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# The size of protein aggregates in relation to their cellular toxicity

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# Foreword

When I started studying biology I was not sure about my choice. It was an exciting choice but in the end it seems that it has been one of the best choices I made in my life. The same goes for this thesis, when I started writing it I had no idea about whether I would produce something worthwhile. I have learned several things about myself during the time I worked on this project. I learned how important it is to be critical and not to leave things open to interpretation. I have also learned that things like writing take time, a lot of time. My planning is something that I can still work on in the future. All in all I think I can be satisfied with the outcome. Even though every time I read through this document, I see another detail that maybe could be improved. I guess that means I am ambitious? Thank you for reading my thesis, it means a lot to me.

# Summary

This thesis focuses on whether the fragmentation of protein aggregates, either amorphous aggregates of highly structured amyloid fibrils, is beneficial for cell survival as compared to not disaggregating these non-functional structures. Many neurodegenerative diseases show that protein aggregation plays a big role in their pathology, these diseases are collectively called “proteinopathies”. Alzheimer’s disease, Parkinson’s disease, Huntington’s disease and Amyotrophic Lateral Sclerosis are well-known proteinopathies. There has been a lot of research on each of these diseases to find a pathological mechanism that could possibly be exploited so that a cure could maybe be established. This research has up until this moment largely been in vain, since a successful cure for these diseases has not yet been found. In this thesis I will cover how protein aggregates form together with several mechanisms and machineries for the disaggregation of protein aggregates such as autophagy and the ubiquitin-proteasome system. Ultimately I will discuss whether the current literature agrees on whether fragmentation of protein aggregates is beneficial for cell survival or whether it has deleterious effects on cell survival. Finally, I will give my own opinion on the matter.

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# Introduction

Alzheimer's disease (AD) and Parkinson's disease (PD) are examples of a class of diseases termed neurodegenerative diseases. As of October 2022, a minimum of 55 million people were believed to be suffering from AD or other forms of dementia. It is expected that this number will almost double every 20 years if no cure is found and if no breakouts occur (National Institutes of Health, 2023). For Parkinson's, the prevalence has doubled in the past 25 years. On a global scale, disability and death due to PD are increasing at a rate bigger than for any other neurological disorder. Estimates made in 2019 suggest that PD caused 329.000 deaths, which is an increase of 100% since 2000 (World Health Organization, 2022). These neurodegenerative diseases are caused by toxic accumulations of tau and □-amyloid, and alpha-synuclein respectively in the brain, which then lead to neuronal cell death. The consequence of the death of these neurons is what is known as the disease (Goedert, 2015; Lashuel et al., 2012, 38-48; Iqbal et al., 2005, 198-210). These accumulations of protein are called protein aggregates, they can form from interactions between misfolded proteins (Holmes et al., 2014, 294-303).

Various molecular machines and mechanisms are capable of either removing protein aggregates or preventing the formation of these aggregates. Autophagy is one of the mechanisms responsible for eradicating aggregates. It describes the process of engulfing cargo in an autophagosome and degradation of cargo after fusion with a lysosome. Multiple types of autophagy exist, among others bulk autophagy, chaperone-mediated autophagy, and selective autophagy (Mizushima, 2007, 2861-2873; Mizushima et al., 2008, 1069-1075; Nakatogawa et al., 2009, 458-467). The ubiquitin-proteasome system is responsible for degrading single proteins in their linear form. It cannot degrade aggregates but is important for the prevention of aggregate formation (Cliffe et al., 2019, 2140-2149). This is similar to the role of molecular chaperones, which can also prevent formation of protein aggregates by aiding in the (re-) folding of proteins into their native state (Hipp et al., 2014, 506-514; Saibil, 2013, 630-642). Furthermore, specific molecular chaperones are capable of disaggregation, which entails untying aggregated proteins and fragmenting them (Shorter, 2011; Rampelt et al., 2012, 4221-4235; Song et al., 2013, 5428-5433; Mattoo et al., 2013, 21399-21411).

Not a lot is known about the effects of protein disaggregation. Is it preferable that protein aggregates are fragmented into smaller accumulations, or does this increase cellular toxicity? In this thesis I will discuss the main processes that are responsible for removal of protein aggregates and/or prevention of formation of these aggregates. Key proteins for these mechanisms will be mentioned and their role will be explained. I will also touch upon several diseases that arise when aggregate removal and prevention are dysregulated. Most importantly, I will focus on what the consequences of protein disaggregation are.

# Formation of protein aggregates

In healthy conditions, mRNA is translated into an amino acid sequence which then, via going through intermediate states, folds into a specific conformation to form a 3D structure known as a protein (Crick, 1958). However, there is also the possibility of an error occurring somewhere in this pathway of protein production. This can lead to the formation of a protein aggregate. More specifically, undesirable interactions between hydrophobic residues and surfaces will lead to the sequestering of other proteins into an aggregate (Lamark & Johansen, 2012; Vendruscolo et al., 2003, 1205-1222). These interactions can either be between hydrophobic surfaces of the same protein or with the exposed surface of another protein. Interactions can be initiated due to multiple causes, which include mutations, protein misfolding after translation, aberrant protein modifications, oxidative stress, failure in assembling protein complexes (e.g. transcriptional complexes) and incomplete translation which leads to the formation of defective ribosomal products (DRiPs) (Lamark & Johansen, 2012).

Protein aggregates are not functional and can have a toxic effect on cells depending on what type of proteins are included in the aggregate (Holmes et al., 2014, 294-303). A protein condensate can also be formed, these structures are, as opposed to aggregates, still functional. Protein condensate formation can result from protein misfolding, but can also be formed on purpose to fulfil a specific function. An example of such a functional protein condensate is the nucleolus, this nuclear condensate comprises proteins, DNA and RNA and functions mainly as the site for ribosome biogenesis (Scheer & Hock, 1999, 385-390).

# Proteinopathies

Proteinopathies are a class of diseases in which depositions of aberrant or structurally abnormal proteins are present (Walker & LeVine, 2000, 83-95; Chiti & Dobson, 2006, 333-366). Alzheimer's disease is probably the most widely-known proteinopathy, together with Parkinson's disease (Walker et al., 2015, 729-748; Ugalde et al., 2016, 162-180). Prions are a form of transmissible proteinopathies, these proteinopathies are highly infectious and require a protein as their main constituent (Legname & Moda, 2017, 147-156). Bovine Spongiform Encephalopathy (mad cow disease) is the most well-known prion disease that was especially prevalent between the years 1990 and 2000 and of which the first case was reported in the United Kingdom in the year 1986 (Pain, 1987). Since this thesis mostly focuses on the removal of protein aggregates in the case of non-prion-like disease, I will from now on only focus on AD and PD.

