Rapid macropinocytic transfer of $\alpha$-synuclein to lysosomes

**Graphical abstract**

**Highlights**
- $\alpha$-Synuclein preformed fibrils (PFFs) enter cells through a rapid form of macropinocytosis
- PFF endocytosis is clathrin independent and circumvents the endosomal pathway
- PFFs are rapidly transported to lysosomes
- The spread of PFF is facilitated by its transport on the surface of exosomes

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**In brief**
Bayati et al. show that the pathogenic preformed fibrils of the Parkinson’s disease protein $\alpha$-synuclein are internalized and transported through a unique set of pathways. Delineation of this pathway contributes to our understanding of Parkinson’s disease pathology and highlights the pathogenic potential of oligomeric and fibril forms of $\alpha$-synuclein.
Rapid macropinocytic transfer of α-synuclein to lysosomes

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INTRODUCTION

A classic hallmark of Parkinson’s disease (PD) is the formation of Lewy bodies (LBs). First discovered in 1912 (Lewy, 1912), LBs are cytoplasmic inclusions composed of fragments of membranous organelles and filamentous proteins (Shahmoradian et al., 2019; Tanaka et al., 2004). LBs are also observed in PD-related disorders such as Lewy body dementia. α-Synuclein (α-syn), encoded by the SNCA gene, is a major component of LBs and is implicated in their formation (Conway et al., 1998; Spillantini et al., 1997). α-Syn appears to function in membrane trafficking and membrane curvature (Fortin et al., 2004; Jao et al., 2004; Nakamura et al., 2008; Vargas et al., 2017), and increased knowledge of its protein structure and the conformation of altered variants has led to an enhanced understanding of α-syn misfolding and aggregation in disease (Lee et al., 2002; Li et al., 2002; Sandal et al., 2008).

Since the early 2000s, the Braak hypothesis has stated that PD develops with the spread of α-syn through the brain (Braak et al., 2003). This model gained traction with the observation that proteinaceous inclusions spread from brain tissue into implanted embryonic stem cells (Li et al., 2008), a result subsequently confirmed in animal models (Desplats et al., 2009; Li et al., 2010; Recasens and Dehay, 2014; Recasens et al., 2018). Propagating pathology is also observed following the injection of the fibril form of α-syn into localized brain regions of mice (Betemps et al., 2014; Luk et al., 2012; Masuda-Suzukake et al., 2014). This tendency to spread, along with the ability of α-syn fibrils to disrupt the conformation of endogenous α-syn, has earned the protein a label as a prion-like (Masuda-Suzukake et al., 2013; Mougenot et al., 2012). However, questions remain regarding the cell biological itinerary of α-syn propagation, notably the mode of cellular entry (Fenyi et al., 2019; Gelpi et al., 2014; Nakamura et al., 2015; Uemura et al., 2018; Yan et al., 2018).

Different mutations in the SNCA locus have varying penetrance that may correlate to the propensity of α-syn to aggregate (Lazar et al., 2016; Rutherford et al., 2014). α-Syn concentration is also a factor in aggregation as increased protein levels enhance aggregation, be it mutant or wild-type protein (Fink, 2006; Manning-Bog et al., 2002; Uversky, 2007). Furthermore, α-syn preformed fibrils (PFFs) and other fibril assemblies of α-syn have the ability to seed, propagate, and amplify in size via the recruitment of α-syn monomers (Alam et al., 2019). PFFs comprise a heterogeneous number of α-syn monomers with various structural conformations (Pieri et al., 2016). Early studies using PFFs revealed their ability to seed and induce PD pathology in cultured cells (Luk et al., 2009); hence, developing a protocol for consistent production of PFFs was a significant contribution (Volpicelli-Daley et al., 2011, 2014).

A key question in PD research relates to the means by which α-syn fibrils enter cells (Bieri et al., 2018). Several studies have concluded that α-syn endocytosis is clathrin dependent, based on the use of dynamin and clathrin inhibitors, dynasore, and pitstop, respectively, and clathrin heavy chain (CHC) colocalization...
Figure 1. Rapid internalization and transport of PFF to lysosomes

(A) U2OS cells were stained with Lyso-Cytopainter and placed in a live-cell imaging chamber at 37°C. Imaging was performed at 1 frame/s. PFF tagged with Alexa 488 at 2 μg/mL was added to cells while imaging. PFF (red) colocalization with lysosomes (gray) can be seen in at 2 min in both low magnification and the inset. Arrowheads point to lysosomes accumulating PFF. Scale bars, 10 μm for low magnification images and 5 μm for insets. Inset location shown at 0 min.

(B) Quantification of PFF uptake in U2OS. Cells were plated on coverslips and transfected with LAMP1-TurboRFP. PFFs were added to each coverslip at 2 μg/mL. Cells were incubated for 0, 2, 10, and 30 min at 37°C following the addition of PFF. Cells were washed with trypsin and fixed. n = 12 for each condition (i.e., n = 48 total).

(C) Manders’ coefficients for LAMP1 and PFF were calculated using the JACoP plugin. n = 12 for each condition (i.e., n = 48 total), from 3 independent experiments. M1 describes the colocalization of LAMP1 with PFFs, while M2 describes the colocalization of PFFs with LAMP1. For both (B) and (C), means ± SDs and data were statistically analyzed using 1-way ANOVA, followed by multiple comparisons Tukey’s test to assess significance from control (0 min). ****p < 0.0001.
(Konno et al., 2012; Liu et al., 2007; Rodriguez et al., 2018). It is generally thought that PFFs are then trafficked to lysosomes via the endosomal system over the course of tens of minutes to multiple hours. We found approximately 40 studies examining α-syn endocytosis with variable conclusions (Table S1). Some of the variability may arise from examining α-syn internalization at longer time courses and not immediately after its addition to cells. Moreover, dynamin inhibition is not synonymous with the inhibition of clathrin-mediated uptake and the specificity of dynasore and pitstop as inhibitors of clathrin-mediated endocytosis (CME) has come under scrutiny as both have off-target effects (Gu et al., 2010; Oh et al., 1998; Park et al., 2013; Peikmans et al., 2002; Preta et al., 2015). Apart from CME, it is suggested that α-syn enters cells via direct permeation of the plasma membrane (Danzer et al., 2007), via the formation of tunneling nanotubes that allow direct connections between cells (Dieriks et al., 2017), or through caveolae-dependent endocytosis (Madeira et al., 2011). Thus, there appears to be no consensus nor definitive evidence regarding the nature of α-syn endocytosis.

We thus sought to examine endocytosis of PFFs immediately after their addition to cells. Surprisingly, PFFs are internalized rapidly and appear in lysosomes within 2 min, bypassing conventional endosomal trafficking pathways. We confirmed this result in multiple cell lines, primary human cells, and neurons derived from induced pluripotent stem cells (iPSCs). The internalization is not dependent on clathrin but instead uses macropinocytosis. We used gold-labeled PFFs and electron microscopy (EM) and discovered PFFs in membrane ruffles that form macropinosomes and in lysosomes. We also detected gold-labeled PFFs on the outer edges of invaginating vesicles and on the outside of vesicles within multivesicular bodies (MVBs). While a portion of PFFs remain in lysosomes for a long period, a smaller portion are transferred to naive cells along with markers of MVBs. Thus, our data unveil a unique form of macropinocytosis that mediates the internalization of PFFs and allows for endocytosis to be coupled to release.

