



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

Aysegul Dilsizoglu Senol, Maura Samarani, Sylvie Syan, Carlos M. Guardia, Takashi Nonaka, Nalan Liv, Patricia Latour-Lambert, Masato Hasegawa, Judith Klumperman, Juan S Bonifacino, et al.

► To cite this version:

Aysegul Dilsizoglu Senol, Maura Samarani, Sylvie Syan, Carlos M. Guardia, Takashi Nonaka, et al.. α -Synuclein fibrils subvert lysosome structure and function for the propagation of protein misfolding between cells through tunneling nanotubes. PLoS Biology, 2021, 19 (7), pp.e3001287. 10.1371/journal.pbio.3001287 . pasteur-03274582

HAL Id: pasteur-03274582

<https://pasteur.hal.science/pasteur-03274582>

Submitted on 30 Jun 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License

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

Aysegul Dilsizoglu Senol^{1§}, Maura Samarani^{1§}, Sylvie Syan¹, Carlos M. Guardia², Takashi
Nonaka³, Nalan Liv⁴, Patricia Latour Lambert⁵, Masato Hasegawa³, Judith Klumperman⁴, Juan
S. Bonifacino², Chiara Zurzolo^{1*}

¹Unité de Trafic Membranaire et Pathogénèse, Département de Biologie Cellulaire et de
l'Infection, Institut Pasteur, Paris, France.

²Neurosciences and Cellular and Structural Division, *Eunice Kennedy Shriver* National
Institute of Child Health and Human Development, National Institutes of Health, Bethesda,
MD, USA.

³Dementia Research Project, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan.

⁴Section Cell Biology, Center for Molecular Medicine, University Medical Center Utrecht,
Utrecht University, Utrecht, Netherlands.

⁵Dynamique des Interaction Hôte-Pathogène, Département de Biologie Cellulaire et de
l'Infection, Institut Pasteur, Paris, France.

[§]These authors contributed equally

*Corresponding Author

E-mail: chiara.zurzolo@pasteur.fr (CZ)

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

amyloidogenic proteins such as prion protein [50–52], huntingtin [53], and tau [52,54,55] use TNTs as highways for transfer to naïve cells, suggesting that TNTs are common routes for the spread of pathogenic proteins between cells. Of particular interest, we showed that α -syn fibrils can be efficiently transferred between neuronal CAD cells, primary neurons, primary astrocytes, and human neural progenitor cells, and between organotypic hippocampal slices and astrocytes. We found that α -syn fibrils are mostly localized inside lysosomes in all of these cell types [19,21,56] and could be transferred inside lysosomes through TNTs from “infected” donor cells to naïve acceptor cells in co-cultures. Importantly, once arrived in the acceptor cells, α -syn fibrils were able to induce the aggregation of soluble α -syn [19]. How this occurs, however, is not clear.

Lysosomes are cytoplasmic, membrane-enclosed, acidic organelles that have long been considered as the “dustbin” and “recycling center” of the cells, as they are responsible for the degradation of both exogenous and intracellular cargos destined to lysosomes via endocytic, phagocytic [57,58], and autophagic pathways [59,60]. However, in recent years, mounting evidence revealed that lysosomes play a more complex role in the regulation of cellular homeostasis, as they are involved in crucial processes such as plasma membrane repair, transcriptional control, nutrient sensing, signaling, and energy metabolism [61–65]. It is therefore not surprising that lysosomal dysfunction is involved in a plethora of pathological conditions, including NDs [66]. Notably, the levels of lysosome-associated membrane proteins 1 and 2 (LAMP1 and LAMP2) and lysosomal enzymes Cathepsin B and D were found to be significantly reduced in PD patient brains, mouse and cell models [67–72]. Moreover, lysosomal membrane permeabilization (LMP) has been reported in cells treated with protein aggregates including α -syn [73,74].

In the present study, we focused on the role of lysosomes in the intercellular spread of α -syn fibrils in neuronal cells. Particularly, we studied how α -syn fibrils affect lysosomes and how these organelles contribute to the spread and propagation of α -syn aggregates through TNTs. We found that α -syn fibrils alter the morphology of lysosomes and compromise lysosomal function. We demonstrated that α -syn fibrils induce the peripheral distribution of the lysosomes exhibiting functional impairment, likely mediated by TFEB, and showed that this peripheral positioning enhances the efficiency of α -syn fibrils’ transfer in a cell-to-cell contact-dependent manner. In addition, we demonstrated that TFEB translocated to the nucleus in the presence of α -syn fibrils, probably contributing to the peripheral distribution of lysosomes. We also found that LMP, which is well described in cells following the uptake of α -syn fibrils from the medium, also occurs in acceptor cells following the transfer of lysosomes bearing α -syn fibrils

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 $74 \pm 1\%$ 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 $60 \pm 1\%$ 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 ($0.43 \pm 0.01 \mu\text{m}$) whereas lysosomes containing α -syn fibrils increased their diameter by 50% ($0.63 \pm 0.01 \mu\text{m}$; 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

were structurally identified as lysosomes by the presence of electron-dense heterogenous luminal content, degraded membranes, and occasional intraluminal vesicles as morphological criteria [77]. Lysosomes not containing α -syn fibrils presented a healthy lysosome morphology with spherical shape and smooth membranes [77,78] (Fig 1C, green square, number 1). Interestingly, most lysosomes containing α -syn fibrils were found to be quite heterogeneous in structure (Fig 1C, red squares numbers 2, 3, and 4). Some had an unusual luminal content and displayed curvy limiting membrane, suggesting that the presence of the fibrils could deform the organelles (Fig 1C, red squares numbers 3, and 4). The aberrant, curvy organelles were completely absent in control cells not treated with α -syn fibrils (S1A Fig). A subset of lysosomes containing α -syn fibrils, as defined by FM, had a healthy morphology (Fig 1C, red square, number 2), which could represent an early stage of fibrillar accumulation.

Next, we used another CLEM approach to analyse the morphology and size of α -syn-positive lysosomes with high-resolution. CAD cells loaded with Alexa 568-tagged α -syn fibrils were prepared for EM following the flat embedding Tokuyasu technique. This technique preserves the fluorescence signal of α -syn fibrils in ultrathin sections prepared for EM, and in addition allows to perform immunolabelling (both gold and fluorescent) of endogenous LAMP1 [79] (Fig 1D). Following a recently developed high-throughput on-section CLEM approach [80], we analysed 50 LAMP1-immunolabelled lysosomes in control cells (S1B Fig), 50 α -syn-positive, and 50 α -syn-negative lysosomes in treated cells. For each lysosome, we correlated EM ultrastructural details (surface area and perimeter) and the presence of LAMP1 to the FM images showing the presence or absence of α -syn fibrils. Using this approach, we confirmed that the lysosomes bearing α -syn fibrils were significantly larger (perimeter: $2.24 \pm 0.14 \mu\text{m}$ and area: $0.16 \pm 0.01 \mu\text{m}^2$) compared to lysosomes devoid of α -syn fibrils (perimeter: $1.16 \pm 0.06 \mu\text{m}$ and area: $0.08 \pm 0.01 \mu\text{m}^2$) and control lysosomes (perimeter: $1.16 \pm 0.04 \mu\text{m}$ and area: $0.08 \pm 0.01 \mu\text{m}^2$; Fig 1D, graphs).

Fig 1. α -Syn fibrils affect the morphology of lysosomes. (A) Representative confocal images of control and Alexa 568-tagged α -syn fibril-treated (18 h) CAD cells, immunolabelled for LAMP1 (green). Colocalization between LAMP1+ puncta and α -syn fibrils is indicated by arrows in the inset of a selected region delimited by a square. % of α -syn fibrils colocalizing with LAMP1+ puncta ($74 \pm 1\%$) and % of LAMP1+ puncta colocalizing with α -syn fibrils ($60 \pm 1\%$) performed by object-based 3D colocalization method (Imaris software) is presented. Mean $\pm$ S.E.M, $n=3$ (30 cells per condition). Scale bar: $10 \mu\text{m}$ (for inset: $2 \mu\text{m}$). **(B)** Super-

resolution (SR) images of control (left panel) and Alexa 568-tagged α -syn fibril-treated CAD cells for 18 h (right panel), immunolabelled for LAMP1 (far-red) antibody (pseudo colored in grey). Merge images of each condition are presented with additional HCS CellMaskTM Blue staining. Magenta and green arrows indicate the selected lysosomes having α -syn fibrils inside the lysosomal lumen and on the lysosomal membrane, respectively, where higher magnifications of these lysosomes and 3D reconstructions are represented in magenta and green squares, respectively. Scale bar: 10 μ m (for insets: 2 μ m, for magenta and green insets: 0.5 μ m). Average diameter (μ m) of lysosomes in control (0.43 ± 0.01) and in α -syn fibril-treated cells, the latter sub-grouped as: lysosomes without α -syn fibrils (0.43 ± 0.01) and lysosomes with α -syn fibrils (0.63 ± 0.01), is presented. Mean $\pm$ S.E.M, lysosomes' diameters were measured in SR images of eight α -syn fibril-treated cells and seven control cells (155 lysosomes per condition). Images were acquired by spinning-disk microscopy with SR module. ns=not significant, ****P<0.0001 by Kruskal-Wallis nonparametric ANOVA test followed by Dunn's multiple comparison tests. **(C)** FM image of LAMP1-GFP transfected and α -syn fibril-treated CAD cells for 18 h (left panel). Enlarged merged images of correlative resin EM and FM images of four selected lysosomes not containing (number 1, indicated by green square) and containing α -syn fibrils (numbers 2, 3, 4, indicated by red squares) are presented. Lysosomes number 1 and 2 are further magnified (blue squares) and presented within the same image. Lyso: lysosome, PM: plasma membrane, LD: lipid droplets. Scale bar: 10 μ m (for red and green insets: 0.5 μ m, for blue insets: 0.2 μ m). **(D)** Overlay of on-section correlative EM images immuno-gold labelled for LAMP1¹⁰ and FM images of LAMP1-GFP transfected and α -syn fibril-treated CAD cells (18 h). Insets of three selected regions (indicated by red, purple, and black squares) are presented. Average perimeter (μ m) of lysosomes in control (1.16 ± 0.04) and in α -syn fibril-treated cells: lysosomes without α -syn fibrils (1.16 ± 0.06) and lysosomes with α -syn fibrils (2.24 ± 0.14 ; upper graph) is presented. ns=not significant, ****P<0.0001 by one-way ANOVA followed by Tukey's multiple comparison tests. Average area (μ m²) of lysosomes in control (0.08 ± 0.01) and in α -syn fibril-treated cells: lysosomes without α -syn fibrils (0.08 ± 0.01) and lysosomes with α -syn fibrils (0.16 ± 0.01 ; lower graph) is presented. Mean $\pm$ S.E.M. (50 cells per condition). ns=not significant, ****P<0.0001 by Kruskal-Wallis nonparametric ANOVA test followed by Dunn's multiple comparison tests. Scale bar: 10 μ m (for insets: 0.5 μ m). The data underlying this Figure may be found in S1 Data.

In spite of these changes in lysosome morphology, the presence of α -syn fibrils had no effect on the abundance of lysosomes. Indeed, the average number of LAMP1-positive lysosomes per cell (control cells: 222 ± 6 ; α -syn fibril-treated cells: 218 ± 7 ; Fig 2A, left panel) and the level of LAMP1 (control: 0.9 ± 0.2 AU; α -syn: 0.7 ± 0.1 AU; Fig 2A, right panel) did not show any significant difference in the presence or absence of α -syn fibrils. Overall, these results indicate that, while the overall amount of lysosomes is not affected by α -syn fibrils, lysosomes containing α -syn exhibit changes in size and morphology.

α -Syn fibrils have been shown to impair lysosomal function [67–72]. Thus, next we addressed their effect on lysosomes in our model system. We first assessed the acidity of the lysosomes in cells treated or not with α -syn fibrils by using a fluorogenic acidotropic dye, LysoTracker Deep Red (LysoTracker DR). The fluorescence intensity of LysoTracker DR was significantly decreased ($\sim 41\%$) in α -syn fibril-treated cells compared to untreated control cells (Fig 2B), suggesting alkalization of lysosomes in the presence of α -syn fibrils. Second, we used a DQ-BSA assay in which the fluorescent signal is detected only when the fluorogenic substrate is hydrolyzed by lysosomal proteases. We observed that the DQ-BSA fluorescence intensity was significantly decreased ($\sim 45\%$) in the presence of α -syn fibrils compared to the control (Fig 2C), suggesting that the activity of the lysosomal proteases was compromised. Finally, we used a more specific approach to evaluate lysosomal function by quantifying the activity of the lysosomal enzyme Cathepsin B (CathB). The intracellular activity of CathB was detected as fluorescent puncta produced by the hydrolysis of the fluorogenic substrate Magic Red in live cells. We observed a significant decrease ($\sim 42\%$) in CathB enzyme activity in cells challenged with α -syn fibrils compared to control cells (Fig 2D). Since the lysosomal pool was similar in control and α -syn fibril-treated cells, these data indicate that the decrease in the fluorescent signal of LysoTracker DR, DQ-BSA, and Magic Red is likely due to the presence of α -syn fibrils.

Altogether, our data indicate that the majority of the α -syn fibrils localize to the lysosomes, where they induce lysosomal enlargement and structural changes, and impair lysosome function.

Fig 2. α -Syn fibrils affect the function of lysosomes. (A) Average number of lysosomes (LAMP1+ puncta) per cell in control (222 ± 6) and in α -syn fibril-treated CAD cells for 18 h (218 ± 7) is presented. Mean $\pm$ S.E.M, $n=5$ (50 cells per condition). ns=not significant by 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 $\pm$ 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 $\pm$ S.E.M, $n=3$ (40 cells per condition). **** $P<0.0001$ by Mann-Whitney U -test. Scale bar: 10 μ m. **(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 $\pm$ S.E.M, $n=3$ (25 cells per condition). **** $P<0.0001$ by Mann-Whitney U -test. Scale bar: 10 μ m. **(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 $\pm$ S.E.M, $n=5$ (100 cells per condition). **** $P<0.0001$ by Mann-Whitney U -test. Scale bar: 10 μ m. 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 μ m (for insets: 5 μ m). **(B)** % of control ($7 \pm 1\%$) and α -syn fibril-treated CAD cells for 18 h ($46 \pm 2\%$) 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 $\pm$ 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 ($38 \pm 4\%$), with LAMP1 ($47 \pm 3\%$), and with both α -syn fibrils and LAMP1 ($35 \pm 3\%$) calculated by object-based 3D colocalization (Imaris software). Mean $\pm$ 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 μ m (for insets: 1 μ m). 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 $76 \pm 5\%$ of cells treated with α -syn fibrils produced newly formed α -syn-GFP aggregates, whereas this percentage was significantly lower ($13 \pm 2\%$) 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

question remains as to whether lysosomes participate to this seeding event and how/where in the cell it occurs. To investigate whether seeding is associated with the lysosomes, we performed super-resolution microscopy in CAD cells transfected with α -syn-GFP, loaded with α -syn fibrils and labelled for LAMP1. We observed three different scenarios: i) lysosomes with both α -syn fibrils and α -syn-GFP aggregates colocalizing at the lysosomal membrane (Fig 4C), ii) lysosomes with α -syn fibrils and α -syn-GFP aggregates colocalizing inside their lumen (Fig 4D), and iii) lysosomes containing both α -syn fibrils and α -syn-GFP aggregates inside their lumen, but not necessarily co-localizing (Fig 4E). In addition, we performed object-based 3D colocalization analysis between newly formed α -syn aggregates (α -syn-GFP puncta), α -syn fibrils and lysosomes. We observed that 69 ± 6 % of the newly formed aggregates colocalized with LAMP1+ lysosomes, 74 ± 7 % colocalized with α -syn fibrils, and 63 ± 6 % of them were positive for both α -syn fibrils and LAMP1+ organelles (Fig 4F), indicating that in CAD cells the majority of the seeding takes place at lysosomal compartments following α -syn fibrils uptake.

To understand whether the localization of α -syn-GFP aggregates to lysosomes was consistent with the beginning of the seeding event and not a post seeding delivery of newly formed fibrils, we monitored the occurrence of seeding by live imaging microscopy. We first transfected CAD cells with soluble α -syn-GFP and then immediately after the addition of α -syn fibrils we started to monitor the seeding event by detecting the formation of green puncta (α -syn-GFP newly formed aggregates) in these cells compared to control cells by live spinning-disk microscopy. We found that the seeding first became apparent about 2 h from the addition of the α -syn fibrils and increased with time (S1 Movie and S5A Fig). We did not observe any newly formed α -syn-GFP puncta in untreated control cells (S2 Movie and S5B Fig). Next, we performed super-resolution microscopy in order to investigate whether these newly formed aggregates were associated with the lysosomes by immunolabelling LAMP1 at different time points (1h, 2h, and 4h). Interestingly, at all these time points (even in the rare events found at 1 hour), we found that newly formed aggregates colocalized with α -syn fibrils and LAMP1 (Fig 4G-I). Therefore, newly formed α -syn-GFP aggregates following α -syn fibrils addition are associated with lysosomes at the very beginning of the seeding event, supporting our hypothesis that lysosomes function as hubs for the seeding of newly formed α -syn aggregates.

Overall, these results indicate that exogenous α -syn fibrils induce *de novo* aggregation of soluble α -syn in association with lysosomes.

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 μ m (for insets: 5 μ m). **(B)** % of control ($13 \pm 2\%$) and α -syn fibril-treated CAD cells for 18 h ($76 \pm 5\%$) that formed α -syn-GFP aggregates (left graph). Mean $\pm$ 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 $\pm$ 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 ($74 \pm 7\%$), α -syn-GFP aggregates and LAMP1+ lysosomes ($69 \pm 6\%$), and α -syn-GFP aggregates with both α -syn fibrils and LAMP1+ lysosomes ($63 \pm 6\%$). Mean $\pm$ 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 μ m (for insets: 1 μ m; 0.5 μ m 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

by α -syn aggregates favors the spread of pathology [19,32]. Lysosomes are dynamic organelles that move bidirectionally between the perinuclear and peripheral regions of the cell [90–92]. As our results indicate that the α -syn fibrils interfere with lysosome morphology and function, we hypothesized that they could also have an impact on lysosome positioning. Therefore, we evaluated the distribution of lysosomes in CAD cells challenged with α -syn fibrils compared to control cells. Cells were labelled for LAMP1, HCS CellMaskTM Blue (cell outline stain), and DAPI (nuclear stain), and the number of lysosomes in the perinuclear and peripheral regions in each condition was quantified (see Materials and Methods; Fig 5A). In agreement with previous findings that lysosomes are mainly located in the perinuclear region in healthy non-polarized cells [93], we detected $57 \pm 1\%$ of the lysosomes at the perinuclear region in control CAD cells (Fig 5A, white arrows, and B). In contrast, in cells treated with α -syn fibrils, the situation was reversed, with the majority of lysosomes ($56 \pm 1\%$) found in the peripheral region (Fig 5A, green, red, yellow arrows, and B).

Since our results indicate that α -syn fibrils affect lysosomal pH and function (Fig 2B-D), to understand whether there is a correlation between lysosome distribution and function, we next evaluated the distribution of lysosomes containing α -syn fibrils and quantified the perinuclear and peripheral distribution of only functional lysosomes positive for LysoTracker DR and Magic Red CathB. We found that α -syn fibril-containing LysoTracker DR positive and Magic Red CathB positive lysosomes were more perinuclear ($54 \pm 2\%$ and $56 \pm 1\%$, respectively; Fig 5C), suggesting that peripheral lysosomes containing α -syn fibrils are less functional.

These results thus show that α -syn fibrils promote redistribution of lysosomes towards the cell periphery. Furthermore, this pool of peripheral lysosomes is less acidic and has less degradative capacity.

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

Our next question was to understand whether the effect of α -syn on lysosome positioning could be relevant for the propagation of the pathology and in which way. Peripheral lysosomes could be more prone to exocytosis and therefore could increase the secretion of α -syn fibrils to the extracellular environment. Alternatively, peripheral lysosomes could be more apt for transfer to healthy neighboring cells through TNTs. To distinguish between these alternative scenarios, we first examined lysosomal exocytosis by comparing the amount of CathB released into the culture medium of control and α -syn fibril-treated CAD cells using an ELISA method. These experiments showed no significant difference in the amount of CathB released by control (70

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 ($9 \times 10^4 \pm 2 \times 10^4$
451 AU) and α -syn fibril-treated cells ($9 \times 10^4 \pm 3 \times 10^4$ 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 ($29 \pm$
461 3% and $18 \pm 2\%$, 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 ($9 \pm 2\%$ and $7 \pm 1\%$ 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 $51 \pm 6\%$ of α -
476 syn fibrils colocalizing with LAMP1-GFP+ lysosomes and $51 \pm 6\%$ of lysosomes colocalizing

with α -syn fibrils, suggesting that half of the lysosomes transferred from donor to acceptor cells carried α -syn fibrils (Fig 5H).

Taken together, our data indicate that lysosomes that are more peripherally located in the presence of α -syn fibrils are not secreted more compared to control cells, but instead are more efficiently transferred to acceptor cells by cell-to-cell contact.

Fig 5. α -Syn-loaded lysosomes localize to the cell periphery, are less functional, and are preferentially transferred to neighboring cells. (A) Representative confocal images of control and Alexa 568-tagged α -syn fibril-treated CAD cells (18 h) labelled for LAMP1-Alexa 488 antibody, HCS CellMaskTM Blue, and DAPI. White arrows indicate the perinuclear lysosome clusters in control cells; green, red, and yellow arrows indicate peripheral lysosomes, α -syn fibrils, and lysosomes colocalizing with α -syn fibrils, respectively. Scale bar: 10 μ m. (B) % of LAMP1+ lysosomes in perinuclear (purple dots) and peripheral regions (black dots) of the control cells and cells challenged with α -syn fibrils is presented. In control cells: % of perinuclear lysosomes: $57 \pm 1\%$, and peripheral lysosomes: $43 \pm 1\%$; in α -syn fibril-treated cells: % of perinuclear lysosomes: $44 \pm 1\%$, and peripheral lysosomes: $56 \pm 1\%$. Mean $\pm$ S.E.M, $n=5$ (40 cells per condition). ns=not significant, **** $P<0.0001$ by one-way ANOVA followed by Tukey's multiple comparison tests. (C) Graph representing the distribution of LysoTracker DR+ and Magic Red CathB+ lysosomes colocalizing with the α -syn fibrils in perinuclear (purple dots) and peripheral region (black dots). % of LysoTracker DR+ lysosomes colocalizing with α -syn fibrils in perinuclear region: $54 \pm 2\%$, and in peripheral region: $46 \pm 2\%$. % of Magic Red CathB+ lysosomes colocalizing with α -syn fibrils in perinuclear region: $56 \pm 1\%$, and in peripheral region: $44 \pm 1\%$. Mean $\pm$ S.E.M, $n=4$ (40 cells per condition). ns=not significant by one-way ANOVA followed by Tukey's multiple comparison tests. (D) Quantity of secreted CathB (ELISA) in the media of control (70 ± 1 ng/mL) and α -syn fibril-treated CAD cells (75 ± 7 ng/mL). Mean $\pm$ S.E.M, $n=3$. ns=not significant by Mann-Whitney U -test. (E) Representative confocal images of control and Alexa 568-tagged α -syn fibril-treated CAD cells for 18 h, immuno-labelled for LAMP1-Alexa 488 antibody in non-permeabilized conditions. Scale bar: 10 μ m. Average Integrated Density of LAMP1 immunofluorescence at the cell surface in control ($9 \times 10^4 \pm 2 \times 10^4$) and in α -syn fibril-treated cells ($9 \times 10^4 \pm 3 \times 10^4$) is presented. Mean $\pm$ S.E.M, $n=3$ (20 cells per condition). ns=not significant by paired t -test. Scale bar: 10 μ m. (F) Representative confocal images of donor and acceptor cells after 24 h of co-culture. 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 μ m. **(G)** % of acceptor cells that received LAMP1+ puncta (lysosomes) from donor cells in control co-culture ($18 \pm 2\%$), in α -syn co-culture ($29 \pm 3\%$), in control secretion test ($9 \pm 2\%$), and in α -syn secretion test ($7 \pm 1\%$) (left graph). Mean $\pm$ 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 $\pm$ 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 ($51 \pm 6\%$) and % of LAMP1-GFP+ puncta colocalizing with α -syn fibrils puncta ($51 \pm 6\%$) is presented. Mean $\pm$ 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: $18 \pm 4\%$; α -syn: $57 \pm 6\%$, 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: $59 \pm 1\%$; TFEB-WT-GFP: $54 \pm 2\%$; CMV-

GFP: $52 \pm 1\%$; Fig 6B). These results indicate that TFEB promotes the peripheral distribution of lysosomes in CAD cells.