## Alzheimer's Disease

Alzheimer's Disease is a progressive neurodegenerative disease in which neurons slowly but progressively die over time. Neuronal cell death starts in the Hippocampus where it leads to memory loss in early stages of the disease, and at the later stages to symptoms such as delusionality, loss of speech and apathy (McKhann et al., 2011, 263-269). In AD, it is believed that there is an association between the formation of □-amyloid plaques and neurofibrillary Tau tangles, and the development of the disease (Bursavich et al., 2016, 7389-7409; Medina & Avila, 2014). Tau protein is an important constituent of microtubules and is thus important for the cytoskeleton of the cell. If Tau misfolds, it can aggregate into string-like accumulations termed neurofibrillary tangles (Wang et al., 2011, 83-95). □-amyloid is an oligomer which is cleaved off of Amyloid Precursor Protein (APP) by beta- and gamma secretase, it accumulates in the form of □-amyloid plaques (Hamley, 2012, 5147-5192). APP usually functions as a neuronal cell surface receptor where it is thought to, among others, regulate synapse formation (Bursavich et al., 2016, 7389-7409). The exact role that these aggregates play in AD development is still unknown.

## Parkinson's Disease

Parkinson's disease is a disease in which the motor area in the brain is impaired. It is also a progressive neurodegenerative disease where neurons die due to the formation of alpha-synuclein inclusion bodies in the brain stem (Ciccocioppo et al., 2020, 850-856). Alpha-synuclein is the most important component of Lewy Bodies, which are intracytoplasmic inclusion bodies that characterise synucleinopathies such as PD. Examples of other synucleinopathies are Lewy Body dementia (where Lewy Bodies accumulate in the cortico-limbic system and the brainstem) or Multiple System Atrophy (where alpha-synuclein aggregates in the basal ganglia, the brainstem, and the cerebellum) (Emamzadeh & Surguchov, 2018).

If we want to effectively remove and/or prevent protein aggregates in proteinopathies like AD and PD, we first need to understand how the associated aggregating proteins behave and how they are built-up. It could well be that alpha-synuclein, Tau tangles, and □-amyloid plaques share similarities in their mechanism of aggregation. A number of studies even suggest that there are biophysical and biochemical similarities between alpha-synuclein,

□-amyloid, and the major component of prions (Baker et al., 1993, 441-454; Hsiao et al., 1996, 99-103; Sturchler-Pierrat et al., 1997, 13287-13292).

# Autophagy

Macroautophagy (hereafter, autophagy) is one of the key players in the PQC system in eukaryotic cells, it describes the mechanism by which cellular components are degraded and recycled, this often occurs as a response to starvation to keep the cell alive or as a response to cell stress (bulk autophagy) (Wollert, 2019, 671-677). Selective autophagy is the form of autophagy which is not induced via starvation, but rather because a specific cargo needs to be degraded (Lamark & Johansen, 2012). In this section the mechanism of autophagy is explained from how it is induced up to fusion of the autophagosome and the lysosome to induce degradation of cargo.

## Autophagosome initiation

The process of autophagy requires the presence and subsequently the fusion of two main components, them being the lysosome and the autophagosome. The lysosome was first discovered in 1955 as a hydrolytic enzyme-containing organelle by Christian de Duve, who was a biologist and Nobel-Prize winner (de Duve et al., 1955, 604-618). By conducting electron microscopy studies, Novikoff and colleagues not much later observed that cells contain dense bodies that enclose cytoplasmic components; these dense bodies were actually lysosomes (Novikoff et al., 1956, 179-184). The formation of the second component, the autophagosome, is most important for the initiation of autophagy. The autophagosome is a double lipid-membrane vesicle, its creation involves a cascade of interactions between autophagy related (ATG) proteins (Zhou et al., 2020, 853-863; Bozic et al., 2020). Autophagosome initiation starts with the formation of a structure called the phagophore, the complete process of autophagosome initiation occurs at the endoplasmic reticulum at a structure called the “Omegasome” which envelops the cargo that is destined for autophagy (Graef et al., 2013, 2918-2931; Suzuki et al., 2013, 2534-2544; Axe et al., 2008, 685-701). Phagophore biogenesis, and thereby autophagosome initiation, specifically requires the ATG1/ULK kinase complex which consists of ULK1, FIP200, ATG13 and ATG101 and phosphorylates the PI 3-kinase complex consisting of BECLIN1, p150, ATG14 and VPS34 (Graef et al., 2013, 2918-2931). Under the conditions where nutrients are present in excess, this ATG1/ULK complex is inhibited by mTORC1, however as soon as starvation is imminent mTORC1 is inhibited by AMPK which leads to the activation of the ATG1/ULK kinase complex and thus phagophore biogenesis (Kamada et al., 2000, 1507-1513). This process is also described in figure 1.

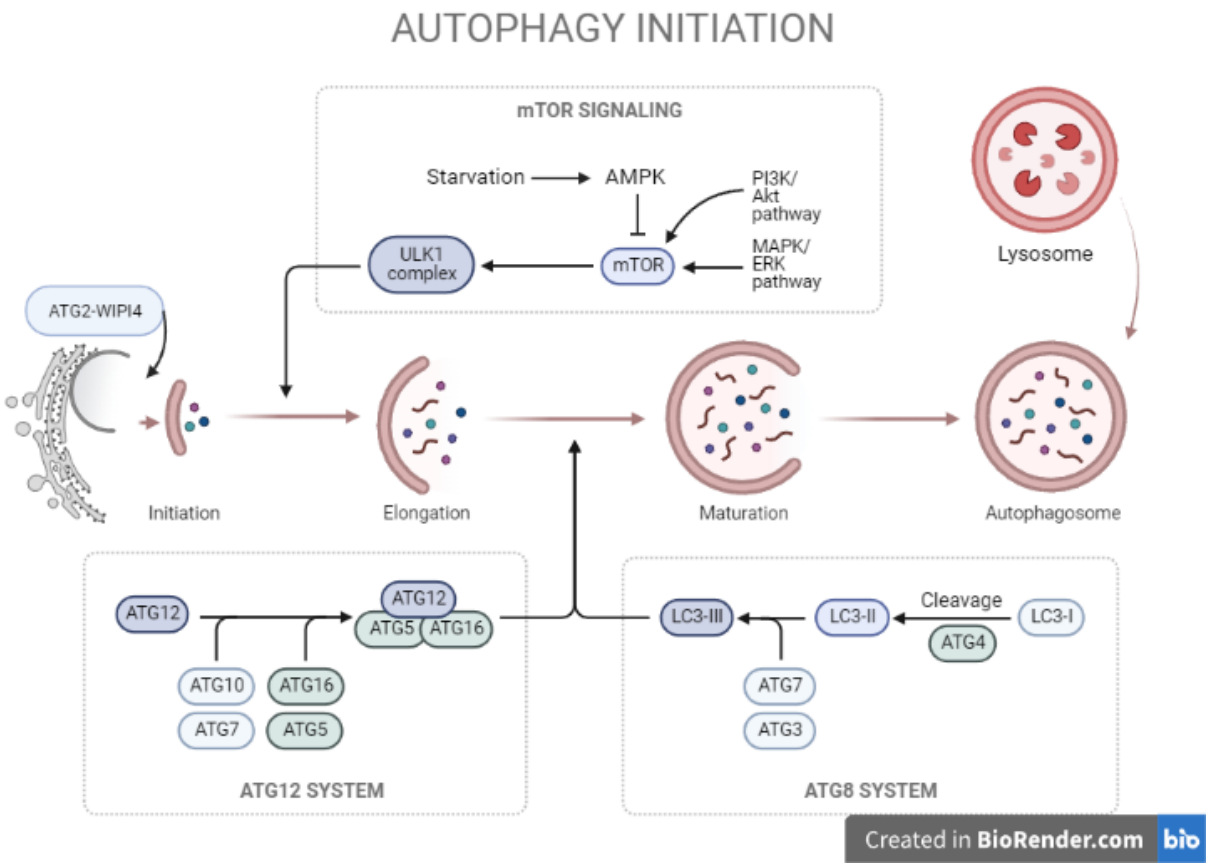



Figure 1.
This figure describes the initiation pathway of autophagy in the case of bulk autophagy. The figure was created with BioRender.com.

