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Understanding the dual nature of lysozyme: part villain – part hero

A *Drosophila melanogaster* model of lysozyme amyloidosis

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Cover: "Owl of Wisdom", Lysozyme WT (red) together with SAP (green)

During the course of the research underlying this thesis, Linda Helmfors was enrolled in Forum Scientium, a multidisciplinary doctoral program at Linköping University, Sweden.

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This too shall pass

Amyloid proteins are a distinct class of proteins that can misfold into β -sheet rich structures that later mature to form the characteristic species known as amyloid fibrils, and accumulate in tissues in the human body. The misfolding event is often caused by mutations (or outer factors such as changes in pH) that destabilize the native protein structure. The mature amyloid fibrils were initially believed to be associated with diseases connected to protein misfolding such as Alzheimer's disease (AD), Parkinson's disease, transthyretin amyloidosis and lysozyme amyloidosis. However, now it is known that many different factors are involved in these diseases such as failure in protein clearance, lysosomal dysfunction and formation of intermediate misfolded protein species, which possess cytotoxic properties, preceding the formation of mature fibrils.

In this thesis the amyloidogenic protein lysozyme has been examined *in vivo* by using *Drosophila melanogaster* (fruit fly) as a model organism. The effects of over-expressing human lysozyme and amyloidogenic variants in *Drosophila* have been investigated both in the absence and presence of the serum amyloid P component (SAP), a protein known to interact with amyloid species. In addition, the role of lysozyme in AD has been investigated by co-expressing human lysozyme and amyloid β in *Drosophila*.

The lysozyme protein is an enzyme naturally found in bodily fluids such as tears, breast milk and saliva. It is engaged in the body's defense and acts by hydrolyzing the cell wall of invading bacteria. Certain disease-associated point mutations in the gene encoding lysozyme destabilize the protein and cause it to misfold which results in systemic amyloidosis. To investigate the *in vivo* misfolding behavior of lysozyme we developed and established a *Drosophila* model of lysozyme amyloidosis. SAP is commonly found attached to amyloid deposits in the body; however, the role of SAP in amyloid diseases is unknown. To investigate the effect of SAP in lysozyme misfolding, these two proteins were co-expressed in *Drosophila*.

The amyloid β peptide is involved in AD, building up the plaques found in AD patient brains. These plaques trigger neuroinflammation and since lysozyme is upregulated during various inflammation conditions, a possible role of lysozyme in AD was investigated by over-expressing lysozyme in a *Drosophila* model of AD. Interaction between lysozyme and the amyloid β protein was also studied by biophysical measurements.

During my work with this thesis, the dual nature of lysozyme emerged; on the one hand a villain, twisted by mutations, causing the lysozyme amyloidosis disease. On the other hand a hero, delaying the toxicity and maybe the neurological damage caused by the amyloid β peptide.

Populärvetenskaplig sammanfattning

Proteiner är viktiga byggstenar i naturen. De bygger upp hud, hår, muskler och organ, påskyndar kemiska reaktioner i kroppen och transporterar till exempel syre och andra molekyler i kroppen. Proteiner byggs upp av aminosyror, liksom pärlor på ett halsband, och den ordning de sitter i avgörs av DNA. DNA är den livskod som finns i varje cell och som beskriver när och hur ett protein skall tillverkas. För att proteinerna ska kunna utföra sina funktioner är det viktigt att de antar rätt form, eller rätt veckning; halsbandet med aminosyrorna ska bli som ett tagliatelle-nystan. Mutationer i DNA kan leda till att en eller flera aminosyror byts ut och detta kan i sin tur leda till att proteinerna felveckas. Om de muterade proteinerna tillhör en särskild klass av proteiner, kallade amyloida proteiner, kan felveckningen leda till att proteinerna aggregerar och bildar fibrer.

I det här arbetet studeras framförallt ett särskilt protein, lysozym, som finns i tårar, saliv och blod. Lysozyms roll i kroppen är att agera som ett första steg i immunförsvaret och förstöra cellväggen hos bakterier. Lysozym är förknippat med en sjukdom som kallas lysozym-amyloidosis och det är en sjukdom som drabbar hela kroppen. För lysozym finns det sju kända mutationer som leder till att proteinet felveckas och bildar amyloida fibrer. Dessa fibrer klumpar ihop sig och fastnar på inre organ som till exempel lever, njurar och tarmar vilket leder till att organen inte kan fungera. Dessa stora klumpar av fibrer dekoreras i princip alltid av ett protein som heter serum amyloid P component (SAP) och detta protein får också mycket uppmärksamhet i den här avhandlingen, där SAPs roll i sjukdomsbilden undersöks.

För att kunna studera sjukdomar utanför provröret (*in vivo*) är det mycket praktiskt att använda en modellorganism, och i det här arbetet användes bananflugor (*Drosophila melanogaster*). Bananflugor används flitigt i proteinforskning på grund av deras korta generationstid, den enkelhet med vilken man kan manipulera deras gener samt att de ger sjukdomssymptom som påminner om de man återfinner hos människan, vilket gör dem mycket väl lämpade som modellsystem.

I den här avhandlingen presenteras den första *Drosophila melanogaster* modellen för lysozym-amyloidosis. Modellen bidrar med kunskap om sjukdomsförloppet, de bakomliggande mekanismerna och kan i framtiden användas för att testa olika behandlingar mot amyloidosis. Genom att lägga till SAP har modellen för lysozym-amyloidosis ytterligare utvecklats. Arbetet i den här avhandlingen visar att SAP kan ha en missuppfattad roll i sjukdomsbilden, istället för att som man tidigare trott binda till färdigbildade fiberklumpar, är det möjligt att SAP binder till felveckat protein i ett tidigt skede och kan hjälpa till att vecka det rätt.

När lysozym uttrycks i dubbel uppsättning i flugorna, dels som normalt så kallat vild-typs protein och dels som en muterad variant (F57I), ansamlas amyloida klumpar i flugorna och de blir mycket sjuka. Detta resultat ger en ökad förståelse för hur lysozym-amyloidosis kan utvecklas och ger ytterligare möjligheter till att studera de intrikata mekanismer som orsakar sjukdomen. I mitt arbete har jag även visat att lysozym kan ha en skyddande roll i en annan amyloidossjukdom, Alzheimers sjukdom, där mina försök visar att lysozym motverkar celldöd orsakat av proteinet amyloid β som är involverad i denna sjukdom. Detta öppnar upp ett nytt sätt att hitta strategier för att kunna bota Alzheimers sjukdom.

List of papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals:

Paper I. Kumita JR*, Helmfors L*, Williams J, Luheshi LM, Menzer L, Dumoulin M, Lomas DA, Crowther DC, Dobson CM and Brorsson AC,

Disease-related amyloidogenic variants of human lysozyme trigger the unfolded protein response and disturb eye development in *Drosophila melanogaster* (2012), FASEB J 26, 192-202

(* These authors contributed equally to this work)

Paper II. Helmfors L, Bergkvist L and Brorsson AC,

SAP to the rescue: Serum amyloid p component ameliorates neurological damage caused by expressing a lysozyme variant in the central nervous system of *Drosophila melanogaster*.
Under revision for resubmission to FEBS Journal

Paper III. Bergkvist L, Helmfors L and Brorsson AC,

Co-expression of a disease-associated lysozyme variant with human lysozyme in *Drosophila melanogaster* causes amyloid deposits and neurodegeneration. Progress report

Paper IV. Helmfors L, Armstrong A, Civitelli L, Sandin L, Nath S, Janefjord C, Zetterberg H, Blennow K, Garner B and Brorsson AC*/Kågedal K*

A protective role of lysozyme in Alzheimer's disease. Pending submission

(* These authors contributed equally to this work)

Paper I:

Linda Helmfors (LH) performed several of the experiments, participated in analyzing the results and participated in the writing of the manuscript.

Paper II:

LH participated in the planning of the project, performed all experiments except ER-stress quantification and analyzed the results. LH was also the main author of the manuscript.

Paper III:

LH participated in the planning of the project, analyzed the results and participated in writing the manuscript.

Paper IV:

LH participated in the planning of the project, performed all the fly work and the MSD experiments. LH participated in analyzing the data. LH participated in writing the manuscript.

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AA	amyloid A amyloidosis
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
AP	alkaline phosphatase
A β PP	amyloid β precursor protein
ATF6	activating transcription factor 6
A β	amyloid β
BSA	bovine serum albumin
CNS	central nervous system
CRP	C-reactive protein
CSF	cerebrospinal fluid
CT	computerized tomography scan
DAM2	<i>Drosophila</i> activity monitor 2
DNA	deoxyribonucleic acid
EGFP	enhanced green fluorescent protein
eIF2 α	eukaryotic translation initiation factor 2 α
EL	equine lysozyme
ELISA	enzyme-linked immuno-sorbent assay
ER	endoplasmic reticulum
ERAD	ER associated degradation
GI	gastrointestinal
HEWL	hen egg white lysozyme
HRP	horseradish peroxidase
HSP	heat-shock protein
IHC	immunohistochemistry
Ire1	inositol requiring kinase 1
LCO's	luminescent conjugated oligothiophenes
LDH	lactate dehydrogenase
MRI	magnetic resonance imaging scan
MSD	meso scale discovery
NFTs	neurofibrillary tangles
PERK	protein kinase RNA-like ER kinase
PET	positron emission tomography scan
PFA	paraformaldehyde
RNA	ribonucleic acid
SAP	serum amyloid P component
SEM	scanning electron microscopy
TEM	transmission electron microscopy
ThT	thioflavine T
T-tau	total-tau
UAS	upstream activation sequence
UPR	unfolded protein response

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Five years have passed since I started working for Anki in the fly-lab with the mission to set up a *Drosophila melanogaster* model of lysozyme amyloidosis. We have struggled with assays and inconclusive results in a never-ending search for amyloid fibrils. If I had known that it would take five years to find the first fibrils I am not sure I would have made it through, luckily, I didn't know and I have happily gone to work every day.

To work in the Department of Physics, Chemistry and Biology at Linköping University, is to be part of an interdisciplinary community where biochemists, biologists and organic chemists cooperate and collaborate. This open environment gives young scientists the opportunity to grow and form networks, not unlike the amyloid "networks" (fibrils) studied in this work, but decidedly non-toxic.

This thesis is focused on the protein lysozyme, which is associated with the disease lysozyme amyloidosis. This very rare misfolding disease has no cure at present, and the mechanism driving the disease progression is not well known. My project aimed at describing the disease using fruit flies to further our understanding of the events underlying the disease. Eventually, it is our hope to be able to provide a treatment for patients suffering from lysozyme amyloidosis. Here I present the dual nature of lysozyme, a villain causing disease in papers I-III and a hero providing comfort and expanding lifespan in paper IV. The introduction is intended to give the reader an understanding as to what lies behind these different sides of the same protein.

With the work that this thesis presents I feel like I have laid down a good foundation for further work on lysozyme amyloidosis and how different amyloidogenic proteins interact. I pass the baton to my successor Liza with my best wishes. In the words of Sir Winston Churchill "Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning."

Linda Helmfors, Linköping, April 2014

1.1 Proteins

Proteins are all around us, they are the building blocks and machinery of life and without them our world would be nothing but water and rock. Proteins are made up of amino acids, like beads on a string, where each bead is an amino acid; this is the primary structure of a protein. There are 20 natural amino acids and they can be integrated into proteins in an infinite amount of combinations. The various amino acids have different properties such as size, polarity, hydrophobicity and charge. Depending on how these amino acids, the beads, interact with each other, the secondary structure is formed. The secondary structure is most commonly divided into elements called α -helix, β -sheet, turns and random coil (1). The amount and order of these elements determine the three-dimensional shape of the protein.

As proteins exist in all areas of life they must also fill a whole host of different roles; proteins make up, for example, our hair, nails, skin, muscles and organs. Beyond the surface, proteins work as catalysts, enzymes, to speed up/enhance/facilitate chemical reactions in the body. Proteins transport oxygen in the bloodstream, carry metal ions, help fight disease (in the form of antibodies), replicate DNA and control cell signaling. Proteins are the cell surface receptors by which cells communicate with one another. The latest release of the human protein knowledge database neXtPro holds (at the time of writing this) 20 135 protein entries, which maybe gives an idea of the huge amount of functions carried out by proteins.