**RESULTS**

**PFFs are rapidly endocytosed to lysosomes**

We used fluorescently labeled PFFs (Del Cid Pellitero et al., 2019; Maneca et al., 2019) (Figures S1A and S1B) with live-cell imaging and a trypan blue exclusion assay (Karpowicz et al., 2017) that quenches extracellular PFF fluorescence (Figure S1D) to examine PFF internalization at the earliest possible time points. PFFs are rapidly internalized in U2OS cells and are targeted to lysosomes, where they colocalize with Lysosomal Cytopainter within 2 min (Figure 1A). Similar results are seen when incubating cells with PFFs at 4°C for 30 min and then transferring the cells to 37°C (Figure S1E). We confirmed the live imaging findings in fixed samples of U2OS cells. PFFs are significantly internalized within 2 min and continue to accumulate within cells for up to 60 min (Figures 1B and S1H). Nearly all labeled PFFs that enter cells are colocalized with LAMP1-TurboRFP by 2 min, and this colocalization remains stable, with all PFF trafficking to lysosomes for up to 60 min in these experiments (Figure 1C). As fixation makes cells permeable to trypan blue, for fixed cell experiments, we used trypsin to proteolyze off extracellular PFFs (Figures S1F and S1G). The rapid uptake of PFFs and transport to lysosomes within 2 min was also observed in U87 (Figures S2A, S2D, and S2E) and U251 (Figures S2B, S2F, and S2G) glioblastoma cells.

To confirm the rapid transport of PFFs to lysosomes, we incubated U2OS cells expressing HA-TMEM192-RFP, a lysosomal protein, with PFFs for various time periods, then lysed the cells and purified lysosomes using hemagglutinin (HA)-magnetic beads (Abu-Remaih et al., 2017). The enrichment of lysosomes was confirmed in the immunoprecipitated fractions, with antibodies recognizing LAMP1 and 2 (Figure 1D). Rab7 was also enriched in the lysosome fractions, whereas Rab5 and LRKK2 were not detected. CD63/LAMP3, a marker of lysosomes and MVBs, was the most highly enriched marker. PFFs were enriched in the lysosome fractions at 2 min, confirming their rapid transport to lysosomes (Figure 1D). The enriched lysosomes were placed on coverslips and visualized through the fluorescently labeled PFFs (Alexa Fluor 488) and TMEM192-RFP (Figure 1E). PFFs were detected in the lumen of the lysosomes, which was most readily seen using stimulated emission depletion (STED) super-resolution microscopy (Figure 1F). Fluorescently conjugated PFFs of various sizes (Figure 1G) were administered to U2OS cells, revealing no significant difference in uptake in samples ranging from 50 to 100 nm (Figure 1H). Thus, PFFs are rapidly endocytosed and transported to lysosomes in as little as 2 min, an unprecedented time frame for lysosomal targeting.

**Rapid transfer of PFFs to lysosomes in nervous system cells, including human dopaminergic neurons**

Human cortical neurons derived from iPSCs (Figure S1I) were incubated with fluorescently labeled PFFs, and colocalization with lysosomes was examined using a fixable form of LysoTracker. PFFs were endocytosed in the neurons with lysosomal colocalization observed at 2 min (Figures S2C, S2H, and S2I), although lysosomes continued to accumulate in PFFs for up to 30 min. In both human dopaminergic neural progenitor cells (NPCs) derived from iPSCs and in dopaminergic neurons derived from the NPC, PFFs were rapidly internalized and are detected at lysosomes within 2 min (Figures S1I, 2A, 2B, 2D, and 2E).
Figure 2. Rapid PFF colocalization with lysosomes in human dopaminergic NPCs, dopaminergic neurons, and astrocytes
(A) Dopaminergic NPCs, stained with LysoTracker, were incubated for 0, 2, 10, and 30 min at 37°C following the addition of Alexa 488-labeled PFF (white) at 2 μg/mL. The cells were then washed lightly and briefly with trypsin and fixed. Scale bar, 20 μm.
(B) NPCs differentiated into dopaminergic neurons were used in the experiments described in (A). Scale bar, 20 μm.
(C) Human astrocytes, grown and mounted on coverslips, were given fluorescently labeled PFFs at a 1-μg/mL concentration. Cells were then incubated for 0, 2, 10, and 30 min at 37°C. Cells were washed with trypsin, fixed, permeabilized, and stained with LAMP1 antibody. Scale bars, 20 μm and 2.5 μm for the insets.
(D and E) Manders’ coefficients were calculated using the JACoP plugin to ascertain the colocalization of LysoTracker with PFFs from experiments described in (A) and (B). n = 6 for NPCs and n = 6 for neurons in each condition (i.e., n = 48 total for NPCs and n = 48 total for neurons), from 3 independent experiments.

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2F). Similar results are seen in human astrocytes (Figures S1, 2C, and 2F). Thus, in multiple cell types, including those from the human nervous system, PFFs are rapidly transported to lysosomes.

To assess the localization of PFFs over longer time periods, U343 glioblastoma cells were incubated with PFFs for 24 h, followed by a change to fresh media. PFFs are seen to traffic to lysosomes and remain predominantly in the organelle, with fluorescent signal detected for up to 10 days (Figure S2J). In contrast, internalized fluorescently tagged epidermal growth factor (EGF) is detected only at very low levels in lysosomes at both 1 and 10 days after addition (Figure S2J). Similar to U343 glioblastoma cells, fluorescent PFFs are readily detected in the lysosomes of human astrocytes at 1 and 7 days (Figure 3A), with the total fluorescent signal slowly decreasing over time (Figure 3B). In human dopaminergic neurons, PFFs are detectable in lysosomes for up to 7 days (Figure 3C).

PFFs are trafficked to lysosomes, avoiding early/recycling endosomes

Considering the speed at which PFFs reach lysosomes, it seems unlikely that PFFs follow endosomal pathways to lysosomes as suggested previously (see Table S1), as endosomal maturation generally takes 10–15 min (Huotari and Helenius, 2011). Transferrin (Tf), a well-studied marker of early and recycling endosomes (Trischler et al., 1999), was added to cells along with PFFs, and there was no colocalization at either 2 or 30 min (Figures S3A and S3D). While a significant co-occurrence of Tf with PFFs was detected at 30 min (approximately 20%; M<sub>1</sub> < 0.2), the co-occurrence of PFFs with Tf was not significant. Moreover, internalized PFFs do not colocalize with EEA1, a marker of early endosomes (Mills et al., 1998), even at time points as early as 2 min (Figures S3B and S3E). PFFs colocalize with the late endosome/lysosome markers Rab9 and LAMP1, but show little colocalization with early and recycling Rabs, 4, 5, and 8 (Figures S3F and S3G). Thus, PFFs appear to reach lysosomes independently of the early and recycling endosomal systems.

Endocytosis of PFFs is clathrin independent

The current consensus is that PFFs enter cells via CME (Uemura et al., 2020). However, we are aware of no mechanism by which cargo that enters cells via CME can gain access to lysosomes in 2 min while bypassing early endosomes. To test whether CME is involved in the endocytosis of PFFs, we used an established genetic approach involving the knockdown (KD) of CHC with previously validated small interfering RNA (siRNA) (Galvez et al., 2007; Kim et al., 2011). Immunoblot reveals effective CHC KD in U2OS cells (Figure 4A). We then plated cells treated with control siRNA or CHC-targeting siRNA as a mosaic in the same well and incubated them with PFFs. Unlike previous studies showing the clathrin dependence of PFF entry (Table S1), we observed no difference in the internalization of PFFs when comparing KD and control cells (Figures 4B and 4C). Thus, it does not appear that CME plays a major role in PFF endocytosis.

