

HtrA1 prevents and reverses α -synuclein aggregation, rendering it non-toxic and seeding incompetent

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Abstract

Parkinson disease (PD) is closely linked to the misfolding and accumulation of α -synuclein (α -syn) into Lewy bodies. HtrA1 is a PDZ serine protease that degrades fibrillar tau, which is associated with Alzheimer disease (AD). Further, inactivating mutations to mitochondrial HtrA2 have been implicated in PD. Here, we establish that HtrA1 inhibits the aggregation of α -syn as well as FUS and TDP-43, which are implicated in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). We demonstrate that the protease domain of HtrA1 is necessary and sufficient for inhibition of aggregation, yet this activity is independent of HtrA1 proteolytic activity. Further, we find that HtrA1 also disaggregates *performed* α -syn fibrils, which may promote their clearance. Treatment of α -syn fibrils with HtrA1 renders α -syn incapable of seeding the aggregation of endogenous α -syn in mammalian biosensor cells. We find that HtrA1 remodels α -syn by specifically targeting the NAC domain, which is the key domain that catalyzes α -syn oligomerization and fibrillization. Finally, in a primary neuron model of α -syn aggregation, we show that HtrA1 and its proteolytically inactive form both detoxify α -syn and prevent the formation of hyperphosphorylated α -syn accumulations. Our findings suggest that HtrA1 prevents aggregation and promotes disaggregation of multiple disease-associated proteins, and may be a therapeutic target for treating a range of neurodegenerative disorders.

Introduction

Protein misfolding is associated with multiple neurodegenerative disorders for which there are no effective disease-modifying therapies, including Parkinson disease (PD), amyotrophic lateral sclerosis (ALS), and frontotemporal dementia (FTD)¹. α -Synuclein (α -syn) is an abundant neuronal protein with several putative roles, including modulation of synaptic transmission². In PD and other synucleinopathies, α -syn undergoes a structural conversion from its native soluble state into β -sheet rich amyloid fibrils^{1,3}. The accumulation of α -syn in cytoplasmic Lewy bodies is the pathological hallmark of PD¹. The misfolding of α -syn may lead to its inactivation and loss of native function. Additionally, accumulation of misfolded α -syn confers a toxic gain of function^{4,5}. α -Syn amyloid fibrils are highly insoluble and resistant to proteases and other denaturants⁶. Further, α -syn fibrils can enter neighboring cells and seed further misfolding of monomeric α -syn^{2,4}. In ALS and FTD, several proteins with prion-like domains can misfold, undergo aberrant phase transitions, and aggregate^{7,8}. These proteins include TDP-43 and FUS, both of which mislocalize from the nucleus to the cytoplasm where they aggregate and are associated with both a toxic gain of function of the misfolded species along with a loss of function due to their sequestration in the cytoplasm⁷. A better understanding of the molecular mechanisms by which cells avert formation of amyloid and other aggregated species, as well as how they might clear misfolded species to avoid further aggregation, is essential for the ultimate development of new therapeutic strategies.

To preserve protein homeostasis (proteostasis), protein quality control systems have evolved to promote the proper folding of proteins, as well as to repair and degrade proteins when necessary⁹. However, a

range of different proteins can adopt a misfolded and insoluble β -sheet amyloid secondary structure which can preclude their clearance by the proteostasis network⁸. Through activation of the heat shock response, chaperone proteins can be upregulated and recruited to aggregates and amyloid. However, chaperones are only able to prevent further aggregation, and are often insufficient to solubilize or clear these accumulations¹⁰. In contrast, protein disaggregases are capable of engaging and dissolving otherwise insoluble amyloid fibrils, pre-amyloid oligomers, and other aggregates^{11,12}. The yeast disaggregase Hsp104 has been demonstrated to eliminate fibrils of not only yeast prions, but also proteins associated with PD, Alzheimer disease (AD), Huntington disease (HD), and other disorders^{13–15}. While humans do not express Hsp104, human disaggregases have been identified, including Hsp110^{16–18}, VCP¹⁹, DAXX²⁰, Kap β 2²¹, and TRIM11²².

High-temperature requirement A (HtrA) proteins are ATP-independent PDZ serine proteases²³. HtrA proteases are conserved and found in bacteria, fungi, plants, and animals, with many organisms expressing more than one HtrA isoform in different cellular compartments²⁴. HtrA proteins are known to function in the stress response, whereby they are thought to bind damaged proteins via their PDZ domain and mediate proteolysis^{25,26}. PDZ domains are known to mediate binding to substrates harboring β -sheets via a β -sheet augmentation mechanism, whereby the β -sheet rich region of the PDZ domain binds the β -sheet of the substrate^{24,27}. Such a mechanism might also promote binding to β -sheet rich amyloid species. Indeed, HtrA1 can disintegrate and proteolyze fibrillar tau aggregates²⁵. HtrA1 is a ubiquitously expressed protein that is secreted into the extracellular matrix²⁴. Intracellularly, HtrA1 is found in the cytoplasm, associated with microtubules, and in the nucleus. HtrA2 is expressed in mitochondria where it is thought to play important roles in mitochondrial proteostasis²³. HtrA2 expression is upregulated by heat shock or activation of the p53 pathway²³. Mice lacking HtrA2 or expressing inactive HtrA2 mutants display a neurodegenerative phenotype, suggesting that HtrA2 may be neuroprotective²³. Further, inactivating mutations in HtrA2 have been implicated in PD^{23,28}. Taken together, we hypothesized that HtrA proteins directly regulate the aggregation and clearance of a range of amyloid proteins.

Amyloid and other misfolded protein aggregates are highly resistant to degradation, and effective therapeutics that clear misfolded proteins are not available for any protein-misfolding disorder¹². Agents that could reverse the formation of toxic α -syn species would be attractive disease-modifying therapies for PD and other synucleinopathies. Such agents could simultaneously reverse a toxic gain of function of the misfolded species and prevent further propagation of pathology via seeding, while also preserving the normal physiological function of α -syn. Here, we show that HtrA1, but not HtrA2, prevents and reverses fibrillization of α -syn, and that this treatment renders preformed α -syn seeds incapable of proteopathic seeding. This activity does not require HtrA1 to be proteolytically active. In contrast with previous studies²⁵, we find that α -syn remodeling does not require the HtrA1 PDZ domain, but can be entirely mediated via the proteolytically-inactive HtrA1 protease domain. Furthermore, we find that HtrA1 confers remodeling by engaging the NAC core of the α -syn fibrils. Upon exposure of cells to preformed α -syn seeds, elevated expression of HtrA1 prevents the triggering of α -syn aggregation, and treatment of α -syn

with HtrA1 renders products that are non-toxic and incapable of seeding α -syn aggregation in primary mouse neurons. We demonstrate that HtrA1 can promote solubilization of a range of otherwise recalcitrant proteins, allowing for clearance of the aggregates.

Results

HtrA1 can proteolyze α -synuclein, TDP-43, and FUS

HtrA1 has been demonstrated to disintegrate fibrillar tau, allowing for its subsequent clearance²⁵. Further, HtrA1 has been shown to associate with microtubules and degrade tubulins, thereby inhibiting cell migration^{25,29}. These findings have led to speculation that HtrA1 specifically regulates tau misfolding. We hypothesized that, because the amyloid fold is highly conserved³⁰, HtrA1 may be active against a range of amyloid and amyloid-like proteins beyond tau. Additionally, we sought to more broadly investigate HtrA1 activity against folded, intrinsically disordered, and fibrillar substrates. We were also curious to test the activity of HtrA2, which resides in the mitochondria. Mitochondrial dysfunction is an important aspect of Parkinson disease (PD) pathophysiology and loss-of-function mutations in HtrA2 have been implicated in PD^{23,28,31}. HtrA1 and HtrA2 are each comprised of an N-terminal domain, a protease domain, and a PDZ domain (Fig. 1A). The N-terminal domain of HtrA1 has a cleavable signal peptide (SP) followed by a fragment of insulin growth factor binding protein 7 (IGFBP), and a Kazal-type protease-inhibitor motif. While HtrA1 is primarily secreted into the extracellular space, approximately 20% of HtrA1 remains in the cytoplasm²⁴. In contrast, the N-terminal domain of HtrA2 harbors a mitochondrial targeting sequence (MTS) followed by a transmembrane anchor that can be removed in processing²⁴.

We first sought to test HtrA1 and HtrA2 for their capacity to proteolyze and/or inhibit the aggregation of α -synuclein (α -syn), as well as TDP-43 and FUS. To directly assay activity, we employed recombinant proteins. Here, we used TDP-43-TEV-MBP-His₆ and GST-TEV-FUS constructs, where MBP and GST function as solubility tags (Fig. 1B)^{32,33}. TDP-43 and FUS remain soluble for many hours with the tags appended, while aggregation proceeds rapidly upon cleavage of the MBP or GST solubility tags with TEV protease. α -Syn was purified as previously described³⁴.

We find that HtrA1 completely digests a 5-fold molar excess of monomeric α -syn within 24h of incubation (Fig. 1C). We also note some autoproteolysis of HtrA1. In contrast, HtrA2 did not digest α -syn. We next purified TDP-43-TEV-MBP and GST-TEV-FUS, which both form amyloid-like aggregates^{32,33,35}. To initiate these reactions, we cleaved with TEV protease to liberate TDP-43 and FUS from the solubility tags, and then added HtrA1, HtrA2, or buffer (Fig. 1D-E). Both HtrA1 and HtrA2 displayed robust proteolysis of TDP-43. In contrast, while HtrA1 proteolyzed FUS, we observed somewhat weaker proteolysis of FUS by HtrA2. We noted no degradation of the free MBP or GST tags following TEV cleavage, indicating that HtrA1 and HtrA2 selectively proteolyze α -syn, TDP-43, and FUS, but not well-folded proteins including MBP and GST.

HtrA1 inhibits TDP-43 and FUS aggregation

We were next curious if HtrA1 harbored chaperone activity toward these substrates in addition to its proteolytic activity. To detect aggregation of TDP-43 and FUS, we employed turbidity assays (Fig. 1F-H). To assay inhibition independently of proteolysis, we included the proteolytically inactive variant HtrA1^{S328A}. We also included aldolase as a control for bulk protein effects. HtrA1 subtly delays TDP-43 aggregation when co-incubated at an equimolar ratio, though this effect is similar to that of aldolase (Fig. 1G and S1). HtrA1^{S328A} and HtrA2 do not modulate TDP-43 aggregation under these conditions. We repeated these assays with a 3-fold and 5-fold molar excess of HtrA1 and observed a dose-dependent increase in inhibition of TDP-43 aggregation by HtrA1 and HtrA1^{S328A} (**Fig S1**). At a 5-fold molar excess of HtrA1, nearly complete inhibition of TDP-43 aggregation is achieved. HtrA1^{S328A} shows a similar, though expectedly weaker, inhibitory effect. In contrast, even a 5-fold molar excess of HtrA2 has minimal effect on TDP-43 aggregation, despite its proteolytic activity against TDP-43 (Fig. 1D and S1).

We then tested inhibition of FUS aggregation and found that HtrA1 activity against FUS is more potent than against TDP-43. Here, an equimolar ratio of HtrA1 achieved complete inhibition of FUS aggregation, while HtrA2 appeared to accelerate aggregation despite its proteolytic activity against FUS (Fig. 1H). Even HtrA1^{S328A}, which lacks proteolytic activity, considerably slowed aggregation (Fig. 1H). We therefore conclude that α -syn is processed for proteolysis by HtrA1, but not by HtrA2, while both HtrA1 and HtrA2 can degrade TDP-43 and FUS. Further, inhibition of TDP-43 and FUS aggregation can occur in a proteolytically-independent fashion, with inhibition of FUS aggregation being more potent. Interestingly, given the differences in proteolysis and inhibition we observed, the HtrA proteins appear to operate via two distinct mechanisms. To further explore these features, we focused on α -syn because inhibition against α -syn aggregation was the most potent.

