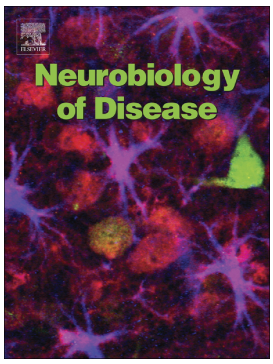

Lewy body-associated proteins: Victims, instigators, or innocent bystanders? The case of AIMP2 and alpha-synuclein

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The case of AIMP2 and alpha-synuclein

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Abstract

Lewy bodies (LBs), one of the neuropathological defining hallmarks of Parkinson's disease (PD), are composed of a complex mixture of alpha-synuclein (aSyn) filaments and hundreds of proteins, lipids, and membranous organelles. However, these proteins' role in aSyn aggregation and the biogenesis of LBs remains poorly understood. Previous studies have focused on investigating the role of these proteins as modifiers of aSyn aggregation, inclusion formation, and toxicity; very often, one protein at a time. In a recent study, Ham *et al*. suggest that one of these proteins, aminoacyl tRNA synthase complex-interacting multifunctional protein 2 (AIMP2), plays a primary role in the initiation of aSyn aggregation and is essential for aSyn inclusion formation and toxicity in cells and several models of synucleinopathies (Ham et al., 2020). Based on *in vitro* aggregation studies, they proposed a model in which AIMP2 self-associates to form amyloid-like aggregates that interact with monomeric aSyn and catalyze/seed the formation of aSyn fibrils and, eventually, LB-like inclusions. Herein, we present a critical analysis of their results and conclusions, review previous studies on AIMP2 aggregation, and reexamine the role of AIMP2 in regulating aSyn inclusion formation and clearance and aSyn-induced neurodegeneration in Parkinson's disease. We conclude by presenting lesson learned and recommendations on experimental factors and approaches that should be considered in future studies aimed at investigating the potential of targeting LBs-associated proteins, including AIMP2, for developing therapies to treat PD and other synucleinopathies.

Background

Alpha-synuclein (aSyn) is a presynaptic protein that is highly expressed in the brain and exhibits an increased propensity to misfold and form amyloid-like fibrils in the brains of individuals affected by several neurodegenerative diseases (Armstrong et al., 1997; Lashuel et al., 2013; Spillantini et al., 1998). The accumulation of aSyn fibrils in the form of intracellular inclusions in neurons (Lewy bodies, LB), Glial cells (Glial cytoplasmic inclusions, GCIs), or nerve cells is one of the defining hallmarks of Parkinson's disease (PD), multiple system atrophy, and dementia with Lewy bodies, also collectively referred to as synucleinopathies (Arima et al., 1998; Goedert et al., 2017; Mckeith et al., 2017; Spillantini et al., 1998). Whether aSyn fibrils and LBs are the cause or consequence of synucleinopathies remains a subject of active research and debate. Answering this question requires 1) a better understanding of the molecular and cellular mechanisms that trigger and drive aSyn misfolding, fibrillization, and LB formation; 2) elucidating the relationship between aSyn fibrillization and LB formation, i.e., whether aSyn fibril formation is a prerequisite step for LB formation; 3) understanding the relative contribution of each stage

on the pathway to LBs/GCIs to neuronal dysfunction and neurodegeneration in multiple cellular and animal models of synucleinopathies; and 4) identifying genetic or pharmacological modifiers that prevent aSyn aggregation and LB formation, promote their clearance, or block their neurotoxic effects.

Recent correlative light electron microscopy (EM) and proteomic studies have shown that LBs consist of a complex mixture of aggregated forms of aSyn and hundreds of other proteins, lipids, and disrupted membranes and membranous organelles (Shahmoradian et al., 2019). The role of these different components in aSyn aggregation and LB formation remains unclear. This is due primarily to a large number of candidate molecules and proteins and the lack of preclinical models that allow for recapitulating the various stages of LB formation, from aSyn misfolding, oligomerization, and fibrillization to LB formation and maturation. Recent studies in a neuronal seeding model, which recapitulated many features of LBs, revealed that aSyn fibrillization occurs before the formation of LB-like inclusions (Mahul-Mellier et al., 2020a). In this model, aSyn fibrils start to form after seven days and interaction with membranous organelles and other proteins occurs from 10-12 days before the appearance of inclusions that bear many of the key biochemical, structural, and organizational features of LBs. However, what triggers aSyn fibrillization in the first place remains a mystery.

The role of AIMP2 and LB formation and PD

In a recent publication, Ham *et al*. suggested that aminoacyl-tRNA synthetase complex interacting multifunctional protein-2 (AIMP2), which has been reported to cause selective and age-dependent degeneration of dopaminergic neurons, plays an essential role in the initiation of aSyn fibrillization and LB formation (Lee et al., 2013). They proposed that AIMP2 first self-assembles to form amyloid like aggregates, which interact with monomeric aSyn and induce its fibrillization *in vitro* as well as the formation of aSyn fibrils and LB-like inclusions in various well-established cellular and animal models of synucleinopathies (Ham et al., 2020).

Several lines of evidence suggest a possible role of AIMP2 in the pathogenesis of PD; 1) AIMP2 was previously identified as a Parkin substrate, an E3 ubiquitin ligase, and its lysosomal-mediated clearance is regulated by VPS35 (Yun et al., 2017), two genes implicated in the pathogenesis of PD (Kitada et al., 1998; Vilariño-Güell et al., 2011); 2) AIMP2 levels are elevated in the cingulate cortex of patients with autosomal recessive juvenile PD and dementia with LBs (Kim et al., 2019); 3) It localizes at the center of LBs in the substantia nigra of PD patients (Ko, 2005; Corti, 2003); 4) Several studies have shown that AIMP2 exhibits a high propensity to aggregate and form inclusions that are immunoreactive for several markers of LBs, including HDAC6 (Takahashi et al., 2018; Wong et al., 2008), ubiquitin, γ-tubulin (Wong et al., 2008), parkin, vimentin, Proteasome 20S, Hsp70, and Hdj-2 (Corti, 2003). Interestingly, co-aggregation of aSyn and AIMP2 was shown to promote the clearance of AIMP2-positive inclusions by autophagy (Wong et al., 2008), whereas AIMP2 aggregates devoid of aSyn were resistant to autophagic degradation. Together, these findings suggest possible interactions between aSyn and AIMP2 that may influence their aggregation and LB formation, maturation or clearance.