The blueprint for a protein is found in the deoxyribonucleic acid (DNA), which is the material that makes up the genome. Craig Venter et al. (2) published the sequence of the human genome in its entirety in 2001. The DNA consists of the four nucleotides (bases): guanine, adenine, thymine and cytosine. Through base-pairing (i.e. adenine - thymine and guanine - cytosine), chains are formed and are held together two and two arranged into a double helix formation (3). The DNA nucleotides are transcribed in triplicate by RNA polymerase and transformed into ribonucleic acid (RNA). This RNA molecule is translated into a primary protein structure by a ribosome complex (4). The ribosome is formed by a large subunit (50S) and a small subunit (30S) and the RNA molecule, called mRNA, is threaded through the middle, facilitating the interaction with tRNAs carrying amino acids covalently bonded to them (4). The tRNA molecule base-pairs with the mRNA molecule and the correct amino acid is added to the growing protein chain until all of the triplicates in the mRNA have been read. The translation is terminated and the protein is released from the ribosome.

1.2 Protein folding and misfolding

With its amino acids like beads on a string, the protein chain folds into its tertiary structure, not unlike a tagliatelle bundle. For any given protein, the number of possible conformations is determined by its amino acid sequence. Each conformation has a certain free energy. Plotting of all free energies versus their corresponding conformations yields a characteristic energy

landscape, with high (unstable) and low (stable) energy states (Fig 1). The landscape is often likened to a funnel because the conformational space accessible to the unfolded protein is reduced as the correct folding is approached (5). The driving force behind the folding is a search for the lowest resting energy, as the protein travels further down the funnel of possible conformations the free energy is lowered. Some protein sequences have a high propensity to form a given type of secondary structure and thus such elements of the protein fold develop early in the folding process, and may be observed as essentially fully formed even before the overall structure is finished (5). Protein folding and unfolding is mostly an “all or none” process that results from a cooperative transition (4). If part of the protein becomes destabilized (due to mutation or unfavorable conditions) and starts to unfold, the interactions between that part and the rest of the protein will be lost. The loss of these interactions will destabilize the rest of the protein, resulting ultimately in total unfolding.

Figure 1. Protein folding energies illustrated with a funnel.

Proteins have to be folded correctly to be able to perform their function as intended; however, misfolding is a common and natural occurrence for proteins. Misfolding can be instigated by mutations or environmental factors (6) or simply due to the complexity of the folding process (7). The natural response in the cell to a misfolded protein is to isolate and degrade it or to inhibit its formation, however, these mechanisms might be unable to cope with large amounts of misfolded protein (8).

Protein misfolding diseases can be divided into three subgroups; i) inability to fold, for example cystic fibrosis; ii) mislocalization due to misfolding, for example familial hypercholesterolemia and the last subgroup; iii) toxic fold, where the amyloid diseases fall (7, 9, 10).

1.2.1 Mutations

Mutations are not necessarily evil; mutation is the mechanism that produces the genetic variation through which evolution moves forward. For instance, the gene FOXP2, which is connected to speech and language, is suggested to be the target of selection during human evolution. Mutations in the FOXP2 gene may be behind the development of human speech and thus one of the things that set us apart from other primates (11).

Mutations occur when the DNA code is changed by one or more nucleotide, and can be divided into hereditary or somatic. Hereditary mutations take place in germ line cells, and the mutation is carried through to generations to come. Somatic mutations take place in non-reproductive cells and are not passed onto offspring. Spontaneous mutations can occur directly as a consequence of replication errors or indirectly due to chemical damage to DNA leading to errors in the correct reading of the damaged DNA (12). The DNA can also be damaged through exposure to ionizing radiation, causing either breaks to the DNA helix, or causing the production of free electrons. Ultraviolet radiation is absorbed by DNA bases and is of enough energy to induce chemical reactions within the DNA helix, causing disruption in the base-pairing (12).

1.3 Amyloid

Diseases such as Alzheimer's disease, Parkinson's disease and lysozyme amyloidosis are named amyloid diseases. Each disease is associated with a specific protein that misfolds and aggregates into amyloid deposits. Around 30 different proteins are connected to amyloid diseases. The reason behind misfolding and aggregation is not always clear, and the mechanism varies depending on which protein is involved. However, the amyloid deposits all share common characteristics, the most prominent being the presence of cross- β -sheets in which the peptide strands are perpendicular to the long axis of the fibril (13). All polypeptide chains have the ability to form β -sheet rich fibrils independent of amino acid sequence, but the conditions required to initiate the formation may differ, for example, high protein concentration and the intrinsic aggregation propensity (9). It has been found that many proteins without any connection to disease, including common proteins such as myoglobin (14), can give rise to fibrillar structures with all the characteristics of those found associated with the clinical amyloidoses. This suggests that the ability to form amyloid fibrils is a generic property of polypeptide chains (15).

An amyloid fibril is defined as "a protein that is deposited as insoluble fibrils, mainly in the extracellular spaces of organs and tissues as a result of a sequence of changes in protein folding that results in a condition known as amyloidosis." according to the Nomenclature Committee of the International Society of Amyloidosis (16).

Another defining feature of amyloid fibrils is that they bind the dye Congo red and give rise to red-green birefringence under the microscope, which means that they first appear red and when the polarization of the microscope is changed they instead appear green (16). A novel

method for detecting amyloid fibrils is to use luminescent conjugated oligothiophenes (LCOs); these molecular dyes spectrally discriminate between plaques of different maturity, different protein deposits and can probe fibril formation in either a fluorescent microscope or using fluorescent spectroscopy (17, 18).

There are a few hypotheses regarding the dangerous species of amyloids; at first the long mature fibers were believed to be the toxic species (19). Since then the smaller dimers and trimers were considered the most toxic species (20). The current understanding in the field implies that oligomers (21) are the most toxic species; however, the presence of mature fibers and plaques is in no way healthy as they can put external pressure on the structures to which they are attached. In Alzheimer’s disease patients, oligomers and the accumulation of plaques and neurofibrillary tangles lead to blockage of the synapses in the brain making long term potentiation difficult or even impossible (20). For patients with lysozyme amyloidosis, the internal organs can be weighed down by up to kilograms of amyloid material, which eventually leads to organ rupture and frequently subsequent death (due to severe blood loss) (22). A general attribute of protein misfolding diseases is the prolonged time before clinical manifestations appear (13). The toxicity of lysozyme and amyloid β ($A\beta$) will be further discussed in sections 1.5 and 1.8.2 respectively.

1.3.1 General mechanism of amyloid formation

The first step toward amyloid formation (Fig 2) is destabilization of the native monomer; this can either be due to a mutation or due to external influences, causing it to unfold slightly (9). This destabilization initiates a so-called nucleation event, where the monomer undergoes rearrangement into a β -sheet rich partly folded molten globule (23). This first part of the

Figure 2. The process of amyloid formation exemplified by lysozyme. Blue, β -sheet structure; red, helical structure; dotted lines, undefined structure. Adapted from (23).

aggregation process is called the lag phase and the nucleation is the rate-limiting step in the aggregation reaction (24).

Figure 3. The fibrillation of a protein followed by ThT fluorescence.

The formed molten globule self-associates through the β -domain and sets in motion fibril formation, this is the growth phase (23). The plateau is reached when a mature fibril is formed and a balance between association and dissociation of monomers is reached (25) (Fig 3). This can be followed by the fluorescent probe Thioflavine T (ThT), where increased fluorescence at 480 nm correlates with increased fibril formation (26). The folding landscape from before can be completed with the amyloid species, and as hydrophobic forces promote aggregation a low resting energy can be seen for the amyloid fibrils (Fig 4).

Figure 4. The folding landscape completed with the species formed on the amyloidogenic pathway. Adapted from (29).

The formed amyloid fibril is characterized by a cross- β X-ray diffraction pattern, with a structural repeat of $4.6\text{\AA}$ along the fiber axis corresponding to the spacing of adjacent β -strands and a $9.8\text{\AA}$ spacing perpendicular to the fiber axis corresponding to the face-to-face separation of the β -sheets. The fiber is held together by hydrogen bonding between the β -strands (27, 28).

1.4 Lysozyme amyloidosis

Several mutations in the gene coding for human lysozyme are connected to non-neuropathic systemic amyloidosis; which is a hereditary disease where large amounts of lysozyme amyloid deposits are formed, ultimately leading to death (30, 31). These deposits can be found in vital organs such as the upper gastrointestinal (GI) tract, throughout the colon and in the kidneys. The deposits cause organ failure, frequently through organ rupture (31, 32). Table 1 outlines the known disease-associated mutations and their respective symptoms.

Table 1. Lysozyme mutations and clinical symptom, adapted from (30).

Mutation	Ethnic background	Clinical symptom	Affected organ	Reference
Y54N	Swedish	Diarrhea, weight loss, abdominal pain, sicca syndrome	GI, eye	(30)
I56T	English	Petechiae, nephropathy	Kidney, liver, spleen, skin	(33)
F57I	Italian	Nephropathy	Kidney	(34)
W64R	French	Sicca syndrome, intestinal infarction, nephropathy, GI hemorrhage, abdominal pain	GI, kidney, liver, salivary gland, eye	(35)
D67H	English	Nephropathy, GI hemorrhage	GI, kidney, liver, spleen	(32, 36)
D68G	American	Nephropathy	Liver	(37)
T70N/W112R	German	GI bleeding, nephropathy	GI, kidney	(38)

In one of the first reported cases of lysozyme amyloidosis (36), a 15-year old boy was presented, he had abdominal pain that progressively got worse; at age 13 he had got a diagnosis of amyloidosis. The patient was in very bad condition and his liver function deteriorated and an emergency liver transplant was needed; however, as lysozyme is produced by macrophages throughout the body, this would only be a temporary solution. Upon removing the liver, several immunohistochemical studies were performed and ultimately lysozyme was found to be a major constituent of the amyloid.

The case described above is unusual, it is more common that patients suffering from lysozyme amyloidosis do not know that they have the disease until it has progressed very far; they generally do not experience any symptoms until they are 50-60 years old. It is not unusual that patients seek care because they experience problems with their kidneys, such as painful urinations and/or blood in the urine, GI symptoms or sicca syndrome (also known as Sjögren’s syndrome, an autoimmune disease) (30). One patient had suffered from violent diarrhea for several years before seeking help (30). Another patient was found to have proteins in the blood (proteinuria) during a routine examination; she was otherwise healthy and her proteinuria and renal function did not worsen, at a one year control they were found to have remained stable (32).

For patients with the D67H variant, it has been demonstrated that, even though the patients are heterozygous for the mutation, the amyloid deposits are composed solely of the full-length variant protein with no contribution of the wild type (WT) protein (23). T70N is a polymorphism found in a population study in England with 0.05% frequency in a normal population, however, no connection has been found between T70N alone and lysozyme amyloidosis (34, 39).

One way of diagnosing lysozyme amyloidosis is to perform a scintigraphy scan, a test where radio labeled reporters, in this case ^{125}I -labeled SAP, are injected and the emitted radiation is captured by an external detector to form an image (40). The image from the scintigraphy reveals amyloid deposits as dark/black areas in the body. These deposits are then biopsied and antibodies are used to determine which protein they consist of, in this case lysozyme. Figure 5 shows a scintigraphic scan from a 51-year-old English woman at her time of diagnosis and amyloid deposits can be seen in the liver, spleen and kidneys of the patient.

Figure 5. Posterior whole-body scintigraphic image after intravenous injection of ^{125}I -labeled SAP. Amyloid deposits (black) observed in liver, spleen and kidneys. Reprinted with permission from (32). Copyright © 1999, Oxford University Press.

Lysozyme amyloidosis is such a rare disease that it is often underdiagnosed or even misdiagnosed, however, there is great need to precisely determine the underlying protein causing the disease as different types of amyloidosis (e.g., serum amyloid a protein, amyloid light chain, transthyretin, lysozyme) can produce similar visceral involvement but prognosis and treatment are completely different (41).

1.5 Lysozyme

Sir Alexander Fleming discovered lysozyme in 1922 when he noticed that nasal mucus from a patient with a head cold inhibited growth of bacteria on an agar plate, and lysozyme has since become one of the most well studied proteins known to man (42). Lysozyme was the first enzyme to have its X-ray crystallographic structure determined (43).

Lysozyme is a 14 kDa glycosidase enzyme that consists of two subunits, one predominantly α -helical and one mainly β -sheet and it has four disulphide bonds, one of which links the two subunits (Fig 6).

Lysozyme is produced in hematopoietic cells where it is found in granulocytes, monocytes and macrophages. Lysozyme is found in bodily fluids such as tears, saliva, blood and breast milk. The normal concentration in plasma is between 4-13 mg/l (44). In the body, lysozyme

Figure 6. The structure of lysozyme with known mutations marked as spheres, non-disease in green and amyloidogenic in red. Pdb structure 2ZIL.

works as the first line of defense against a bacterial attack through its action as a 1,4-β-N-acetylmuramidase cleaving the glycosidic bond between the C-1 of N-acetylmuramic acid (Mur NAc) and the C-4 of N-acetylglucosamine (Glc NAc) in the peptidoglycan layer of the bacterial cell wall (Fig 7) (45, 46).

