

***In situ* architecture of neuronal α -Synuclein inclusions**

V.A. Trinkaus et al.

Supplementary Files

Contents

Supplementary Table 1

Supplementary Table 2

Supplementary Fig. 1

Supplementary Fig. 2

Supplementary Fig. 3

Supplementary Fig. 4

Supplementary Fig. 5

Supplementary Fig. 6

Supplementary Fig. 7

Supplementary Methods

Supplementary Table 1 | Statistics of cryo-ET experiments on mouse neurons.

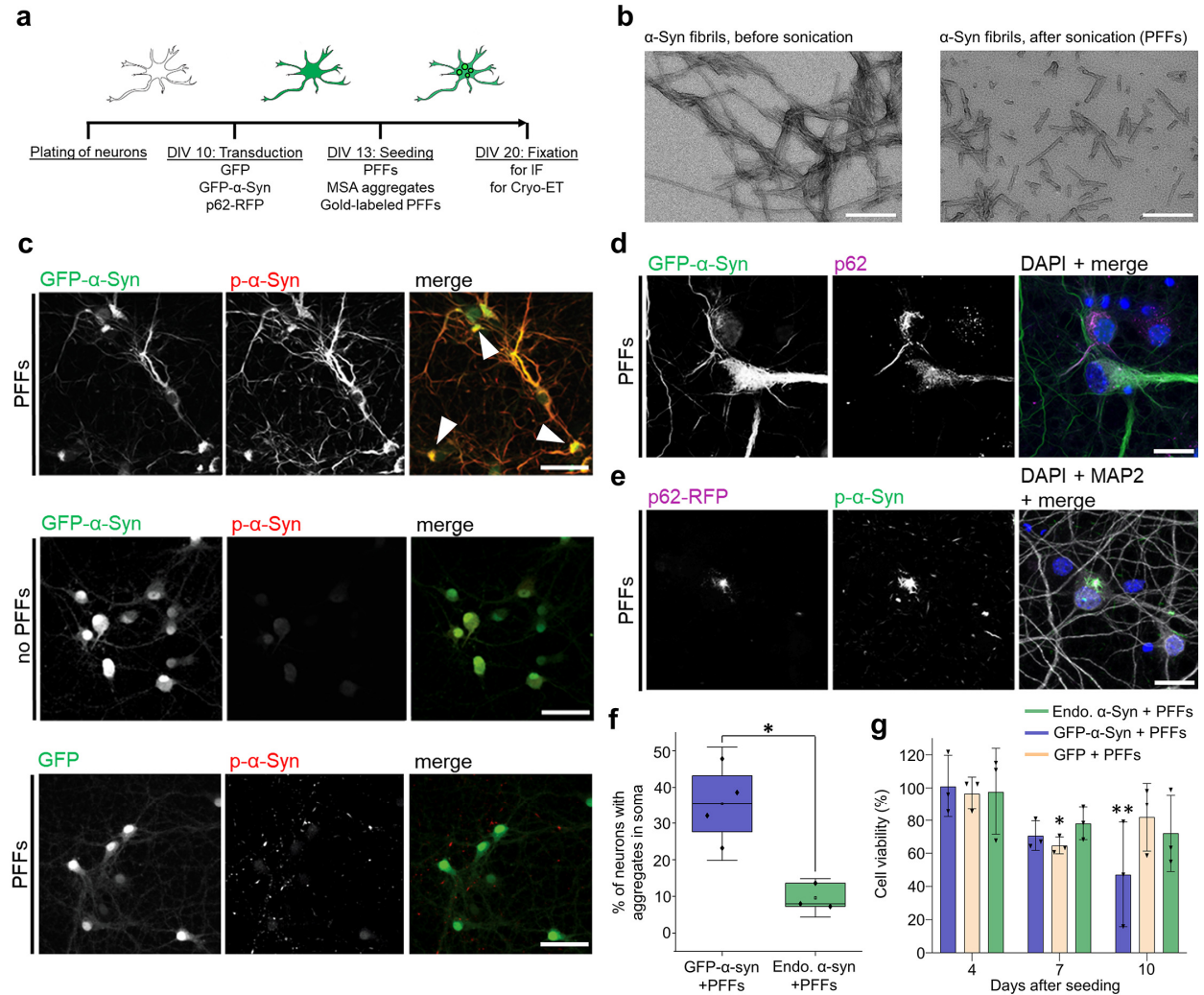
Condition	Experiments	Analyzed tomograms	Analyzed filaments	Analyzed membrane area (μm^2)
GFP- α -syn + PFFs	3	6	1592	4.11
Endogenous α -syn + PFFs	3	4	220	2.87
GFP- α -syn + MSA	3	5	721	3.70
Untransduced - PFFs	2	5	-	3.67

Neurons were either transduced with GFP- α -syn and seeded with PFFs (“GFP- α -syn + PFFs”), transduced with p62-RFP and seeded with PFFs (“Endogenous α -syn + PFFs”), transduced with GFP- α -syn and seeded with aggregates derived from a MSA patient brain (“GFP- α -syn + MSA”), or untransduced and unseeded as control (“Untransduced - PFFs”). The column “Experiments” lists biologically independent replicates. “Analyzed filaments” includes all filaments analyzed in Fig. 2d, e, Fig. 4 and Supplementary Fig. 7d. “Analyzed membrane area” includes all membranes analyzed in Fig. 4 and Supplementary Fig. 7e.

Supplementary Table 2 | List of primers.

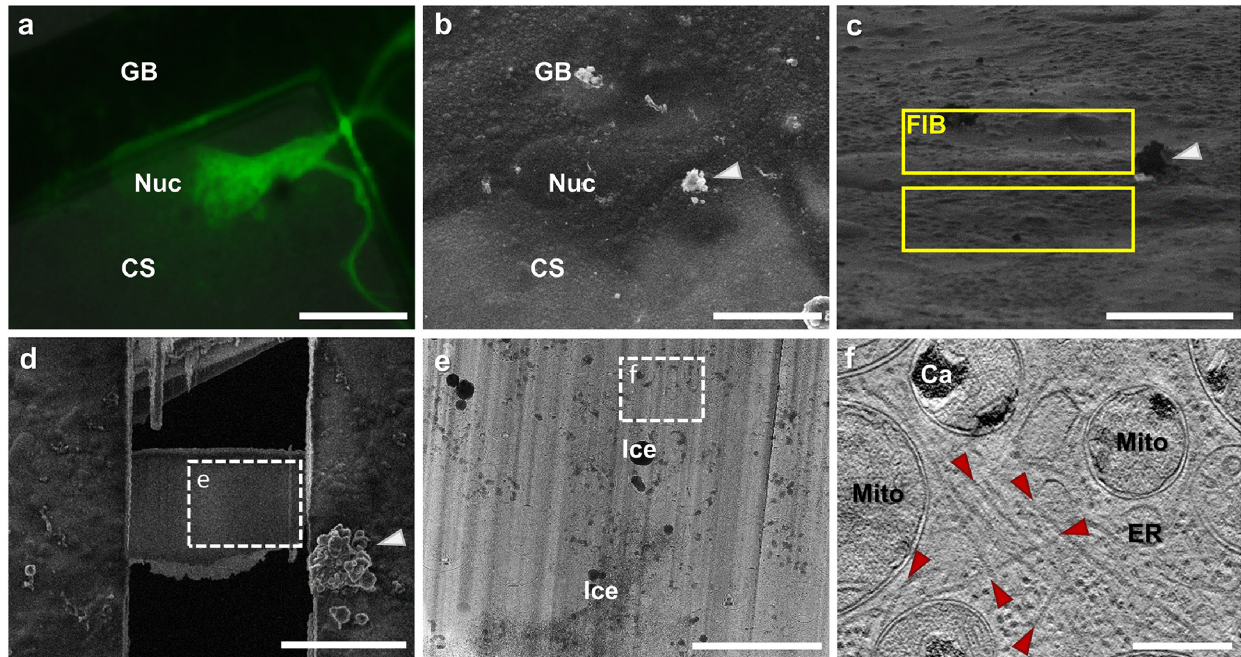
Primer name	Sequence
Forward primer pFhSynW2	GCA GTC GAG AGG ATC CCG GGC CCA CCA TGG TGA GCA
GFP-synA53T-Flag	AGG GCG AG
Reverse primer pFhSynW2	CCG CTC TAG AGC TAG CTT ATT TAT CGT CGT CAT CCT
GFP-synA53T-Flag	TGT AAT CGG CTT CAG GTT CGT AGT CTT GAT AC
Forward primer pFhSynW2	GAG CGC AGT CGA GAG GAT CCC CCA CCA TGG ATT ACA
Flag-GFP	AGG ATG ACG ACG ATA AGC CCG GGA TGG TGA GCA AGG GCG AG
Reverse primer pFhSynW2	GCT TGA TAT CGA ATT CTT ACT TGT ACA GCT CGT CCA
Flag-GFP	TGC

Primers used for cloning the pFhSynW2 GFP-synA53T-Flag and pFhSynW2 Flag-GFP constructs.