Lysosomal transfer of PFFs does not depend on phagocytosis

Due to the rapid transport of PFF to lysosomes, we tested whether the internalization of PFFs uses phagocytosis. RAW 264.7 macrophage cells were activated with lipopolysaccharide (LPS) (1 µg/mL) and after 24 h, cells were given fluorescent latex beads FluoSpheres (FS), along with PFFs. Little colocalization was observed between FS and PFFs (Figures S4A and S4B), indicating that phagocytosis is not involved in the uptake of PFF.

Internalization of PFFs occurs through a unique form of macropinocytosis

Holmes et al. (2013) demonstrated that tau fibrils use macropinocytosis for cellular entry and that fibril α-syn colocalizes with tau during uptake, suggesting a potential role for macropinocytosis in the internalization of fibril α-syn. Similarly, Zeineddine et al. (2015) found that α-syn fibrils induce membrane ruffling, an early step in forming macropinosomes. Although macropinocytic cargo generally follows the endosomal pathway (Mayor and Pagano, 2007), we sought to test whether macropinocytosis is involved in the internalization of PFFs. We first examined whether 5-(N-ethyl-N-isopropyl) amiloride (EIPA), an established inhibitor of macropinocytosis (Commissou et al., 2014; Koivusalo et al., 2010; Nakase et al., 2015) influences the internalization of PFFs. EIPA disrupts the Na<sup>+</sup>/H<sup>+</sup> exchanger, decreasing cytosolic pH and inhibiting the activation of Cdc42 and Rac1, which is required for macropinocytosis (Koivusalo et al., 2010). At a concentration of 20 µM, EIPA inhibits the uptake of PFFs in human astrocytes (Figures 4D and 4E). Latrunculin B (LatB), which inhibits macropinocytosis by disrupting actin polymerization (Williams and Kay, 2018), had a similar block on the uptake of PFFs when used at 5 µM (Figures 4D and 4E). In contrast, neither drug influenced the uptake of EGF (Figures 4D and 4F), which at the concentration used, enters cells via CME (Sigismund et al., 2005). In addition to astrocytes, we confirmed our findings in dopaminergic NPCs. LatA, which, like LatB, is a potent actin polymerization inhibitor (Fujisawa et al., 2018), demonstrated a dose-dependent inhibition in the uptake of PFFs in dopaminergic NPCs, with uptake reduced by 81% compared to control at 2 µM (Figure 4G).

Consistent with a role for macropinocytosis in the endocytosis of PFFs, PFFs induce the formation of actin-rich membrane ruffles, the precursors of macropinosomes (Condon et al., 2018) (Figures S5A and S5B). More specifically, PFFs induce the recruitment of Rac1 to actin-rich membranes on the cell surface, a characteristic of macropinocytosis (Grimmer et al., 2002) (Figure S5A). The ability of PFF to induce membrane ruffling is also observed by EM (Figure S5C). As a control, we examined the influence of EGF, a documented inducer of membrane ruffles, even though it does not use macropinocytosis itself for internalization (Bryant et al., 2007).
We also examined Tf, which is not known to induce membrane ruffling or macropinocytosis. As expected, EGF causes the recruitment of Rac1 to the surface, where it colocalizes with F-actin, whereas no Rac1 recruitment is seen upon the addition of Tf (Figure S5D). Thus, PFFs appear to stimulate membrane ruffles and use macropinocytosis to gain direct access to lysosomes.

Figure 3. PFFs remain in lysosomes days after exposure in dopaminergic neurons and astrocytes

(A) Human astrocytes were mounted on coverslips and were given PFF488 at 2 μg/mL on ice for 1 h. Cells were removed from ice, given fresh media, and were then incubated for 0 h, 1 day, or 7 days at 37°C. Media was changed 24 h following the addition of PFFs. Following incubation, cells were lightly trypsin washed and fixed. Arrowheads point to LAMP1 and PFF colocalization. Scale bars, 20 μm for low magnification and 5 μm for insets. (B) Adult human astrocytes at passage 7 were plated on coverslips at 50% confluence and incubated with PFFs for 24 h. The cells were then trypsin washed and given fresh media. Some cells were fixed for 1 day, while others were incubated for an additional 6 or 13 days before fixation. Scale bar, 20 μm. PFF fluorescence intensity was then quantified. n = 5 for each condition (n = 15 total), collected from 5 independent experiments. Data were then analyzed using 1-way ANOVA, and Tukey’s test was conducted post hoc to compare differences between means. **p < 0.001; p > 0.05 was set to be not significant, denoted as ns. (C) Dopaminergic neurons derived from human iPSCs mounted onto coverslips and treated as in (A). Lysosomes were stained using LAMP1 (red) antibody. PFF (blue) remains localized to lysosomes 7 days following its addition to the cell. Scale bars, 20 μm for low magnification and 5 μm for insets.

Dextrons are fluid-phase endocytic markers that can use macropinocytosis (Li et al., 2015). Dextrons of different molecular weights were added to cells alongside PFFs. Colocalization of dextrons with PFFs (M1) was ~25%, while PFF colocalization with dextran (M2) was less than 20% (Figures S5E and S5F). Thus, PFFs appear to use a unique form of macropinocytosis that only partially overlaps with conventional bulk entry.

Trafficking itinerary of PFFs revealed by immunogold EM

To observe the trafficking itinerary of PFF directly, we labeled the fibrils with gold (Figure S1C) and followed their trafficking by EM. At 2–3 min following the addition of PFFs, astrocytes were fixed and processed for EM. Gold-labeled PFFs appear under membrane ruffles and within intracellular macropinosomes formed by ruffle closures (Figures 5Ai and 5Aii). The immunogold-labeled PFFs remain in the lumen of the macropinosomes/endothosomes that begin to demonstrate inward invagination of vesicles (Figures 5Aiii and 5Aiv). At 3–5 min, the PFFs can be found in the lumen of intracellular membranes that
Figure 4. PFF internalization is unaffected by CHC KD but decreased using macropinocytic inhibitors

(A and B) U2OS cells previously transfected with EBFP2-LAMP1 were transfected with CHC siRNA or control siRNA (A). Cell lysates were immunoblotted with antibodies recognizing the indicated proteins. (B) At 24 h, control and CHC siRNA-treated cells were replated as a mosaic onto coverslips. PFFs were added to each coverslip at 2 μg/mL. Cells were incubated for 0, 2, 10 min at 37°C following the addition of PFFs. Cells were washed with trypsin and fixed. Arrowheads show large LAMP1+ (red)- and PFF+ (white) vesicles in both CHC+ (blue) and CHC− cells, outlined by the CHC antibody. KD cells are outlined with dashed lines. Scale bar, 20 μm.

(C) Internalization of PFF in CHC KD versus control cells at 2 min were quantified from experiments as in (B), except that for quantification, KD and control cells were mounted on separate coverslips. n = 6 for each condition (i.e., n = 12 total), from 3 independent experiments. Individual data points shown and means ± SDs. Data were analyzed using a 2-sample t test; p > 0.05 denoted as ns for not significant.

(D) Astrocytes were grown and mounted on coverslips. Cell media was replaced with serum-free media containing 20 μM EIPA, 5 μM LatB, or DMSO (vehicle control) for 30 min. Fluorescently labeled PFF or EGF (green) was added to each coverslip (at 2 μg/mL and 0.2 μg/mL, respectively) for 0, 2, 10, and 30 min at 37°C. Cells were trypsin washed and fixed. Cell nuclei were stained with DRAQ7 (blue). Scale bar, 20 μm.