Figure 7. The enzymatic activity of lysozyme.

Lysozymes are well conserved throughout nature and can be found in mammals, reptiles, insects and plants (45, 47). Folding of lysozyme takes place in the oxidizing environment of the endoplasmic reticulum (ER) before secretion by the Golgi apparatus. The disease-associated deposits are extracellular, making it probable that the amyloidogenic variants are synthesized correctly and secreted in significant quantities (48). The disease-associated variants are functional as enzymes when they are monomeric, meaning that they are still able to break down cell walls as a part of our innate immunity (49). *Ex vivo* fibrils of D67H that were dissolved in 6M GuHCl and refolded in water, resulted in lysozyme monomers that were enzymatically active (23).

In vitro studies of variant lysozyme suggest that amyloid formation is a consequence of a reduction in the native state stability relative to the WT protein, resulting in the population of a transient, partially unfolded species, and eventually the formation of amyloid fibrils (Table 2) (52).

Table 2. Lysozyme variants and native-state stability based on thermal denaturation, reported in (50, 51).

Lysozyme variant	Native-state stability (°C)
F57I	60.4 ± 1.1
I59T	70.1 ± 1.3
D67H	66.0 ± 2.0
Wild type	79.2 ± 1.4

However, there appears to be a balance between being too destabilized and cleared by the quality control system of the cell, and being destabilized enough to populate the transiently formed partially unfolded species necessary to form fibrils. The variant T70N is only slightly destabilized compared to WT and does not appear to form fibrils *in vivo*, suggesting that the stability range from which fibrils can result is quite narrow (9, 48, 52). In addition to reducing the stability of the protein, the amyloidogenic mutations reduce the cooperativity of the folding process (48). In the partially folded intermediary species, the β -domain and the adjacent C-helix (Fig 2, p4) are cooperatively unfolded while the rest of the protein remains in a native-like state (52). The mutations I56T, W64R and F57I are located in the hinge region between the α - and β - domains, whereas D67H and T70N are located in the long loop of the β domain (53). Indeed, the residue I56 is critical for the structural integrity of the lysozyme fold and connects the two domains together (23). The disruption between the α - and β -domain is a crucial event in deciding the amyloidogenicity of the lysozyme variants (52). A three-stage process makes up the pathogenic behavior of the amyloidogenic lysozyme variants. First, the variants fold well enough to evade the quality control system and be secreted in significant amounts into the extracellular space. Second, the variants decrease the stability and cooperativity of the protein, making it possible for the protein to unfold and populate an intermediate state. And third, the partly folded, intermediate species that is formed is highly aggregation prone. Taken together these events lead to the formation of amyloid fibrils (48).

Using equine (EL) and hen egg white (HEWL) lysozymes, many important conclusions regarding *in vitro* cytotoxicity can be drawn. Monomeric and fibrillar equine lysozyme does not affect the viability of cell lines (i.e. primary murine neurons, primary fibroblasts and neuroblastoma cells), but soluble amyloid oligomers cause cell death of the same cell lines (54). The cytotoxicity of lysozyme oligomers depend on their size (54).

Studies with HEWL have shown that oligomers and fibrils act in different ways to induce cell death; oligomers induce apoptosis-like cell death and fibrils lead to necrosis-like death (55). In addition to the different pathways for cell death, oligomers and fibrils also elicit responses in different time-scales; samples exposed to fibrils react with a faster and less specific response (55).

1.5.1 Lysozyme in *Drosophila*

Drosophila flies express seven different forms of intrinsic lysozyme in different parts of the

body; for example, LysP is expressed in the adult salivary gland, LysD is expressed in midgut of larvae and adults, LysS is mainly expressed in the intestines and LysX is expressed in the midgut wall. The lysozymes in *Drosophila* are not upregulated as response to bacterial exposure (as in humans), but rather, presumed to aid in the digestion of bacteria that are ingested through food due to the expression of the lysozymes in the digestive tract. One of the lysozymes, LysX is expressed at two times, right before and right after pupariation and may be involved in clearing bacteria from the larval gut before metamorphosis (56, 57).

1.6 Serum Amyloid P Component

Serum amyloid P component (SAP) is a glycoprotein that is produced in the hepatocytes in the liver and circulates in the blood of humans at concentrations $\sim 40 \mu\text{g/ml}$ (58). SAP (also known as PTX2) is a part of the pentraxin family, their major function is to bind microbial pathogens or cellular debris during infection and inflammation and contribute to the clearance of pathogens through complement activation (59). No deficiency of SAP has been reported in humans (60). The pentraxin family is highly conserved throughout nature and evolution and is named from the Greek words penta (five) and ragos (berries) based on its shape (Fig 8) (61, 62). Pentraxins are characterized by the cyclic pentameric structure and calcium-dependent ligand binding (63). The SAP molecule consists of five identical 23kDa subunits, each of 204 amino acids and circulates in the serum as a single pentamer (64); however, two pentamer rings are able to interact face-to-face (65).

Figure 8. The pentameric structure of SAP. Pdb structure 1SAC.

The most well studied pentraxin is C-reactive protein (CRP), which shares 60% sequence homology with SAP (60). CRP and SAP both bind two Ca^{2+} through two overlapping Ca^{2+} -binding sites on each subunit (65).

CRP is an acute phase protein, which SAP is not, and is upregulated in response to inflammation (62). The prototype pentraxin PTX3, one of the long pentraxins, and the short pentraxins SAP and CRP recognize non-overlapping ligands (66). SAP, but not CRP, binds to

histones, chromatin and DNA (67-69). More specifically, SAP binds to and stabilizes DNA in chromatin that has migrated to the extracellular space due to apoptosis or necrosis, thereby protecting it from degradation (70). In the absence of SAP, aggressive degradation of exposed chromatin may enhance its immunogenicity (71). Mice with a homozygous knock-down of the SAP gene developed spontaneous autoimmunity with autoantibodies against chromatin, DNA and histones from 3 months with an acute inflammation of the kidneys. However, this did not lead to increase in mortality or shortened lifespan (71, 72).

SAP and CRP are able to activate the classical pathway of the complement system through C1q binding (69). The complement system is made up of more than 30 proteins and the activation leads to a chain reaction of enzymatic activity and a range of physiological responses (73). Pathogens are recognized by the complement system and opsonized, which facilitates phagocytosis (74). The complement system was first believed to work only as a part of the innate immunity where a rapid response against pathogens is required; it has since been established that the complement system is involved in the adaptive immunity with antibodies (75).

The activation of innate pentraxins during infection or tissue damage is much more rapid than the activation of antibodies (59). SAP is considered a non-specific marker of infection and inflammation, where increased levels in serum follow IL-6 mediated inflammatory response, and SAP is also found at sites of inflammation (76, 77).

SAP binds to late apoptotic cells, independent of chromatin, and presents them to C1q, which in turn activates the complement system and elicits the removal of the apoptotic cells (78).

To summarize, the complement system has a monitoring role and works against infections, links innate and acquired immunity and removes immune complexes and inflammatory products with SAP playing an important role as an activator (79).

In 2000 Coker et al. published a possible new role for SAP, as a molecular chaperone; where denatured lactate dehydrogenase (LDH) was allowed to refold in the absence and presence of SAP, addition of SAP increased the yield of active LDH (80). The authors speculate that SAP binds to intermediates in the refolding landscape and redirects them through more productive routes. SAP may have a general capacity to stabilize structures to which it binds. It has been suggested that SAP has a surveillance role *in vivo*, binding to misfolded species and preventing the seeding of larger aggregates (80).

1.6.1 SAP in amyloidosis

SAP is severely entangled in amyloidosis but the exact role is not clear; indeed, different studies give contradictory results, ranging from inhibiting to prohibiting fibril formation. This section will go through some of these studies.

SAP binds to all forms of amyloid fibrils and is universally present in amyloid deposits (65). ¹²³I-labeled SAP has even been used for scintigraphic detection of lysozyme deposits *in vivo*

(33, 81). SAP is thought to prevent proteolytic cleavage by binding and stabilizing aggregates (82). SAP itself is protected from proteolysis in the presence of calcium and this is possibly the underlying mechanism behind the persistent amyloid deposits (65). The SAP molecule that interacts with amyloid deposits is unchanged, compared to the molecule in circulation, and this might disguise the fibril, further preventing degradation (83, 84).

In an important article, Janciauskiene et al. show that the addition of SAP to $A\beta_{42}$ *in vitro* resulted in a dose-dependent inhibition of fibril formation. At a peptide to SAP ratio of 5:1 the fibril formation was completely inhibited. The authors speculate that SAP affects the packing mechanism of $A\beta_{42}$ and disturbs the typical uniformity of the fibrils (85)

However, in an article published the same year (1995) Hamazaki et al. show the opposite; that SAP promoted *in vitro* aggregation of $A\beta_{40}$ at physiological concentrations (86). Furthermore, SAP has been implicated in the fibrillogenesis of $\beta 2$ -microglobulin, in a study from Myers et al. *in vitro* $\beta 2$ -microglobulin does not easily produce amyloid-like fibrils at physiological conditions without detergents, organic solvents or urea. The authors found that monomeric $\beta 2$ -microglobulin incubated with seeds in the presence of SAP at pH 7.0 increased the ThT fluorescence signal, corresponding to increased fibril formation. This effect was found to be both calcium- and concentration dependent. The authors speculate that the binding of SAP stabilizes the seeds and provides a surface for further assembly (87).

The role of SAP in amyloid deposition has been examined by Botto et al. using mice with the gene for SAP knocked down homozygously or heterozygously. The mice were injected with casein five days per week for 66 days and the amyloid build-up was determined using ^{125}I -labeled SAP. The results revealed that mice with SAP deficiency developed significantly less amyloid deposits and that the deposition was delayed, compared to the mice that expressed one copy of SAP (heterozygously). Thus, Botto et al. conclude that SAP is a strong contributor to amyloid deposition *in vivo* (84).

In contrast, a recent study by Andersson et al. proposes that the decoration of amyloid fibrils and their pre-aggregated precursor states with SAP could be another defense mechanism against formation of toxic aggregates. The study found that by co-expressing transthyretin V14N/V16E (denoted TTR-A) and SAP in the eye of *Drosophila* a complete protection from the degenerative changes induced by the variant TTR-A was achieved. The authors also noted that SAP neither promotes nor prevents aggregation of TTR-A (88).

This proposed action for SAP correlates with the observations from Coker et al., where SAP acts as a molecular chaperone and Coker et al. suggest that SAP binds to misfolded species in an attempt to facilitate refolding but that SAP is overwhelmed and in a calcium dependent way binds to mature fibrils, stabilizing them and protecting them from degradation (80).

Finally, SAP has been suggested as a target for treatment against systemic amyloidosis, using an approach that combines anti-SAP antibodies and a compound called CPHPC ((R)-1-[6-[(R)-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2 carboxylic acid) (89). CPHPC was developed to specifically target SAP and inhibit binding to amyloid deposits; the compound

was found to bind two SAP molecules and form face-to-face decamers. Circulating SAP binds to CPHPC and forms complexes that are rapidly cleared by the liver, leading to almost complete depletion of plasma SAP (90). CPHPC on its own removed about 90% of the SAP bound to amyloid in deposits, in patients that had received 80 mg of CPHPC daily for 41 weeks (91). In a study in mice with induced amyloid A amyloidosis (AA), Bodin et al. combined CPHPC with anti-SAP antibodies. The mice were first treated with CPHPC for five days clearing circulating SAP and then injected once with anti-SAP antibodies; substantially less amyloid was found after the treatment and no unfavorable biochemical effects or deaths were observed. The antibody clearance was found to be complement dependent and macrophage derived. The authors suggest this as a possible treatment for systemic amyloidosis where minimizing amyloid load is important (89).

1.7 Molecular chaperones

A molecular chaperone is defined as any protein that interacts, stabilizes or helps a non-native protein to achieve its native confirmation but is not itself part of that final functional structure (29, 92). Molecular chaperones are a set of protein families where most of the chaperones use cycles of ATP-binding to take their action on nascent polypeptide chains, enabling their folding or unfolding (93). Other chaperones protect the newly formed subunits during assembly, the so called "holdases" (93, 94). The chaperone families are distinguished by their unrelated structures and the differing conditions that cause them to be upregulated (29, 95, 96). Many of the chaperones are known as stress proteins or heat-shock proteins (HSPs), depending on what causes them to be upregulated, and they are classified according to their molecular weight (e.g. HSP70, HSP90 and small HSPs) (94). Other chaperones play a role in regular cell maintenance, in normal conditions of non-stress (97).