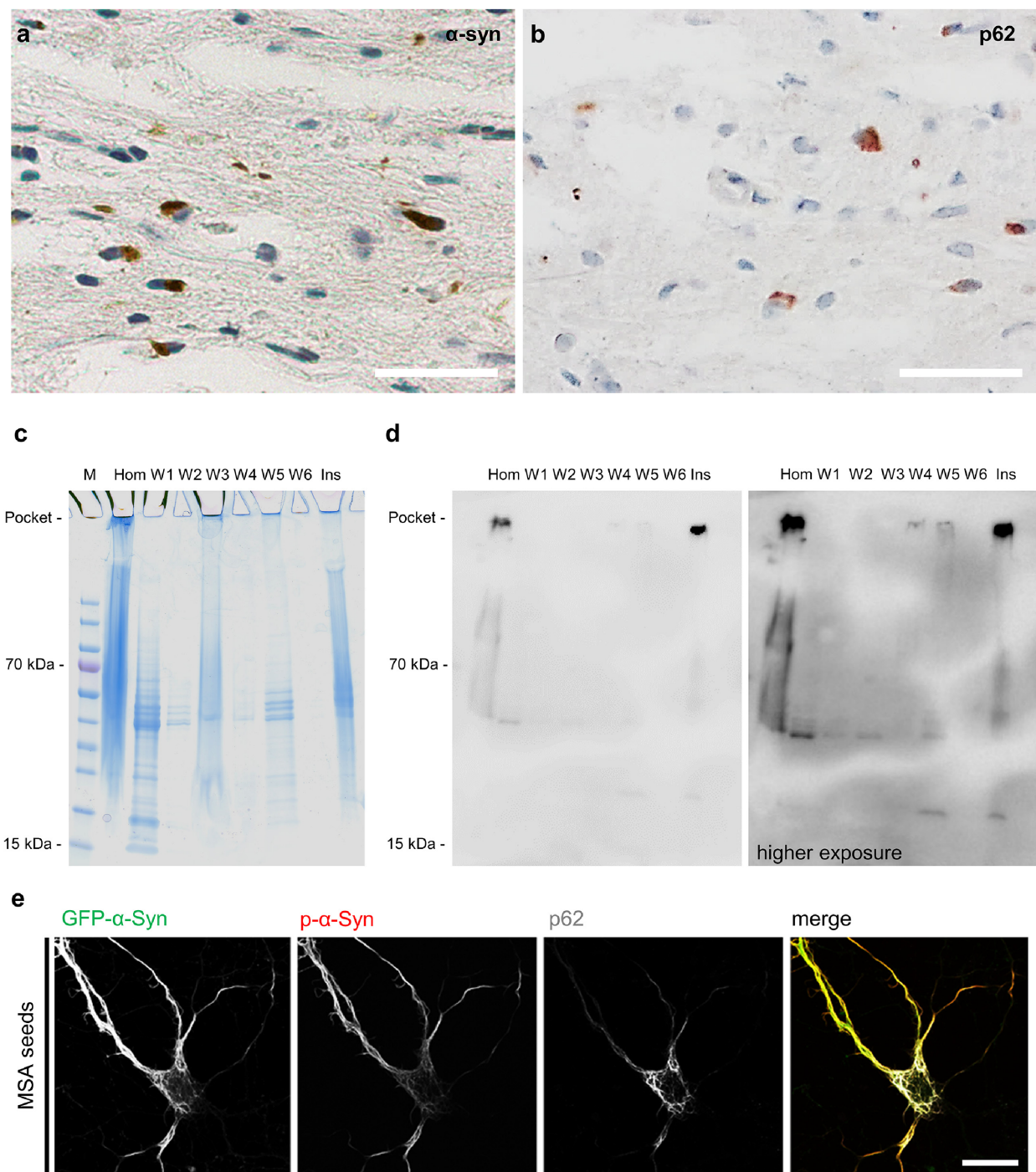


Supplementary Fig. 1 | Seeding of α -Syn aggregates in neurons. **a**, Schematic of the seeding of α -Syn aggregates in primary neurons. Primary mouse neurons were transduced at day *in vitro* (DIV) 10 with GFP, GFP- α -Syn or p62-RFP. Seeds (PFFs or MSA brain-derived) were applied at DIV 13, and α -Syn inclusions were studied at DIV 20 by light microscopy or cryo-ET upon chemical or cryo-fixation, respectively. For light microscopy imaging, GFP signal was enhanced by staining with an antibody against GFP. **b**, Negative stain images of α -Syn fibrils before (left) and after (right) sonication. Sonicated seeds were used for all seeding experiments. Scale bars: 250 nm. Two biologically independent experiments were performed. **c**, Immunofluorescence imaging of α -Syn aggregates, as detected by an antibody against phosphorylated α -Syn Ser129 (p- α -Syn). Top: aggregate formation (arrowheads) upon seeding cells expressing GFP- α -Syn with exogenous PFFs. Middle: no aggregate formation in cells expressing GFP- α -Syn in the

absence of PFFs. Bottom: PFFs seed smaller aggregates in cells with endogenous α -Syn levels that express GFP only as control (see Supplementary Fig. 1f for quantification). Scale bars: 50 μ m. Two biologically independent experiments were performed. **d**, Immunofluorescence imaging of GFP- α -Syn aggregates detected by an antibody against p62. The merged image shows a superposition of the GFP- α -Syn (green), p62 (magenta) and DAPI (blue) channels. An arrowhead indicates the colocalization of GFP- α -Syn and p62. Scale bar: 20 μ m. Two biologically independent experiments were performed. **e**, Immunofluorescence imaging of endogenous α -Syn aggregates positive for p- α -Syn colocalizing with p62-RFP. The merged image shows a superposition of the p62-RFP (magenta), phospho- α -Syn (green), the neuronal marker MAP2 (gray) and DAPI (blue) channels. Scale bar: 20 μ m. Two biologically independent experiments were performed. **f**, Quantification of the percentage of neurons with aggregates in the soma upon treatment with PFFs of cells transduced with GFP- α -Syn (blue) or untransduced (green; endogenous α -Syn). The horizontal lines of each box represent 75% (top), 50% (middle) and 25% (bottom) of the values, and a black square the average value. Whiskers represent 1.5x standard deviation and black diamonds the individual data points. * indicates $p = 0.011$ by two-tailed unpaired t-test with Welch's correction, $n = 4$ (GFP- α -Syn + PFFs) and 3 (endogenous α -Syn + PFFs) biologically independent experiments. **g**, Quantification of neuronal viability upon seeding with PFFs for cells expressing endogenous α -Syn (Endo. α -Syn + PFFs), or transduced with GFP- α -Syn (GFP- α -Syn + PFFs) or with GFP only (GFP + PFFs) relative to untransduced and unseeded control cells. Bars represent average values, the error bars the standard deviation and black triangles the individual data points. * and ** respectively indicate $p = 0.04$ and $p = 0.002$ by two-way ANOVA and Dunnett's multiple comparison test, $n = 3$ biologically independent experiments for all conditions. Representative images are shown in **b**, **c**, **d**. Source data for **f**, **g** are provided as a Source Data file.