Overall, the large body of data presented by Ham and colleagues demonstrates that AIMP2 aggregates bind preferentially to monomeric aSyn and exhibit a high tendency to colocalize with pS129 aSyn aggregates and inclusions in several models of synucleinopathies. In two different mouse models of synucleinopathy (a model based on AAV-mediated overexpression of aSyn and inoculation with preformed fibrils (PFFs) and 6-OHDA injection), AIMP2 knockdown resulted in a marked reduction in pS129 aSyn aggregate formation, neuronal loss, bradykinesia, and anxiety-like phenotype. Based on these findings, the authors concluded that AIMP2 is required for aSyn aggregation and the formation of LB-like inclusions. They suggested that targeting AIMP2 oligomerization and/or its interactions with aSyn may provide alternative strategies for preventing aSyn pathology formation and could open new avenues to develop therapies for PD and other synucleinopathies. However, careful review and analysis of their results and interpretations reveal a possible alternative explanation for their results and experimental gaps that are worth considering in follow-up studies or before embarking on new research programs aimed at targeting AIMP2 polymerization. Our assessment is that the data presented in the manuscript do not provide compelling evidence supporting the hypothesis that AIMP2 forms amyloid-like aggregates or LB-like inclusions or that AIMP2 aggregation is a primary driver of aSyn aggregation or inclusion formation. Instead, we believe that their results could also be explained by AIMP2 recruitment or binding to aSyn aggregates after they form or other yet-to-be-discovered AIMP2-dependent mechanisms. Below, we present a more in-depth analysis of the data, highlighting experimental gaps, and suggesting possible experiments that could pave the way to elucidating how interactions between these two proteins and other LB-associated proteins may influence aSyn aggregation and LB formation and contribute to the pathogenesis of PD.

Evidence for the presence of AIMP2 in LBs

In a previous study, Corti et al. reported, using immunofluorescence techniques (Figure 1), the presence of AIMP2 staining in the core of classical LBs in the SN and, to a lesser extent, in ubiquitin-positive neurites (Corti, 2003). Interestingly, there was limited or no overlap between the ubiquitin and AIMP2 signals in LBs, whereas the two signals overlapped, extensively in neurites. The authors interpreted the localization of AIMP2 in the core of LBs suggesting that they exist in aggregated forms and must play a central role in the early stages of LB formation. They also proposed that parkin-mediated ubiquitinylation of AIMP2 represents an important early event in LB formation. Additionally, Ko et al. reported the detection and colocalization of AIMP2 in the center of LBs by immunofluorescence and in frontal cortical tissues of three of eight PD/DLBD brains by western blot (WB), mainly those with a defective parkin function as reflected by higher levels of S-nitrosylated parkin (Ko, 2005). These findings suggest that AIMP2 is not a key component of all LBs. Ham et al. also provided immunohistochemical and immunogold labeling data showing significant colocalization of pS129-aSyn and AIMP2 in LBs and the presence of AIMP2 in the insoluble fractions from

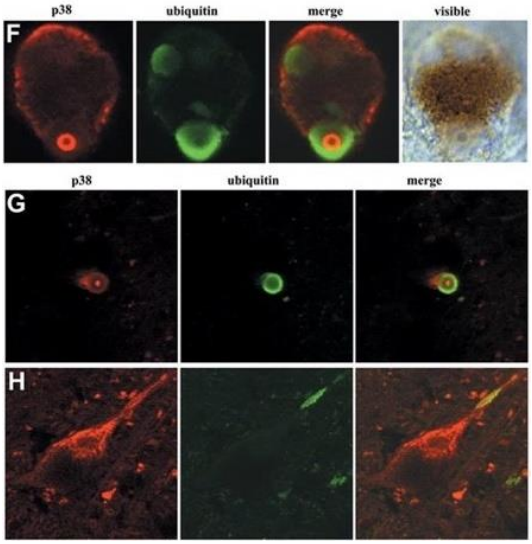

Figure 1: Immunohistochemical analysis of the distribution of p38 in the human SN adult post-mortem and its localization in LBs. Adapted with permission from (Corti, 2003).

substantia nigra obtained from the brains of patients with PD. There was no correlation between the levels of pS129-aSyn and AIMP2 in these fractions (Ham et al., 2020).

Several mass spectrometry-based proteomics studies have sought to unravel the composition of LBs with the aim of identifying disrupted pathways, aSyn interacting proteins, and modifiers of aSyn aggregation and LB formation (Datta et al., 2017; Killinger et al., 2020; Leverenz et al., 2007; Licker et al., 2014; Ping et al., 2018; Van Dijk et al., 2012; Xia, 2008). Interestingly, in the majority of these studies, AIMP2 was not identified as one of the LB-associated proteins. These include proteomic studies ranging from those on cortical LBs purified from patients diagnosed with the Lewy body variant of Alzheimer's disease (Xia, 2008) or isolated by laser capture microdissection from patients with cortical LB disease (Leverenz et al., 2007) to those of proteins enriched in isolated LBs from multiple brain regions, including SN (Licker et al., 2014), locus coeruleus (Van Dijk et al., 2012), and the cortex (Datta et al., 2017). Furthermore, a recent study by Killinger B. et al., using biotinylation by antibody-recognition (BAR), identified 815 enriched aSyn pathology-associated proteins extracted from a formalin-fixed PD and DLB brain (Killinger et al., 2020), but remarkably, AIMP2 was not identified among these proteins. In one study by Ping et al. using Tandem Mass Tag (TMT) isobaric labeling and synchronous precursor selection-based MS3 (SPS-MS3) mass spectrometry, AIMP2 was detected as one of the components of LBs found in the brain tissues of AD, PD, and co-morbid AD/PD cases (Ping et al., 2018). Nevertheless, it is important to note that AIMP2 levels tend to vary in patients' samples (Ham et al., 2020; Ko et al., 2010). Overall, these studies suggest that AIMP2 is not an essential component of LBs and might be present in only some LBs or aSyn pathological aggregates.

Evidence of AIMP2 aggregate in cells?

AIMP2 is part of the multiaminoacyl tRNA synthetase complex, where it acts as a scaffold subunit to stabilize the whole complex. This complex plays a pivotal role in protein biosynthesis by catalyzing the esterification of amino acids with their corresponding tRNA (Robinson et al., 2000). In this regard, its function suggests that it possess multiple protein-protein interaction surfaces. Ham and colleagues reported that AIMP2 is prone to self-aggregation, but what triggers AIMP2 aggregation in the first place remains unknown. They also proposed that defective processing and clearance of AIMP2 by the PD-linked proteins parkin and/or VSP35 could result in its accumulation and aggregation. However, the stage during their aggregation and inclusion formation when aSyn and AIMP2 interacted with each other, and the role of this interaction in the pathophysiology of PD, remained unclear.

In the absence of overexpression, AIMP2 exhibits a homogenous distribution of small cytoplasmic puncta/granules, suggesting that it accumulates either in vesicles or in complexes with other proteins or cellular structures. Under physiological conditions, AIMP2 is ubiquitinated by parkin and degraded by the proteasome (Ko et al., 2010; Yun et al., 2017). When the expression of AIMP2 is upregulated, it tends to aggregate and form inclusion bodies that are positive for HDAC6 (Takahashi et al., 2018; Wong et al., 2008), ubiquitin, γ-tubulin, p62 (Wong et al., 2008), parkin, vimentin, Proteasome 20S, Hsp70, and Hdj-2 (Corti, 2003). In addition, AIMP2 accumulates in sporadic cases of PD, in which it localizes in neurites and intracellular and extracellular LBs (Corti, 2003). Despite the

consistent observation of AIMP2 inclusions in various cellular models, none of the published studies sought to assess the aggregation state or the biochemical properties of AIMP2 in these inclusions. Therefore, the conclusions that AIMP2 is capable of self-assembly into amyloid-like aggregates are supported mainly by its ability to form these foci structures in cells. Its presence in LBs or colocalization with other LB or aggresome-associated proteins merely suggests its association with these processes and does not prove that it exists in aggregated forms or that it drives LB formation in PD. It is also interesting to note that AIMP2 aggregates are resistant to autophagic clearance, whereas AIMP2-aSyn co-aggregates are cleared by autophagy (Lee et al., 2013). This effect is due to a failure in recruiting key components of the autophagic/lysosomal system, despite AIMP2 aggregates being positive for p62 and ubiquitination (Wong et al., 2008). On the other hand, AIMP2-aSyn aggregates can recruit mTOR and increase the engagement of inclusions with LAMP1.