Chaperonins (HSP60s) are large cylindrical complexes that function by confining unfolded protein molecules, one at a time, in a binding pocket so that folding can take place without the risk of aggregation (29). This is one of the distinctions between chaperones and chaperonins, chaperones release the protein to finish folding in bulk solution instead of in the protected environment of the pocket (94).

Chaperones act by binding to the unfolded protein molecule and preventing aggregation, reducing the population of partially folded intermediates and thereby smoothing the energy landscape and guiding aggregation-prone intermediates towards the native state (29, 98).

Both types of chaperones increase the yield of folded protein, but through different processes. Heat shock, or stress, chaperones increase the folding yield by binding to the unfolded protein, immobilizing it and then releasing it, the yield is increased by preventing the formation of unfavorable intermediates. The steady-state chaperones act in two ways; i) by binding to aggregation-prone sites on the protein and reducing the time the protein spends unprotected; ii) by increasing the rates of folding (97).

1.8 Alzheimer's disease

Alzheimer's disease (AD) was first described by Alois Alzheimer in 1901, in a 51-year old woman, Auguste D., who could not remember her last name, only that it began with a "D" (99). Alzheimer studied her case until she died in 1906 and published his paper in 1907: *Über eine eigenartige Erkrankung der Hirnrinde*. Alzheimer writes about how "A woman of 51 years presented with ideas of jealousy toward her husband as the first apparent illness sign. Soon, a rapidly worsening memory weakness was noticeable; she could no longer negotiate her way around her dwelling; dragged objects back and forth and hid them; and at times she believed she was about to be murdered and started yelling loudly." (100).

AD patients commonly suffer from: memory impairment; disordered cognitive function; altered behavior including paranoia, delusions and loss of social appropriateness; and a progressive decline in language function (101). The average time course of AD dementia is 7-10 years and inevitably the illness culminates in death (102). Clinically, symptomatic AD begins after age 65 years in most cases and the frequency of disease occurrence increases with age. More than 99% of all AD cases are what is called "sporadic" or "late-onset" AD; a hereditary form known as familial AD make up the remaining per cent (<1%) and for these patients dementia onset occurs earlier in life (30-60 years of age) (102).

After Auguste passed away Alzheimer was able to obtain some sections of her brain for histological evaluation, he notes: "The section yielded an evenly atrophic brain without macroscopic foci. The larger brain vessels show atherosclerotic changes. In preparations that were stained according to the silver method of Bielschowsky, very peculiar changes of neurofibrils are observable." (100).

The classical pathological hallmark of Alzheimer's disease is the accumulation of amyloid plaques and the associated neurofibrillary tangles (NFTs) associated with brain shrinkage to almost half of the original size (Fig 9) (103).

The amyloid plaques and NFTs begin to accumulate many years before the clinical symptoms

Figure 9. A normal aged brain (left) compared to the brain of a person with Alzheimer's disease.

and signs of very mild dementia appear.

A major factor in AD is amyloid β ($A\beta$), a peptide resulting from cleavage of the amyloid β precursor protein ($A\beta$ PP) (104, 105). Within plaques, $A\beta$ is present in aggregated (insoluble) forms including fibrils as well as oligomers (101). Generation of the $A\beta$ peptide will be described in more detail in section 1.8.1. NFTs are intracellular structures predominantly composed of a hyperphosphorylated aggregated form of the microtubule-binding protein tau. Data strongly suggest that NFTs contribute to neuronal dysfunction and correlates with the clinical progression of AD (102, 106).

Alzheimer's diagnosis of Auguste has since been confirmed by re-examination of his original histological slides. These analyses verified the loss of neurons in various areas of the cortex as well as the presence of large numbers of typical NFTs and amyloid plaques in her cerebral cortex, exactly as had been described and depicted by Alzheimer himself (107).

To diagnose AD, a physician commonly performs a physical exam checking reflexes, sense of sight and hearing, coordination and balance. The mental status is tested through different exercises and questions and specific brain changes can be detected using Computerized tomography- (CT), Magnetic resonance imaging- (MRI) or Positron emission tomography- (PET) scans (108).

1.8.1 $A\beta$ peptide

Amyloid β precursor protein ($A\beta$ PP) is synthesized in most tissues of the body as a transmembrane protein, the function of which is not entirely clear, but would seem to be important for neuronal and synaptic function (109, 110). *Drosophila* flies that lack the APP equivalent APPL are viable and fertile, but show subtle behavioral defects that can be partially rescued by human APP, this demonstrates functional conservation between the different species (109, 111). Further studies of these flies revealed reduced synaptic bouton numbers at the neuromuscular junction (109).

Figure 10. APP processing.

APP undergoes cleavage by at least three enzymes: α - β - and γ -secretase (109). This processing is divided into two pathways, the non-amyloidogenic pathway and the amyloidogenic pathway (Fig 10).

The prevalent non-amyloidogenic pathway starts with APP being cleaved by α -secretase, producing sAPP α , a large domain that is secreted into the extracellular space. The remaining C83-fragment is attached to the membrane and cleaved by γ -secretase, resulting in a 3 kDa product (p3) (Fig 10) (101).

The amyloidogenic pathway is initiated when β -secretase, named BACE1, cleaves APP, resulting in sAPP β and C99. The C99 fragment is also attached to the membrane and cleaved by γ -secretase between residues 37 and 43, giving rise to A β peptides of varying length (101, 112) (Fig 10). The most common A β peptide is 40 amino acids long, A β_{40} ; around 10% of the peptides are 42 amino acids long and these A β_{42} peptides are more aggregation prone than A β_{40} (103).

1.8.2 A β toxicity

The involvement of A β in AD is historically anchored, in 1984 the peptide was identified and in 1985 the peptide was found to be the primary component of plaques from AD patient brain (104, 113).

The effects of A β have been studied both *in vitro* and *in vivo*. Hippocampal neurons cultured in the absence and presence of A β_{42} show significant neuronal degeneration and death in the presence of A β_{42} (114). Rats injected with cell medium containing oligomeric and monomeric A β_{42} but not fibrils revealed significant inhibition of long-term potentiation (20). The memory of a learned behavior in normal rats is disrupted if the rats are injected with solubilized A β from AD brain (115). *Drosophila* flies that express A β_{42} show deposits of A β together with neuronal dysfunction, revealed by abnormal locomotor behavior and reduced longevity to a degree that closely correlates with the aggregation propensity of A β_{42} (116).

The exact mechanism behind toxicity of the A β peptide is shrouded in darkness, however, several theories exist; this section will go through some of the most prominent.

The so-called amyloid cascade hypothesis can be summarized as follows; production of A β_{42} is increased for some reason, the peptide starts to accumulate and oligomerize and finally deposits of A β are formed. Oligomers formed during the aggregation process affect synapses and activate microglia and astrocytes leading to damage to the neurons. The damaged neurons give rise to oxidative injury that in turn affects various kinase and phosphatase activities leading to plaques. The neuronal damage is now extensive and cell death occurs, ultimately leading to dementia (19, 117).

A different theory considers only the oligomers of A β_{42} to be the toxic species, as only a weak correlation between insoluble A β_{42} deposits and dementia have been found (112). The proponents of this theory suggest that species other than fibrils must contribute to cognitive

deficits or neurodegeneration. Studies in mice exposed to cell medium containing A β_{42} of various sizes found the active species resulting in significant cognitive deficits to be oligomeric rather than monomeric or fibrillar (118). This theory has gained ground since it explains how extracellular A β_{42} peptides may be toxic without a correlation between deposits of insoluble A β_{42} and neuronal loss. However, the exact nature of the oligomers is not well defined (119).

An alternative hypothesis considers a general toxicity by various sizes of A β_{42} acting simultaneously by binding to membrane proteins, causing oxidative stress and changing membrane properties (112). This general toxicity theory is supported by the suggestion that the nucleation-dependent aggregation process itself promotes toxicity as there seems to be structures that are similar between different amyloid forming proteins (15, 120-122). Neuronal cells treated with either fibrillar A β_{42} or soluble A β_{42} showed no cell death, suggesting that the toxicity may be a dynamic process depending on protein aggregation (123). Indeed, cells treated with prefibrillar oligomeric species of the A β_{42} E22G variant are 60% less viable compared to control cells (124).

1.9 Endoplasmic Reticulum stress and the Unfolded Protein Response

The Endoplasmic Reticulum (ER) is an organelle in the cell responsible for protein synthesis, modification, protein folding and protein sorting.

When proteins misfold they can cause a buildup in the cell, specifically in the ER and when the ER becomes overwhelmed by accumulation of unfolded or misfolded protein, it is said to be in a stressed state, so called ER stress. ER stress can also be initiated by decreased chaperone function and abnormal ER calcium content (125).

To relieve this stress, the ER deploys a defense mechanism called the Unfolded Protein Response (UPR). The UPR is a homeostasis striving mechanism, its role is to remove the misfolded proteins and prepare the ER to function as normal. The UPR concept was introduced 1988 in a study where misfolded proteins in the ER induce synthesis of the ER chaperones BiP and GRP98 (126)

The UPR is divided into three parts (or arms); activating transcription factor 6 (ATF6), inositol requiring kinase 1 (Ire1) and protein kinase RNA-like ER kinase (PERK); these three parts work complementary and in parallel (127), if one is activated, all will be activated and if one of them is impaired, the others are upregulated but the different parts may be specialized in responding to different conditions (128). The UPR has several different tools in the toolbox, and the relief of stress can be divided into four stages (Fig 11). The first response to ER stress is to lessen the burden of unfolded or misfolded proteins by decreasing influx of proteins into the ER (129) and by halting translation (130). In the second stage, ER-resident chaperones and ER associated degradation (ERAD) are upregulated and the size of the ER is increased (131). If the ER stress is sustained, a stage of chronic stress is entered and signaling from the UPR is now changed from prosurvival to pro-apoptotic; the redox state of the cell is changed and the cell is sensitized to apoptosis (132). In the fourth and last stage, if the ER stress is not relieved

and the UPR activation allowed to persist, the cell undergoes apoptosis (133).

Ire1, PERK and ATF6 are associated with BiP under normal and “un-stressed” conditions and rendered inactive (135); unfolded proteins in the ER titrate BiP from the sensors and activates them (136). PERK is thought to be the first arm to be activated in the UPR, followed by ATF6 and Ire1 being the last (137). Both PERK and ATF6 signals are prosurvival and aim to counteract ER stress. If the ER stress is maintained, Ire1 becomes active and the signaling is switched towards pro-apoptotic. Signaling from PERK persists under prolonged ER stress while signaling for both ATF6 and Ire1 decrease over time (Fig 11).

Figure 11. Kinetics of UPR signaling and cell fate. PERK and Ire1 are simultaneously activated and signaling is prosurvival. During chronic stress, ATF6 signaling is attenuated whereas PERK signaling is sustained. Ire1 activation indicates a switch from prosurvival to pro-apoptotic. After prolonged stress Ire1 is turned off. Modified from (134).

Upon activation, PERK phosphorylates the eukaryotic translation initiation factor 2α (eIF2 α), which halts general protein translation (Fig 12). This halt in translation decreases the load of proteins in the ER, as short-lived proteins are cleared, and decreases the burden on ER chaperones. To allow the cell to recover from ER stress, translation inhibition is transient (140). Phosphorylated eIF2 α increases the expression of ATF4 and the downstream targets CHOP and GADD34 (133). CHOP and GADD34 are involved in the de-phosphorylation of eIF2 α in a negative feedback loop (141).

ATF6 occurs as a monomer, dimer and oligomer in unstressed ER through inter- and intramolecular disulfide bridges (144). After BiP dissociates, ATF6 is reduced in response to ER stress, through an unknown mechanism, and monomeric ATF6 translocates to the Golgi where the luminal domain is cleaved and the cytosolic bZIP domain is released (142-144). ATF6 is solely responsible for transcription of ER chaperones and induces expression of Xbp1 (141) (Fig 12). ATF6 together with Xbp1 forms a heterodimer that upregulates ERAD-related genes (131). ATF6 also upregulates autophagy in an attempt to achieve homeostasis by removing the misfolded/unfolded proteins and also to degrade damaged or enlarged ER (136). PERK is necessary for ATF6, as it promotes synthesis of full-length ATF6, which is required since it is cleaved when activated; PERK also facilitates translocation to the Golgi (145).

