeLa cells transiently transfected with soluble α-syn-GFP, we again observed the aggregation of soluble α-syn-GFP (S7C Fig, green arrows) in acceptor HeLa cells that received α-syn fibrils from donor CAD cells (S7C Fig, red arrows). Taken together, these results indicate that α-syn fibrils can be efficiently transferred in a cell-to-cell contact manner and can induce seeding in the acceptor cells following their transfer also in a heterotypic mouse-human co-culture assay.

As we could recognize the origin of lysosomes, we then used this system to better quantify the transfer of all lysosomes and of lysosomes under LMP from donor to acceptor cells. To this end, CAD cells were either loaded or not with α-syn fibrils and co-cultured with HeLa cells expressing Gal3-Turquoise. Cells in co-culture were then immunolabelled with the antibody to mouse LAMP1 to detect the transfer of lysosomes originated from donor cells (Fig 10A). The percentage of acceptor HeLa cells that received donors' lysosomes was significantly higher in α-syn co-culture ($52 \pm 4\%$) compared to control co-culture ($32 \pm 5\%$), whereas the average number of transferred lysosomes (LAMP1+ puncta) did not change (3 ± 0.3 in both co-cultures;

837 Fig 10B). Furthermore, we observed that in α-syn co-culture both lysosomes containing α-syn

838 fibrils (Figs 10A, yellow arrows, and S7G, red arrow) or not (Figs 10A, white arrows, and S7G,

839 white arrow) were transferred to acceptor cells.

840 Next, to evaluate the amount of lysosomes having LMP that were transferred from CAD cells

841 to HeLa cells, we quantified the occurrence of LMP in CAD cells in our heterotypic co-culture

842 condition. Thus, we counted the number of Gal3-GFP puncta in donor CAD cells transfected

843 with Gal3-GFP, challenged or not with α-syn fibrils, and co-cultured with HeLa-Gal3-

844 Turquoise cells (S7D Fig, green arrows). As expected, the percentage of donor CAD cells

845 having LMP was significantly higher in α-syn loaded donor cells (11 ± 4%) compared to control

846 donors (2 ± 2%), whereas the average number of Gal3-GFP puncta per cell did not show any

847 statistically significant change (10 ± 2 and 14 ± 4 in control and α-syn donors, respectively;

848 S7E Fig). These findings indicate that, in co-culture conditions, donor CAD cells challenged

849 with α-syn fibrils undergo LMP similar to what we showed earlier (Fig 3A and B). Next, we

850 evaluated the transfer of donors' lysosomes with LMP by quantifying the presence of Gal3-

851 GFP puncta in acceptor HeLa-Gal3-Turquoise cells both in control and α-syn co-cultures (Fig

852 10C). We found a significantly higher percentage of HeLa cells with Gal3-GFP puncta in the

853 α-syn co-culture (13 ± 2%; Fig 10C, green arrows, and D, left graph) compared to control co-

854 culture (4 ± 1%; Fig 10D, left graph). We did not observe a significant difference between the

855 average number of Gal3-GFP puncta per acceptor cell in the two co-cultures (2 ± 0.2 and 2 ±

856 0.3 in control and α-syn co-cultures, respectively; Fig 10D, right graph). These data indicate

857 that CAD cells challenged with α-syn fibrils transfer more damaged lysosomes (i.e., undergoing

858 LMP) to acceptor cells compared to control CAD cells. In addition, we were able to detect

859 donors' lysosomes colocalizing both with α-syn fibrils and Gal3-GFP puncta in acceptor HeLa

860 cells (Fig 10E), demonstrating that damaged lysosomes carrying α-syn fibrils are transferred

861 into the acceptor cells.

862 By taking advantage of HeLa cells stably expressing Gal3-Turquoise as acceptor cells, we also

863 evaluated the occurrence of LMP in acceptor cells by assessing the Gal3-Turquoise puncta (Fig

864 10F, turquoise arrows). We found that 18 ± 3% of acceptor cells undergo LMP with an average

865 of 10 ± 1 Gal3-Turquoise puncta per cell in α-syn co-culture (Fig 10G). LMP in the acceptor

866 cells of control co-culture was significantly lower (3 ± 1%) with an average of 9 ± 1 Gal3-

867 Turquoise puncta per cell (Fig 10G). In addition, by using antibody to human LAMP1, we also

868 detected colocalization with Gal3-Turquoise puncta in acceptor cells (S7F Fig, arrow). These

869 results suggest that α-syn fibrils derived from CAD cells are able to induce LMP in the acceptor

870   HeLa cells following their transfer. Interestingly, in very few cases we could also detect
871   colocalization between Gal3-Turquoise puncta and donors' lysosomes labelled with anti-mouse
872   LAMP1 antibody in acceptor HeLa cells (S7G Fig, white arrow), suggesting that damaged
873   donors' lysosomes can undergo LMP upon arrival to the acceptor cells.
874   Finally, to evaluate lysosome transfer from acceptor to donor cells, the CAD-HeLa co-culture
875   was immunolabelled with anti-human LAMP1 antibody and donor CAD cells were evaluated
876   for the presence of acceptors' lysosomes (Fig 10H). The percentage of donor cells that received
877   acceptors' lysosomes in control co-culture was $14 \pm 4\%$ whereas in α-syn co-culture this
878   percentage was significantly higher ($34 \pm 5\%$; Fig 10I, left graph). The average number of
879   acceptors' lysosomes per donor cell in both co-culture conditions was similar (2 ± 0.3 and $2 \pm$
880   0.1 in control and α-syn co-cultures, respectively; Fig 10I, right graph). To understand whether
881   lysosomes transferred from acceptor to donor cells were damaged, we quantified the presence
882   of Gal3-Turquoise puncta in donor CAD cells in both in the α-syn and control co-cultures (S7H
883   Fig). Only $4 \pm 1\%$ of donor cells resulted positive for Gal3-Turquoise puncta in α-syn co-
884   culture, while we could barely ($0.3 \pm 0.3\%$) observe donor cells positive for Gal3-Turquoise
885   puncta in control co-culture (S7I Fig). Taken together, these results demonstrate that donor cells
886   challenged with α-syn fibrils receive healthy lysosomes from acceptor cells, while the transfer
887   of damaged lysosomes from the acceptor cells is a rare event. This suggests the occurrence of
888   a rescue mechanism towards donor cells overloaded with α-syn, similar to what was previously
889   reported in the case of a lysosomal disease [106].
890
891   **Fig 10. Intercellular lysosome transfer is reciprocal. (A)** Representative confocal image of
892   CAD-HeLa co-culture where donor mouse CAD cells were treated with Alexa 568-tagged α-
893   syn fibrils (18 h) and co-cultured with acceptor human HeLa stable Gal3-Turquoise cells for 24
894   h, labelled for anti-LAMP1 mouse antibody and DAPI. In acceptor HeLa Gal3-Turquoise cells,
895   arrows indicate donors' lysosomes containing (yellow) or not (white) α-syn fibrils. Inset
896   represents a selected region (white square) of one acceptor cell containing donors' lysosomes.
897   Scale bar: 10 μm (for the inset: 2 μm). **(B)** % of acceptor HeLa cells that received lysosomes
898   from donor CAD cells in control ($32 \pm 5\%$) and in α-syn ($52 \pm 4\%$) co-cultures (left graph).
899   **P=0.006 by two-tailed *t*-test. Average number of lysosomes transferred from donor to
900   acceptor cells in control (3 ± 0.3) and in α-syn (3 ± 0.3) co-cultures (right graph). Mean $\pm$
901   S.E.M. ns= not significant by Mann-Whitney *U*-test. **(C)** Representative confocal image of
902   CAD-HeLa co-culture where Gal3-GFP transfected donor CAD cells were treated with Alexa

903   568-tagged α-syn fibrils for 18 h and co-cultured with HeLa Gal3-Turquoise for 24 h, labelled

904   with DAPI. In acceptor HeLa Gal3-Turquoise cells, red arrows indicate α-syn fibrils, green

905   arrows indicate Gal3-GFP puncta (lysosomes under LMP received from donor cells). Scale bar:

906   10 μm. **(D)** % of acceptor HeLa cells that received Gal3-GFP puncta from donor cells in control

907   ($4 \pm 1\%$) and in α-syn ($13 \pm 2\%$) co-cultures (left graph); average number of Gal3-GFP puncta

908   per acceptor HeLa cell in control (2 ± 0.3) and in α-syn (2 ± 0.2) co-cultures (right graph). Mean

909   $\pm$ S.E.M. ns= not significant, *P=0.04 by Mann-Whitney *U*-test. **(E)** Acceptor HeLa Gal3-

910   Turquoise cell that received a donor's lysosome containing α-syn fibrils and under LMP.

911   Colocalization between LAMP1 (labelled with anti-LAMP1 mouse antibody), α-syn fibrils, and

912   Gal3-GFP is presented in the inset of the selected area (white square). Scale bar: 10 μm (for the

913   inset: 1 μm). **(F)** Representative confocal image of LMP in HeLa Gal3-Turquoise (pseudo

914   colored in yellow) acceptor cell in CAD-HeLa co-culture. In acceptor HeLa Gal3-Turquoise

915   cells, red arrows indicate the α-syn fibrils, turquoise arrows indicate the Gal3Turquoise puncta.

916   Scale bar: 10 μm. **(G)** % of acceptor HeLa cells under LMP in control ($3 \pm 1\%$) and in α-syn

917   ($18 \pm 3\%$) co-cultures is presented (left graph); average number of Gal3-Turquoise puncta per

918   acceptor cell under LMP in control (9 ± 1) and in α-syn (10 ± 1) co-cultures is presented (right

919   graph). Mean $\pm$ S.E.M. ns= not significant, *P=0.03 by Mann-Whitney *U*-test. **(H)**

920   Representative confocal image of CAD-HeLa co-culture where donor CAD cells were treated

921   with Alexa 568-tagged α-syn fibrils for 18 h and co-cultured with HeLa Gal3-Turquoise for 24

922   h, labelled for anti-LAMP1 human antibody and DAPI. In donor CAD cells, white arrows

923   indicate acceptors' lysosomes. Inset of a selected region of a donor CAD cell (white square)

924   showing acceptors' lysosomes in higher magnification. Scale bar: 10 μm (for the inset: 5 μm).

925   **(I)** % of acceptors' lysosomes transferred from acceptor to donor cells in control ($14 \pm 4\%$) and

926   in α-syn ($34 \pm 5\%$) co-cultures (left graph). **P=0.002 by two-tailed *t*-test. Average number of

927   acceptors' lysosomes per donor cell in control (2 ± 0.3) and in α-syn (2 ± 0.1) co-cultures (right

928   graph). Mean $\pm$ S.E.M. ns= not significant by Mann-Whitney *U*-test. In CAD-HeLa co-culture

929   model, data was collected from seven independent experiments in which four and three of them

930   were labelled with anti-LAMP1 mouse and human antibodies, respectively. According to the

931   experiment, in average 100 acceptor or donor cells were analysed for each condition per

932   experiment. The data underlying this Figure may be found in S1 Data.

Discussion

α-Syn plays a crucial role in the generation and progression of synucleinopathies including PD. Indeed, the pathology correlates with the presence of α-syn inclusions spreading through interconnected brain regions in a "prion-like" manner [5,16,107]. By using a co-culture system, we previously demonstrated that α-syn fibrils can be transferred from donor to acceptor cells inside of lysosomes through TNTs and induce the seeding in the recipient cells [19]. However, fundamental questions regarding how lysosomes participate in this process remained open. In the current study, we focused on the role of lysosomes and the crosstalk between α-syn fibrils and lysosomes in the context of intercellular spread of α-syn fibrils and the subsequent seeding mechanism.

By using super-resolution microscopy, we looked precisely at the subcellular localization of the α-syn fibrils and we found them mainly in the lysosomal lumen or at the lysosomal membrane (Fig 1B). In both cases, lysosomes associated with α-syn fibrils had a larger diameter compared to control lysosomes. Lysosomal enlargement in the presence of α-syn fibrils has been reported before [70,108]; however, here we showed that this morphological change is specific to the lysosomes containing α-syn fibrils. Indeed, in α-syn fibril-treated cells, the diameter of the lysosomes devoid of fibrils was comparable to that of the control lysosomes (Fig 1B, graph). This finding was supported by correlative EM analysis that indicated an α-syn-dependent enlargement of lysosomes both in the perimeter and area (Fig 1D, graphs). In addition, we found that lysosomes containing α-syn fibrils exhibit structural alterations (e.g., enlargement in size, aberrant luminal content), directly supporting that α-syn fibrils cause changes in lysosome morphology (Fig 1C). The exact mechanism of lysosomal enlargement needs to be further investigated; in agreement with studies demonstrating lysosome dilation accompanied by lysosomal dysfunction [70,108,109], one possibility is that dysfunction results from accumulation of undegraded α-syn fibrils. Consistently, while the total number of lysosomes did not vary, we detected lysosomal dysfunction in α-syn fibril-treated cells compared to control cells. Specifically, in the presence of α-syn fibrils, we observed disturbance of lysosomal pH and decrease of lysosomal enzyme activities (Fig 2B and D). These changes could be a consequence of the LMP induced by α-syn fibrils that we and others detected in α-syn fibril-treated cells [73,74]. Indeed, LMP causes the release of cathepsins and other hydrolases from the lysosomal lumen to the cytosol [72,110].