Is AIMP2 oligomerization necessary for aSyn aggregation and inclusion formation?

The results from Ham et al. suggest that modulation of AIMP2 levels significantly influences aSyn aggregation, inclusion formation, and toxicity. For example, their data point to a strong association between AIMP2 and pS129 immunoreactive aSyn aggregates in several well-established cellular and animal models of synucleinopathies. In addition, knockdown of AIMP2 or a reduction of its levels significantly attenuates aSyn aggregation and toxicity in both genetic and toxin models of synucleinopathies. However, we disagree with their interpretations and conclusion that AIMP2 oligomerization is the primary mechanism by which this protein influences aSyn aggregation and toxicity. The evidence in support of their model is derived primarily from *in vitro* aggregation studies, which were not supported by data demonstrating the formation of AIMP2 oligomers or amyloid-like aggregates in any of the synucleinopathies models used in their studies. This is reflected in the absence of data on the characterization of the AIMP2 puncta or aggregate-like structures observed in these models. For example, it was suggested that overexpression of Myc-AIMP2 in SH-SY5Y cells results in AIMP2 aggregate formation. Although the data show the AIMP2 accumulation and formation of AIMP2 foci, no additional data were presented to support the claim that these foci represent AIMP2 amyloid-like aggregates/oligomers. Even when AIMP2 inclusions were reported in neurons or in vivo, no supporting data were presented to corroborate the claim that these inclusions correspond to amyloid fibrils or oligomers. For example, it remains unclear whether these inclusions of AIMP2 bind to Thioflavin T/S (ThT/S) or other amyloid dyes. Characterization of AIMP2 inclusion formation was limited to reporting on puncta formation, which the authors refer to as “LB-like inclusions” without providing immunohistochemical or biochemical data to show that they exhibit LB-like features.

Western blot analysis of AIMP2 protein in the soluble and insoluble fractions did not show the formation of high molecular weight (HMW) SDS-resistant bands typically seen for amyloid aggregates formed by aSyn and other proteins. In fact, all the data on AIMP2 aggregation were limited to assessing changes in the level of the monomeric AIMP2 band. Interestingly, in both soluble and insoluble fractions, AIMP2 runs as a single monomeric band, which suggests that all the AIMP2 aggregates are SDS-sensitive and break down to monomers or that the AIMP2 in the identified puncta/foci structures represent the accumulation of soluble forms of the protein. In contrast, in the case of aSyn, both levels of aSyn monomers and HMW species were evaluated. Surprisingly, no further studies (e.g.,

size exclusion chromatography, in-cell crosslinking, or amyloid-dye binding studies) were performed to assess the aggregation state or amyloid-like properties of AIMP2 in any of the cellular and animal models used in this study or previous AIMP2 studies. These observations seem consistent with AIMP2 co-accumulation with pS129 aSyn but are not sufficient as evidence that AIMP2 forms amyloid-like oligomers or fibrils in cells/neurons. Finally, although a mechanism was proposed suggesting that AIMP2 aggregates first and then catalyzes/seeds the aggregation of aSyn, no experiments were performed to test this hypothesis or to investigate the temporal relationship between AIMP2 oligomer formation and aSyn fibrillization and inclusion formation.

Does AIMP2 knockdown or depletion inhibit aSyn aggregation and inclusion formation or protect against aSyn toxicity?

The most compelling evidence in support of an important role of AIMP2 in the aggregation of aSyn is the finding that the AIMP2 knockdown prevented aSyn aggregation, inclusion formation, and aSyn-induced toxicity in several cellular and animal models of synucleinopathies. Using a neuronal seeding-based cellular model, they showed that knockdown of AIMP2 (by approximately 50%) resulted in nearly complete inhibition of the formation of pS129 immunoreactive species—an effect that could be reversed by restoring AIMP2 levels. They also reported that treatment of these neurons with PFFs induced the accumulation of AIMP2 aggregates in close proximity to the pS129 immunoreactive aSyn aggregates, with extensive but not complete overlap between the two proteins. In neurons treated with PBS, AIMP2 exhibited a diffuse distribution with the rare occurrence of foci structures. Similar findings were observed using a mouse combinatorial model based on both PFF seeding and viral-mediated overexpression of aSyn. In this model, knockdown of AIMP2 reversed aSyn aggregation and aSyn-induced neurodegeneration and motor deficit.

To further validate their findings in these genetic models, the effect of AIMP2 knockdown in a toxin (6-OHDA)-based cellular and animal models of PD was also assessed. In SH-SY5Y cells, treatment with 6-OHDA induced the formation of insoluble and pS129-aSyn immunoreactive inclusions. This was accompanied by a decrease in soluble aSyn and a concomitant increase in aSyn HMW species in the insoluble fractions. Similar results were obtained in the 6-OHDA striatal injection model. Although western blot analysis of the insoluble fraction showed the presence of several SDS-resistant aSyn immunoreactive HMW species, the aggregation state of AIMP2 in the insoluble fractions was not assessed. AIMP2 was found to co-accumulate with aggregated aSyn in the insoluble fraction from brains injected with 6-OHDA. However, it remains unclear whether this is due to the aggregation of AIMP2 or simply to the recruitment of AIMP2 into aSyn aggregates. Surprisingly, no data were reported on whether or not 6-OHDA treatment alters the aggregation or cellular properties of AIMP2 and no attempts were made to assess the aggregation state of AIMP2 in these models, beyond demonstrating that it exists in the insoluble fractions. The colocalization of AIMP2 and aSyn or pS129-aSyn was not assessed in this model. Altogether, these findings suggest that knockdown of AIMP2 or a significant lowering of its levels is sufficient to prevent aSyn seeding and formation of new aSyn aggregates, but the underlying mechanisms remain unclear. Four possibilities could explain these observations: 1) treatment with PFFs induce the accumulation or aggregation of AIMP2, which then seed the aggregation of endogenous aSyn; 2) aSyn and AIMP2 aggregation occurs in a concerted manner and the interaction between the two proteins is a pre-requisite for aSyn

aggregation; 3) AIMP2 is slowly recruited to newly formed aSyn aggregates during post-PFF seeding events; and 4) AIMP2 pathology inhibition and neuroprotective effects are mediated by other mechanisms, independent of its direct interactions with aSyn. For example, it has been shown that depletion of AIMP2 increases resistance to DNA damage-induced apoptosis via activation of p53 (Han et al., 2008). Therefore, it can be postulated that the neuroprotective effect against 6OHDA-induced neurodegeneration displayed in the heterozygous knockout (HET KO) AIMP2 mice could be due to the blockade of the AIMP2-p53 axis rather than AMIP2 modulation of aSyn aggregation.