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gradually acquire electron density, indicating that they are likely lysosomes (Figures 5B, 5C, S6A, and S6B).

Remarkably, gold-labeled PFFs are often found in close association with regions of membrane curvature as they begin to bud inward into electron-lucent organelles approximately 300–500 nm in diameter (Figure 6A). Thus, PFFs may contribute to the formation of MVBs. In fact, PFFs are readily detected on the surface of vesicles in MVBs (Figure 6B). Fluorescently labeled PFFs were also detected in LAMP1+ MVBs using STED microscopy (Figure 6C). Live-cell studies were performed to examine the dynamics of these structures, which appear to undergo multiple rounds of fusion and membrane budding (Figures 6D and 6E). Thus, PFFs enter cells via macropinocytosis and appear rapidly in lysosomes and MVBs.

**PFFs are transferred to naive cells using exosomes**

The spread of PFFs throughout the nervous system requires that the fibrils escape cells and be transferred to neighboring naive cells. Exosomes provide a mechanism for cell-to-cell transfer of protein, lipids, and other cellular molecules, and the presence of PFFs in MVBs suggests a potential mechanism for cellular release. To test this hypothesis, U2OS cells with the stable expression of CD63-GFP were incubated with fluorescently labeled PFFs for 24 h and then washed with trypsin and buffer before replating with PFF-naive cells with stable expression of LAMP1-red fluorescent protein (RFP). From 12 to 24 h following the start of co-culture, PFFs were observed to transfer...
Figure 6. PFF trafficking to lysosomes and MVBs

(A) Astrocytes administered PFFs for 10 min localize PFFs in larger newly forming MVBs, always in proximity to the vesicular membrane, sometimes at invaginations, potentially signifying the early stages of exosome formation. Scale bars, 100 nm for low magnification, 80 nm for moderate magnification, and 50 nm for highest magnification inset.

(B) Localization of PFFs outside of vesicles in electron-dense MVBs signifies the progression of MVB maturation. Scale bars, 100 nm and 50 nm for inset.

(C) Similar findings regarding the formation of MVB (LAMP1⁺; white) containing PFF (red) was confirmed using STED microscopy. Scale bars, 20 μm for low-magnification images and 2.5 μm for high-magnification images.

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from the CD63+ cells to the LAMP1+ cells (Figures 7A and 7C). Moreover, we observed the transfer of CD63 (Figures 7A and 7B), suggesting that the transfer of PFFs involves exosomes. Similar results are seen with the transfer to naive cells expressing CD9-RFP (Figure S7A). Notably, the transfer of both CD63 and PFFs is blocked by the addition of manumycin A, a compound that disrupts exosome release (Figures 7A–7C).

We next took CD63-expressing donor cells and incubated them with fluorescently labeled PFFs for 24 h before extensive trypsin/buffer wash. The cells were then replated in serum-free media and incubated for 36 h before the media was collected and spun at low speeds to remove any cells or cellular debris. The supernatant containing exosomes was transferred to naive cells. Extracellular vesicles (with CD63-GFP and PFF-Alexa 633) were detected in PFF naive cells using confocal microscopy (Figure 7D). The appearance of these fluorescent signals in the naive cells was blocked by using EIPA (Figures 7D and 7E). We then followed a similar procedure but used gold-labeled PFFs. We detected exosomes decorated with PFFs in contact with naive cells, sometimes seemingly near sites of membrane ruffles and forming macropinosomes (Figure S7C).

We next used a series of centrifugation steps to isolate exosomes (Chhoy et al., 2021) from the culture media of U2OS cells that were exposed to unlabeled PFFs for 24 h. EM analysis revealed fibril-like structures on their surface while exosomes isolated from the media of cells not exposed to PFFs did not (Figure S7B). A portion of the isolated exosomes from the PFF-treated samples were trypsinized to determine whether PFFs remain exclusively on the exosomal surface. Immunoblotting of the exosomal samples not only confirmed the identity of the exosomes (CD63+, GM130−) but additionally showed that the trypsinized exosomes did not contain any α-syn; hence, PFF resides on their surface (Figure S7C). In summary, PFFs that have entered cells by macropinocytosis are trafficked to MVBs and appear to be transported on the outer surface of exosomes to neighboring cells.

**DISCUSSION**

The mechanisms underlying PFF uptake remain incomplete (Bieri et al., 2018; Grozdanov and Danzer, 2018). Most studies investigating the internalization of α-syn or PFFs evaluate uptake hours following addition to cells (Desplats et al., 2009; Hansen et al., 2011; Konno et al., 2012; Lee et al., 2008; Liu et al., 2007; Luk et al., 2009, 2016; Madeira et al., 2011; Rodriguez et al., 2018; Sung et al., 2001; Volpicelli-Daley et al., 2011, 2014). While such uptake assays are valuable for genetic screening, they lack the temporal resolution to identify the pathways involved in endocytosis, an early event. We performed a detailed analysis of early events in the endocytosis of PFFs and were surprised to discover a unique uptake mechanism that allows the protein to reach lysosomes within 2 min, bypassing the early endosomal system.

The endocytosis of PFFs has been thought to follow canonical CME pathways, entering cells via clathrin-coated pits and vesicles followed by trafficking through endosomes to lysosomes (Konno et al., 2012; Lee et al., 2008). However, we found no evidence for the trafficking of PFFs through early or recycling endosomes, which is inconsistent with a CME pathway. Moreover, using a previously established pool of CHC siRNA (Bayati et al., 2021; Galvez et al., 2007; Kim et al., 2011), we attained ~95% KD efficiency, yet PFF endocytosis remained unperturbed. Thus, our data do not support a role for CME in the uptake of PFFs.

PFFs induce the formation of actin- and Rac1-rich membrane ruffles, precursors of macropinocytic vesicles (Cox et al., 1997; Grimmer et al., 2002). Furthermore, inhibitors of macropinoctosis (Commissio et al., 2014; Koivusalo et al., 2010; Zwartkruis and Burgering, 2013), including EIPA, LatA, and LatB (Erami et al., 2017; Furstner et al., 2007; Morton et al., 2000; Wakatsuki et al., 2001), significantly inhibited the uptake of PFFs, while not affecting EGF uptake. Our findings point to the endocytosis of PFF through a form of macropinocytosis, where macropinosomes either mature into MVBs and lysosomes or fuse with preexisting LAMP1+ compartments (i.e., late endosomes and lysosomes). Recent papers show promising results regarding the role of actin in PFF internalization (Underwood et al., 2020; Zhang et al., 2020).

Aspects of our findings corroborate previous studies. First, although slower, previous research demonstrates the direct fusion of macropinosomes with lysosomes (Yoshida et al., 2019). Second, the findings of previous studies on dynamin inhibition and reduction in the uptake of PFFs can be due to the role of dynamin in some forms of macropinocytosis and its involvement in actin remodeling (Gu et al., 2010; Krueger et al., 2003; Mulherkar et al., 2011). In fact, at high concentrations, we observed that dynasore does block PFF internalization (data not shown). Finally, amilorides, such as EIPA, which block the uptake of PFFs have been shown to have neuroprotective effects in PD (Anas et al., 2008).

Although previous studies attempted to examine the localization of exogenous α-syn using immuno-EM (Volpicelli-Daley et al., 2011), we conjugated PFFs directly with gold. Many antibodies to α-syn cannot distinguish between different conformations of the protein (Kumar et al., 2020), making the direct conjugation of gold to PFFs a more specific method. Consistent with previous literature (Vargas et al., 2017), PFFs were almost always in close association with membranes, whether at the cell surface, during internalization, or while in MVBs and lysosomes.