HtrA1 prevents α -synuclein amyloidogenesis and preserves α -synuclein solubility

We next sought to elucidate the activity of HtrA1 and HtrA2 in antagonizing α -syn misfolding. To monitor amyloid formation, we used the amyloid-binding dye ThioflavinT (ThT) to detect α -syn amyloidogenesis. α -Syn assembles into fibrils rapidly, with fibrillization complete after approximately 48h of agitation. When α -syn fibrillization is conducted in the presence of HtrA1, with a 5-fold molar excess of α -syn, the ThT signal only reaches approximately 20% of that achieved in the absence of HtrA1 or with an aldolase control (Fig. 2A). Addition of the proteolytically inactive HtrA1^{S328A} variant achieved a similar level of inhibition, indicating that inhibition of amyloidogenesis by HtrA1 can proceed in a protease-independent fashion. HtrA2 activity is notably weaker, achieving ~ 50% inhibition of α -syn amyloidogenesis as compared to HtrA1. Similar effects were achieved after a 72h incubation (**Fig S2A**). To determine whether the anti-amyloidogenic activity of HtrA1 is specific to the amino acid sequence of α -syn, we performed similar experiments using α -Syn^{A53T}, which contains a missense mutation linked to familial PD that accelerates α -syn fibrillization³⁶. HtrA-mediated inhibition of α -Syn^{A53T} fibrillization follows similar trends, although surprisingly, inhibition of amyloidogenesis is weaker by HtrA1 than by HtrA1^{S328A} (Fig. 2B). Our

results suggest that both wild-type and α -syn^{A53T} can be substrates for HtrA1, but that HtrA2 is inactive, and HtrA1 has somewhat diminished activity against the α -syn^{A53T} variant.

We hypothesized that the decreased formation of ThT-reactive species was due to preserved solubility of the α -syn monomer. To probe this, we first monitored solubility using sedimentation assays where solubility was assessed in the presence or absence of HtrA. Here, a 5-fold molar excess of α -syn monomer was incubated with HtrA1, HtrA1^{S328A}, HtrA2, aldolase, or a buffer control (Fig. 2C-D). After 24h of incubation, whereas 20% of α -syn ordinarily partitions to the insoluble fraction, HtrA1 co-incubation prevents any detectable accumulation of insoluble α -syn (Fig. 2D, S2B-C). This protective effect persisted for 72h of incubation, with less than 10% of α -syn accumulating in the insoluble fraction, by which time nearly 60% of α -syn was found in the pellet in the absence of HtrA1 (Fig. 2D). However, we did note proteolysis of both HtrA1 and α -syn (Fig. 2C). To assess solubilization independently of proteolysis, we also assayed HtrA1^{S328A} and find that α -syn is retained in the soluble fraction even when there is no proteolytic cleavage, with approximately 80% of α -syn remaining soluble after 72h (Fig. 2C-D). Addition of either HtrA2 or aldolase has no inhibitory effect on α -syn aggregation, although HtrA2 undergoes complete auto-proteolysis within the 72h incubation (Fig. 2C). We were surprised to note some accumulation of HtrA1^{S328A} in the insoluble fraction, so we also explored HtrA solubility in the absence of substrate (Fig. 2E). We find that both HtrA1 and HtrA1^{S328A} largely partition to the insoluble fraction when incubated without substrate (Fig. 2E), while nearly all HtrA1 and HtrA1^{S328A} are found in the soluble fraction in the presence of α -syn (Fig. 2C). These results suggest that α -syn and HtrA1 form a stable complex that preserves the solubility of both proteins.

Using negative stain transmission electron microscopy, we confirmed that treatment with HtrA1 prevents the formation of α -syn fibrils, with just a small amount of amorphous material accumulating (Fig. 2F). HtrA1^{S328A} also modulates α -syn fibrillization, whereby the treated products are less abundant and appear more diffuse as compared to the tightly-packed appearance of the untreated fibrils, correlating with the ThT results which indicate decreased amyloid content of the treated products (Fig. 2A). Treatment with HtrA2 did not result in any apparent changes to α -syn fibril morphology. Thus we conclude that HtrA1, but not HtrA2, can prevent α -syn^{WT} and α -syn^{A53T} from forming amyloid fibrils, and that this α -syn – HtrA1 interaction preserves the solubility of both proteins. Further, this activity is proteolytically-independent and mediated via a direct interaction between the two proteins.

HtrA1 treatment renders α -synuclein seeding incompetent

When preformed fibrillar (PFF) α -syn is added exogenously to mammalian cell cultures or via intrastriatal inoculation of mice, these PFFs enter the cell and initiate the seeding and aggregation of endogenous soluble α -syn^{4,5,37}. To monitor this process, we used a HEK293T FRET biosensor cell line engineered to stably co-express cyan fluorescent protein (CFP)-tagged α -syn and yellow fluorescent protein (YFP)-tagged α -syn³⁸. Upon addition of α -syn PFFs to the cell culture medium, PFFs trigger aggregation of the

fluorescent α -syn, which can be observed by fluorescence microscopy and measured by FRET³⁸. Based on our findings that co-incubation of α -syn with HtrA1 prevents α -syn fibrillization and preserves α -syn solubility, we hypothesized that HtrA1 treatment would also render α -syn incapable of forming seeding competent PFFs. To test this idea, we formed α -syn PFFs in the presence of HtrA1, HtrA1^{S328A}, aldolase, or buffer with a 5-fold molar excess of α -syn. We then applied the reaction products to HEK293T α -syn FRET biosensor cells. Cells were analyzed by fluorescence microscopy or flow cytometry to assess α -syn aggregation (Fig. 3A). Application of 50nM PFFs was sufficient to induce robust seeding of the biosensor cells, with abundant puncta throughout the cell population and a strong FRET signal as detected by flow cytometry (Fig. 3B-D). However, pre-treatment with HtrA1 or HtrA1^{S328A} nearly completely abolished PFF seeding capacity (Fig. 3B-D). Application of higher concentrations of 100, 200, and 400nM PFFs gave similar results (**Fig S3A**). Quantification of these effects by flow cytometry indicates that HtrA1 treatment renders the PFFs nearly completely seeding incompetent (Fig. 3C, S3B-C). Treatment with HtrA1^{S328A} also markedly reduced the seeding competence of the α -syn PFFs. In contrast, aldolase treatment had no significant effect (Fig. 3C). Similar results were achieved when treating α -syn^{A53T} with HtrA1 or HtrA1^{S328A} (Fig. 3D, S3D). Thus, HtrA1 robustly inhibits the conversion of α -syn and α -syn^{A53T} to a seeding competent form, and this activity does not depend on the proteolytic activity of HtrA1. Furthermore, the products of HtrA1 remodeling and proteolysis cannot serve as seeds to nucleate and propagate α -syn aggregation.

The protease domain of HtrA1 is necessary and sufficient for remodeling of α -syn.

HtrA proteases share many features with classical serine proteases including trypsin and chymotrypsin²⁴. However, HtrA proteases are unique because their activity is finely tuned and can be reversibly switched on and off, unlike classical serine proteases. This distinct structural and functional plasticity is thought to be mediated by the PDZ domain of HtrA, and it is thought that HtrA activity is regulated by the binding of peptides to the PDZ domain^{24,26}.

To explore how this mechanism ultimately dictates HtrA activity against α -syn, we employed a series of constructs with the protease or PDZ domain deleted (Fig. 4A). To probe a possible direct interaction between HtrA and α -syn as suggested by our sedimentation assay results (Fig. 2), we allowed fibrillization to proceed with HtrA1, HtrA1^{S328A}, or HtrA2 and monitored complex formation using native PAGE (**Fig S4A**). In the presence of HtrA1^{S328A}, we note a distinct smear at a higher molecular weight than that of HtrA1^{S328A} or α -syn monomer alone, corresponding to likely complex formation between HtrA1^{S328A} and α -syn. We observe a sharp band for HtrA2 at its expected molecular weight, indicating no complex formation with α -syn, though there does appear to be some protein trapped in the wells of the gel, suggestive of formation of some higher order complexes. In the presence of α -syn, HtrA1^{WT} also forms a smear, but at an intermediate molecular weight, and of decreased intensity. We excised this band from the gel and confirmed the presence of both HtrA1 and α -syn by mass spectrometry. This suggests that HtrA1^{WT} or HtrA1^{S328A}, but not HtrA2, form a stable complex with α -syn. Further, this interaction with HtrA1 appears to partially protect α -syn from proteolysis.

Using a fluorescein isothiocyanate (FITC)-casein model substrate, we next tested if the PDZ domain was required for proteolysis (Fig. 4B). Here, FITC fluorescence is quenched due to fusion to casein and upon degradation of casein, this self-quenching is diminished and FITC fluorescence increases. We observe that both HtrA1 and the protease domain alone (HtrA1 ProD) robustly digest the FITC-casein substrate, though digestion is more efficient with full-length HtrA1. We confirmed these results also using α -syn as a substrate (Fig. 4C). We hypothesized that due to the prominent role of PDZ domains in mediating protein-protein interactions²⁷, the PDZ domain would be essential for binding and suppressing amyloidogenesis. Surprisingly, HtrA1 ProD can completely inhibit the formation of ThT-reactive species (Fig. 4D) and preserve α -syn solubility (Fig. 4E). These effects do not require HtrA1 proteolytic activity. Further, the isolated PDZ construct (HtrA1PDZ) had no effect on amyloidogenesis and only weakly preserved α -syn solubility (Fig. 4D-E). To further corroborate these results, we applied these remodeled products to the FRET biosensor cells and found that again, HtrA1ProD restricts the formation of seeding-competent species in a proteolysis-independent fashion while HtrA1PDZ only weakly inhibits seeding (Fig. 4F). Finally, to investigate if this activity is mediated by a direct interaction, we monitored binding with pull-down assays. Supporting our earlier results, we again note strong binding by HtrA1^{S328A} and HtrA1ProD^{SA}, while HtrA1PDZ only weakly binds α -syn. Further, although HtrA2 has only limited remodeling activity against α -syn, it binds α -syn with similar affinity to HtrA1^{S328A}, indicating that chaperone activity observed by the HtrA proteins is not merely due to the effects of binding (Fig. 4G-H). These results are in contrast to those observed in the native PAGE assays (**Fig S4A**), where no complex formation is observed. This is possibly due to the differing timescales of the two experiments, and suggests that while HtrA2 can bind α -syn, binding alone is insufficient to prevent aggregation, and aggregation can still occur on a longer timescale despite complex formation. Further, these results suggest that binding to α -syn is insufficient to inhibit aggregation, but that HtrA1 is instead conferring a distinct remodeling activity. We can conclude that HtrA1 chaperoning of α -syn relies on direct interaction between the protease domain and α -syn, and that the protease domain is necessary and sufficient for this interaction. Further, this activity does not depend upon the proteolytic activity of this domain.

HtrA1 dissolves preformed α -synuclein fibrils and renders them seeding incompetent

We next aimed to assess if HtrA1 could not just prevent α -syn from forming seeding competent species, but also dissolve α -syn PFFs and diminish their seeding capacity. Here, we treated mature PFFs with HtrA proteins and monitored the biophysical properties of the treated PFFs as well as their seeding capacity (Fig. 5A). Treatment of α -syn PFFs with HtrA decreased the ThT signal by approximately 60% for HtrA1 and 40% for HtrA1^{S328A} (Fig. 5B). Disaggregation was also assessed by sedimentation assay (Fig. 5C-D). Here, following the treatment of PFFs with HtrA1, the reaction products were partitioned to a soluble and insoluble fraction. Upon treatment with HtrA1, we noted a decrease in total α -syn, presumably due to proteolysis. However, this decrease in α -syn was primarily in the soluble fraction and not the pellet fraction. In contrast, upon treatment with HtrA1^{S328A}, we note a decrease primarily in the insoluble pellet fraction. This suggests that HtrA1 disaggregase activity is preferential for the insoluble species, while soluble species are favored for proteolysis. Remodeling was also noted by electron microscopy. HtrA1

treatment led to the fibrils adopting a more diffuse appearance while HtrA1^{S328A} treatment yielded amorphous accumulations that did not resemble fibrils (Fig. 5E). HtrA1 or HtrA1^{S328A} treatment reduced seeding capacity of these products by approximately 30–40% when applied to FRET biosensor cells (Fig. 5F, S5). Thus we conclude that HtrA1 can remodel α -syn PFFs, decreasing their amyloid content, disrupting their morphology, and decreasing their seeding capacity. Further, this remodeling activity does not require HtrA proteolytic activity.