AIMP2 aggregates accelerate aSyn aggregation, but do aSyn PFFs also seed that aggregation of AIMP2?

Based on their findings, Ham et al. proposed a model in which AIMP2 oligomerization plays an essential role in the initiation of aSyn aggregation and LB formation. The primary evidence supporting this model comes mainly from in vitro studies demonstrating that 1) AIMP2 forms oligomers and ThT/S positive aggregates; and 2) AIMP2 aggregates, but not monomers, bind to monomeric aSyn and accelerate its fibrillization. Interestingly, aSyn PFFs were shown to bind to both aggregated and monomeric AIMP2. However, whether aSyn PFFs could seed the aggregation of AIMP2 in vitro or in cells was not investigated. This is important, as aSyn fibrils' ability to bind AIMP2 could offer an alternative explanation for the co-accumulation of AIMP2 and pS129 aSyn aggregates and inclusions, i.e., it is plausible that AIMP2 monomers are recruited to aSyn aggregates after their formation rather than the other way around. This model could explain why the great majority of AIMP2 found in aSyn aggregates ran as a single monomeric band when the insoluble fractions from the various cellular and animal models of synucleinopathies were analyzed by WBs. Finally, the mechanisms by which aggregated forms of AIMP2 induce the misfolding and aggregation of aSyn also were not investigated.

The lack of proper control experiments and minimal characterization of the aggregation states of aSyn and AIMP2 in the cellular and animal models makes it difficult to draw any conclusions about the role of AIMP2 aggregation in PFF-mediated seeding and aggregation of aSyn. No experiments were performed to investigate whether aSyn PFFs are capable of triggering AIMP2 aggregation or to assess the aggregation state of the AIMP2 associated with the pS129 immunoreactive aSyn aggregates. This could be accomplished by investigating the effects of aSyn PFFs inoculation or treatment on AIMP2 levels or aggregation in SNCA KO mice or primary neurons derived from these mice. These experiments would enable an investigation of the mechanism of PFFs-induced AIMP2 aggregation, independent of the aggregation of endogenous aSyn. The aggregation state of AIMP2 could also be assessed using a combination of imaging (immunofluorescence, ThS staining) and biochemical methods (sedimentation assays and/or SEC). Interestingly, in a separate experiment, they showed that viral-mediated overexpression of AIMP2 after AIMP2 knockdown resulted in the formation of AIMP2 puncta. Unfortunately, they did not assess whether this was sufficient to induce the aggregation of endogenous aSyn. To investigate whether AIMP2 is involved in regulating aSyn aggregation and/or the dynamics of LB formation or maturation, the effect of AIMP2 knockdown at different days post-seeding and after newly formed fibrils start to form but before they convert into LB-like structures could be assessed. In this regard, the neuronal seeding model is ideal, as aSyn

fibrillization occurs within 5-7 days post-PFF treatment, whereas the transition to LB-like inclusion occurs between 10-20 days later.

Does AIMP2 form amyloid-like oligomers?

To investigate the aggregation propensity of AIMP2 *in vitro*, the authors generated various AIMP2 proteins and assessed the aggregation of some of these proteins using multiple techniques, including WB in denatured gels, circular dichroism (CD), EM and ThT. Two different recombinant proteins purified from *E. coli* were used: one with both C-terminal (GST-) and N-terminal (-FLAG) tags (GST-AIMP2-FLAG) and a second with a C-terminal-FLAG tag (AIMP2-FLAG). Interestingly, different AIMP2 constructs were used in the cellular (Myc-tagged AIMP2) and animal (untagged native AIMP2) models of synucleinopathies used in this study. The assumption here is that the fusion of these peptide/protein tags to AIMP2 does not influence its biochemical, biophysical, aggregation, and cellular properties, although this was never assessed.

The use of different AIMP2 proteins bearing different peptides (FLAG and Myc) and protein tags (GST) in the various *in vitro* studies and synucleinopathies model makes it difficult to compare findings from different studies. Furthermore, the paper does not present data on the purity or the native oligomeric state of the native AIMP2 or AIMP2-FLAG proteins. The only EM images demonstrating AIMP2 aggregation shown are for the GST-AIMP2-FLAG and display primarily the formation of amorphous and prefibrillar-like structures. This raises the question of whether the presence of the GST protein at the N-terminus of AIMP2 could be responsible for its increased propensity to form oligomers. Unfortunately, without proper characterization of each protein's biophysical properties, it is not possible to answer this question.

Although AIMP2 fusion proteins (GST-AIMP2-FLAG and AIMP2-FLAG) exhibit a tendency to self-aggregate *in vitro*, the mechanism of AIMP2 aggregation and the structural and morphological properties of the aggregates formed by these proteins remain poorly defined. Ham et al showed, using CD, the time-dependent formation of a β-sheets-rich structure that correlates with an increased ThS signal, suggesting that AIMP2-FLAG aggregates possess amyloid-like properties. Unfortunately, no EM experiments were carried out on these samples to determine whether these changes correlate with the formation of AIMP2-FLAG oligomers or fibrils or directly compare the morphology of the aggregates formed by AIMP2-FLAG and GST-AIMP2-FlAG proteins. Amyloid is a term that is usually used to describe fibrillar aggregates that bind to other amyloid-specific dyes such as ThT/S, Congo red, or others, are rich in β-sheet structure, and possess the classical cross-β-sheet structures that characterize the amyloid structure of nearly all amyloid-forming proteins. Indeed, neither GST-AIM2P-FLAG nor AIMP2-FLAG were shown to form fibrillar structures or aggregates that possess a cross-β sheet structure. It is also noteworthy that oligomeric aggregates of aSyn and other amyloid-forming proteins bind weakly to ThT/S compared to the fibrillar forms of these proteins (Chen et al., 2015). Therefore, the high ThS binding to the GST-AIMP2-FLAG amorphous/non-fibrillar aggregates is, indeed, intriguing and worthy of further investigation.