To determine whether TFEB participates in peripheral redistribution of lysosomes upon α -syn loading, we silenced the expression of TFEB in CAD cells by RNA interference (Fig 6C). CAD cells transfected with scrambled siRNA (control) or siTFEB were challenged with α -syn fibrils for 18 h. Then, we quantified the positioning of lysosomes containing α -syn fibrils (Fig 6D). As expected, control cells showed more peripheral α -syn-loaded lysosomes ($52.1 \pm 1\%$). In contrast, in siTFEB-treated CAD cells α -syn-loaded lysosomes were slightly but significantly more perinuclear ($50.7 \pm 1\%$). These findings indicate that silencing of endogenous TFEB reduced the peripheral redistribution of lysosomes upon α -syn loading, suggesting the involvement of TFEB in this event.

Fig 6. The role of TFEB in lysosome redistribution upon α -syn loading. (A) % of cells overexpressing TFEB-WT-GFP having nuclear TFEB in control CAD cells ($18 \pm 4\%$) and α -syn fibril-treated CAD cells for 18 h ($57 \pm 6\%$). Mean $\pm$ S.E.M, $n=3$ (50 cells per condition). **** $P<0.0001$ by Unpaired t test. (B) % of LAMP1+ lysosome distribution in perinuclear (purple dots) and peripheral regions (black dots) of cells overexpressing CMV-GFP (control), TFEB-WT-GFP, or TFEB-mut-GFP. In control CMV-GFP cells: % of perinuclear lysosomes: $48 \pm 1\%$, and peripheral lysosomes: $52 \pm 1\%$; in TFEB-WT-GFP cells: % of perinuclear lysosomes: $46 \pm 2\%$, and peripheral lysosomes: $54 \pm 2\%$; in TFEB-mut-GFP cells: % of perinuclear lysosomes: $41 \pm 1\%$, and peripheral lysosomes: $59 \pm 1\%$. Mean $\pm$ S.E.M, $n=3$ (30 cells per condition). *** $P<0.001$ by one-way ANOVA followed by Tukey's multiple comparison tests. (C) Western blot prepared from cell extracts collected from control, scrambled siRNA (sicontrol)-, and siTFEB-pretreated CAD cells for 60 h, showing TFEB and GAPDH (loading control) expression. (D) Graph representing the distribution of lysosomes containing α -syn fibrils in perinuclear (purple dots) and peripheral region (black dots) of α -syn fibril-treated sicontrol and siTFEB cells. % of lysosomes containing α -syn fibrils of sicontrol cells in perinuclear region: $49 \pm 1\%$, and in peripheral region: $52 \pm 1\%$. % of lysosomes containing α -syn fibrils of siTFEB cells in perinuclear region: $51 \pm 1\%$, and in peripheral region: $49 \pm 1\%$. Mean $\pm$ S.E.M, $n=4$ (30 cells per condition). ns=not significant, * $P=0.0288$ by one-way ANOVA followed by Tukey's multiple comparison tests. The data underlying this Figure may be found in S1 Data.

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).

Furthermore, we silenced the expression of Arl8b in CAD cells by RNA interference (Fig 7C). Briefly, we pretreated donor cells with siRNA targeting Arl8b (siArl8b) and scrambled siRNA (sicontrol) for 60 h prior to loading with α -syn fibrils for 18 h; in parallel, untreated control cells were challenged with α -syn fibrils. These donors were then co-cultured with acceptor cells transiently transfected with H2B-GFP (Figs 7D and S3E). In control co-culture and in siicontrol co-culture, the percentage of acceptor cells that received α -syn fibrils was $55 \pm 4\%$ and $60 \pm 3\%$, respectively, whereas in siArl8b co-culture we observed a significant lower percentage of $28 \pm 3\%$ (Fig 7E, left graph). We did not detect any significant difference in α -syn transfer through secretion ($6 \pm 1\%$, $9 \pm 1\%$, and $6 \pm 1\%$ in control, siicontrol, and siArl8b secretion conditions, respectively; Fig 7E, left graph). The average number of α -syn puncta detected in acceptor cells in co-cultures and secretion conditions was not significantly changed (1-2 puncta per cell in all the conditions; Fig 7E, right graph).

To further test the importance of lysosome positioning in α -syn fibrils transfer, we performed similar experiments in HeLa cells with knock out (KO) of components of the lysosome-positioning machinery. These components included the myrlysin (also known as BORCS5) subunit of BORC, which promotes recruitment of Arl8b to membranes [100], and the kinesins KIF1B and KIF5B [101–105], which drive lysosome movement towards the cell periphery [90]. KO of these proteins impairs lysosome movement towards the cell periphery [90,100]. These KO donor cells, in parallel to control donors (WT HeLa) were loaded with α -syn fibrils and co-cultured with WT HeLa cells expressing H2B-GFP (Figs S3F and S6C). Acceptor cells were then analysed for the presence of α -syn fibrils. The percentage of acceptor cells that received α -syn fibrils in control co-culture was $29 \pm 2\%$, whereas in myrlysin-KO and KIF1B-KIF5B-double-KO (KIF1B-5B dKO) co-cultures this percentage was significantly decreased to $16 \pm 2\%$ and $12 \pm 2\%$, respectively (S6D Fig, left graph). α -Syn fibrils' transfer through secretion was similar in all the conditions ($5 \pm 2\%$, $6 \pm 1\%$, $6 \pm 1\%$ in control, myrlysin-KO, and KIF1B-5B dKO secretion conditions, respectively; S6D Fig, left graph). We did not observe a significant difference in the average number of transferred α -syn puncta in any of the co-cultures/secretion conditions (1-2 puncta per cell in all the conditions; S6D Fig, right graph). Overall, these data indicate that the contact-dependent transfer of α -syn is affected by the position of lysosomes, with more transfer when lysosomes are peripheral and less transfer when lysosomes are perinuclear.

As we have shown that lysosomes containing α -syn are transferred from acceptor cells inside TNTs [52] (S2 Fig), we wondered whether TNT formation was affected by the manipulations

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 ($45 \pm 3\%$) and Arl8b-GFP-overexpressing cells ($46 \pm 2\%$; Fig 7F, left graph). However, in the presence of α -syn fibrils the percentage of TNT-connected cells was significantly increased to $60 \pm 5\%$ in control and $64 \pm 5\%$ in Arl8b-GFP-overexpressing cells (Fig 7F, left graph). Similarly, the percentage of TNT-connected cells remained unchanged between control ($44 \pm 4\%$), sicontrol ($45 \pm 4\%$), and siArl8b ($43 \pm 4\%$) pretreated cells, whereas in the presence of α -syn fibrils the percentage of TNT-connected cells significantly increased to $62 \pm 4\%$ in control, $62 \pm 5\%$ in sicontrol, and $66 \pm 3\%$ 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™ 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 μ m. **(B)** % of acceptor cells received α -syn fibrils in control co-culture ($49 \pm 4\%$), in Arl8b-GFP co-culture ($65 \pm 4\%$), in control secretion ($8 \pm 2\%$), and in Arl8b-GFP secretion ($10 \pm 2\%$) (left graph). Mean $\pm$ 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 $\pm$ S.E.M, $n=3$ (90 acceptor cells per condition). ns=not significant, by one-way ANOVA followed by

Tukey's multiple comparison tests. **(C)** Western blot prepared from cell extracts collected from control, scrambled siRNA (sicontrol)-, and siArl8b-pretreated CAD cells for 60 h, showing Arl8b and α -tubulin (loading control) expression. Integrated Density of Arl8b protein expression levels normalized to α -tubulin in control (0.80 ± 0.03), sicontrol- (0.82 ± 0.02), and siArl8b-pretreated cells (0.19 ± 0.09) are presented in Arbitrary Units (AU). Mean $\pm$ S.E.M, $n=3$. ns= not significant, *** $P=0.0004$ by one-way ANOVA followed by Tukey's multiple comparison tests. **(D)** Representative confocal images of donor and acceptor cells after 24 h of co-culture. Donor CAD cells were pretreated with sicontrol or siArl8b for 60 h or left untreated prior to be challenged with Alexa 568-tagged α -syn fibrils (referred to as control, sicontrol, and siArl8b co-cultures, respectively). Acceptor cells were transfected with H2B-GFP. Co-cultures were labelled with HCS CellMaskTM 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 μ m. **(E)** % of acceptor cells that received α -syn fibrils in control co-culture ($55 \pm 4\%$), sicontrol co-culture ($60 \pm 3\%$), siArl8b co-culture ($28 \pm 3\%$), control secretion ($6 \pm 1\%$), sicontrol secretion ($9 \pm 1\%$), and siArl8b secretion ($6 \pm 1\%$) (left graph). Mean $\pm$ S.E.M, $n=4$ (80 acceptor cells per condition). ns= not significant, **** $P<0.0001$ by one-way ANOVA followed by Tukey's multiple comparison tests. Average number of α -syn puncta per acceptor cell in control co-culture (2 ± 0.2), sicontrol co-culture (2 ± 0.1), siArl8b co-culture (2 ± 0.3), control secretion (1 ± 0.2), sicontrol secretion (2 ± 0.2), and siArl8b secretion (2 ± 0.3) (right graph). Mean $\pm$ S.E.M, $n=4$ (80 acceptor cells per condition). ns= not significant by Kruskal-Wallis nonparametric ANOVA test followed by Dunn's multiple comparison tests. **(F)** Control, Arl8b-GFP transfected, and sicontrol- or siArl8b-pretreated CAD cells for 60 h were challenged or not with α -syn fibrils (18 h) and labelled with WGA for TNT counting experiments. % of TNT-connected cells in control ($45 \pm 3\%$), Arl8b-GFP ($46 \pm 2\%$), control- α -syn ($60 \pm 5\%$), and Arl8b-GFP- α -syn ($64 \pm 5\%$) conditions. Mean $\pm$ S.E.M, $n=4$ (115 cells per condition). ns= not significant (left graph). % of TNT-connected cells in control ($44 \pm 4\%$), sicontrol ($45 \pm 4\%$), siArl8b ($43 \pm 4\%$), control- α -syn ($62 \pm 4\%$), sicontrol- α -syn ($62 \pm 5\%$), and siArl8b- α -syn ($66 \pm 3\%$) is presented (right graph). Mean $\pm$ S.E.M, $n=3$ (90 cells per condition). ns= not significant, * $P<0.05$, ** $P<0.01$ by one-way ANOVA followed by Tukey's multiple comparison tests. The data underlying this Figure may be found in S1 Data.

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

Our data show that more than 50% of α -syn fibrils in acceptor cells colocalize with LAMP1, indicating that they are still associated with lysosomes after the transfer (Fig 5H). Moreover, we have previously reported that α -syn fibrils can induce seeding of soluble α -syn once they are transferred to acceptor cells [19]. Based on the results presented here one possible explanation for this observation would be that α -syn fibrils also trigger LMP in acceptor cells following their transfer, similar to what we have observed in donor cells (Fig 3A and B). To assess this hypothesis, we performed co-culture experiments where donor cells were either transiently transfected with H2B-mCherry (control co-culture) or treated with α -syn fibrils for 18 h (α -syn co-culture), and co-cultured with acceptor cells transiently transfected with Gal3-GFP (Figs 8A and S3G number 1). Acceptor cells were then evaluated for the presence of Gal3-GFP puncta. The percentage of acceptor cells exhibiting LMP (Fig 8A, white arrows) was significantly higher in the α -syn co-culture ($33 \pm 5\%$) compared to control co-culture ($6 \pm 2\%$) (Fig 8B, upper graph). We also quantified the average number of Gal3-GFP puncta per acceptor cell and we found a significant increase in α -syn co-culture (14 ± 1) compared to control co-culture where some LMP occurs spontaneously (8 ± 1 ; Fig 8B, lower graph). We also performed super-resolution microscopy in α -syn co-culture labelled for lysosomes with antibody to LAMP1 and observed Gal3-GFP and α -syn fibrils puncta both in close proximity inside the lysosomal lumen (Fig 8C) and colocalizing on the lysosomal membrane (Fig 8D). These results thus show that, following their transfer, α -syn-containing lysosomes undergo LMP in acceptor cells.

Fig 8. α -Syn fibrils induce LMP upon arrival in the acceptor cells. (A) Representative confocal images of donor and acceptor cells after 24 h of co-culture. Donor cells were transfected with H2B-mCherry (control co-culture) or treated with Alexa 568-tagged α -syn fibrils (α -syn co-culture). Acceptor cells were transfected with Gal3-GFP. Co-cultures were labelled with HCS CellMaskTM Blue. Red arrows indicate the α -syn fibrils received by acceptor cells, white arrows indicate the Gal3-GFP puncta formation (LMP) in the acceptor cells that received α -syn fibrils. Scale bar: 10 μ m. (B) % of acceptor cells under LMP in control ($6 \pm 2\%$) and α -syn ($33 \pm 5\%$) co-cultures (upper graph). Average number of Gal3-GFP puncta formation in acceptor cells under LMP in control (8 ± 1) and α -syn (14 ± 1) co-cultures (lower graph). Mean $\pm$ S.E.M, $n=5$ (50 acceptor cells per condition). * $P=0.04$, **** $P<0.0001$ by Mann-Whitney U - test. (C) SR image of a co-culture where donor cells were treated with α -syn fibrils for 18 h and acceptor cells were transfected with Gal3-GFP, labelled for LAMP1-Alexa 647

antibody (pseudo colored in grey). A selected region where Gal3-GFP puncta were observed (indicated by a white square in the acceptor cell of the co-culture image), was further magnified to observe one lysosome (indicated by the small white square) having α -syn fibrils puncta and recruited Gal3-GFP. Image was acquired with a Spinning Disk microscope with SR module. Scale bar: 10 μ m (for the inset: 0.2 μ m). **(D)** SR image of an acceptor cell demonstrating Gal3-GFP puncta recruited at the lysosomal membrane where α -syn fibrils were detected in close proximity. Selected lysosome is indicated by a white square. Image was acquired by SR SIM (Zeiss, LSM 780-Elyra PS.1). Scale bar: 5 μ m (for the inset: 0.5 μ m). In C, the signal was boosted to make the selected lysosome visible; in D, the acceptor cell was presented with HCS CellMaskTM Blue labelling as this cell was poorly transfected but accumulation of Gal3-GFP puncta was clearly detectable. The data underlying this Figure may be found in S1 Data.

α -Syn fibrils induce seeding after their transfer into acceptor cells and this event is preferentially associated with lysosomes.

To further investigate the seeding mechanism in acceptor cells that received lysosomes containing α -syn fibrils, we used again our α -syn-GFP soluble fluorescent reporter expressed in acceptor cells co-cultured with donor cells, either challenged with α -syn fibrils or transfected with H2B-mCherry (α -syn co-culture and control co-culture respectively; Figs 9A and S3G number 2). We then quantified the presence of α -syn-GFP puncta (indicating aggregation of the soluble protein; Fig 9A, white arrows) in the acceptor cells that received α -syn fibrils (Fig 9A, red arrows). We found that 27 ± 4 % of acceptor cells contained 7 ± 1 α -syn-GFP puncta in α -syn co-culture, whereas in control co-culture only 10 ± 4 % of the acceptor cells had 2 ± 0.2 α -syn-GFP puncta (Fig 9B).

Next, to address whether the seeding in acceptor cells was associated with lysosomes, we performed super-resolution microscopy. After labelling the cells in co-culture with antibody to LAMP1, we examined the subcellular localization of α -syn-GFP aggregates in acceptor cells. We observed four different scenarios: i) lysosomes containing α -syn fibrils and colocalizing with α -syn-GFP puncta surrounding the lysosomal membrane (Fig 9C), ii) lysosomes filled with α -syn fibrils with discrete α -syn-GFP deposits on their membrane (Fig 9D), iii) lysosomes containing α -syn-GFP puncta with α -syn fibrils in close proximity (Fig 9E), and iv) lysosomes containing both α -syn fibrils and α -syn-GFP puncta partially colocalizing in their lumen (Fig 9F). In addition, we performed 3D object-based colocalization analysis between α -syn-GFP

puncta, α -syn fibrils, and LAMP1+ lysosomes. We observed that $60 \pm 7\%$ of the newly formed α -syn-GFP aggregates colocalized with LAMP1+ lysosomes (Fig 9G). Overall, these data indicate that the majority of the seeding of endogenously expressed soluble α -syn takes place at lysosomal compartments in acceptor cells after the transfer of α -syn fibrils.

Fig 9. α -syn fibrils induce seeding after their transfer into acceptor cells and this event is frequently associated with lysosomes. (A) Representative confocal images of donor and acceptor cells after 24 h of co-culture. Donor cells were transfected with H2B-mCherry (control co-culture) or treated with Alexa 568-tagged α -syn fibrils for 18 h (α -syn co-culture). Acceptor cells were transfected with α -syn-GFP. Inset of acceptor cells (indicated by white squares) is presented at the right top corner of acceptor cells' panel. White arrows indicate α -syn-GFP aggregate formation, red arrows indicate α -syn fibrils received by acceptor cells in α -syn co-culture. Scale bar: 10 μ m (for the inset: 5 μ m). (B) % of acceptor cells that formed α -syn-GFP aggregates in control ($10 \pm 4\%$) and in α -syn ($27 \pm 4\%$) co-cultures (left graph). Average number of α -syn-GFP puncta detected in acceptor cells in control (2 ± 0.2) and in α -syn (7 ± 1) co-cultures is presented (right graph). Mean $\pm$ S.E.M, $n=3$ (150 acceptor cells per condition). * $P=0.01$ and ** $P=0.003$ for % of aggregates and average number of aggregates in control versus α -syn co-cultures, respectively, by Mann Whitney U -test. (C) SR image showing a lysosome containing α -syn fibrils puncta colocalizing with α -syn-GFP+ aggregate at the lysosomal membrane. (D) SR image showing a lysosome containing α -syn fibrils where α -syn-GFP+ aggregate is located mostly in a restricted portion of the lysosomal membrane. (E) SR image showing a lysosome having α -syn-GFP+ aggregate where α -syn fibrils puncta were found to be in close proximity of the lysosomes. (F) SR image showing a lysosome where both α -syn fibrils and α -syn-GFP aggregates are located to the lumen of the lysosome and partially colocalizing. (G) Object-based 3D analysis (Imaris software) of colocalization between α -syn-GFP and LAMP1+ lysosomes ($60 \pm 7\%$). Mean $\pm$ S.E.M, $n=2$ (15 super-resolution images, ~3000 lysosomes were analysed). Insets of images in C, D (acquired with Spinning-Disk microscope with SR module) and E, F (acquired with SR SIM, Zeiss, LSM 780-Elyra PS.1) from four selected regions (white squares) and from different acceptor cells transfected with α -syn-GFP and that received α -syn fibrils, labelled for LAMP1 Alexa 647 antibody (pseudo colored in grey). Scale bars: 10 μ m (for the insets: 1 μ m). The data underlying this Figure may be found in S1 Data.

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;

Fig 10B). Furthermore, we observed that in α -syn co-culture both lysosomes containing α -syn fibrils (Figs 10A, yellow arrows, and S7G, red arrow) or not (Figs 10A, white arrows, and S7G, white arrow) were transferred to acceptor cells.

Next, to evaluate the amount of lysosomes having LMP that were transferred from CAD cells to HeLa cells, we quantified the occurrence of LMP in CAD cells in our heterotypic co-culture condition. Thus, we counted the number of Gal3-GFP puncta in donor CAD cells transfected with Gal3-GFP, challenged or not with α -syn fibrils, and co-cultured with HeLa-Gal3-Turquoise cells (S7D Fig, green arrows). As expected, the percentage of donor CAD cells having LMP was significantly higher in α -syn loaded donor cells ($11 \pm 4\%$) compared to control donors ($2 \pm 2\%$), whereas the average number of Gal3-GFP puncta per cell did not show any statistically significant change (10 ± 2 and 14 ± 4 in control and α -syn donors, respectively; S7E Fig). These findings indicate that, in co-culture conditions, donor CAD cells challenged with α -syn fibrils undergo LMP similar to what we showed earlier (Fig 3A and B). Next, we evaluated the transfer of donors' lysosomes with LMP by quantifying the presence of Gal3-GFP puncta in acceptor HeLa-Gal3-Turquoise cells both in control and α -syn co-cultures (Fig 10C). We found a significantly higher percentage of HeLa cells with Gal3-GFP puncta in the α -syn co-culture ($13 \pm 2\%$; Fig 10C, green arrows, and D, left graph) compared to control co-culture ($4 \pm 1\%$; Fig 10D, left graph). We did not observe a significant difference between the average number of Gal3-GFP puncta per acceptor cell in the two co-cultures (2 ± 0.2 and 2 ± 0.3 in control and α -syn co-cultures, respectively; Fig 10D, right graph). These data indicate that CAD cells challenged with α -syn fibrils transfer more damaged lysosomes (i.e., undergoing LMP) to acceptor cells compared to control CAD cells. In addition, we were able to detect donors' lysosomes colocalizing both with α -syn fibrils and Gal3-GFP puncta in acceptor HeLa cells (Fig 10E), demonstrating that damaged lysosomes carrying α -syn fibrils are transferred into the acceptor cells.

By taking advantage of HeLa cells stably expressing Gal3-Turquoise as acceptor cells, we also evaluated the occurrence of LMP in acceptor cells by assessing the Gal3-Turquoise puncta (Fig 10F, turquoise arrows). We found that $18 \pm 3\%$ of acceptor cells undergo LMP with an average of 10 ± 1 Gal3-Turquoise puncta per cell in α -syn co-culture (Fig 10G). LMP in the acceptor cells of control co-culture was significantly lower ($3 \pm 1\%$) with an average of 9 ± 1 Gal3-Turquoise puncta per cell (Fig 10G). In addition, by using antibody to human LAMP1, we also detected colocalization with Gal3-Turquoise puncta in acceptor cells (S7F Fig, arrow). These results suggest that α -syn fibrils derived from CAD cells are able to induce LMP in the acceptor

HeLa cells following their transfer. Interestingly, in very few cases we could also detect colocalization between Gal3-Turquoise puncta and donors' lysosomes labelled with anti-mouse LAMP1 antibody in acceptor HeLa cells (S7G Fig, white arrow), suggesting that damaged donors' lysosomes can undergo LMP upon arrival to the acceptor cells.