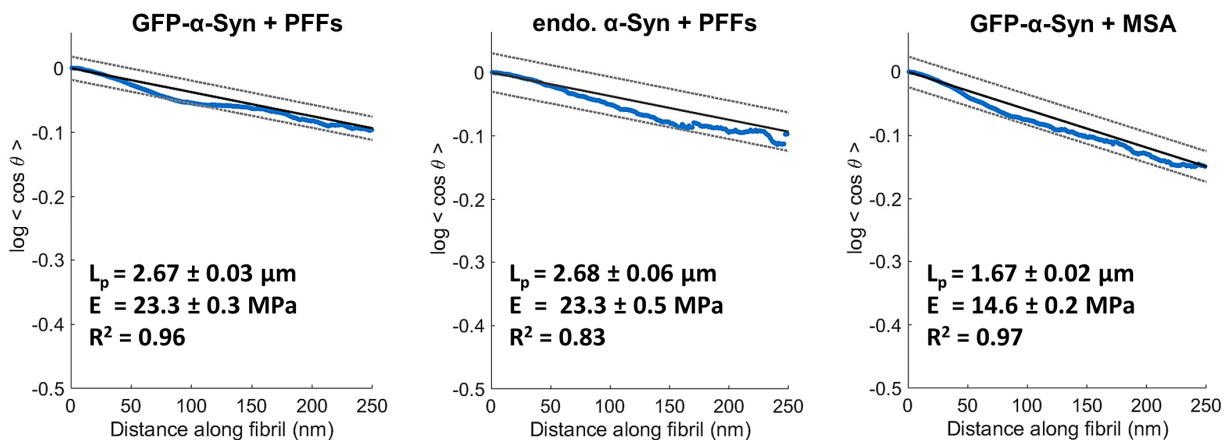
Supplementary Fig. 2 | Cryo-ET workflow. **a**, Cryo-light microscopy imaging of GFP fluorescence in a primary neuron grown on the carbon support (CS) of an EM grid. The cell was transduced with GFP- α -Syn at DIV 10 and aggregate formation was seeded at DIV 13. The grid was vitrified at DIV 20. GB: grid bar, Nuc: nucleus. Scale bar: 25 μ m. **b**, Correlative scanning electron microscopy imaging of the same cell within the cryo-FIB instrument upon coordinate transformation. A white arrowhead marks a piece of ice crystal contamination that can also be found in panels **c** and **d** as visual reference. Scale bar: 25 μ m. **c**, FIB-induced secondary electron image of the same cell. Yellow boxes indicate the regions to be milled away by the FIB during lamella preparation. Scale bar: 15 μ m. **d**, Scanning electron microscopy imaging of the same cell upon preparation of a 150 nm-thick electron transparent lamella. The white rectangle marks the region of the lamella shown in **e**. Scale bar: 10 μ m. **e**, Low magnification transmission electron microscopy image of the area of the lamella marked in **d**. Ice: ice crystal contamination on the lamella surface. The white rectangle marks the region shown in **f**. Scale bar: 3 μ m. **f**, A tomographic slice (thickness 1.4 nm) recorded in the area indicated in **e**. Ca: mitochondrial calcium stores, ER: endoplasmic reticulum, Mito: mitochondrion. Red arrowheads indicate α -Syn fibrils. Scale bar: 300 nm. The number of tomograms and biologically independent cryo-ET experiments is listed in Supplementary Table 1. Representative images are shown for all panels.



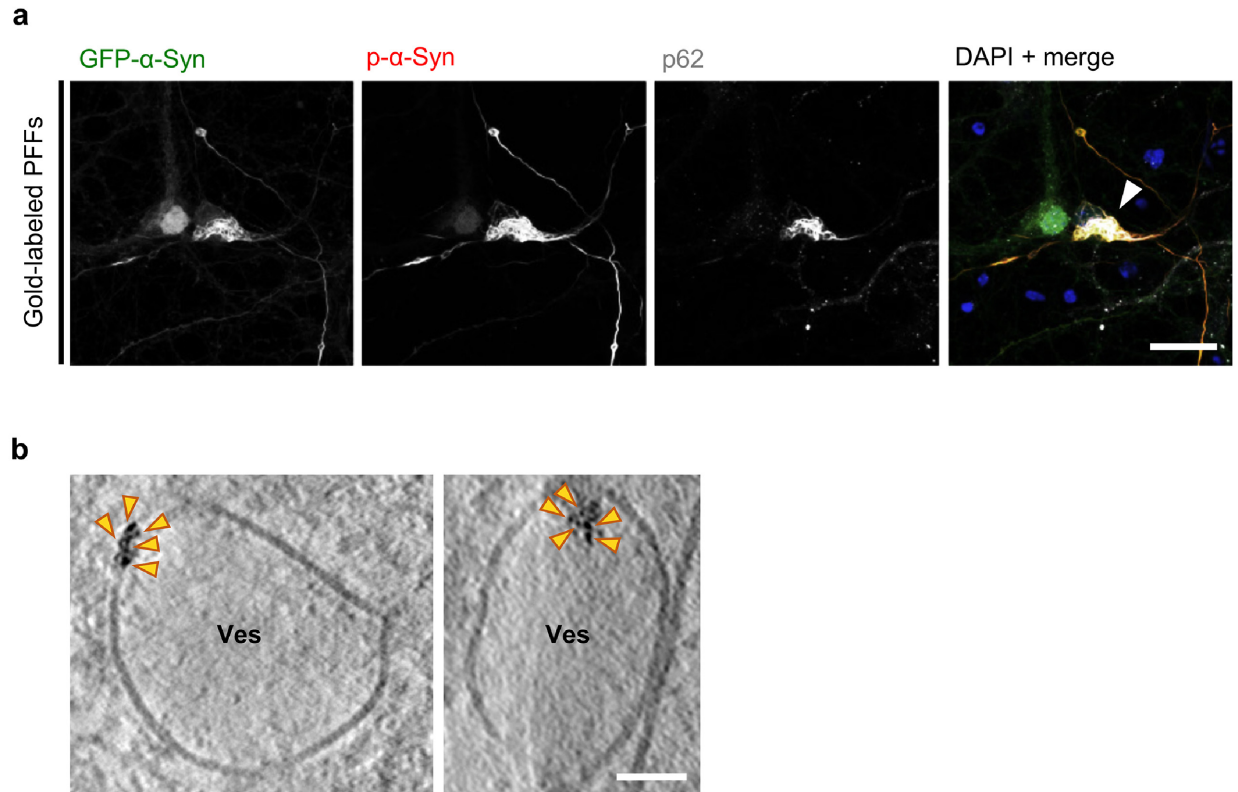
Supplementary Fig. 3 | Purification of α -Syn aggregates from MSA patient brain.

a, b, Immunohistochemistry staining showing cytoplasmic inclusions (brown) positive for α -Syn (**a**) and p62 (**b**) in the basilar part of the pons of the brain of an MSA patient. Nuclei are stained in blue. Aggregates for seeding neurons for cryo-ET imaging were purified from the same region

(**c**, **d**). Scale bars: 50 μ m. Experiment was performed once. **c**, **d**, Purification of α -Syn aggregates from the MSA patient brain shown in **a**, **b**. Coomassie staining (**c**) and anti-phospho- α -Syn western blot (**d**) of SDS PAGE gels loaded with brain homogenate (Hom), washing fractions (W1-6) and the final sarkosyl-insoluble fraction (Ins) at low (left) and high (right) exposure levels. M: molecular weight marker. Note the aggregated material in the stacking gel. For gel source images, see Supplementary Fig. 2. Experiment was performed once. **e**, Immunofluorescence images of a GFP- α -Syn-expressing neuron seeded with the sarkosyl-insoluble fraction from MSA patient brain, showing aggregates positive for phospho- α -Syn and p62. GFP signal was enhanced by staining with an antibody against GFP. The merged image shows a superposition of the GFP- α -Syn (green), phospho- α -Syn (red) and p62 (gray) channels. Scale bar: 20 μ m. Two biologically independent experiments were performed. Representative images are shown for all panels. Source data for **c**, **d** are provided as a Source Data file.