In all the data provided on the extent of AIMP2 aggregation, there is a lack of correlation between the amount of monomeric protein remaining in solution and the amount of HMW species formed. In almost all the *in vitro* aggregation studies, the increase in high molecular weight species with time or in the insoluble fractions was observed without any changes in the levels of the aSyn or AIMP2 monomeric bands. One would expect to see a decrease in monomers' levels with the increased formation of HMW aggregates. Instead, we see a significant increase in the amounts of both monomers and oligomers on day 5, which is unexpected. Indeed, the monomer band's intensity is almost twice that seen at the baseline, suggesting that the increase in the level of oligomers is due to the loading of more proteins rather than a time-dependent increase in AIMP2 oligomerization.
Previous studies have shown that GST tends to self-oligomerize and could induce artificial oligomerization when fused to the N- or C-terminus of different proteins (Park et al., 2002). In the case of AIMP2, it is not clear whether its increased tendency to form dimers, trimers, or oligomers reflects an intrinsic property of the protein or is driven by the presence of GST. As a proof of concept that GST-tagged proteins tend to oligomerize, we previously showed that GST-aSyn exists as a mixture of oligomers and monomeric aSyn is reformed immediately upon GST's removal (Fauvet et al., 2012). In this regard, the lack of further characterization and comparison of the biophysical properties of the AIMP2 proteins with and without GST makes it challenging to answer this question. It would have been more appropriate to evaluate the oligomerization properties of the native (untagged) AIMP2. Furthermore, no EM images were included for AIMP2-FLAG or aSyn fibrils formed in the absence of GST or AIMP2 proteins. Without this data, it is difficult to determine the GST tag's impact on AIMP2 aggregation and directly compare the morphology of aSyn aggregates formed in the presence or absence of GST-AIMP2-FLAG.

Before comparing the aggregation propensity of proteins, it is crucial to first establish the purity of the proteins and their initial conformational properties and oligomeric state. Unfortunately, no data were provided to establish the chemical purity or conformation and oligomerization state of the native proteins (untagged AIMP2, GST-AIMP2-FLAG, or AIMP2-FLAG). The only data provided is an SDS-PAGE analysis of the GST-AIMP2-FLAG, which showed the presence of some impurities. Therefore, it is not clear whether the proteins used were pure, properly folded, or homogeneous (monomers, oligomers, or a mixture of different species). This information is crucial not only for proper interpretation of the results but also to enable reproducibility of the results.

Lesson learned and recommendation

Experimental approach: One of the major strengths of this work is that the authors aimed to apply an integrative approach that combines *in vitro* aggregation studies and the use of multiple cellular and animal models of aSyn aggregation and toxicity. This approach provided strong evidence supporting an important role of AIMP2 in regulating aSyn aggregation and aSyn-induced neurodegeneration. However, the limited characterization of the aggregation state and biochemical properties of aSyn and AIMP2 inclusions in these models limits mechanistic insight into how the interactions between these proteins could play a role in the pathogenesis of PD. Although the *in vitro* aggregation studies could have helped address this gap, the constant switching between the various protein constructs, the absence of some important control experiments, and the limited characterization of the

proteins used in these studies make it difficult to confidently draw any conclusions about the mechanism of AIMP2 aggregation and the stage in the pathway of aSyn fibrillization when AIMP2 exerts its effects. Furthermore, the fact that the proteins used in the *in vitro* aggregation studies differ significantly from those used in the *in vivo* studies presents additional challenges for using the results from *in vitro* aggregation studies to explain experimental observations in the cellular and animal models. Finally, while the amyloid-like properties of the aSyn aggregates in SH-SY5Y cells were assessed with ThT, this was not done for the pS129 immunoreactive inclusions in the 6-OHDA injected mice brain tissues. This gap in the analysis and lack of consistency in characterizing aSyn and AMIP2 aggregation in all the models used in this study make it difficult to compare data across the different models.

Antibodies: To assess the nature of the aSyn aggregates, the authors conducted a biochemical assessment (WB) of the aSyn species in the insoluble fraction of brain extracts using the MJFR14 antibody, which they refer to as a filament-specific antibody. Interestingly, WB analysis showed mainly two SDS-resistant bands (~47 and 125 KDa) in the insoluble fractions from the 6-OHDA treated mice and the absence of any streaks that are usually observed in samples containing aSyn fibrils. Thus, it is surprising that such bands are detected by this antibody. This could be explained by recent studies from our group and others that have shown that this antibody does not differentiate between oligomers and fibrils and detects very prominently denatured monomeric aSyn (Kumar et al., 2020). In response to our findings, the providers of this antibody no longer refer to it as a filament-specific antibody and do not recommend its use in WBs (Abcam's Recombinant Anti-Alpha-synuclein aggregate antibody [MJFR-14-6-4-2] Conformation-Specific).

Animal models: The authors employed a mouse model based on intranigral injection of rAAV2, under an unspecified promoter, to induce the overexpression of aSyn and AIMP2. The main caveats of these studies are the lack of neurodegeneration and the absence of a pS129 aSyn signal, which is in contrast to previous reports using the same model (Bourdenx et al., 2015; Oliveras-Salvá et al., 2013; St Martin et al., 2007). For example, it has been previously reported that viral-mediated overexpression of aSyn in the mouse substantia nigra results in increased aSyn phosphorylation at serine 129, the formation of insoluble aSyn aggregates, and striatonigral neurodegeneration (Oliveras-Salvá et al., 2013). It can be argued that this is due to the low titer of virus injected; however, this is difficult to decipher, as the titer of the viruses employed was not specified. Supplementary information shows that the transgene can transduce efficiently and quite extensively, although the image quality does not make it possible to evaluate the extent of expression in TH-positive neurons. Furthermore, the majority of the animal studies are based on a very small sample size (4 or less) that might lead to wrong assumptions due to a lack of robust statistics (Anderson and Vingrys, 2001).

Finally, we identified a few discrepancies between the results in the 6-OHDA mouse model reported by the authors and what has been reported in the literature using the same model. For example, the level of neurodegeneration displayed in this model (50% reduction) is not consistent with previous studies by Stott and colleagues, who failed to observe nigral neurodegeneration up to 6 days post-injection even when they employed a higher dose of 6-OHDA (10 vs 8 µg) (Stott and Barker, 2014). Furthermore, previous studies using this

model have consistently reported the absence of aSyn pS129-positive aggregates or LB-like inclusions, even though it has been shown that 6-OHDA does interact with aSyn (Blandini et al., 2008; Lindgren et al., 2012; Zeng et al., 2018).

AIMP2 constructs: One of the major limitations of the *in vitro* studies is the use of different AIMP2 constructs with different peptide/protein tags at the N- or C-terminus of the protein. This, combined with the lack of biophysical characterization of the proteins used in the aggregation studies, makes it difficult to interpret their findings and proposed mechanisms for the interactions between aSyn and AIMP2 and how they modulate each other's aggregation. In fact, in a previous study by the same group, overexpression of the FLAG-AIMP2 protein in SH-SY5Y cells resulted in diffuse staining of AIMP2 and no evidence of aggregation or inclusion formation (Lee et al., 2013). One possible explanation for this discrepancy is the use of a FLAG peptide instead of a Myc tag at the N-terminus of the protein. If this is indeed the case, this would suggest that the fusion of these non-native sequences significantly alters the behavior of AIMP2.

It is important to always keep in mind that PD, and synucleinopathies in general, are multi-faceted diseases, which encompasses several physiological changes (Gopalakrishna and Alexander, 2015). For this reason, in order to fully dissect the various pathophysiological aspects of the disease, the scientific community needs a robust and reliable model. However, there is not a single model that recapitulates all aspects of PD, thus when embarking on a new study to answer a specific scientific question, it is crucial to employ the most suitable model and be aware of its strengths and weaknesses (Lashuel, 2021). In this regard, it is also important to always thoroughly characterize aSyn pathology in each of the models employed using multiple immunohistochemical and biochemical approaches, irrespective of what the original publications show as the properties of the aggregates could change due to experimental manipulation, including modulating the expression levels of aSyn interacting proteins.