HtrA1 disaggregates α -synuclein fibrils by specifically targeting the NAC domain

To better understand the mechanism by which HtrA1 remodels α -syn at higher resolution, we performed proteolysis experiments followed by identification of the cleavage products by liquid chromatography/mass spectrometry (LC/MS). First, we incubated α -syn monomer with HtrA1 and analyzed the cleavage pattern by LC/MS (Fig. 6A). We find that cleavage occurs throughout the α -syn sequence, with cleavage enriched in the nonamyloid component (NAC) domain, residues 61–95, a domain known to play a critical role in catalyzing α -syn oligomerization and fibrillization³⁹. The cleavage sites we identified are consistent with previously reported trends in HtrA1 proteolytic cleavage, where HtrA1 preferentially cleaves following residues such as valine and threonine⁴⁰. Next, to better understand how conversion to the amyloid form modulates HtrA1 activity, we performed similar experiments with α -syn PFFs. Here, to more clearly identify the key cleavage sites, we pre-treated the PFFs with HtrA1^{S328A} to render the PFFs more susceptible to proteolysis by active HtrA1 and then analyzed the fragments by LC/MS. We first analyzed the number of fragments produced and found that pre-treatment with HtrA1^{S328A} rendered the PFFs more susceptible to fragmentation than treatment with HtrA1 alone (Fig. 6B-C). Analysis of the fragmentation pattern indicated that pre-treatment with HtrA1^{S328A} resulted in more cleavage sites and greater overall fragmentation than treatment with HtrA1 alone (Fig. 6D-E). Furthermore, analysis of the specific cleavage products indicated that treatment with HtrA1 WT alone resulted in cleavage at three primary positions in the α -syn sequence, all outside the NAC domain that is otherwise susceptible to cleavage when α -syn is in the monomeric form. In contrast, the addition of an HtrA1^{S328A} pre-treatment step resulted in several new cleavage sites within the NAC domain (Fig. 6E). When PFFs are not pre-treated with HtrA1^{S328A}, this region remains resistant to cleavage. This suggests that HtrA1 preferentially cleaves α -syn monomer in the NAC domain, and treatment with HtrA1^{S328A} mediates disaggregation by engagement of the NAC domain. However, upon amyloidogenesis, this region becomes protected and resistant to HtrA1 cleavage. To enable cleavage even in the fibrillar state, HtrA1^{S328A} directly engages this aggregation-prone region of α -syn to mediate disaggregation, thereby allowing proteolytic cleavage to proceed.

Overexpression of HtrA1 prevents α -syn PFFs from seeding endogenous α -syn aggregation

We next sought to determine if HtrA1 expression could protect against α -syn seeding, or if the PFFs required pretreatment with HtrA1. We transfected HEK293T biosensor cells with plasmids to transiently overexpress HtrA1 and HtrA1^{S328A}. Cells treated with PFFs showed robust seeding, while cells overexpressing HtrA1 or HtrA1^{S328A} that were subsequently treated with PFFs showed an apparent decrease in puncta accumulation, particularly in regions where transfection efficiency was higher (Fig. 7A). We transfected these constructs at two different levels to monitor any dose dependence and quantified these effects by flow cytometry (Fig. 7B-C, S6). Both HtrA1 and HtrA1^{S328A} decreased seeding by approximately 40%, with a dose-dependent increase in inhibitory activity at higher HtrA expression levels. Thus we conclude that in the cellular environment, HtrA1 can protect against α -syn seeding, and pre-treatment with HtrA1 is not required. This activity is likely due to HtrA1 inhibiting α -syn aggregation and/or preventing the uptake of seeds.

HtrA1 treatment renders α -synuclein non-toxic and incompetent of seeding formation of pathological α -syn inclusions in primary mouse neurons

To evaluate the effect of HtrA1 treatment on α -syn PFF-induced seeding in mouse primary neurons, we incubated α -syn alone or with HtrA1, HtrA1^{S328A}, HtrA2, or aldolase and applied the products to primary mouse hippocampal neurons. It has been shown that α -syn PFFs can be taken up by neurons, seed, and convert soluble α -syn into Lewy Body-like inclusions, which are also associated with hyperphosphorylation of α -syn^{5,34}. 24h following treatment, toxicity was measured by MTT assay, and 1 week following treatment the neurons were processed for phosphorylated α -syn by ICC and imaged by confocal microscopy (Fig. 8A). Application of untreated PFFs decreases neuronal viability, and only approximately 70% of neurons remained viable. However, pre-treatment of α -syn with HtrA1 or HtrA1^{S328A} was partially protective, restoring viability to approximately 85% and 81%, respectively. This viability level is similar to that achieved when nontoxic monomeric α -syn is applied. Treatment with HtrA2 or aldolase did not modulate viability in a statistically significant manner (Fig. 8B).

To confirm that the mechanism of toxicity suppression is due to decreased seeding of intracellular α -syn, we next tested whether HtrA1 affected PFF-induced aggregation and hyperphosphorylation of α -syn. Here, reactions were prepared as described previously and neurons were treated for 1 week with the reaction products. Cells were then processed and immunostained for phosphorylated α -syn (Fig. 8C). Here, upon transduction of α -syn PFFs formed in the absence of HtrA1, we observe the accumulation of Lewy Body-like inclusions comprised of hyperphosphorylated α -syn in the cytosol and mislocalization of α -syn to the axons. Phosphorylated α -syn is a highly specific marker of α -syn pathology⁴¹, and we can confirm that these inclusions are comprised of endogenous α -syn because the recombinant PFFs were not phosphorylated prior to transduction. Transduction of products formed in the presence of HtrA2 or aldolase induced similar accumulation of hyperphosphorylated inclusions. However, when products were formed in the presence of HtrA1 or HtrA1^{S328A}, we observe no accumulation of phosphorylated α -syn inclusions. Thus we conclude that treatment with HtrA1 renders α -syn seeds non-toxic in neurons. These

products are also incapable of seeding endogenous α -syn and restrict the formation of pathological, hyperphosphorylated Lewy Body-like inclusions of α -syn.

Discussion

Here, we establish that HtrA1 can both prevent the amyloidogenesis of α -synuclein and dissolve preformed α -syn amyloid fibrils. HtrA1 restricts α -syn from forming amyloid species and renders it incapable of seeding the aggregation of endogenous α -syn. Further, HtrA1 treatment prevents α -syn from forming neurotoxic species and also prevents formation of the characteristic α -syn amyloid conformers that drive aggregation of phosphorylated α -syn inclusions in neurons. This activity is not limited to the prevention of amyloidogenesis, as HtrA1 can also solubilize *preformed* α -syn amyloid fibrils, thereby decreasing their seeding capacity. Most known protein quality control systems are comprised of multicomponent machines that require ATP hydrolysis for remodeling¹². In contrast, HtrA1 remodeling does not require collaboration with other proteins or ATP hydrolysis. This ATP-independent disaggregase activity is not unique to HtrA1, and has also recently been discovered in DAXX²⁰, TRIM proteins²², and nuclear-import receptors such as karyopherin- β 2 (Kap β 2)²¹.

We find that HtrA1-mediated disaggregase activity is independent of HtrA1 proteolytic activity, and that disaggregation can be facilitated via the protease domain alone, suggesting that HtrA proteins leverage distinct mechanisms under different circumstances. Further, HtrA1 and HtrA2 appear to operate via distinct mechanisms. Because mitochondrial dysfunction is implicated in PD³¹, and inactivating mutations in the mitochondrial HtrA2 gene have been implicated in PD^{23,28}, we anticipated that HtrA2 might be the principal HtrA isoform that mediates α -syn disaggregation. However, although we find that HtrA2 binds α -syn with similar affinity to HtrA1^{S328A}, HtrA2 has only limited remodeling activity against α -syn. These results suggest that physical association alone does not account for the remodeling conferred by HtrA1 in detoxifying α -syn. Similar phenomena were described for the mechanism of Kap β 2, which disaggregates FUS²¹. In those studies, an antibody that binds FUS in the same region as Kap β 2 did not mediate disaggregation of FUS fibrils. With HtrA1, our findings give further support to the concept that disaggregation and proteolysis operate via distinct mechanisms to regulate proteopathic aggregates. In future studies it will be important to further explore these differences.

Amyloid species are generally characterized as protease-resistant, and so we aimed to better understand how HtrA1 dissolves these species. We demonstrate that HtrA1 disaggregase activity can function in a proteolytically-independent fashion to solubilize otherwise recalcitrant species. Surprisingly, the proteolytic activity of HtrA1 appears to restrict its remodeling activity, as the HtrA1^{S328A} protease-inactive mutant was more protective in most settings. Further, while PDZ domains typically mediate protein-protein interactions and bind β -sheet rich proteins through a β -sheet augmentation mechanism²⁴, we surprisingly find that the PDZ domain is not required for mediating the HtrA1– α -syn binding interaction and that the PDZ domain is dispensable for disaggregase activity. Instead, the protease domain binds substrate, mediates remodeling, and, when active, can proteolyze the product.

To further explore this mechanism, we probed these reactions using mass spectrometry. We find that HtrA1 ordinarily cleaves monomeric α -syn at many different positions throughout the α -syn sequence. However, upon fibrillization, the NAC domain of α -syn PFFs becomes inaccessible to HtrA1 proteolysis, with no cleavage occurring in the NAC domain. Instead, cleavage was restricted to the N- and C-terminal regions. However, upon addition of HtrA1^{S328A}, we observe several new cleavage sites are present, and these sites are particularly enriched in the NAC domain. Thus, we propose a mechanism whereby HtrA1 ordinarily mediates cleavage of α -syn monomer in the NAC domain. Upon fibrillization, HtrA1 binds the NAC domain of α -syn fibrils via its protease domain, stabilizing this region of α -syn, and thereby promoting disaggregation. Once the NAC domain is solubilized, it becomes susceptible to proteolysis. These findings are contrary to previous studies suggesting that the PDZ domain is essential for substrate binding by HtrA1²⁵. However, in this earlier study by Poepsel et al., the authors demonstrated that a PDZ domain deletion variant still maintained high proteolytic activity, with just subtle impairment as compared to the full length protein, suggesting that the PDZ domain is instead dispensable. Further, they found that the HtrA1 PDZ domain alone could not solubilize tau fibrils²⁵, while we find that the HtrA1 protease domain alone retains chaperone activity against α -syn. In future studies, it will be important to better understand these features and how HtrA1 operates via a distinct mechanism as compared to canonical proteases.

Beyond the remodeling activity of HtrA1 against α -syn and tau, HtrA1 can also proteolyze and inhibit the aggregation of the amyloid-like proteins TDP-43 and FUS. However, HtrA1 appears tuned to the properties of amyloid, as its activity is more potent against α -syn than the amyloid-like proteins TDP-43 or FUS, and it does not proteolyze well-folded proteins including GST and MBP. However, we find that both HtrA1^{WT} and HtrA1^{S328A} are somewhat less effective in countering the misfolding of the PD-associated mutant α -syn^{A53T}. We propose that HtrA1 has native inhibitory and disaggregase activity against α -syn, but that this activity may be insufficient to overcome α -syn^{A53T} amyloidogenesis, which proceeds more rapidly and is associated with early-onset PD³⁶. Furthermore, because HtrA1 is also secreted, it may function in preventing cell-to-cell propagation of amyloid seeds. In the future, it may be possible to engineer HtrA1 variants with enhanced disaggregation activity against α -syn^{A53T} and other substrates implicated in neurodegeneration. Such a strategy has been successful with the yeast amyloid disaggregase Hsp104, which has been engineered to counter the misfolding of α -syn, TDP-43, FUS, and other disease-associated proteins^{42–48}.