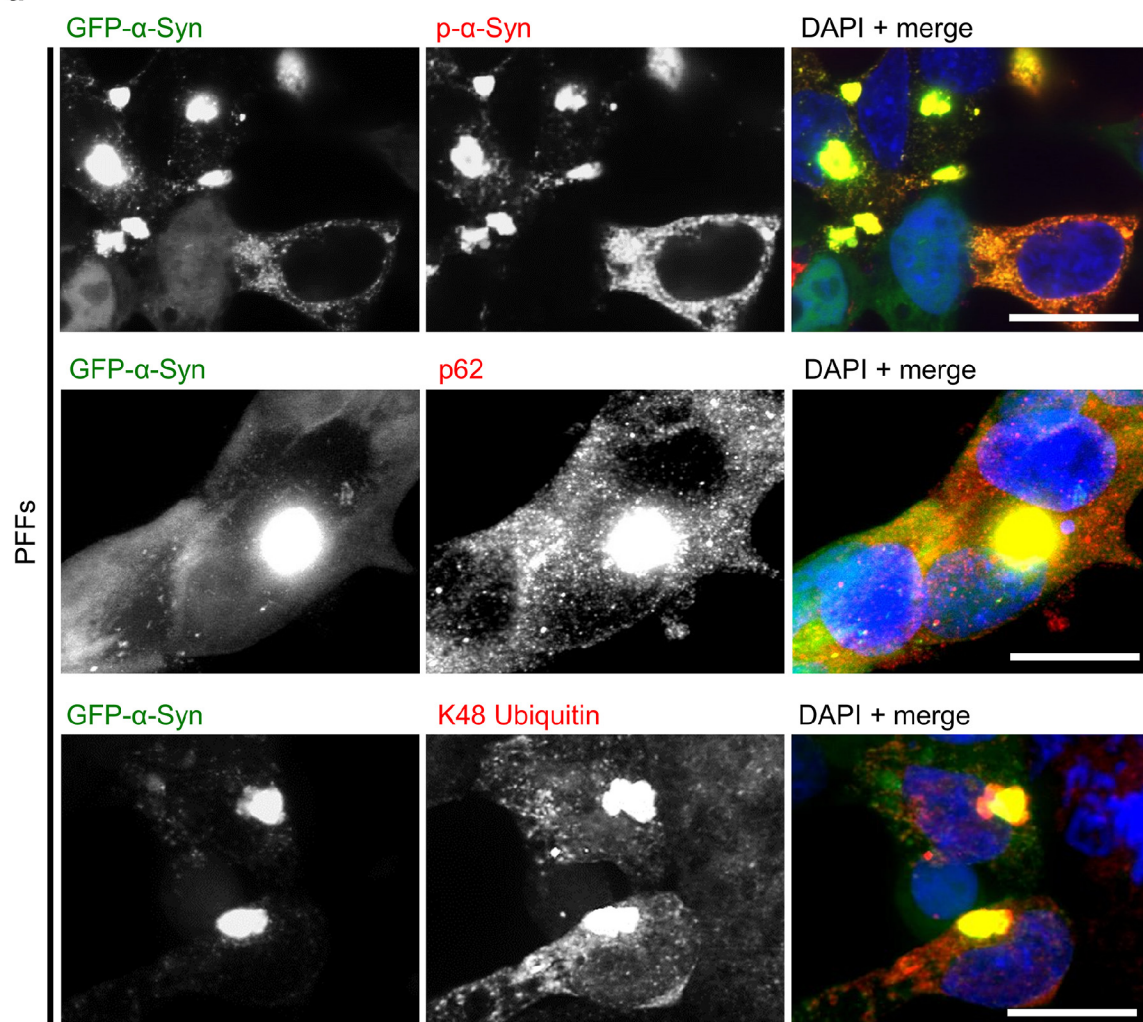


Supplementary Fig. 4 | Persistence length of α -Syn fibrils. Linear fit of the total persistence length for all fibrils analyzed. $n = 1295$ (GFP- α -Syn + PFFs), 220 (endogenous α -Syn + PFFs) and 721 (GFP- α -Syn + MSA) fibrils in total over two (GFP- α -Syn + PFFs) or three (endogenous α -Syn + PFFs and GFP- α -Syn + MSA) biologically independent experiments. The blue curves represent the original data. 95% confidence interval (dotted lines) and the values of the persistence length (L_p), Young's modulus (E) and coefficients of determination (R^2) are indicated. Note that the values are almost identical for GFP- α -Syn and endogenous α -Syn seeded with PFFs, but lower for GFP- α -Syn seeded with MSA patient aggregates. Source data are provided as a Source Data file.

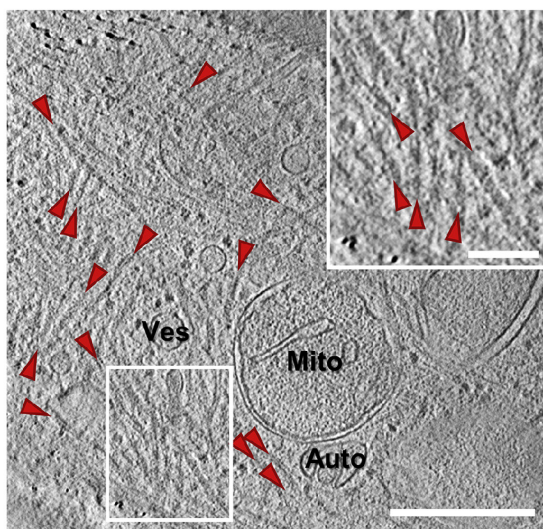


Supplementary Fig. 5 | Seeding of α -Syn aggregates in neurons by gold-labeled PFFs. a, Immunofluorescence images of a GFP- α -Syn-expressing neuron seeded with gold-labeled PFFs. The cells develop α -Syn aggregates, as detected by antibodies against phosphorylated α -Syn Ser129 and p62. GFP signal was enhanced by staining with an antibody against GFP. The merged image shows a superposition of the GFP- α -Syn (green), phospho- α -Syn (red), p62 (gray) and DAPI (blue) channels. An arrowhead indicates the GFP- α -Syn aggregates. Scale bar: 20 μ m. **b,** Tomographic slices (thickness 1.4 nm) showing accumulations of gold particles (orange arrowheads) at the membrane (left) or in the lumen (right) of intracellular vesicles. Ves: vesicles. Scale bar: 50 nm. Two biologically independent experiments were performed in all cases. Representative images are shown for all panels.

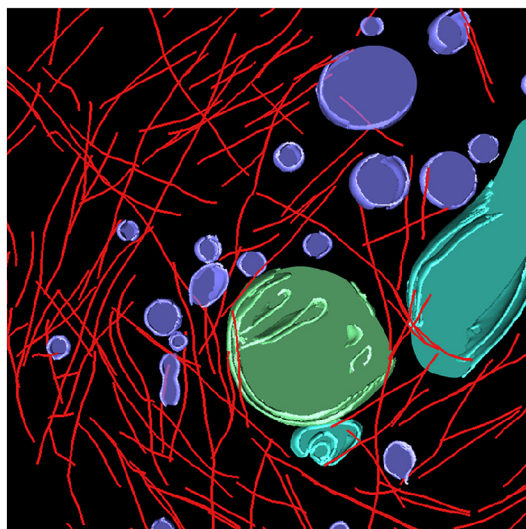
a



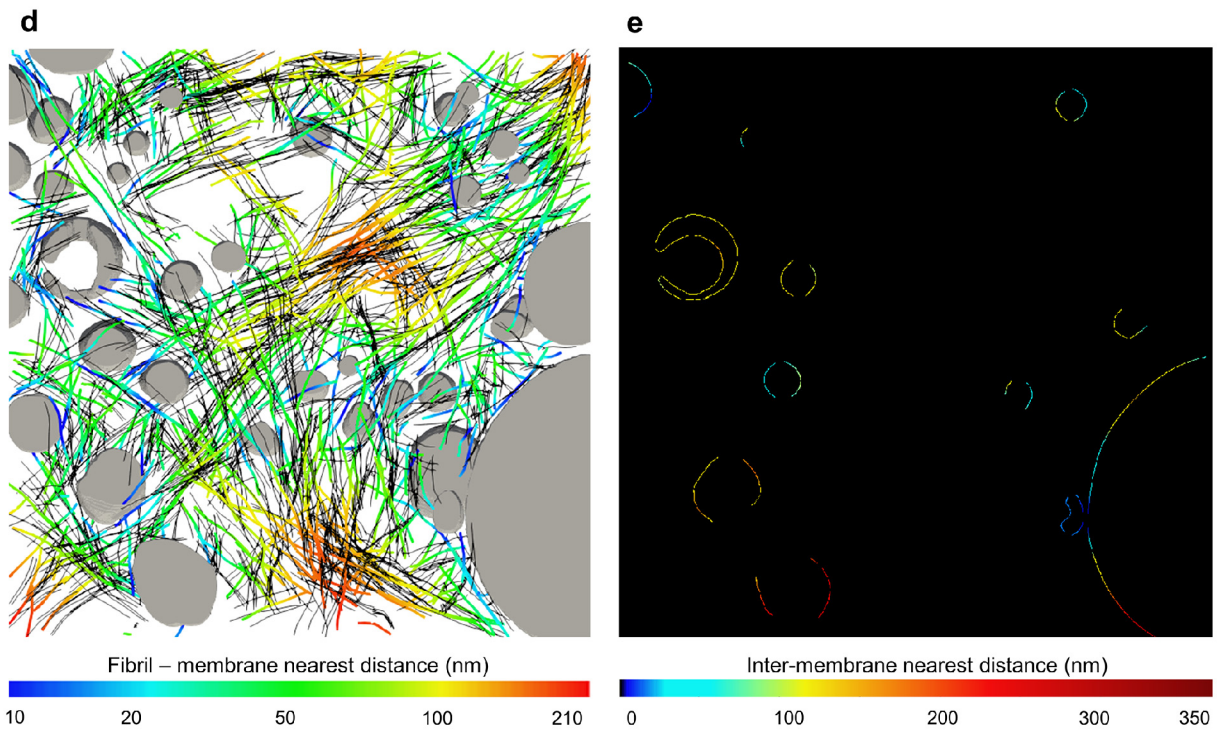
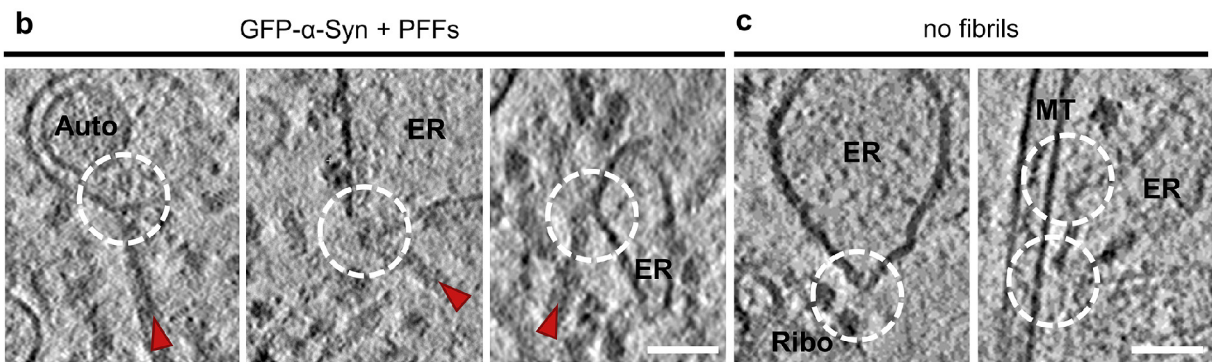
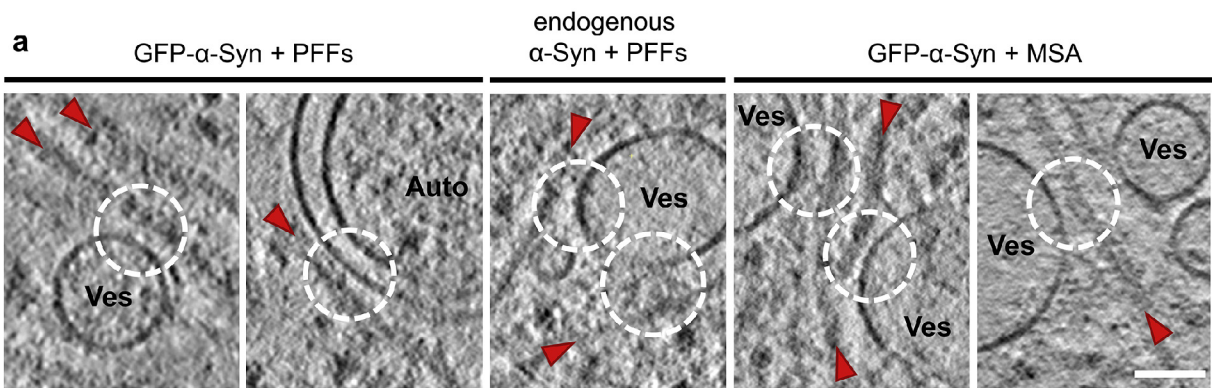
b