Our laboratory has recently published a review article that carefully dissects over 200 papers that employ cellular and animal models of aSyn aggregation and LB formation (Fares et al., 2021). In this extensive review, we focused on highlighting to which extent these models reproduce aSyn pathology and LB formation by categorizing aSyn pathology upon 8 different methods (refer to Supplementary Table 2 for cellular models and Supplementary Table 3 for animal models). Based on such analysis, researchers should assess which pathological features the model they wish to employ should recapitulate.

What is next?

Our analysis highlights the critical importance of conducting more detailed biochemical/biophysical studies to characterize the conformational, oligomerization, and aggregation properties of untagged AIMP2 and/or establish that the peptide/protein fused AIMP2 does not alter its native state. Furthermore, further studies are essential to determine whether AIMP2 aggregates on its own in cells and the extent to which these aggregates exhibit some of the well-established amyloid staining (ThT/S), immunohistochemical and biochemical (insolubility and presence of SDS-resistant HMW species) properties of LBs or other brain-derived aSyn aggregates (e.g., colocalization with LB-markers (p62, Ubiquitin, pS129). Given that aSyn PFF fibrils also bind to AIMP2

monomers and fibrils, it is important to systematically dissect the interactions between aSyn and AIMP2 at different stages of aSyn aggregation in well-established models of aSyn aggregation and inclusions. In this regard, the aSyn neuronal seeding model is ideal. It allows for monitoring of the transition of aSyn monomers to fibrils (0-7 days) and conver of fibrils to LB-like inclusions (7-21 days).

The existing data suggest that AIMP2 could be part of a vicious pathogenic cycle that amplifies aSyn aggregation and cell-death pathways in PD (Figure 2) and suggests that it could provide another link between different signaling pathways (e.g., aSyn and Parkin pathways). AIMP2 degradation relies on the activity of parkin (Lee et al., 2013), which can be altered or inactivated by pathological mutations, abnormal activity of the VPS35 (Yun et al., 2017), or c-Abl activation (Ko et al., 2010).

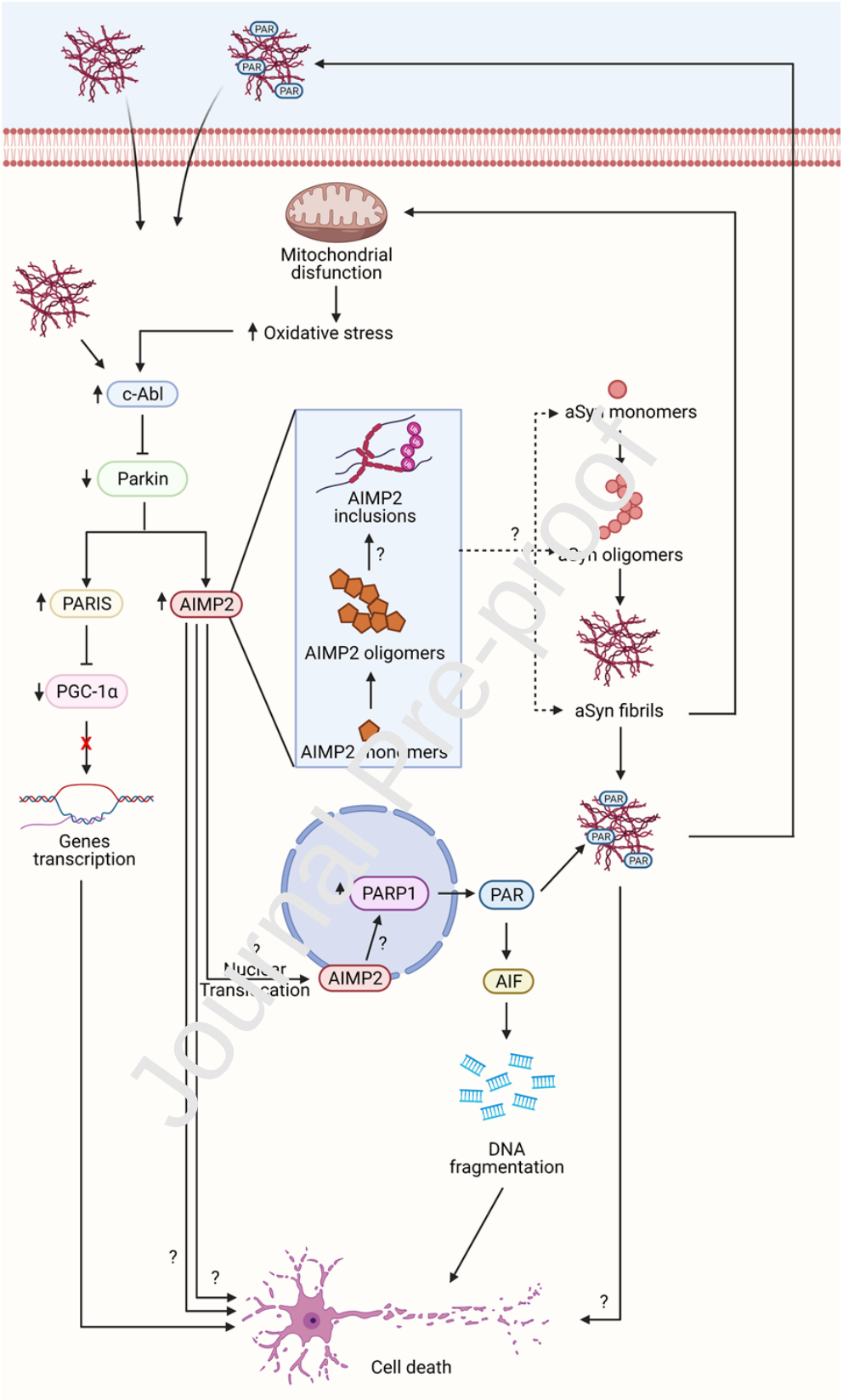

Figure 2: Schematic depiction of the cell death's pathways involving AIMP2 and aSyn. Further studies are needed to determine which forms of AIMP2 contribute to the various interactions and mechanisms outlined in this pathway. The native state of AIMP2 remains to be assessed, including its PTM profile and whether its aggregation could seed or initiate the aggregation of aSyn and potentially other amyloid-forming proteins, e.g., Tau or TDP-43. Adapted from (Brahmachari et al., 2019) with modifications to incorporate the potential role of AIMP2 aggregation and AIMP2-mediated aSyn-independent mechanisms of toxicity.