At early time points after addition to cells, we observed gold-PFFs on inward invaginating vesicles within MVBs. At longer time points, gold-PFFs were seen to be transported on exosomes, which confocal microscopy demonstrated were CD63+. Two important discoveries were made as a result of this: (1) PFF transmission is at least partly due to the exosomal transport of PFF and (2) PFFs reside on the surface of exosomes.
Figure 7. PFFs containing exosomes play a role in the transmission of PFFs from donor to acceptor cells

(A) U2OS cells stably expressing CD63-GFP were exposed to PFFs or PBS (vehicle control) for 24 h. CD63-GFP (donor cells; blue) were then trypsin washed 3 times, pelleted, trypsinized again, pelleted and PBS washed, before being co-plated with acceptor cells (PFF naive cells stably expressing LAMP1-RFP; red). Donor and acceptor cells were then incubated for 12 or 24 h. Half of the 24 h sample were given manumycin A (MA) at 1.2 μM, while the other half was given DMSO (vehicle control) at the time of plating. Both 12- and 24-h samples show PFF (white) and CD63 (blue) fluorescence in acceptor cells, while the acceptor cells in the PBS- and the MA-treated condition show very little PFF and CD63 fluorescence. Arrows point to LAMP1+ compartments in acceptor cells that contain both CD63 and PFF fluorescence within them. Scale bars, 10 μm for low magnification and 5 μm for insets.

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rather than being contained within their lumen. Furthermore, exosomes isolated from cells exposed to PFFs contained PFFs on their outer surface. Biochemically, we were able to show that PFFs can be removed from exosomes through trypsinization; this would only be possible if PFFs resided on the surface of exosomes. We then used manumycin A, a drug that blocks the release of CD63\(^*\) exosomes (Datta et al., 2017), and found that manumycin A disrupts PFF transmission from cell to cell. We suspect that since PFFs reside on the outside of exosomes, drugs like EIPA, LatA, and LatB that blocked their initial uptake can be used to block the spread of PFFs. Our hypothesis was confirmed with our experiment using EIPA, showing the inhibition in the uptake of PFF-transporting exosomes by PFF-naive cells.

Although all of the evidence presented indicates that PFFs use macropinocytosis to gain access into cells, we are also cognizant that the properties of \(\alpha\)-syn itself could drive membrane curvature and protrusions. As previously observed, PFFs associate and may even drive membrane curvature (Vargas et al., 2017; Westphal and Chandra, 2013). This could mean that PFFs drive their own internalization into the cell by causing membrane curvature, and then, once trafficked to MVBs/lysosomes, they drive membrane invaginations, resulting in intraluminal vesicle formation, leading to their eventual release via exosomes. Once released, PFFs then go on to disrupt other cells, all the while remaining on the outer surface exosomes, allowing them to drive membrane curvature, enabling their internalization into neighboring cells. Although PFFs are only an oligomer, they certainly have many prion-like characteristics. Above all else, PFFs may be taking advantage of the interaction of \(\alpha\)-syn with membranes to drive their own endocytosis, exocytosis, and the production of more aggregated \(\alpha\)-syn. It is this evolutionary drive for PFFs to create more fibrillated forms of \(\alpha\)-syn that truly makes them more than just an oligomer and more like a prion.

In conclusion, our data demonstrate that PFFs enter cells via macropinocytosis, with seemingly direct transfer to MVBs and lysosomes, bypassing early endosomal pathways. It remains unclear whether this represents a fusion of macropinosomes with MVBs and lysosomes or maturation of macropinosomes into these structures. A portion of the PFFs that enter the cells are subsequently secreted on exosomes, providing a mechanism for cell-to-cell transport; however, our data do not explain how fibrillar \(\alpha\)-syn interacts with \(\alpha\)-syn in the cytosol to allow prion-like propagation. Regardless, the inhibition of macropinocytic pathways may prove useful in limiting the progression of PD and other synucleinopathies.

**Limitations of this study**

While our experiments demonstrate the rapid transport of \(\alpha\)-syn PFFs to lysosomes through a unique form of macropinocytosis in a clathrin-independent manner, we acknowledge that our study has several limitations. First, amilorides and their inhibitive activity are not limited exclusively to macropinocytosis, and their addition to cells can have other non-specific effects (Arias et al., 2008; Levenson et al., 1980; Mine et al., 2015; Vila-Carriles et al., 2006). Second, it is important to note that while we attempt to examine the internalization of \(\alpha\)-syn preformed fibrils, PFFs may not be the true pathogenic component seen in synucleinopathies, as there are several forms of pathogenic \(\alpha\)-syn that may be responsible (Alam et al., 2019; Emadi et al., 2009). In addition, we acknowledge that there are multiple strains of \(\alpha\)-syn fibrils (Bouisset et al., 2013; Peng et al., 2018) that may use different endocytic pathways. Third, although we see gold-labeled PFFs in both MVBs and lysosomes, we have not established the relationship between these two pools of internalized \(\alpha\)-syn; furthermore, our knowledge regarding the amount of PFFs in these two compartments is limited. Lastly, while our data suggest the role macropinocytosis could play in the spread of \(\alpha\)-syn PFFs, further work needs to be done to address the relationship between internalized PFFs and their influence on endogenous \(\alpha\)-syn, a key component in understanding the pathophysiology of PD.

**STAR Methods**

Detailed methods are provided in the online version of this paper and include the following:

- **Key Resources Table**
- **Resource Availability**
  - Lead contact
  - Materials availability
  - Data and code availability
- **Experimental Model and Subject Details**
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  - Production, characterization, and Nano-gold labeling of PFF
  - IPSC culturing
  - Plasmids and lentivirus
- **Method Details**
  - Trypan blue exclusion assay
  - PFF live internalization assay
  - Live MVB assay
  - Lysosomal staining in fixed samples

(B and C) Quantification of CD63 and PFF fluorescence in acceptor cells. \(n = 8\) for each condition (i.e., \(n = 32\) total), from 3 independent experiments, means ± SDs. No PFF, 12- and 24-h conditions were statistically compared (2-sample t test) to the 24-h samples with MA; ****\(p < 0.0001\), **\(p < 0.01\), and \(p > 0.05\) denoted as ns for not significant. There was a significant reduction in CD63 and PFF in acceptor cells with MA.

(D) At 24 h following the exposure of donor cells (CD63\(^*\)) to PFF, cells were thoroughly trypsin washed, passaged onto plates, and incubated with serum-free media for 36 h. The media was then collected, centrifuged for 5 min at 1,000 rpm to pellet any floating cells, and given to acceptor cells (wild-type U2OS) some of which were treated with DMSO (control) and others treated with EIPA. The cells were fixed following 6 h of incubation. Donor cells in control showed both PFF (blue) and CD63 processed for EM. Insets show extracellular vesicles with PFF-gold on their surface. Scale bars, 100 nm for low-magnification images and 100 nm for inset.

(E) Quantification of PFF and CD63 fluorescence in (D). Multiple paired t test was used to statistically compare CD63 and PFF fluorescence in acceptor cells (control versus EIPA); \(n = 6\) per condition (i.e., \(n = 12\) total) from 3 independent experiments. ****\(p < 0.001\).