## Phagophore elongation

After initiation of phagophore biogenesis, elongation of the phagophore is required. This is mediated via ATG2-WIPI complexes and two ubiquitination-like systems (Chowdhury et al., 2018, 9792-9801). Current literature speculates that the ATG2-WIPI4 complex plays an important role in the connection between the omegasome and the forming phagophore by tethering PI3P-containing and non-PI3P-containing membranes (Chowdhury et al., 2018, 9792-9801; Otomo et al., 2018). It is hypothesised that ATG2 binds to the phagophore edge at the N-terminus of the protein while a highly-conserved, specific cysteine-alanine-aspartic acid triad (CAD-domain) on the other end of the protein binds WIPI4 which in turn then binds PI3P to connect the ATG2-WIPI4 complex to the omegasome (Chowdhury et al., 2018, 9792-9801). However Chowdhury et al. also conclude that more research is required to make a definitive statement regarding the role of ATG2-WIPI4 complexes in phagophore biogenesis. Dooley et al. have shown that WIPI2 is another important factor in phagophore elongation, specifically for recruitment of the LC3-conjugating ATG12-5-16L1 complex. This complex is required for LC3 lipidation which stimulates correct double-membrane autophagosome formation. WIPI2 is mostly involved in starvation induced autophagy and binds to PI3P bound to the phagophore, WIPI2 then recruits ATG16L1 + ATG5 + ATG12. After recruitment of this complex, ATG3 binds to the complex and LC3 binds to ATG3

(Dooley et al., 2014, 238-252). Lipidation of LC3 can now take place. Of note: the mechanism of action of WIPI2 described above occurs in starvation-induced autophagy.

## Autophagosome completion and autolysosome formation

The two ubiquitination-like systems are critical components for the recruitment and conversion of LC3, which, after the activation of the PI 3-kinase complex, binds to a DFCP1-PI3P conjugate (Nakatogawa et al., 2007, 165-178). LC3 is a protein that is part of the LC3 subfamily (LC3A, -B, -C), which is yet again part of a larger subfamily called ATG8 homologues together with the GABARAP subfamily and GABARAPL2/GATE-16 (Shpilka et al., 2011; Bozic, 2020). ATG8 homologues thus share similarities regarding their role in autophagy. Besides being a key player in the autophagy mechanism, LC3 is also an autophagosomal marker. Recruitment and conversion of LC3 first requires a conjugation reaction between ATG12 and ATG5, which leads to the formation of an ATG5-ATG12-ATG16L structure. This structure then undergoes another conjugation reaction to link the LC3 subfamily to phosphatidylethanolamine (PE) and connect them to the phagophore membrane (Mizushima et al., 1998, 395-398). LC3 is known to further assemble the phagophore until complete and transport the correct cargo into the phagophore during the phagophore elongation step by acting as specific surface receptor proteins on both sides of the phagophore membrane (Nakatogawa et al., 2007, 165-178). Subsequently the complete autophagosome containing cargo is transported to the microtubule-organising centre (MTOC) along microtubules, at the MTOC the autophagosome fuses with either a late endosome or a lysosome via SNARE proteins to finalise the autophagy process (Johnston et al., 1998, 1883-1898).

## Selective autophagy

There are multiple forms of selective autophagy, in the case where protein aggregates act as cargo this is called aggrephagy. This section will focus on several important receptors in selective autophagy, which are for instance p62 and NBR1 which can bind to LC3 and other ATG8 homologues and mediate the process of selective autophagy through interacting with targeted substrates (Kirkin et al., 2009, 505-516; Pankiv et al., 2007, 24131-24145). P62 is an example of a soluble selective autophagy receptor (SAR), it binds to ubiquitinated cargo and is therefore ubiquitin dependent. When bound to the ubiquitinated cargo, p62 also binds NBR1 and the relevant ATG machinery for phagophore biogenesis, autophagosome completion and ultimately autolysosome formation (Pankiv et al., 2007, 24131-24145). TAX1BP1 is another soluble SAR which is ubiquitin-dependent, it binds ubiquitinated aggregates to tag them for autophagy, is recruited by NBR1 and recruits FIP200 (Kirkin et al., 2009, 505-516; Lamark & Johansen, 2021, 143-169; Bjørkøy et al., 2005, 603-614 Turco et al., 2021). FIP200 interacts with NBR1 and p62 bodies so that it stays close to the aggregate/condensate; it also recruits autophagy machinery for the formation of the autophagosome and therefore induces autophagy initiation (Turco et al., 2021). Alfy is a facilitator protein for SARs and is suspected to aid in the formation of p62 bodies as a coreceptor for ubiquitin-dependent autophagy initiation (Clausen et al., 2010, 330-344). According to literature, Alfy is required for recruitment of ubiquitinated proteins to p62 bodies and therefore an important stimulator for aggrephagy as well (Clausen et al., 2010, 330-344).

Through successful aggrephagy, protein aggregates are degraded and recycled. This naturally is beneficial for patients who suffer from proteinopathies such as Alzheimer's, Huntington's and Parkinson's and the goal should therefore be to optimise the functionality of this specific form of autophagy. However, optimisation of autophagy in general should be done with extreme caution, for dysregulated autophagy has been shown to be associated with the pathogenesis of cancer and neurological disorders (Yang & Klionsky, 2020, 858-871). The process of selective autophagy can be seen in figure 2.