LMP can be induced by different protein aggregates (e.g., α-syn, tau, huntingtin), and it has been proposed to be a common mechanism used by exogenous fibrils to spread through the

966 cells [74]. Specifically, LMP has been described in several cell types challenged with α-syn

967 fibrils, such as the neuronal cell lines SH-SY5Y and N27, as well as human dopaminergic

968 neurons derived from induced pluripotent stem cells [73,74]. Here, we showed that α-syn fibrils

969 induce LMP also in neuronal CAD cells (Fig 3A and B). By using SIM, we observed the

970 recruitment of Gal3 to the damaged lysosomes displaying α-syn fibrils in their lumen or limiting

971 membrane (Fig 3D and E). Importantly, we demonstrated that α-syn fibrils trigger LMP also in

972 the recipient cells following their intercellular transfer (Fig 8A and B). LMP in acceptor cells

973 has been only reported in a co-culture system where donor N27 cells expressing α-syn were

974 pretreated with the mitochondrial toxin MPP$^+$ (1-methyl-4-phenylpyridine) to induce PD-like

975 pathology and co-cultured with acceptor cells stably expressing Gal3 [73]. Our results indicate

976 that, without inducing a PD-like pathology in donor cells, the transfer of α-syn fibrils 'per se'

977 is sufficient to induce LMP in acceptor cells both in homotypic (Fig 8A and B) and heterotypic

978 co-culture systems (Fig 10F and G). In addition, by performing super-resolution microscopy,

979 we also detected the recruitment of Gal3 to damaged lysosomes bearing α-syn fibrils either in

980 their lumen or at their membrane in the acceptor cells, similar to what we observed in donor

981 cells (Fig 8C and D). Interestingly, our results show that lysosomes containing α-syn fibrils are

982 not targeted for lysophagy [84] (S4 Fig), possibly suggesting a rescue mechanism such as the

983 one orchestrated by the ESCRT machinery [111,112]. Considering the evidence supporting

984 autophagy impairment in neurodegenerative diseases and more specifically following α-syn

985 accumulation [55,113], and the recent findings that impaired autophagic flux contributes to the

986 occurrence of LMP [114], it will be interesting to assess the contribution of autophagy to LMP,

987 lysosomal dysfunction, and propagation of α-syn.

988 The underlying mechanism of LMP induction by α-syn fibrils is unclear. Several studies have

989 reported the ability of α-syn protofibrils to permeabilize membranes [115–117]; thus, as

990 suggested by Freeman et al., the luminal α-syn fibrils could induce membrane curvature causing

991 the rupture of lysosomes [73]. Our correlative EM data show a wavy limiting membrane in

992 lysosomes containing α-syn fibrils compared to lysosomes devoid of α-syn fibrils, supporting

993 this hypothesis. Moreover, oxidative stress could also compromise lysosomal integrity [118].

994 Consistently, we have previously reported that α-syn fibrils increase ROS levels in CAD cells

995 [19], and enhanced ROS levels have been found in PD mouse models and PD patients [68,119].

996 Since α-syn fibrils located to the lysosomes need to directly interact with the soluble cytosolic

997 α-syn in order to induce the seeding, LMP has been proposed as a relevant mechanism to allow

998 the escape of α-syn fibrils from the lysosomes [19,85]. Co-localization between Gal3 and

999 aggregated α-syn has been reported in the basal forebrain of Diffused Lewy Body Disease

1000   patients but not in normal, age-matched controls, supporting the role of lysosome rupture in α-

1001   syn pathology and progression [85]. In the current study, we detected efficient seeding of

1002   soluble α-syn both in donor cells treated with α-syn fibrils and in acceptor cells that received

1003   the fibrils inside lysosomes from donor cells (Figs 4A and B and 9A and B). By using super-

1004   resolution microscopy, we observed that 69 ± 6% and 60 ± 7% of the newly formed α-syn

1005   aggregates colocalize with LAMP1+ lysosomes in the donor and acceptor cells, respectively.

1006   These data together with live imaging show that the seeding event begins shortly after the

1007   internalization of α-syn fibrils and occurs preferentially at the lysosomes (Figs 4C-I, 9C-G,

1008   S5A, and S1 Movie). It has been shown that specific environments inside membranous and

1009   vesicular structures favor the aggregation of the α-syn protein [120,121]; thus, our results

1010   indicate that lysosomes can create a confined environment facilitating the conversion of soluble

1011   α-syn.

1012   Do lysosomes contribute to the spread of the disease? Lysosomes are highly dynamic organelles

1013   that move bidirectionally along microtubules between the center and the periphery of the cell

1014   [122]. In non-polarized cells, lysosomes are mostly clustered at the perinuclear region, but a

1015   more dynamic pool is located at the cell periphery [90,92,100]. Here, we demonstrated that α-

1016   syn fibrils act on lysosome positioning by inducing their peripheral redistribution (Fig 5A and

1017   B). We also found that peripheral α-syn-containing lysosomes exhibit lysosomal dysfunction

1018   (i.e., disturbed pH and decreased lysosomal enzyme activities; Fig 5C). In addition, we showed

1019   that donor cells challenged with α-syn fibrils, thus having more peripheral lysosomes, transfer

1020   lysosomes more efficiently to the recipient cells compared to untreated donor cells (Fig 5F and

1021   G). Moreover, the peripheral distribution of lysosomes positively affects α-syn fibrils' transfer

1022   both in CAD and HeLa cells (Figs 7A and B and S6A and B).

1023   The induction of LMP has been recently shown to promote nuclear translocation of TFEB

1024   [123], which influences lysosomal motility and exocytosis [95]. Furthermore, TFEB

1025   overexpression promotes α-syn clearance [124]. Thus, we investigated whether the loading of

1026   lysosomes with α-syn fibrils triggers TFEB nuclear translocation and whether this translocation

1027   participates in α-syn-induced distribution of lysosomes to the cell periphery. We found that α-

1028   syn fibrils induced TFEB translocation to the nucleus (Fig 6A). Moreover, expression of active

1029   TFEB correlates with more peripheral distribution of lysosomes (Fig 6B), while silencing of

1030   the endogenous TFEB prevents the peripheral redistribution of lysosomes upon α-syn loading

1031   (Fig 6D) suggesting the involvement of TFEB in this event. However, how this occurs is not

1032   yet clear. Previous reports suggested that TFEB-regulated transcription induced lysosomal

1033   docking to the plasma membrane, thus promoting the peripheral distribution of lysosomes in

1034   HeLa cells [95]. However, TFEB and the related transcription factor TFE3 have been reported

1035   to contribute to perinuclear positioning of lysosomes through transcriptional activation of the

1036   lysosomal protein TMEM55B under starvation conditions in HeLa cells [96]. It has also been

1037   reported that overexpression of TFEB promotes perinuclear positioning of the lysosomes in

1038   mouse fibroblast cells defective for the lysosomal transporter cystinosin [97]. Given these

1039   controversies in the literature, the exact role of TFEB in the regulation of lysosome positioning

1040   remains to be addressed in the future.

1041   To our knowledge, this is the first report showing that α-syn fibrils influence lysosome

1042   positioning, favoring α-syn fibrils transfer in a cell-to-cell contact-dependent manner via

1043   lysosomes. These findings suggest that lysosomes play an important role in the intercellular

1044   trafficking of α-syn fibrils. It is worth noting that α-syn fibrils do not affect lysosomal

1045   exocytosis (Fig 5D and E). In addition, in the secretion tests used in each co-culture experiment,

1046   we never detected any significant difference in the transfer of α-syn fibrils and/or lysosomes

1047   through the cell supernatant. Although this was not the aim of our study, these results further

1048   confirm that transfer of α-syn fibrils primarily relies on TNT- and/or cell-to-cell-contact-

1049   dependent mechanisms in accordance with our previous findings [19,20,22]. The mechanism

1050   by which α-syn fibrils enhance TNT formation is still largely unknown. However, we and others

1051   demonstrated that cells form more TNTs under stress, such as when they are treated with H_2O_2

1052   [125,126]. In line with these findings, we previously reported that CAD cells bearing α-syn

1053   fibrils exhibit sustained increase in ROS levels [19]. Therefore, α-syn fibrils can indeed trigger

1054   stress in the cells in order to induce TNT formation. In addition, it has been reported that α-syn

1055   could induce membrane remodeling, such as membrane expansion and formation of cylindrical

1056   tubes, and that the extent of membrane expansion correlated linearly with the density of α-syn

1057   [127]. Thus, further studies are essential to address how α-syn fibrils participate in the formation

1058   of TNTs.

1059   Lysosome transfer through TNTs has been shown to be bidirectional [106]. However, there is

1060   no study on the directionality/origin and the nature (whether they are healthy or damaged) of

1061   the lysosomes transferred between cells in the presence of the α-syn fibrils. Thus, in the current

1062   study, we studied the transfer of both healthy and damaged (i.e., under LMP) lysosomes

1063   between CAD (donor) and HeLa (acceptor) cells and found that the transfer of both types of

1064   lysosomes is bidirectional (Fig 10A, B, H, and I). We showed that α-syn fibril-treated donor

1065   cells transfer more lysosomes (both healthy and damaged) to acceptor cells compared to

1066   untreated control donor cells. Interestingly, we observed the recruitment of acceptor's Gal3 to

1067   the donor's lysosomes transferred in an acceptor cell (S7G Fig), suggesting that the rupture of

1068   donors' lysosomes containing α-syn fibrils could occur after their transfer in the recipient cells.

1069   Focusing on the transfer of lysosomes from acceptors to donors, we found that α-syn fibril-

1070   treated donor cells received more healthy acceptors' lysosomes compared to control untreated

1071   donor cells (Fig 10H and I). We also detected damaged acceptors' lysosomes in α-syn fibril-

1072   treated donor cells although this was a very rare event (S7H and I Fig). Based on these data we

1073   suggest that in the context of α-syn spreading, donor cells loaded with α-syn fibrils, try to get

1074   rid of α-syn fibrils through the transfer of lysosomes, thus promoting the spreading of the fibrils.

1075   On the other hand, donor cells receive healthy lysosomes from acceptor cells, suggesting a

1076   potential rescue mechanism. A similar rescue mechanism has been previously reported in

1077   cystinosin-deficient fibroblasts, which receive cystinosin-bearing lysosomes from healthy

1078   macrophages through TNTs [106].

1079   In light of these findings, we propose a working model on how lysosomes contribute to the

1080   transfer and propagation of α-syn fibrils (Fig 11). After internalization, α-syn fibrils localize to

1081   lysosomes (Fig 11, number 1), where they induce LMP and recruit Gal3 (Fig 11, number 2). α-

1082   Syn fibrils escape from damaged lysosomes and seed soluble α-syn in donor cells, an event that

1083   occurs mostly in association with the lysosomes (Fig 11, number 3). Following α-syn fibrils

1084   internalization, TFEB translocates to the nucleus (Fig 11, number 4) and participates in the

1085   redistribution of lysosomes containing α-syn fibrils, likely under LMP or functionally impaired,

1086   towards the cell periphery (Fig 11, number 5). Here, lysosomes are not exocytosed (Fig 11,

1087   number 6), but are more prone to be transferred to other cells through TNTs. Indeed, cells

1088   loaded with α-syn fibrils transfer more lysosomes (both under LMP or not) to acceptor cells

1089   compared to healthy cells through TNTs (Fig 11, number 7). Once lysosomes reach the acceptor

1090   cells, α-syn fibrils escape from lysosomes already under LMP or from lysosomes that undergo

1091   LMP when they arrive in the acceptor cells. This again allows α-syn fibrils to seed soluble α-

1092   syn in the acceptor cells, mostly in association with the lysosomes (Fig 11, number 8) and

1093   further triggering LMP (Fig 11, number 9). In an attempt to rescue α-syn-overloaded donor

1094   cells, acceptor cells transfer healthy lysosomes to donor cells (Fig 11, number 10).

1095   In conclusion, our data explain how lysosomes are involved in the transfer and propagation of

1096   α-syn aggregates, and the crucial role that they play in this process. In agreement with these

1097   findings, we have previously reported that α-syn fibrils can be efficiently transferred between

1098   neurons, primary astrocytes, neurons and astrocytes, astrocytes and organotypic hippocampal

1099   slices and between iPSCs and they were associated to lysosomes in these co-culture systems

1100   [19,21,56]; however we have used here undifferentiated neuronal CAD cells line, for their easy

1101   manipulation. Although our results contribute to a better understanding of the mechanism of

1102 development and progression of α-syn pathology, opening new avenues for possible therapeutic

1103 approaches against synucleinopathies, it will be necessary in the future to investigate the

1104 transfer of α-syn containing lysosomes in differentiated primary neurons and/or in ex-vivo/in-

1105 vivo systems to further validate our data and overcome the limitation of this study.