c



Supplementary Fig. 6 | α -Syn aggregates in SH-SY5Y cells. **a**, Immunofluorescence images of SH-SY5Y cells stably expressing GFP- α -Syn and seeded with PFFs. The cells develop α -Syn inclusions, as detected by antibodies against phosphorylated α -Syn Ser129 (top), p62 (middle) and K48-linked ubiquitin (bottom). The merged images show a superposition of the respective green and red channels plus DAPI (blue). Scale bars: 15 μ m. **b**, A tomographic slice (thickness 1.8 nm) of an inclusion seeded by PFFs in a SH-SY5Y cell expressing GFP- α -Syn. Auto: autophagosome; Mito: mitochondrion; Ves: vesicles. Fibrils are marked by red arrowheads. Scale bars: 350 nm (main panel) and 100 nm (inset). **c**, 3D rendering of the tomogram depicted in **b** showing α -Syn fibrils (red), autophagosomes (cyan), mitochondria (green) and various vesicles (purple). Three biologically independent experiments were performed in all cases. Representative images are shown for all panels. Source data are provided as a Source Data file.



Supplementary Fig. 7 | Proximity of α -Syn fibrils and cellular membranes. **a**, Gallery of tomographic slices showing close proximity events (dashed white circles) between α -Syn fibrils (red arrowheads) and different cellular membranes with no apparent interactions. Auto: autophagosome, Ves: vesicles. Tomographic slices are 1.8 nm (GFP- α -Syn + PFFs) or 1.4 nm (endogenous α -Syn + PFFs and GFP- α -Syn + MSA) thick. Scale bar: 50 nm. **b**, Gallery of tomographic slices (thickness 1.8 nm) showing apparent contacts between α -Syn fibrils and different cellular membranes at sites of high membrane curvature (dashed white circles), within inclusions seeded by PFFs in neurons expressing GFP- α -Syn. ER: endoplasmic reticulum. Scale bar: 50 nm. **c**, Tomographic slices showing sites of high membrane curvature (dashed white circles) in the absence of α -Syn fibrils in neurons expressing p62-RFP and seeded with PFFs. MT: microtubule; Ribo: ribosome. Tomographic slices are 1.4 nm thick. Scale bar: 60 nm. **d**, 3D rendering shown in Fig. 1d and Fig. 4a with α -Syn fibrils color-coded according to their distance to the nearest cellular membrane (gray). To elucidate whether the events of close proximity between fibrils and membranes were caused by chance or mediated by molecular interactions, random shifts (by 10 – 20 nm) and rotations (between 0 and 10°) were performed to the experimentally determined location of the fibrils. Black lines show 5 simulations for 50 randomly chosen fibrils. **e**, Measurements of inter-membrane distances for a 2D slice of the tomogram shown in **d**. The number of tomograms and biologically independent cryo-ET experiments is listed in Supplementary Table 1. Representative images are shown.

Supplementary Methods | Fibril-membrane and inter-membrane distance calculation.

Fibril-membrane distance

The algorithm computing fibril-membrane nearest distances can be summarized as follows:

For each tomogram:

1. Use the segmentation of organelle lumina to compute the distance transform tomogram¹, which calculates the Euclidean distance from each background voxel to the nearest segmented one.
2. For each fibril:
 - 2.1. The curve defined by Amira's Xtracing module during segmentation is sampled uniformly each 5 nm (i.e. similar to the fibril radius).
 - 2.2. For each point in the fibril:
 - 2.2.1. To achieve subvoxel precision, get the interpolated value of the distance transform tomogram at the coordinates of that point.
 - 2.2.2. Add this value to a list of fibril-membrane nearest distances.

The probability density was computed as the normalized histogram of the list of fibril-membrane nearest distances.

To test whether these fibril-membrane nearest distances resulted from random or specific interactions, we compared the experimentally determined distances with those of simulated fibrils. These simulated fibrils were created by randomly shifting and rotating the experimentally measured fibrils as follows:

For each tomogram, generate 200 synthetic tomograms:

1. Take randomly an input experimental fibril as reference.
2. Shift the reference fibril in respect to its center at a random distance in a range of [10, 20] nm.
3. Rotate the fibril randomly with respect to the fibril center with Euler angles selected randomly in the range of [0, 10] degrees.
4. Try to insert the resulting fibril in the synthetic tomogram. The insertion fails in the following cases:

- 4.1. The fibril intersects with another one, considering that fibrils have a cross-section radius of 5 nm.
- 4.2. The fibril intersects with a segmented membrane.
- 4.3. Part of the fibril is out of the tomogram boundaries.
5. Iterate until 50 fibrils are inserted or 5000 tries are reached.

Inter-membrane distance

The algorithm for computing inter-membrane nearest distances can be summarized as follows:

For each tomogram:

1. Assign labels for the lumen of each organelle.
2. Associate segmented membranes and lumina by a proximity criterion. For each voxel in a membrane segmentation, the label of the nearest lumen voxel is determined. The lumen is then associated to the membrane segmentation most frequently found.
3. For each lumen:
 - 3.1. Compute the distance transform tomogram¹ from all lumina.
 - 3.2. Erase the current lumen.
 - 3.3. For each pixel on the membrane segmentation associated to the current lumen:
 - 3.3.1. Get the interpolated value of the distance transform tomogram at the coordinates of that point.
 - 3.3.2. Add this value to a list of inter-membrane nearest distances.

Probability densities were computed as described for fibril-membrane nearest distances.

References

- 1 van der Walt, S., Colbert, S. C. & Varoquaux, G. The NumPy Array: A Structure for Efficient Numerical Computation. *Comput Sci Eng* **13**, 22-30, doi:10.1109/mcse.2011.37 (2011).

Reviewer #1 (Remarks to the Author):

The authors used cryo-electron tomography to image seeded alpha-synuclein filaments in primary neurons (untransfected or transfected) and SH-SY5Y cells (transfected). The seeds were either sonicated preformed fibrils (PFFs) assembled from human recombinant wild-type or mutant A53T alpha-synuclein, gold-labelled or not, or prepared from the pons of a case of neuropathologically confirmed multiple system atrophy (MSA) using sarkosyl extraction and sonication.

The tomography part of this work is of high quality and wide interest. However, the description of seed production lacks in detail (see point 3 of major comments). Moreover, discussion of some of the background and implications is inadequate and needs changing.