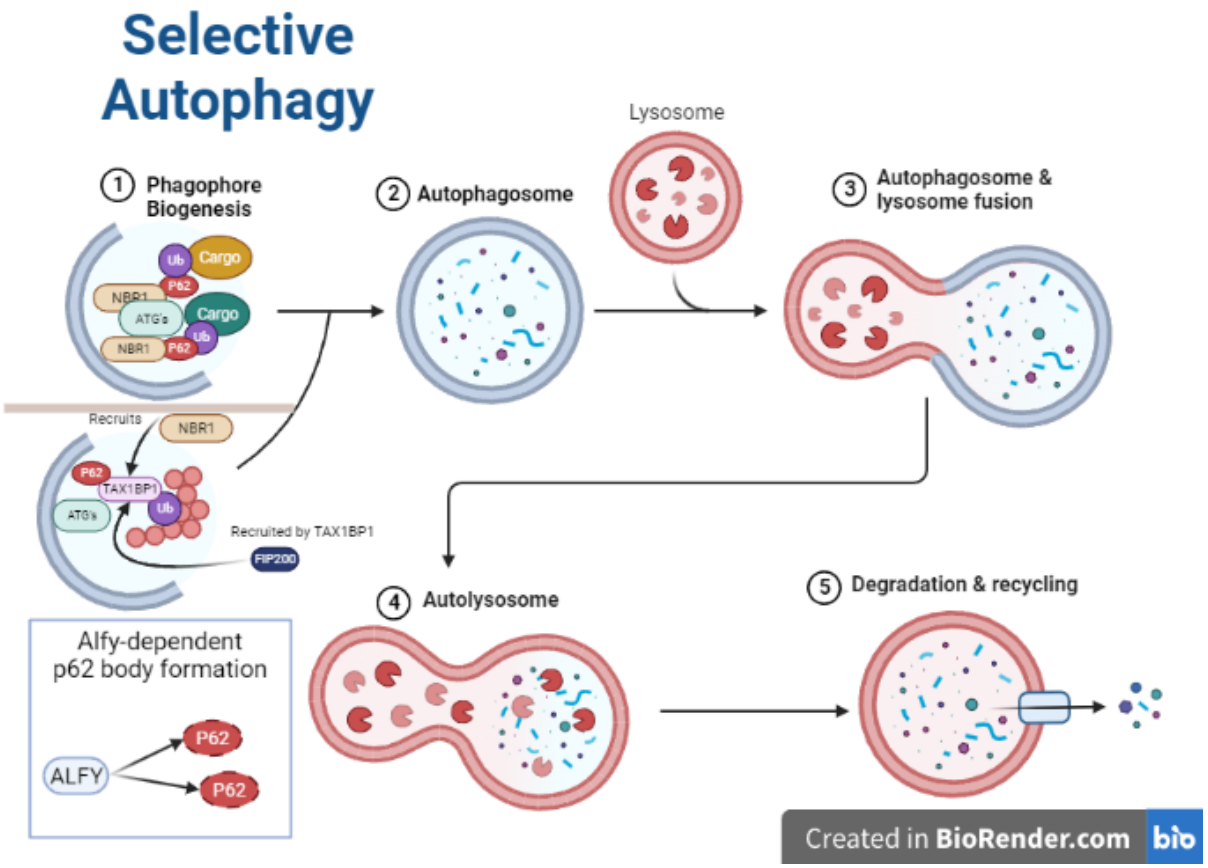



Figure 2.
This figure shows the mechanism of selective autophagy. The phagophore initiation can be induced via NBR1 or via TAX1BP1 in the case of protein aggregates as cargo. In the bottom left you can see the Alfy-dependent formation of p62 bodies. This figure was created with BioRender.com.

# Removal and prevention of protein aggregates

Protein disaggregation entails the fragmentation of protein aggregates. Removal and prevention of aggregates or aberrant proteins can occur along various pathways such as autophagy (bulk autophagy), selective autophagy (aggrephagy), microautophagy, chaperone-mediated autophagy (CMA), the ubiquitin-proteasome system (UPS), and via interactions with molecular chaperones such as HSP70 and DNA-J proteins (Mauthe et al., 2022, 216-228; den Brave et al., 2020). It is important that this removal is well-regulated, for a dysregulated or impaired protein degradation mechanism may trigger increased protein aggregate formation and sequestration which can have toxic effects on a cell (Holmes et al., 2014, 294-303). In this section I will mostly focus on the various activities of protein aggregate-removal and -disaggregation machinery and the fate of the proteins that are subjected to disaggregation. A visual representation of these processes can also be found in figure 3.

## Autophagy

Multiple forms of autophagy are important for protein aggregate removal, these include CMA, bulk autophagy, selective autophagy, microautophagy but also chaperone-associated selective autophagy (CASA). As stated above, bulk autophagy is mostly activated via inhibition of the mTORC in conditions of cell starvation which leads to phosphorylation of the ATG1/ULK kinase complex, the latter also applies to selective autophagy (in this case aggrephagy). SARs such as NBR1 and p62 bind ubiquitin which is bound to the target cargo and with aid of molecular chaperones (e.g. HSP70) or facilitator proteins (e.g. Alfy), the client aggregates are translocated to autophagosomes and possibly endosomes in the case of selective microautophagy (Mauthe et al., 2022, 216-228). Important to note: the target cargo does not necessarily have to be amorphous or amyloid protein aggregates but can also be a single, linear protein. Ubiquitinated cargo is a prerequisite for aggrephagy since it acts as a signal and a substrate for SARs, membrane-bound SARs are not relevant for aggrephagy but only in other forms of selective autophagy when the cargo contains a membrane (e.g. mitophagy) (Mauthe et al., 2022, 216-228).

CMA and CASA slightly deviate from the classical autophagy pathway in that they are more dependent on the activity of molecular chaperones for their mechanism of action. CMA is highly dependent on heat shock cognate 70 (HSC70) and HSP70 in a way that HSC70 recognises and subsequently binds a specific KFERQ peptide sequence which proteins may exhibit, then HSP70 aids in the translocation of this protein into lysosomal lumens (Kaushik & Cuervo, 2018, 365-381). This KFERQ motif consists of five peptides which are respectively lysine, phenylalanine, glutamic acid, arginine, and glutamine. The sequence always has an adjacent glutamine on one of the sides and is required but sufficient for CMA. This means we can conclude that CMA is a fully ubiquitin-independent form of autophagy. HSC70 is a molecular chaperone with a size of 71 kDa that aids in protein folding of unfolded or misfolded proteins, it does so by binding to the hydrophobic regions of the un- or misfolded proteins. As opposed to binding hydrophobic regions for the facilitation of correct protein folding, HSC70 binds KFERQ-like motifs to tag proteins that are destined for degradation (Kaushik & Cuervo, 2018, 365-381). Besides in CMA, HSC70 plays an important role in two other forms of autophagy in mammals; them being endosomal microautophagy and CASA. In endosomal microautophagy, HSC70 binds KFERQ-like motifs

to target proteins for degradation, which is similar in CMA. However, for CASA this is different, here HSC70 cooperates with BAG1 and BAG3, which are part of the co-chaperones BAG family molecular chaperone regulator 1 to induce autophagy (Arndt et al., 2010, 143-148). I will come back to CASA in a later section.

Translocation across lysosomal membranes in CMA is achieved via multimerised channels consisting of the lysosomal integral membrane protein 2A (LAMP2A), and once in the lysosome, the cargo is hydrolysed by degradative enzymes (Kaushik & Cuervo, 2018, 365-381). Cargo is internalised into lysosomes via binding of the HSC70-substrate complex to LAMP2A and subsequent unfolding of the substrate. Then the substrate is translocated through the CMA translocation complex and degraded by hydrolases in the lysosome lumen, this process is mediated by lysosomal HSC70 (Cuervo & Dice, 1996, 501-503). LAMP2A is solely required for lysosomal docking of HSC70 in CMA and not for CASA or endosomal microautophagy, which makes it an excellent marker for determining whether protein degradation occurs via CMA (Kaushik & Cuervo, 2018, 365-381). LAMP2A is encoded for by the LAMP2 gene, it is one of the three splice variants of the gene, the other two being LAMP2B and LAMP2C (Eskelinen et al., 2005, 1058-1061).