1106

1107 **Fig 11. Working Model. 1)** α-Syn fibrils localize to lysosomes following their uptake. **2)** α-

1108 Syn fibrils induce LMP (i.e., recruit Gal3 to the ruptured lysosomes) in donor cells following

1109 their uptake, allowing their escape from lysosomes. **3)** α-Syn fibrils interact with soluble α-syn

1110 and induce seeding in donor cells. **4)** Following α-syn fibrils internalization, TFEB translocates

1111 to the nucleus. **5)** Lysosomes (possibly under LMP, as their pH is higher and lysosomal

1112 activities is lower) having the α-syn fibrils move towards the cell periphery. **6)** Peripheral

1113 lysosomes are not preferentially exocytosed**. 7)** Lysosomes having α-syn (both under LMP or

1114 not) are transferred to acceptor cells along with healthy lysosomes. **8)** α-Syn fibrils induce LMP

1115 following their transfer to acceptor cells. **9)** α-Syn fibrils induce further seeding following their

1116 transfer to acceptor cells. **10)** Acceptor cells transfer healthy lysosomes to donors.

Materials and Methods

Cell culture, transfection, siRNA treatment

The mouse catecholaminergic neuronal cells (CAD cells) generated from a brain tumour of transgenic mice by targeted oncogenesis (kind gift of Hubert Laude; Institut National de la Recherche Agronomique, Jouy-en-Josas, France), were cultured in Opti-MEM^R GlutaMAX^{TM} (Gibco) including 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep). CAD cells express neuronal properties, but they lack neuronal morphology, thus stay undifferentiated in the presence of serum in their medium. This situation can be reversed by serum starvation and differentiation can be induced [128]. In this study, we used CAD cells in their undifferentiated state as this condition is optimal to study TNTs [19,125,129–131]. HeLa cells WT, HeLa cells myrlysin-KO and KIF1B-KIF5B double KO [100,132] and HeLa cells stably expressing Gal3-Turquoise were cultured in DMEM-GlutaMAX^{TM} including 4.5 g/L D glutamine and pyruvate (Gibco) supplemented with 10% of FBS. CAD and HeLa cells were transiently transfected with Gal3-GFP, Arl8b-GFP, fluorescently tagged human histone H2B (H2B-GFP or H2B-mCherry) plasmids (final concentration: 1 µg for 150,000 cells/35mm Ibidi µ-dishes, 3 µg for 800.000 cells/T25 flasks) by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

To downregulate Arl8b and TFEB, 30 pmol of unique 27mer siRNA duplex against mouse Arl8b and TFEB (CliniSciences and OriGene respectively) was introduced to CAD cells in parallel to siRNA scramble (sicontrol) by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions, and incubated 60h prior to be used in co-culture experiments and in Western blot analysis.

To induce lysophagy, CAD cells were treated with 1 mM L-Leucyl-L-Leucine methyl ester (LLOMe) for 3h.

Generation of stable HeLa mTurquoise-Galectin3 cell line

In order to generate stable HeLa cell line expressing a mTurquoise-Galectin3 fluorescent reporter, we used a well-established Sleeping Beauty (SB) transposable system that originates from an inactive transposon in salmonid fish and belongs to the Tc1/mariner-type transposon family, as previously described [133,134]. Briefly, the insert sfiI-mTurquoise-Galectin3-sfiI was amplified from pE mTurquoise-Galectin3 plasmid by polymerase chain reaction (PCR) where SfiI-mTurquoise-forward (AGG CCT CTG AGG CCG CCA CCA TGG TGA GCA

AGG G) and Gal3-SfiI-reverse (AGG CCT GAC AGG CCT TAT CAT GGT ATA TGA AGC AC) primers (IDT-DNA Company) were used with Flash Phusion High Fidelity PCR Master Mix (ThermoFisher Scientific). Next, the insert was cut by sfiI enzyme and inserted into the constitutive vector pSBbi-PUR, a gift from Eric Kowarz, (Addgene Plasmid #60523) that was cut by sfiI enzyme and dephosphorylated by Rapid DNA Dephos & Ligation Kit (Roche). Transformation was performed with on stBL3 competent bacteria following the ligation of sfiI-pSBbi-PUR-sfiI vector and insert sfiI-mTurquoise-Galectin3-sfiI, and colonies were selected on LB broth agar plates containing amphicilin. Clones were then sequenced (Eurofins Genomic) in order to verify the existence of the insert and maxiprep for the pSBbi-PUR-mTurquoise-Galectin3 plasmid was performed by using the Invitrogen™ PureLink™ HiPure Plasmid Maxiprep Kit. Next, HeLa cells seeded on 6 well plates (300.000 cells per well) were co-transfected with 9 µg of pSBbi-PUR-mTurquoise-Galectin3 and 100 ng SB transposase expression vector pCMV (CAT)T7-SB100, a gift from Eric Kowarz (Addgene plasmid # 34879) by using XtremGene® transfection reagent (Roche). 24 h following the transfection, cells were subjected to puromycin (1 µg/mL). Selection was carried out for 2 to 10 days. Cells were then cultivated for four weeks without selection medium and monoclonal selection was performed on 96 well plates (1 cell/well). Clones exhibited expected mTurquoise fluorescent were further tested for classical behavior for HeLa cells (low Galectin-3 fluorescent in the whole cell and a brilliant signal upon vacuole rupture) and used in the experiments described in the current study.

Preparation and internalization of α-syn fibrils

Human wild-type α-syn in pRK172, a construct containing α-syn that lacks cysteine because of mutagenesis of codon 136 (TAC to TAT) was transformed into *Escherichia coli* BL21 (DE3) as previously reported. Expression and purification were performed as described previously [86]. Briefly, the protein concentrations of monomeric α-syn were measured by RP-HPLC as described previously [86,135]. Purified recombinant α-syn monomers (~5 mg/mL) containing 30 mM Tris-HCl, pH 7.5, 10 mM DTT, and 0.1% sodium azide were incubated at 37°C with shaking using a horizontal shaker (TAITEC) at 200 rpm. Following the incubation for 7 days, the samples were ultracentrifuged at 100.000 g for 20 min at room temperature (RT), and the ppt fraction was recovered as α-syn fibrils. They were re-suspended in saline prior to be ultracentrifuged again. The pellets were re-suspended in saline and sonicated using a cup horn sonicator (Sonifier SFX, Branson) at 35% power for 180 s (total 240 s, 30 s on, 10 s off). The

1183 fibrils were labelled with Alexa Fluor 488 or 568 Protein Labelling Kit (Invitrogen) following

1184 the manufacturer's instructions. After the incubation of fibrils with Alexa Fluor dyes, samples

1185 were ultracentrifuged again and the pellets were re-suspended in 30 mM Tris-HCl, pH 7.5 prior

1186 to be ultracentifuged one more time. Finally, labelled α-syn fibrils were resuspended in saline

1187 containing 0.1% sodium azide and the protein concentration of the fibrils was determined by

1188 RP-HPLC as described before [86,135]. Seeding activity of the fibrils were checked by adding

1189 the labelled fibrils (3 µg) to 100 µL of 1 mg/mL α-syn monomer solution in 30 µM Thioflavin

1190 T and 80 mM Hepes, pH 7.5. Amyloid-like fibril formation was continuously monitored by

1191 measuring the Thioflavin T fluorescence (excitation 442 nm, emission 485 nm) with a

1192 microplate reader (Infinite 200, TECAN).

1193 0.5 µM Alexa 488 or Alexa 568-tagged α-syn fibrils were diluted in the appropriate medium

1194 and sonicated for 5 min at 80% amplitude with a pulse cycle of 5 s on and 2 s off in an ultrasonic

1195 water bath Vibra-Cell 75,041 (BioBlocks Scientific). Fibrils were then introduced to cells

1196 immediately and incubated for 18 h. Before cells were processed for further procedures (e.g.,

1197 co-culture preparation, fixation, etc.), they were washed 3 times with diluted trypsin (1/3) in

1198 phosphate buffered saline (PBS). In parallel, control cells were subjected to the same treatments

1199 as described above without the addition of α-syn fibrils.

1200

1201 **Immunocytochemistry for lysosomes**

1202 CAD and/or HeLa cells and co-cultures (CAD-CAD and CAD-HeLa) were fixed with 4%

1203 paraformaldehyde (PFA) for 20 min at room temperature (RT). After 3 washes with PBS, cells

1204 were permeabilized with 0.1% Triton X-100 in PBS for 3 min at RT. Cells were then washed

1205 with PBS and nonspecific antibody binding sites were blocked by using 10% of goat serum

1206 (GS) in PBS for 30 min. Cells were then incubated with primary rat monoclonal anti-mouse

1207 LAMP1 (1D4B) or anti-human LAMP1 (H4A3) primary antibodies with the dilution of 1/200

1208 in blocking solution for 1 h. Antibodies were purchased from Developmental Studies

1209 Hybridoma Bank (DSHB), Iowa City, IA, USA. Cells were then washed 3 times with PBS and

1210 incubated for secondary antibodies (anti-rat Alexa-647 or anti-rat Alexa-488 purchased from

1211 Invitrogen, Carlsbad, CA) diluted 1:500 in blocking solution for 2 h at RT. After 3 washes with

1212 PBS, cells were further stained with HCS CellMask™ Blue (Invitrogen) for 15 min at RT

1213 (diluted 1:5000 in PBS). Cells were then washed 3 times with PBS and further stained for DAPI

1214 (diluted 1:5000 in PBS) for 5 min. Finally, cells were washed and mounted with Aqua-

1215 Poly/Mount (Polysciences, Inc.). Image acquisitions were performed by confocal microscopy

1216 with identical settings.

1217

1218 **Immunocytochemistry for lysophagy**

1219 CAD cells were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature (RT).

1220 After 3 washes with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 3 min

1221 at RT. Cells were then washed with PBS and nonspecific antibody binding sites were blocked

1222 by using 2% of bovine serum albumin (BSA) in PBS for 30 min. Cells were then incubated

1223 with primary rabbit anti-mouse LC3 (1:500, MBL International), mouse anti-Ubiquitin (1:1000,

1224 FK2, Enzo), and rat monoclonal anti-mouse LAMP1 (1D4B) (1:100, DSHB) in blocking

1225 solution for 1 h. Cells were then washed 3 times with PBS and incubated for secondary

1226 antibodies (anti-rabbit Alexa 488, anti-mouse Alexa-647 from Invitrogen, Carlsbad, CA; and

1227 anti-rat CF™405M from Sigma) diluted 1:500 in blocking solution for 1 h at RT. Finally, cells

1228 were washed and mounted with Aqua-Poly/Mount (Polysciences, Inc.). Image acquisitions

1229 were performed by confocal microscopy with identical settings.

1230

1231 **LysoTracker Deep Red staining, DQ-BSA assay, and Cathepsin B activity assay**

1232 CAD cells were plated on 35 mm Ibidi μ-dishes (100.000 cells/dish). Alexa 568-tagged α-syn

1233 fibrils were introduced or not (control) to the cell culture medium 24 h after plating. Following

1234 18 h of α-syn fibril uptake, 20 nM LysoTracker Deep Red (Invitrogen) was prepared in culture

1235 medium and cells were incubated for 20 min at 37°C. Cells were then fixed with 4% PFA at

1236 RT and stained for HCS CellMask™ Blue and DAPI as described before. Image acquisitions

1237 were performed by confocal microscopy with identical settings.

1238 To detect Cathepsin B (CathB) activity, CAD cells were plated on 35 mm Ibidi μ-dishes

1239 (100.000 cells/dish). Alexa 488-tagged α-syn fibrils were introduced or not (control) to the cell

1240 culture medium 24 h after plating. Following 18 h of α-syn fibril uptake, cells were treated with

1241 1X Magic Red Cathepsin solution, provided by Magic Red Cathepsin Assay kit

1242 (ImmunoChemistry Technologies LLC) and prepared through the manufacturer's

1243 recommendation, for 15 min at 37°C. Cells were then immediately imaged by confocal

1244 microscopy in live condition. The intracellular activity of CathB was detected as red fluorescent

1245 puncta produced by the hydrolysis of the fluorogenic substrate introduced. All images were

1246 acquired with the identical settings. For both experiments integrated density of LysoTracker

1247 Deep Red and CathB signal were measured by using FiJi software after a region of interest

1248   (ROI) is manually created by the freehand selection tool and the measurements were expressed
1249   in arbitrary unit (AU).

1250   For DQ-BSA assay, CAD cells were plated on 35 mm Ibidi μ-dishes (130.000 cells/dish). Alexa
1251   568-tagged α-syn fibrils were introduced or not (control) to the cell culture medium 24 h after.
1252   Following 18 h of α-syn fibrils uptake, 10 μg/mL DQ Green BSA (Thermo Fisher Scientific)
1253   was prepared in culture medium and cells were incubated for 90 min at 37°C. Cells were then
1254   fixed with 4% PFA at RT. Image acquisitions were performed by confocal microscopy with
1255   identical settings. Integrated density of DQ-BSA signal were measured by using FiJi software
1256   after a region of interest (ROI) is manually created by the freehand selection tool and the
1257   measurements were expressed in arbitrary unit (AU).