(F) U2OS cells were given media from donor cells treated with PFF-gold) isolated using the same protocol as in (D) and incubated for 12 h. Cells were then processed for EM. Insets show extracellular vesicles with PFF-gold on their surface. Scale bars, 100 nm for low-magnification images and 100 nm for inset.
ACKNOWLEDGMENTS

We acknowledge the Neuro Microscopy Imaging Center, the Advanced Biomaging Facility, and the Facility for Electron Microscopy Research at McGill University. We thank Dr. Michael Davidson and Dr. Paul Luzio for the LAMP1 and C6D3 plasmids, respectively. We also thank Drs. Sabatini and Zoncu for the HA-TMEM192 plasmids. A.B. is supported by Fonds de recherche du Québec doctoral award and a studentship from the Parkinson Society of Canada. This work was supported by a grant from the Canada First Research Excellence Fund, Healthy Brain, Healthy Lives, McGill University, awarded to P.S.M. P.S.M. is a Distinguished James McGill Professor and Fellow of the Royal Society of Canada.

AUTHOR CONTRIBUTIONS

A.B. planned and conducted the experiments and wrote the manuscript with P.S.M. E.B. performed lysosomal immunoprecipitation experiments, along with analyzing protein expression through Western blotting. C.H. provided NPC and differentiated iPSCs into neurons. Preprint at bioRxiv. A.B. is supported by Fonds de recherche du Québec doctoral award and a studentship from the Parkinson Society of Canada. This work was supported by a grant from the Canada First Research Excellence Fund, Healthy Brain, Healthy Lives, McGill University, awarded to P.S.M. P.S.M. is a Distinguished James McGill Professor and Fellow of the Royal Society of Canada.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


Lewy, F. (1912). Handbuch der Nervenkrankheiten (Julius Springer).


### STAR★METHODS

#### KEY RESOURCES TABLE

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Critical commercial assays

- Mycoplasma detection kit: Biotool Cat# B39038
- Cytotune reprogramming kit: ThermoFisher Cat# A34546

Deposited data

- Raw data and analysis: This paper https://doi.org/10.17632/nh3cvm3m3p.5

Experimental models: Cell lines

- HeLa: ATCC Cat# CRM-CCL-2
- U-2 OS: ATCC HTB-96
- U-87: ATCC HTB-14
- U-343: ATCC Discontinued
- RAW 264.7: ATCC TIB-71
- U-251: Sigma Aldrich 09063001

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**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to the Lead Contact, Dr. Peter Scott McPherson (peter.mcpherson@mcgill.ca) and will be fulfilled.

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
- All the raw data used in this paper have been deposited at Mendeley Data and are publicly available as of the date of publication. DOIs are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Cell lines**
HeLa, U2OS, U87, U-343, RAW 264.7 were obtained from American Type Culture Collection (Cat# CRM-CCL-2, HTB-96, HTB-14, discontinued, TIB-71, respectively). U-251 cells were obtained from Sigma (Cat# 09063001). Human fetal and adult astrocytes were obtained from Cell Applications (Cat# 882AK-05f and 882AK-05a, respectively). For studies with iPSCs, we used the line AIW002-2 obtained from the Neuro’s C-BIG Biorepository. This line was reprogrammed from peripheral blood mononuclear cells of a healthy donor with the Cytotune reprogramming kit (ThermoFisher, Cat# A34546). The process of reprogramming and quality control profiling for this iPSC was outlined in a previous study (Chen et al., 2021). The use of iPSCs in this project is approved by the McGill University Health Centre Research Ethics Board (DURCAN_IPSC / 2019-5374).

All cells were cultured in DMEM high-glucose (GE Healthcare, Cat# SH30081.01) containing 10% bovine calf serum (GE Healthcare, Cat# SH30072.03), 2 mM L-glutamate (Wisent, Cat# 609065, 100 IU penicillin and 100 μg/ml streptomycin (Wisent cat# 450201). Cell lines were routinely checked for mycoplasma contamination using the mycoplasma detection kit (Biotool cat# B39038).

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Production, characterization, and Nano-gold labeling of PFF

Production and characterization of recombinant α-syn monomers and PFF have been described previously (Del Cid Pellitero et al., 2019; Maneca et al., 2019). Both electron microscopy and dynamic light scattering were used for the characterization of α-syn monomers and PFF (Figures S1A and S1B). Previously characterized PFF, was then conjugated with 5 nm gold beads (Cytodiagnostics, Cat# CGNSK-5-2), immediately before experimental use. The Cytodiagnostics conjugation protocol was optimized to conjugate gold onto PFF. Following conjugation, some PFF was collected for characterization on carbon-covered grids (Electron Microscopy Sciences, Cat# FCF400CU50) (Figure S1C).

IPSC culturing

AIW002-2 hiPSC cultures were maintained as feeder-free cultures following the protocol described previously (Chen et al., 2021). AIW002-2 hiPSCs were plated onto Matrigel (Corning, Cat# 354277)-coated plates containing mTeSR1 medium (Stemcell Technologies, Cat# A1110501) into single-cell suspensions. 50,000 cells were plated onto coated coverslips in 24-well plates with neural progenitor plating medium (DMEM/F12 supplemented with N2, B27 supplement; ThermoFisher, Cat# A4192001, 17502001, 17504044). To further differentiate into dopaminergic neurons, neural progenitor medium was switched to dopaminergic neural differentiation medium (Brainphys Neuronal medium, STEMCELL Technologies, Cat# 05790) supplemented with N2A Supplement A (STEMCELL Technologies; Cat# 07152), Neurocult SM1 Neuronal Supplement (STEMCELL Technologies; Cat# 05711), BDNF (20 ng/mL; Peprotech, Cat# 450-02), GDNF (20 ng/mL; Peprotech, Cat# 450-10), Compound E (0.1 μM; STEMCELL Technologies, Cat# 73954), db-cAMP (0.5 mM; Carbosynth, Cat# ND07996), Ascorbic acid (200 μM; Sigma Aldrich, Cat# A5960) and laminin (1 μg/mL, Sigma Aldrich, Cat# L2020).

Plasmids and lentivirus

EBFP2-Lysosomes-20, tdTurboRFP-Lysosomes-20 were gifts from Michael Davidson (Addgene plasmid# 55246 and 58061). pL JM1-Tmem192-mRFP-3xHA was a gift from Roberto Zoncu (Addgene, plasmid# 134631). pLJC5-Tmem192-3xHA was a gift from David Sabatini (Addgene plasmid # 102930). CD63-pEGFP C2 was a gift from Paul Luzio (Addgene, Cat# 62964). LAMP1-RFP lentivirus was obtained from Applied Biological Materials Inc. (Cat# LVP719).

METHOD DETAILS

Trypan blue exclusion assay

HeLa (ATCC, Cat# CRM-CCL-2) cells were grown on poly-L-lysine coated 35 mm glass-bottom dishes (MatTek, Cat# P35G-1.5-14-C-GRD) for 48 h to 50% confluency. When ready for imaging, cells were stained with Hoescht (Invitrogen, Cat# H3570) for 30 min and dishes were placed in the pre-heated live imaging chamber of the Zeiss LSM-880 confocal microscope. Imaging was commenced before the addition of PFF tagged with Alexa Fluor 488 (PFF488) at a speed of 1 frame/sec. PFF was added during live imaging.