With the use of all these components, CMA has been shown to play a role in the prevention of protein aggregation. CMA cannot degrade protein aggregates, but can degrade single proteins that are damaged or (partially) unfolded (Cuervo et al., 2004, 1292-1295). This suggests that CMA acts more as a first barrier of defence against protein aggregation rather than a mechanism of protein aggregate removal. Proteostasis failure can be a result of CMA blockage or increased stressors such as oxidative stress or lipid challenge, this leads to increased protein aggregation in tissues that are CMA incompetent (Schneider et al., 2015, 249-264). However, blockage of CMA does not necessarily lead to loss of proteostasis in every case. In the liver for instance, upregulation of (selective) autophagy and the UPS will compensate for the degradation of the increased amount of CMA substrates (Schneider et al., 2014, 417-432). CMA is therefore important, but not strictly required for the prevention of protein aggregation. Compensatory systems are able to take over when necessary.

CASA is a type of selective autophagy that, as stated before, depends on the cooperation between HSC70 and BAG3. The mechanism is initiated by the chaperone-associated ubiquitin ligase CHIP, which ubiquitinates proteins that should be degraded, and by p62 binding to the ubiquitinated proteins (Arndt et al., 2010, 143-148). Thereafter, BAG3 coordinates the activity of HSC70 and the small heat shock protein HSPB8, which together bind and translocate the client towards the site of autophagosome initiation. This mainly takes place at a stress-induced inclusion body called the aggresome which is located near the MTOC, protein aggregates are deposited here by the CASA complex (contains HSPA, STUB1-BAG3, and HSPB8) before autophagosome initiation (Johnston et al., 1998, 1883-1898; Tedesco et al., 2023, 1619-1641). Actual formation of the autophagosome in CASA is dependent on BAG3 interacting with Synaptopodin-2 (Arndt et al., 2010, 143-148). Degradation of the cargo is achieved via either fusion of the autophagosome with a lysosome, or upon entering of the substrate into the proteasome (Tedesco et al., 2023, 1619-1641). Once the protein aggregates are degraded and fragmented, remaining single, linear proteins or small peptide chains can be refolded and/or recycled.

CASA and the CASA complex can induce degradation of misfolded proteins so that the products can be recycled (Tedesco et al., 2023, 1619-1641). It can therefore prevent the aggregation of proteins to an extent, and thus contributes to PQC. HSPB8 and BAG3 expression has also been shown to increase in the presence of aggregation-prone proteins, likely stimulating autophagic removal of aggregating substrates (Crippa et al., 2010, 3440-3456). However, it can not degrade already aggregated proteins and is thus not relevant for protein aggregate degradation. Mutant BAG-3 can even aggregate and impair CASA, which in one case lead to the onset of myofibrillar myopathy (Lee et al., 2012, 394-398).

## The ubiquitin-proteasome system

The UPS is another major degradation system in the cell that can take care of amorphous or amyloid protein aggregates and also conglomerates. The 26S proteasome is probably the most important structure in the UPS, it consists of multiple protein complexes that together form one cylindrical structure that is capable of degrading single, linear proteins (Lamark & Johansen, 2012). The 20S particle is the core particle of proteasomes, where the degradation of clients occurs, it assembles into a functional 26S proteasome with two 19S regulatory particles (Tanaka, 2009, 12-36). Essential for degradation is that the 19S substructure of the proteasome undergoes a conformational change which allows the ubiquitinated protein to enter the proteasome and be degraded. This conformational change can be compared to a lid opening up and closing again. Recognition of clients destined for degradation by the proteasome is achieved via tagging the substrates with a minimum of 4 polyubiquitin moieties. Ubiquitin proteins contain 7 lysine residues (K6, K11, K27, K29, K33, K48, and K63) which can be linked to form polyubiquitin chains (Komander, 2009, 937-953; Ikeda & Dikic, 2008, 536-542). In most cases, K48-linked polyubiquitin chains represent the tag for proteasomal degradation. However, K11-linked chains and, in some cases, K63-linked chains also function as tags for degradation by the proteasome (Wong & Cuervo, 2010). Proteases are the other key players in the UPS, these enzymes aid in preparing the substrates for degradation by ubiquitinating them and transporting the substrates to the proteasome. After degradation the remaining products can, again, be recycled and used for the synthesis of new proteins.

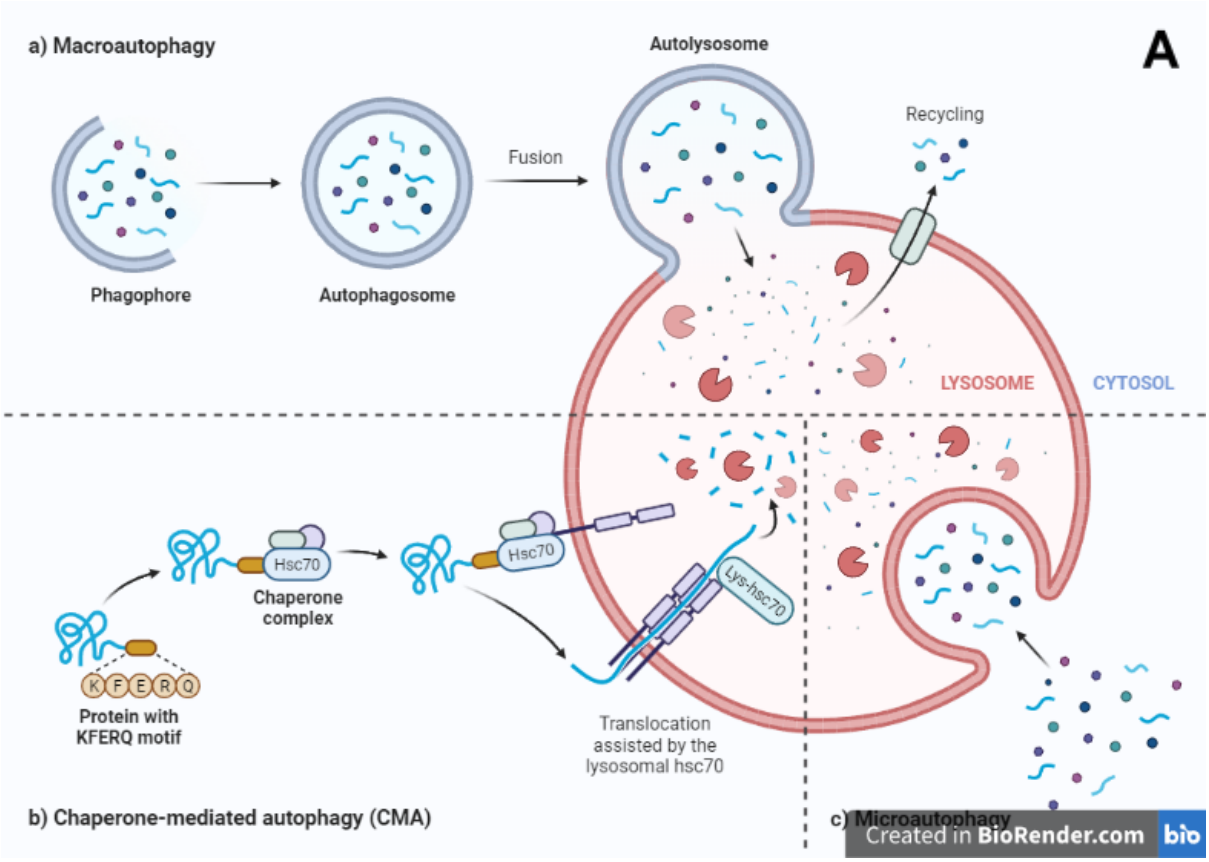
Since the UPS only degrades linear proteins in their unfolded, soluble state, it does not contribute to aggregate degradation in any way. It does however, similar to CMA and CASA, contribute to protein aggregation prevention by degrading damaged, soluble proteins. It has been shown that mutants of both alpha-synuclein and Cu,Zn-superoxide dismutase (SOD1) are degraded by the proteasome in mice, substantiating the hypothesis that the UPS contributes to the prevention of protein aggregation (Webb et al., 2003, 25009-25013; Kabuta et al., 2006, 30524-20533).