1258

1259   **TNT labelling**

1260   To preserve the structure of TNTs, cells were fixed by a two-step fixation protocol as described
1261   previously [20,129]. Briefly, cells were first fixed with fixative solution-1 (2% PFA, 0.05%
1262   glutaraldehyde (GA) and 0.2 M HEPES in PBS) for 20 min at 37°C, then with fixative solution-
1263   2 (4% PFA and 0.2 M HEPES in PBS) for an additional 20 min at 37°C. After several washes,
1264   cells were labelled with 3.3 μg/μL Wheat Germ Agglutinin (WGA)-Alexa Fluor-488 nm
1265   conjugate solution (Life Technologies) for 20 min at RT for membrane detection. As there is
1266   no specific marker for TNTs, WGA labelling was used to detect TNTs as described in detail in
1267   the quantification section.

1268

1269   **Lysosomal exocytosis analysis**

1270   *Evaluation of released Cathepsin B by ELISA*

1271   CAD cells were plated in B12-wells (80.000 cells/well). Cells were then treated with Alexa
1272   568-tagged α-syn fibrils or not for 18 h. Soluble lysosomal Cathepsin B (CathB) released in
1273   culture medium was evaluated by using Human Cathepsin B ELISA Kit (Abcam) following the
1274   manufacturer's instructions. Briefly, aliquots of culture medium derived from control cells and
1275   cells challenged with α-syn fibrils were incubated in 96-well plates precoated with a CathB
1276   specific mouse monoclonal antibody for 90 min at 37°C. Biotinylated CathB-specific goat
1277   polyclonal antibody was then added on the cells for 60 min at 37°C. Cells were then washed
1278   with tris-buffered saline (TBS) prior to the addition of Avidin-Biotin-Peroxidase Complex for
1279   30 min at 37°C. Unbound conjugates were washed away with TBS buffer five times. 3,3′,5,5′-

1280 Tetramethylbenzidine was then used to visualize the horse radish peroxidase (HRP) enzymatic
1281 reaction (O.D. absorbance: 450 nm). Released CathB content is expressed as nanograms of
1282 CathB/mL of culture medium.
1283

1284 *Evaluation of LAMP1 at the plasma membrane*

1285 CAD cells were plated in 35 mm Ibidi μ-dishes (120.000 cells/dish). Cells were then treated
1286 with Alexa 568-tagged α-syn fibrils or not for 18 h. Live cells were the incubated with the
1287 primary rat monoclonal anti-mouse Lamp1 (1D4B) antibody recognizing an epitope at the
1288 luminal region of the protein, diluted in complete medium, for 30 min at 4°C. After washing
1289 with PBS, cells were fixed in 2% PFA in PBS for 10 min at RT. Next, cells were incubated
1290 with the Alexa Fluor 488–conjugated goat anti-rat secondary antibody diluted in PBS for 30
1291 min at RT. Cells were then imaged by confocal microscopy.
1292

1293 **Detection of LMP and seeding**

1294 CAD cells were plated on 35 mm Ibidi μ-dishes (100.000 cells/dish) and transiently transfected
1295 with 1 μg of Gal3-GFP (for LMP) or 1 μg of α-syn-GFP plasmids (for seeding). 24 h later cells
1296 were either treated with Alexa 568-tagged α-syn fibrils for 18 h or left untreated. In co-culture
1297 experiments, cells transiently transfected with Gal3-GFP or α-syn-GFP plasmids were co-
1298 cultured for 24 h with donor cells previously treated for 18 h with Alexa 568-tagged α-syn
1299 fibrils or not. Cells were then fixed with 4% PFA for 20 min at RT and stained for DAPI.
1300 Following image acquisition with confocal microscopy, cells were quantified for the presence
1301 of Gal3-GFP or α-syn-GFP puncta by using "Spot detector" tool of the ICY software. Note that
1302 a threshold of five puncta is applied to all images analysed and cells bearing puncta above this
1303 threshold were considered as having LMP.
1304

1305 **Co-culture experiments**

1306 Donor cells were plated on 35 mm well plates (400.000/well) and transfected with suitable
1307 plasmids according to the experiment (LAMP1-GFP, Arl8b-GFP, Gal3-GFP, H2B-mCherry)
1308 or pre-treated with siRNA Arl8b or siRNA-scrambled for 60 h. Myrlysin-KO and KIF1B-5B
1309 dKO HeLa cells were directly used as donors. Donor cells (in parallel to untreated control cells)
1310 were then challenged with Alexa 568-tagged α-syn fibrils for 18 h (or not according to the

1311   experiment). They were then washed once with PBS and 3 times with 1:3 diluted trypsin before

1312   co-culture preparation.

1313   Acceptor cells were plated on T25 flasks (800.000/flask) and transfected with suitable plasmids

1314   according to the experiment (Gal3-GFP, H2B-mCherry, H2B-GFP, α-syn-GFP). HeLa Gal3-

1315   Turquoise cell line was used directly as acceptor cell population. Both donor and acceptor cells

1316   were then detached (mechanically and by trypsinization for CAD and HeLa cells respectively)

1317   and counted. Co-culture was prepared in a 1:1 ratio (100.000 donor and 100.000 acceptors on

1318   35 mm Ibidi μ-dishes) for 24 h. Cells were then fixed with 4% PFA for 20 min at RT and stained

1319   for HCS CellMask^{TM} Blue and DAPI as described before and mounted. Schematic presentation

1320   of different co-culture preparations was presented in S3 Fig.

1321   Of note, co-cultures were named with the predominant treatment of the donor cells: i) co-

1322   cultures having donor cells treated with α-syn fibrils or not were referred to as "α-syn co-

1323   culture" and "control co-culture" respectively; ii) in the cases where all donor populations were

1324   treated with α-syn fibrils, in order to emphasize the different treatments between donor cells,

1325   co-cultures were named by these treatments (e.g., Arl8b-GFP over-expressing or siArl8b

1326   pretreated donors were referred to as "Arl8b-GFP co-culture" and "siArl8b co-culture"

1327   respectively).

1328

1329   *Secretion test*

1330   In all co-culture conditions, donor cells and acceptor cells were also plated separately during

1331   the co-culture preparation (100.000 cells/35 mm Ibidi μ-dishes) for the total duration of co-

1332   culture incubation (24 h). Then the conditioned medium collected from donor cells were

1333   transferred onto acceptor cells. Acceptor cells were then quantified for the presence of specific

1334   cargos (e.g., lysosomes, α-syn fibrils) to assess the cargo transfer through secretion in each co-

1335   culture condition. Secretion tests for particular experiments were referred to as predominant

1336   treatment of the donor cells as described above (e.g., "control secretion", "α-syn secretion",

1337   "Arl8b-GFP secretion").

1338

1339   **Confocal microscopy**

1340   After fixation and immunostaining, images were acquired with an inverted laser scanning

1341   confocal microscope LSM700 (Zeiss), with a 63X objective (zoom 1.0). Images were acquired

1342   using the ZEN acquisition software (Zeiss). In all experiments, we acquired Z-stacks covering

the whole volume of cells. For the experiments where integrated density was measured in control and α-syn treated cells (LysoTracker Deep Red, Magic Red CathB, DQ-BSA assays, and evaluation of LAMP1 at the plasma membrane in non-permeabilized condition), all settings (including the laser power and exposure line time) were kept identical. In some experiments line averaging were also used in order to improve the signal to noise ratio.

Super-Resolution (SR) Microscopy

Sample preparation

24-well plates containing sterile cover slips (Deckglaser 12 mm) were coated with fibronectin (25 µg/mL) diluted in PBS and kept at 37°C for 1 h. Cover slips were then washed 3 times with PBS. CAD cells were then plated on cover slips (20.000 cells/coverslip). Gal3-GFP or α-syn-GFP transfected and α-syn treated cells or mixture of donor and acceptor cells (co-cultures prepared for LMP and seeding experiments) were then plated on coverslips. Cells were then fixed with 4% PFA for 20 min at RT and immunolabelled with primary rat monoclonal anti-mouse LAMP1 (1D4B) and secondary anti-rat Alexa 647 antibodies as described before. Cells were then stained with DAPI and HCS CellMask™ Blue and mounted with Fluoromount-G (Southern Biotech) mounting medium.

Structured Illumination Microscopy (SIM)

SIM was performed on a Zeiss LSM780 Elyra PS1 microscope (Carl Zeiss, Germany) using 100X/1.4 oil Plan Apo objective with a 1.518 refractive index oil (GE Healthcare Life Science) and an EMCCD Andor Ixon 887 1 K camera for the detection. SIM images were processed with ZEN software and then aligned with ZEN using 100-nm TetraSpeck microspheres (ThermoFisher Scientific) embedded in the same conditions as the sample.

Spinning Disk- Live Super-Resolution (SR) Confocal Microscopy

Images were acquired with an inverted Eclipse Ti Nikon microscope equipped with a CSU- X1 spinning disk confocal scanning unit (Yokogawa), with an EMCCD Camera (Evolve 512 Delta, Photometrics), and with Live SR super-resolution module (Gataca systems), using a x100 or x60 1.4 NA PL-APO VC oil objective lenses controlled by MetaMorph software. SR images were denoised by using Safir software [136].

Live imaging microscopy

Time-lapse microscopy imaging was performed on an inverted Spinning Disk microscope (Elipse Ti microscope system, Nikon Instruments, Melville, NY, U.S.A.) using 60X 1.4 NA CSU oil immersion objective lens and Laser illumination 488 only or together with 561. For live cell imaging, the 37°C temperature was controlled with an Air Stream Stage Incubator, which also controlled humidity. Cells were plated in Ibidi μ-Dish 35 mm and incubated with 5% CO_2 during image acquisition. Image processing and movies were realized using MetaMorph and ImageJ/Fiji software.

Electron Microscopy

Correlative Resin Electron Microscopy

For correlation of FM and resin-embedded EM of CAD cells, FM imaging was performed prior to sample preparation in EM. Cells were transiently transfected with LAMP1-GFP using Lipofectamine 2000 transfection reagent for 24 h and seeded on carbon-coated, gridded coverslips prepared as described before [76]. 24 h later, they were treated with α-syn- Alexa568 fibrils for 18 h. Cells were then washed in 0.1 M phosphate buffer (PB) and fixed in 4% formaldehyde in 0.1 M PB buffer after which fluorescent Z-stacks of cells of interest were obtained for the GFP and Alexa 568 signal. The position of cells relative to the pattern etched in the coverslip was registered using polarized light. After FM imaging, the same specimens were prepared for EM by fixing with 2.5% glutaraldehyde + 2% formaldehyde in 0.1 M PB buffer for 2 h, and post fixation with osmium tetroxide and uranyl acetate, Fixed cells were dehydrated using a graded ethanol series, and embedded in Epon resin which was polymerized for 48 h at 65°C. After polymerization, the glass coverslip was removed from the Epon block by dissolving it in hydrogen fluoride. The exposed Epon surface was thoroughly cleaned with distilled water and left to harden overnight at 63°C. Areas of the resin block containing the cells previously imaged by FM were cut out using a clean razor blade, and glued to empty Epon sample stubs, with the basal side of the cells facing outwards [75,76]. From these blocks, 70 nm sections were cut and collected on formvar and carbon coated copper 50 mesh support grids. Thin EPON sections were imaged in a Tecnai 12 TEM (Thermo Fischer Scientific, Eindhoven, The Netherlands) equipped with a Veleta 2k×2k CCD camera (EMSIS, Munster, Germany), operating at 80 kV. For correlation of FM and EM data, the TEM image of a ROI was overlayed with FM data using Photoshop. Multiple corresponding spots (e.g. nuclei) on images were

1407   manually selected, after which the correct scaling and transformation steps were performed and

1408   high-precision overlays of FM and EM data were generated. We used only linear transformation

1409   options to achieve the overlays shown in the Figures.

1410

1411   *On-section Correlative Microscopy of thawed cryosections*

1412   Similarly, CAD cells were grown on carbon-coated, gridded coverslips, incubated with α-syn

1413   Alexa 568-tagged fibrils for 18 h and fixed in 0.1 M PB (pH 7.4) containing 4% PFA that was

1414   added to an equal volume of medium. Fixed cells were flat embedded in 12% gelatin by keeping

1415   their orientation, cryoprotected in 2.3 M sucrose, and plunge frozen according to previous

1416   protocols [137,138]. 70 nm cryosections were picked up on formvar coated copper grids, after

1417   which they were immunolabelled for LAMP1 with a secondary Alexa 488-tagged rabbit

1418   antibody and 10 nm gold-conjugated Protein A (Cell Microscopy Core, UMC Utrecht, The

1419   Netherlands). Finally, sections were labelled using Hoechst 33342 to outline nuclei for

1420   correlation. The grids were washed with dH2O and sandwiched between a microscope slide

1421   and a no. 1 coverslip in 2% methylcellulose in dH$_2$O. Sections were imaged in a Deltavision

1422   RT wide field fluorescence microscope (GE Healthcare, U.S.A.) equipped with a Cascade II

1423   EM-CCD camera (Photometrics, U.S.A.). Grids were first imaged at 40× magnification to form

1424   a map of the section, after which regions of interest (ROIs) were selected using 100×

1425   magnification. After imaging the grids were removed from the microscope slide, thoroughly

1426   rinsed with H$_2$O and contrasted for EM and embedded in methylcellulose containing uranyl

1427   acetate. After drying, ROIs were imaged using a Tecnai 12 TEM (Thermo Fischer Scientific)

1428   operating at 80 kV, equipped with a Veleta 2k×2k CCD camera (EMSIS, Munster, Germany)

1429   camera, and running serialEM software. Registration of thin section FM and EM data was

1430   performed using Adobe Photoshop, similar to above.