When proteins form amyloid, this is typically viewed as an irreversible transition, whereby the proteins become resistant to processing and clearance from the cell. This is problematic due to the possible loss of function of the misfolded protein, as well as the accumulation of potentially toxic species. Our work suggests that HtrA1 may function as a chaperone that maintains proteostasis by regulating the folding of α -syn. However, it is unclear whether deficits in HtrA1-mediated disaggregase activity contribute to pathologic α -syn aggregation in PD. In developing new therapeutic strategies for neurodegeneration, it is possible that small molecule therapeutics will be insufficient to prevent or actively clear accumulations of amyloid and amyloid-like misfolded species. Disaggregases are a promising alternative therapeutic

approach. They have the capacity to engage and remodel misfolded and amyloid species, countering both a possible loss of function or gain of toxic function¹². Modulation of HtrA1 is particularly intriguing in this regard, as it could be harnessed to dissolve misfolded species and allow for either their reactivation or degradation. In the future, the development of small molecule modulators of HtrA1 or the engineering of HtrA1 variants with such properties could be a promising new avenue for the development of tailored therapeutics to modulate protein quality control^{11,49}.

Methods

Protein Purification

All proteins were expressed and purified from *E. coli* BL21-CodonPlus(DE3)-RIL cells (Agilent) and purified under native conditions unless otherwise noted. Plasmids containing the HtrA1 Δ NTD (residues 156–480) or the protease-inactive HtrA1 Δ NTD^{S328A} gene with a C-terminal 6-His tag in the pET21a plasmid were obtained from the Saghatelian lab⁵⁰. The truncations: HtrA1ProD (residues 156–379), HtrA1PDZ (residues 380–480), and HtrA1ProDSA (residues 156–379) were generated by site-directed mutagenesis, with sequences confirmed by Sanger sequencing. The HtrA2 Δ NTD (residues 134–458) gene with a C-terminal 6-His tag in the pET21d plasmid was obtained from Genscript. To generate recombinant protein, *E. coli* cells were induced at OD₆₀₀ = 0.6 with 0.4mM IPTG for 18h at 16°C. Cell pellets were resuspended in HtrA wash buffer (50mM Tris, pH 8.0, 1M NaCl, and 30mM imidazole) supplemented with lysozyme (20mg per L of initial culture) and protease inhibitors (cOmplete, EDTA free, Roche). Cells were lysed by sonication and the lysate was cleared by centrifugation. The supernatant was then incubated with Fast flow nickel sepharose (GE Healthcare) for 2h at 4°C, and all subsequent steps occurred at 4°C. The resin was then transferred to a column, washed with HtrA wash buffer, and eluted with HtrA elution buffer (50mM Tris, pH 8.0, 100mM NaCl, and 500mM imidazole). The protein was then buffer exchanged into HtrA storage buffer (50mM Tris, pH 8.0, 100mM NaCl, 10% glycerol) and concentrated to approximately 5–10mg/mL. The protein was then flash frozen in liquid nitrogen before storage at -80°C. Protein was stored in the freezer for no longer than three months to minimize auto-proteolysis.

Plasmids for expression of α -synuclein were from Peter Lansbury⁵¹. α -Synuclein was purified as described³⁴. Briefly, α -syn was expressed in *E. coli* BL21-DE3-RIL cells (Invitrogen), where expression was induced at OD₆₀₀ = 0.6 with 1mM IPTG for 2h at 37°C. Cell pellets were resuspended in osmotic shock buffer (30mM Tris, pH 7.2, 2mM EDTA, 40% sucrose). Cells were lysed by incubating in osmotic shock buffer for 10min at room temperature, centrifuged, and resuspended in 0.84mM MgCl₂. Lysate was then cleared by centrifugation. Nucleic acids were removed via streptomycin sulfate precipitation. The supernatant was then boiled for 10min, after which most proteins precipitate while α -syn remains soluble following boiling. Protein was then loaded onto a bed of DEAE Sepharose for anion-exchange. The column was washed with wash buffer (20mM Tris, pH 8, 1mM EDTA) and eluted with elution buffer (20mM Tris, pH 8.0, 300mM NaCl, 1mM EDTA). The eluate was then dialyzed into α -syn fibrillization buffer (20mM Tris, pH 8.0, 100mM NaCl), flash frozen, and stored at -80°C until use.

GST-FUS was expressed and purified as described³³. Briefly, GST-FUS was expressed in *E. coli* and expression was induced at OD₆₀₀ = 0.6 with addition of 0.5mM IPTG for 18h at 16°C. Cell pellets were resuspended in FUS wash buffer (PBS supplemented with 2mM DTT, 100μM PMSF, 10μM pepstatin A, and cOmplete protease inhibitors) supplemented with lysozyme. Lysis was completed with sonication or homogenization and lysates were cleared by centrifugation. The lysate was then incubated with Glutathione Sepharose Fast Flow (GE Healthcare) resin for 1h at 4°C, washed with FUS wash buffer, and eluted with FUS elution buffer (50mM Tris, pH 8.0, 200mM trehalose, and 20mM glutathione). Protein was then flash frozen and stored at -80°C until use.

A TDP-43-MBP-His₆ construct was obtained from Addgene and purified as described⁵². Briefly, TDP-43-MBP was expressed in *E. coli* and expression was induced at OD₆₀₀ = 0.5 with addition of 1mM IPTG for 18h at 16°C. Cell pellets were resuspended in TDP-43 lysis buffer (50mM HEPES, pH 7.4, 0.5M NaCl, 30mM imidazole, 10% glycerol, 2mM DTT, 100μM PMSF, 10μM pepstatin A, and cOmplete EDTA-free protease inhibitors) supplemented with lysozyme. Lysis was completed by sonication and lysates were cleared by centrifugation. The lysate was then incubated with Fast flow nickel sepharose (GE Healthcare) for 1-1.5 h at 4°C. The resin was then washed with TDP-43 wash buffer (50mM HEPES, pH 7.4, 0.5M NaCl, 30mM imidazole, 10% glycerol, 2mM DTT) and eluted in buffer (50mM HEPES, pH 7.4, 0.5mM NaCl, 0.5mM imidazole, 10% glycerol, 2mM DTT, 100μM PMSF, 10μM pepstatin A, and cOmplete protease inhibitors). The eluent was then added to amylose resin (NEB) and incubated for 90 min at 4°C, washed with amylose wash buffer (50mM HEPES, pH 7.4, 0.5M NaCl, 10% glycerol, 2mM DTT), and eluted with amylose elution buffer (50mM HEPES, pH 7.4, 0.5M NaCl, 10% glycerol, 2mM DTT, 100μM PMSF, 10μM pepstatin, 10mM maltose, and cOmplete protease inhibitors). The eluent was concentrated to approximately 40μM using a 30kDa molecular weight cutoff filter, flash frozen, and stored at -80°C until use.

Proteolysis Assays

α-Syn (25μM) was treated with buffer, HtrA1, or HtrA2 (5μM) for the indicated time at 37°C. Samples were then processed by SDS-PAGE. GST-FUS (10μM) or TDP-43-MBP-His₆ (10μM) were treated with TEV protease for 1h at 37°C to liberate free FUS and TDP-43. Following cleavage, buffer, HtrA1, or HtrA2 (2μM) was added for 24h at 37°C. Samples were then processed by SDS-PAGE.

Inhibition of FUS and TDP-43 aggregation

FUS aggregation reactions were prepared by mixing GST-TEV-FUS (10μM) in FUS assembly buffer (50mM HEPES, pH 7.4, 10% glycerol, 1mM DTT) supplemented with 1mM DTT with the indicated HtrA construct (10μM) or buffer control. Reactions were initiated by addition of TEV protease and reactions were monitored for turbidity by continuously measuring absorbance at 395nm at 25°C without agitation in a BioTek Epoch plate reader.

TDP-43 aggregation reactions were prepared in a similar way in TDP-43 assembly buffer (50mM HEPES, pH 7.4, 10% glycerol, 1mM DTT). Here TDP-43-TEV-MBP (10 μ M) was mixed with varying concentrations of HtrA1. Reactions were initiated by addition of TEV protease and reactions were monitored for turbidity by continuously measuring absorbance at 395nm at 30°C with agitation in a BioTek Epoch plate reader.

Preparation of α -Syn preformed fibrils (PFFs)

To prepare α -syn preformed fibrils, monomeric α -syn was filtered through a 0.2 μ M filter. Monomer (5mg/mL) was then diluted in fibrillization buffer (20mM Tris, pH 8, 100mM NaCl) and incubated at 37°C with agitation at 1,500 rpm in an Eppendorf Thermomixer for 7 days to produce mature fibrils. The resulting mixture was centrifuged at 15,000 rpm for 30 min at room temperature. The supernatant was then removed and a BCA assay was used to determine the concentration of the fibrils. PFFs were resuspended in fibrillization buffer to 5mg/mL.

α -Syn inhibition and disassembly reactions

For inhibition reactions, α -synuclein monomer (25 μ M) was incubated in fibrillization buffer (20mM Tris, pH 8.0, 100mM NaCl) with or without HtrA1, HtrA1^{S328A}, or aldolase (5 μ M) at 37°C with agitation at 1500rpm in an Eppendorf Thermomixer.

For disassembly reactions, α -syn PFFs (10 μ M) were incubated in fibrillization buffer with or without HtrA1, HtrA1^{S328A}, or aldolase (100 μ M) at 37°C with gentle shaking at 350rpm in an Eppendorf Thermomixer for 24h.

Fibril assembly and disassembly was monitored by ThioflavinT (ThT) fluorescence. Here, ThT (10 μ M) was mixed with α -synuclein (0.5 μ M). Fluorescence at 482nm was measured after excitation at 450nm using a Tecan Spark plate reader.

Sedimentation assays

To monitor inhibition of α -syn fibrillization, reactions were prepared as above were taken at the indicated time points. Reactions were then centrifuged at 15,000rpm for 30 min at room temperature to separate the soluble and insoluble fractions. Following centrifugation, the supernatant and pellet were resuspended in sample buffer (60mM Tris, pH 6.8, 5% glycerol, 2% SDS, 4% β -mercaptoethanol). The total, soluble, and pellet fractions were then resolved by SDS-PAGE and stained with Coomassie Brilliant Blue.

To monitor PFF disassembly, α -syn PFFs (5 μ M) were incubated in fibrillization buffer with the indicated HtrA1 variant (100 μ M) at 37°C for 48h. The total, soluble, and pellet fractions were then spotted on nitrocellulose membranes and probed with α -syn antibody.

The amount in either fraction was determined by densitometry using the Image Lab software on a Bio-Rad Gel Doc EZ Imaging system.

Electron Microscopy

Samples of α -synuclein incubated with or without HtrA1 as described above were applied to 200 mesh, pure carbon, copper grids (Ted Pella #0184-F). The grids were then washed with water five times, and stained with 2% uranyl acetate for 1min.

Images were obtained using a JEOL JEM-1400 120 kV transmission electron microscope.

Pull down assay

Interactions between HtrA constructs and α -synuclein were examined by His-mediated pull-down assays. Recombinant HtrA-6His (0.15 mg) was immobilized to 50 μ L of Ni-Sepharose resin (GE Healthcare Cytiva, cat: 45002985) and then incubated with 0.15 mg of wild-type α -synuclein at room temperature in assay wash buffer (20mM Tris, 100mM NaCl, 10mM Imidazole, pH 8.0). The incubated mixture was washed five times with wash buffer, and eluted with 500mM imidazole. Protein samples were collected prior to the wash step as 'Input'. For Western blot analysis, proteins were transferred to nitrocellulose membrane and probed with anti-syn1 antibody (BD Science, Cat 610787). Membranes were imaged using a Li-COR Odyssey FC Imaging system and the amount of protein in 'input' and 'bound' fractions was determined by densitometry using Image Studio Lite software.