The increased levels of AMIP2 lead to its translocation to the nucleus (Lee et al., 2013), which activates poly(ADP-ribose) polymerase-1 (PARP1). This enhances the production of poly(ADP-ribose) (PAR1), which polymerizes and translocates from the nucleus to mitochondria and binds apoptosis-inducing factor (AIF). This binding facilitates the release of AIF from mitochondria to the nucleus, ultimately leading to cell death via parthanatos (Lee et al., 2013). PAR1 also plays a role in aSyn aggregation and toxicity, as aSyn PFFs activate a feed-forward loop by over-activating PARP1, which, in turn, increases the production and translocation of PAR1. PAR1 was reported to bind to aSyn leading to the formation of a more toxic aSyn strain which contributes to increased aSyn transmission and toxicity (Kam et al., 2018). Furthermore, matrix metalloproteinases are released from aSyn-activated microglia and further exacerbate microglia activation via PAR1 (Lee et al., 2010).

Although several studies have proposed that an increase in levels of AIMP2 could lead to its aggregation, more robust studies are needed to establish that AIMP2 indeed aggregates and to characterize the nature of AIMP2 foci and accumulations detected in cells. It is possible that AIMP2, through phase-separation, forms different types of condensates that could be reversible or become irreversible upon interaction with or recruitment and aggregation of aSyn. It is also possible that AIMP2 exerts its effects by interaction with or recruitment of other proteins to aSyn inclusions. Alternatively, AIMP2 aggregates could directly induce the activation of other PD-related cell death pathways. Establishing whether AIMP2 forms pathological aggregates or condensates could pave the way for new studies to decipher the mechanisms and signaling pathways responsible for triggering its aggregation, which could create new opportunities for targeting pathogenic mechanisms in PD.

Future studies aimed at investigating the mechanisms of action of AIMP2 should therefore pay more attention to elucidating its biochemical properties and aggregation state and the mechanisms by which it interacts with aSyn and LBs. It is also important to explore whether AIMP2 exerts its effects by other mechanisms and signaling pathways independent of Parkin or aSyn aggregation and LB formation (Figure 2). This knowledge is essential to elucidate the role of AIMP2 in the pathogenesis of PD and to develop effective strategies for targeting AIMP2.

In this regard, AIMP2-focused studies should address whether the parkin-mediated cell death involves a common pathway between the AIMP2-mediated cell toxicity and the Parkin-PARIS-PGC 1α axis, or whether these are separated pathways that can be modulated in a completely independent manner, as postulated by Lee et al. Furthermore, it would be important to assess the mechanism of AIMP2 nuclear translocation and which form exerts its toxic effect.

Implications for dissecting the role of LB-associated proteins in pathology formation and PD

LBs and other pathological inclusions are composed of hundreds of proteins (Datta et al., 2017; Licker et al., 2014; Van Dijk et al., 2012; Xia, 2008). Some of these proteins may play a role in different stages of pathological aggregate formation or maturation. Others may simply be innocent bystanders that become entrapped in pathological aggregates due to specific or non-specific interactions with other proteins. For decades, the detection of proteins in LBs and other aSyn inclusions has prompted studies to investigate their

interactions with aSyn and their role in aSyn aggregation and LB formation. Very often, the only results published are those in which there are some positive effects on aSyn aggregation or toxicity in a limited number of assays or model systems. Despite the large number of LB-associated proteins that have been reported to modify aSyn aggregation and pathology formation or protect against aSyn toxicity, none has emerged as a druggable therapeutic target or advanced to clinical trials. We believe that elucidating the role of these proteins in pathology formation and neurodegeneration in synucleinopathies requires a more systematic approach to establish the link between these proteins and pathology formation in the brains of patients with synucleinopathies. This should be followed by investigating the biophysical and cellular properties of these proteins and their interactions with aSyn species on the pathway of LB formation in multiple cell cultures and animal models of aSyn de novo and seeding mediated aggregation and LB formation. Below we outline our proposal for an integrative approach for systematic investigation of the role of LB-associated proteins in the pathogenesis of PD and synucleinopathies.

1. **Establishing the link:** Before investing valuable time and resources into a specific protein candidate, it is crucial to confirm the association with LBs and to determine whether its presence in LBs or pathological inclusions is relevant to disease development or progression. This could be accomplished by using multiple antibodies against the protein of interest (POI) and confirming its presence and association with pathological aSyn aggregates and LBs using immunohistochemical, immunofluorescence, and biochemical approaches. Furthermore, it is important to determine whether the POI has been described in previous studies that examined the proteome of LB using mass-spectrometry approaches (Datta et al., 2017; Killinger et al., 2020; Leverenz et al., 2007; Licker et al., 2014; Ping et al., 2018; Van Dijk et al., 2012; Xia, 2008).

2. **Disease relevance:** To gain insight into the possible roles of the POI in pathology formation and/or neurodegeneration, it is important to assess whether the levels, biochemical properties, and aggregation state of the candidate protein change during the development of PD. This could be achieved by quantifying the levels of these proteins in the soluble and insoluble fractions of post-mortem brain tissues from the affected brain regions or in biological fluids (e.g., CSF) from patients with PD and related synucleinopathies, in comparison to age-matched control cases. Given the heterogeneity of PD and other synucleinopathies, the studies in 1 and 2 must be performed in a larger sample set of brain tissues/samples from different patients (~10), instead of the commonly used sample size of 3-5.

3. **Using the right protein:** Very often, once a candidate protein is identified in LB, the full-length version of the protein is produced in E. coli, and its interactions with aSyn and ability to modify aSyn aggregation properties are assessed. This approach assumes that the protein identified, usually using antibodies, exists in one form. We recommend more precise biochemical studies to determine whether the POI is post-translationally modified or exists in different forms. This could be achieved by mass spectrometry techniques or using multiple antibodies targeting different modified forms of the proteins to assess the presence of this protein in Lewy bodies employing immunocytochemical and biochemical approaches. This will help identify which forms

of the POI are relevant and should be used to investigate the possible mechanisms by which the it contributes to pathology formation. In addition, once the most relevant form of the POI is produced, its oligomeric state and propensity to self-aggregate should be thoroughly assessed.

4. **Role in aSyn aggregation and pathology formation:** To understand the role of specific proteins in the process of LB formation, it is important to gain insight into the stage along the pathway to LBs during which these proteins exert their effect. Do they act at the level of monomeric synuclein and trigger or prevent its aggregation, or do they act at an intermediate stage to delay or accelerate the transition to fibrils or LBs? These questions could be easily answered *in vitro* because we have reliable protocols for preparing well-defined aSyn species (monomers, oligomers, and fibrils) and an extensive set of tools to monitor the transitions between the different stages of aSyn fibrillization (Kumar et al., 2020). However, this is not the case for cellular models because there is no single cellular model of synucleinopathies that reproduces *de novo* aSyn oligomerization, fibrillization, seeding, and LB formation. Neuronal models of *de novo* aSyn aggregation show the formation of fibrillar-like aSyn structures but not LBs (Fares et al., 2016). On the other hand, neuronal seeding models show the formation of fibrillar aSyn aggregates and LB-like inclusion (Mahul-Mellier et al., 2020b). However, in the seeding models, the seeding-mediated aggregation process, driven by the addition of PFFs, dominates, and the early events associated with *de novo* aSyn fibrillization are rarely detected. Therefore, putative modifiers of aSyn pathology formation should always be assessed using multiple cellular and animal models of aSyn oligomerization, fibrillization, seeding, and LB formation.