1431

1432   **Western immunoblots**

1433   For the Western blot analysis, cells were rinsed with PBS and then scraped in RIPA buffer (50

1434   mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1%

1435   SDS). The resulting soluble fractions were centrifuged at 18,000 g for 5 min at 4°C, and then,

1436   the supernatants were taken as the whole cell lysate, mixed with Laemmli sample buffer (1 M

1437   Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, 5% ß-mercaptoethanol, and 0.02% bromophenol

1438   blue), and boiled for 5 min. For Western blots, equal amounts of proteins (30 µg/lane) were

1439   separated on SDS-PAGE gels and transferred to nitrocellulose membranes (GE Healthcare Life

sciences), following standard procedures. Membranes were then blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% non-fat dried milk for 1 h and probed overnight at 4°C with primary antibodies. The membranes were then washed and exposed for 1h at RT to the anti-rabbit/anti-mouse peroxidase-conjugated antibodies (1:1000) The specific protein bands were visualized using the ECL-immunoblotting chemiluminescence system (GE Healthcare Life sciences) and the ImageQuant LAS 500TM camera (GE Healthcare Life sciences). Primary antibodies used in this study: rat anti-Lamp1 (1:100, DSHB), anti-Arl8b (1:100), rabbit anti-TFEB (1:1000, Bethyl Laboratories), mouse anti-tubulin (1:1000), rabbit anti-GAPDH (1:5000, Boster). To determine the apparent molecular weights of the protein bands, a PageRuler plus prestained protein ladder (Thermo Fisher Scientific) was used.

Quantification analysis

Quantification for lysosomal positioning

We performed 2D analysis with the Z-projection where we used a two-step detection protocol in ICY software in order to assess the lysosomal positioning. In the first step, we used a script to detect two ROIs: i) the limiting membrane of whole cell that was detected with HCS CellMask™ Blue staining; ii) the nuclear region that was detected by DAPI staining. Important to note that in the cases where perinuclear region of the cells including microtubule organizing center (MTOC) was not detected in Z-projection, this ROI was manually corrected. In the second step of the protocol another script was used where "Spot detector" and "Colocalization" modules were combined. Thus, we were able to detect the number of lysosomes, the number of α-syn fibrils puncta, and the number of lysosomes colocalizing with the α-syn fibrils. Number of peripheral lysosomes were calculated by simply subtracting the number of centrally located lysosomes from the whole number of lysosomes and the percentages central and peripheral lysosomes were calculated accordingly. Same protocols were used to quantify the LysoTracker Deep Red and MagicRed Cathepsin B positive lysosomes where HCS CellMask™ Blue and Cell Tracker Blue (Invitrogen) staining were used in addition to DAPI staining respectively.

TNT counting

A semi-automated "TNT counting" tool in ICY software was used for TNT counting experiments as previously described [20,129]. Briefly, total number of cells were counted in each image and the connection between each cell pair was evaluated by scanning through the Z-stacks. Cell pairs that are connected by a protrusion that fits to certain criteria that are used

1473   to discriminate TNTs from other cellular protrusions (such as having thin, straight, and

1474   uninterrupted connections that are not touching to the substratum) were counted as "TNTs" and

1475   connected by a line using a free-hand line tool. Total number of cells and number of TNT-

1476   connected cells were automatically counted by the tool. Thus, data were presented as percentage

1477   of TNT connected cells.

1478

1479   *Lysosome size (on-section CLEM)*

1480   FM and EM images from the same section were overlaid and correlated to each other. LAMP1

1481   immunogold labelled organelles (lysosomes) were selected blindly and their size was measured

1482   using the "lasso" tool in Fiji. Afterwards the lysosomes were clustered based on presence of α-

1483   syn fibrils, and their size distribution was plotted.

1484

1485   *Lysosome size (SR microscopy)*

1486   Diameter of lysosomes were measured by using free-hand straight line tool of FiJi software.

1487   For each condition (control lysosomes and lysosomes containing α-syn or not), SR images were

1488   zoomed, and diameter of randomly selected lysosomes was measured.

1489

1490   *Lysosome number*

1491   The average number of lysosomes in control cells and in cells treated with α-syn fibrils were

1492   counted by using the "Spot detector" tool in ICY software.

1493

1494   *Object-based 3D colocalization analysis (Imaris software)*

1495   ROI for each was created by using "Surface" tool in the Surpass mode in Imaris. For each cell

1496   puncta of interest (Gal3-GFP, α-syn-GFP, α-syn fibrils and LAMP1+ puncta) in different colors

1497   were detected by "Spots" tool. Then colocalization was performed first for each pair of interest,

1498   and then for triple presence of the puncta by using "Colocalization" tool.

1499

1500   **Statistical analysis**

1501   Data collected at least from 3 independent experiments was presented as Mean ± SEM. Exact

1502   number of experiments and number of cells analysed per condition for each experiment is

1503   presented in the Figure legends. All data were first subjected to a D'Agostino-Pearson omnibus

1504   normality test. Values having Gaussian distribution were further analysed by two-tailed *t*-test

1505   and one-way ANOVA followed by Tukey's multiple comparison tests for paired and multiple

1506   comparisons respectively. Values having non-Gaussian distribution were analysed by Whitney-

1507   Mann *U*-test and Kruskal-Wallis nonparametric ANOVA test followed by Dunn's multiple

1508   comparison tests for paired and multiple comparisons respectively. Paired set of data were

1509   analysed by paired-*t* test.

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Supporting information

S1 Fig. EM images of control CAD cells. (A) Resin embedded EM images of control CAD cells. Three examples of control lysosomes (indicated by blue squares) selected from different regions (indicated by red squares) of three different cells are presented. Scale bar for the cells: 5 μm, for the selected regions: 1 μm, and for lysosomes: 0.2 μm. **(B)** On-section EM images of control CAD cells immunogold labelled with LAMP1[10]. Two examples of lysosomes were presented in insets selected from regions indicated by red and blue squares. Scale bar: 1 μm (for the insets: 0.2 μm).

S2 Fig. α-Syn fibrils are transferred from donor to acceptor cells inside of lysosomes through TNTs. Representative image of a TNT having a lysosome containing α-syn fibrils which is formed between α-syn loaded donor cell for 18 h (D) and H2B-GFP transfected acceptor cell (A) in 24 h of CAD-CAD co-culture. Z projection of donor and acceptor cells and lysosomes labelled with LAMP1 is presented (upper panels). Z projection of merged image with an additional staining of HCS CellMask™ Blue, a bottom section (section no:3) where TNT is not visible and an upper section (section no:8) where TNT is visible are presented (middle panels). Arrows in the orthogonal view of the upper section and in insets are indicating the lysosome containing α-syn fibrils inside of the TNT (lower panels). Scale bar: 10 μm (for insets: 5 μm).

S3 Fig. Schematic presentation of experimental designs. (A) CAD cells were transiently transfected with Gal3-GFP (1), α-syn-GFP (2), or TFEB-WT-GFP (3) and treated with Alexa 568- tagged α-syn fibrils for 18 h; cells were then analysed for the presence of Gal3-GFP puncta, α-syn-GFP puncta, or nuclear TFEB respectively. **(B)** Donor CAD cells were transiently transfected with LAMP1-GFP and either treated with Alexa 568-tagged α-syn fibrils for 18 h (co-culture prepared from these donors was referred to as "α-syn co-culture") or left untreated (co-culture prepared from these donors was referred to as "control co-culture"). Donor cells were then co-cultured with acceptor CAD cells transiently transfected with H2B-mCherry for 24 h. Efficiency of the LAMP1-GFP+ lysosome transfer was measured in each condition. **(C)** Donor CAD cells were transiently transfected with Arl8b-GFP (co-culture prepared from these donors was referred to as "Arl8b co-culture") or not (co-culture prepared from these donors was referred to as "control co-culture") prior to be loaded with Alexa 568-

tagged α-syn fibrils for 18 h. Arl8b-GFP expressing donors (having more peripheral lysosomes) and control donors were then co-cultured with acceptor CAD cells that were transiently transfected with H2B-GFP for 24 h. Efficiency of the α-syn fibrils' transfer was measured in each condition. **(D)** Donor HeLa cells were transiently transfected with Arl8b-GFP (co-culture prepared from these donors was referred to as "Arl8b co-culture") or not transfected (co-culture prepared from these donors was referred to as "control co-culture") prior to be loaded with Alexa 568-tagged α-syn fibrils for 18 h. Arl8b-GFP expressing donors (having more peripheral lysosomes) and control donors were then co-cultured with acceptor HeLa cells that were transiently transfected with H2B-GFP for 24 h. Efficiency of the α-syn fibrils' transfer was measured in each condition. **(E)** Three donor CAD cell populations were prepared: untreated control cells, cells pretreated with sicontrol (scramble) or with siArl8b. Co-cultures were referred to as "control", "sicontrol", and "siArl8b" co-cultures to distinguish the treatments applied to the donor cells. Donors having perinuclear lysosomes (siArl8b pretreated) and control donors (sicontrol pretreated or untreated) were loaded with Alexa 568-tagged α-syn fibrils for 18 h prior to be co-cultured with acceptor CAD cells transfected with H2B-GFP for 24 h. Efficiency of the α-syn fibrils' transfer was measured in each condition. **(F)** Donor control cells (WT Hela) or myrlysin-KO HeLa cells or KIF1B-5B dKO HeLa cells were treated with Alexa 568-tagged α-syn fibrils for 18 h. Control donors (WT HeLa) and donors having perinuclear lysosomes (myrlysin-KO and KIF1B-5B dKO HeLa cells) were then co-cultured with the acceptor WT HeLa cells transiently transfected with H2B-GFP for 24 h. Co-cultures were referred to as "control", "myrlysin-KO", and "KIF1B-5B dKO" co-cultures to distinguish the treatments applied to the donor cells. Efficiency of the α-syn fibrils' transfer was measured in each condition. **(G)** Donor CAD cells were either transfected with H2B-mCherry (co-culture prepared from these donors was referred to as "control co-culture") or treated with Alexa 568-tagged α-syn fibrils for 18 h (co-culture prepared from these donors was referred to as "α-syn co-culture") were co-cultured with acceptor CAD cells were either transiently transfected with Gal3-GFP (1) or α-syn-GFP (2). Donor and acceptor cells were then co-cultured for 24 h. Acceptors cells were analysed for the presence of Gal3-GFP or α-syn-GFP puncta, respectively. **(H)** Donor CAD cells transiently transfected with Gal3-GFP and treated with Alexa 568-tagged α-syn fibrils for 18 h were co-cultured with acceptor HeLa cells stably expressing Gal3-Turquoise for 24 h. Co-cultures were either immunolabelled with LAMP1 (mouse) antibody or LAMP1 (human) antibody to detect lysosomes derived from donor and acceptor cells, respectively. **(I)** Secretion test: in parallel to co-culture preparations where donor and acceptor cells were mixed in 1:1 ratio, donors and acceptor cells were also plated separately and medium

1989   of acceptor cells were replaced by the conditioned medium collected from donor cells. Acceptor

1990   cells were then analysed for the presence of different cargos (e.g., α-syn fibrils, lysosomes) in

1991   order to evaluate cargo transfer from donor to acceptor cells through secretion.

1992

1993   **S4 Fig. α-Syn fibrils do not induce lysophagy.** Representative confocal images of CAD cells

1994   control, treated with 1 mM LLOMe for 3 h, or treated with Alexa 568-tagged α-syn fibrils for

1995   18 h and immunolabelled with LC3-Alexa 488, Ubiquitin FK2-Alexa 647, and Lamp1-

1996   CF™405M (pseudo colored in grey) antibodies. Light blue arrows indicate lysosomes under

1997   lysophagy. *n*=3 (60 cells analysed per condition). Scale bar: 10 μm.

1998

1999   **S5 Fig. Timeframes of the S1 and S2 Movies of CAD cells overexpressing soluble α-syn-**

2000   **GFP treated or not with Alexa 568-tagged α-syn fibrils up to 4 h. (A)** Timeframes at

2001   0/1/2/3/4 hours of the S1 Movie of CAD cells overexpressing soluble α-syn-GFP monitored

2002   after the administration of α-syn fibrils. **(B)** Timeframes at 0/1/2/3/4 hours of the S2 Movie of

2003   CAD cells overexpressing soluble α-syn-GFP.

2004

2005   **S6 Fig. Lysosome positioning affects the efficiency of α-syn fibrils' transfer in HeLa cells.**

2006   **(A)** Representative confocal images of donor and acceptor HeLa cells after 24 h of co-culture.