Figure 12. Overview of the unfolded protein response. Upon activation, each sensor elicits downstream responses with the aim to restore ER function. Adapted from (137-139).

The Ire1 sensor is the most well studied branch of the UPR and is preserved among eukaryotes (146). In mammals two isoforms of Ire1 can be found; Ire1 α , which is expressed in all cells and Ire1 β , which is expressed in the GI and respiratory tracts (133). When the UPR was first introduced, the regulation of activation for Ire1 was suggested to be the dissociation of BiP from Ire1 (126). As more studies have been done in the field, a new mechanism for UPR activation and regulation has been proposed, that Ire1 is able to bind directly to unfolded proteins (147). BiP still plays a role in this new theory, as a means to fine-tune the activation and also to help turn off Ire1.

Ire1 is a transmembrane protein with a luminal part and cytoplasmic kinase and RNase parts (Fig 12). As a result of activation, either through the release of BiP or binding to unfolded proteins, Ire1 dimerizes, oligomerizes and an RNase domain is activated through autophosphorylation (134). The activated RNase domain cleaves Xbp1 mRNA to remove an intron (139). The cleaved Xbp1 mRNA is then spliced together. Translated Xbp1 upregulates chaperone activity and ERAD. During persistent stress, Ire1 signaling is attenuated and pro-apoptosis pathways induced (137, 148). If the ER stress is relieved, Ire1 resets and becomes ready for reactivation (149).

Continuous ER stress might contribute to various forms of cancer (150), diabetes (136), inflammation (151) and neurodegeneration (AD, Parkinson's and amyotrophic lateral sclerosis (ALS)) (152). The functional significance of UPR in neurodegenerative disease is not well understood, activation might promote neuronal protection by increasing folding efficiency or it may cause neuronal cell death through apoptosis, or it is a late event in extensive neuronal damage and non-essential for disease progression (152, 153). Selective regulation of the UPR might be a viable treatment when the process is more well understood (154). In one example, the UPR was modified, in a mouse study where SOD1 mice were made Xbp1-deficient and as a result had increased lifespan, and exhibited delayed ALS disease onset (155).

1.9.1 UPR in *Drosophila*

The UPR machinery in *Drosophila* is regulated by two pathways, PEK-1 and Ire-1; the ATF6 pathway is not functional in *Drosophila* even though the genome contains a gene for atf-6 (146).

Drosophila flies are very useful in the study of UPR and misfolding protein disorders as the mechanisms between human and flies are mostly conserved (156, 157).

Casas-Tinto et al. expressed A β in the retina of *Drosophila* and observed a rough-eye phenotype; this rough-eye was avoided if A β was co-expressed with Xbp1. Interestingly this rescue effect was achieved without altering A β accumulation or misfolding. Upon further investigation it was found that Xbp1 overexpression limited the activation of caspases in response to stimuli from A β oligomers (158). Loewen and Feany show that the UPR protects against tau neurotoxicity in a model where tau is expressed in the neurons of *Drosophila*. By reducing the levels of Xbp1, the activity of the UPR is reduced and flies were significantly more affected by neurotoxicity (159).

1.10 Antibodies

Antibodies are found in the blood and other body fluids and are used by the immune system to recognize and neutralize unknown objects such as bacteria and viruses. Antibodies are produced in B cells and belong to a group of proteins called immunoglobulins, which recognize a specific part of the foreign object, called an antigen and bind to specific sites on the antigen; these sites are called epitopes (160). Antibodies are Y-shaped proteins consisting of two heavy chains and two small light chains. Experimentally there are two types of antibodies being used; monoclonal and polyclonal. Monoclonal antibodies are raised against a single epitope on the protein of interest whereas polyclonal antibodies are a mix of several antibodies that detect several different epitopes on the same protein (161).

Baron Kitsato Shibasaburo initiated the field of immunology when he showed that animals injected with soluble tetanus toxin developed “antitoxin” and that this antitoxin was specific (162). We now know this antitoxin to be antibodies. The ability of antibodies to specifically recognize a protein of interest has been used in the lab since the mid 1950s in different types of immunoassays, such as enzyme-linked immuno-sorbent assay (ELISA) and immunohistochemistry, which will both be described later in this thesis.

Commercial antibodies are raised in mammals, most commonly in rabbit, mouse, goat, horse and sheep (161). In this thesis, a camelid anti-lysozyme antibody has been used. The antibodies raised in camelids differ from those from other mammals in that they only have heavy chains, and it is possible to express them recombinantly in *Escherichia coli* (163, 164).

The quality of an immunoassay is highly dependent on the quality of the antibodies used (161) and serious thought should be given regarding the choice of antibodies. Monoclonal antibodies are lot-to-lot consistent and lack the inherent variability of polyclonal antibodies, however, polyclonal antibodies are more specific as they are produced by a large number of B cell clones each reacting to a specific epitope on the same protein of interest (165). Polyclonal antibodies are more stable over a broad pH and salt concentration whereas monoclonal antibodies can be very sensitive to both (165).

1.11 The fly as a model system

During the past 20 years *Drosophila* flies have been used in the study of human disease and they are faithful employees; in the service of man, they reveal the secrets behind many disorders by showing effects on various behaviors such as locomotor activity, they develop plaques, tangles, memory deficits and even die (166, 167). The genome of *Drosophila* was sequenced in the year 2000 and 75% of all human disease genes were found to have related sequences in *Drosophila* (168, 169).

The different types of disease that can be modeled using *Drosophila* are as diverse as blindness (170), neurodegenerative diseases (171), cancer (172), cardiac disease (173) and immunological disorders (174). Among the neurodegenerative diseases models have been established for

AD (175, 176), Parkinson's (177-179), Huntington's (180), prion disease (181), transthyretin amyloidosis (182) and ALS (183).

There are several approaches towards investigating disease using *Drosophila*, three stand out as the most common: i) expression of a human disease gene in its wild-type or mutant form; ii) loss and gain of function of the *Drosophila* homolog of a human disease; iii) genetic screens to identify enhancers and inhibitors that are able to modify one or more phenotypes caused by approach (i) and/or (ii) (184). The first approach is relatively straightforward if the disease-causing gene is known, express the gene in specific tissues and analyze the effects (185). The second method is equally important, as understanding the normal function of the disease genes can give valuable information into the underlying reason for disease and suggest possible disease mechanisms (166). In addition to these methods for furthering understanding of disease, the *Drosophila* fly has a blood-brain-barrier, making it excellent for conducting high-throughput screens for new treatments of human diseases (186).

Modeling human disease in *Drosophila* gives phenotypic effects that are remarkably similar to the disease manifestation in human; for example, flies expressing A β ₄₂ in the nervous system exhibit extracellular deposits and vacuole formation, decreased lifespan and locomotor dysfunction (176). Neurodegeneration is typically a late event in the fly life, with the first signs noticeable in the late pupal stage or after eclosion in adult flies, mimicking human disease progression (187).

While *Drosophila* flies provide reliable models for human neurodegenerative diseases as mentioned above, it is important to remember that not all aspects of human disease progression can be robustly mimicked in flies. For instance, flies do not have an adaptive immune system, brain infarcts and brain hemorrhage cannot be analyzed because the vessels are not there and they have simpler circulatory systems (188-190). Nevertheless, *Drosophila* models of disease can give valuable insight into gene function, reveal mechanisms of neural maintenance and test new approaches for the treatment of human disease (166).

2 Aims of the research

The overall aim of the research has been to establish a *Drosophila* model of lysozyme amyloidosis to be used as a model system to study the *in vivo* behavior of the amyloidogenic lysozyme variants.

More specifically the aims were to:

- Characterize *Drosophila* flies expressing WT lysozyme and disease-associated variants.
- Find out the impact of SAP on *in vivo* lysozyme aggregation and toxicity using the fly model.
- Examine the *in vivo* consequences of co-expressing WT lysozyme together with the variant F57I.
- Investigate the role of lysozyme in Alzheimer's disease.

3.1 *Drosophila melanogaster* as a research tool

The first detailed record of *Drosophila melanogaster*'s usefulness in research came from Thomas H Morgan in the late 1910s (191) when he used fruit flies to study heredity, and he was awarded the Nobel Prize in physiology or medicine in 1933 for his work on *Drosophila* genetics.

Drosophila is a great resource in the research lab, with many advantages such as short generation time (10 days at 25°C), they are cheap to rear, large amounts of offspring are relatively easy to accumulate and there is no need for an ethical permission. The major advantage is that the fruit fly genome is very well studied, and many molecular mechanisms, including apoptosis (192), autophagy (193, 194) and the unfolded protein response (156), are conserved between human and fly.

The life cycle of a fly can be divided into four phases; egg, larval, pupation and adult life. After fertilization the female fly lays eggs, which after ~1 day hatch into larvae, a state that remains for ~6 days after which pupation occurs. After 3 days as a pupa metamorphosis takes place and an adult fly ecloses (hatch). The fruit fly has four pairs of chromosomes (195), one pair of sex chromosomes and 3 autosomes, which can be used for targeted gene expression, where it is possible to insert one or several genes of interest.

The *Drosophila* flies offer a great variety of different read-outs to choose from when studying disease such as changes in wing morphology, retinal disruption, color change in the eye or even the whole body, affected lifespan and changes in locomotor behavior. Some of these phenotypes will be addressed in more detail later in this thesis.

3.2 Expression of genes in *Drosophila*: UAS-Gal4 system

As discussed in the previous section, the fruit fly with its many possible disease-associated read-outs is a good choice as a model system to study human diseases. When creating a *Drosophila* disease model, very often the disease-associated gene is inserted into the fly genome to achieve protein expression of the gene. This is possible using the UAS-Gal4 system, introduced in 1993 by Brand and Perrimon (196), which uses a yeast transcription factor (*Gal4*) and an upstream activation sequence (UAS) to direct expression to a specific tissue. This two-

Figure 13. Illustration of the UAS-Gal4 system, which allows tissue-specific expression of genes of interest.

part system uses a driver line and a reporter line that are combined to initiate expression of the protein of interest. In the absence of Gal4 the gene of interest remains dormant, which makes it possible to keep stable parental stocks for toxic proteins (196). Figure 13 shows the principle for this system, either the male or the female is the driver line, which expresses Gal4 in a tissue specific manner, and the other fly in the cross is called the reporter line and carries the UAS coupled to the gene of interest. The offspring from this cross will express Gal4 that binds to the UAS and promotes expression of the protein in the specific tissue of choice.

3.3 Fly lines

In this thesis four variants of lysozyme were used: WT, F57I, I59T and D67H. The F57I mutation is the most destabilized and I59T is a non-natural variant of intermediate stability compared to WT and the disease-associated variants. The D67H mutation was chosen because it was the first mutation to be discovered. To direct expression to specific areas different Gal4-driver lines were used; first, *gmr-Gal4* which directs expression to the retina of the fly making it possible to assess if a rough-eye phenotype occurs due to the expression of the disease-associated protein. Secondly, a ubiquitous driver, *Act5C-Gal4*, was used to mimic the human whole body expression of lysozyme in the flies. Thirdly, expression was directed to the CNS of the flies using either *elav-Gal4* or *nsyb-Gal4* to be able to perform the behavioral studies. For experiments regarding the UPR and ER-stress, *Xbp1-EGFP* flies were used, which together with a driver fly line and the lysozyme variants reveal the presence of ER-stress in the tissue of interest (Described in more detail in section 3.4 *Xbp1-EGFP* flies). Table 3 summarizes the flies used in this thesis.

Table 3. Summary of flies used.

Fly name	Expresses
A11, control	Offspring from A11 and a driver line expresses Gal4
<i>Act5C-Gal4</i>	Gal4 expressing driver fly that gives ubiquitous expression of the transgene (197)
A β_{42}	Transgenic for human A β_{42} peptide (176)
<i>elav-Gal4</i>	Gal4 expressing driver fly that directs expression of the transgene to the CNS of the fly (198)
<i>gmr-Gal4</i>	Gal4 expressing driver fly that directs expression of the transgene to the eye (199)
LysD67H	Transgenic for human lysozyme with the disease-associated mutation D67H (51)
LysF57I	Transgenic for human lysozyme with the disease-associated mutation F57I (51)
LysI59T	Transgenic for human lysozyme with the disease-associated mutation I59T (51)
LysWT	Transgenic for human lysozyme WT (51)
<i>nsyb-Gal4</i>	Gal4 expressing driver fly that directs expression of the transgene to the CNS of the fly (Thor, unpublished)
<i>Xbp1-EGFP</i>	Marker fly that expresses EGFP in the presence of ER-stress (157)

3.4 Xbp1-flies

In order to probe the flies for activation of the UPR, a special marker fly was used. This fly is constructed so that Xbp1 is connected to the Enhanced Green Fluorescent Protein (EGFP), which in the unstressed state is out of read frame (Fig 14). When Ire1 detects stress in the ER, Xbp1 is cleaved and the fragments are spliced back together without the intron, and EGFP comes into frame and is expressed. EGFP is naturally fluorescent, however, the signal is very faint in the samples examined here. EGFP can be detected using a couple of different methods; the methods used in this thesis are immunohistochemistry (in paper I) and a protein assay (in paper II). Both methods employ antibodies against EGFP and a secondary antibody, which either gives a fluorescent signal (for immunohistochemistry) or gives an electrochemiluminescent signal (for the protein assay).