Native-PAGE analysis

For native-PAGE analysis of protein complex formation, inhibition reactions following 48h incubation were prepared in native-PAGE sample buffer (62.5mM Tris-HCl, 40% glycerol, pH 6.75) at a 1:1 ratio. Protein complexes were then separated on a 4–20% non-denaturing gradient polyacrylamide gel and stained with Coomassie Brilliant Blue.

HEK293T cell culture

HEK293T biosensor cells were obtained from Tritia Yamasaki⁵³. Cells were grown in Dulbecco's modified high glucose Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. Control cell lines, HEK293T, HEK293T- α -syn-CFP and HEK293T- α -syn-YFP lines were cultured in the same conditions. For FRET seeding assays, the biosensor cells (HEK293T- α -syn-CFP/ α -syn-YFP) were plated in 96-well plates at a density of 35K cells per well. Inhibition and disassembly reactions were prepared as described above and used following 48h (inhibition) or 24h (disassembly) treatment with HtrA or control. Samples were then sonicated in a cup horn water bath sonicator (QSonica) at 65amp for 3 min, packaged with 0.5 μ L Lipofectamine 3000 (Invitrogen), and transduced

into the biosensor cells. Here, 24h following plating, treated samples were added dropwise to achieve a final concentration of α -syn of 50nM (inhibition) or 10nM (disassembly) in each well. Cells were then harvested after 48h (inhibition) or 24h (disassembly) and processed for flow cytometry analysis. For flow cytometry, cells were detached with 0.05% Trypsin/EDTA, followed by fixation with 4% paraformaldehyde for 15 min at 4°C in the dark. Cells were then resuspended in MACSQuant Flow Running buffer for analysis in a MACSQuant VYB flow cytometer. Fluorescence compensation was performed with control cell lines (HEK293T- α -syn-CFP and HEK293T- α -syn-YFP) each time prior to sample analysis. Following excitation of the CFP donor fluorophore with a 405nm laser, FRET signal was monitored from the YFP acceptor fluorophore at 525nm with a 50nm bandpass filter. All data analysis was performed with FlowJo V10 software to determine the percent of FRET positive cells and median FRET fluorescence intensity for each sample. The percent of FRET positive cells was then multiplied by the median FRET intensity to calculate integrated FRET density, which was then normalized to a vehicle control.

HtrA transient transfection and α -Syn seeding assay

For transient expression of HtrA1 or HtrA1^{S328A}, cells were plated in 6-well plates at 100,000 cells per cm². Plasmids containing HtrAs with C-terminal Myc-DDK tags in the pCMV6 vector were obtained from Origene. Transfections were performed 16-24h after plating, at 70% confluence, using Lipofectamine 3000 (Invitrogen, Carlsbad, CA). Following two days of HtrA expression, α -syn PFFs were transduced as described above. Following an additional day of PFF treatment, cells were harvested (3 days post-HtrA1 transfection, 1 day post PFF transduction). Populations of cells were then split into two fractions for flow cytometry analysis or immunoblotting. Flow cytometry sample preparation was performed as described above. For immunoblotting, cells were pelleted and lysed by vortexing in modified RIPA buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.5% TX-100, 0.5% deoxycholate, cOmplete protease inhibitors). Crude lysates were then centrifuged at 1,000 xg for 10 min at 4°C. Total protein was quantified by BCA assay and equal amounts of total protein from each sample were prepared in 1xLaemmli sample buffer and boiled for 5 min. Lysates were then separated by SDS-PAGE (4–20% gradient, BioRad) and transferred to a PVDF membrane. Membranes were blocked in Odyssey Blocking Buffer (LI-COR) for at least 1h. Primary antibody incubations were performed at 4°C overnight. Primary antibodies used: anti-Myc (Proteintech Cat No. 60003-2-Ig), anti- α -Syn (BD Bioscience), anti-GAPDH (Proteintech). Membranes were then incubated with 680RD anti-Rabbit IgG (LI-COR CAT#926-68071) and 800CW Goat anti-Mouse IgG (LI-COR CAT#926-32210). Membranes were imaged using a Li-COR Odyssey FC Imaging system.

For immunocytochemistry, cells were fixed in paraformaldehyde (4%) for 15 min, followed by permeabilization and blocking with 3% BSA/0.1% TX-100 for 15 min. Cells were then labeled with primary antibody at 4°C overnight. Cells were washed with PBS and then incubated with secondary antibody (Alexa-488/Alexa-568) for 1h at room temperature. Primary antibodies used: anti-pSyn (Abcam MJFR13), anti-Tau (Sigma T49), and anti-Myc (Proteintech Cat#60003-2-Ig). Nuclei were stained with DAPI for 5 min. Cells were mounted onto slides using with Prolong Gold mounting solution. Images were acquired using a Nikon Eclipse Te200-E microscope and processed with ImageJ.

Peptide sample preparation for mass spectrometry

To prepare samples for mass spectrometry analysis, α -synuclein monomer monomer (25 μ M) was incubated with HtrA1^{WT} (5 μ M) at 37°C with shaking at 350rpm for 3hr in an Eppendorf Thermomixer. The samples were reduced with 5mM DTT, centrifuged at 15,000rpm for 30min at room temperature to clear any insoluble material, and then subjected to C18 desalting.

For analysis of the fibrillar proteolysis, α -syn PFFs (5 μ M) were pre-treated with HtrA1^{S328A} (50 μ M) or buffer (50mM Tris, pH 8.0, 100mM NaCl) at 37°C with shaking at 350rpm for 2h, followed by the addition of HtrA1^{WT} (2.5 μ M). Samples were then incubated for an additional 3h at 37°C with shaking in an Eppendorf Thermomixer. Samples were then solubilized in 6M urea supplemented with 10% formic acid at 60°C for 30 min with shaking at 600rpm. The samples were then reduced with 5mM DTT, and centrifuged at 15,000rpm for 30min at room temperature to clear any insoluble material. The supernatant was transferred to a fresh protein low-bind tube and desalted with a C18 spec tip (Varian, cat# A57203). After C18 desalting, samples were dried under speedvac and resuspended (0.1% formic acid, 3% acetonitrile) prior to LC/MS analysis.

LC/MS analysis of α -synuclein peptides

Peptide mixtures were analyzed by LC/MS by using a UHPLC system coupled to an Orbitrap ID-X Tribrid mass spectrometer (Thermo Fisher Scientific). The following electrospray ionization conditions were used: sheath gas flow 32 arbitrary units (Arb), auxiliary gas flow 5 Arb, sweep gas flow 0 Arb, ion transfer tube temperature 325°C, and vaporizer temperature 125°C. The RF lens value was 60%. Data were acquired in positive polarity with a spray voltage of 3.5kV. MS1 data were acquired at a resolution of 60K with an automatic gain control (AGC) target of 4e5 and a maximum injection time of 100 ms. MS/MS spectra were collected on [M + H]⁺ ions in positive polarity for each sample by using DDA. The MS/MS isolation window was set to 1.6 *m/z*. A normalized collision energy (NCE) of 30% was used. MS/MS data were acquired with 15K resolution, an AGC target of 1.25e4, a maximum injection time of 86 ms, and a dynamic exclusion of 10 s. The intensity threshold was set to 2.5e4. Samples were randomized before analysis. Negative control sample containing only α -Synuclein fibrils were injected and analyzed to preclude the identification of α -Synuclein fragments resulting from protein purification. In addition, a quality-control (QC) sample was injected to monitor signal stability of the instrument.

MaxQuant (Version 2.0.3.0) was used to annotate data. All data files were then analyzed in Skyline-daily (Version 22.2.1.351) to obtain peak areas for relative quantification of peptide abundance. Peaks were extracted for each target peptide under consideration of retention times. For data analysis, low-abundance peptides with peak areas below 10,000 mAU were excluded from the data set. Further, all peptides included in the analysis were identified at least twice among three biological replicates. The relative abundance of percent fragmentation at specific residues was normalized to the sum of the total peptide area identified for each sample.

Primary neuron dissection and culturing

Primary hippocampal neurons were obtained from E18 CD-1 mice. Hippocampi were dissected in Hanks' Balanced Salts with 10mM HEPES and penicillin/streptomycin, followed by digestion with 0.25% Trypsin-EDTA / 0.02mg/mL DNase at 37°C for 15 min and mechanical dissociation by trituration through a fire-polished Pasteur pipette. Neurons were then resuspended in plating medium (MEM supplemented with glucose, L-glutamine, 10% heat-inactivated horse serum, and penicillin/streptomycin) at a density of 25k cells/cm² on poly-L-Lysine coated coverslips in 24-well plates for ICC or at 80k cells/cm² in 96-well plates for viability assays. The media was then changed to neuronal maintenance medium (Neuro basal medium with L-glutamine and B27 supplement and penicillin/streptomycin) after 2–4 h. Neurons were then treated with α -syn inhibition reactions on DIV 18–21. Here, samples from inhibition reactions were taken at the 48h time point, sonicated, and applied to the neurons (1 μ g for ICC or 7.2 μ g for viability assays). Neuronal viability was assessed by MTT assay after 1 day, while aggregation was assessed by immunocytochemistry as described above after 1 week. Images were acquired using a Leica Sp8 Single Photon Confocal microscope and processed with ImageJ.

Neuronal Viability

Cell viability was assessed by MTT assay. Here, 24h following addition of inhibition reactions (DIV 19–22), viability was assessed by MTT Cell Proliferation Assay (ATCC) according to the manufacturer's protocol. Absorbance readings were taken at 570nm with a reference filter of 630nm on a BioTek EPOCH2 microplate reader.