2007   Donor cells were either treated with Alexa 568-tagged α-syn fibrils for 18 h (referred as "control

2008   co-culture") or they were transfected with Arl8b-GFP prior to be treated with α-syn fibrils

2009   (referred as "Arl8b-GFP co-culture"). Acceptor cells were transfected with H2B-GFP. 24 h

2010   later, co-cultures were labelled with HCS CellMask™ Blue and DAPI. Arrows indicate the α-

2011   syn fibrils received by acceptor cells and asterisks indicate the cells having α-syn fibrils in each

2012   co-culture condition. Scale bar: 10 μm. **(B)** % of acceptor cells received α-syn fibrils in control

2013   co-culture ($28 \pm 3\%$), Arl8b-GFP co-culture ($60 \pm 5\%$), control secretion ($1 \pm 1\%$), and Arl8b-

2014   GFP secretion ($8 \pm 3\%$) is presented (left graph); average number of α-syn puncta in control

2015   co-culture (3 ± 1), Arl8b-GFP co-culture (5 ± 1), control secretion (1 ± 0.0), and in Arl8b-GFP

2016   secretion (2 ± 0.2) is presented (right graph). Mean $\pm$ S.E.M, *n*=3 (70 acceptor cells per

2017   condition). ns=not significant, ***P=0.0001 by Kruskal-Wallis nonparametric ANOVA test

2018   followed by Dunn's multiple comparison tests. **(C)** Representative confocal images of donor

2019   and acceptor HeLa cells after 24 h of co-culture. WT, myrlysin-KO, and KIF1B-5B dKO HeLa

2020   cells were loaded with Alexa 568-tagged α-syn fibrils (referred as control, myrlysin-KO, and

2021   KIF1B-5B dKO co-cultures, respectively) and co-cultured with acceptor cells transfected with

2022   H2B-GFP. Co-cultures were labelled with HCS CellMask^{TM} Blue and DAPI. Arrows indicate

2023   the α-syn fibrils received by acceptor cells and asterisks indicate the cells having α-syn fibrils

2024   in each co-culture condition. Scale bar: 10 μm. **(D)** % of acceptor cells received α-syn fibrils in

2025   control co-culture (29 ± 2%), myrlysin-KO co-culture (16 ± 2%), KIF1B-5B dKO co-culture

2026   (12 ± 2%), control secretion (5 ± 2%), myrlysin-KO secretion (6 ± 1%), and KIF1B-5B dKO

2027   secretion (6 ± 1%) is presented (left graph); average number of α-syn in control co-culture (2 ±

2028   0.3), myrlysin-KO co-culture (2 ± 0.3), KIF1B-5B dKO co-culture (2 ± 0.4), control secretion

2029   (1 ± 0.4), myrlysin-KO secretion (1 ± 0.2), and KIF1B-5B dKO secretion (1 ± 0.2) is presented

2030   (right graph). Mean ± S.E.M, *n*=3 (90 acceptor cells per condition). ns=not significant,

2031   **P=0.016, ****P<0.0001 by Kruskal-Wallis nonparametric ANOVA test followed by Dunn's

2032   multiple comparison tests. The data underlying this Figure may be found in S1 Data.

2033

2034   **S7 Fig. Validation of CAD-HeLa co-culture system with additional information. (A)**

2035   Representative confocal image of donor CAD cells loaded with Alexa 568-tagged α-syn fibrils

2036   (18 h) co-cultured with HeLa Gal3-Turquoise acceptor cells for 24 h. In acceptor HeLa cells,

2037   arrows indicate α-syn fibrils. Scale bar: 10 μm. **(B)** % of acceptor HeLa cells received α-syn

2038   fibrils in co-culture (65 ± 3%) and in secretion test (7 ± 2%) is presented (left graph). Mean ±

2039   S.E.M. ****P<0.0001 by two-tailed *t*-test. Average number of α-syn fibrils puncta per acceptor

2040   cell in co-culture (4 ± 0.2) and in secretion test (2 ± 0.2) is presented (right graph). ns=not

2041   significant by Mann-Whitney *U*-test. **(C)** Representative confocal image of donor CAD cells

2042   loaded with Alexa 568-tagged α-syn fibrils (18 h) co-cultured with acceptor WT HeLa cells

2043   transfected with α-syn-GFP for 24 h. Red arrows indicate α-syn fibrils, green arrows indicate

2044   α-syn-GFP puncta formation (seeding) in acceptor HeLa cells. Scale bar: 10 μm. **(D)**

2045   Representative confocal image of Gal3-GFP transfected and Alexa 568-tagged α-syn fibril

2046   loaded donor CAD cells (18 h) co-cultured with HeLa Gal3-Turquoise cells for 24 h. In donor

2047   CAD cell, green arrows indicate Gal3-GFP puncta formation. Scale bar: 10 μm. **(E)** % of donor

2048   CAD cells under LMP in control (2 ± 2%) and in α-syn (11 ± 4%) co-cultures is presented (left

2049   graph); average number of Gal3-GFP puncta in donor cells under LMP in control (10 ± 2) and

2050   in α-syn (14 ± 4) co-cultures is presented (right graph). Mean ± S.E.M. ns=not significant, by

2051   *P=0.04 Mann-Whitney *U*-test. **(F)** HeLa Gal3-Turquoise acceptor cell under LMP labelled for

2052   LAMP1 human Alexa 647 antibody (pseudo colored in grey). Colocalization between Gal3-

2053   Turquoise puncta and LAMP1 (indicated by arrows) is presented in a selected region indicated

2054   by the square. Scale bar: 10 μm (for the inset: 1 μm). **(G)** HeLa Gal3-Turquoise acceptor cell

under LMP labelled with LAMP1 mouse Alexa 647 antibody (pseudo colored in grey). Colocalization between Gal3-Turquoise puncta and donor's lysosome (white arrows), and colocalization between α-syn fibrils puncta and donor's lysosome (red arrows) are presented in a selected region indicated by a square. Scale bar: 10 μm (for the inset: 1 μm). **(H)** Representative confocal image of Alexa 568-tagged α-syn fibril-treated donor CAD cells for 18 h co-cultured with HeLa Gal3-Turquoise cells (pseudo colored in yellow) for 24 h. Higher magnification of a selected region (white square) in donor CAD cell is presented. Arrows indicate a Gal3-Turquoise puncta detected in a donor CAD cell. "Merge" image is saturated for the better visualization of the Gal3-Turquoise puncta. Scale bar: 10 μm (for the inset: 2 μm). **(I)** % of donor CAD cells received Gal3-Turquoise puncta in control (0.3 ± 0.3%) and in α-syn (4 ± 1%) co-cultures. Mean ± S.E.M. **P=0.003 by Mann-Whitney *U*- test. In CAD-HeLa co-culture model, data was collected from seven independent experiments in which four and three of them were labelled with anti-LAMP1 mouse and human antibodies, respectively. According to the experiment, in average 100 acceptor or donor cells were analysed for each condition per experiment. The data underlying this Figure may be found in S1 Data.

S1 Movie. Seeding of α-syn-GFP aggregates in CAD cells overexpressing soluble α-syn-GFP treated with α-syn fibrils up to 4 h. Timeframes of the max projection of 36 slides (step size: 0.20 μm) with a total physical thickness of 7.2 μm, with 5 minutes of interval time. Video was acquired using laser 488 in a Spinning Disk microscope.

S2 Movie. Monitoring of CAD cells overexpressing soluble α-syn-GFP up to 4 h. Timeframes of the max projection of 21 slides (step size: 0.48 μm) with a total physical thickness of 9.55 μm, with 15 minutes of interval time. Video was acquired using laser 488 in a Spinning Disk microscope.