Figure 14. Illustration of principle for Xbp1-EGFP marker.

In the immunohistochemistry assay it was necessary to enhance the naturally fluorescent signal of EGFP further to facilitate detection; for this purpose an anti-EGFP antibody followed by a fluorescently labeled secondary antibody was used. The secondary antibody was chosen to give a red color to only present a signal where the primary antibodies had bound. The samples were analyzed using a LSM 780 confocal microscope (Zeiss) and for each sample several images were captured. The micrographs were then coded so that it was impossible to determine which genotype they belonged to, and then examined for the presence of GFP-positive “spots”. The spots were counted and the values entered into GraphPad prism (GraphPad Software, San Diego, CA, USA) where a one-way ANOVA statistical test was used to determine if the samples had statistically differing mean values. After this, the samples were de-coded and assigned the right genotypes.

To get a quantitative measure of the amount of ER-stress, a protein assay was developed that measures the amount of EGFP in the flies. This protein assay uses the Meso Scale Discovery technique that will be described further in section 3.8.

3.5 Longevity assay

The aim of a longevity assay is to determine if there are any differences in lifespan between healthy control flies (not expressing any transgene but only Gal4) and flies that are expressing a disease-associated transgene of interest (184, 200, 201). For example, by comparing the lifespan of different transgenic A β fly variants the toxicity of the protein has been shown to correlate with the aggregation propensity of the protein (116).

A longevity assay is performed by ageing 100 mated female flies per genotype in tubes with 10 flies in each tube on agar food supplemented with yeast paste. The numbers of live flies are counted and the live flies are transferred onto fresh food three times a week until no flies are left alive. The numbers are then analyzed using Kaplan-Meier log rank statistics (202) and tested for statistical significance using GraphPad Prism (GraphPad Software, San Diego, CA, USA). A measurement that is commonly used in longevity assays to determine the health status of the flies is the median survival; this corresponds to the time when half of the flies for a genotype have died.

3.6 Locomotor assay

The longevity assay described above is a rather blunt method for determining the health of flies, as it only takes into account if the fly is alive or not; the flies can be less healthy and show lower activity but still be alive. Thus, a locomotor assay, which probes the movement of the flies, gives a better understanding of the fly health (203).

There are a few different assays to choose from when examining the locomotor behavior of flies, for example a climbing assay, which probes how many flies that reach a certain point of the vial in a specific time (201) and the *Drosophila* Activity Monitor2 (DAM2)-system, which measures the number of beam breaks per fly per tube. However, these two methods are only one dimensional (204). In this project the iFly system, developed in Cambridge by Professor Damian C Crowther and co-workers (205), has been used. The iFly is a 3D-system which tracks fly movement and calculates velocity, slope of end-to-end distance, angle of movement and interaction angle.

Both the climbing assay and the iFly system take advantage of the knowledge that flies are strongly negatively geotactic, meaning that they strive to reach the top of the vial, which is an innate escape response shown to decline with age (203, 206). In addition, healthy flies move in a vertical path from the bottom toward the top of the measuring vial, as a consequence of wanting to reach the top as quickly as possible.

The iFly system consists of a video camera, and a box with mirrors and a software package. For each genotype 10 flies are transferred into a measuring vial, the vial is then dropped into the iFly-box to ensure that the flies start at the bottom of the vial, and a movie is recorded. Every 30 seconds the vial is dropped down again, causing the flies to fall to the bottom (re-activating the locomotor behavior) and to start their climbing again. After 90 seconds the recording is stopped and the acquiring is finished. This is repeated three times per genotype, yielding three video files with three clips of 30 seconds. The video files are processed using the first part of the iFly software that plots a trajectory for each fly in the vial and calculates the 3D parameters for each fly at each given time during the movie. These 3D parameters are then plugged into the second part of the iFly software, that from these data calculates the velocities, angle of movement and the other output parameters mentioned above. The output data is plotted in GraphPad prism (GraphPad Software, San Diego, CA, USA) and compared between different genotypes using a one-way ANOVA statistical test.

The velocity is a measurement of how fast the fly moves, in mm/s. As the flies get sicker and older they start to move more slowly. This is the most used parameter as it gives a very clear read-out.

The angle of movement quantifies if the fly moves from the bottom of the vial to the top in a vertical line; healthy flies move in a vertical line from the bottom of the vial to the top and typical values lie around 50°. As the health of the flies deteriorates this value tends to rise towards 100°, because the sick flies travel in a rather zigzag pattern towards the top. The hypothesis is that this measurement reflects damage to the fly's behavioral brain functions and affect orientation and movement direction. In the subsequent analysis of the data, flies are deemed to be "sick" when the angle of movement reaches 80° and the day the flies reach this point is compared between different genotypes.

Drosophila has been the subject of many studies regarding the circadian rhythm, since the first clock mutant was discovered in 1971 (207). These studies have demonstrated that in standard conditions of 12:12 light:dark cycles at 25°C, *Drosophila* flies are bimodal and crepuscular, with two activity peaks. The first activity peak occurs in the "morning", which is the lights-off-to-lights-on transition and the second activity peak occurs in the mid afternoon in the transition from lights-on-to-lights-off (204). In this thesis, the locomotor behavior of the flies has been assayed during the mid afternoon activity peak, consistently over the course of the measurement and for all assayed genotypes.

3.7 ELISA

A common method for analyzing proteins is an ELISA, Enzyme-Linked Immuno-Sorbent Assay, which is an antibody based method of detecting a specific protein from a liquid crude sample, for example blood, cerebrospinal fluid (CSF) or fly juice, performed in a microtiter plate. The first immunoassay was described in 1960 by Yalow and Berson, where they measured the levels of insulin in humans in a quantitative way using a radioisotope (208). As the name implies, the ELISA uses enzymes to develop a signal from the protein of interest. The assay from 1960 was refined and presented again in 1971 by two Swedes, Engvall and Perlmann, where the radioisotopes were replaced by enzymes to simplify the measuring and to create more stable complexes that can be used for a long time (209).

Signal development is conventionally done via either alkaline phosphatase (AP) or horseradish peroxidase (HRP) enzyme and a substrate, which results in a colored product that can be measured as a changed absorbance using a spectrometer. There are several different types of ELISA, the most common are direct-, indirect- and sandwich-ELISA (Fig. 15).

In the direct ELISA, the target antigen is adsorbed in the well and a labeled primary antibody is allowed to bind giving rise to a colorimetric signal. Drawbacks of the direct method are that every antibody used needs to be labeled and it is not possible to enhance the signal from the antibody as is possible with the use of a secondary antibody.

In the indirect ELISA, the target antigen is adsorbed in the well as for the direct method, and

again a primary antibody is allowed to bind; however, for the indirect ELISA a labeled secondary antibody is needed. This secondary antibody binds to the primary antibody and gives rise to a signal (210).

Figure 15. Schematic overview of ELISA principle.

Sandwich-ELISAs are excellent for crude samples with other contaminating proteins in the mix. An antibody against the target protein is coated in the wells; the crude sample is added and incubated to allow the target protein to bind and a primary antibody that recognizes the target protein but at a different epitope is added. In a sandwich-ELISA, this primary antibody can either be labeled with an enzyme or a secondary labeled antibody is needed (210). In paper I, a colorimetric sandwich-ELISA was used.

It is possible to make an ELISA quantitative if the target protein is available in recombinant form, a standard curve can then be prepared and the signal from unknown samples can be compared against it.

3.8 Meso Scale Discovery Protein Assay

In the remaining papers in this thesis, protein level determination was performed using Meso Scale Discovery (MSD) protein assay, which is an electrochemiluminescence assay similar to ELISA (Fig 16). The MSD assay is also antibody based and performed in a microtiter plate with a carbon electrode surface. The MSD assay is most commonly used in a sandwich setup comparable to the conventional ELISA but instead of using an enzyme-linked secondary antibody, a Sulfo-Tagged (Meso Scale Discovery, Rockville, MD, USA) secondary antibody is used. The Sulfo-Tag reacts with the Ru(bpy)₃-substrate, which transfers electrons to the working electrode and a light signal is emitted.

Figure 16. Schematic overview of MSD protein assay.

There are several advantages with using the MSD assay over a conventional ELISA: minimal background signal and higher signal to background ratio and the assay is not temperature sensitive. MSD sandwich protein assays were used in papers II, III and IV.

3.9 Immunohistochemistry - Antibody staining

Immunohistochemistry (IHC) is a method for looking at tissue samples with the help of antibodies; it is possible to determine which proteins are present, where they are located, to some extent the amount of protein, the morphology of the sample and if two different proteins co-localize. The method was first reported in 1941 when Coons et al. successfully coupled a fluorescent molecule to an antibody and saw that the agglutinated mass was brilliantly fluorescent in ultraviolet light (211). This method was further developed and in 1950 the first protocol for immunofluorescent staining was presented (212).

When preparing tissue samples from *Drosophila* for IHC, thin (10-20 μm) sections are cut from fly heads using cryosectioning (213), which is a method where the sample is embedded in a gel, frozen and cut into sections in a cold chamber. These thin sections are placed on glass slides.

IHC commonly starts with fixation of the sample, most commonly by paraformaldehyde (PFA) (161), to ensure that the tissue maintains its cellular components, prevent autolysis and stabilize cellular material. This is followed by blocking with, for example, non-fat dry milk or bovine serum albumin (BSA) to minimize unspecific interactions between the sample and the antibodies. The following step is the use of primary antibodies, which can be one or more antibodies against the protein or proteins of interest. These antibodies can either be unlabeled or labeled with an enzyme (resulting in a brown or blue product upon addition of substrate) or a fluorescent (resulting in a light signal) label. In this thesis only fluorescence-based detection has been used. After an unlabeled primary antibody, a labeled secondary antibody, which is raised against a specific species, is added, for example goat-anti-mouse, which is raised in goat and recognizes antibodies from mouse, and a fluorescent signal can be detected.

The use of this two-step method with a primary and a secondary antibody has many advantages, the signal is enhanced as at least two secondary antibodies can bind to the primary giving a higher signal than if a single labeled primary antibody gives rise to the signal. The same secondary antibody can be used against several different primary antibodies and the two-step system gives flexibility regarding which fluorescent colors are used.

In the following section, the principal findings in each of the papers included in this thesis are summarized and discussed.

4.1 Paper I

The aim of the first paper was to generate a novel *Drosophila melanogaster* model for lysozyme amyloidosis and to investigate the *in vivo* behavior of disease-associated variants. Four variants of lysozyme were used: wild type (WT), F57I, I59T and D67H. The genes for these variants were inserted into the *Drosophila* genome to generate transgenic *Drosophila* flies under control of the Gal4/UAS system. This new set of flies was then characterized using several different techniques. We used qRT-PCR to determine mRNA levels, ELISA to probe lysozyme levels, scanning electron microscopy for eye phenotype classification and immunohistochemistry to detect the UPR activation.

Two *Drosophila* drivers were used in this paper, *Act5C-Gal4* (ubiquitous expression) and *gmr-Gal4* (retinal expression) to examine the effects of expressing lysozyme in the fly. In this first paper two different *Gal4*-responsive vectors were used, pUAST and pBS. The pUAST vector was frequently used before the pBS vector was designed, however, insertion into the genome using the pUAST vector is pseudorandom and cannot be controlled. The pBS vector has since then been established as a more precise vector where the insertion site is predefined. As lysozyme is an extracellular protein, a signal peptide was fused to the lysozyme gene to achieve efficient secretion of the protein (Fig 1 in paper I). Next, the mRNA levels of the lysozyme variants were determined using qRT-PCR. All four fly lines generated using the pBS vector (WT, F57I, I59T and D67H) were found to have equal levels of mRNA and for the flies generated using the pUAST vector (WT and D67H), two lines were found that yielded equivalent mRNA levels (WT^b and D67H^c, Fig 2 in paper I). The soluble protein levels of fly lines with equal mRNA expression were investigated using an ELISA assay.