Declarations

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Figures

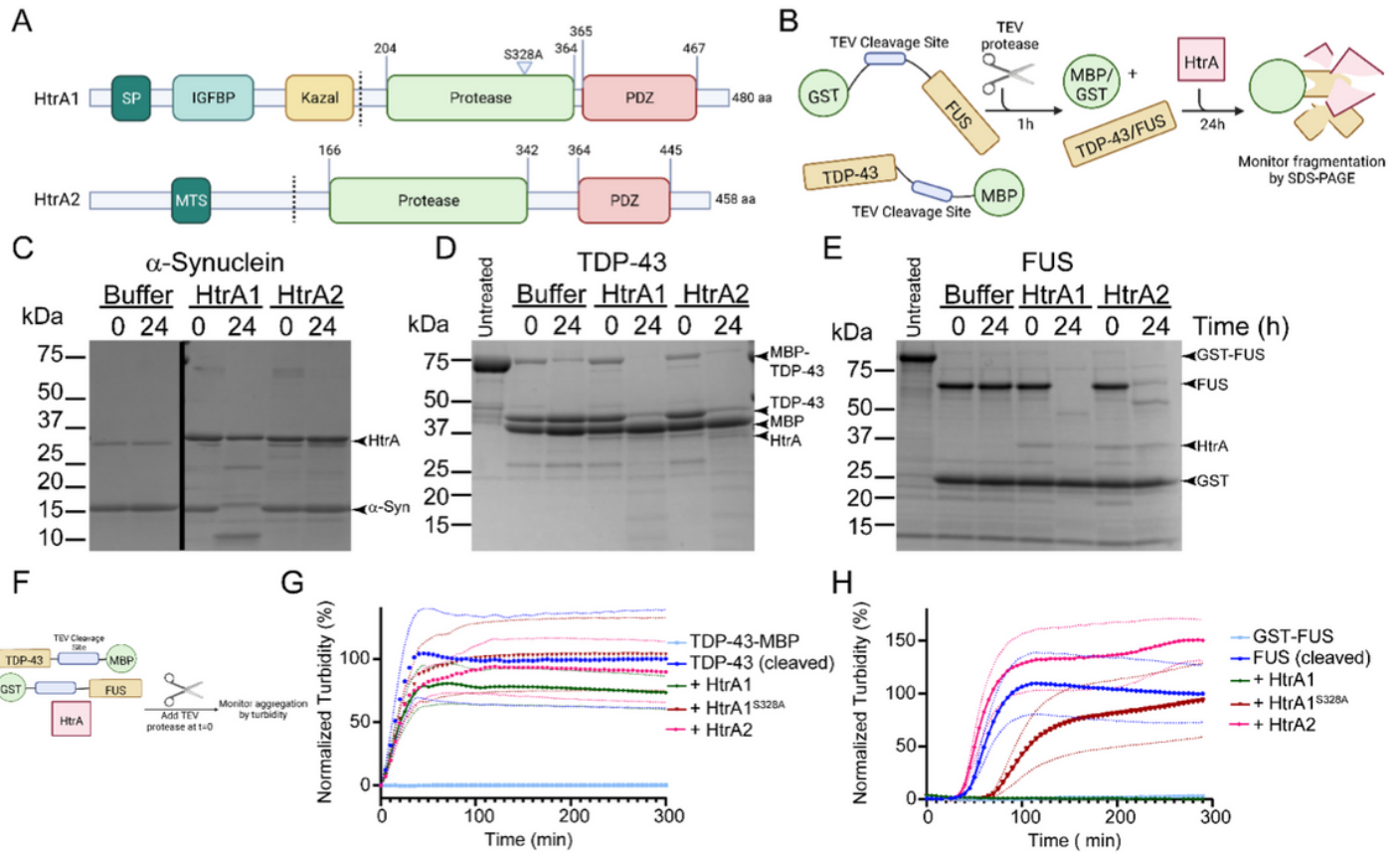


Figure 1

HtrA1 can process diverse substrates for proteolysis. (A) Domain architecture of HtrA1 and HtrA2. Signal peptide (SP), insulin-like growth factor binding protein (IGFBP), Kazal-like domain, mitochondrial targeting sequence (MTS). Dashed lines indicate sites where Δ NTD constructs used in our studies begin. (B) Experimental setup for proteolysis experiments for TDP-43 and FUS. (C) α -synuclein (25 μ M) was treated with buffer, HtrA1, or HtrA2 (5 μ M) for 24h at 37°C. Samples were then separated by SDS-PAGE and visualized with Coomassie blue staining. (D) TDP-43-TEV-MBP (10 μ M) was treated with TEV protease for 1h at 37°C, followed by treatment with buffer, HtrA1, or HtrA2 (2 μ M) for 24h at 37°C. Samples were then processed by SDS-PAGE. Untreated lane is TDP-43-TEV-MBP with no added TEV or HtrA. (E) GST-TEV-FUS (10 μ M) was treated with TEV protease for 1h at 37°C, followed by treatment with buffer, HtrA1, or HtrA2 (2 μ M) for 24h at 37°C. Samples were then processed by SDS-PAGE. Untreated lane is GST-TEV-FUS with no added TEV or HtrA. (F) Schematic of turbidity assay design. (G) TDP-43-TEV-MBP (10 μ M) was incubated with buffer, HtrA1, HtrA1^{S328A}, or HtrA2 (10 μ M). Reactions were initiated by addition of TEV protease at t=0 and aggregation was monitored by turbidity. (N = 3, means are shown as large symbols, SEM is shown as smaller symbols of the same color). (H) GST-TEV-FUS (10 μ M) was incubated with buffer, HtrA1, HtrA1^{S328A}, or HtrA2 (10 μ M). Reactions were initiated by addition of TEV protease and then aggregation was monitored by turbidity. (N = 3, means are shown as large symbols, SEM is shown as smaller symbols of the same color).

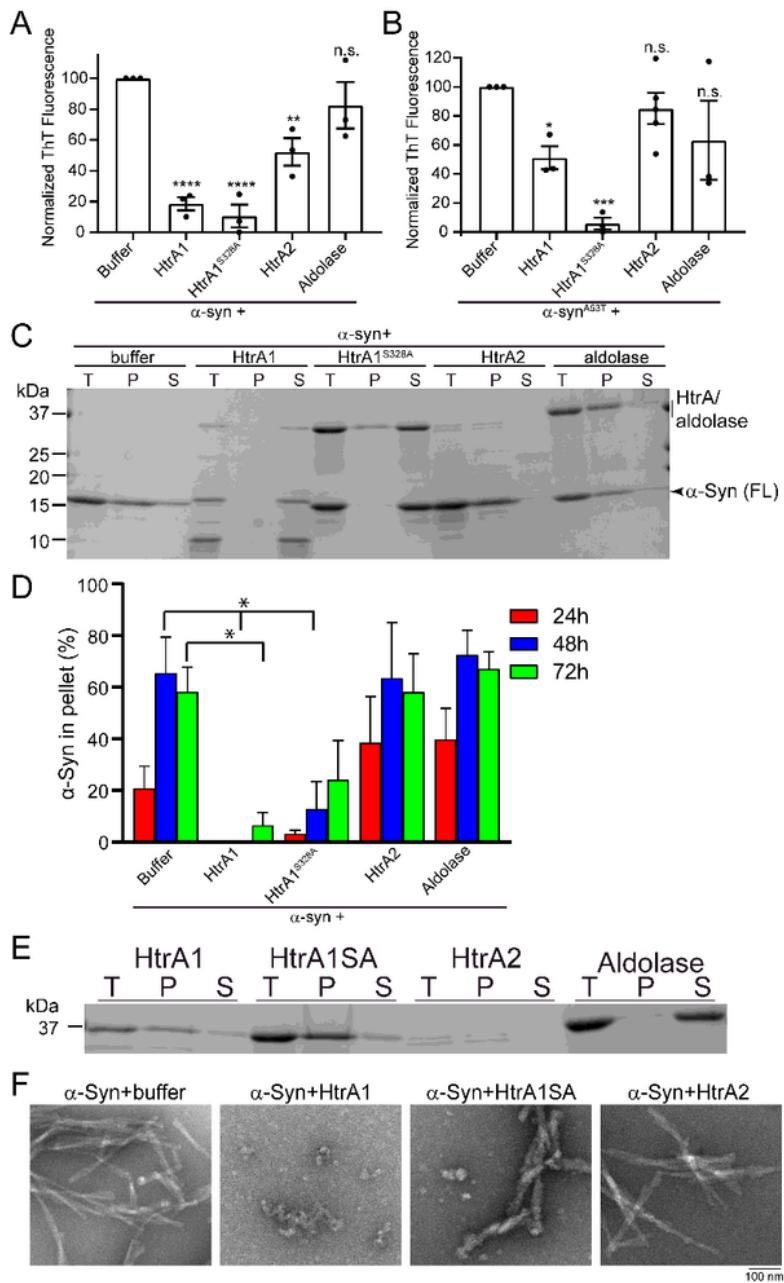


Figure 2

HtrA1 prevents α -Syn amyloidogenesis and preserves α -Syn solubility. (A) α -Syn monomer (25 μ M) was incubated with buffer, HtrA1, HtrA1^{S328A}, HtrA2, or aldolase (5 μ M) for 48h. Amyloid content was assessed by ThioflavinT (ThT) fluorescence. Values are compared to buffer treatment using a one-way ANOVA with a Dunnett's multiple comparisons test (N = 3, biological replicates are shown as dots, bars are means $\pm$ SEM, *p<0.1, **p<0.05, ***p<0.01, ****p<0.001). (B) Experiments were performed as in A but with α -

syn^{A53T}. (C) α -Syn (25 μ M) was incubated with HtrA1, HtrA1^{S328A}, HtrA2, or aldolase (5 μ M) at 37°C. At the indicated time points, samples were removed and fibrillization was assessed by sedimentation. (T = total, P = pellet, S = soluble). SDS-PAGE gel for 72h time point is shown. (D) Quantification of sedimentation analysis from C. Values at 24, 48, or 72h were compared to buffer control at the same time point using a one-way ANOVA with a Dunnett's multiple comparisons test (N = 4, bars are means $\pm$ SEM, *p<0.05). (E) Experiments were performed as in C, but in the absence of α -syn to monitor solubility of HtrA independently. SDS-PAGE gel for 24h time point is shown. (F) Fibrillization reactions performed as in (A-C) and processed for EM. Representative images are shown. Scale bar, 100nm.

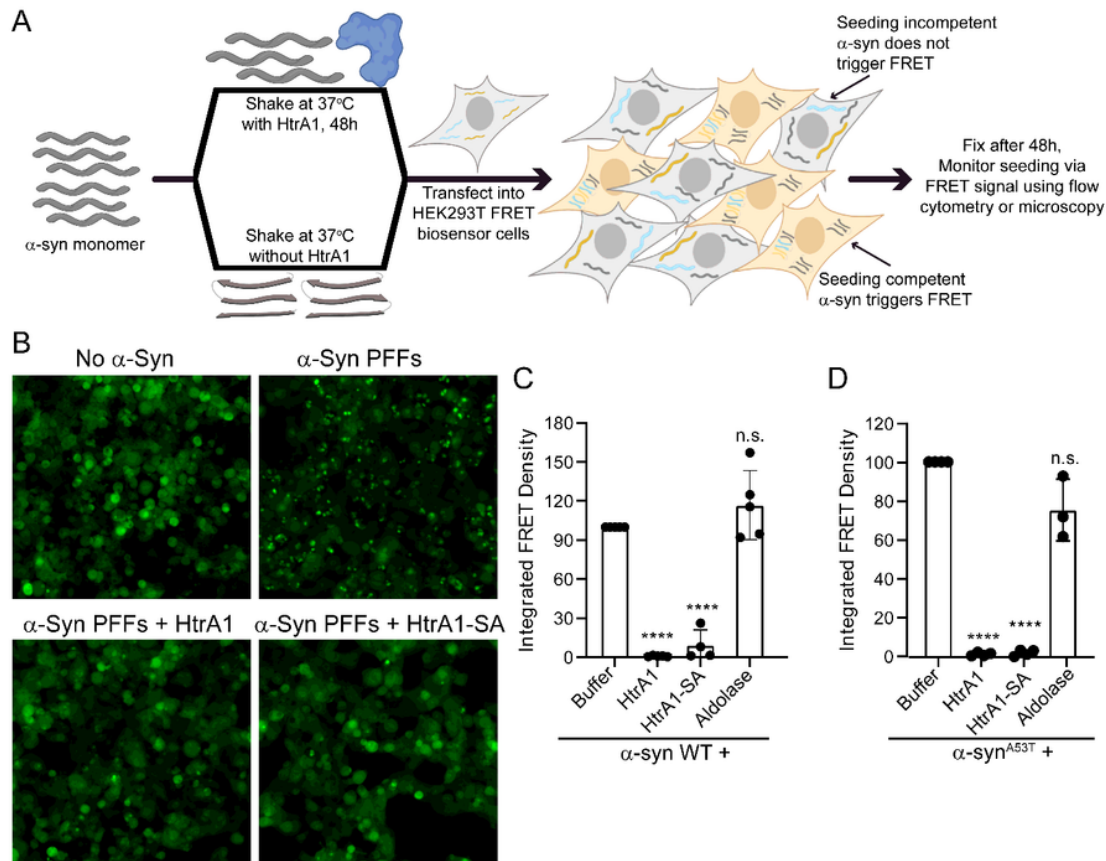


Figure 3

HtrA1 prevents α -Syn from forming seeding competent species. (A) Schematic showing experimental design. (B) α -Syn (25 μ M) was incubated with buffer, HtrA1, or HtrA1^{S328A} (5 μ M) at 37°C for 48h. Reaction products were transfected into HEK293T biosensor cells (50nM α -syn in media). Cells were assessed by microscopy 48h following treatment. (C) Cells from (B) were analyzed by flow cytometry and integrated FRET density was calculated. Values were compared to control reactions with α -syn alone using a one-way ANOVA with a Dunnett's multiple comparisons test (N $\geq$ 3, biological replicates are shown as dots,

bars represent means $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$). (D) Experiments were performed as in (C) but with α -syn^{A53T}.

