HHS Public Access

Author manuscript

*Nat Rev Drug Discov*. Author manuscript; available in PMC 2016 November 01.

Published in final edited form as:

*Nat Rev Drug Discov*. 2015 November ; 14(11): 759–780. doi:10.1038/nrd4593.

# Targeting Protein Aggregation for the Treatment of Degenerative Diseases

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## Abstract

The aggregation of specific proteins is hypothesized to underlie several degenerative diseases, collectively called amyloid disorders. However, the mechanistic connection between the process of protein aggregation and tissue degeneration is not yet fully understood. Here, we review current and emerging strategies to ameliorate aggregation-associated degenerative disorders, with a focus on disease-modifying strategies that prevent the formation of and/or eliminate protein aggregates. Persuasive pharmacologic and genetic evidence now support protein aggregation as the cause of post-mitotic tissue dysfunction or loss. However, a more detailed understanding of the factors that trigger and sustain aggregate formation, as well as the structure-activity relationships underlying proteotoxicity are needed to develop future disease-modifying therapies.

Transthyretin (TTR)[1], immunoglobulin light chain (LC)[2], serum amyloid A (SAA)[3], and amyloid-β (Aβ)[4] are examples of more than thirty human proteins whose misfolding and/or misassembly into a variety of aggregate structures appear to cause a spectrum of degenerative disorders[5]. These so-called amyloid diseases are named after the cross-β-sheet aggregates, or amyloid fibrils, that are the pathological hallmarks of these maladies[6, 7]. Amyloid fibrils in a specific disease are generally composed predominantly of one protein[5]. Amyloid fibrils from different diseases and composed of different proteins exhibit similar structural features[6].

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In affected patient tissues, protein aggregation and deposition mainly occurs at the normal extracellular or intracellular location of the aggregation-prone protein. However, there is increasing evidence for the presence of both intra- and extracellular aggregates in nearly all of the aggregation-associated degenerative diseases[8-10]. Moreover, evidence indicates that aggregates can travel between intracellular and extracellular locations, suggesting that intracellular toxicity might also contribute to the pathology once thought to result exclusively from extracellular aggregation, e.g., Aβ aggregates in Alzheimer's disease (AD)[11-16]. Furthermore, cellular uptake and release of protein aggregates appears to contribute to their spreading within a multicellular organism and the associated pathology and tissue damage[17-20]. However, the mechanism(s) by which the process of intra- and/or extracellular aggregation cause pathology remains unclear.

Strong genetic, pharmacologic, biochemical and pathologic evidence support the hypothesis that human amyloid diseases result from the process of protein aggregation or amyloidogenesis (Fig. 1)[21-28]. By the “process of protein aggregation” we are referring to aggregation in a multicellular organism wherein physical chemical forces and biological modifiers together influence the aggregate structural ensembles afforded. It is important to recognize that there is an incomplete understanding of aggregation, both *in vitro* and in a multicellular organism, because probes to monitor the different types of aggregates formed or the structures afforded during this dynamic process are not available. In the absence of more detailed information about the ensemble of aggregate structures present in a patient, it is probably useful to think about aggregates as a spectrum of structures ranging from small relatively unstructured oligomers to structurally well-defined cross-β-sheet amyloid fibrils, recognizing that some structures may only be significantly populated in an organism or in certain cellular compartments. It is also unclear, which of the aggregate types are toxic and what the mechanism of cytotoxicity is. A current hypothesis suggests that smaller diffusible oligomers, exhibiting a spectrum of structures, rather than the insoluble cross-β-sheet amyloid fibrils are driving the degenerative pathology[29]. However, these diffusible oligomers could result from fragmentation of fibrils into small pieces no longer capable of supporting a cross-β-sheet amyloid structure or from unsuccessful degradation of amyloid by the lysosome or proteasome. Thus, we hypothesize that it is important to consider protein aggregation *in vivo* as a dynamic process with many players. Even with this incomplete knowledge of the aggregated structures present in patients, preventing active protein aggregation and/or removing diffusible proteotoxic aggregates, as well as ameliorating the toxic effects of aggregates while maximizing the physiological function of these proteins, are the focus of therapeutic strategies currently being developed[22-27, 30-32].

## Amyloidogenesis: The Process of Protein Aggregation

There are two categories of amyloidogenic proteins (Fig. 1). In the case of proteins that initially adopt a well-defined, folded, three-dimensional structure (category 1), substantial evidence supports the idea that a partial loss of this well-defined structure is required for their aggregation[33-36]. Early studies on TTR and LC demonstrate that conformational changes alone are sufficient to enable these proteins to misassemble into a spectrum of aggregate structures, including amyloid fibrils[33, 34]. These observations provide substantial evidence supporting the “conformation change hypothesis” as the basis for their

aggregation. The same forces that drive intramolecular protein folding, including hydrogen bonding and the hydrophobic effect, also mediate intermolecular aggregation or concentration-dependent amyloidogenesis when the misfolded protein is present at sufficient concentrations[33, 37].

Intrinsically disordered polypeptides are the second category of amyloidogenic proteins (category 2)[38, 39]. Generally speaking, category 2 polypeptides undergo thermodynamically linked aggregation and conformational changes, affording more β-sheet-rich structures, often requiring a nucleation step (Fig. 2). Category 2 polypeptides can be further separated into two subcategories. The first of these, category 2a, are intrinsically disordered polypeptides that result from the endoproteolysis of a large precursor protein. The best-studied example is the amyloid-β protein (Aβ), which is actually a family of peptides arising from β- and γ-secretase endoproteolysis of the amyloid precursor protein (APP)[40, 41]. Under pathological conditions, Aβ forms extracellular aggregates in the brain parenchyma and/or the walls of blood vessels in the brain, and, to a lesser extent, inside of brain neurons—these processes have been linked to AD and cerebral β-amyloid angiopathy. The SAA peptide, derived from endoproteolysis of the SAA protein, that is produced in high concentration as a consequence of chronic inflammation, is also a well-studied intrinsically disordered category 2a protein whose aggregation causes AA amyloidosis[3]. The aggregation propensity of intrinsically disordered proteins, such as SAA and Aβ, is strongly influenced by their concentration, post-translational modifications, and the exact sequence afforded by the endoproteolytic processing event(s).