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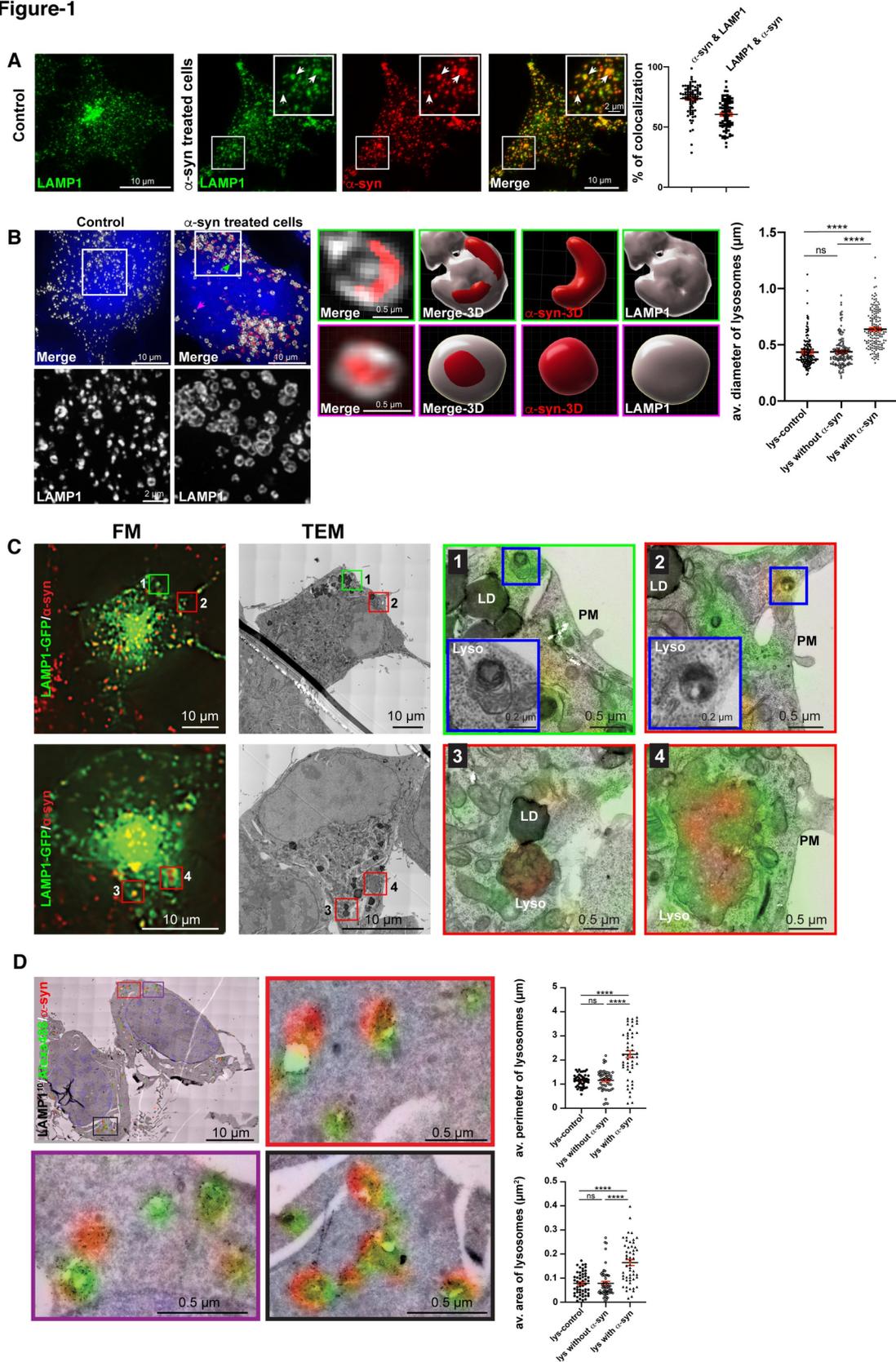
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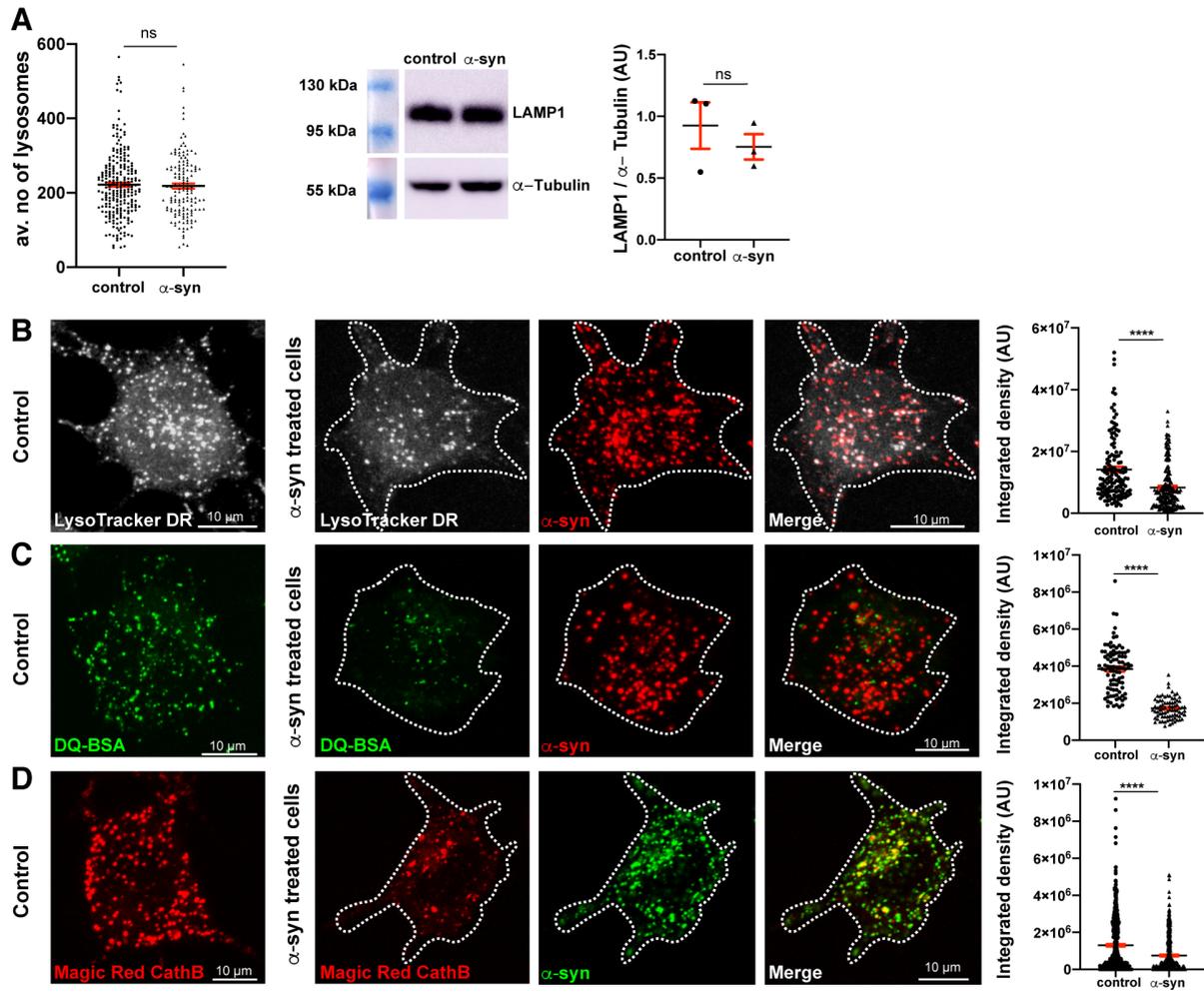
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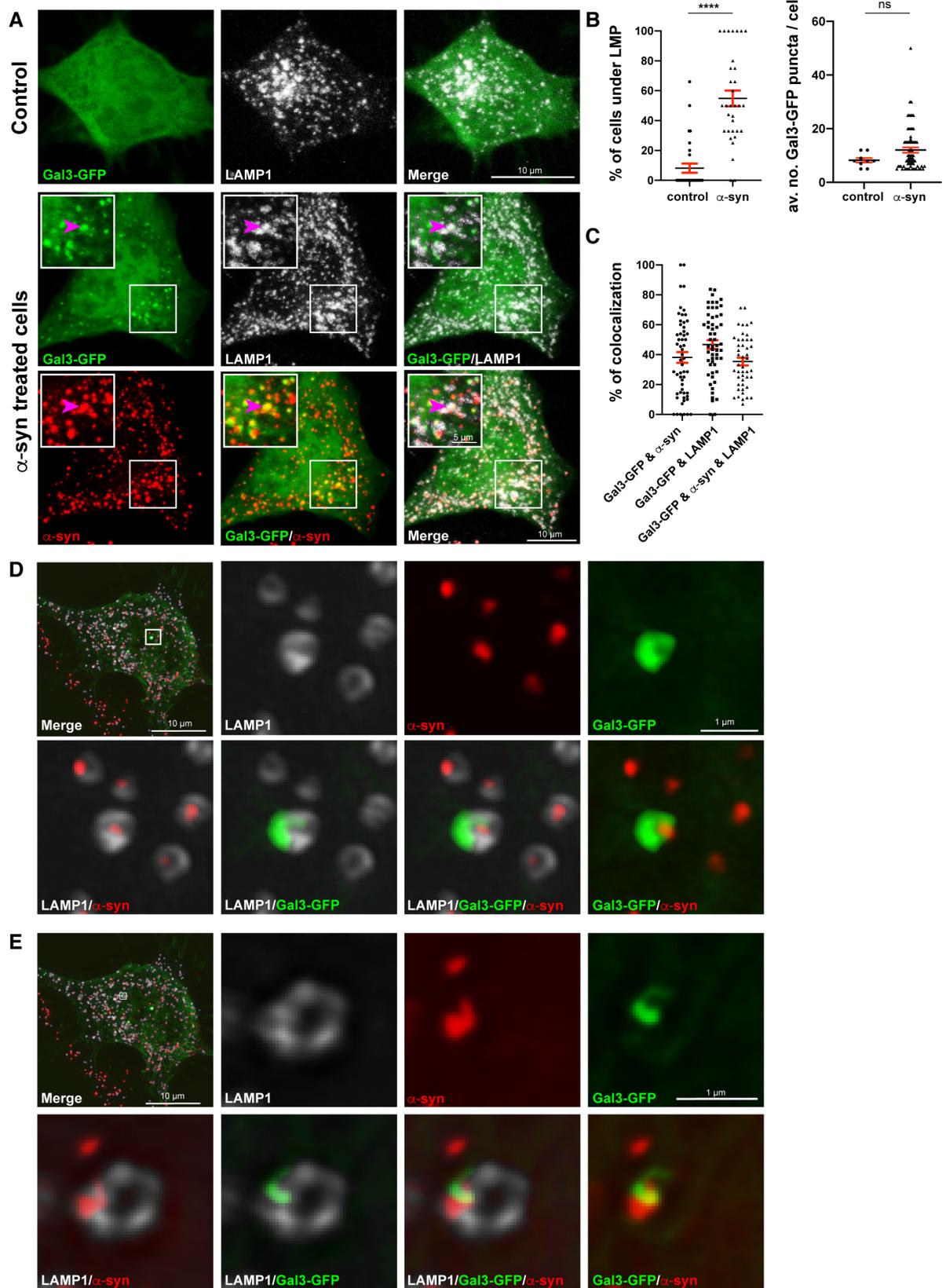
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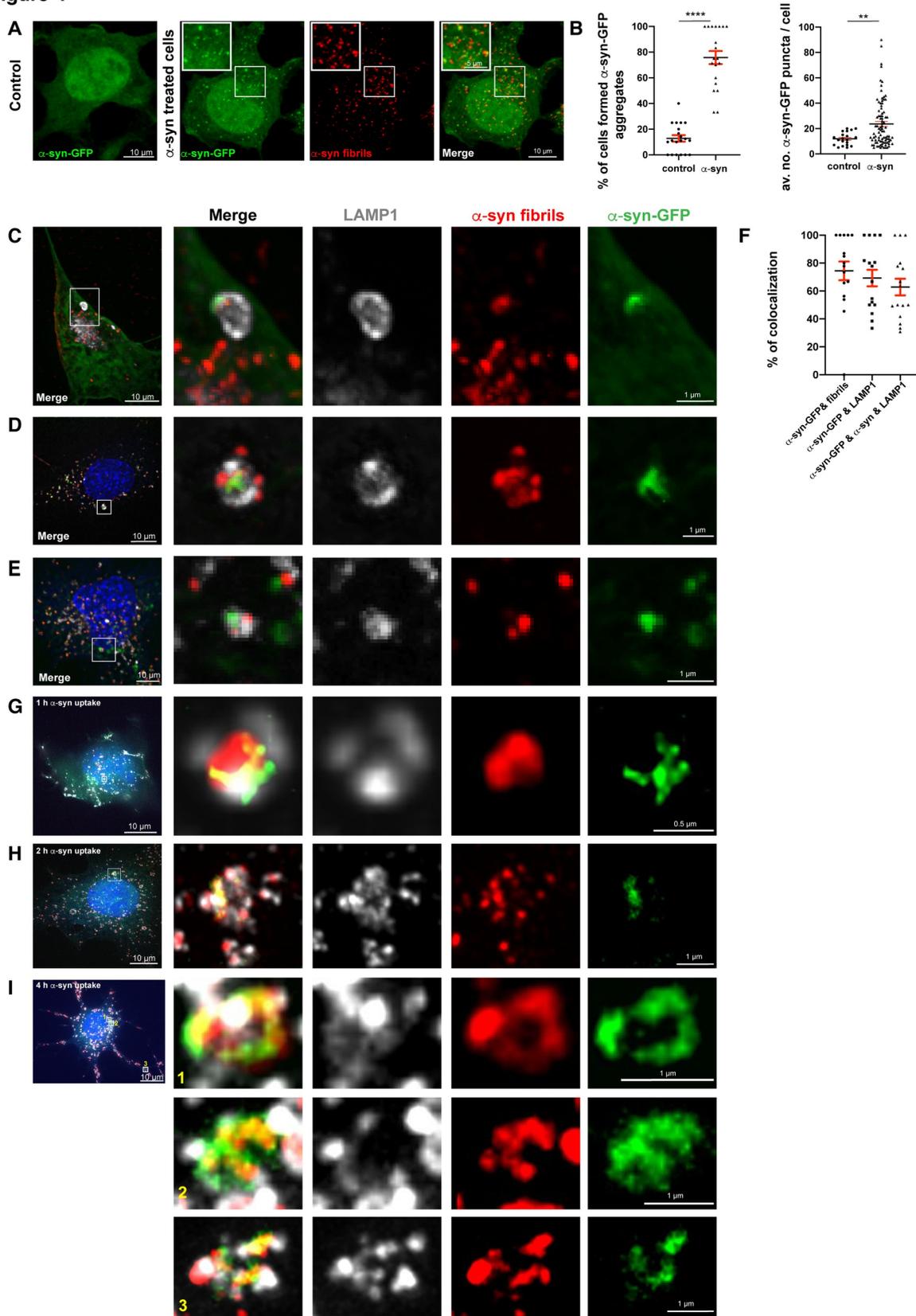
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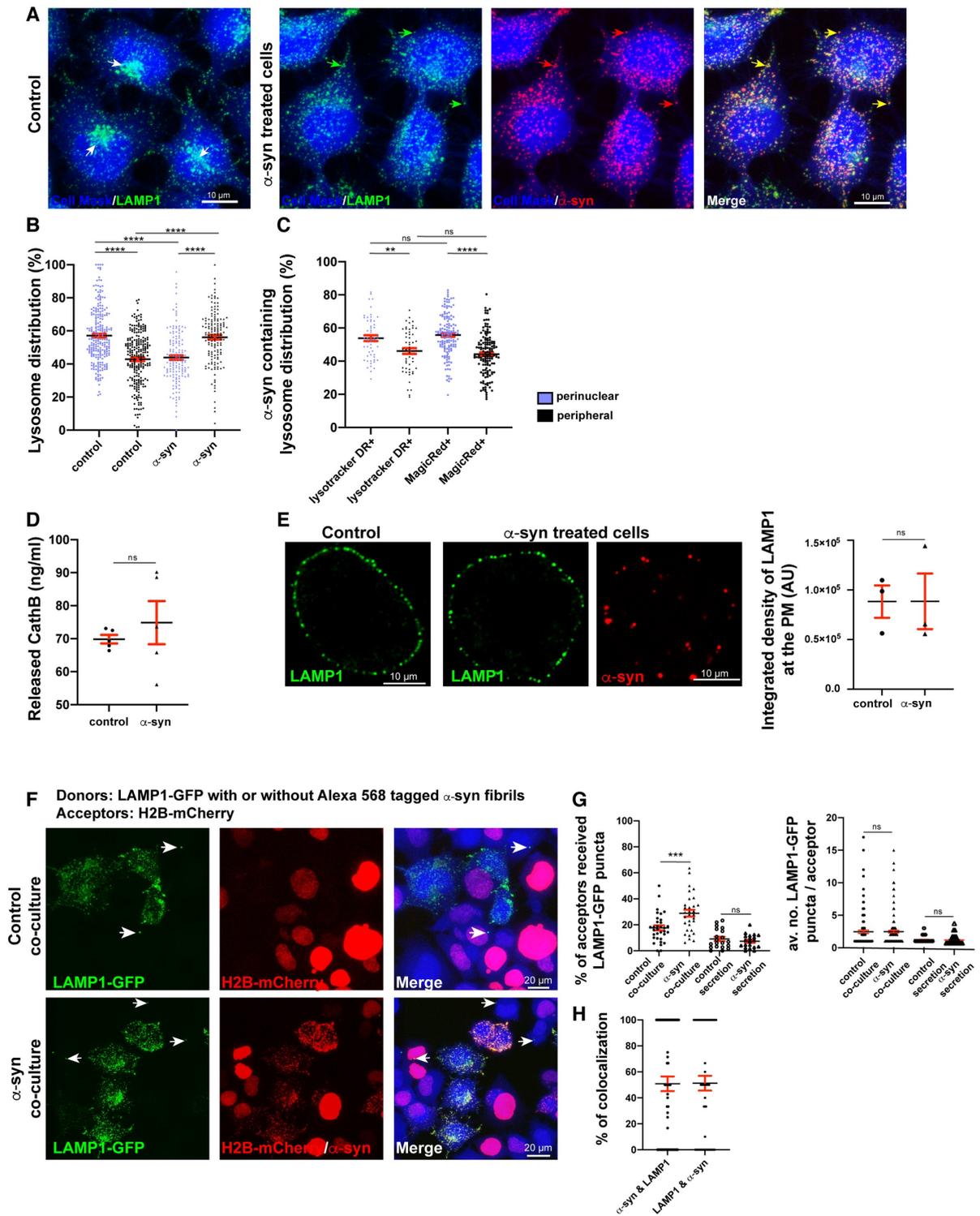
Figure-6