Finally, to evaluate lysosome transfer from acceptor to donor cells, the CAD-HeLa co-culture was immunolabelled with anti-human LAMP1 antibody and donor CAD cells were evaluated for the presence of acceptors' lysosomes (Fig 10H). The percentage of donor cells that received acceptors' lysosomes in control co-culture was $14 \pm 4\%$ whereas in α -syn co-culture this percentage was significantly higher ($34 \pm 5\%$; Fig 10I, left graph). The average number of acceptors' lysosomes per donor cell in both co-culture conditions was similar (2 ± 0.3 and 2 ± 0.1 in control and α -syn co-cultures, respectively; Fig 10I, right graph). To understand whether lysosomes transferred from acceptor to donor cells were damaged, we quantified the presence of Gal3-Turquoise puncta in donor CAD cells in both in the α -syn and control co-cultures (S7H Fig). Only $4 \pm 1\%$ of donor cells resulted positive for Gal3-Turquoise puncta in α -syn co-culture, while we could barely ($0.3 \pm 0.3\%$) observe donor cells positive for Gal3-Turquoise puncta in control co-culture (S7I Fig). Taken together, these results demonstrate that donor cells challenged with α -syn fibrils receive healthy lysosomes from acceptor cells, while the transfer of damaged lysosomes from the acceptor cells is a rare event. This suggests the occurrence of a rescue mechanism towards donor cells overloaded with α -syn, similar to what was previously reported in the case of a lysosomal disease [106].

Fig 10. Intercellular lysosome transfer is reciprocal. (A) Representative confocal image of CAD-HeLa co-culture where donor mouse CAD cells were treated with Alexa 568-tagged α -syn fibrils (18 h) and co-cultured with acceptor human HeLa stable Gal3-Turquoise cells for 24 h, labelled for anti-LAMP1 mouse antibody and DAPI. In acceptor HeLa Gal3-Turquoise cells, arrows indicate donors' lysosomes containing (yellow) or not (white) α -syn fibrils. Inset represents a selected region (white square) of one acceptor cell containing donors' lysosomes. Scale bar: 10 μ m (for the inset: 2 μ m). **(B)** % of acceptor HeLa cells that received lysosomes from donor CAD cells in control ($32 \pm 5\%$) and in α -syn ($52 \pm 4\%$) co-cultures (left graph). **P=0.006 by two-tailed *t*-test. Average number of lysosomes transferred from donor to acceptor cells in control (3 ± 0.3) and in α -syn (3 ± 0.3) co-cultures (right graph). Mean $\pm$ S.E.M. ns= not significant by Mann-Whitney *U*-test. **(C)** Representative confocal image of 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 μ m. **(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 μ m (for the
 913 inset: 1 μ m). **(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 μ m. **(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 μ m (for the inset: 5 μ m).
 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

cells [74]. Specifically, LMP has been described in several cell types challenged with α -syn fibrils, such as the neuronal cell lines SH-SY5Y and N27, as well as human dopaminergic neurons derived from induced pluripotent stem cells [73,74]. Here, we showed that α -syn fibrils induce LMP also in neuronal CAD cells (Fig 3A and B). By using SIM, we observed the recruitment of Gal3 to the damaged lysosomes displaying α -syn fibrils in their lumen or limiting membrane (Fig 3D and E). Importantly, we demonstrated that α -syn fibrils trigger LMP also in the recipient cells following their intercellular transfer (Fig 8A and B). LMP in acceptor cells has been only reported in a co-culture system where donor N27 cells expressing α -syn were pretreated with the mitochondrial toxin MPP⁺ (1-methyl-4-phenylpyridine) to induce PD-like pathology and co-cultured with acceptor cells stably expressing Gal3 [73]. Our results indicate that, without inducing a PD-like pathology in donor cells, the transfer of α -syn fibrils ‘per se’ is sufficient to induce LMP in acceptor cells both in homotypic (Fig 8A and B) and heterotypic co-culture systems (Fig 10F and G). In addition, by performing super-resolution microscopy, we also detected the recruitment of Gal3 to damaged lysosomes bearing α -syn fibrils either in their lumen or at their membrane in the acceptor cells, similar to what we observed in donor cells (Fig 8C and D). Interestingly, our results show that lysosomes containing α -syn fibrils are not targeted for lysophagy [84] (S4 Fig), possibly suggesting a rescue mechanism such as the one orchestrated by the ESCRT machinery [111,112]. Considering the evidence supporting autophagy impairment in neurodegenerative diseases and more specifically following α -syn accumulation [55,113], and the recent findings that impaired autophagic flux contributes to the occurrence of LMP [114], it will be interesting to assess the contribution of autophagy to LMP, lysosomal dysfunction, and propagation of α -syn.

The underlying mechanism of LMP induction by α -syn fibrils is unclear. Several studies have reported the ability of α -syn protofibrils to permeabilize membranes [115–117]; thus, as suggested by Freeman et al., the luminal α -syn fibrils could induce membrane curvature causing the rupture of lysosomes [73]. Our correlative EM data show a wavy limiting membrane in lysosomes containing α -syn fibrils compared to lysosomes devoid of α -syn fibrils, supporting this hypothesis. Moreover, oxidative stress could also compromise lysosomal integrity [118]. Consistently, we have previously reported that α -syn fibrils increase ROS levels in CAD cells [19], and enhanced ROS levels have been found in PD mouse models and PD patients [68,119]. Since α -syn fibrils located to the lysosomes need to directly interact with the soluble cytosolic α -syn in order to induce the seeding, LMP has been proposed as a relevant mechanism to allow the escape of α -syn fibrils from the lysosomes [19,85]. Co-localization between Gal3 and aggregated α -syn has been reported in the basal forebrain of Diffused Lewy Body Disease

patients but not in normal, age-matched controls, supporting the role of lysosome rupture in α -syn pathology and progression [85]. In the current study, we detected efficient seeding of soluble α -syn both in donor cells treated with α -syn fibrils and in acceptor cells that received the fibrils inside lysosomes from donor cells (Figs 4A and B and 9A and B). By using super-resolution microscopy, we observed that $69 \pm 6\%$ and $60 \pm 7\%$ of the newly formed α -syn aggregates colocalize with LAMP1+ lysosomes in the donor and acceptor cells, respectively. These data together with live imaging show that the seeding event begins shortly after the internalization of α -syn fibrils and occurs preferentially at the lysosomes (Figs 4C-I, 9C-G, S5A, and S1 Movie). It has been shown that specific environments inside membranous and vesicular structures favor the aggregation of the α -syn protein [120,121]; thus, our results indicate that lysosomes can create a confined environment facilitating the conversion of soluble α -syn.

Do lysosomes contribute to the spread of the disease? Lysosomes are highly dynamic organelles that move bidirectionally along microtubules between the center and the periphery of the cell [122]. In non-polarized cells, lysosomes are mostly clustered at the perinuclear region, but a more dynamic pool is located at the cell periphery [90,92,100]. Here, we demonstrated that α -syn fibrils act on lysosome positioning by inducing their peripheral redistribution (Fig 5A and B). We also found that peripheral α -syn-containing lysosomes exhibit lysosomal dysfunction (i.e., disturbed pH and decreased lysosomal enzyme activities; Fig 5C). In addition, we showed that donor cells challenged with α -syn fibrils, thus having more peripheral lysosomes, transfer lysosomes more efficiently to the recipient cells compared to untreated donor cells (Fig 5F and G). Moreover, the peripheral distribution of lysosomes positively affects α -syn fibrils' transfer both in CAD and HeLa cells (Figs 7A and B and S6A and B).

The induction of LMP has been recently shown to promote nuclear translocation of TFEB [123], which influences lysosomal motility and exocytosis [95]. Furthermore, TFEB overexpression promotes α -syn clearance [124]. Thus, we investigated whether the loading of lysosomes with α -syn fibrils triggers TFEB nuclear translocation and whether this translocation participates in α -syn-induced distribution of lysosomes to the cell periphery. We found that α -syn fibrils induced TFEB translocation to the nucleus (Fig 6A). Moreover, expression of active TFEB correlates with more peripheral distribution of lysosomes (Fig 6B), while silencing of the endogenous TFEB prevents the peripheral redistribution of lysosomes upon α -syn loading (Fig 6D) suggesting the involvement of TFEB in this event. However, how this occurs is not yet clear. Previous reports suggested that TFEB-regulated transcription induced lysosomal docking to the plasma membrane, thus promoting the peripheral distribution of lysosomes in

HeLa cells [95]. However, TFEB and the related transcription factor TFE3 have been reported to contribute to perinuclear positioning of lysosomes through transcriptional activation of the lysosomal protein TMEM55B under starvation conditions in HeLa cells [96]. It has also been reported that overexpression of TFEB promotes perinuclear positioning of the lysosomes in mouse fibroblast cells defective for the lysosomal transporter cystinosis [97]. Given these controversies in the literature, the exact role of TFEB in the regulation of lysosome positioning remains to be addressed in the future.

To our knowledge, this is the first report showing that α -syn fibrils influence lysosome positioning, favoring α -syn fibrils transfer in a cell-to-cell contact-dependent manner via lysosomes. These findings suggest that lysosomes play an important role in the intercellular trafficking of α -syn fibrils. It is worth noting that α -syn fibrils do not affect lysosomal exocytosis (Fig 5D and E). In addition, in the secretion tests used in each co-culture experiment, we never detected any significant difference in the transfer of α -syn fibrils and/or lysosomes through the cell supernatant. Although this was not the aim of our study, these results further confirm that transfer of α -syn fibrils primarily relies on TNT- and/or cell-to-cell-contact-dependent mechanisms in accordance with our previous findings [19,20,22]. The mechanism by which α -syn fibrils enhance TNT formation is still largely unknown. However, we and others demonstrated that cells form more TNTs under stress, such as when they are treated with H_2O_2 [125,126]. In line with these findings, we previously reported that CAD cells bearing α -syn fibrils exhibit sustained increase in ROS levels [19]. Therefore, α -syn fibrils can indeed trigger stress in the cells in order to induce TNT formation. In addition, it has been reported that α -syn could induce membrane remodeling, such as membrane expansion and formation of cylindrical tubes, and that the extent of membrane expansion correlated linearly with the density of α -syn [127]. Thus, further studies are essential to address how α -syn fibrils participate in the formation of TNTs.

Lysosome transfer through TNTs has been shown to be bidirectional [106]. However, there is no study on the directionality/origin and the nature (whether they are healthy or damaged) of the lysosomes transferred between cells in the presence of the α -syn fibrils. Thus, in the current study, we studied the transfer of both healthy and damaged (i.e., under LMP) lysosomes between CAD (donor) and HeLa (acceptor) cells and found that the transfer of both types of lysosomes is bidirectional (Fig 10A, B, H, and I). We showed that α -syn fibril-treated donor cells transfer more lysosomes (both healthy and damaged) to acceptor cells compared to untreated control donor cells. Interestingly, we observed the recruitment of acceptor's Gal3 to the donor's lysosomes transferred in an acceptor cell (S7G Fig), suggesting that the rupture of

donors' lysosomes containing α -syn fibrils could occur after their transfer in the recipient cells. Focusing on the transfer of lysosomes from acceptors to donors, we found that α -syn fibril-treated donor cells received more healthy acceptors' lysosomes compared to control untreated donor cells (Fig 10H and I). We also detected damaged acceptors' lysosomes in α -syn fibril-treated donor cells although this was a very rare event (S7H and I Fig). Based on these data we suggest that in the context of α -syn spreading, donor cells loaded with α -syn fibrils, try to get rid of α -syn fibrils through the transfer of lysosomes, thus promoting the spreading of the fibrils. On the other hand, donor cells receive healthy lysosomes from acceptor cells, suggesting a potential rescue mechanism. A similar rescue mechanism has been previously reported in cystinosis-deficient fibroblasts, which receive cystinosis-bearing lysosomes from healthy macrophages through TNTs [106].

In light of these findings, we propose a working model on how lysosomes contribute to the transfer and propagation of α -syn fibrils (Fig 11). After internalization, α -syn fibrils localize to lysosomes (Fig 11, number 1), where they induce LMP and recruit Gal3 (Fig 11, number 2). α -Syn fibrils escape from damaged lysosomes and seed soluble α -syn in donor cells, an event that occurs mostly in association with the lysosomes (Fig 11, number 3). Following α -syn fibrils internalization, TFEB translocates to the nucleus (Fig 11, number 4) and participates in the redistribution of lysosomes containing α -syn fibrils, likely under LMP or functionally impaired, towards the cell periphery (Fig 11, number 5). Here, lysosomes are not exocytosed (Fig 11, number 6), but are more prone to be transferred to other cells through TNTs. Indeed, cells loaded with α -syn fibrils transfer more lysosomes (both under LMP or not) to acceptor cells compared to healthy cells through TNTs (Fig 11, number 7). Once lysosomes reach the acceptor cells, α -syn fibrils escape from lysosomes already under LMP or from lysosomes that undergo LMP when they arrive in the acceptor cells. This again allows α -syn fibrils to seed soluble α -syn in the acceptor cells, mostly in association with the lysosomes (Fig 11, number 8) and further triggering LMP (Fig 11, number 9). In an attempt to rescue α -syn-overloaded donor cells, acceptor cells transfer healthy lysosomes to donor cells (Fig 11, number 10).

In conclusion, our data explain how lysosomes are involved in the transfer and propagation of α -syn aggregates, and the crucial role that they play in this process. In agreement with these findings, we have previously reported that α -syn fibrils can be efficiently transferred between neurons, primary astrocytes, neurons and astrocytes, astrocytes and organotypic hippocampal slices and between iPSCs and they were associated to lysosomes in these co-culture systems [19,21,56]; however we have used here undifferentiated neuronal CAD cells line, for their easy manipulation. Although our results contribute to a better understanding of the mechanism of

development and progression of α -syn pathology, opening new avenues for possible therapeutic approaches against synucleinopathies, it will be necessary in the future to investigate the transfer of α -syn containing lysosomes in differentiated primary neurons and/or in ex-vivo/in-vivo systems to further validate our data and overcome the limitation of this study.

Fig 11. Working Model. **1)** α -Syn fibrils localize to lysosomes following their uptake. **2)** α -Syn fibrils induce LMP (i.e., recruit Gal3 to the ruptured lysosomes) in donor cells following their uptake, allowing their escape from lysosomes. **3)** α -Syn fibrils interact with soluble α -syn and induce seeding in donor cells. **4)** Following α -syn fibrils internalization, TFEB translocates to the nucleus. **5)** Lysosomes (possibly under LMP, as their pH is higher and lysosomal activities is lower) having the α -syn fibrils move towards the cell periphery. **6)** Peripheral lysosomes are not preferentially exocytosed. **7)** Lysosomes having α -syn (both under LMP or not) are transferred to acceptor cells along with healthy lysosomes. **8)** α -Syn fibrils induce LMP following their transfer to acceptor cells. **9)** α -Syn fibrils induce further seeding following their 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 GlutaMAXTM (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-GlutaMAXTM 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 ampicillin. 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

fibrils were labelled with Alexa Fluor 488 or 568 Protein Labelling Kit (Invitrogen) following the manufacturer's instructions. After the incubation of fibrils with Alexa Fluor dyes, samples were ultracentrifuged again and the pellets were re-suspended in 30 mM Tris-HCl, pH 7.5 prior to be ultracentrifuged one more time. Finally, labelled α -syn fibrils were resuspended in saline containing 0.1% sodium azide and the protein concentration of the fibrils was determined by RP-HPLC as described before [86,135]. Seeding activity of the fibrils were checked by adding the labelled fibrils (3 μ g) to 100 μ L of 1 mg/mL α -syn monomer solution in 30 μ M Thioflavin T and 80 mM Hepes, pH 7.5. Amyloid-like fibril formation was continuously monitored by measuring the Thioflavin T fluorescence (excitation 442 nm, emission 485 nm) with a microplate reader (Infinite 200, TECAN).

0.5 μ M Alexa 488 or Alexa 568-tagged α -syn fibrils were diluted in the appropriate medium and sonicated for 5 min at 80% amplitude with a pulse cycle of 5 s on and 2 s off in an ultrasonic water bath Vibra-Cell 75,041 (BioBlocks Scientific). Fibrils were then introduced to cells immediately and incubated for 18 h. Before cells were processed for further procedures (e.g., co-culture preparation, fixation, etc.), they were washed 3 times with diluted trypsin (1/3) in phosphate buffered saline (PBS). In parallel, control cells were subjected to the same treatments as described above without the addition of α -syn fibrils.

Immunocytochemistry for lysosomes

CAD and/or HeLa cells and co-cultures (CAD-CAD and CAD-HeLa) were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature (RT). After 3 washes with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 3 min at RT. Cells were then washed with PBS and nonspecific antibody binding sites were blocked by using 10% of goat serum (GS) in PBS for 30 min. Cells were then incubated with primary rat monoclonal anti-mouse LAMP1 (1D4B) or anti-human LAMP1 (H4A3) primary antibodies with the dilution of 1/200 in blocking solution for 1 h. Antibodies were purchased from Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA. Cells were then washed 3 times with PBS and incubated for secondary antibodies (anti-rat Alexa-647 or anti-rat Alexa-488 purchased from Invitrogen, Carlsbad, CA) diluted 1:500 in blocking solution for 2 h at RT. After 3 washes with PBS, cells were further stained with HCS CellMaskTM Blue (Invitrogen) for 15 min at RT (diluted 1:5000 in PBS). Cells were then washed 3 times with PBS and further stained for DAPI (diluted 1:5000 in PBS) for 5 min. Finally, cells were washed and mounted with Aqua-

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

Immunocytochemistry for lysophagy

CAD cells were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature (RT). After 3 washes with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 3 min at RT. Cells were then washed with PBS and nonspecific antibody binding sites were blocked by using 2% of bovine serum albumin (BSA) in PBS for 30 min. Cells were then incubated with primary rabbit anti-mouse LC3 (1:500, MBL International), mouse anti-Ubiquitin (1:1000, FK2, Enzo), and rat monoclonal anti-mouse LAMP1 (1D4B) (1:100, DSHB) in blocking solution for 1 h. Cells were then washed 3 times with PBS and incubated for secondary antibodies (anti-rabbit Alexa 488, anti-mouse Alexa-647 from Invitrogen, Carlsbad, CA; and anti-rat CFTM405M from Sigma) diluted 1:500 in blocking solution for 1 h at RT. Finally, cells were washed and mounted with Aqua-Poly/Mount (Polysciences, Inc.). Image acquisitions were performed by confocal microscopy with identical settings.

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

CAD cells were plated on 35 mm Ibidi μ -dishes (100.000 cells/dish). Alexa 568-tagged α -syn fibrils were introduced or not (control) to the cell culture medium 24 h after plating. Following 18 h of α -syn fibril uptake, 20 nM LysoTracker Deep Red (Invitrogen) was prepared in culture medium and cells were incubated for 20 min at 37°C. Cells were then fixed with 4% PFA at RT and stained for HCS CellMaskTM Blue and DAPI as described before. Image acquisitions were performed by confocal microscopy with identical settings.

To detect Cathepsin B (CathB) activity, CAD cells were plated on 35 mm Ibidi μ -dishes (100.000 cells/dish). Alexa 488-tagged α -syn fibrils were introduced or not (control) to the cell culture medium 24 h after plating. Following 18 h of α -syn fibril uptake, cells were treated with 1X Magic Red Cathepsin solution, provided by Magic Red Cathepsin Assay kit (ImmunoChemistry Technologies LLC) and prepared through the manufacturer's recommendation, for 15 min at 37°C. Cells were then immediately imaged by confocal microscopy in live condition. The intracellular activity of CathB was detected as red fluorescent puncta produced by the hydrolysis of the fluorogenic substrate introduced. All images were acquired with the identical settings. For both experiments integrated density of LysoTracker Deep Red and CathB signal were measured by using FiJi software after a region of interest

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

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

TNT labelling

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

Lysosomal exocytosis analysis

Evaluation of released Cathepsin B by ELISA

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

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

Evaluation of LAMP1 at the plasma membrane

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

Detection of LMP and seeding

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

Co-culture experiments

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

experiment). They were then washed once with PBS and 3 times with 1:3 diluted trypsin before co-culture preparation.

Acceptor cells were plated on T25 flasks (800.000/flask) and transfected with suitable plasmids according to the experiment (Gal3-GFP, H2B-mCherry, H2B-GFP, α -syn-GFP). HeLa Gal3-Turquoise cell line was used directly as acceptor cell population. Both donor and acceptor cells were then detached (mechanically and by trypsinization for CAD and HeLa cells respectively) and counted. Co-culture was prepared in a 1:1 ratio (100.000 donor and 100.000 acceptors on 35 mm Ibidi μ -dishes) for 24 h. Cells were then fixed with 4% PFA for 20 min at RT and stained for HCS CellMaskTM Blue and DAPI as described before and mounted. Schematic presentation of different co-culture preparations was presented in S3 Fig.

Of note, co-cultures were named with the predominant treatment of the donor cells: i) co-cultures having donor cells treated with α -syn fibrils or not were referred to as “ α -syn co-culture” and “control co-culture” respectively; ii) in the cases where all donor populations were treated with α -syn fibrils, in order to emphasize the different treatments between donor cells, co-cultures were named by these treatments (e.g., Arl8b-GFP over-expressing or siArl8b pretreated donors were referred to as “Arl8b-GFP co-culture” and “siArl8b co-culture” respectively).

Secretion test

In all co-culture conditions, donor cells and acceptor cells were also plated separately during the co-culture preparation (100.000 cells/35 mm Ibidi μ -dishes) for the total duration of co-culture incubation (24 h). Then the conditioned medium collected from donor cells were transferred onto acceptor cells. Acceptor cells were then quantified for the presence of specific cargos (e.g., lysosomes, α -syn fibrils) to assess the cargo transfer through secretion in each co-culture condition. Secretion tests for particular experiments were referred to as predominant treatment of the donor cells as described above (e.g., “control secretion”, “ α -syn secretion”, “Arl8b-GFP secretion”).

Confocal microscopy

After fixation and immunostaining, images were acquired with an inverted laser scanning confocal microscope LSM700 (Zeiss), with a 63X objective (zoom 1.0). Images were acquired 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 μ g/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].

1374

1375 **Live imaging microscopy**

1376 Time-lapse microscopy imaging was performed on an inverted Spinning Disk microscope
1377 (Elipse Ti microscope system, Nikon Instruments, Melville, NY, U.S.A.) using 60X 1.4 NA
1378 CSU oil immersion objective lens and Laser illumination 488 only or together with 561. For
1379 live cell imaging, the 37°C temperature was controlled with an Air Stream Stage Incubator,
1380 which also controlled humidity. Cells were plated in Ibidi μ -Dish 35 mm and incubated with
1381 5% CO₂ during image acquisition. Image processing and movies were realized using
1382 MetaMorph and ImageJ/Fiji software.

1383

1384 **Electron Microscopy**

1385 *Correlative Resin Electron Microscopy*

1386 For correlation of FM and resin-embedded EM of CAD cells, FM imaging was performed prior
1387 to sample preparation in EM. Cells were transiently transfected with LAMP1-GFP using
1388 Lipofectamine 2000 transfection reagent for 24 h and seeded on carbon-coated, gridded
1389 coverslips prepared as described before [76]. 24 h later, they were treated with α -syn- Alexa568
1390 fibrils for 18 h. Cells were then washed in 0.1 M phosphate buffer (PB) and fixed in 4%
1391 formaldehyde in 0.1 M PB buffer after which fluorescent Z-stacks of cells of interest were
1392 obtained for the GFP and Alexa 568 signal. The position of cells relative to the pattern etched
1393 in the coverslip was registered using polarized light. After FM imaging, the same specimens
1394 were prepared for EM by fixing with 2.5% glutaraldehyde + 2% formaldehyde in 0.1 M PB
1395 buffer for 2 h, and post fixation with osmium tetroxide and uranyl acetate. Fixed cells were
1396 dehydrated using a graded ethanol series, and embedded in Epon resin which was polymerized
1397 for 48 h at 65°C. After polymerization, the glass coverslip was removed from the Epon block
1398 by dissolving it in hydrogen fluoride. The exposed Epon surface was thoroughly cleaned with
1399 distilled water and left to harden overnight at 63°C. Areas of the resin block containing the cells
1400 previously imaged by FM were cut out using a clean razor blade, and glued to empty Epon
1401 sample stubs, with the basal side of the cells facing outwards [75,76]. From these blocks, 70
1402 nm sections were cut and collected on formvar and carbon coated copper 50 mesh support grids.
1403 Thin EPON sections were imaged in a Tecnai 12 TEM (Thermo Fischer Scientific, Eindhoven,
1404 The Netherlands) equipped with a Veleta 2k $\times$ 2k CCD camera (EMSIS, Munster, Germany),
1405 operating at 80 kV. For correlation of FM and EM data, the TEM image of a ROI was overlayed
1406 with FM data using Photoshop. Multiple corresponding spots (e.g. nuclei) on images were

manually selected, after which the correct scaling and transformation steps were performed and high-precision overlays of FM and EM data were generated. We used only linear transformation options to achieve the overlays shown in the Figures.