PFF live internalization assay

U2OS (ATCC, HTB-96) cells were grown on Poly-L-Lysine (Sigma Aldrich, Cat# A-005-M) coated 35 mm glass-bottom dish (MatTek, Cat# P35G-1.5-14-C-GRD) for 72 h to 70% confluency. Before imaging, cells were stained with Lysosomal Cytopainter (Abcam, Cat# ab176827). Dishes were placed in the pre-heated live imaging chamber of the Zeiss LSM-880 confocal microscope. Imaging was commenced before the addition of PFF at a speed of 1 frame/sec using the Airy imaging mode for higher resolution (~1.3x resolution of conventional confocal microscopy). PFF488 was added during imaging.

Live MVB assay

These live experiments were carried out the same way as above. For the hollow lysosome morphology, the “Find Edges” processing was used on ImageJ (NIH, https://imagej.net/software/fiji/).

Lysosomal staining in fixed samples

Lysosomal staining was achieved in one of the following ways: transfection with plasmids stated above with the aid of Lipofectamine 3000 (ThermoFisher, Invitrogen, Cat# L3000015), fixable Lysotracker (ThermoFisher, Invitrogen, Cat# L7528) for staining of lysosomes in neurons and neural progenitor cells, or with the use of LAMP1 antibody (Cell Signaling, Cat# 9091S). Transfection was done 24 h prior to experimentation. Staining with Lysotracker was done 30 min before experimentation. LAMP1 antibody staining was done following fixation and permeabilization.

PFF endocytosis assays

Cells mounted on coverslips were plated in 24 well plates at 37°C with 200 μL of media in each well. Immediately prior to experimentation, cells were taken out of incubators and placed in the cell culture hood. PFF aliquots were then removed from dry ice, diluted
with serum-free media, and added to each well. Cells were then incubated at 37°C for 0, 2, 10, 30, or longer. Cells were then placed on ice and washed with trypsin (Wisent, Cat# 325-052-EL) for 90 s (to remove extracellular PFF). Following 2 washes with PBS (Wisent, Cat# 311-010-CL), cells were fixed. In experiments where trypsin was not used, cells were washed three times with PBS and fixed. In experiments using iPSC neurons, trypsinization was avoided and only PBS was used to wash cells. Same protocol was followed for samples prepared for EM, except for plating cells on Nunc 8-well dishes (Lab-Tek, Nunc, Thermo Scientific, Cat# 177445) and fixation with Glutaraldehyde and not PFA.

**PFF long-term endocytosis assays**

Cell plating and culturing was done as described above. Cells were then incubated at 37°C for 24 h. Following 24 h, cells were trypsin washed three times, pelleted, resuspended and re-plated onto fresh coverslips. For iPSC neurons, trypsinization was avoided and only PBS was used to remove any remaining serum. Serum-free media was added with a final concentration of either 20 μM of EIPA, 0–2 μM of LatA, or 5 μM of LatB. Cells were then incubated at 37°C for 30 min before experimentation for EIPA and LatB, and 1 h for LatA. Due to the autofluorescence of EIPA, DRAQ7 (abcam, Cat# ab109202) was used to stain cell nuclei.

**Macropinocytic inhibitors**

Ethylisopropyl amiloride (EIPA; R&D Systems, Tocris, Cat# 3378), Latrunculin A (LatA; Cayman Chemical, Cat# 10010630), and Latrunculin B (LatB, respectively; abcam, Cat# ab144920, ab144291) were the macropinocytic inhibitors used in this study. Cells normally incubated in media with serum were washed three times with serum-free media to remove any remaining serum. Serum-free media was added with a final concentration of either 20 μM of EIPA, 0–2 μM of LatA, or 5 μM of LatB. Cells were then incubated at 37°C for 30 min before experimentation for EIPA and LatB, and 1 h for LatA. Due to the autofluorescence of EIPA, DRAQ7 (abcam, Cat# ab109202) was used to stain cell nuclei.

**LatA inhibition in dopaminergic NPCs**

Dopaminergic NPCs were seeded in polyornithin/laminin coated 384 well plates (Corning, Cat# 353962) at 4000 cells/well. After 24 h, Latrunculin A (LatA; Cayman Chemical, Cat# 10010630) was added. After 1 h, Alexa488-PFF was added. After another 24 h incubation period, the wells were washed twice with PBS (Wisent, Cat# 311-010-CL) and the cells fixed with 2% FA/PBS. Cells were counterstained with Hoechst 33342 (ThermoFisher, Cat# H3570) and imaged on a high content imager (CellInsight CX7, ThermoFisher Scientific). The amount of intracellular Alexa488-PFF was measured as the total intensity of Alexa488 fluorescent stain in the perinuclear area. Nuclei count and nuclear area (px²) were also obtained as indicators for cell toxicity. All data was normalized against DMSO (vehicle) only controls. Data represents the mean and standard deviation of 3 independent experiments.

**Lysosomal immunoprecipitation**

U2OS cells expressing 3xHA-TMEM192-RFP were incubated with PFF for varying lengths of time, washed with trypsin (Wisent, Cat# 325-052-EL) three times to remove extracellular PFF, and then trypsinized and pelleted. Lysosomes were immunoprecipitated based on the protocol described in Abu-Remaileh et al. (2018). Briefly, cells were washed twice with KPBS and centrifuged at 4°C for 2 min at 1000 x g. Pelleted cells were resuspended in lysis buffer (20 mM HEPES pH 7.4, 100 mM NaCl, and protease and phosphatase inhibitor cocktail) and manually homogenized with 25 up and down strokes. Homogenates were centrifuged for 2 min at 4°C at 1000 x g and 10% of the total volume for each IP was reserved for starting material (SM) fractions. Homogenates were incubated with gentle rocking at 4°C for 3 min with 100 μL anti-HA magnetic beads (ThermoFisher, Thermo Scientific, Cat# 88836) pre-washed with KPBS. After collecting the total IP volume for the unbound materials (UM), immunoprecipitates were washed three times with KPBS using a DynaMag-2 Magnet (ThermoFisher, Invitrogen, Cat# 12321D) before resuspension in 1X SDS-PAGE sample buffer. Protein aliquots were then analyzed by SDS-PAGE and Immunoblot.

For samples to be processed via confocal microscopy, 20 μL pre-washed anti-HA magnetic beads were used in the IP and were resuspended using PFA. The sample was then pelleted and resuspended with PBS (Wisent, Cat# 311-010-CL) and mounted onto slides.

**PFF Inter cellular (with contact) Transfer assay**

U2OS cells were transfected with CD63-EGFP or LAMP1-tdTurboRFP using Lipofectamine 3000 transfection reagent and incubated for 24 h. Cell media was then changed, and cell selection process was initiated through the addition of Neomycin (Gibco Cat# LS21810031). U2OS cells stably expressing CD63-GFP were exposed to PFF or PBS (vehicle control) for 24 h. CD63-GFP (donor cells) were then trypsin washed three times, pelleted, trypsinized again, pelleted and PBS washed, prior to being co-plated with acceptor cells (PFF naïve cells stably expressing LAMP1-tdTurboRFP). Donor and acceptor cells were then incubated for 12 or 24 h. Half of the 24 h sample were given Manumycin A (MA; Cayman Chemical, Cat# 10010497) at 1.2 μM while the other half was given DMSO (vehicle control) at the time of plating. Cells were then fixed, mounted and imaged.
PFF Intercellular (without contact) Transfer assay
U2OS cells were stably expressing with CD63-EGFP, the cells were exposed to PFF, for 24 h then trypsin washed three times and replated prior to the addition of serum-free media. The media was collected following 36 h of incubation at 37°C and given to naïve U2OS cells. Some of the cells given this media were exposed to EIPA while others were given DMSO as a vehicle control. For fluorescent microscopy samples, the naïve cells were plated on coverslips, while for samples to be examined with EM, naïve cells were plated on Nunc 8-well plates (Lab-Tek, Nunc, Thermo Scientific, Cat# 177445).