Major comments

1. The authors repeatedly mention the paper by Shahmoradian et al. (2019), which questioned the relevance of alpha-synuclein filaments for Lewy pathology. The present work cannot say anything definitive about this controversy, because it is unclear how seeded aggregation using PFFs relates to Lewy pathology. As the authors mention, the structures of filaments made from recombinant protein may differ from those found in human brain. The other seeds were prepared from MSA brain. But MSA is not characterized by Lewy pathology. So, although this work deals with seeded aggregation of alpha-synuclein, it cannot say anything specifically about Lewy bodies. It is incorrect to call the assemblies observed here 'Lewy body-like'. This must be changed. This also applies to the claim that, based on the current work, the filaments seen by Shahmordian et al. were alpha-synuclein filaments. The paper by Mahul-Mellier, which is directly relevant for the mechanisms underlying Lewy body formation, must be discussed and cited (PNAS 117, 4971-4982, 2020). Perhaps it is all only question of time downstream of filament assembly. The work by Shahmoradian et al. may or may not be relevant for human diseases. One way to link what is going on in human diseases with what is described here would be to use labels that are positive in both. This may go beyond the scope of this manuscript, but the possibility should be mentioned. Such labels could be thioflavins, luminescent conjugated oligothiophenes or silver stains.

2. The work showing polarised growth of alpha-synuclein filaments is interesting. The authors show that upon internalisation of gold-labelled seeds made of wild-type alpha-synuclein PFFs, seeded GFP-alpha-synuclein filaments were only decorated at one end by gold particles. But the experiments used recombinant assembled proteins and the reference (Guerrero-Ferreira et al., 2019) refers to the cryo-EM structures of filaments assembled from recombinant (1-121) alpha-synuclein. As mentioned above, the structures of recombinant alpha-synuclein filaments are probably different from those found in human brain. The authors ignore the evidence (from over 20 years ago) suggesting polarised growth of alpha-synuclein filaments in human brain (dementia with Lewy bodies, MSA, Parkinson's disease). References 5 and 6, as well as Crowther et al. Neuroscience Letters 292, 128-130 (2000) must be cited in this respect. The authors must discuss how they see polarised growth of alpha-synuclein filaments in MSA, taking into account the cryo-EM structures of alpha-synuclein filaments from MSA described in reference 26.

3. The authors say that nucleation-relevant seeds consist of oligomeric alpha-synuclein. They should explain this statement. Is a major point of seeded aggregation not that it is nucleation-independent (no lag phase)? In the Methods it says that seeds were sonicated. The authors must show negative stain pictures of their seeds after sonication and describe how sonication was carried out. This is a

crucial piece of information. How can they exclude that what they call oligomers were in fact short filaments? If they cannot do so, they must mention the possibility that at least some seeds were short filaments of alpha-synuclein. The paper by Pieri et al. (Scientific Reports 6: 24526, 2016) must be discussed and cited. Can tomography distinguish between large oligomers and small filaments?

4. This study depends on seeded aggregate formation. It is important to point out that there is no evidence to suggest that the high-resolution structures of seeds and seeded aggregates are the same, which is a hidden assumption behind much of the discussion.

Minor comments

1. Duffy and Tennyson (JNEN 24, 398-414, 1965) described Lewy body filaments before Roy and Wolman.

2. Cross-reaction of alpha-synuclein antibodies with neurofilaments is not a general problem. Reference 12 and its discussion must be removed.

3. The work of Araki et al. (PNAS 116, 17963-17969, 2019) must be discussed and cited. It looks at human disease and disagrees with the conclusions of Shahmoradian et al.

Reviewer #2 (Remarks to the Author):

Review for Trinkhaus et al

Trinkhaus et al present a methodologically sound, well-controlled study showing a higher resolution picture of the cellular and molecular organisation of Lewy bodies, neuronal inclusions common to several neurodegenerative diseases, including Parkinson's Disease and multiple systems atrophy. Previous studies - most notably a room temperature correlative light and electron microscope and tomography study - have shown that alpha-synuclein-positive Lewy bodies consist of crowded environments of membranes of disfigured organelles and vesicles, as well as unidentified filamentous structures. The latter have been proposed to consist of alpha-synuclein, but their identity has not been unequivocally confirmed due to lower resolution of the employed imaging method.

There is very little to fault this study, as it is fairly coherent and well-designed. Only the following minor points -

- figure 3a – beautiful images. How does the labelling relate / agree with the atomic structure of Syn reported by Henning Stahlberg?

- “Our measurements are also consistent with single-particle studies reporting a higher twist, indicative of higher flexibility, for MSA-derived fibrils compared to recombinant”

Could you provide numbers for twists, diameters, maybe even an image comparison with the downloaded EMDB structure?

- Perhaps use of red and green in figures could be prevented? I suggest to change to cyan and magenta throughout the manuscript.
- L286: The caption reads '3D rendering of a)', but should be '3D rendering of tomogram depicted in a)', as the description for a) reads 'tomographic slice', which cannot be 3D-rendered.
- L304: 'Cytosolic fibril density': It is not directly obvious what is meant by this term as it is only explained in the methods section. Perhaps this could be explained in the main text?
- L556: 'cameras' -> 'camera'

We would like to thank the reviewers for their comments, which we found very helpful in improving the manuscript.

Reviewer #1 (Remarks to the Author):

The authors used cryo-electron tomography to image seeded alpha-synuclein filaments in primary neurons (untransfected or transfected) and SH-SY5Y cells (transfected). The seeds were either sonicated preformed fibrils (PFFs) assembled from human recombinant wild-type or mutant A53T alpha-synuclein, gold-labelled or not, or prepared from the ponds of a case of neuropathologically confirmed multiple system atrophy (MSA) using sarkosyl extraction and sonication.

The tomography part of this work is of high quality and wide interest. However, the description of seed production lacks in detail (see point 3 of major comments). Moreover, discussion of some of the background and implications is inadequate and needs changing.

Major comments

1. The authors repeatedly mention the paper by Shahmoradian et al. (2019), which questioned the relevance of alpha-synuclein filaments for Lewy pathology. The present work cannot say anything definitive about this controversy, because it is unclear how seeded aggregation using PFFs relates to Lewy pathology. As the authors mention, the structures of filaments made from recombinant protein may differ from those found in human brain. The other seeds were prepared from MSA brain. But MSA is not characterized by Lewy pathology. So, although this work deals with seeded aggregation of alpha-synuclein, it cannot say anything specifically about Lewy bodies. It is incorrect to call the assemblies observed here 'Lewy body-like'. This must be changed. This also applies to the claim that, based on the current work, the filaments seen by Shahmoradian et al. were alpha-synuclein filaments. The paper by Mahul-Mellier, which is directly relevant for the mechanisms underlying Lewy body formation, must be discussed and cited (PNAS 117, 4971-4982, 2020). Perhaps it is all only question of time downstream of filament assembly. The work by Shahmoradian et al. may or may not be relevant for human diseases. One way to link what is going on in human diseases with what is described here would be to use labels that are positive in both. This may go beyond the scope of this manuscript, but the possibility should be mentioned. Such labels could be thioflavins, luminescent conjugated oligothiophenes or silver stains.

We no longer refer to the α -Syn inclusions observed in our cellular system as "Lewy body-like". We have also rephrased the statements on the disease-relevance of our findings and refer to Mahul-Mellier et al. (ref 31), e.g. (lines 198-200): "Altogether, we show that neuronal α -Syn aggregates consist of both α -Syn fibrils and various cellular membranes. In agreement with a recent report³¹, our findings suggest that the fibrils observed in pathological α -Syn inclusions^{12-14,16} are indeed α -Syn fibrils."

Moreover, we have included additional references illustrating the similarities between seeded aggregation with PFFs and Lewy bodies, including thioflavin staining (ref 26, lines 81-83): "We performed cryo-ET on neuronal α -Syn aggregates using a well-established seeding paradigm that recapitulates inter-neuronal spreading and key neuropathological features of Lewy bodies, including the ability to bind amyloid dyes^{1,25-27}."

2. The work showing polarised growth of alpha-synuclein filaments is interesting. The authors show that upon internalisation of gold-labelled seeds made of wild-type alpha-synuclein PFFs, seeded GFP-alpha-synuclein filaments were only decorated at one end by gold particles. But the experiments used recombinant assembled proteins and the reference (Guerrero-Ferreira et al., 2019) refers to the cryo-EM structures of filaments assembled from recombinant (1-121) alpha-synuclein. As mentioned above, the structures of recombinant alpha-synuclein filaments are probably different from those found in human brain. The authors ignore the evidence (from over 20 years ago) suggesting polarised growth of alpha-synuclein filaments in human brain (dementia with Lewy bodies, MSA, Parkinson's disease). References 5 and 6, as well as Crowther et al. *Neuroscience Letters* 292, 128-130 (2000) must be cited in this respect. The authors must discuss how they see polarised growth of alpha-synuclein filaments in MSA, taking into account the cryo-EM structures of alpha-synuclein filaments from MSA described in reference 26.