On-section Correlative Microscopy of thawed cryosections

Similarly, CAD cells were grown on carbon-coated, gridded coverslips, incubated with α -syn Alexa 568-tagged fibrils for 18 h and fixed in 0.1 M PB (pH 7.4) containing 4% PFA that was added to an equal volume of medium. Fixed cells were flat embedded in 12% gelatin by keeping their orientation, cryoprotected in 2.3 M sucrose, and plunge frozen according to previous protocols [137,138]. 70 nm cryosections were picked up on formvar coated copper grids, after which they were immunolabelled for LAMP1 with a secondary Alexa 488-tagged rabbit antibody and 10 nm gold-conjugated Protein A (Cell Microscopy Core, UMC Utrecht, The Netherlands). Finally, sections were labelled using Hoechst 33342 to outline nuclei for correlation. The grids were washed with dH₂O and sandwiched between a microscope slide and a no. 1 coverslip in 2% methylcellulose in dH₂O. Sections were imaged in a Deltavision RT wide field fluorescence microscope (GE Healthcare, U.S.A.) equipped with a Cascade II EM-CCD camera (Photometrics, U.S.A.). Grids were first imaged at 40 $\times$ magnification to form a map of the section, after which regions of interest (ROIs) were selected using 100 $\times$ magnification. After imaging the grids were removed from the microscope slide, thoroughly rinsed with H₂O and contrasted for EM and embedded in methylcellulose containing uranyl acetate. After drying, ROIs were imaged using a Tecnai 12 TEM (Thermo Fischer Scientific) operating at 80 kV, equipped with a Veleta 2k $\times$ 2k CCD camera (EMSIS, Munster, Germany) camera, and running serialEM software. Registration of thin section FM and EM data was performed using Adobe Photoshop, similar to above.

Western immunoblots

For the Western blot analysis, cells were rinsed with PBS and then scraped in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS). The resulting soluble fractions were centrifuged at 18,000 g for 5 min at 4°C, and then, the supernatants were taken as the whole cell lysate, mixed with Laemmli sample buffer (1 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 5% β -mercaptoethanol, and 0.02% bromophenol blue), and boiled for 5 min. For Western blots, equal amounts of proteins (30 μ g/lane) were 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-automatized “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

to discriminate TNTs from other cellular protrusions (such as having thin, straight, and uninterrupted connections that are not touching to the substratum) were counted as “TNTs” and connected by a line using a free-hand line tool. Total number of cells and number of TNT-connected cells were automatically counted by the tool. Thus, data were presented as percentage of TNT connected cells.

Lysosome size (on-section CLEM)

FM and EM images from the same section were overlaid and correlated to each other. LAMP1 immunogold labelled organelles (lysosomes) were selected blindly and their size was measured using the “lasso” tool in Fiji. Afterwards the lysosomes were clustered based on presence of α -syn fibrils, and their size distribution was plotted.

Lysosome size (SR microscopy)

Diameter of lysosomes were measured by using free-hand straight line tool of Fiji software. For each condition (control lysosomes and lysosomes containing α -syn or not), SR images were zoomed, and diameter of randomly selected lysosomes was measured.

Lysosome number

The average number of lysosomes in control cells and in cells treated with α -syn fibrils were counted by using the “Spot detector” tool in ICY software.

Object-based 3D colocalization analysis (Imaris software)

ROI for each was created by using “Surface” tool in the Surpass mode in Imaris. For each cell puncta of interest (Gal3-GFP, α -syn-GFP, α -syn fibrils and LAMP1+ puncta) in different colors were detected by “Spots” tool. Then colocalization was performed first for each pair of interest, and then for triple presence of the puncta by using “Colocalization” tool.

Statistical analysis

Data collected at least from 3 independent experiments was presented as Mean $\pm$ SEM. Exact number of experiments and number of cells analysed per condition for each experiment is presented in the Figure legends. All data were first subjected to a D’Agostino-Pearson omnibus 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.

1510 **Acknowledgements**

1511 The authors thank all the lab members for useful discussion. We are grateful to Institut Pasteur
1512 Image Analysis Hub (IAH) for their help in quantitative image analysis, Audrey Salles from
1513 Unit of Technology and Service Photonic BioImaging platform (UTechS PBI, Institut Pasteur)
1514 and Cécile Leduc from Cell Polarity, Migration and Cancer unit, Institut Pasteur, for their help
1515 in SIM acquisitions. UTechS PBI, part of the France–BioImaging infrastructure network (FBI)
1516 acknowledges Agence Nationale de la Recherche (ANR-10-INSB-04), ANR/FBI and the
1517 Région Ile-de-France Domaine d’Intérêt Majeur-Malinf program for the use of SIM. We thank
1518 Alexandre Dufour for writing the script for the lysosomal positioning analysis, Eric Kowarz for
1519 the pSBbi-PUR and pCMV (CAT) T7-SB100 plasmids, Andrea Ballabio (TIGEM) for TFEB-
1520 WT-GFP and TFEB-mut-GFP plasmids, and Jost Enninga (Institut Pasteur) and Kristine
1521 Schauer (Institut Curie) for discussion. We also acknowledge Cilia de Heus (UMCU) for her
1522 assistance in CLEM experiments.

References

1. Soto C, Pritzkow S. Protein misfolding, aggregation, and conformational strains in neurodegenerative diseases. *Nat Neurosci.* 2018;21: 1332–1340. doi:10.1038/s41593-018-0235-9
2. Gan L, Cookson MR, Petrucelli L, La Spada AR. Converging pathways in neurodegeneration, from genetics to mechanisms. *Nat Neurosci.* 2018;21: 1300–1309. doi:10.1038/s41593-018-0237-7
3. Poewe W. Nature Reviews Disease Primers. *Nat Rev Dis Primer.* : 52.
4. Volpicelli-Daley LA, Gamble KL, Schultheiss CE, Riddle DM, West AB, Lee VM-Y. Formation of α -synuclein Lewy neurite-like aggregates in axons impedes the transport of distinct endosomes. Martin TFJ, editor. *Mol Biol Cell.* 2014;25: 4010–4023. doi:10.1091/mbc.e14-02-0741
5. Braak H, Tredici KD, Rüb U, de Vos RAI, Jansen Steur ENH, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging.* 2003;24: 197–211. doi:10.1016/S0197-4580(02)00065-9
6. Takamatsu Y, Fujita M, Ho GJ, Wada R, Sugama S, Takenouchi T, et al. Motor and Nonmotor Symptoms of Parkinson's Disease: Antagonistic Pleiotropy Phenomena Derived from α -Synuclein Evolvability? *Park Dis.* 2018;2018: 5789424. doi:10.1155/2018/5789424
7. McCann H, Stevens CH, Cartwright H, Halliday GM. α -Synucleinopathy phenotypes. *Parkinsonism Relat Disord.* 2014;20: 62–67. doi:10.1016/S1353-8020(13)70017-8
8. Taguchi K, Watanabe Y, Tsujimura A, Tanaka M. Brain region-dependent differential expression of alpha-synuclein: α -Synuclein Differential Expression. *J Comp Neurol.* 2016;524: 1236–1258. doi:10.1002/cne.23901
9. Chandra S, Chen X, Rizo J, Jahn R, Südhof TC. A Broken α -Helix in Folded α -Synuclein. *J Biol Chem.* 2003;278: 15313–15318. doi:10.1074/jbc.M213128200
10. Varkey J, Isas JM, Mizuno N, Jensen MB, Bhatia VK, Jao CC, et al. Membrane Curvature Induction and Tubulation Are Common Features of Synucleins and Apolipoproteins. *J Biol Chem.* 2010;285: 32486–32493. doi:10.1074/jbc.M110.139576
11. Meade RM, Fairlie DP, Mason JM. Alpha-synuclein structure and Parkinson's disease – lessons and emerging principles. *Mol Neurodegener.* 2019;14:29: 10.1186/s13024-019-0329–1. doi:10.1186/s13024-019-0329-1
12. Huang M, Wang B, Li X, Fu C, Wang C, Kang X. α -Synuclein: A Multifunctional Player in Exocytosis, Endocytosis, and Vesicle Recycling. *Front Neurosci.* 2019;13: 28. doi:10.3389/fnins.2019.00028

- 1558 13. Burré J, Sharma M, Tsetsenis T, Buchman V, Südhof TC. α -Synuclein Promotes SNARE-
1559 Complex Assembly in vivo and in vitro. *Science*. 2011; 1663–1667.
- 1560 14. Sun J, Wang L, Bao H, Premi S, Das U, Chapman ER, et al. Functional cooperation of α -
1561 synuclein and VAMP2 in synaptic vesicle recycling. *Proc Natl Acad Sci*. 2019;116:
1562 11113–11115. doi:10.1073/pnas.1903049116
- 1563 15. Man WK, Tahirbegi B, Vrettas MD, Preet S, Ying L, Vendruscolo M, et al. The docking of
1564 synaptic vesicles on the presynaptic membrane induced by α -synuclein is modulated
1565 by lipid composition. *Nat Commun*. 2021;12: 927. doi:10.1038/s41467-021-21027-4
- 1566 16. Braak H, Rub U, Gai WP, Del Tredici K. Idiopathic Parkinson's disease: possible routes by
1567 which vulnerable neuronal types may be subject to neuroinvasion by an unknown
1568 pathogen. *J Neural Transm*. 2003;110: 517–536. doi:10.1007/s00702-002-0808-2
- 1569 17. Bae E-J, Yang N-Y, Song M, Lee CS, Lee JS, Jung BC, et al. Glucocerebrosidase depletion
1570 enhances cell-to-cell transmission of α -synuclein. *Nat Commun*. 2014;5:
1571 doi:10.1038/ncomms5755. doi:10.1038/ncomms5755
- 1572 18. Domert J, Sackmann C, Severinsson E, Agholme L, Bergström J, Ingelsson M, et al.
1573 Aggregated Alpha-Synuclein Transfer Efficiently between Cultured Human Neuron-
1574 Like Cells and Localize to Lysosomes. Witt SN, editor. *PLOS ONE*. 2016;11: e0168700.
1575 doi:10.1371/journal.pone.0168700
- 1576 19. Abounit S, Bousset L, Loria F, Zhu S, Chaumont F, Pieri L, et al. Tunneling nanotubes
1577 spread fibrillar α -synuclein by intercellular trafficking of lysosomes. *EMBO J*. 2016;35:
1578 2120–2138. doi:10.15252/embj.201593411
- 1579 20. Dilsizoglu Senol A, Pepe A, Grudina C, Sassoon N, Reiko U, Bousset L, et al. Effect of
1580 tolytoxin on tunneling nanotube formation and function. *Sci Rep*. 2019;9: 5741.
1581 doi:10.1038/s41598-019-42161-6
- 1582 21. Loria F, Vargas JY, Bousset L, Syan S, Salles A, Melki R, et al. α -Synuclein transfer
1583 between neurons and astrocytes indicates that astrocytes play a role in degradation
1584 rather than in spreading. *Acta Neuropathol (Berl)*. 2017;134: 789–808.
1585 doi:10.1007/s00401-017-1746-2
- 1586 22. Vargas JY, Loria F, Wu Y, Córdova G, Nonaka T, Bellow S, et al. The Wnt/Ca²⁺ pathway is
1587 involved in interneuronal communication mediated by tunneling nanotubes. *EMBO J*.
1588 2019;38: e101230. doi:10.15252/embj.2018101230
- 1589 23. Desplats P, Lee H-J, Bae E-J, Patrick C, Rockenstein E, Crews L, et al. Inclusion formation
1590 and neuronal cell death through neuron-to-neuron transmission of α -synuclein. *Proc*
1591 *Natl Acad Sci*. 2009;106: 13010–13015. doi:10.1073/pnas.0903691106
- 1592 24. Luk KC, Kehm V, Carroll J, Zhang B, O'Brien P, Trojanowski JQ, et al. Pathological -
1593 Synuclein Transmission Initiates Parkinson-like Neurodegeneration in Nontransgenic
1594 Mice. *Science*. 2012;338: 949–953. doi:10.1126/science.1227157

- 1595 25. Recasens A, Dehay B, Bové J, Carballo-Carbajal I, Dovero S, Pérez-Villalba A, et al. Lewy
1596 body extracts from Parkinson disease brains trigger α -synuclein pathology and
1597 neurodegeneration in mice and monkeys: LB-Induced Pathology. *Ann Neurol*. 2014;75:
1598 351–362. doi:10.1002/ana.24066
- 1599 26. Kordower JH, Chu Y, Hauser RA, Freeman TB, Olanow CW. Lewy body–like pathology in
1600 long-term embryonic nigral transplants in Parkinson’s disease. *Nat Med*. 2008;14:
1601 504–506. doi:10.1038/nm1747
- 1602 27. Masuda-Suzukake M, Nonaka T, Hosokawa M, Kubo M, Shimozaawa A, Akiyama H, et al.
1603 Pathological alpha-synuclein propagates through neural networks. 2014;2:
1604 <http://www.actaneurocomms.org/content/2/1/88>.
- 1605 28. Ayers JL, Brooks MM, Rutherford NJ, Howard JK, Sorrentino ZA, Riffe CJ, et al. Robust
1606 Central Nervous System Pathology in Transgenic Mice following Peripheral Injection of
1607 α -Synuclein Fibrils. *J Virol*. 2017;91: e02095-16.
- 1608 29. Brundin P, Melki R, Kopito R. Prion-like transmission of protein aggregates in
1609 neurodegenerative diseases. *Nat Rev Mol Cell Biol*. 2010;11: 301–307.
1610 doi:10.1038/nrm2873
- 1611 30. Masuda-Suzukake M, Nonaka T, Hosokawa M, Oikawa T, Arai T, Akiyama H, et al. Prion-
1612 like spreading of pathological α -synuclein in brain. *Brain*. 2013;136: 1128–1138.
1613 doi:10.1093/brain/awt037
- 1614 31. Rey NL, Wesson DW, Brundin P. The olfactory bulb as the entry site for prion-like
1615 propagation in neurodegenerative diseases. *Neurobiol Dis*. 2018;109: 226–248.
1616 doi:10.1016/j.nbd.2016.12.013
- 1617 32. Victoria GS, Zurzolo C. The spread of prion-like proteins by lysosomes and tunneling
1618 nanotubes: Implications for neurodegenerative diseases. *J Cell Biol*. 2017;216: 2633–
1619 2644. doi:10.1083/jcb.201701047
- 1620 33. Angot E, Steiner JA, Lema Tomé CM, Ekström P, Mattsson B, Björklund A, et al. Alpha-
1621 Synuclein Cell-to-Cell Transfer and Seeding in Grafted Dopaminergic Neurons In Vivo.
1622 Mosley RL, editor. *PLoS ONE*. 2012;7: e39465. doi:10.1371/journal.pone.0039465
- 1623 34. Hansen C, Angot E, Bergström A-L, Steiner JA, Pieri L, Paul G, et al. α -Synuclein
1624 propagates from mouse brain to grafted dopaminergic neurons and seeds aggregation
1625 in cultured human cells. *J Clin Invest*. 2011;121: 715–725. doi:10.1172/JCI43366
- 1626 35. Vargas JY, Grudina C, Zurzolo C. The prion-like spreading of α -synuclein: From in vitro to
1627 in vivo models of Parkinson’s disease. *Ageing Res Rev*. 2019;50: 89–101.
1628 doi:10.1016/j.arr.2019.01.012
- 1629 36. Dieriks BV, Park TI-H, Fourie C, Faull RLM, Dragunow M, Curtis MA. α -synuclein transfer
1630 through tunneling nanotubes occurs in SH-SY5Y cells and primary brain pericytes from
1631 Parkinson’s disease patients. *Sci Rep*. 2017;7: 42984. doi:10.1038/srep42984

- 1632 37. Rustom A. Nanotubular Highways for Intercellular Organelle Transport. *Science*.
1633 2004;303: 1007–1010. doi:10.1126/science.1093133
- 1634 38. Abounit S, Zurzolo C. Wiring through tunneling nanotubes - from electrical signals to
1635 organelle transfer. *J Cell Sci*. 2012;125: 1089–1098. doi:10.1242/jcs.083279
- 1636 39. Marzo L, Gousset K, Zurzolo C. Multifaceted Roles of Tunneling Nanotubes in
1637 Intercellular Communication. *Front Physiol*. 2012;3: doi: 10.3389/fphys.2012.00072.
1638 doi:10.3389/fphys.2012.00072
- 1639 40. Gerdes H-H, Bukoreshtliev NV, Barroso JFV. Tunneling nanotubes: A new route for the
1640 exchange of components between animal cells. *FEBS Lett*. 2007;581: 2194–2201.
1641 doi:10.1016/j.febslet.2007.03.071
- 1642 41. Cordero Cervantes D, Zurzolo C. Peering into tunneling nanotubes—The path forward.
1643 *EMBO J*. 2021 [cited 29 Mar 2021]. doi:10.15252/embj.2020105789
- 1644 42. Chinnery HR, Pearlman E, McMenamin PG. Cutting Edge: Membrane Nanotubes In
1645 Vivo: A Feature of MHC Class II⁺ Cells in the Mouse Cornea. *J Immunol*. 2008;180:
1646 5779–5783. doi:10.4049/jimmunol.180.9.5779
- 1647 43. Lou E, Fujisawa S, Morozov A, Barlas A, Romin Y, Dogan Y, et al. Tunneling Nanotubes
1648 Provide a Unique Conduit for Intercellular Transfer of Cellular Contents in Human
1649 Malignant Pleural Mesothelioma. Yang P-C, editor. *PLoS ONE*. 2012;7: e33093.
1650 doi:10.1371/journal.pone.0033093
- 1651 44. Pasquier J, Guerrouahen BS, Al Thawadi H, Ghiabi P, Maleki M, Abu-Kaoud N, et al.
1652 Preferential transfer of mitochondria from endothelial to cancer cells through
1653 tunneling nanotubes modulates chemoresistance. *J Transl Med*. 2013;11: 94.
1654 doi:10.1186/1479-5876-11-94
- 1655 45. Osswald M, Jung E, Sahm F, Solecki G, Venkataramani V, Blaes J, et al. Brain tumour
1656 cells interconnect to a functional and resistant network. *Nature*. 2015;528: 93–98.
1657 doi:10.1038/nature16071
- 1658 46. Seyed-Razavi Y, Hickey MJ, Kuffová L, McMenamin PG, Chinnery HR. Membrane
1659 nanotubes in myeloid cells in the adult mouse cornea represent a novel mode of
1660 immune cell interaction. *Immunol Cell Biol*. 2013;91: 89–95. doi:10.1038/icb.2012.52
- 1661 47. Alarcon-Martinez L, Villafranca-Baughman D, Quintero H, Kacerovsky JB, Dotigny F,
1662 Murai KK, et al. Interpericyte tunnelling nanotubes regulate neurovascular coupling.
1663 *Nature*. 2020;585: 91–95. doi:10.1038/s41586-020-2589-x
- 1664 48. Sartori-Rupp A, Cordero Cervantes D, Pepe A, Gousset K, Delage E, Corroyer-Dulmont S,
1665 et al. Correlative cryo-electron microscopy reveals the structure of TNTs in neuronal
1666 cells. *Nat Commun*. 2019;10: <https://doi.org/10.1038/s41467-018-08178-7>.
1667 doi:10.1038/s41467-018-08178-7

- 1668 49. Zurzolo C. Tunneling nanotubes: Reshaping connectivity. *Curr Opin Cell Biol.* 2021;71:
1669 139–147. doi:10.1016/j.ceb.2021.03.003
- 1670 50. Gousset K, Schiff E, Langevin C, Marijanovic Z, Caputo A, Browman DT, et al. Prions
1671 hijack tunnelling nanotubes for intercellular spread. *Nat Cell Biol.* 2009;11: 328–336.
1672 doi:10.1038/ncb1841
- 1673 51. Victoria GS, Arkhipenko A, Zhu S, Syan S, Zurzolo C. Astrocyte-to-neuron intercellular
1674 prion transfer is mediated by cell-cell contact. *Sci Rep.* 2016;6: 20762.
1675 doi:10.1038/srep20762
- 1676 52. Abounit S, Wu JW, Duff K, Victoria GS, Zurzolo C. Tunneling nanotubes: A possible
1677 highway in the spreading of tau and other prion-like proteins in neurodegenerative
1678 diseases. *Prion.* 2016;10: 344–351. doi:10.1080/19336896.2016.1223003
- 1679 53. Costanzo M, Abounit S, Marzo L, Danckaert A, Chamoun Z, Roux P, et al. Transfer of
1680 polyglutamine aggregates in neuronal cells occurs in tunneling nanotubes. *J Cell Sci.*
1681 2013;126: 3678–3685. doi:10.1242/jcs.126086
- 1682 54. Tardivel M, Bégard S, Bousset L, Dujardin S, Coens A, Melki R, et al. Tunneling nanotube
1683 (TNT)-mediated neuron-to neuron transfer of pathological Tau protein assemblies.
1684 *Acta Neuropathol Commun.* 2016;4: 117. doi:10.1186/s40478-016-0386-4
- 1685 55. Chastagner P, Loria F, Vargas JY, Tois J, I Diamond M, Okafo G, et al. Fate and
1686 propagation of endogenously formed Tau aggregates in neuronal cells. *EMBO Mol*
1687 *Med.* 2020;12. doi:10.15252/emmm.202012025
- 1688 56. Grudina C, Kouroupi G, Nonaka T, Hasegawa M, Matsas R, Zurzolo C. Human NPCs can
1689 degrade α -syn fibrils and transfer them preferentially in a cell contact-dependent
1690 manner possibly through TNT-like structures. *Neurobiol Dis.* 2019;132: 104609.
1691 doi:10.1016/j.nbd.2019.104609
- 1692 57. Luzio JP, Parkinson MDJ, Gray SR, Bright NA. The delivery of endocytosed cargo to
1693 lysosomes. *Biochem Soc Trans.* 2009;37: 1019–1021. doi:10.1042/BST0371019
- 1694 58. Luzio JP, Pryor PR, Bright NA. Lysosomes: fusion and function. *Nat Rev Mol Cell Biol.*
1695 2007;8: 622–632. doi:10.1038/nrm2217
- 1696 59. Singh R, Cuervo AM. Autophagy in the Cellular Energetic Balance. *Cell Metab.* 2011;13:
1697 495–504. doi:10.1016/j.cmet.2011.04.004
- 1698 60. Rabinowitz JD, White E. Autophagy and Metabolism. *Science.* 2010;330: 1344–1348.
1699 doi:10.1126/science.1193497
- 1700 61. Settembre C, Fraldi A, Medina DL, Ballabio A. Signals from the lysosome: a control
1701 centre for cellular clearance and energy metabolism. *Nat Rev Mol Cell Biol.* 2013;14:
1702 283–296. doi:10.1038/nrm3565