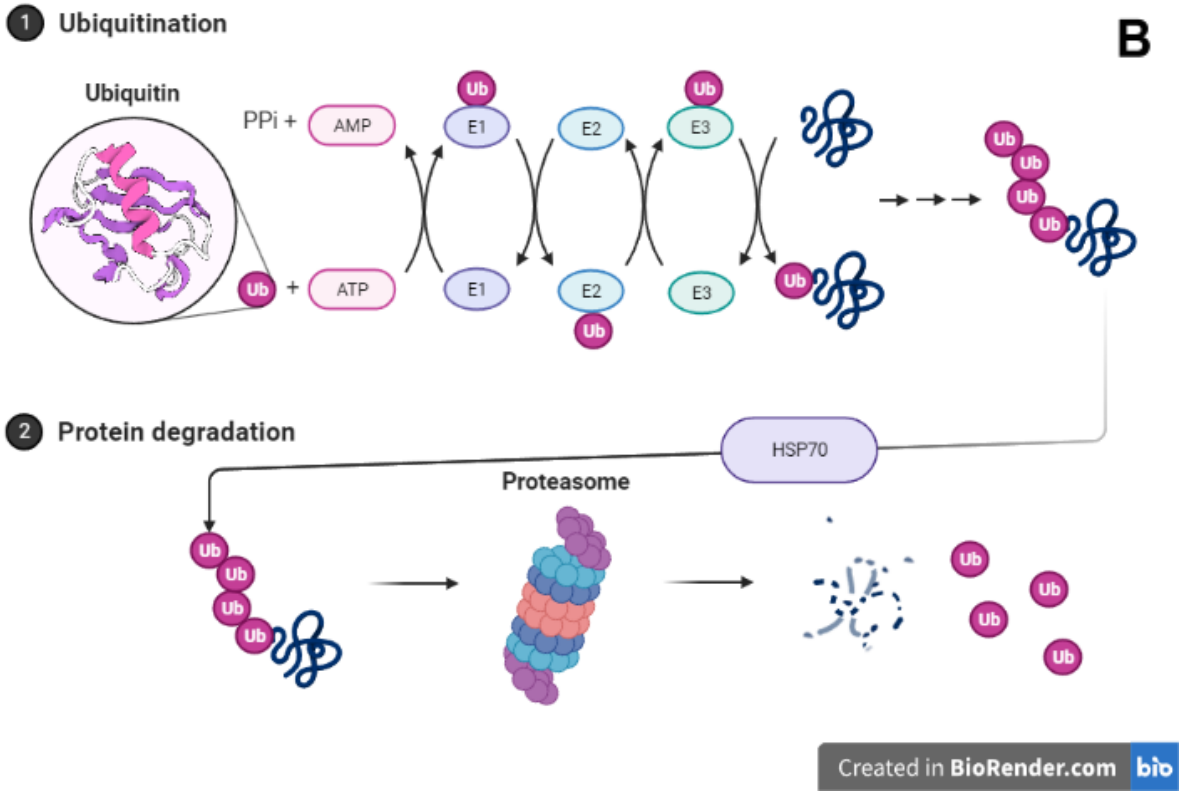
## Molecular chaperones

Molecular chaperones such as the heat shock proteins and various co-chaperones play an important role in the PQC system. Although chaperones (e.g. HSP70 and the HSP90 complex) and co-chaperones (e.g. Cdc37) do not possess the ability to degrade protein aggregates, they can interfere with the process of aggregation by stimulating refolding of misfolded or unfolded proteins, this is mostly done by HSP90 (Pratt et al., 2010, 278-289). If

a protein is rendered misfolded or not properly processed after possible interactions with molecular chaperones and co-chaperones, the proteins are delivered to the endoplasmic reticulum for ER-associated degradation (ERAD), where the proteins are degraded mainly by the UPS. This delivery mechanism for degradation depends on the activity of the molecular chaperone HSP70 (Nakatsukasa et al., 2008, 101-112). Until degradation of the substrate takes place, an associated BAG6 complex mediating chaperone holdase activity is required to keep the ERAD substrates unfolded but soluble (Wang et al., 2011, 758-770). Degradation of misfolded proteins that cannot be refolded is generally done by the UPS, CMA and/or selective autophagy as mentioned earlier (Tanaka, 2009, 12-36).
In the case of degradation by the UPS, misfolded proteins are recognised by the ER luminal HSP70 homologue BiP/Grp78 and translocalisation of the substrates into the cytoplasm is mediated by a complex of p97/VCP and Derlin-1 (Lilley & Ploegh, 2004, 834-840; Ye et al., 2004, 841-847). Once in the cytoplasm, E3 ubiquitin ligases such as Hrd1 and gp78 ubiquitinate the proteins that need to be degraded (Hirsch et al., 2009, 7237). P97/VCP also mediates segregation of ubiquitinated mitochondrial outer membrane proteins and nuclear or cytoplasmic protein complexes by the UPS (Tanaka et al., 2010, 1367-1380). We can therefore conclude that p97/VCP is a key player in the mechanism of client degradation via the UPS.
For CMA, CASA, and selective autophagy it has been shown that it is mostly HSP70 and HSP90 in combination with Cdc37 that are important for the PQC of newly synthesised proteins and for adequate transport of substrates to the degradation machinery (Mandal et al., 2007, 319-328). LAMP2A is a key player in CMA, as was mentioned before (Kaushik & Cuervo, 2018, 365-381). ALFY, p62 bodies and NBR1 are important for respectively recruitment of p62 bodies and tagging of substrates for degradation by selective autophagy (Clausen et al., 2010, 330-344; Kirkin et al., 2009, 505-516). CASA specifically requires CHIP for ubiquitination of substrates, BAG3 and HSPB8 for client translocation, and p62 bodies for cargo tagging (Lamark & Johansen, 2012; Arndt et al., 2010, 143-148).