The suggested references and discussion regarding the polarized growth of α -Syn fibrils are now included (lines 153-158): "Interestingly, cryo-ET analysis of inclusions seeded by gold-labeled PFFs showed GFP- α -Syn fibrils with one end decorated by 3-10 gold particles (Fig. 3b, c), indicating that exogenous seeds trigger the fibrillation of cellular α -Syn in a polarized manner, consistent with the polar structures of recombinant α -Syn fibrils³⁴. Although patient-derived α -Syn fibrils are polar as well^{7,8,33,40}, it remains to be established whether disease-related seeds also trigger unidirectional fibril growth in cells."

3. The authors say that nucleation-relevant seeds consist of oligomeric alpha-synuclein. They should explain this statement. Is a major point of seeded aggregation not that it is nucleation-independent (no lag phase)? In the Methods it says that seeds were sonicated. The authors must show negative stain pictures of their seeds after sonication and describe how sonication was carried out. This is a crucial piece of information. How can they exclude that what they call oligomers were in fact short filaments? If they cannot do so, they must mention the possibility that at least some seeds were short filaments of alpha-synuclein. The paper by Pieri et al. (*Scientific Reports* 6: 24526, 2016) must be discussed and cited. Can tomography distinguish between large oligomers and small filaments?

We have now modified the corresponding sections, avoiding the term "nucleation".

Given that our seeds were produced by sonication of mature fibrils, we agree that it is more likely that the small seeding-competent species correspond to small fibrils, rather than oligomers. This has now been corrected throughout. This conclusion is consistent with the findings of Pieri et al., which we now cite (ref 41, lines 158-160): "Our data also show that, in our experimental conditions, small α -Syn fibrils are the most seeding-competent species, in agreement with previous results⁴¹."

Additional information on the sonication procedure is now included in the Methods (lines 332-333, 338-339), and negative stain images are shown in Supplementary Fig. 1b.

4. This study depends on seeded aggregate formation. It is important to point out that there is no evidence to suggest that the high-resolution structures of seeds and seeded aggregates are the

same, which is a hidden assumption behind much of the discussion.

The following sentence has been added to clarify this point (lines 165-168): “Although α -Syn strains can be transmitted between cells *in vitro* and *in vivo*³, the cellular environment may also modify the strain characteristics⁴². Thus, higher resolution data are needed to elucidate to what extent the structure of the seed is templated in the aggregates seeded within cells.”

Minor comments

1. Duffy and Tennyson (JNEN 24, 398-414, 1965) described Lewy body filaments before Roy and Wolman.

We thank the Reviewer for pointing us to this study, which is now also cited in our manuscript (ref 11, lines 58-59): “Early electron microscopy (EM) studies suggested that the Lewy bodies⁹⁻¹² and glial cytoplasmic inclusions^{13,14},”

2. Cross-reaction of alpha-synuclein antibodies with neurofilaments is not a general problem. Reference 12 and its discussion must be removed.

Done.

3. The work of Araki et al. (PNAS 116, 17963-17969, 2019) must be discussed and cited. It looks at human disease and disagrees with the conclusions of Shahmoradian et al.

We now cite Araki et al. in the Introduction (ref 15, lines 62-63): “Intriguingly, X-ray diffraction measurements confirmed the presence of amyloid fibrils only in some Lewy bodies¹⁵”

Reviewer #2 (Remarks to the Author):

Review for Trinkhaus et al

Trinkhaus et al present a methodologically sound, well-controlled study showing a higher resolution picture of the cellular and molecular organisation of Lewy bodies, neuronal inclusions common to several neurodegenerative diseases, including Parkinson's Disease and multiple systems atrophy. Previous studies - most notably a room temperature correlative light and electron microscope and tomography study - have shown that alpha-synuclein-positive Lewy bodies consist of crowded environments of membranes of disfigured organelles and vesicles, as well as unidentified filamentous structures. The latter have been proposed to consist of alpha-synuclein, but their identity has not been unequivocally confirmed due to lower resolution of the employed imaging method.

There is very little to fault this study, as it is fairly coherent and well-designed. Only the following minor points –

We thank the Reviewer for the overall positive comments.

- figure 3a – beautiful images. How does the labelling relate / agree with the atomic structure of Syn reported by Henning Stahlberg?

We measured an average distance between gold bead centers of 3.5 ± 0.4 nm (mean $\pm$ sd) along α -Syn fibrils. Given that the typical distance between beta strands is ~ 4.8 Å across various high-resolution structures of α -Syn fibrils (PMID 32112991, 32461689), we estimate that the gold beads label approximately every 7-8 beta strands. This is now mentioned in the legend of Fig. 3 (lines 247-249). The cartoon in Fig. 3c has also been re-scaled accordingly.

- “Our measurements are also consistent with single-particle studies reporting a higher twist, indicative of higher flexibility, for MSA-derived fibrils compared to recombinant”
Could you provide numbers for twists, diameters, maybe even an image comparison with the downloaded EMDB structure?

We now mention in the text that the diameter of our fibrils was ~ 10 nm, similar to all recombinant and patient-derived structures published to date (lines 109-111): “The fibrils were ~ 10 nm in diameter, similar to recombinant and patient-derived α -Syn fibrils^{33,34} and clearly distinct from neurofilaments (Fig. 1g).”

Approximate values for the twists of the published structures are also listed (lines 138-141): “Our measurements are also consistent with single-particle studies reporting a higher twist, indicative of higher flexibility³⁸, for MSA-derived fibrils³³ (~ 60 nm) than for most structures of recombinant fibrils³⁴ (90-120 nm).”

However, despite extensive image processing efforts, we could not determine an average structure of our fibrils with sufficient resolution, or even reliably calculate their twist, possibly due to their structural variability. Therefore, we would find it premature to present an image comparison at this point.

- Perhaps use of red and green in figures could be prevented? I suggest to change to cyan and magenta throughout the manuscript.

Thank you for the suggestion. We have checked all figures with a color-blind simulator:

<https://www.color-blindness.com/coblis-color-blindness-simulator/>

Furthermore, for all light microscopy images, we show the different channels separately as black and white images.

- L286: The caption reads '3D rendering of a)', but should be '3D rendering of tomogram depicted in a)', as the description for a) reads 'tomographic slice', which cannot be 3D-rendered.

Thank you for pointing this out. We have corrected it in all relevant legends (Fig. 1d, Fig. 1h, Fig. 2b, Fig. 3d, Fig. 3e, Supplementary Fig. 6c).

- L304: 'Cytosolic fibril density': It is not directly obvious what is meant by this term as it is only explained in the methods section. Perhaps this could be explained in the main text?

We have now added this explanation in the main text (lines 129-131: "However, fibril density within inclusions, defined as the fraction of cytosolic volume occupied by fibrils, was significantly higher in cells expressing GFP- α -Syn (Fig. 2e, Supplementary Table 1).") and the legend of Fig. 2 (lines 236-237).

- L556: 'cameras' -> 'camera'

"Camera" is used in plural as it refers to both K2 and K3 cameras.

Reviewer #1 (Remarks to the Author):

The authors have answered my previous questions. I have no additional comments.

Reviewer #2 (Remarks to the Author):

The authors have answered all my questions, happy to recommend publication.

Tanmay Bharat

Description of Additional Supplementary Files

File Name: Supplementary Movie 1 | Tomogram and 3D rendering of an α -Syn aggregate in a neuron seeded by PFFs.

Description: Tomographic volume and 3D rendering of an inclusion seeded by PFFs in a neuron expressing GFP- α -Syn, shown in Fig. 1a, d. The field of view is 1.6 x 1.6 μ m. The rendering depicts α -Syn fibrils (red), an autophagosome (cyan), ER (yellow), mitochondria (green) and various vesicles (purple).

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see our [Editorial Policies](#) and the [Editorial Policy Checklist](#).