- 1703 62. Settembre C, Ballabio A. Lysosomal Adaptation: How the Lysosome Responds to
1704 External Cues. *Cold Spring Harb Perspect Biol.* 2014;6: a016907–a016907.
1705 doi:10.1101/cshperspect.a016907
- 1706 63. Lim C-Y, Zoncu R. The lysosome as a command-and-control center for cellular
1707 metabolism. *J Cell Biol.* 2016;214: 653–664. doi:10.1083/jcb.201607005
- 1708 64. Perera RM, Zoncu R. The Lysosome as a Regulatory Hub. *Annu Rev Cell Dev Biol.*
1709 2016;32: 223–253. doi:10.1146/annurev-cellbio-111315-125125
- 1710 65. Shin HR, Zoncu R. The Lysosome at the Intersection of Cellular Growth and Destruction.
1711 *Dev Cell.* 2020;54: 226–238. doi:10.1016/j.devcel.2020.06.010
- 1712 66. Fraldi A, Klein AD, Medina DL, Settembre C. Brain Disorders Due to Lysosomal
1713 Dysfunction. *Annu Rev Neurosci.* 2016;39: 277–295. doi:10.1146/annurev-neuro-
1714 070815-014031
- 1715 67. Chu Y, Dodiya H, Aebischer P, Olanow CW, Kordower JH. Alterations in lysosomal and
1716 proteasomal markers in Parkinson’s disease: Relationship to alpha-synuclein
1717 inclusions. *Neurobiol Dis.* 2009;35: 385–398. doi:10.1016/j.nbd.2009.05.023
- 1718 68. Dehay B, Bove J, Rodriguez-Muela N, Perier C, Recasens A, Boya P, et al. Pathogenic
1719 Lysosomal Depletion in Parkinson’s Disease. *J Neurosci.* 2010;30: 12535–12544.
1720 doi:10.1523/JNEUROSCI.1920-10.2010
- 1721 69. Mazzulli JR, Zunke F, Isacson O, Studer L, Krainc D. α -Synuclein–induced lysosomal
1722 dysfunction occurs through disruptions in protein trafficking in human midbrain
1723 synucleinopathy models. *Proc Natl Acad Sci.* 2016;113: 1931–1936.
1724 doi:10.1073/pnas.1520335113
- 1725 70. Hoffmann A-C, Minakaki G, Menges S, Salvi R, Savitskiy S, Kazman A, et al. Extracellular
1726 aggregated alpha synuclein primarily triggers lysosomal dysfunction in neural cells
1727 prevented by trehalose. *Sci Rep.* 2019;9: 544. doi:10.1038/s41598-018-35811-8
- 1728 71. Mazzulli JR, Xu Y-H, Sun Y, Knight AL, McLean PJ, Caldwell GA, et al. Gaucher Disease
1729 Glucocerebrosidase and α -Synuclein Form a Bidirectional Pathogenic Loop in
1730 Synucleinopathies. *Cell.* 2011;146: 37–52. doi:10.1016/j.cell.2011.06.001
- 1731 72. Boya P. Lysosomal Function and Dysfunction: Mechanism and Disease. *Antioxid Redox*
1732 *Signal.* 2012;17: 766–774. doi:10.1089/ars.2011.4405
- 1733 73. Freeman D, Cedillos R, Choyke S, Lukic Z, McGuire K, Marvin S, et al. Alpha-Synuclein
1734 Induces Lysosomal Rupture and Cathepsin Dependent Reactive Oxygen Species
1735 Following Endocytosis. Kahle PJ, editor. *PLoS ONE.* 2013;8: e62143.
1736 doi:10.1371/journal.pone.0062143
- 1737 74. Flavin WP, Bousset L, Green ZC, Chu Y, Skarpathiotis S, Chaney MJ, et al. Endocytic
1738 vesicle rupture is a conserved mechanism of cellular invasion by amyloid proteins.
1739 *Acta Neuropathol (Berl).* 2017;134: 629–653. doi:10.1007/s00401-017-1722-x

- 1740 75. Jongsma ML, Bakker J, Cabukusta B, Liv N, Elsland D, Fermie J, et al. SKIP - HOPS recruits
1741 TBC 1D15 for a Rab7-to-Arl8b identity switch to control late endosome transport.
1742 EMBO J. 2020;39. doi:10.15252/embj.2019102301
- 1743 76. Fermie J, Liv N, ten Brink C, van Donselaar EG, Müller WH, Schieber NL, et al. Single
1744 organelle dynamics linked to 3D structure by correlative live-cell imaging and 3D
1745 electron microscopy. *Traffic*. 2018;19: 354–369. doi:10.1111/tra.12557
- 1746 77. Meel E, Klumperman J. Imaging and imagination: understanding the endo-lysosomal
1747 system. *Histochem Cell Biol*. 2008;129: 253–266. doi:10.1007/s00418-008-0384-0
- 1748 78. Klumperman J, Raposo G. The Complex Ultrastructure of the Endolysosomal System.
1749 *Cold Spring Harb Perspect Biol*. 2014;6: a016857–a016857.
1750 doi:10.1101/cshperspect.a016857
- 1751 79. Mohammadian S, Fokkema J, Agronskaia AV, Liv N, de Heus C, van Donselaar E, et al.
1752 High accuracy, fiducial marker-based image registration of correlative microscopy
1753 images. *Sci Rep*. 2019;9: 3211. doi:10.1038/s41598-019-40098-4
- 1754 80. Cortese K, Vicidomini G, Gagliani MC, Boccacci P, Diaspro A, Tacchetti C. High Data
1755 Output Method for 3-D Correlative Light-Electron Microscopy Using Ultrathin
1756 Cryosections. In: Sousa AA, Kruhlak MJ, editors. *Nanoimaging*. Totowa, NJ: Humana
1757 Press; 2013. pp. 417–437. doi:10.1007/978-1-62703-137-0_23
- 1758 81. Calafate S, Flavin W, Verstreken P, Moechars D. Loss of Bin1 Promotes the Propagation
1759 of Tau Pathology. *Cell Rep*. 2016;17: 931–940. doi:10.1016/j.celrep.2016.09.063
- 1760 82. Papadopoulos C, Kirchner P, Bug M, Grum D, Koerver L, Schulze N, et al. VCP /p97
1761 cooperates with YOD 1, UBXD 1 and PLAA to drive clearance of ruptured lysosomes by
1762 autophagy. *EMBO J*. 2017;36: 135–150. doi:10.15252/embj.201695148
- 1763 83. Oku Y, Murakami K, Irie K, Hoseki J, Sakai Y. Synthesized A β 42 Caused Intracellular
1764 Oxidative Damage, Leading to Cell Death, via Lysosome Rupture. *Cell Struct Funct*.
1765 2017;42: 71–79. doi:10.1247/csf.17006
- 1766 84. Maejima I, Takahashi A, Omori H, Kimura T, Takabatake Y, Saitoh T, et al. Autophagy
1767 sequesters damaged lysosomes to control lysosomal biogenesis and kidney injury.
1768 *EMBO J*. 2013;32: 2336–2347. doi:10.1038/emboj.2013.171
- 1769 85. Jiang P, Gan M, Yen S-H, McLean PJ, Dickson DW. Impaired endo-lysosomal membrane
1770 integrity accelerates the seeding progression of α -synuclein aggregates. *Sci Rep*.
1771 2017;7: 7690. doi:10.1038/s41598-017-08149-w
- 1772 86. Nonaka T, Watanabe ST, Iwatsubo T, Hasegawa M. Seeded Aggregation and Toxicity of
1773 α -Synuclein and Tau: CELLULAR MODELS OF NEURODEGENERATIVE DISEASES. *J Biol*
1774 *Chem*. 2010;285: 34885–34898. doi:10.1074/jbc.M110.148460

- 1775 87. Shrivastava AN, Bousset L, Renner M, Redeker V, Savistchenko J, Triller A, et al.
1776 Differential Membrane Binding and Seeding of Distinct α -Synuclein Fibrillar
1777 Polymorphs. *Biophys J*. 2020;118: 1301–1320. doi:10.1016/j.bpj.2020.01.022
- 1778 88. Yang X, Williams JK, Yan R, Mouradian MM, Baum J. Increased Dynamics of α -Synuclein
1779 Fibrils by β -Synuclein Leads to Reduced Seeding and Cytotoxicity. *Sci Rep*. 2019;9:
1780 17579. doi:10.1038/s41598-019-54063-8
- 1781 89. Sangwan S, Sahay S, Murray KA, Morgan S, Guenther EL, Jiang L, et al. Inhibition of
1782 synucleinopathic seeding by rationally designed inhibitors. *eLife*. 2020;9: e46775.
1783 doi:10.7554/eLife.46775
- 1784 90. Guardia CM, Farías GG, Jia R, Pu J, Bonifacino JS. BORC Functions Upstream of Kinesins
1785 1 and 3 to Coordinate Regional Movement of Lysosomes along Different Microtubule
1786 Tracks. *Cell Rep*. 2016;17: 1950–1961. doi:10.1016/j.celrep.2016.10.062
- 1787 91. Pu J, Guardia CM, Keren-Kaplan T, Bonifacino JS. Mechanisms and functions of
1788 lysosome positioning. *J Cell Sci*. 2016;129: 4329–4339. doi:10.1242/jcs.196287
- 1789 92. Cabukusta B, Neefjes J. Mechanisms of lysosomal positioning and movement. *Traffic*.
1790 2018;19: 761–769. doi:10.1111/tra.12587
- 1791 93. Jongsma MLM, Berlin I, Wijdeven RHM, Janssen L, Janssen GMC, Garstka MA, et al. An
1792 ER-Associated Pathway Defines Endosomal Architecture for Controlled Cargo
1793 Transport. *Cell*. 2016;166: 152–166. doi:10.1016/j.cell.2016.05.078
- 1794 94. Bajaj L, Lotfi P, Pal R, Ronza A di, Sharma J, Sardiello M. Lysosome biogenesis in health
1795 and disease. *J Neurochem*. 2019;148: 573–589. doi:10.1111/jnc.14564
- 1796 95. Medina DL, Fraldi A, Bouche V, Annunziata F, Mansueto G, Spampanato C, et al.
1797 Transcriptional Activation of Lysosomal Exocytosis Promotes Cellular Clearance. *Dev*
1798 *Cell*. 2011;21: 421–430. doi:10.1016/j.devcel.2011.07.016
- 1799 96. Willett R, Martina JA, Zewe JP, Wills R, Hammond GRV, Puertollano R. TFEB regulates
1800 lysosomal positioning by modulating TMEM55B expression and JIP4 recruitment to
1801 lysosomes. *Nat Commun*. 2017;8: 1580. doi:10.1038/s41467-017-01871-z
- 1802 97. Zhang J, Johnson JL, He J, Napolitano G, Ramadass M, Rocca C, et al. Cystinosin, the
1803 small GTPase Rab11, and the Rab7 effector RILP regulate intracellular trafficking of the
1804 chaperone-mediated autophagy receptor LAMP2A. *J Biol Chem*. 2017;292: 10328–
1805 10346. doi:10.1074/jbc.M116.764076
- 1806 98. Hofmann I, Munro S. An N-terminally acetylated Arf-like GTPase is localised to
1807 lysosomes and affects their motility. *J Cell Sci*. 2006;119: 1494–1503.
1808 doi:10.1242/jcs.02958
- 1809 99. Bagshaw RD, Callahan JW, Mahuran DJ. The Arf-family protein, Arl8b, is involved in the
1810 spatial distribution of lysosomes. *Biochem Biophys Res Commun*. 2006;344: 1186–
1811 1191. doi:10.1016/j.bbrc.2006.03.221

- 1812 100. Pu J, Schindler C, Jia R, Jarnik M, Backlund P, Bonifacino JS. BORC, a Multisubunit
1813 Complex that Regulates Lysosome Positioning. *Dev Cell*. 2015;33: 176–188.
1814 doi:10.1016/j.devcel.2015.02.011
- 1815 101. Nakata T, Hirokawa N. Point mutation of adenosine triphosphate-binding motif
1816 generated rigor kinesin that selectively blocks anterograde lysosome membrane
1817 transport. *J Cell Biol*. 1995;131: 1039–1053. doi:10.1083/jcb.131.4.1039
- 1818 102. Rosa-Ferreira C, Munro S. Arl8 and SKIP Act Together to Link Lysosomes to Kinesin-1.
1819 *Dev Cell*. 2011;21: 1171–1178. doi:10.1016/j.devcel.2011.10.007
- 1820 103. Korolchuk VI, Saiki S, Lichtenberg M, Siddiqi FH, Roberts EA, Imarisio S, et al. Lysosomal
1821 positioning coordinates cellular nutrient responses. *Nat Cell Biol*. 2011;13: 453–460.
1822 doi:10.1038/ncb2204
- 1823 104. Matsushita M, Tanaka S, Nakamura N, Inoue H, Kanazawa H. A Novel Kinesin-Like
1824 Protein, KIF1B β 3 Is Involved in the Movement of Lysosomes to the Cell Periphery in
1825 Non-Neuronal Cells: Novel Kinesin Motor for Lysosomal Movement. *Traffic*. 2004;5:
1826 140–151. doi:10.1111/j.1600-0854.2003.00165.x
- 1827 105. Bentley M, Decker H, Luisi J, Banker G. A novel assay reveals preferential binding
1828 between Rabs, kinesins, and specific endosomal subpopulations. *J Cell Biol*. 2015;208:
1829 273–281. doi:10.1083/jcb.201408056
- 1830 106. Naphade S, Sharma J, Gaide Chevonnay HP, Shook MA, Yeagy BA, Rocca CJ, et al. Brief
1831 Reports: Lysosomal Cross-Correction by Hematopoietic Stem Cell-Derived
1832 Macrophages Via Tunneling Nanotubes: Lysosomal Cross-Correction by Stem Cells Via
1833 TNTs. *STEM CELLS*. 2015;33: 301–309. doi:10.1002/stem.1835
- 1834 107. Del Tredici K, Braak H. Review: Sporadic Parkinson's disease: development and
1835 distribution of α -synuclein pathology. *Neuropathol Appl Neurobiol*. 2016;42: 33–50.
1836 doi:10.1111/nan.12298
- 1837 108. Usenovic M, Tresse E, Mazzulli JR, Taylor JP, Krainc D. Deficiency of ATP13A2 Leads to
1838 Lysosomal Dysfunction, α -Synuclein Accumulation, and Neurotoxicity. *J Neurosci*.
1839 2012;32: 4240–4246. doi:10.1523/JNEUROSCI.5575-11.2012
- 1840 109. Chen PM, Gombart ZJ, Chen JW. Chloroquine treatment of ARPE-19 cells leads to
1841 lysosome dilation and intracellular lipid accumulation: possible implications of
1842 lysosomal dysfunction in macular degeneration. *Cell Biosci*. 2011;1: 10.
1843 doi:10.1186/2045-3701-1-10
- 1844 110. Boya P, Kroemer G. Lysosomal membrane permeabilization in cell death. *Oncogene*.
1845 2008;27: 6434–6451. doi:10.1038/onc.2008.310
- 1846 111. Radulovic M, Schink KO, Wenzel EM, Nähse V, Bongiovanni A, Lafont F, et al. ESCRT -
1847 mediated lysosome repair precedes lysophagy and promotes cell survival. *EMBO J*.
1848 2018;37. doi:10.15252/embj.201899753

- 1849 112. Skowyra ML, Schlesinger PH, Naismith TV, Hanson PI. Triggered recruitment of ESCRT
1850 machinery promotes endolysosomal repair. *Science*. 2018;360: eaar5078.
1851 doi:10.1126/science.aar5078
- 1852 113. Lynch-Day MA, Mao K, Wang K, Zhao M, Klionsky DJ. The Role of Autophagy in
1853 Parkinson's Disease. *Cold Spring Harb Perspect Med*. 2012;2: a009357–a009357.
1854 doi:10.1101/cshperspect.a009357
- 1855 114. Cui L, Zhao L-P, Ye J-Y, Yang L, Huang Y, Jiang X-P, et al. The Lysosomal Membrane
1856 Protein Lamp2 Alleviates Lysosomal Cell Death by Promoting Autophagic Flux in
1857 Ischemic Cardiomyocytes. *Front Cell Dev Biol*. 2020;8: 31.
1858 doi:10.3389/fcell.2020.00031
- 1859 115. Lashuel HA, Petre BM, Wall J, Simon M, Nowak RJ, Walz T, et al. α -Synuclein, Especially
1860 the Parkinson's Disease-associated Mutants, Forms Pore-like Annular and Tubular
1861 Protofibrils. *J Mol Biol*. 2002;322: 1089–1102. doi:10.1016/S0022-2836(02)00735-0
- 1862 116. Tosatto L, Andrichetti AO, Plotegher N, Antonini V, Tessari I, Ricci L, et al. Alpha-
1863 synuclein pore forming activity upon membrane association. *Biochim Biophys Acta*
1864 *BBA - Biomembr*. 2012;1818: 2876–2883. doi:10.1016/j.bbamem.2012.07.007
- 1865 117. Volles MJ, Lansbury PT. Vesicle Permeabilization by Protofibrillar α -Synuclein Is
1866 Sensitive to Parkinson's Disease-Linked Mutations and Occurs by a Pore-like
1867 Mechanism [†]. *Biochemistry*. 2002;41: 4595–4602. doi:10.1021/bi0121353
- 1868 118. Butler D, Bahr BA. Oxidative Stress and Lysosomes: CNS-Related Consequences and
1869 Implications for Lysosomal Enhancement Strategies and Induction of Autophagy.
1870 *Antioxid Redox Signal*. 2006;8: 185–196. doi:10.1089/ars.2006.8.185
- 1871 119. Perier C, Tieu K, Guegan C, Caspersen C, Jackson-Lewis V, Carelli V, et al. Complex I
1872 deficiency primes Bax-dependent neuronal apoptosis through mitochondrial oxidative
1873 damage. *Proc Natl Acad Sci*. 2005;102: 19126–19131. doi:10.1073/pnas.0508215102
- 1874 120. Bourdenx M, Dehay B. What lysosomes actually tell us about Parkinson's disease?
1875 *Ageing Res Rev*. 2016;32: 140–149. doi:10.1016/j.arr.2016.02.008
- 1876 121. Trinkaus VA, Riera-Tur I, Martínez-Sánchez A, Guo Q, Arzberger T, Baumeister W, et al.
1877 In situ architecture of neuronal α -Synuclein inclusions. : 46.
- 1878 122. Matteoni R, Kreis TE. Translocation and Clustering of Endosomes and Lysosomes
1879 Depends on Microtubules. *J Cell Biol*. 1987;105: 1253–1265.
- 1880 123. Nakamura S, Shigeyama S, Minami S, Shima T, Akayama S, Matsuda T, et al. LC3
1881 lipidation is essential for TFEB activation during the lysosomal damage response to
1882 kidney injury. *Nat Cell Biol*. 2020;22: 1252–1263. doi:10.1038/s41556-020-00583-9
- 1883 124. Decressac M, Mattsson B, Weikop P, Lundblad M, Jakobsson J, Bjorklund A. TFEB-
1884 mediated autophagy rescues midbrain dopamine neurons from α -synuclein toxicity.
1885 *Proc Natl Acad Sci*. 2013;110: E1817–E1826. doi:10.1073/pnas.1305623110

- 1886 125. Gousset K, Marzo L, Commere P-H, Zurzolo C. Myo10 is a key regulator of TNT
1887 formation in neuronal cells. *J Cell Sci.* 2013;126: 4424–4435. doi:10.1242/jcs.129239
- 1888 126. Wang Y, Cui J, Sun X, Zhang Y. Tunneling-nanotube development in astrocytes depends
1889 on p53 activation. *Cell Death Differ.* 2011;18: 732–742. doi:10.1038/cdd.2010.147
- 1890 127. Shi Z, Sachs J, Rhoades E, Baumgart T. Biophysics of α -synuclein induced membrane
1891 remodelling. 2015; 19.
- 1892 128. Qi Y, Wang JKT, McMillian M, Chikaraishi DM. Characterization of a CNS Cell Line, CAD,
1893 in which Morphological Differentiation Is Initiated by Serum Deprivation. *J Neurosci.*
1894 1997;17: 1217–1225. doi:10.1523/JNEUROSCI.17-04-01217.1997
- 1895 129. Abounit S, Delage E, Zurzolo C. Identification and Characterization of Tunneling
1896 Nanotubes for Intercellular Trafficking. *Curr Protoc Cell Biol.* 2015;67: doi:
1897 10.1002/0471143030.cb1210s67. doi:10.1002/0471143030.cb1210s67
- 1898 130. Zhu S, Abounit S, Korth C, Zurzolo C. Transfer of disrupted-in-schizophrenia 1
1899 aggregates between neuronal-like cells occurs in tunnelling nanotubes and is
1900 promoted by dopamine. *Open Biol.* 2017;7: 160328. doi:10.1098/rsob.160328
- 1901 131. Delage E, Cervantes DC, Pénard E, Schmitt C, Syan S, Disanza A, et al. Differential
1902 identity of Filopodia and Tunneling Nanotubes revealed by the opposite functions of
1903 actin regulatory complexes. *Sci Rep.* 2016;6: 39632. doi:10.1038/srep39632
- 1904 132. Jia R, Guardia CM, Pu J, Chen Y, Bonifacino JS. BORC coordinates encounter and fusion
1905 of lysosomes with autophagosomes. *Autophagy.* 2017;13: 1648–1663.
1906 doi:10.1080/15548627.2017.1343768
- 1907 133. Kowarz E, Löscher D, Marschalek R. Optimized Sleeping Beauty transposons rapidly
1908 generate stable transgenic cell lines. *Biotechnol J.* 2015;10: 647–653.
1909 doi:10.1002/biot.201400821
- 1910 134. Mátés L, Chuah MKL, Belay E, Jerchow B, Manoj N, Acosta-Sanchez A, et al. Molecular
1911 evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable
1912 gene transfer in vertebrates. *Nat Genet.* 2009;41: 753–761. doi:10.1038/ng.343
- 1913 135. Nonaka T, Iwatsubo T, Hasegawa M. Ubiquitination of α -Synuclein[†]. *Biochemistry.*
1914 2005;44: 361–368. doi:10.1021/bi0485528
- 1915 136. Boulanger J, Kervrann C, Bouthemy P, Elbau P, Sibarita J-B, Salamero J. Patch-Based
1916 Nonlocal Functional for Denoising Fluorescence Microscopy Image Sequences. *IEEE*
1917 *Trans Med Imaging.* 2010;29: 442–454. doi:10.1109/TMI.2009.2033991
- 1918 137. Oorschot V, de Wit H, Annaert WG, Klumperman J. A Novel Flat-embedding Method to
1919 Prepare Ultrathin Cryosections from Cultured Cells in Their In Situ Orientation. *J*
1920 *Histochem Cytochem.* 2002;50: 1067–1080. doi:10.1177/002215540205000809
- 1921 138. Slot JW, Geuze HJ. Cryosectioning and immunolabeling. *Nat Protoc.* 2007;2: 2480–2491.
1922 doi:10.1038/nprot.2007.365

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 LAMP1¹⁰. 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 CellMaskTM 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

of acceptor cells were replaced by the conditioned medium collected from donor cells. Acceptor cells were then analysed for the presence of different cargos (e.g., α -syn fibrils, lysosomes) in order to evaluate cargo transfer from donor to acceptor cells through secretion.

S4 Fig. α -Syn fibrils do not induce lysophagy. Representative confocal images of CAD cells control, treated with 1 mM LLOMe for 3 h, or treated with Alexa 568-tagged α -syn fibrils for 18 h and immunolabelled with LC3-Alexa 488, Ubiquitin FK2-Alexa 647, and Lamp1-CFTM405M (pseudo colored in grey) antibodies. Light blue arrows indicate lysosomes under lysophagy. $n=3$ (60 cells analysed per condition). Scale bar: 10 μ m.