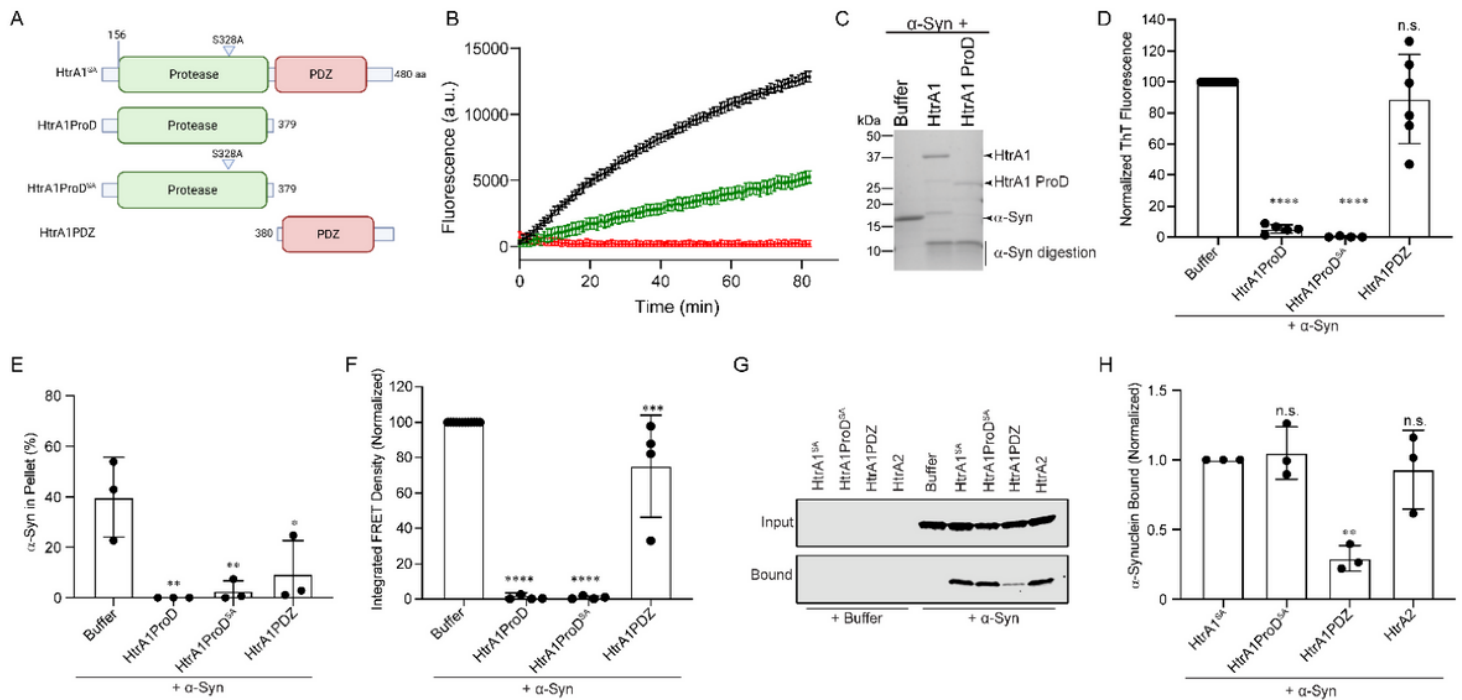


Figure 4

The protease domain of HtrA1 is necessary and sufficient for remodeling of α -syn. (A) Schematic showing constructs used in these experiments. (B) HtrA1 (Black), HtrA1ProD (green), or HtrA1PDZ (red) (2 μ M) were incubated with FITC-casein (10 μ M). Degradation of FITC-casein was monitored by an increase in fluorescence. Representative data of a technical triplicate from one of three biological replicates are shown. Error bars show SEM for this technical triplicate. (C) α -Syn (25 μ M) was treated with buffer, full-length HtrA1, or the protease domain alone (HtrA1ProD) (5 μ M) for 24h at 37°C. Samples were then processed by SDS-PAGE to assess α -syn proteolysis. (D) α -Syn monomer (25 μ M) was incubated with buffer or the indicated HtrA1 truncation construct (5 μ M) for 48h. Amyloid content was assessed by ThioflavinT (ThT) fluorescence. Values are compared to buffer using a one-way ANOVA with a Dunnett's multiple comparisons test ($N \geq 4$, biological replicates are shown as dots, bars are means $\pm$ SEM (**** $p < 0.0001$). (E) Sedimentation assays were performed as described with α -syn (25 μ M) and the indicated HtrA1 construct (5 μ M) at 37°C. After 48h, samples were removed and fibrillization was assessed by sedimentation and quantified by SDS-PAGE. Values are compared to control reactions with α -syn alone using a one-way ANOVA with a Dunnett's multiple comparisons test ($N \geq 4$, biological replicates are shown as dots, bars represent means $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$). (F) Samples from E were transduced into HEK293T biosensor cells (50nM α -syn in media). FRET was assessed by flow cytometry 48h following treatment and integrated FRET density was calculated. Values are compared to control reactions with α -syn alone using a one-way ANOVA with a Dunnett's multiple comparisons test ($N \geq 4$, biological replicates are shown as dots, bars represent means $\pm$ SEM, *** $p < 0.001$, **** $p < 0.0001$). (G) The

indicated His-tagged HtrA constructs (10μM) were immobilized on Ni-NTA resin and incubated with α-syn (20μM) overnight. Beads were then washed and the input and bound fractions were processed by immunoblotting using an α-syn antibody. (H) Experiments from G were quantified and normalized to the HtrA1^{SA} condition. Values are compared to control reactions with HtrA1^{SA} using a one-way ANOVA with a Dunnett's multiple comparisons test (N = 3, biological replicates are shown as dots, bars represent means ± SEM, **p< 0.01, n.s. p > 0.05).

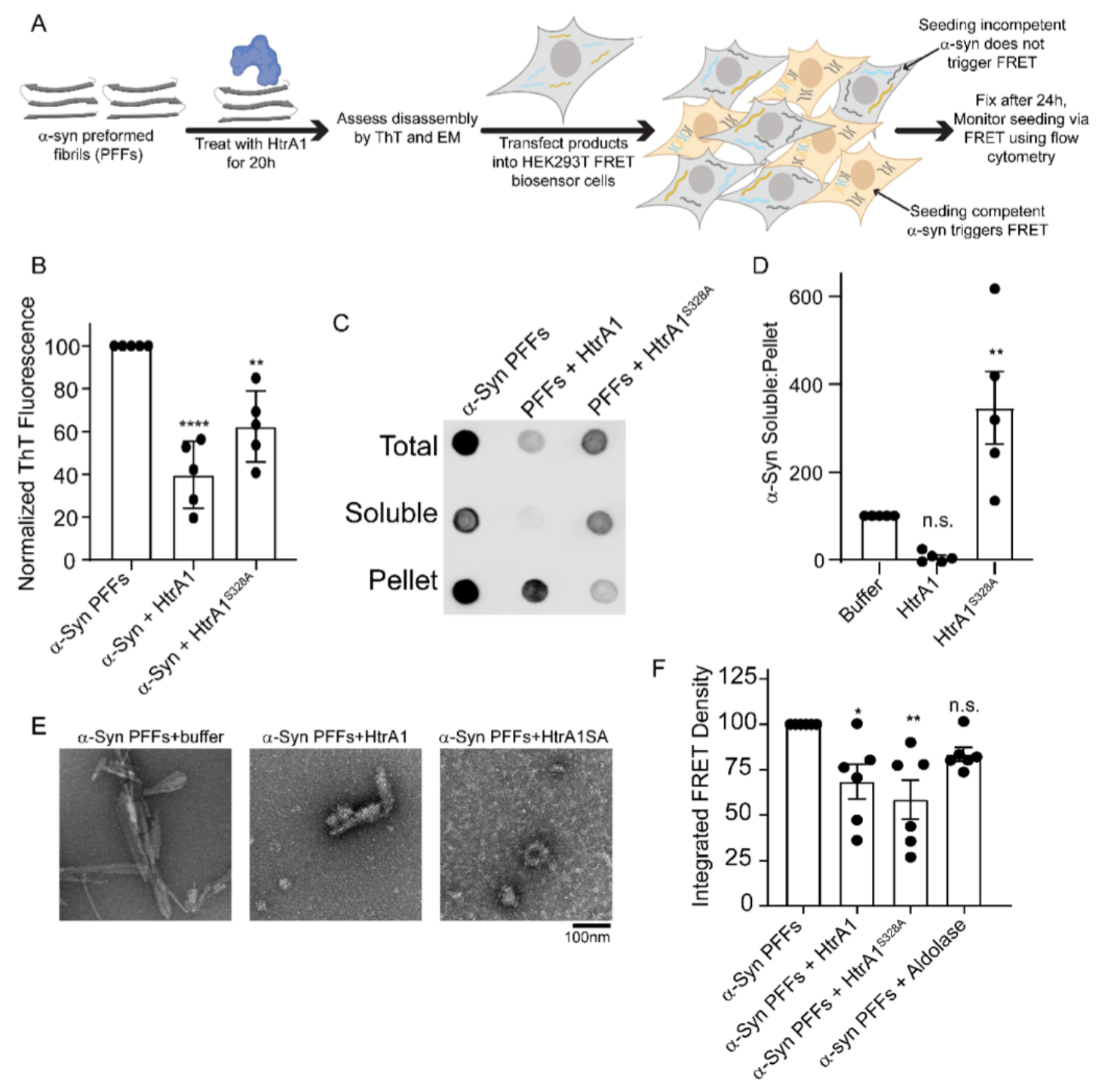


Figure 5

HtrA1 treatment remodels α -Syn PFFs to a seeding incompetent form. (A) Schematic showing experimental design. (B) α -Syn PFFs (10 μ M) were treated with buffer, HtrA1, or HtrA1^{S328A} (100 μ M) for 24h and amyloid content was assessed by ThioflavinT (ThT) fluorescence. Values are compared to buffer treatment using a one-way ANOVA with a Dunnett's multiple comparisons test (N = 5, biological replicates are shown as dots, bars are means $\pm$ SEM, * p <0.05, ** p <0.01, **** p <0.0001). (C) α -Syn PFFs (5 μ M) were treated with buffer, HtrA1, or HtrA1^{S328A} (100 μ M) for 48 h and then partitioned into total, soluble, and insoluble fractions. Samples were then spotted onto a nitrocellulose membrane and α -syn was visualized by immunoblotting. Representative image is shown. (D) Quantitation of experiments shown in C. Values are normalized to α -syn alone condition and compared to this treatment using a one-way ANOVA with a Dunnett's multiple comparisons test (N=4, biological replicates are shown as dots, bars are means $\pm$ SEM, ** p <0.01). (E) Reactions from B were processed for EM. Scale bar, 100nm. (F) α -Syn PFFs were treated as in B and the remodeled products were transduced into HEK293T biosensor cells (10nM PFFs) using lipofectamine. 24h after transduction, cells were harvested and integrated FRET density was measured by flow cytometry. Values are compared to buffer treatment using a one-way ANOVA with a Dunnett's multiple comparisons test (N = 3, biological replicates are shown as dots, bars represent means $\pm$ SEM, * p < 0.05, ** p < 0.01).