Fixation and antibody staining following PFF uptake
Fixation was done with 2% freshly made paraformaldehyde (PFA; Thermo Fisher Scientific, A1131322) for 10–15 min on ice. In experiments where antibody staining was done, cells were then blocked and permeabilized for 30 min using 0.05% Triton X-100 (Sigma, Cat# X100-1L) in Phosphate-buffered saline (PBS; Wisent, Cat# 311-010-CL) along with 5% BSA (Wisent, Cat# 800-095). Coverslips were then transferred into a wet chamber and incubated with 1:500 dilution of the antibody in 0.01% Triton X-100 and 5% BSA. Cells were incubated with the diluted antibody for 2 h at room temperature. Coverslips were then gently washed 3 times with PBS, and 1:500 dilution of secondary antibody was added in 0.01% Triton X-100 and 5% BSA. Cells were then washed 2 more times with PBS and stained with DAPI (ThermoFisher, Invitrogen, Cat# D1306) for 10 min at 1 μg/mL concentration. Coverslips were then mounted on a glass slide using Fluorescent Mounting Medium (Dako, Agilent, Cat#S3023). All fixed samples were then imaged using a Leica TCS SP8 confocal microscope. STED samples were imaged using Abberior STED super-resolution nanoscope.

CHC knockdown
U2OS cells at 60% confluency were transfected with siRNA retrieved from Dharmacon (SMARTpool, ONTARGETplus, see KRT) or control siRNA (Dharmacon; ON-TARGETPLUS CONTROL) using Lipofectamine 3000 (ThermoFisher, Invitrogen, Cat# L3000015). At 24 h following transfection, cells were passage, with some cells retrieved for imaging experiments and mounted onto coverslips. At 48 h, cells were collected in HEPES lysis buffer (20 mM HEPES, 150 mM sodium chloride, 1% Triton X-100, pH 7.4) accompanied with protease inhibitors. Cells in lysis buffer were then transferred into a wet chamber and incubated with 1:500 dilution of the antibody in 0.01% Triton X-100 and 5% BSA. Cells were incubated with the diluted antibody for 2 h at room temperature. Coverslips were then gently washed 3 times with PBS, and 1:500 dilution of secondary antibody was added in 0.01% Triton X-100 and 5% BSA. Cells were then washed 2 more times with PBS and stained with DAPI (ThermoFisher, Invitrogen, Cat# D1306) for 10 min at 1 μg/mL concentration. Coverslips were then mounted on a glass slide using Fluorescent Mounting Medium (Dako, Agilent, Cat#S3023). All fixed samples were then imaged using a Leica TCS SP8 confocal microscope. STED samples were imaged using Abberior STED super-resolution nanoscope.

FluoSpheres and dextran uptake assays
RAW 264.7 cells (ATCC, Cat# TIB-71) were plated onto coverslips treated with Poly-L-Lysine (Sigma, Cat# A-005-M). FluoSpheres (ThermoFisher, Invitrogen, Cat# F13082), latex beads with 1 μm diameter, and orange fluorescence were incubated with PFF. Cells were then washed with trypsin (Wisent, Cat# 325-052-EL), washed twice with PFF, and fixed. Cells were then counterstained with DAPI prior to mounting using Fluorescence Mounting Medium (Dako, Agilent, Cat#S3023). U2OS cells were used in the dextran and PFF uptake experiment. U2OS cells were plated and prepared as described above. Three different dextran (10,000 MW, 70,000 MW, 2,000,000 MW; ThermoFisher, Cat# D1817, D1818, D7139) were added to cells alongside PFF.

Membrane ruffling assay
Cellular response to PFF addition was done using human fetal astrocytes (Cell Applications, 882AK-05f). Cells were treated with PFF, and their recruitment of Rac1 (Emd Millipore Corporation, Cat# 16319) to the cell surface and colocalization with F-actin (Phalloidin; Abcam, Cat# A22287) was analyzed. For negative and positive control, Transferrin (Tf; ThermoFisher, Invitrogen, Cat# T13342) Epidermal growth factor (EGF; ThermoFisher, Invitrogen, Cat# E13345) were added to cells, respectively. A null condition was also in place, where cells were administered PBS (a vehicle control for PFF).

Exosomal isolation
For exosomal isolation, a previously published protocol was followed (Chhoy et al., 2021). Following isolation, samples were prepared for immunoblotting and EM. For immunoblotting, a portion of exosomes retrieved from PFF treated cells were treated with trypsin for 5 min. The exosomes were then re-pelleted at 10,000 g’s, and the supernatant removed. These trypsinized exosomes were run alongside exosomes collected from PBS and PFF treated cells, to compare PFF content.

TEM
Human astrocytes and U2OS cells were plated onto 8 well chamber slides (Lab-Tek, Nunc, Thermo Scientific, Cat# 177445) and were administered PFF conjugated with gold. Cells were then fixed with glutaraldehyde 2.5% in 0.1M sodium cacodylate buffer (Electron Microscopy, Cat# 1653715), post-fixed with 1% OsO4 and 1.5% potassium ferrocyanide in sodium cacodylate buffer. Cells were then en bloc stained with 4% uranyl acetate. Post-embedding, some grids were stained with uranyl acetate for enhanced membrane staining. Samples were viewed with a Tecnai Spirit 120 kV electron microscope and captured using a Gatan Ultrascan 4000 camera.
QUANTIFICATION AND STATISTICAL ANALYSIS

Colocalization analysis
Colocalization analysis was conducted using JACoP plugin (Bolte and Cordelieres, 2006) in ImageJ (imagej.net). This plugin was used to calculate the Manders’ Colocalization Coefficients (MCC) as performed previously. Unlike Pearson’s correlation coefficient, MCC provides a direct measure colocalization: the ratio of with which one probe co-occurs with a second probe (Dunn et al., 2011), hence we found it to be the most appropriate measure of colocalization for our study. Due to the sensitivity of MCC to background noise, a 20% threshold was used for all colocalization analysis to minimize the effect of fluorescent noise and bleed-through on our colocalization analysis. M₁ coefficient represents the ratio of the sum of pixel intensities in probe 1 for which probe 2 has an intensity above zero compared to the total intensity of probe 1. M₂ represents the same concept for probe 2. For consistency, M₁ and M₂ were calculated so that M₁ would always represent the ratio to which lysosomes (LAMP1/Lysotracker) colocalize with PFF (i.e., the portion of lysosomes that contain PFF) while M₂ always represents the ratio in which PFF colocalizes with lysosomes (i.e., the portion of PFF that colocalize with lysosomes). In experiments where different markers or cargo were used alongside PFF, M₂ still represents the degree in which PFF colocalizes to the other probe.

Quantification and statistics
For all quantifications, including uptake and colocalization, the Leica LAS X and ImageJ (imagej.net) software were used. Readout from ImageJ for signal intensity and colocalization (using the JACoP plugin) was then used to tabulate results. In each experiment, images were selected from a large field imaged at low quality using the Leica SP8 spiral scan. From the large field, regions were selected that contained a minimum of 4 cells. Graphs were then prepared using GraphPad Prism 9 software. For statistical comparisons, two-sample t test and one-way ANOVA were used. When significance was detected under ANOVA, multiple comparisons Tukey’s test was conducted to find significant differences across means. All data is shown as mean ± SD, with only a few exceptions. For statistical significance, p < 0.05 was used.