S5 Fig. Timeframes of the S1 and S2 Movies of CAD cells overexpressing soluble α -syn-GFP treated or not with Alexa 568-tagged α -syn fibrils up to 4 h. (A) Timeframes at 0/1/2/3/4 hours of the S1 Movie of CAD cells overexpressing soluble α -syn-GFP monitored after the administration of α -syn fibrils. (B) Timeframes at 0/1/2/3/4 hours of the S2 Movie of CAD cells overexpressing soluble α -syn-GFP.

S6 Fig. Lysosome positioning affects the efficiency of α -syn fibrils' transfer in HeLa cells. (A) Representative confocal images of donor and acceptor HeLa cells after 24 h of co-culture. Donor cells were either treated with Alexa 568-tagged α -syn fibrils for 18 h (referred as "control co-culture") or they were transfected with Arl8b-GFP prior to be treated with α -syn fibrils (referred as "Arl8b-GFP co-culture"). Acceptor cells were transfected with H2B-GFP. 24 h later, co-cultures were labelled with HCS CellMaskTM 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 μ m. (B) % of acceptor cells received α -syn fibrils in control co-culture ($28 \pm 3\%$), Arl8b-GFP co-culture ($60 \pm 5\%$), control secretion ($1 \pm 1\%$), and Arl8b-GFP secretion ($8 \pm 3\%$) is presented (left graph); average number of α -syn puncta in control co-culture (3 ± 1), Arl8b-GFP co-culture (5 ± 1), control secretion (1 ± 0.0), and in Arl8b-GFP secretion (2 ± 0.2) is presented (right graph). Mean $\pm$ S.E.M, $n=3$ (70 acceptor cells per condition). ns=not significant, *** $P=0.0001$ by Kruskal-Wallis nonparametric ANOVA test followed by Dunn's multiple comparison tests. (C) Representative confocal images of donor and acceptor HeLa cells after 24 h of co-culture. WT, myrlysin-KO, and KIF1B-5B dKO HeLa cells were loaded with Alexa 568-tagged α -syn fibrils (referred as control, myrlysin-KO, and KIF1B-5B dKO co-cultures, respectively) and co-cultured with acceptor cells transfected with

H2B-GFP. Co-cultures were labelled with HCS CellMask™ 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 μ m. **(D)** % of acceptor cells received α -syn fibrils in control co-culture ($29 \pm 2\%$), myrlysin-KO co-culture ($16 \pm 2\%$), KIF1B-5B dKO co-culture ($12 \pm 2\%$), control secretion ($5 \pm 2\%$), myrlysin-KO secretion ($6 \pm 1\%$), and KIF1B-5B dKO secretion ($6 \pm 1\%$) is presented (left graph); average number of α -syn in control co-culture (2 ± 0.3), myrlysin-KO co-culture (2 ± 0.3), KIF1B-5B dKO co-culture (2 ± 0.4), control secretion (1 ± 0.4), myrlysin-KO secretion (1 ± 0.2), and KIF1B-5B dKO secretion (1 ± 0.2) is presented (right graph). Mean $\pm$ S.E.M, $n=3$ (90 acceptor cells per condition). ns=not significant, $**P=0.016$, $****P<0.0001$ by Kruskal-Wallis nonparametric ANOVA test followed by Dunn's multiple comparison tests. The data underlying this Figure may be found in S1 Data.

S7 Fig. Validation of CAD-HeLa co-culture system with additional information. (A) Representative confocal image of donor CAD cells loaded with Alexa 568-tagged α -syn fibrils (18 h) co-cultured with HeLa Gal3-Turquoise acceptor cells for 24 h. In acceptor HeLa cells, arrows indicate α -syn fibrils. Scale bar: 10 μ m. **(B)** % of acceptor HeLa cells received α -syn fibrils in co-culture ($65 \pm 3\%$) and in secretion test ($7 \pm 2\%$) is presented (left graph). Mean $\pm$ S.E.M. $****P<0.0001$ by two-tailed t -test. Average number of α -syn fibrils puncta per acceptor cell in co-culture (4 ± 0.2) and in secretion test (2 ± 0.2) is presented (right graph). ns=not significant by Mann-Whitney U -test. **(C)** Representative confocal image of donor CAD cells loaded with Alexa 568-tagged α -syn fibrils (18 h) co-cultured with acceptor WT HeLa cells transfected with α -syn-GFP for 24 h. Red arrows indicate α -syn fibrils, green arrows indicate α -syn-GFP puncta formation (seeding) in acceptor HeLa cells. Scale bar: 10 μ m. **(D)** Representative confocal image of Gal3-GFP transfected and Alexa 568-tagged α -syn fibril loaded donor CAD cells (18 h) co-cultured with HeLa Gal3-Turquoise cells for 24 h. In donor CAD cell, green arrows indicate Gal3-GFP puncta formation. Scale bar: 10 μ m. **(E)** % of donor CAD cells under LMP in control ($2 \pm 2\%$) and in α -syn ($11 \pm 4\%$) co-cultures is presented (left graph); average number of Gal3-GFP puncta in donor cells under LMP in control (10 ± 2) and in α -syn (14 ± 4) co-cultures is presented (right graph). Mean $\pm$ S.E.M. ns=not significant, by $*P=0.04$ Mann-Whitney U -test. **(F)** HeLa Gal3-Turquoise acceptor cell under LMP labelled for LAMP1 human Alexa 647 antibody (pseudo colored in grey). Colocalization between Gal3-Turquoise puncta and LAMP1 (indicated by arrows) is presented in a selected region indicated by the square. Scale bar: 10 μ m (for the inset: 1 μ m). **(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 μ m (for the inset: 1 μ m). **(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 μ m (for the inset: 2 μ m). **(I)** % of donor CAD cells received Gal3-Turquoise puncta in control ($0.3 \pm 0.3\%$) and in α -syn ($4 \pm 1\%$) co-cultures. Mean $\pm$ 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 μ m) with a total physical thickness of 7.2 μ m, 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 μ m) with a total physical thickness of 9.55 μ m, with 15 minutes of interval time. Video was acquired using laser 488 in a Spinning Disk microscope.