5. **Interaction with aSyn:** Characterization and quantification of aSyn aggregation should not be limited to immunofluorescence-based approaches or quantifying levels of pS129. At the cellular or tissue level, additional studies should be conducted to determine whether the pS129 immunoreactive species or inclusions possess the biophysical properties of aSyn fibril (e.g., the presence of fibrils or binding to amyloid-specific dyes) and whether they share many of the staining features of LBs (immunoreactivity to LB markers, e.g., ubiquitination, p62). Furthermore, analyses of the soluble and insoluble fractions from brain tissue or cellular extracts by SEC or WB should enable assessment of the relative distribution of different types of aSyn aggregate (e.g., oligomers vs. fibrils) and the aggregation state of the POI. All of these experiments do not require the use of special equipment and can easily be performed in any laboratory equipped with basic biochemistry and cell biology setups.

6. **Employment of fusion proteins:** To the extent possible, the use of fusion proteins should be avoided unless the POI expresses at low levels or is unstable, as this approach might lead to confounding data and misinterpretations. Under these circumstances, the proper control experiments should be performed to determine whether the addition of fusion proteins/peptides influences the native state and cellular properties of the POI.

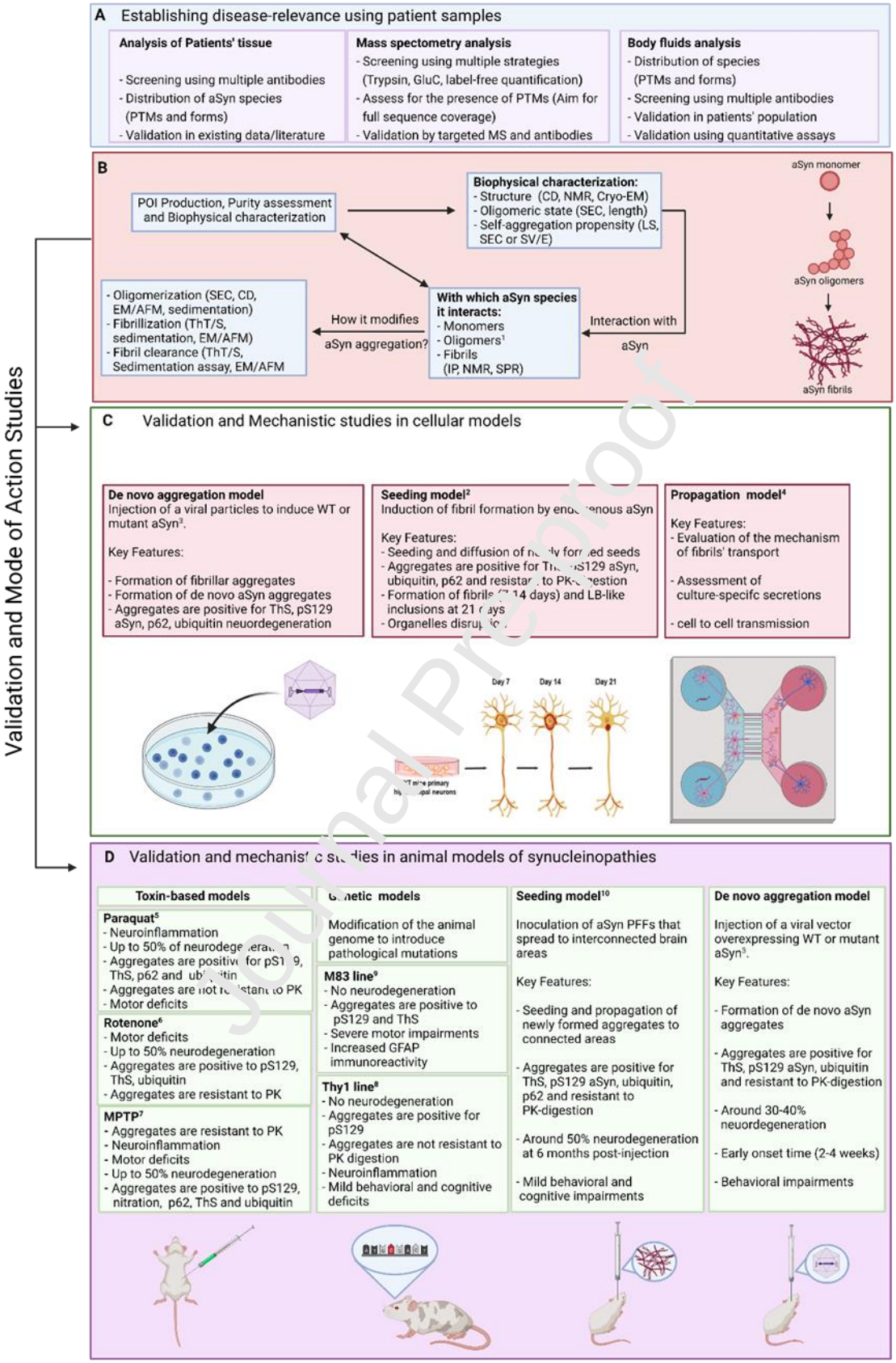

Figure 3: Diagram depicting our proposed workflow for establishing the disease relevance and mode of putative proteins involved in LBs formation. Each study aiming at investigating putative components of Lewy Bodies. This process should start by validating the presence of the POI and its PTM profile in a large sample set of human PD brain tissues, extracts, and biofluids and careful assessment of existing literature and PD/synucleinopathies data (e.g., genetic studies and gene expression or proteomics data)(A). Once the POI is identified, it should be produced in the relevant expression system and highly pure proteins should be used to characterize its biophysical properties and to determine with which forms aSyn it interacts and how it modifies aSyn aggregation properties (B). Studies to determine relevance to PD/Synucleinopathies pathogenesis should be carried out in multiple cellular (C) or animal models (D) that reproduce well-characterized features of disease pathology. The employment of one model over the other depends on the properties of the protein of interest, its putative contribution to LB disease and the nature of the question to be answered. Abbreviations : PTMs = Post-Translational Modifications, CD = Circular Dichroism, SEC = Size-exclusion chromatography, IP = Immuno-precipitation, SPR = surface plasmon resonance, SV = sedimentation velocity, SE = sedimentation equilibrium, AFM= atomic force microscopy, EM = electron microscopy, ThT/S = Thioflavin T/S, PFF = Pre-Formed Fibrils, PK = Proteinase K, GFAP = Glial fibrillar acidic protein. References: 1 (Kumar et al., 2020), 2 (Mahul-Mellier et al., 2020b), 3 (Fares et al., 2016), 4 (Freundt et al., 2012), 5 (Fernagut et al., 2007), 6 (Cannon et al., 2009), 7 (Fornai et al., 2005), 8 (Chesselet et al., 2012), 9 (Unger et al., 2006) and 10 (Burtscher et al., 2019).

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