We found that the levels of lysozyme differed dramatically between WT lysozyme and

Figure 17. ELISA analysis of lysozyme levels in *Drosophila* at day 0. A) Flies crossed with *Act5C-Gal4*, which drives expression ubiquitously. B) Flies crossed with *gmr-Gal4*, which drives expression in the retina of *Drosophila* eyes. Means ± SD. Reprinted with permission from (51). © 2012 FASEB J.

variants. The protein levels correlated well with the native-state stability of the proteins; the highest level was found for WT, the level of I59T was about half compared to WT, while the level of the D67H, and F57I variants were very low (Fig 17).

We then investigated if the soluble protein levels of the variants were low due to the variants being trapped as insoluble species within the flies by adding an additional extraction step with DMSO before the ELISA analysis, however, no significant level of insoluble protein was detected for either variant, suggesting that the variants are cleared by the degradation system. The lysozyme variants were expressed in the retina of the flies using *gmr-Gal4* and the eye phenotype was analyzed using scanning electron microscopy (SEM). For all the variants, a rough-eye phenotype correlating with the increasing instability of the protein was discovered (Fig 18).

Figure 18. SEM analysis of rough-eye phenotype. Scale bars = 50 μ m. Reprinted with permission from (51). © 2012 FASEB J.

Control, WT and I59T flies had normal ommatidia, arranged into straight lines. For D67H the organization of the ommatidia was disrupted, and for F57I the ommatidia were fused together. These results suggested that the expression of the disease-associated variants in the fly eye is

Figure 19. Immunohistological analysis of UPR activation. Red stain shows GFP signal enhanced by Alexa 594-nm secondary antibody. Blue stain shows staining of cell nuclei by DAPI. A) Control flies, B) WT, C) I59T, D) D67H, E) F57I, F) colocalization between DAPI and GFP in the cell nuclei. Scale bars = 50 μ m. G) Scatter plot for GFP-positive cells for each variant of lysozyme. ** $p < 0.01$, *** $p < 0.001$. Reprinted with permission from (51). © 2012 FASEB J.

toxic, despite the low levels of protein present in the flies.

To find out if the low level of lysozyme in these flies could be due to the protein being cleared by the degradation system, a marker for ER stress, xbp1-EGFP, was co-expressed with the lysozyme variants in the fly retina to probe activation of the UPR. The xbp1-EGFP specifically probes the Ire1 pathway of UPR activation, and the resulting EGFP was detected using fluorescence microscopy. A low signal was detected for control flies, revealing a basal level of UPR activation (Fig 19G). However, a significantly increased signal was found for the amyloidogenic variants D67H and F57I showing that the UPR is activated for the most destabilized variants, which also show a rough-eye phenotype (Fig 19).

The results from paper I show that the amyloidogenic variants D67H and F57I are destabilized enough to activate the UPR, which clears away a large proportion of the proteins, to a degree that correlates with the destabilization of the variant. The toxicity observed in these flies, despite the low levels of protein, is most probably due to the prolonged activation of the UPR, which in turn induces apoptosis. From our findings in paper I, we speculate that the onset of familial amyloid disease may be linked to an inability of the UPR to detect and target for degradation the entire population of the amyloidogenic variants prior to secretion, allowing a proportion of these destabilized species to enter circulation and eventually to aggregate and accumulate in the body as intractable deposits.

4.2 Paper II

Using the *Drosophila* model of lysozyme amyloidosis that was established in paper I, the effects of expressing WT and the disease-associated lysozyme variant F57I in central nervous system (CNS) in the absence and presence of Serum amyloid P component (SAP) were investigated.

We first examined the locomotor activity of the flies (assessed by iFly) and their lifespan and found that flies expressing the WT lysozyme were as healthy as control flies (not expressing any lysozyme) and that the flies expressing F57I, in the absence of SAP, both moved slower and showed bigger deviations from a straight path and died at a significantly increased rate compared to WT and control flies. However, co-expression of F57I and SAP extended the

Figure 20. Survival trajectories for flies expressing WT lysozyme or the F57I variant in the CNS, with and without co-expression of SAP, and for *nsyb*-Gal4 control flies with and without expression of SAP. Kaplan-Meier graph showing per cent survival vs age of flies in days *** $p < 0.001$, **** $p < 0.0001$.

lifespan of the F57I flies (Fig 20) and restored their locomotor activity (Fig 2, paper II) to a level comparable to control and WT flies.

By using a MSD protein assay, the levels of lysozyme in the *Drosophila* heads were analyzed at three different time-points (day 0, day 25 and day 35); the samples were divided into soluble and insoluble fractions to examine the distribution between these fractions in the flies with and without SAP. Both in the absence and in the presence of SAP, the total level of lysozyme in WT-expressing flies was significantly higher than in F57I-expressing flies at all time points (Fig 21A & D). In the absence of SAP, the total level of lysozyme increased over time in both WT- and F57I-expressing flies. In WT-expressing flies, the levels of soluble and insoluble lysozyme were similar at day 0, but over time, the level of insoluble lysozyme increased (Fig 21B). In the F57I-expressing flies, only soluble lysozyme was detected at day 0, but over time the insoluble fraction increased and at day 35 most of the lysozyme detected was insoluble (Fig 21C). When WT lysozyme was co-expressed with SAP, the protein level was evenly distributed between the soluble and insoluble fraction at day 0; over time, the amount of insoluble protein was decreasing while the soluble remained stable (Fig 21E). When co-

expressing F57I with SAP, most of the lysozyme was soluble at all time-points (Fig 21F).

Figure 21. (A) Total amounts of WT and F57I lysozyme in fly samples without SAP co-expression, (B) Amounts of soluble and insoluble WT protein, (C) Amounts of soluble and insoluble F57I protein, (D) Total amounts of WT and F57I lysozyme in fly samples with SAP co-expression, (E) Amounts of soluble and insoluble WT protein with SAP co-expression, (F) Amounts of soluble and insoluble F57I protein with SAP co-expression. Normalized values: nanograms of lysozyme detected per unit total protein content (mg/ml) in the samples after subtracting unspecific signals from control flies. Bars represent means ± s.e.m. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

Thus, in the absence of SAP both WT and F57I lysozyme accumulate as insoluble forms over time; notably co-expression with SAP prevented accumulation of these insoluble forms of lysozyme in both WT and F57I flies.

To investigate if lysozyme and SAP co-localize in the CNS, sections of *Drosophila* brains (day 0) were stained with both anti-lysozyme and anti-SAP antibodies and counterstained with DAPI. SAP was detected in samples from control-SAP flies, which only express SAP, and in flies co-expressing SAP with WT (WT-SAP) or F57I (F57I-SAP) (Fig 4 in paper II). A clear signal from lysozyme was detected in WT-SAP brains but not in the F57I-SAP brains, corresponding to the MSD measurements showing a substantial higher level of lysozyme in WT flies compared to the lysozyme level in the F57I flies, which is very low. In the samples were both lysozyme and SAP could be detected, i.e. WT-SAP, almost total co-localization of lysozyme and SAP was observed.

In paper I we found that expression of the amyloidogenic variants triggers the UPR; here we wanted to investigate if the UPR was activated also in the CNS and if that activation varied over time. Using the marker xbp1-EGFP from paper I and a MSD anti-GFP assay, a quantitative measure of EGFP was achieved reflecting the degree of UPR upregulation (Fig 22). A basal level of UPR activation was detected for control flies (only expressing *Gal4* and *xbp1-EGFP*). In

the absence of SAP, the EGFP levels were low at day 0 for all of the fly variants, and increased over time. Accumulation of EGFP for F57I was most pronounced, revealing that expression of F57I in the CNS enhances UPR upregulation. In the presence of SAP, the level of EGFP decreased over time for control flies, suggesting that SAP counteracts the background stress observed for flies without SAP. A similar reduction in EGFP levels was observed for WT-expressing flies. In contrast, for F57I-expressing flies, the EGFP level increased over time, revealing that despite the presence of SAP the UPR is upregulated. However, in the presence of SAP the increase in EGFP over time was lower compared to in the absence and the level at day 35 was lower in the presence of SAP than without SAP (Fig 22C), demonstrating that SAP

Figure 22. UPR activity in WT- and F57I-expressing flies without (A) and with (B) SAP co-expression, based on nanograms of EGFP detected per unit total protein content (mg/ml) in the samples. Bars represent means $\pm$ s.e.m. (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$) (C) Trend lines for EGFP accumulation in F57I-expressing flies over time in the absence (solid line) and presence (dashed line) of SAP.

can suppress ER stress in flies caused by expression of the amyloidogenic variant. In other *Drosophila* models, activation of the UPR is commonly protective. However, in our model of lysozyme amyloidosis, we speculate that upregulation of the UPR in the CNS neurons caused by accumulation of misfolded F57I species in the ER, is too severe for the UPR mechanism to counter. Hence apoptosis results from the prolonged exposure of the ER to misfolded F57I species and when a sufficient proportion of neurons are apoptotic the health of the fly critically deteriorates, resulting in the observed lifespan deficiency for F57I flies. It is highly likely that SAP can retard accumulation of misfolded F57I in the ER and thereby postpone upregulation of the UPR and prevent apoptosis of the nerve cells. SAP has previously been shown to act as a molecular chaperone *in vitro* (80), enhancing the refolding yield of denatured LDH. Accordingly, we propose that SAP may act as a molecular chaperone towards F57I, binding and stabilizing the misfolded or unfolded forms and thereby reducing the crowding of the ER.

In conclusion, we suggest that SAP can prevent cytotoxic effects of expressing F57I in fly CNS by retaining F57I in a soluble form and preventing crowding of misfolded F57I in the ER. If we speculate regarding the action of SAP in lysozyme amyloidosis, is it possible that SAP is able to bind to a portion of the amyloidogenic variants that escaped the UPR and thereby hinder aggregation and accumulation. However, over time a substantial amount of unstable lysozyme variants are neither degraded by the UPR nor captured by SAP, allowing them to aggregate and give rise to the disease.

4.3 Paper III

In paper I and II the lysozyme variants were expressed individually in the flies, however, patients affected by lysozyme amyloidosis are heterozygous in the gene, and carry one allele for the WT protein and one allele for the disease associated variant. Therefore we created double transgenic flies to investigate the effects of co-expressing WT and F57I in the fly CNS. The first analysis performed was the locomotor assay using iFly, which showed that the

Figure 23. A) Velocities recorded over time for WT, F57I, WT-F57I and control flies. B) Angle of movement recorded over time for WT, F57I, WT-F57I and control flies.

movements of control flies, WT, F57I and WT-F57I flies were similar at the beginning of the experiment. However, while the velocity remained essentially unchanged for control, WT and F57I flies over the course of the experiment, the velocity for the WT-F57I flies decreased substantially after 10 days and after 15 days the movement of the flies was so limited that the analysis could not be continued (Fig 23A). Also the angle of movement, which probes the flies' deviation from a straight path, showed that the WT-F57I flies differ from the other genotypes and deviate more from a straight path (Fig 23B). The data from the locomotor assay demonstrate that co-expressing WT and F57I in the CNS of the fly causes neurological damage leading to dysfunctional mobility.

The lifespan of the flies was also examined; WT-expressing flies had a median survival similar to that of controls (34 vs. 38 days) while the F57I flies had a substantially shorter median

Figure 24. Survival trajectories for flies expressing WT, F57I, WT-F57I and nsyb-Gal4 control flies. Kaplan-Meier graph showing per cent survival vs age of flies in days **p<0.01, ****p<0.0001.

survival (23 days), in line with previous survival data in paper II. However, the WT-F57I flies had the shortest median survival, only 16 days, demonstrating that co-expression of both lysozymes causes a higher toxic effect compared to expressing F57I alone (Fig 24).

When performing MSD-analyses on the flies to examine the soluble and insoluble levels of lysozyme at day 0, the levels of WT and F57I, when expressed individually, were found to be in coherence with the MDS data in paper II; WT was evenly distributed between soluble and insoluble and more abundant than F57I, and lysozyme was found mostly in the insoluble fraction for the F57I flies (Fig 3, paper III). The protein levels for WT-F57I-expressing flies were higher than for WT alone in the soluble fraction and similar in the insoluble fraction. This suggests that the lysozymes in WT-F57I flies are not degraded, as is the case for F57I flies (Fig 3, paper III).

Figure 25. Brain section stained with pFTAA (green, amyloid structures) and ToPro3 (red, cell nuclei) for control, WT, F57I and WT-F57I flies at day 0 and day 15. Normalized emission spectra from pFTAA fluorescence detected in WT-F57I flies at days 0 and 15. Pictures captured at 20x and 40x magnification.