Figure-1

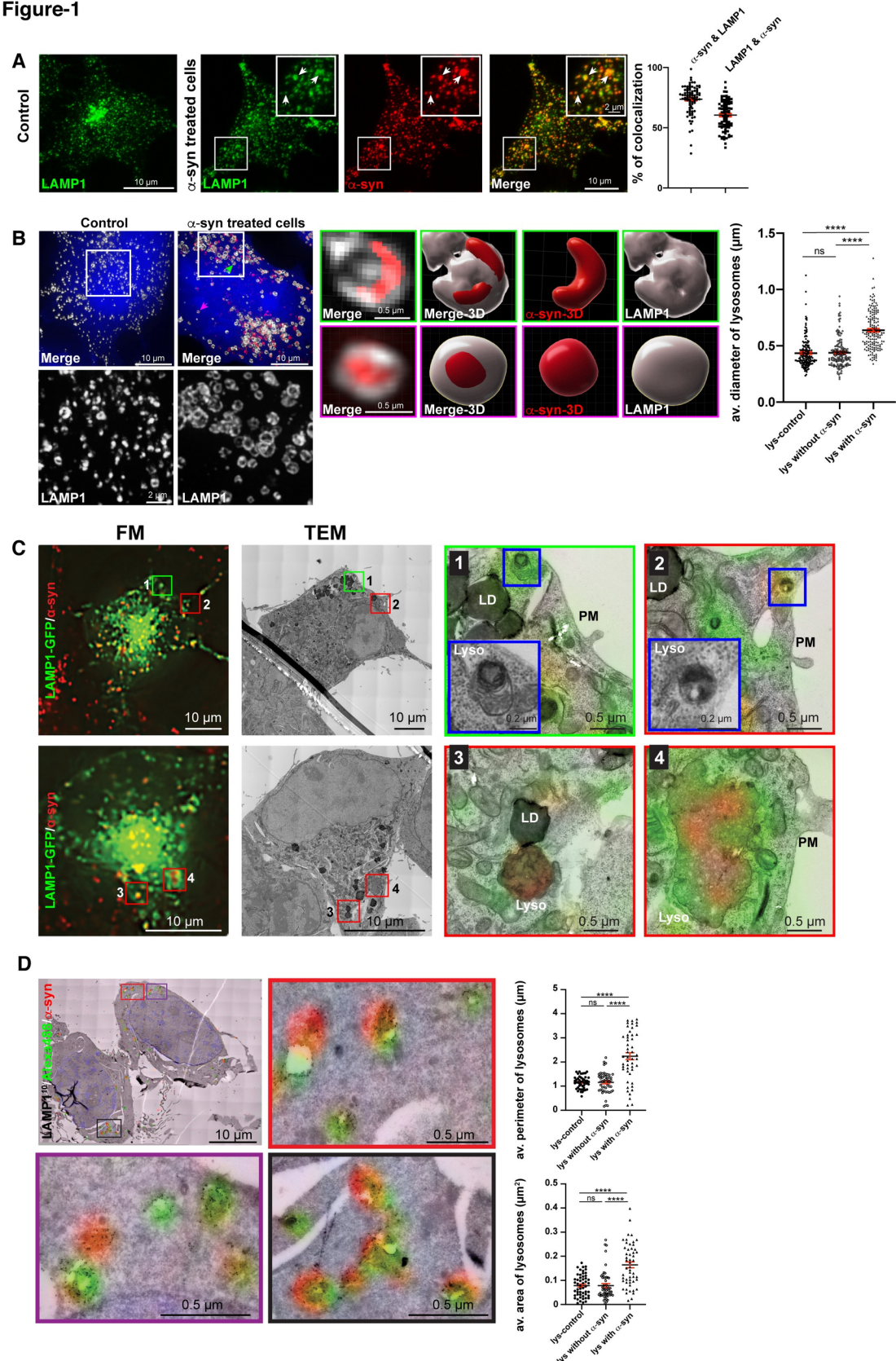


Figure-2

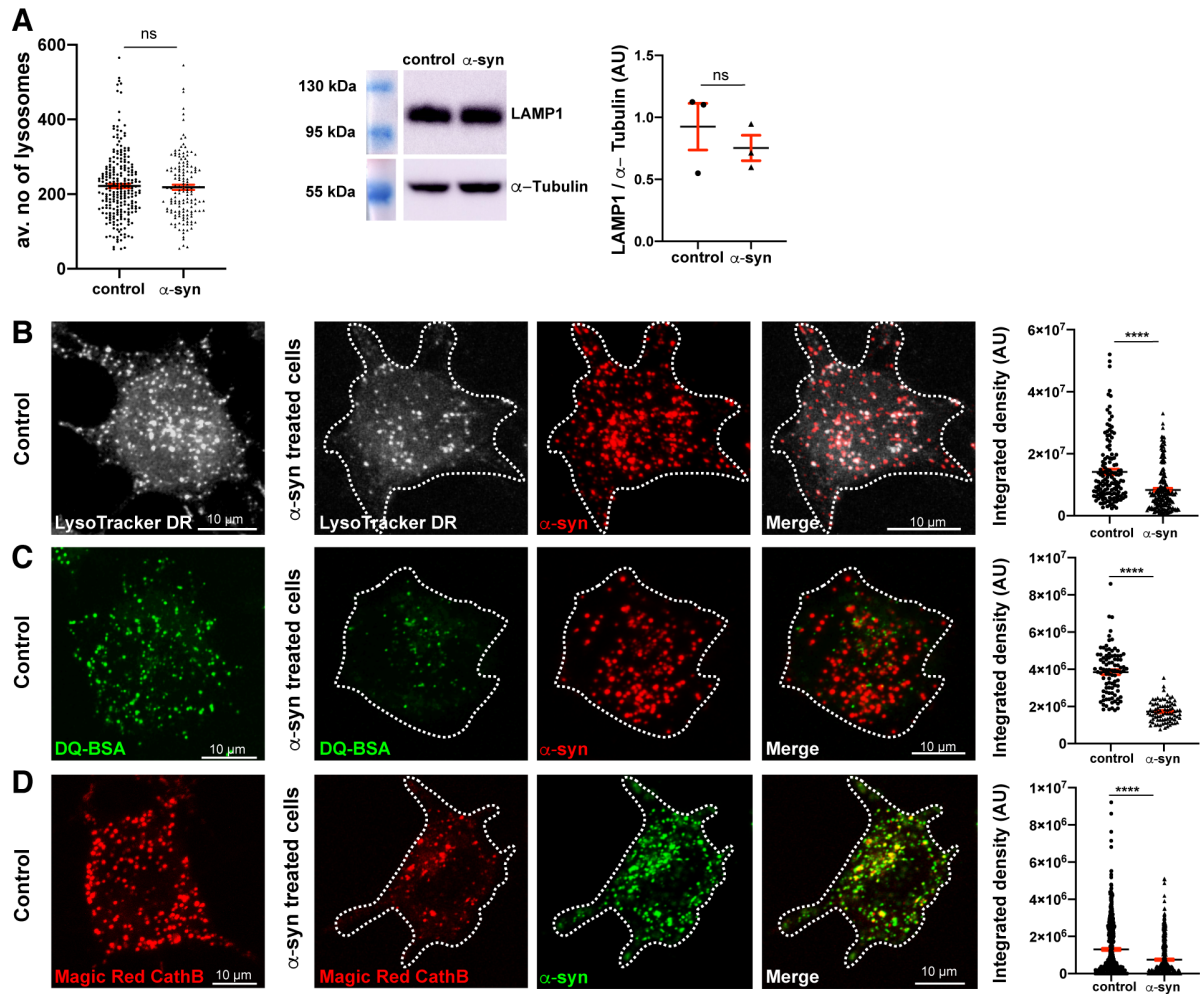


Figure-3

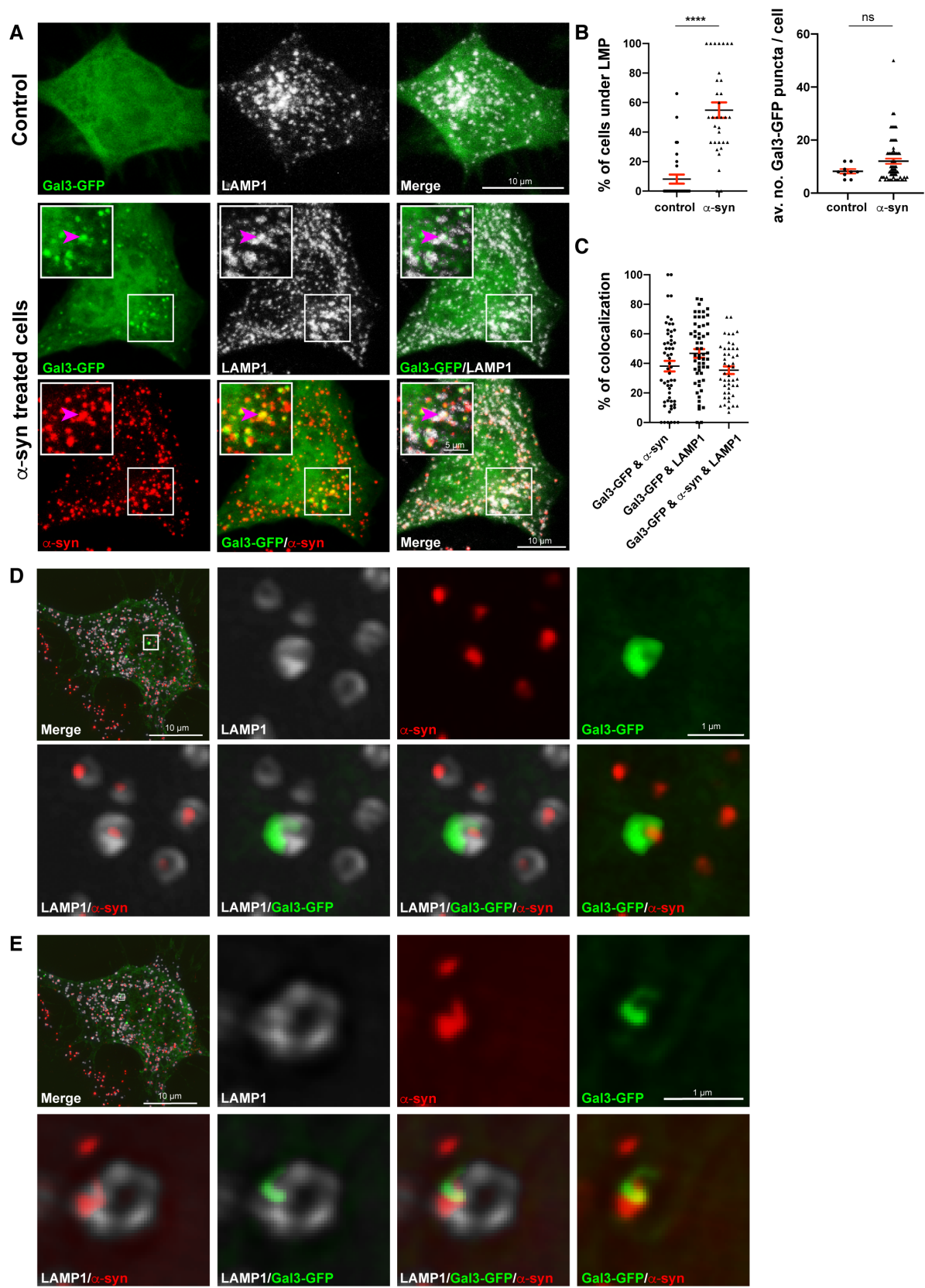


Figure-4

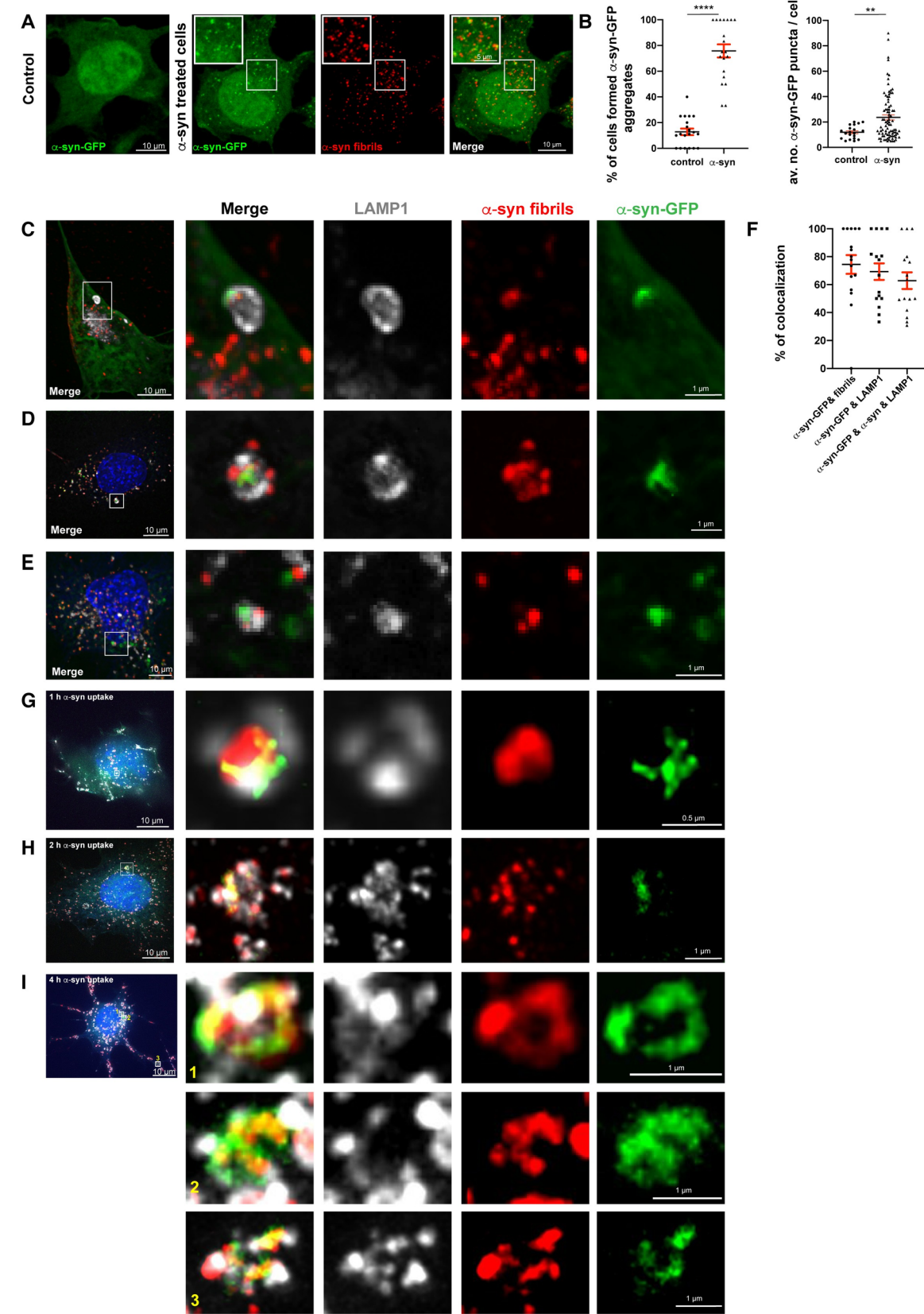


Figure-5

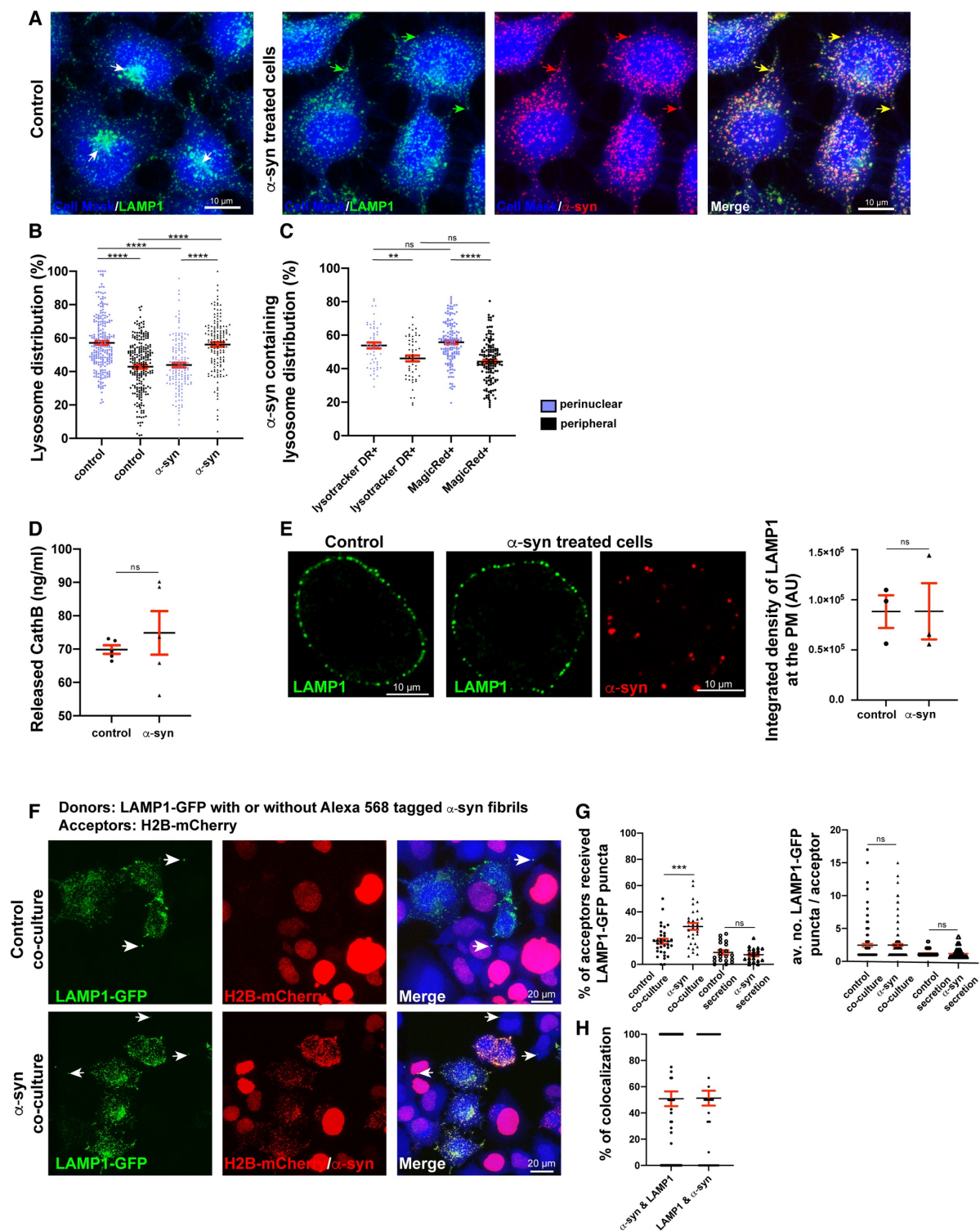


Figure-6

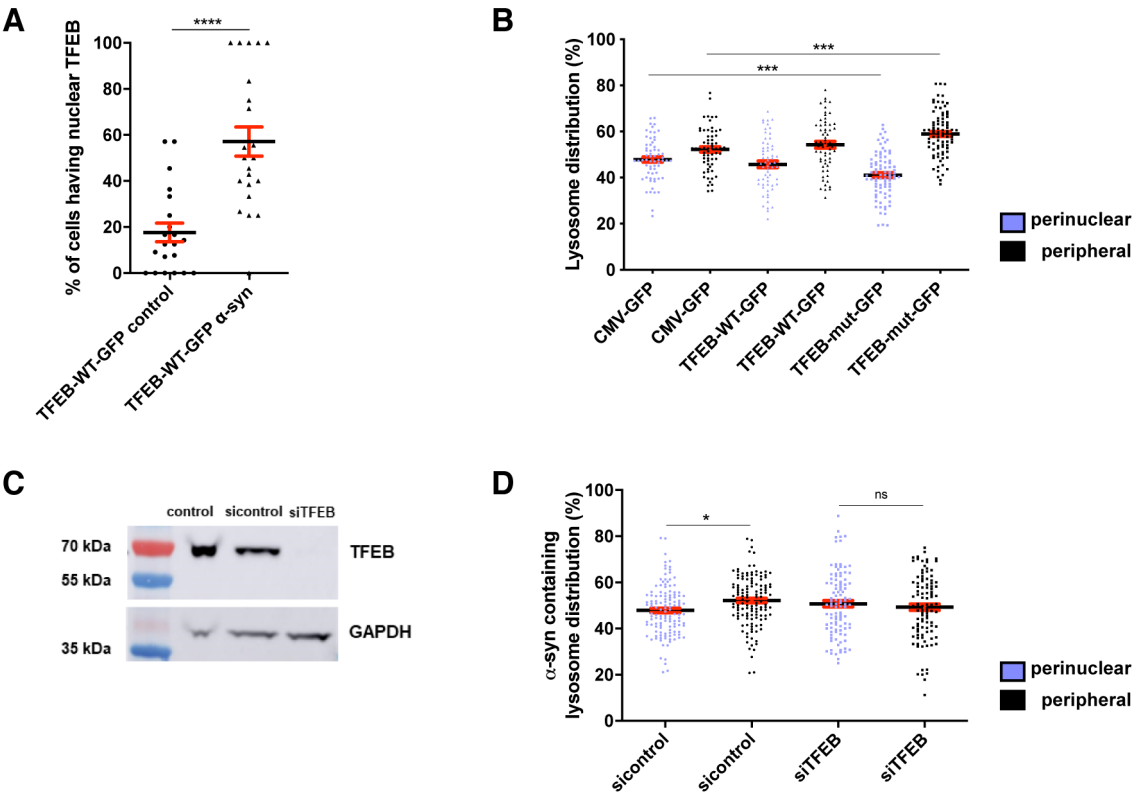


Figure-7

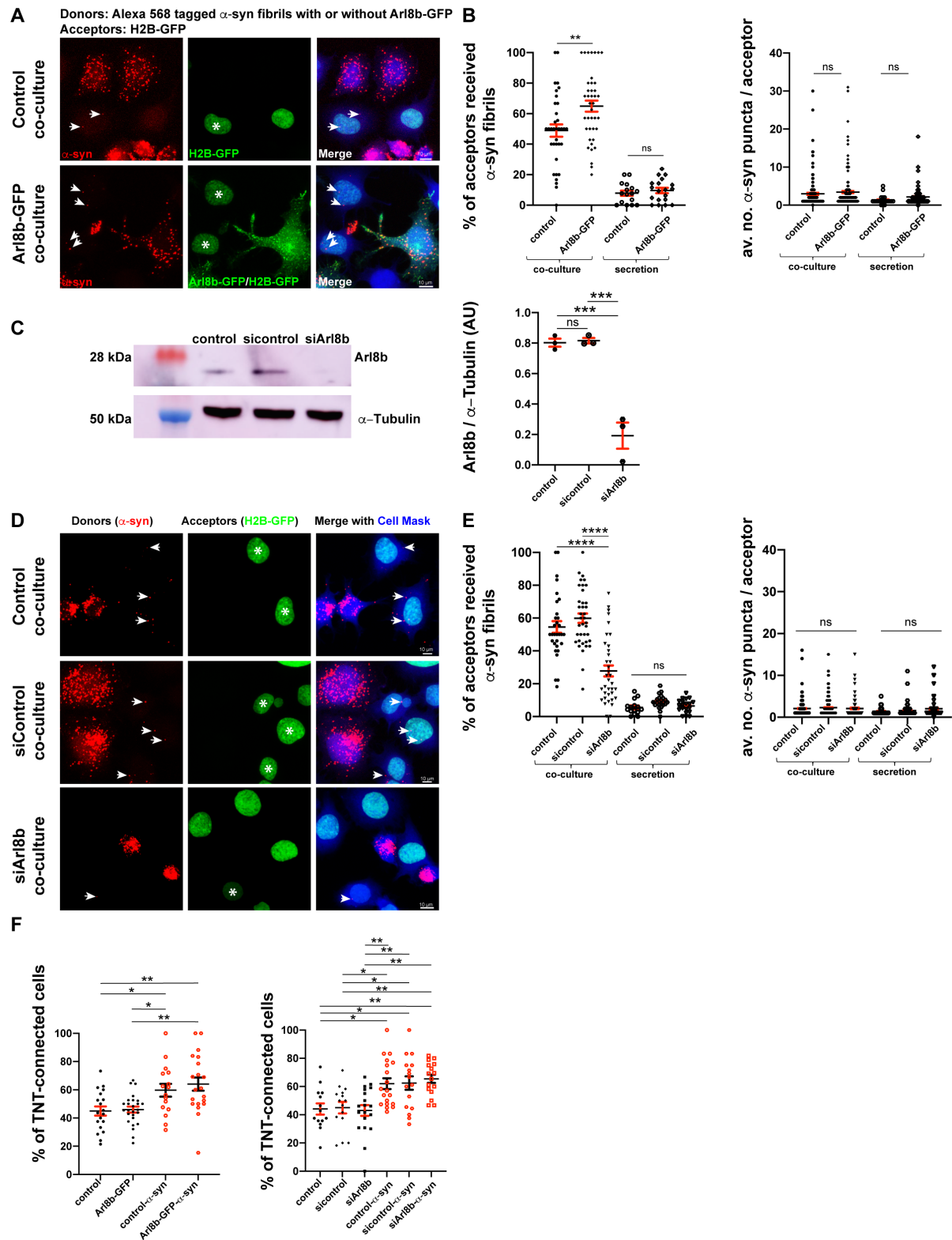


Figure-8

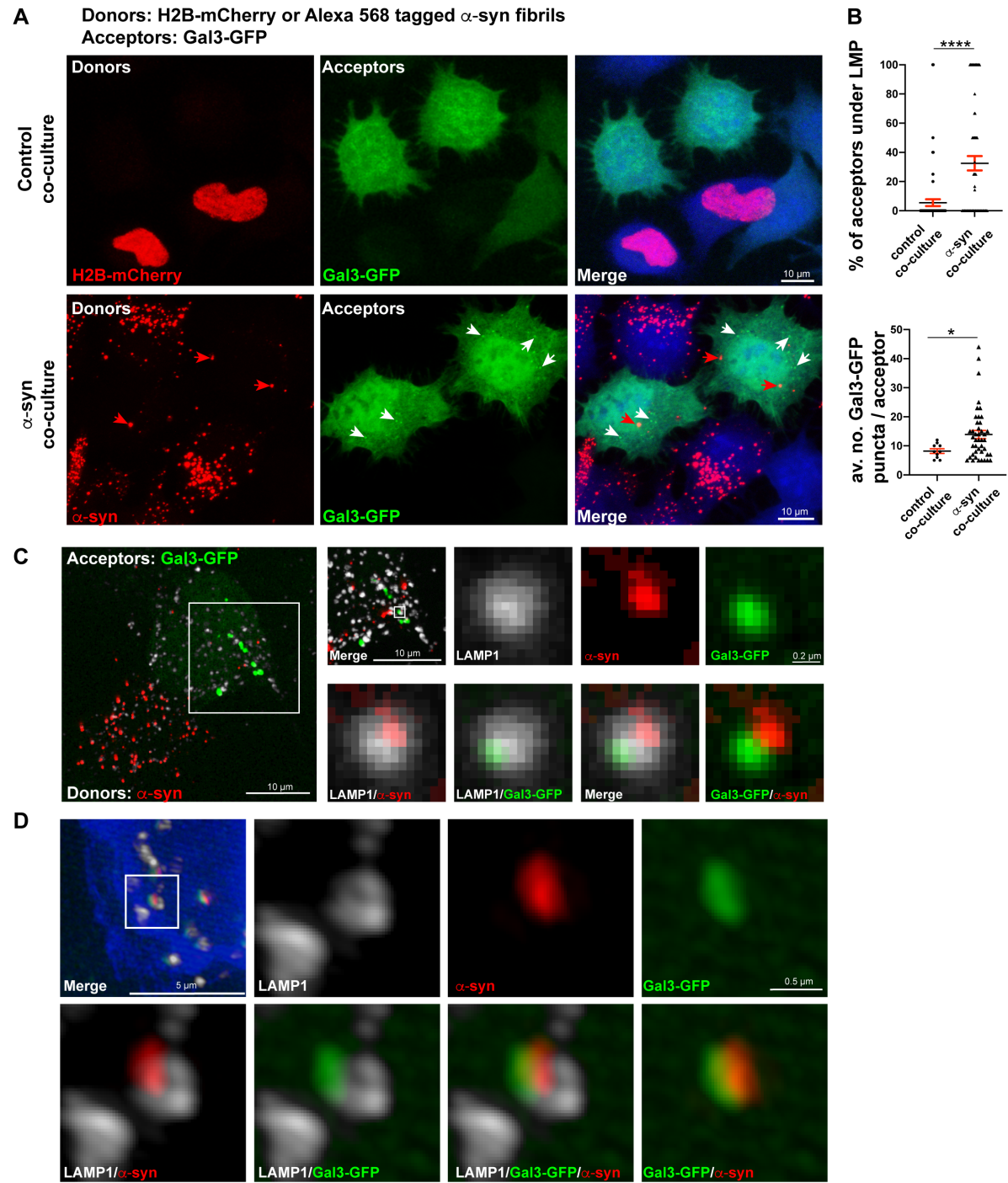


Figure-9

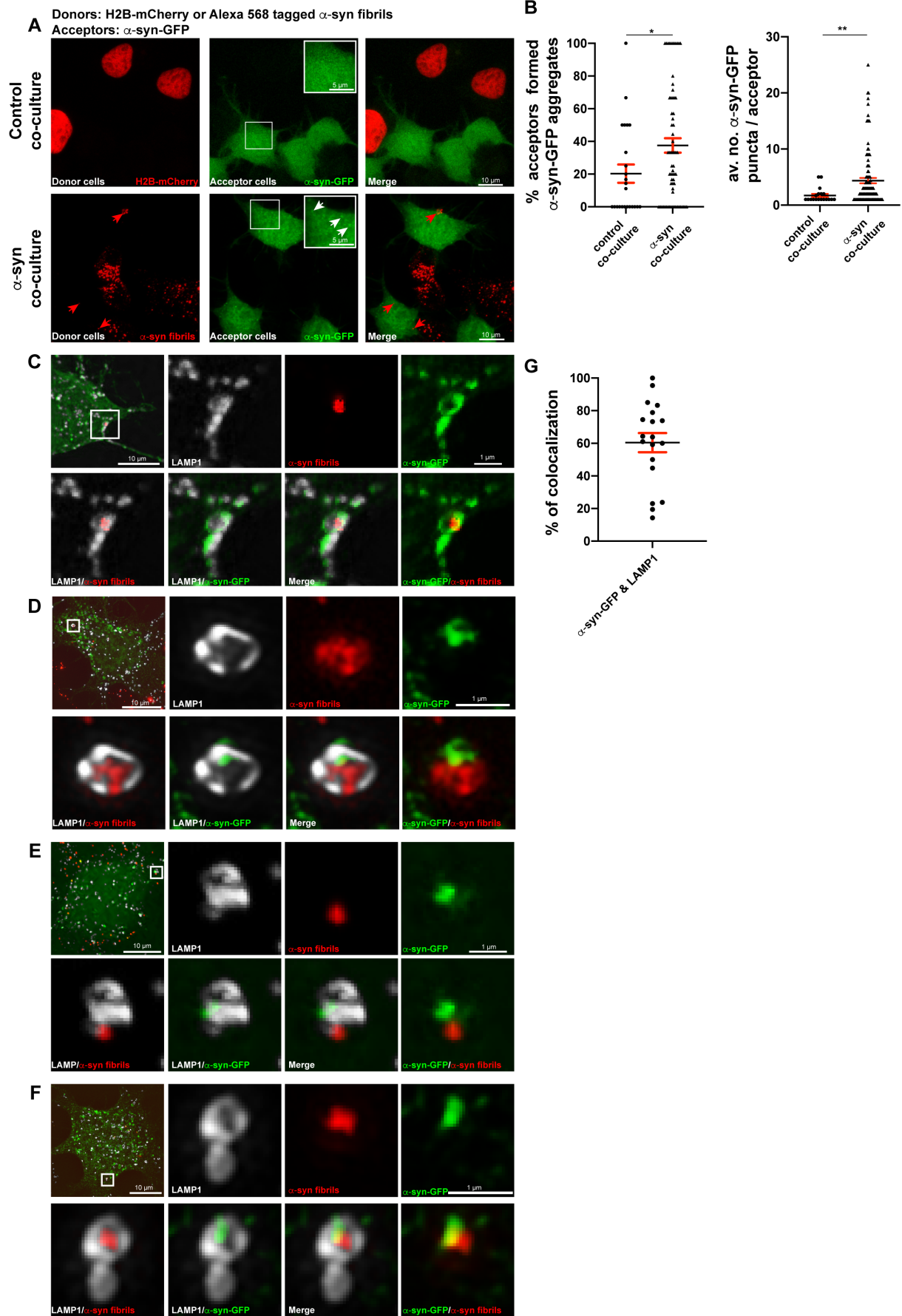


Figure-10

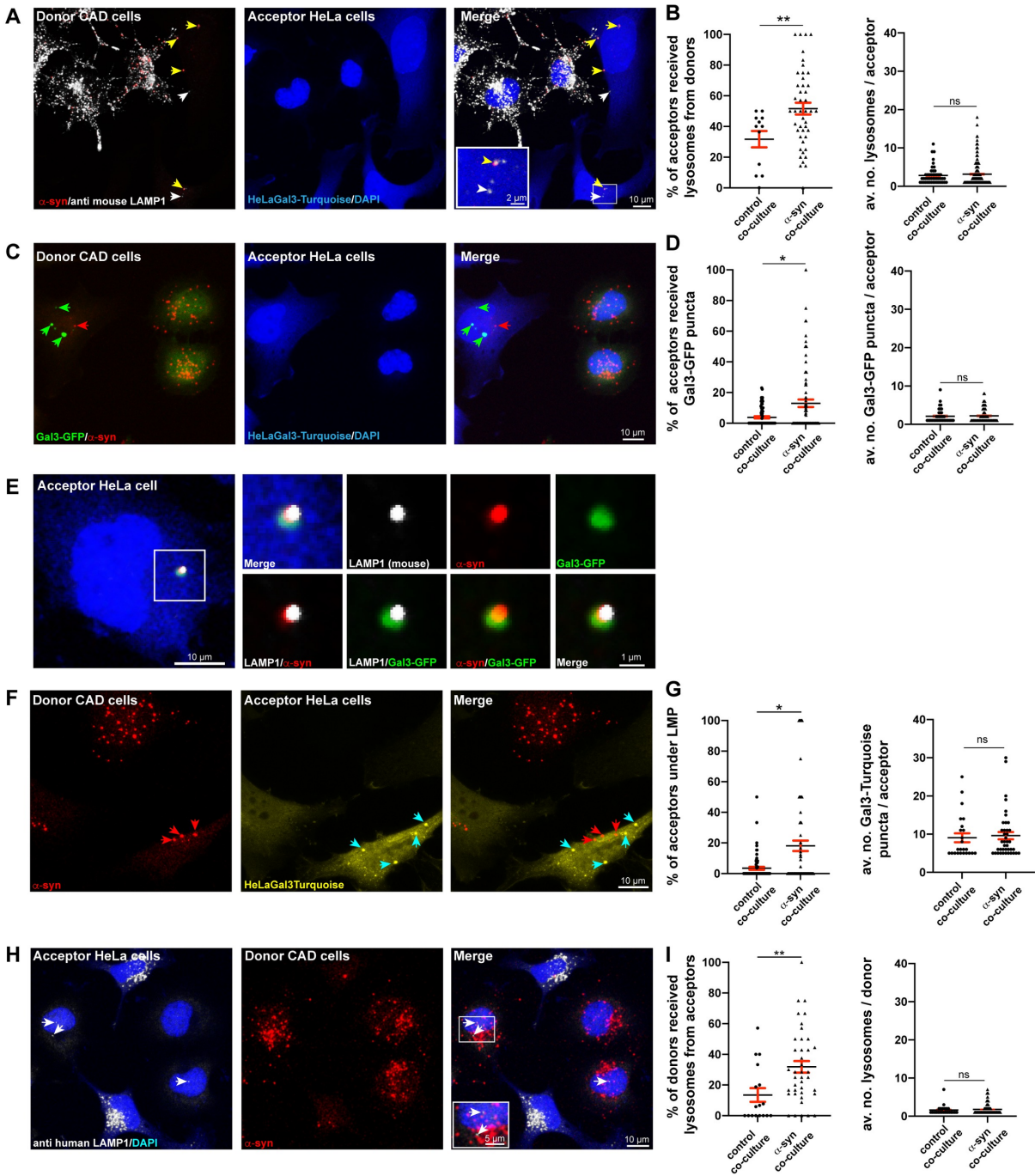


Figure-11

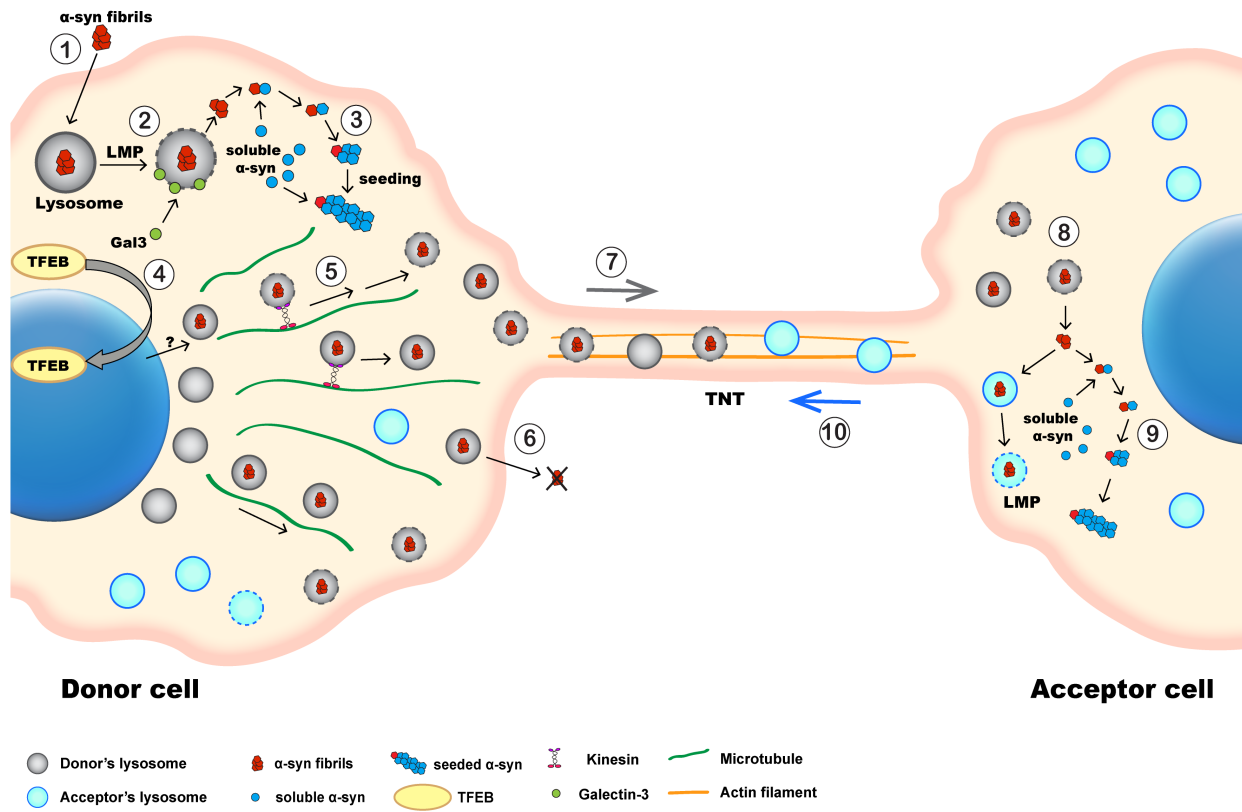


Figure S1

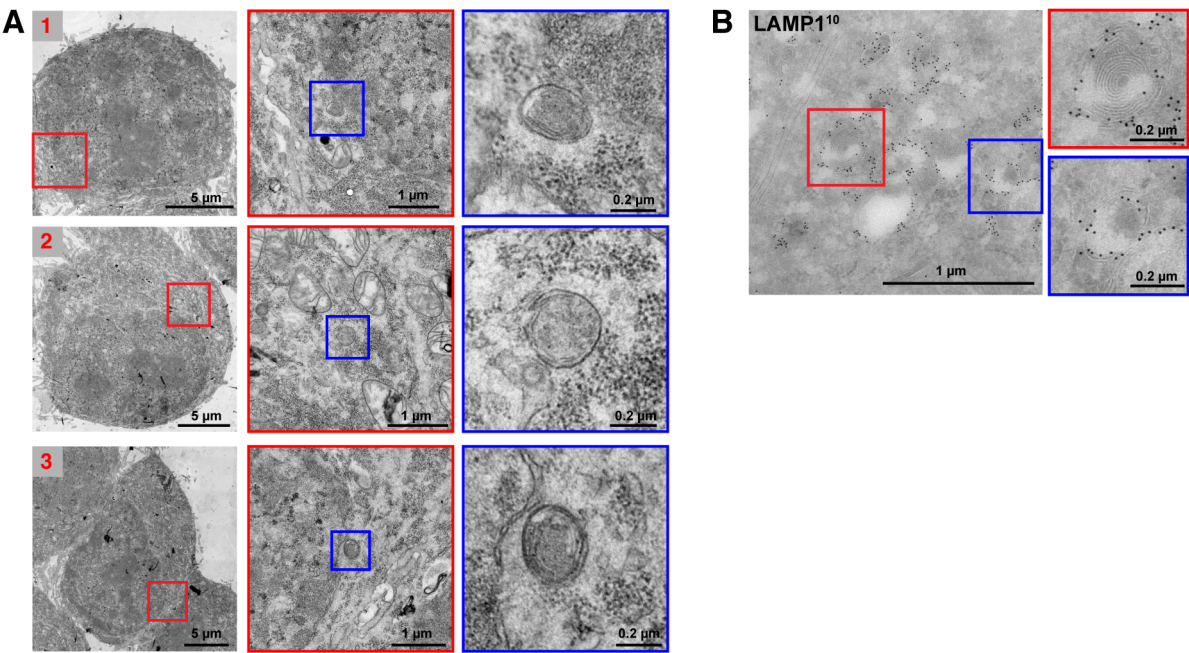


Figure S2

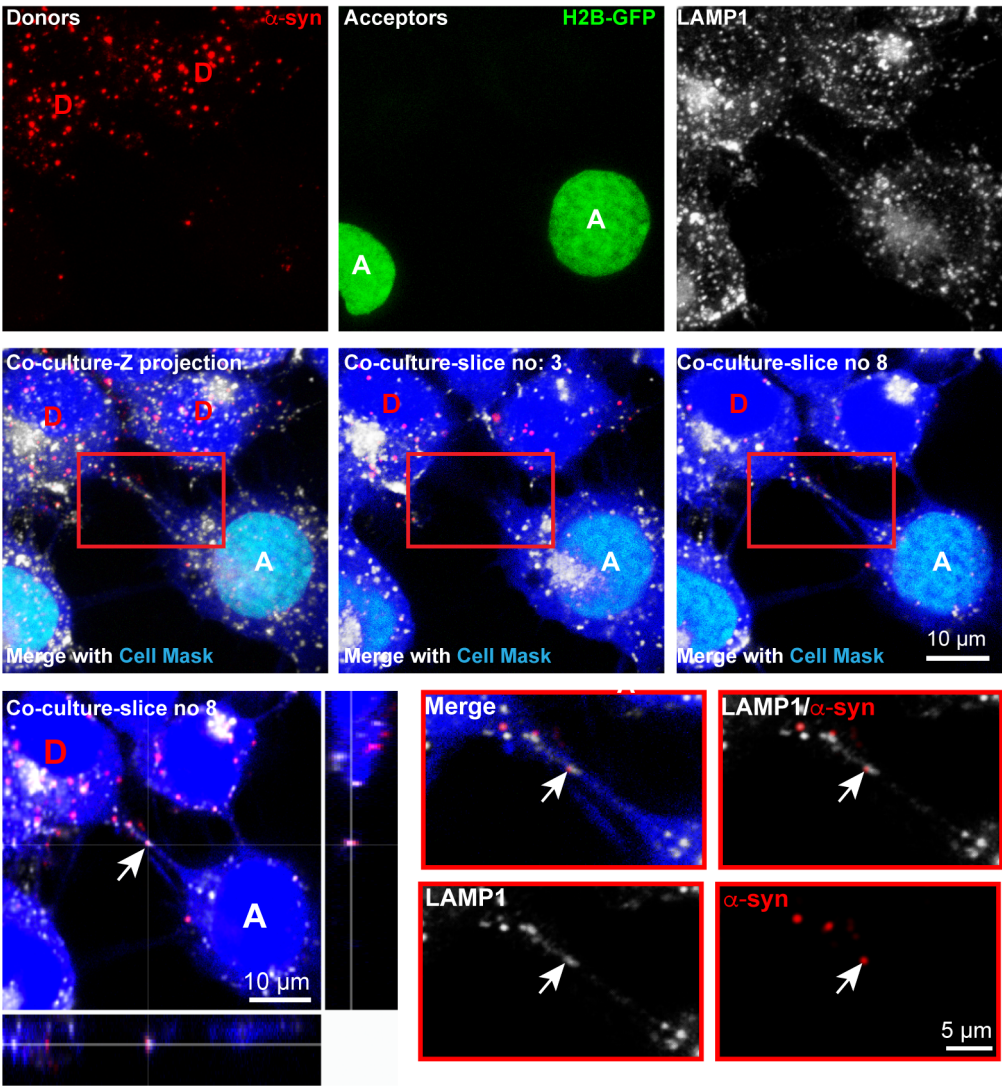


Figure S3

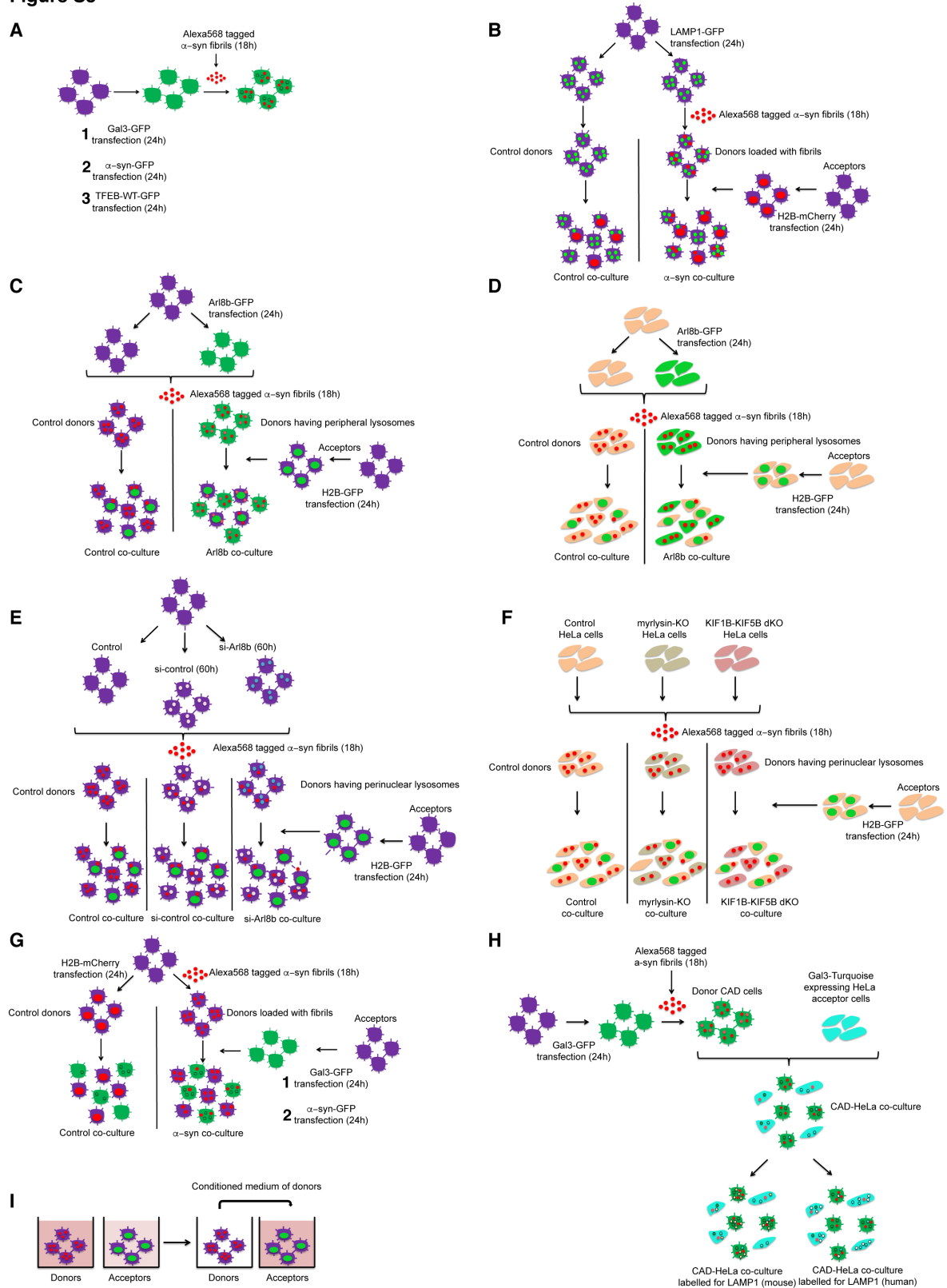


Figure S4

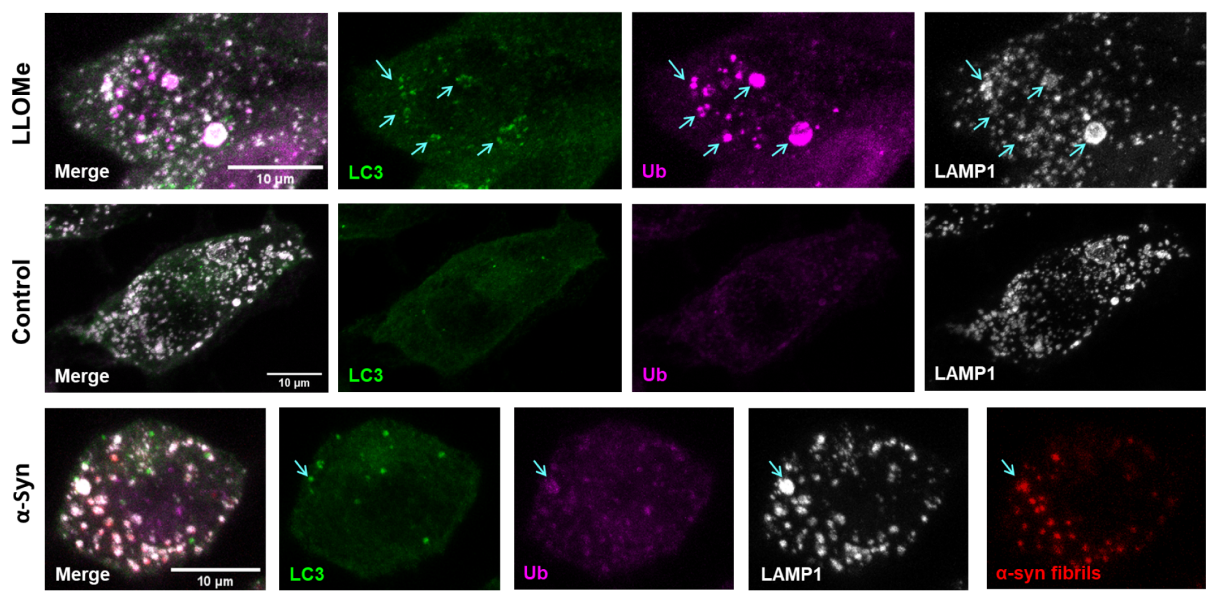


Figure S5

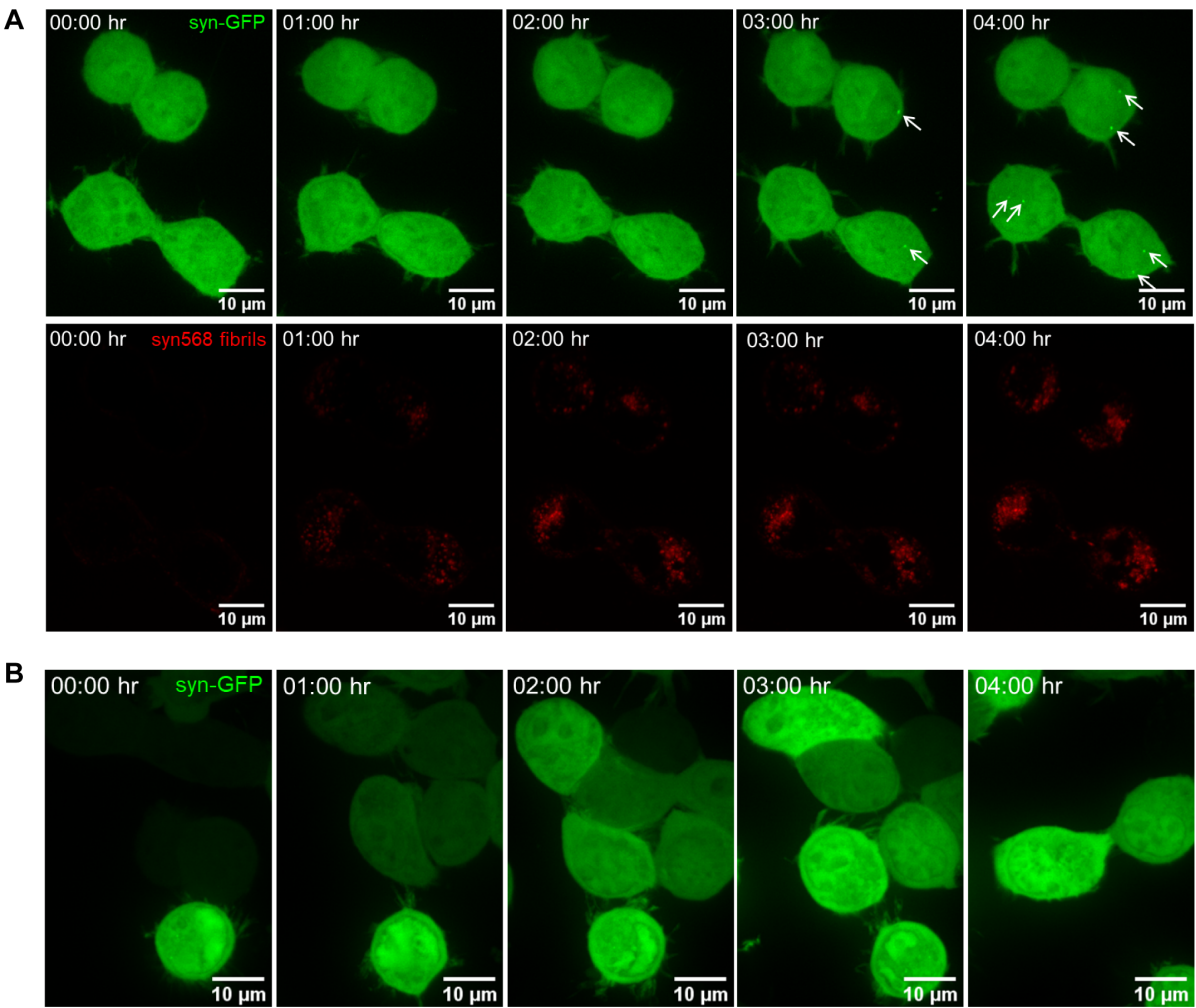


Figure S6

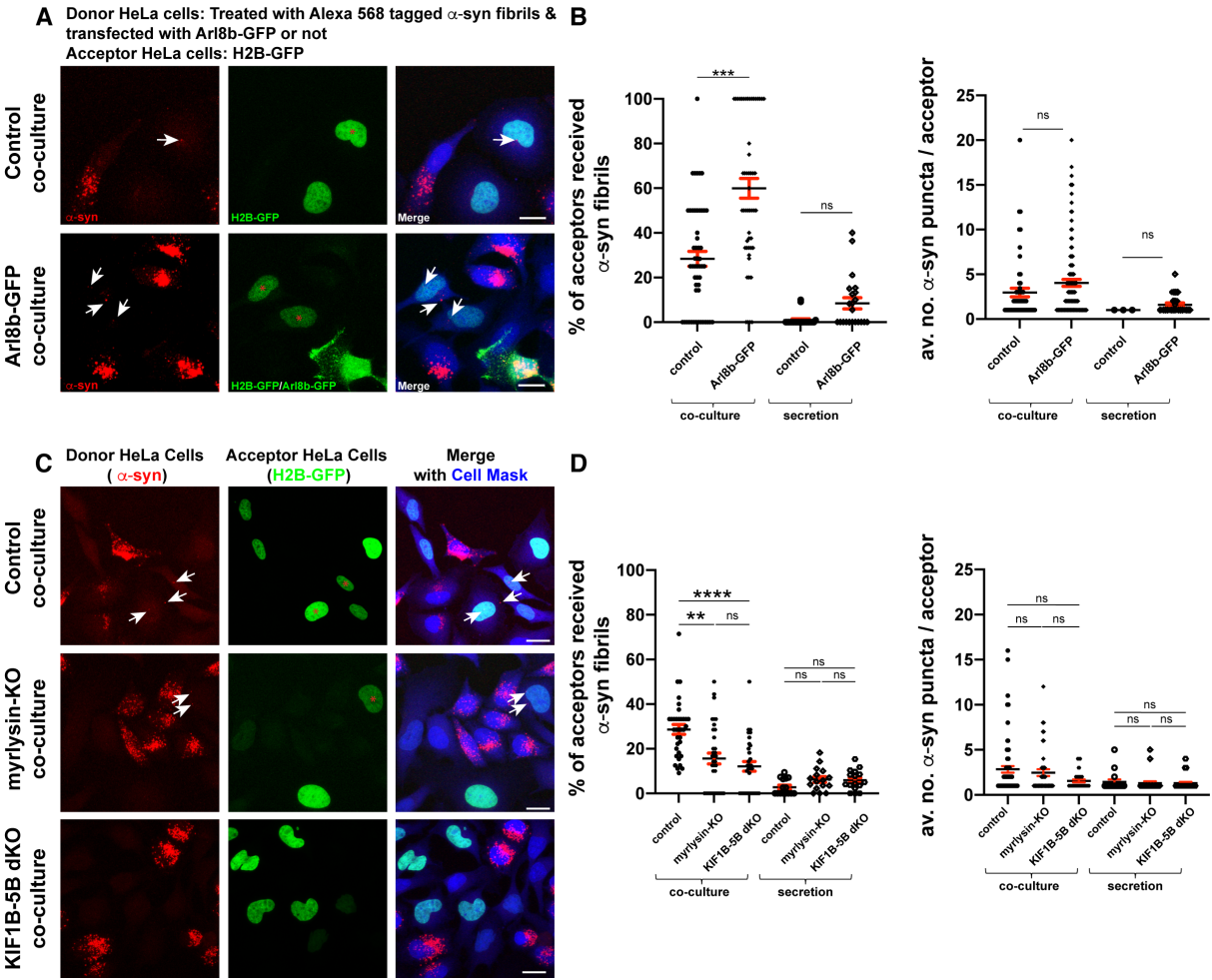
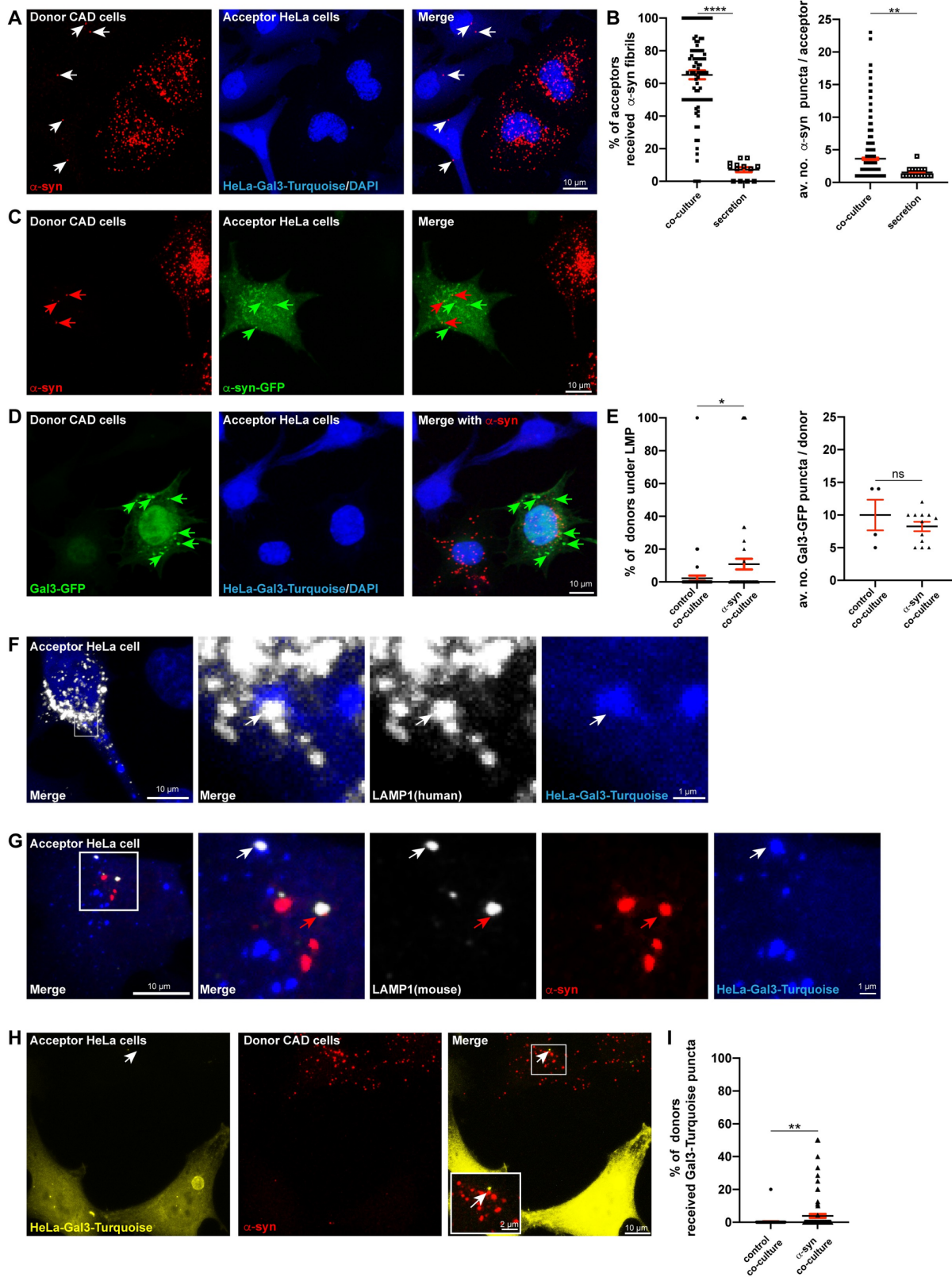


Figure S7



Funding