The molecular chaperones that are addressed above mainly play a role in the refolding of aberrant proteins. There are several other PQC machines that are important for the disaggregation of protein aggregates specifically. The HSP100 molecular chaperones possess the ability to disaggregate a wide variety of substrates including pre-amyloid oligomers, phase-transitioned gels, but also amorphous aggregates and prions (Shorter & Southworth, 2019). These proteins are encoded for by the HSP100 family genes (Fassler et al., 2021). HSP104 is an example of such a disaggregase in yeast, it contains two nucleotide binding domains that use energy from ATP hydrolysis to disassemble protein aggregates by disentangling proteins from these amorphous structures (Deville et al., 2017; Gates et al., 2017, 273-279). The disaggregation occurs via translocation of the trapped substrate through the axial channel of HSP104, this gives the opportunity for the substrates to refold into their correct state after dissociation with HSP104 and ultimately leads to the removal of the protein aggregate (Glover & Lindquist, 1998, 73-82; Goloubinoff et al., 1999, 13732-13737). In metazoans, no form of HSP104 or ClpB (a bacterial disaggregase) is present. Instead, cooperation of molecular machines such as HSP110, HSP70, HSP40, DNAJ-proteins and small heat shock proteins (sHPS) is required for disaggregation of amorphous aggregates and amyloid fibrils (Schneider et al., 2021; Saha et al., 2023; Scior et al., 2017, 282-299; Nillegoda et al., 2015, 247-251). By disentangling these protein aggregates efficiently, molecular chaperones can contribute significantly to removal of protein aggregates.


a) Macroautophagy
Autolysosome
A
Recycling
Fusion
Phagophore
Autophagosome
LYSOSOME
CYTOSOL
Hsc70
Chaperone complex
Hsc70
Lys-hsc70
K F E R Q
Protein with KFERQ motif
Translocation assisted by the lysosomal hsc70
b) Chaperone-mediated autophagy (CMA)
c) Microautophagy
Created in BioRender.com bio


1 Ubiquitination
B
Ubiquitin
PPi +
AMP
Ub
E1
E2
E3
Ub + ATP
2 Protein degradation
HSP70
Proteasome
Created in BioRender.com bio

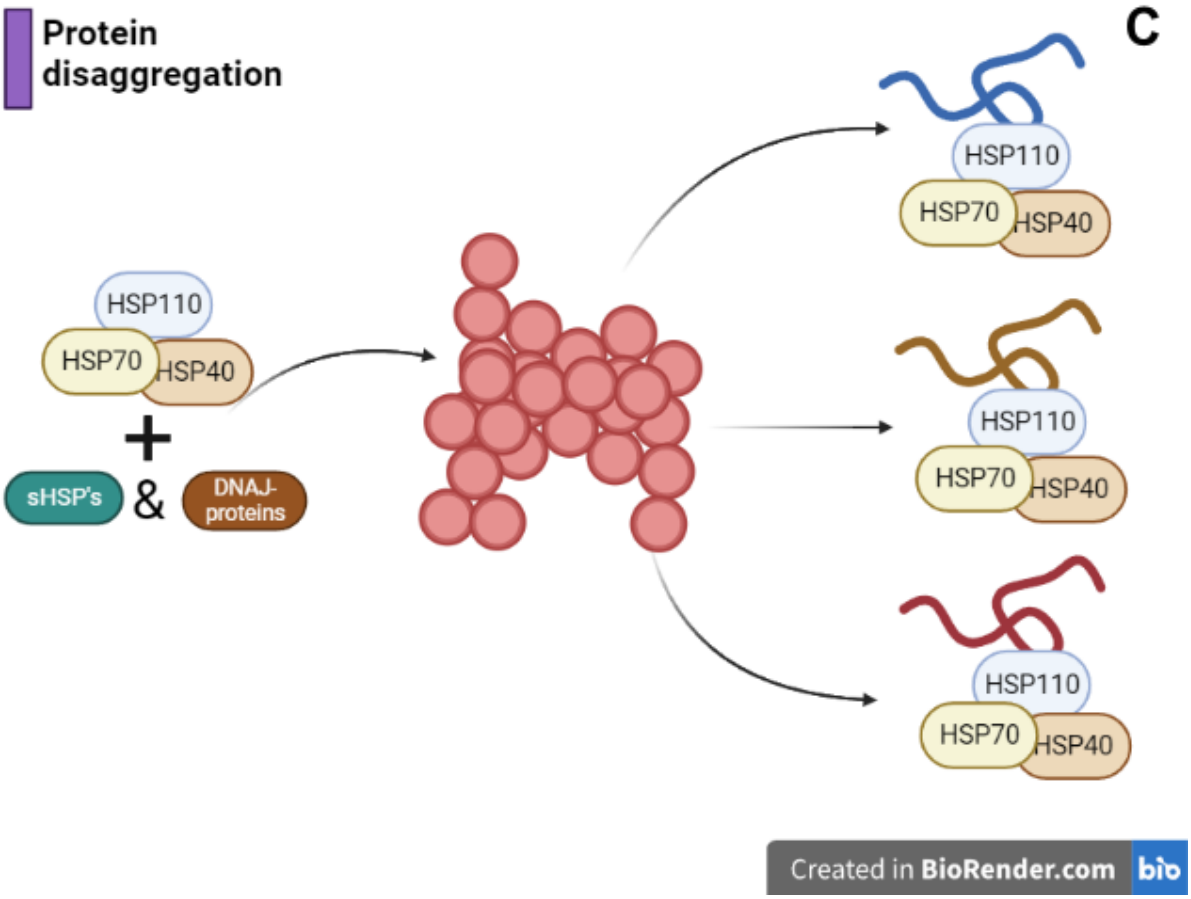



Figure 3.
a) Three types of autophagy are shown, CASA works in a similar way to macroautophagy. b) The mechanism of the UPS is visualised here. c) The process of protein disaggregation is shown, this depends on cooperation of various molecular chaperones. This figure was created with BioRender.com.

# Is aggregate fragmentation beneficial or unfavourable for cells?

An array of aggregate degradation and disaggregation mechanisms has been discussed in the sections prior to this one. Among them are autophagy, molecular chaperones, and the UPS. Up until this point I only addressed possible mechanisms that possess disaggregating properties, there was no focus on whether this would be favourable for a cell or not. This is what will be discussed in the following sections. Does protein aggregate disaggregation yield benefits for cell survival? Or is it preferable to leave the aggregates in their larger, non-fragmented state?