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- | | |
|-------------------------------------|--|
| n/a | Confirmed |
| ☒ | ☒ The exact sample size (<i>n</i>) for each experimental group/condition, given as a discrete number and unit of measurement |
| ☒ | ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☒ | ☒ The statistical test(s) used AND whether they are one- or two-sided
<i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i> |
| ☒ | ☐ A description of all covariates tested |
| ☒ | ☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ☒ | ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| ☒ | ☒ For null hypothesis testing, the test statistic (e.g. <i>F</i> , <i>t</i> , <i>r</i>) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted
<i>Give P values as exact values whenever suitable.</i> |
| ☒ | ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ☒ | ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☒ | ☐ Estimates of effect sizes (e.g. Cohen's <i>d</i> , Pearson's <i>r</i>), indicating how they were calculated |

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	SerialEM 3.7.0 MAPS 2.1 Las X 3.5.7.23225 CellSens 2.1 FACSDiva Version 6.1.3
Data analysis	MotionCor2 1.2.1 IMOD 4.9.0 tom_deconv (https://github.com/dtegunov/tom_deconv) tomoSegMemTV (https://github.com/anmartinezs/pyseg_system/tree/master/code/tomosegmmtv) Amira 6.2 Matlab 2017a ImageJ 2.0.0 Prism 6 Origin 2019b PySeg (https://github.com/anmartinezs/pyseg_system/tree/master/code/pyorg/scripts/filaments ; https://doi.org/10.5281/zenodo.4429140) Persistence Length measurement (https://github.com/FJBauerlein/Huntington ; https://doi.org/10.5281/zenodo.4428865)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Data availability

Source Data for Fig. 2d, e, Fig. 4b, c, Supplementary Fig. 1f, g, Supplementary Fig. 3c, d, Supplementary Fig. 4 and Supplementary Fig. 6 are available with the online version of this paper. The individual values for the average graphs shown in Fig. 2d, Fig. 4b, c and Supplementary Fig. 4 are available at the Edmond repository: <https://edmond.mpdl.mpg.de/imeji/collection/rnVkl2lwG8loNXOi>. The tomograms shown in Fig. 1 and Fig. 2 are available in EMPIAR through accession codes EMD-11401 (Fig. 1a; <https://www.ebi.ac.uk/pdbe/entry/emdb/EMD-11401>), EMD-11417 (Fig. 1e; <https://www.ebi.ac.uk/pdbe/entry/emdb/EMD-11417>) and EMD-11416 (Fig. 2a; <https://www.ebi.ac.uk/pdbe/entry/emdb/EMD-11416>). All other data are available from the corresponding authors upon reasonable request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- ☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample sizes were selected based on previous experience to obtain statistical significance and reproducibility (see e.g. Table S1 in Collado et al., Dev Cell 2019)
Data exclusions	Tomograms with insufficient signal-to-noise-ratio were excluded for all conditions.
Replication	Replicates were conducted for all experiments quantified as described in the Figure legends and Supplementary Table 1
Randomization	Mouse embryos of both sexes were chosen randomly for neuronal cell cultures. The MSA patient was selected based on histopathological brain analyses (randomization did not apply). Experiments on SH-SY5Y cells were performed comparing different experimental treatments on the same cell line (randomization did not apply).
Blinding	No blinding was applied. All experiments were performed comparing various treatments on otherwise comparable samples, and as such it was necessary for the researchers to be aware of the treatment applied (e.g. PBS, PFFs or MSA aggregates). Appropriate controls were included in each experimental replication.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☐	☒ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

The following primary antibodies were used: GFP (A10262, Thermo Fisher, 1:500; RRID: AB_2534023), K48-linked ubiquitin (05-1307, Millipore; 1:500; RRID: AB_1587578), MAP2 (NB300-213, Novus Biologicals; 1:500; RRID: AB_2138178), p62 (ab56416, Abcam; 1:200; RRID: AB_945626), phospho S129 α -Syn (ab51253, Abcam; 1:500 for immunofluorescence, 1:2500 for western blot; RRID: AB_945626).

AB_869973), α -Syn (610787, BD Biosciences; 1:1000; RRID: AB_398108) and p62 Ick ligand (610832, BD Biosciences; 1:100; RRID: AB_398151).

The following secondary antibodies were used: Alexa Fluor 488 AffiniPure Donkey Anti-Chicken (703-545-155, Jackson ImmunoResearch; 1:250), Alexa Fluor 647 AffiniPure Donkey Anti-Chicken (703-605-155, Jackson ImmunoResearch; 1:250), Cy3 AffiniPure Donkey Anti-Rabbit (711-165-152, Jackson ImmunoResearch; 1:250), Alexa Fluor 488 AffiniPure Donkey Anti-Mouse (715-545-150, Jackson ImmunoResearch; 1:250), Cy3-conjugated AffiniPure Goat Anti-Mouse IgG (115-165-003, Jackson ImmunoResearch; 1:1000), Cy3-conjugated AffiniPure Goat anti-rabbit (111-165-045, Dianova; 1:1000; RRID: AB_2338003), HRP-conjugated Goat Anti-Rabbit (A9169, Sigma; 1:5000; RRID: AB_258434).

Validation

All antibodies have been previously used for the application and species described here. The GFP Antibody has been validated for ICC in mice (<https://www.thermofisher.com/antibody/product/GFP-Antibody-Polyclonal/A10262>). The K48-linked ubiquitin Antibody has been validated for IF in human cell culture (https://www.merckmillipore.com/DE/de/product/Anti-Ubiquitin-Antibody-Lys48-Specific-clone-Apu2-rabbit-monoclonal,MM_NF-05-1307). The MAP2 antibody has been validated for IF in mouse primary cultures (https://www.novusbio.com/products/map2-antibody_nb300-213). The p62 antibody has been validated for IF in human cell culture (<https://www.abcam.com/sqstm1--p62-antibody-autophagosome-marker-ab56416.html>). The phospho S129 α -Syn antibody has been validated for WB with mouse brain lysates and has been used for IF in human cell culture (<https://www.abcam.com/alpha-synuclein-phospho-s129-antibody-ep1536y-ab51253.html> ; <https://europepmc.org/backend/ptpmcrender.fcgi?accid=PMC7212628&blobtype=pdf>). The α -Syn antibody has been validated for IHC in humans (<https://www.bdbiosciences.com/eu/applications/research/stem-cell-research/ectoderm-markers/human/purified-mouse-anti--synuclein-42-synuclein/p/610787>). The p62 Ick ligand antibody has been validated for IHC in humans (<https://www.bdbiosciences.com/us/reagents/research/antibodies-buffers/cell-biology-reagents/cell-biology-antibodies/purified-mouse-anti-p62-ick-ligand-3p62-ick-ligand/p/610832>).

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s) SH-SY5Y cells were a gift from Konstanze Winklhofer and Joerg Tazelt (10.1038/emboj.2011.86) and originally purchased at DSMZ (ACC209)
HEK293T cells were purchased from Takara (632180)

Authentication No authentication was performed

Mycoplasma contamination No contamination was found by PCR, electron and light microscopy.

Commonly misidentified lines (See [ICLAC](#) register) None

Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

Laboratory animals Primary cortical neurons were prepared from E15.5 CD-1 wild type mouse embryos of both sexes (breeding line MpiCrlIcr:CD-1). Mice were housed in a specific pathogen free facility at 22 $\pm$ 1.5 $^{\circ}$ C, 55 $\pm$ 5% humidity, 14-hour light / 10-hour dark cycle.

Wild animals no

Field-collected samples no

Ethics oversight All experiments involving mice were performed in accordance with the relevant guidelines and regulations of the Government of Upper Bavaria (Germany).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about [studies involving human research participants](#)

Population characteristics A brain sample was collected from a male patient who died at the age of 54, 6 years after being diagnosed with a cerebellar type of MSA

Recruitment The patient donated brain tissue to the Neurobiobank Munich. This sample was selected due to the abundant alpha-synuclein inclusions in the pons region

Ethics oversight MSA patient brain tissue was obtained from Neurobiobank Munich. All autopsy cases of the Neurobiobank Munich are collected on the basis of an informed consent according to the guidelines of the ethics commission of the Ludwig-Maximilians-University Munich, Germany. The experiments performed in this paper were approved by the Max Planck Society's Ethics Council.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

- ☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☒ All plots are contour plots with outliers or pseudocolor plots.
- ☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

To create a stable cell line expressing EGFP- α -SynA53T, SH-SY5Y cells were transfected using Lipofectamine 2000 (Thermo Fisher). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Biochrom) supplemented with 10 % fetal bovine serum (FBS, GIBCO), 2 mM L-glutamine (GIBCO) and 2,000 μ g/ml geneticin for selection. Polyclonal cell lines were generated by fluorescence-activated cell sorting. Upon selection, cells were cultured in medium supplemented with 200 μ g/ml geneticin (Thermo Fisher) and penicillin/streptomycin (Thermo Fisher).

Negative control for FACS: untransfected SH-SY5Y cells

Instrument

BD FACS Aria III with 375nm, 405nm, 488nm, 561nm, 633nm lasers

Software

FACSDiva Version 6.1.3

Cell population abundance

All sorted cells showed GFP signal

Gating strategy

Control cells (untransfected SH-SY5Y cells): FITC-A: 120 FSC-A: 150.000
Transfected SH-SY5Y cells: FITC-A: 10.000 FSC-A: 150.000

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.