Association pour la Recherche sur la Sclérose Latérale Amyotrophique et Autres Maladies du Motoneurone (ARSLA 2016), INNOV 39-2019 Flash Start (Institut Pasteur) supported ADS, Access to Correlative Light and Electron Microscopy Flagship Node was supported by Euro-BioImaging grant (EuBI_AYDI109) for ADS,

MS was supported by France Alzheimer (S-FB17026) and the Marie Skłodowska-Curie Action H2020-MSCA-IF-2019 EU project 897378

Agence Nationale de la Recherche (ANR-16-CE16-0019-01), Fondation pour la Recherche Médicale (FRM-2016-DEQ20160334896) and LECMA-Vaincre Alzheimer (2016 / FR-16020) and France Alzheimer (AAP SM 2017#1674) foundations financially supported CZ,

Intramural Program of NICHD (project ZIA HD001607) supported JSB, Grant-in-Aids for Scientific Research (JP26117005), CREST, JST (JP18071300) and AMED Brain/MINDS (JP18dm0207019) supported MH, Brain Science Foundation supported TN, NL was supported by the Netherlands Organization for Scientific Research (NWO) through a ZonMW-TOP grant (91216006) to JK.

Agence Nationale de la Recherche (ANR-10-INSB-04), ANR/FBI and the Région Ile-de-France Domaine d'Intérêt Majeur-Malinf program supported the use of structured illumination microscopy (SIM) at the BioImagerie Photonic platform at Institut Pasteur