Huntington's Disease comes forth from aggregating proteins that gain a toxic function and subsequently damage neurons, however Arrasate et al. (2004) claim that the formation of inclusion bodies in the brain reduces levels of mutant huntingtin. Besides, they claim that the formation of inclusion bodies in neurons leads to a reduction in neuronal cell death (Arrasate et al., 2004, 805-810). In this study, among other things, PolyQ expansion was induced in neurons and neurons were tested for inclusion body levels related to diffuse huntingtin levels elsewhere in the cell. Results of these experiments yielded that the levels of diffuse huntingtin rapidly fell after an inclusion body was formed, which does not seem very striking. It would be expected that diffuse huntingtin levels reduce after formation of an inclusion body, since the mutant huntingtin now probably resides in the inclusion body. Furthermore, results yielded that neurons which formed inclusion bodies in either the nucleus or the cytoplasm exhibited a decrease in the risk of death compared to neurons with the same PolyQ expansion which did not form inclusion bodies (Arrasate et al., 2004, 805-810). This would suggest that mutant huntingtin inclusion bodies induce a beneficial effect for cell survival. However, in a different figure the opposite is shown. Of note: this result is less significant. Various other studies show that formation of these inclusion bodies promotes neurotoxicity and thereby neuronal cell death, instead of inclusion bodies being protective for cells (Kontopoulos et al., 2006, 3012-3023; Kadowaki et al., 2005, 19-24; Weston et al., 2022). Specifically the formation of nuclear inclusion bodies induces neurotoxicity and cell death, this result was obtained by using neuroblastoma cells transfected with alpha-synuclein that localises to the nucleus (Kontopoulos et al., 2006, 3012-3023). Similar findings have been reported in the case of extended polyglutamine protein inclusion body formation, where these inclusions also promote cellular toxicity and have adverse effects on cell survival (Warrick et al., 1998, 939-949).

The concept of functional amyloid structures has been introduced by Stefani & Dobson (2003) and Uversky & Fink (2004). These structures appear to have no association with the development of proteinopathies and might have functional properties for cells. It is important to appreciate that most of these functional amyloid structures, with the exception of one, are present in either yeast or prokaryotes. The intraluminal domain of Pmel17 is the non-pathological amyloid-like fibril in humans. It functions to stimulate growth of melanin granules upon fibrous striations within melanosomes (Chiti & Dobson, 2006, 333-366). The conclusion that can be drawn from this study is that amyloid fibril-like structures can be used as functional entities in living systems, there is one such a structure that has functional properties in humans.

It has also been reported that fragmented protein aggregates trigger cell lysis (Cliffe et al., 2019, 2140-2149). This study tested whether cell lysis would be an effect of adding a toxic protein aggregate to the extracellular environment in which cells resided. Indeed, this experiment was done *in vitro* and yielded that fragmented tau aggregates resulted in significantly higher levels of cell lysis compared to when cells were exposed to intact aggregated tau protein (Cliffe et al., 2019, 2140-2149). This therefore suggests that aggregate fragmentation is more toxic to cells than the presence of intact aggregates *in vitro*. Tittelmeier et al. (2020) have shown *in vivo* that HSP110/HSP70 disaggregation machinery produces toxic alpha-synuclein species that exhibit prion-like behaviour due to their ability to spread. This again substantiates that fragmentation of protein aggregates is less desirable regarding cellular toxicity than the presence of intact, unfragmented aggregates. The organism that was used for this study was *C. elegans* (Tittelmeier et al., 2020).

Removal of protein aggregates is beneficial for mitigating the effects of neurodegenerative disease. In the case of AD, it has been shown that autophagy ameliorates the effects of disease in a human AD brain by clearing □-amyloid aggregates (Harris et al., 2020, 755-766). Cheng et al. (2020) found that a microglial knockout of ATG5 led to disturbed cognitive recognition and impairment of motor coordination. This phenotype is resemblant of the phenotype in PD. Besides, it has been reported that mutation of alpha-synuclein induces dysregulated autophagy (Senkevich & Gan-Or, 2020, 60-71). This strongly relates to PD development and progression. We can therefore also conclude that removal of these aggregates is beneficial for cell health.

Collectively, these results suggest that fragmentation of protein aggregates is not beneficial for cell survival and appears to increase cellular toxicity. Important to note is that the results discussed above show little to no association with humans (Arrasate et al., 2004, 805-810; Uversky & Fink, 2004, 131-153; Cliffe et al., 2019, 2140-2149). Also, the study that focused on the relation between increased inclusion body levels and decreased risk of cell death shows contradictory results (Arrasate et al., 2004, 805-810). Furthermore, the results show that removal of these protein aggregates is beneficial for cell survival. Therefore I find the conclusion that fragmented protein aggregates increase cellular toxicity compared to large, intact aggregates, quite a plausible one.

# Concluding remarks

The aggregation of proteins into non-functional amorphous aggregates or amyloid fibrils, can lead to the development of a variety of proteinopathies. Alzheimer's disease, Parkinson's disease, Huntington's disease and ALS are four of the most well-known proteinopathies that all involve a different aggregating protein in their pathology. Formation of protein aggregates is induced by interactions between misfolded proteins (Vendruscolo et al., 2003, 1205-1222). Hydrophobic groups that generally are oriented inward of a correctly folded protein, are now exposed and interact with other hydrophobic groups from other proteins (Holmes et al., 2014, 294-303; Vendruscolo et al., 2003, 1205-1222). These interactions make proteins "stick together" and form an aggregate. Various cellular mechanisms exist to eradicate or prevent these protein aggregates, among which autophagy, a molecular chaperone network, and the UPS.

Protein disaggregation, and thereby fragmentation, appears to be a natural cellular response to the formation of aggregates. However, various literature suggests that this response is not optimised and therefore leads to enhanced toxicity of fragmented protein aggregates (Cliffe et al., 2019, 2140-2149; Chiti & Dobson, 2006, 333-366). Some studies even posit that larger, intact aggregates are more beneficial to cell survival than fragmented ones (Arrasate et al., 2004, 805-810; Uversky & Fink, 2004, 131-153). I personally support the hypothesis that fragmented aggregates are more harmful to cell health and survival, than larger, intact aggregates. The idea of functional amyloid was proposed by Uversky & Fink (2004), however this was focused mainly around yeast and prokaryotes. Up until now, one functional amyloid structure exists in humans and therefore we cannot conclude that functional amyloid is a relevant concept in humans. More evidence is required to substantiate this hypothesis. Similar conclusions can be drawn based on a study that tested beneficial aspects of inclusion bodies on cell survival (Arrasate et al., 2004, 805-810), and a study that tested levels of cell lysis induced by fragmented tau aggregates *in vitro* (Cliffe et al., 2019, 2140-2149) and *in vivo* (Tittelmeier et al., 2020).

To finalise, it seems we can conclude that the fragmentation of protein aggregates (either amorphous or in the form of amyloid fibrils) leads to increased cellular toxicity and thereby impaired cellular health. However, most results that were discussed are not relevant for humans and correlations between results are not always overly significant. Protein disaggregation is a concept that should be focused on more in research for the upcoming years. It strikes me as a potential target for therapeutics yet we do not know enough about it at this moment to be able to use it to our advantage. By performing experiments that focus even more on the differences in cellular health between cells with fragmented aggregates versus intact aggregates, we might uncover more about whether aggregate size is associated with cellular toxicity.

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