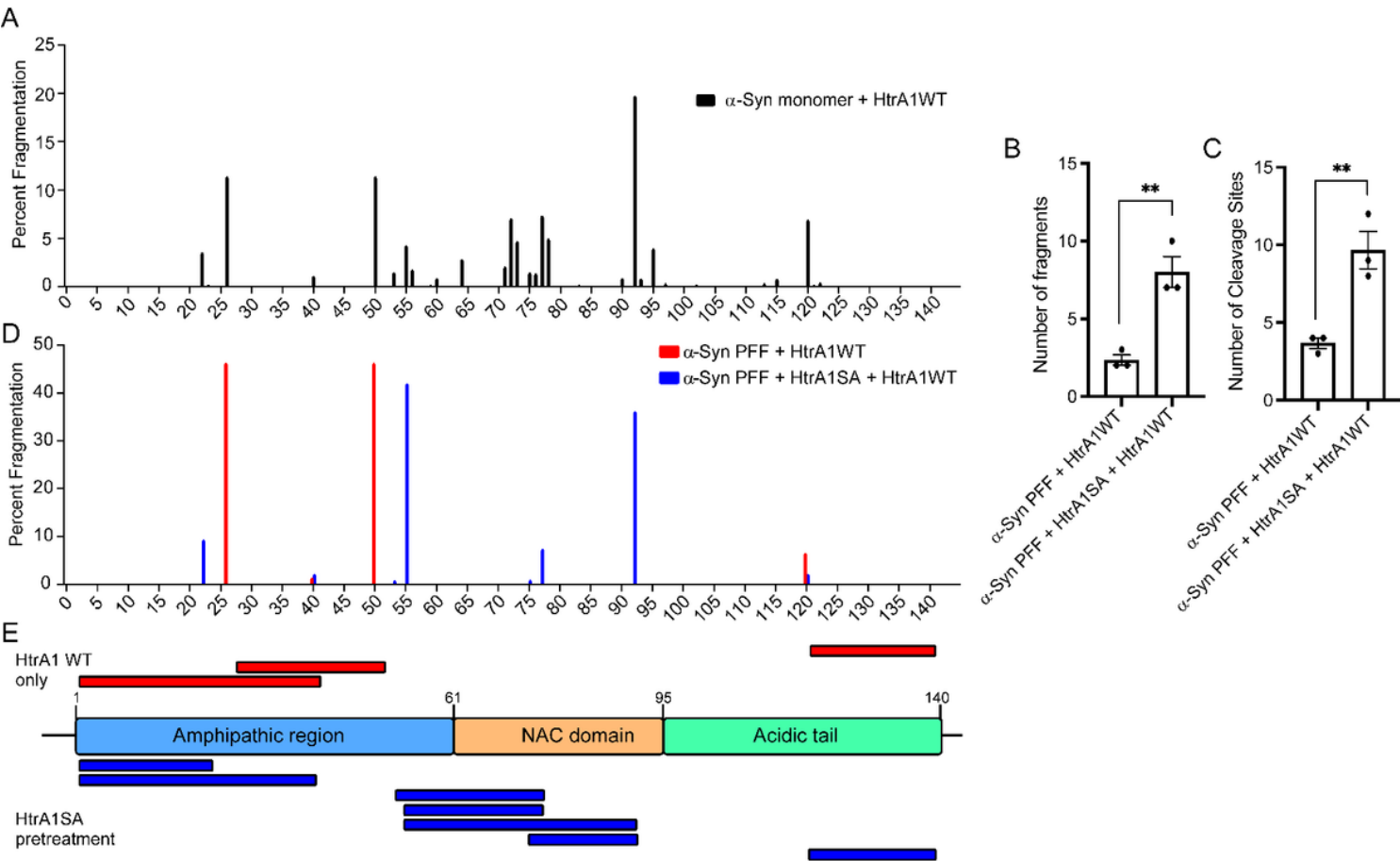


Figure 6

HtrA1 disaggregation promotes solubilization and proteolysis of the NAC domain. (A) α -Syn monomer (25 μ M) was treated with HtrA1 (5 μ M) for 3h. Samples were then analyzed by LC/MS. (B) α -Syn fibrils (5 μ M) were pre-treated with HtrA1 or HtrA1^{S328A} (50 μ M) for 2h, followed by addition of HtrA1 (2.5 μ M) for 3h. Samples were then analyzed by LC/MS. The total number of fragments detected or (C) total number of cleavage sites are shown. (N=3, biological replicates are shown as dots, bars represent means $\pm$ SEM, ** $p < 0.01$). (D) Quantification of the relative abundance of fragmentation at specific cleavage sites from experiments shown in B-C. Percent fragmentation at specific sites was normalized to the sum of the peak areas for each condition (N=3, representative data from one replicate is shown). (E) Map of α -syn PFF proteolysis. Fragments identified upon cleavage with HtrA1 alone (red) and after adding a pre-treatment step with HtrA1^{S328A} prior to proteolysis (blue).

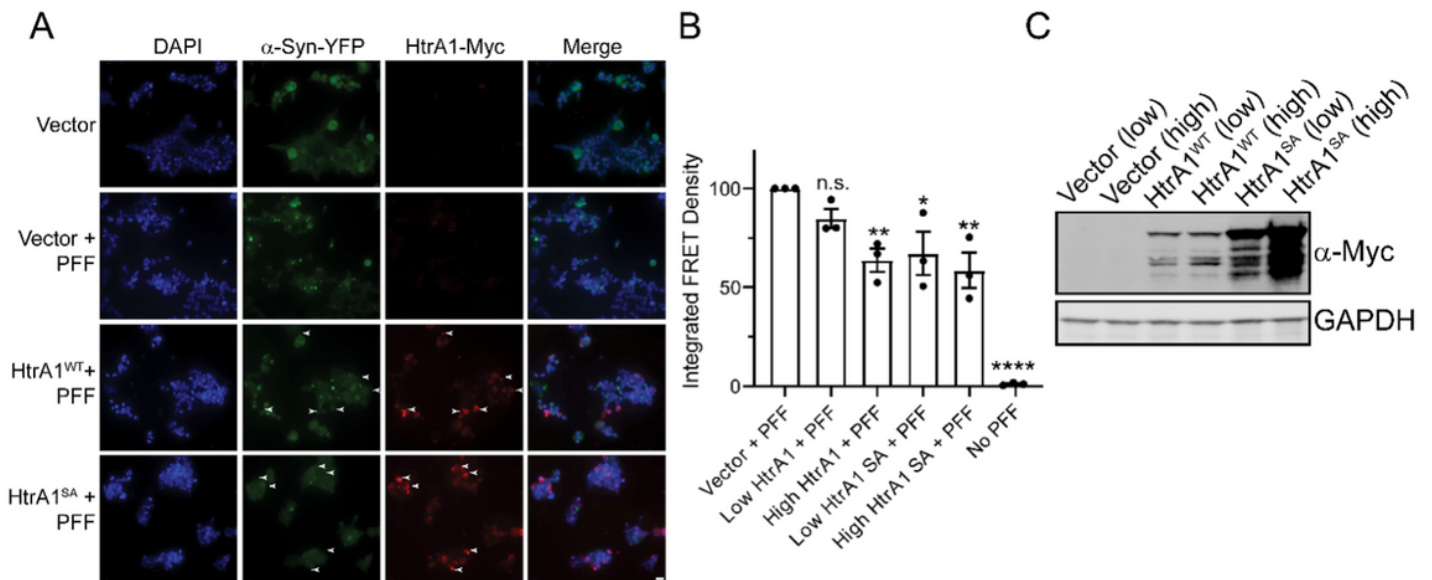


Figure 7

Overexpression of HtrA1 prevents α -syn PFFs from seeding aggregation. (A) HEK293T biosensor cells were transfected with HtrA1-Myc, HtrA1^{S328A}-Myc plasmid, or a vector control. 2 days following transfection, α -syn PFFs were added to media (50nM). After 1 day of treatment, cells were fixed and stained for DAPI (blue) or Myc (HtrA1, red). α -Syn was imaged via the YFP tag. Arrowheads indicate areas with high HtrA expression and low α -syn puncta formation, suggesting HtrA-mediated inhibition of aggregation. Scale bar, 20 μ m. (B) HEK293T biosensor cells were transfected, fixed, and analyzed by flow cytometry and integrated FRET density was calculated. Values were compared to the control expressing vector alone using a one-way ANOVA with a Dunnett's multiple comparisons test (N $\geq$ 3, biological replicates are shown as dots, bars represent means $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$). (C) Cells from B were lysed and immunoblotted.

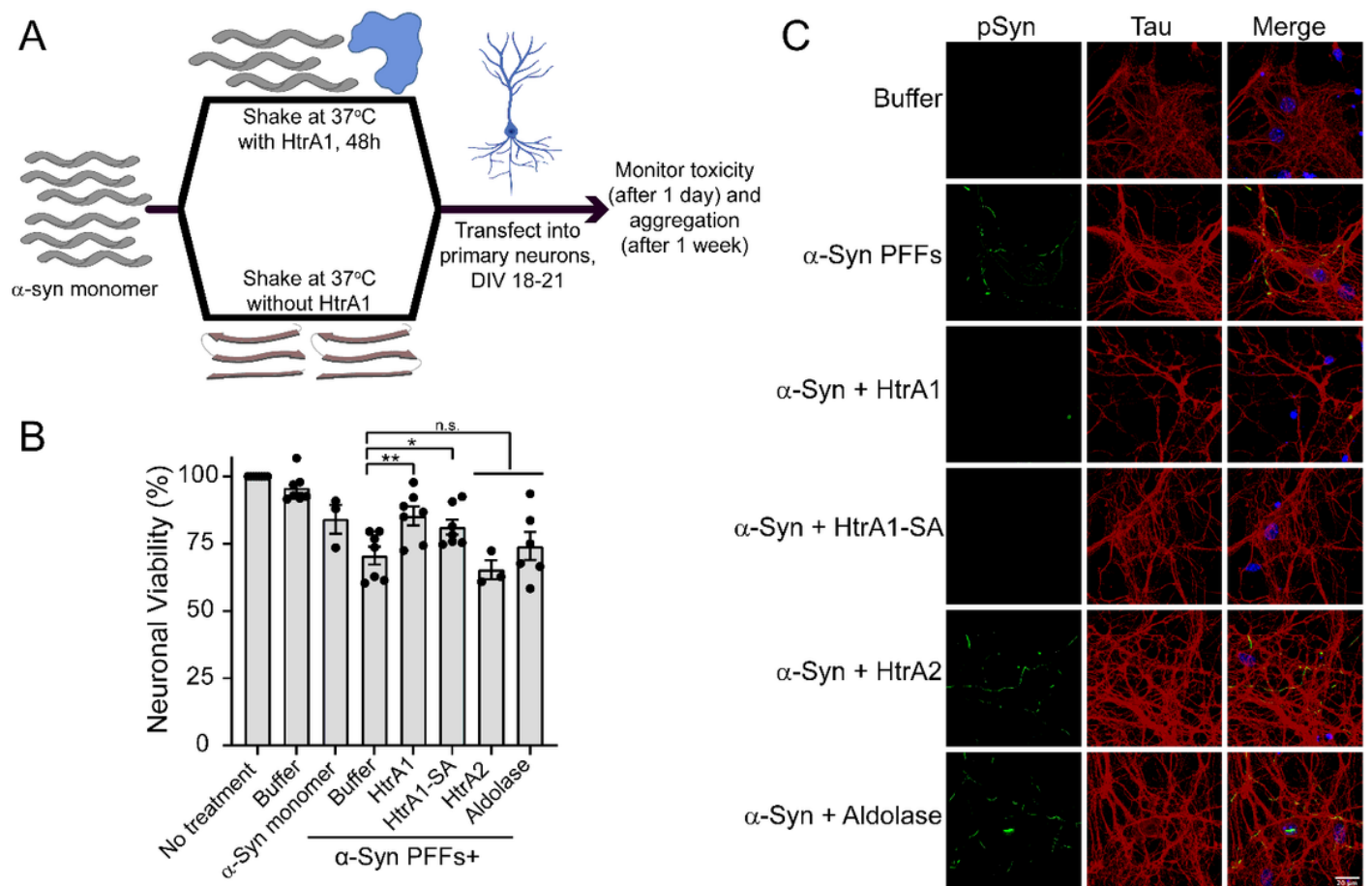


Figure 8

HTRA1 treatment renders α-Syn non-toxic and seeding incompetent in primary mouse neurons. (A) Schematic showing experimental design. (B) Fibrillization of α-syn (100μM) was conducted by shaking for 48h at 37°C in the presence of HtrA1, HtrA1^{S328A}, HtrA2, buffer, or Aldolase (20μM). Reaction products were applied to mouse primary hippocampal neurons at DIV 28-21. 24h following treatment, cell viability was assessed by MTT assay. Each value is compared directly to α-syn + no treatment control using a series of Welch's t tests (N ≥ 3, biological replicates are shown as dots, bars represent means ± SEM, *p < 0.05, **p < 0.01). (C) Immunocytochemistry of neurons as treated in part B, one week following addition of α-syn/HtrA reactions. Endogenous phosphorylated-α-syn (green), tau (red), DAPI (blue). Scale bar, 20μm.

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