Figure-7

Figure-8

A Donors: H2B-mCherry or Alexa 568 tagged α-syn fibrils
Acceptors: Gal3-GFP

Control co-culture

Donors | Acceptors | Merge
H2B-mCherry | Gal3-GFP | Merge
10 µm

α-syn co-culture

Donors | Acceptors | Merge
α-syn | Gal3-GFP | Merge
10 µm

B

% of acceptors under LMP
****
control co-culture | α-syn co-culture

av. no. Gal3-GFP puncta / acceptor
*
control co-culture | α-syn co-culture

C Acceptors: Gal3-GFP

Donors: α-syn
10 µm

Merge 10 µm | LAMP1 | α-syn | Gal3-GFP 0.2 µm
LAMP1/α-syn | LAMP1/Gal3-GFP | Merge | Gal3-GFP/α-syn

D

Merge 5 µm | LAMP1 | α-syn | Gal3-GFP 0.5 µm
LAMP1/α-syn | LAMP1/Gal3-GFP | LAMP1/Gal3-GFP/α-syn | Gal3-GFP/α-syn

Figure-9

A Donors: H2B-mCherry or Alexa 568 tagged α-syn fibrils
Acceptors: α-syn-GFP

Control co-culture

Donor cells — H2B-mCherry | Acceptor cells — α-syn-GFP | Merge

α-syn co-culture

Donor cells — α-syn fibrils | Acceptor cells — α-syn-GFP | Merge

B

% acceptors formed α-syn-GFP aggregates

control co-culture | α-syn co-culture

av. no. α-syn-GFP puncta / acceptor

control co-culture | α-syn co-culture

C LAMP1 | α-syn fibrils | α-syn-GFP
LAMP1/α-syn fibrils | LAMP1/α-syn-GFP | Merge | α-syn-GFP/α-syn fibrils

D LAMP1 | α-syn fibrils | α-syn-GFP
LAMP1/α-syn fibrils | LAMP1/α-syn-GFP | Merge | α-syn-GFP/α-syn fibrils

E LAMP1 | α-syn fibrils | α-syn-GFP
LAMP/α-syn fibrils | LAMP1/α-syn-GFP | Merge | α-syn-GFP/α-syn fibrils

F LAMP1 | α-syn fibrils | α-syn-GFP
LAMP1/α-syn fibrils | LAMP1/α-syn-GFP | Merge | α-syn-GFP/α-syn fibrils

G

% of colocalization

α-syn-GFP & LAMP1

75

Figure-10

Figure-11

Figure S1
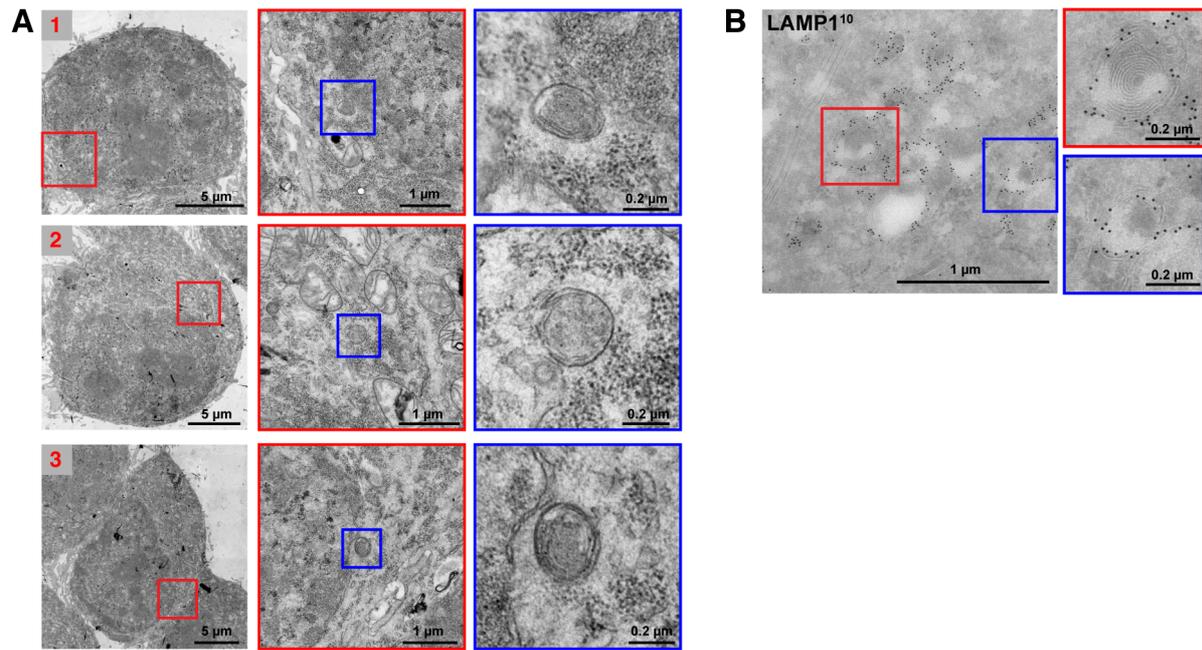
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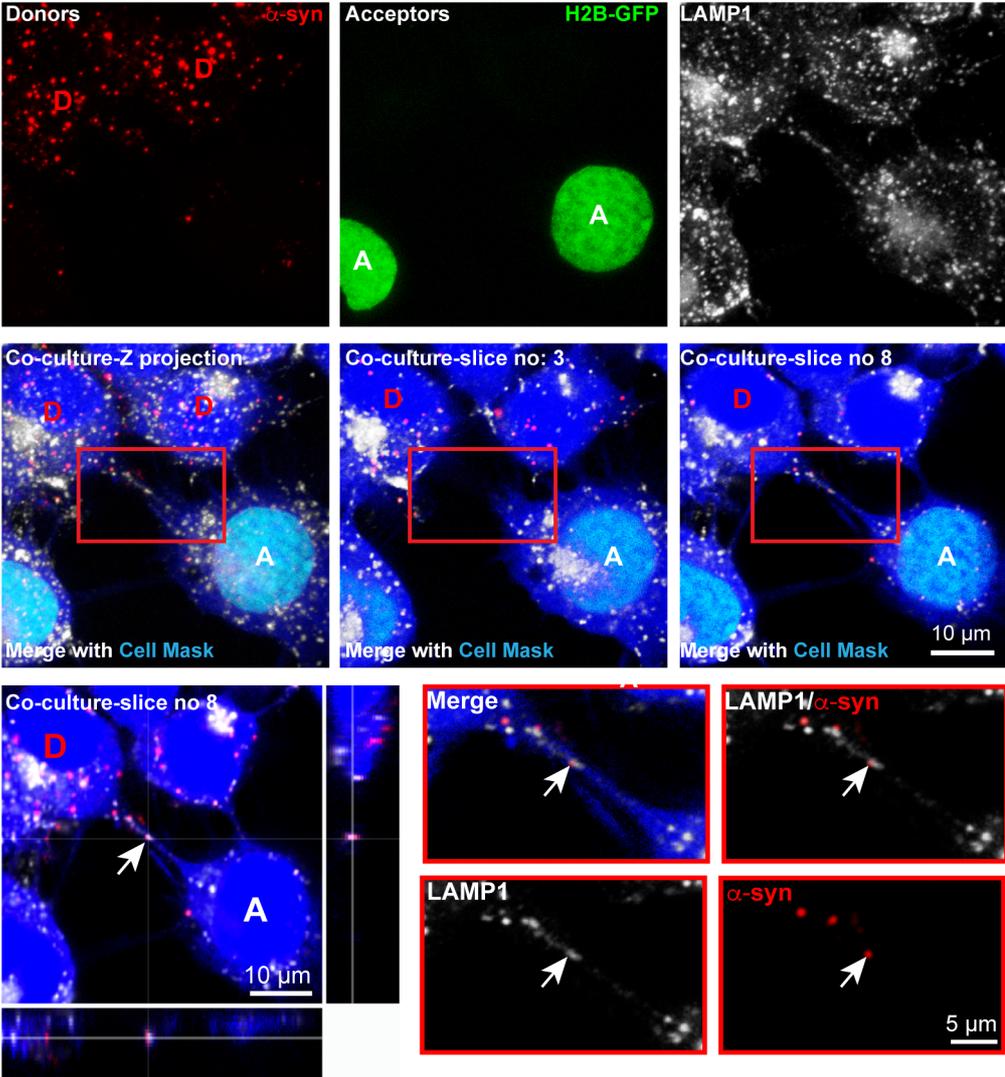
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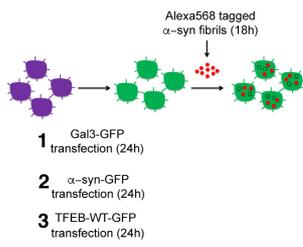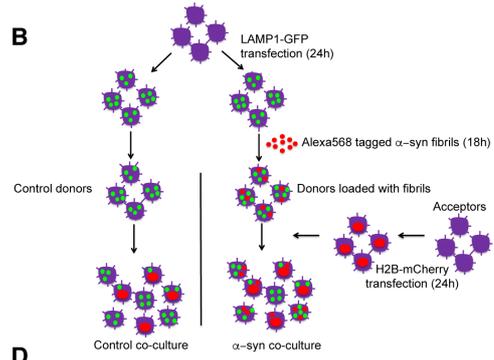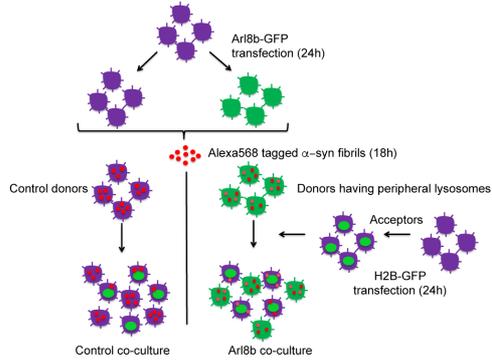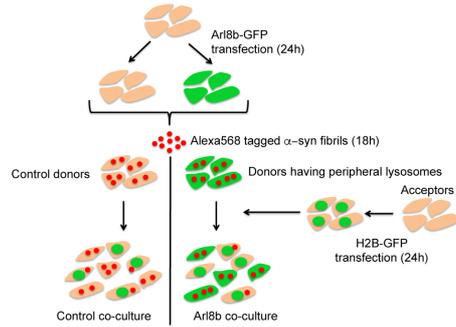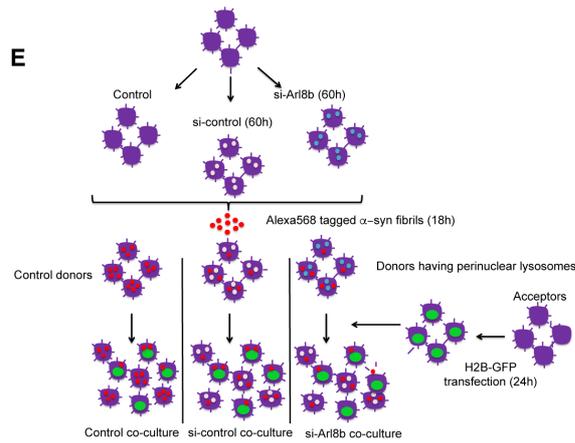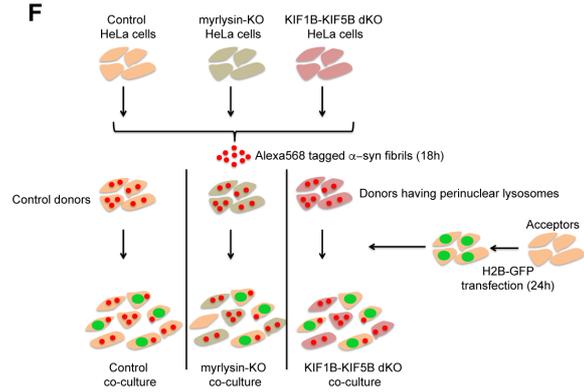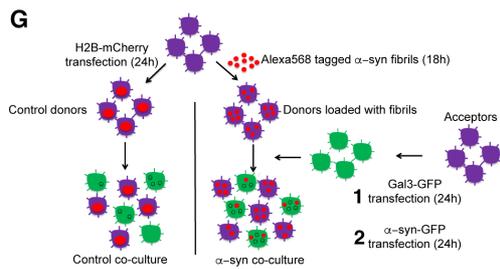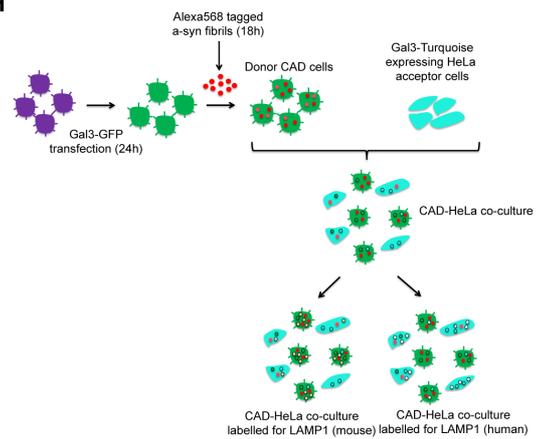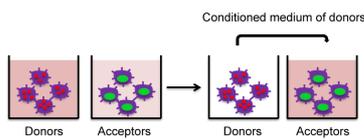
Figure S4

Figure S5

Figure S6

A Donor HeLa cells: Treated with Alexa 568 tagged α-syn fibrils & transfected with Arl8b-GFP or not
Acceptor HeLa cells: H2B-GFP

B

C

D

Figure S7

Funding