The considerable amount of insoluble lysozyme in the WT-F57I expressing flies together with the locomotor and survival deficiencies lead us to believe that the neurological effects are due to a cytotoxic species formed in these flies. To investigate if these insoluble species might be amyloid fibrils, the dye pFTAA was used to stain the species. pFTAA is a Luminescent Conjugated Oligothiophene (LCO), a dye specifically designed to recognize amyloid structures. Flies expressing the different lysozymes were sectioned and stained with pFTAA and the nuclei marker ToPro3, at day 0 and day 15. The sections were examined using a fluorescent microscope. No pFTAA positive aggregates were detected for control, WT and F57I flies at the two tested time points; however for the WT-F57I flies, accumulation of pFTAA positive aggregates could be detected at day 15. (Fig 25).

In conclusion our study shows that co-expression of WT lysozyme and the amyloidogenic variant F57I results in neurological damage and is required for accumulation of amyloid deposits, which is characteristic for the disease observed in humans. Our data suggest that

insoluble amyloid species or intermediate species, formed on the pathway toward amyloid species, may be cytotoxic and thus contribute to the impaired neurological functions observed for the WT-F57I flies.

This work is still ongoing and the next step will be to further characterize these insoluble amyloid and possibly toxic species that accumulate in WT-F57I flies and to find out how they differ in structure from the insoluble species formed in WT-expressing flies that do not possess cytotoxicity. This might give new insight into the mechanism how cytotoxic effects are mediated by aggregated lysozyme species that can help finding novel therapeutic strategies for this amyloid disease.

4.4 Paper IV

The aim of paper IV was to investigate if lysozyme could play a role in AD, since it is known that A β deposits triggers neuroinflammation and that lysozyme is increased in CSF during inflammation (214-216).

The level of lysozyme in CSF from control and AD patients was measured and lysozyme was found to be significantly increased in CSF from AD patients (Fig 26A). The levels of lysozyme in serum from control and AD patients were measured and no differences were detected between control and AD patients (Fig 26B). To rule out that the upregulation of lysozyme was due to neurodegeneration in general, CSF from a set of patients with normal A β_{42} and P-tau_{181P} levels but elevated total tau (T-tau) was analyzed. No difference in lysozyme levels could be detected between control and high T-tau samples, indicating that the upregulation of lysozyme in AD patient CSF is not due to general neurodegeneration (Fig 26C). Human neuroblastoma cells were treated with oligomeric A β_{42} and the levels of lysozyme were probed using western blot to investigate if A β could cause a change in lysozyme expression; indeed, lysozyme was found to be upregulated in the cells exposed to A β_{42} (Fig 26D).

Figure 26. A) CSF level of lysozyme (Lys) from ten controls (C) and ten AD patients were analyzed using western blot and by densitometric quantification of the scanned western blots. The protein levels are normalized to the mean of the control. B) Levels of lysozyme in CSF and serum from ten controls and ten AD patients were analyzed using Meso Scale Discovery. C) The lysozyme level in five control samples and five samples with high T-tau levels (High T-tau) were analyzed using western blot and by densitometric quantification of the scanned western blots. D) Intracellular levels of lysozyme in differentiated SH-SY5Y cells treated with 1 μ M oligomeric A β for 24 and 48 h. The bars represent the mean $\pm$ SD, **p < 0.01.

Figure 27. A) Aggregation of 10 μM A β with or without 10 or 40 μM lysozyme (Lys) for 24 h probed by ThT. A representative ThT fluorescence curve from three experiments are shown B) TEM images of A β (10 μM) aggregated alone or in the presence of 10 or 40 μM lysozyme. Images were taken at the end of the aggregation experiment in A. Scale bar = 500 nm. C) Fluorescence from anti-A β antibody (red) and from anti-lysozyme antibody (green) in samples analyzed at the end of the aggregation experiment in A. D) ELISA analysis of oligomeric A β at the end of the aggregation experiment in A. E) Viability of differentiated SH-SY5Y cells analyzed by XTT after 3 days exposure to A β (10 μM) aggregated for 6 or 24 h alone or in the presence 40 μM lysozyme. The bars represent the mean $\pm$ SD, * $p < 0.05$, ** $p < 0.01$ and $n=4$.

The binding properties between lysozyme and A β_{42} were measured using FRET steady state fluorescence revealing a moderate binding between lysozyme and monomeric A β (Fig 2, paper IV). To investigate if binding between lysozyme and A β has any effect on the A β aggregation process, A β_{42} was allowed to aggregate in the presence of lysozyme at ratios 1:1 and 1:4 and the process followed by Thioflavine T (ThT) fluorescence. For A β alone a characteristic sigmoidal fibrillation curve was detected (Fig 27A), in the presence of 1:1 lysozyme:A β the kinetics was substantially slowed down and in the presence of four times more lysozyme than A β , the increase in ThT fluorescence was very low (Fig 27A). The end-point of aggregation for each sample was examined using transmission electron microscopy (TEM); fibrils were formed from A β alone as expected. In the presence of lysozyme at equal amounts, much smaller aggregated species were formed together with fibrillar species (Fig 27B). TEM for A β and four times more lysozyme was dominated by aggregated non-fibrillar structures. The samples were examined for possible co-localization using antibodies against lysozyme and A β . In the sample with A β alone only fluorescence from the anti-A β -antibody could be detected (red) and in the sample with lysozyme alone only fluorescence from anti-lysozyme-antibody could be detected (green). In the samples with both lysozyme and A β_{42} aggregates could be found, with different morphology depending on the ratio. For 1:1 lysozyme:A β large yellow flecks,

were A β and lysozyme co-localize could be detected along with some small spheres of A β alone and amorphous species of lysozyme alone (Fig 27C). Product formed from the 1:4 ratio of A β and lysozyme appeared as small yellow spheres with almost complete co-localization (Fig 27C).

The effect of lysozyme on cytotoxicity was examined in cells exposed to A β aggregated with or without lysozyme. Even though the TEM images suggest that smaller non-fibrillar species with possible oligomeric toxicity is formed when A β aggregates with lysozyme at 1:4, no enhanced cell toxicity could be detected demonstrating that the formed structures do not possess any cytotoxic properties. Instead the presence of lysozyme abolished formation of toxic A β species.

Figure 28. A) Survival trajectories for flies expressing A β in the CNS in the absence and in the presence of lysozyme (Lys:A β) and for control flies (only expressing Gal4). Kaplan-Meier graph showing percent survival vs. age of flies in days (**p<0.01). Locomotor assay performed by using iFly: B) velocities and C) angle of movement recorded over time of A β , lysozyme-A β (Lys:A β) and control flies.

The *in vivo* implications of lysozyme on A β cytotoxicity was investigated using a novel AD/lysozyme *Drosophila* model where A β_{42} and human lysozyme were co-expressed in fly CNS. The survival assay showed that expression of the A β peptide caused a reduction in the fly survival by 4 days compared to control flies; this reduction was completely prevented by co-expressing lysozyme with A β (Fig 28A). A β -expressing flies also showed a reduction in velocity compared to control flies, reaching a predetermined cut-off value of 4 mm/s at day 20

compared to day 35 for control flies. Upon co-expression with lysozyme the cut-off value was reached 6 days later compared to flies expressing A β alone (Fig 28B). The angle of movement showed the same pattern as survival and velocity; in the absence of lysozyme, flies expressing A β reached a cut-off value of 80° at day 14. In the presence of lysozyme this value was not reached until day 21 (Fig 28C).

Sections of *Drosophila* brains were probed with anti-lysozyme and anti-A β antibodies, in flies co-expressing lysozyme and A β a strong co-localization was detected (Fig 29).

Figure 29. Sections (20 μ m) of *Drosophila* brains (zoomed in to superior medial protocerebrum and fan-shaped body, inset figure) (day 20) from A β , lysozyme (Lys), lysozyme-A β (Lys:A β) and control flies (only expressing Gal4) co-stained with anti-A β (red) and anti-lysozyme (green) antibodies followed by counterstaining of cell nuclei by DAPI (blue). Co-localization is shown in yellow for the Lys:A β flies. Micrographs were taken at 40x magnification. Scale bars = 50 μ m.

To investigate if expression of lysozyme affects the levels of A β in the *Drosophila* brains, the levels of A β were measured at day of eclosion (day 0) and when the flies were aged for 20 days. The insoluble fraction of A β was found to be higher than the soluble fraction at both days 0 and 20 and the insoluble fractions accumulated over time. In aged flies, both the soluble and insoluble levels of A β were significantly lower in lysozyme-A β flies compared to flies expressing A β alone (Fig 6 in paper IV).

In conclusion, the presence of lysozyme resulted in altered aggregation properties for A β , reduced toxicity of A β in a cell model and a protective effect in a *Drosophila* model of AD. These *in vitro* and *in vivo* effects might be mediated by the binding affinity of lysozyme to A β . This

study suggests that when lysozyme binds to monomeric A β , the pool of free monomeric A β peptides, which can aggregate and form insoluble cytotoxic species, is reduced in favor of the formation of a lysozyme-A β complex. This complex is able to aggregate to form non-toxic and non-fibrillar structures, composed of both lysozyme and A β . The formation of these lysozyme-A β aggregates is likely to occur on a pathway that differs from the A β fibril aggregation pathway. Previous studies show that lysozyme has anti-inflammatory and anti-oxidative properties and we demonstrate an inhibitory effect of A β aggregation and cytotoxicity. These properties make lysozyme an interesting new drug target for AD.

From the work presented in this thesis the following overall conclusions can be drawn:

- We successfully established a *Drosophila* model of lysozyme amyloidosis, resulting in a rough-eye phenotype that correlates with the amyloidogenicity of the lysozyme variants. The amyloidogenic variants D67H and F57I are destabilized enough to activate the UPR resulting in degradation of the proteins while WT lysozyme does not activate the UPR and is allowed to accumulate. This unique *Drosophila* model provides an initial foundation for generating further insights into the mechanism by which amyloidogenic lysozyme variants are able to evade the quality control system to an extent that the protein can aggregate in patients with these genetic mutations.
- Expressing F57I in the CNS decreases lifespan with 8 days compared to WT lysozyme flies and significantly upregulates UPR over time. Co-expression of SAP and F57I can prevent the cytotoxic effects of expressing F57I in the fly CNS, resulting in a restored lifespan. The rescue effect may occur via a 2-fold mechanism. Firstly, interaction between SAP and lysozyme retains F57I in the soluble fraction, preventing formation of toxic species. Secondly, SAP acts as a molecular chaperone, binding to misfolded F57I and assisting proper folding. This prevents crowding of misfolded proteins in the ER, reducing ER stress and avoiding prolonged UPR upregulation, thereby protecting the cells from apoptosis.
- Co-expressing WT and F57I in the CNS significantly reduces the lifespan compared to WT but also compared to F57I alone. Insoluble species formed in WT-F57I flies are cytotoxic whereas insoluble species from WT flies are not. Sections of *Drosophila* brains show that the insoluble species formed WT-F57I flies is amyloid (pFTAA positive). Our data suggest that insoluble amyloid species, or intermediate species formed on the pathway towards amyloid species, may be cytotoxic and thus contribute to the impaired neurological functions observed for the WT-F57I flies.
- In AD patients the levels of lysozyme are upregulated. Flies co-expressing lysozyme and $A\beta_{42}$ have a significantly improved lifespan and locomotor behavior compared to flies expressing $A\beta_{42}$ alone, suggesting that lysozyme might have a protective role against $A\beta$ toxicity. Also *in vitro*, lysozyme prevents aggregation of $A\beta_{42}$ into amyloid structures and formation of cytotoxic species. Little is known of the implication of lysozyme in AD. Our finding that lysozyme is upregulated in CSF from AD patients might be caused as a cellular compensation for decreased lysosomal function in the AD brain, by upregulation of lysosomal proteins such as lysozyme. The upregulation of lysozyme might also be an attempt to modulate the inflammatory response.

There is still much work to be done using this *Drosophila* model of lysozyme amyloidosis, for example:

- To try and determine the action by which SAP is able to, prevent accumulation of UPR in F57I flies, resulting in the rescue from neurodegeneration for these flies.
- To characterize the WT-F57I amyloid plaques in more detail; to find out if they are composed of both WT and F57I together or F57I alone.
- Use the WT-F57I flies to screen for different therapeutic compounds that can reduce or prevent the formation of toxic lysozyme species.
- To further analyze the interaction between lysozyme and A β and to study the species formed by the lysozyme:A β complex.

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