The huntingtin (htt) protein[42] - whose aggregation underlies Huntington's disease - is also thought to be a category 2a protein. The majority of publications suggest that an N-terminal fragment of htt, including the polyglutamine (polyQ) stretch, is released following proteolysis and/or aberrant RNA splicing, triggering the formation of aggregates, and is more toxic than the full-length htt[43, 44]. Other mechanisms underlying htt cytoxicity have been suggested, including altered protein structure of the polyQ stretch within the full-length htt and possibly altered protein-protein interactions of full-length htt. Other polyQ disorders may result from the misfolding and misassembly of category 1 proteins, e.g., spinal and bulbar muscular atrophy, a neuromuscular disorder apparently caused by the aggregation of an expanded polyQ tract in the androgen receptor[45]. It is clear that more thorough observations are needed with regard to whether full-length and/or fragment polyQ proteins are driving pathology. Nonetheless, the number of CAG repeats in several polyQ expanded proteins, including htt, correlates with the age of onset and disease severity, suggesting that the increased probability for misassembly correlates with proteotoxicity[46].

Category 2b polypeptides are full-length intrinsically disordered proteins that aggregate without the requirement for endoproteolysis. This classification includes proteins such as tau[47] whose aggregation is associated with forms of frontotemporal dementia and AD, and α-synuclein[48] whose amyloidogenesis is thought to cause Parkinson's disease. These category 2b proteins aggregate intracellularly. The propensity of tau and α-synuclein to aggregate is influenced principally by concentration, and by post-translational modifications like phosphorylation and nitrosylation at certain residues in sporadic forms of these diseases or by point mutations in familial categories of these maladies[49, 50]. It should be noted that

recent findings suggest that the α-synuclein protein may also adopt a quaternary structure within neurons under specific conditions, thus challenging the previous notion that α-synuclein is solely an intrinsically disordered protein[51-53].

Typically, the misfolding and/or aggregation of category 1 or 2 proteins are thought to cause a “gain-of-proteotoxicity” phenotype, disturbing cellular function(s) and initiating toxicity cascades (Fig. 1). However, it is worth noting that loss of function may also contribute to aggregation disease pathogenesis. This seems especially relevant for nuclear proteins like TDP-43 (TAR DNA binding protein of 43kDa) and FUS (Fused in Sarcoma, an RNA binding protein) involved in amyotrophic lateral sclerosis and frontotemporal lobar degeneration type dementia. Both TDP-43 and FUS relocate to and aggregate in the cytoplasm, where they can also be recruited to stress granules, and cause translational alterations[54, 55]. In the case of C9orf72 hexanucleotide repeat expansion-associated amyotrophic lateral sclerosis and frontotemporal dementia, it appears that sense and antisense RNA aggregates as well as aggregation of proteins resulting from unconventional translation into polydipeptides are jointly responsible for the neurodegenerative phenotypes[56]. Importantly, protein aggregates can sequester folded proteins and RNA, altering additional cellular functions[57]. In addition, the buildup of protein aggregates can impair proteostasis network capacity and thereby compromise the quality of other cellular protein components.

We define the process of protein aggregation or amyloidogenesis as encompassing all misassembled structures formed, irrespective of their conformations. Aggregates include low n oligomers of heterogeneous structure, rather disordered micelle-type structures, amorphous aggregates of various sizes, β-sheet-rich structures, and cross-β-sheet amyloid structures (Fig. 1)[29, 58, 59]. Misfolding of the monomer could also contribute to proteotoxicity and should not be overlooked. At least three distinct mechanisms of protein aggregation are supported by the literature (Fig. 2). These mechanisms are largely based on *in vitro* data and their consistency with mathematical models of protein aggregation.

The first mechanism posits that protein aggregation occurs by a nucleated polymerization, wherein a high-energy, sparsely populated species (typically oligomeric, but monomers have also been implicated) is formed that can subsequently efficiently add monomers to form a thermodynamically more stable aggregate structure[60-62] (Fig. 2A). A nucleus can have a cross-β-sheet structure, or it can have an alternative structure that may or may not later conformationally convert to a cross-β-sheet structure[63]. As mentioned above, nucleation is often required for the aggregation of both category 1 and 2 proteins[64, 65]. The requirement for nucleus formation can be bypassed by adding aggregate seeds (often cross-β-sheet aggregates) of a particular protein, such as Aβ, to a solution of monomers of the same protein (Fig. 2A). This enables efficient aggregation by exogenous seeding–thereby accelerating aggregation[62]. Indeed, there is now ample evidence that this type of seeding mechanism might be an important step for the progression and spreading of aggregation in neurodegenerative disorders (discussed in more detail below). Thus, aggregation could start in a single rogue cell or tissue and spread throughout the brain by cellular uptake of seeds followed by cellular secretion of the amplified aggregates either by canonical or alternative pathways[8, 17, 66, 67]. Based on *in vitro* and *ex vivo* data, it is commonly assumed that seeded

polymerizations afford only structures identical to the seeds and not other structures that currently cannot easily be monitored. However, this may not be true in a multicellular organism where biological modifiers influence the process. In fact, it is hard to explain why oligomers can be isolated from tissue containing amyloid fibrils if seeded polymerizations only afford cross-β-sheet amyloid fibrils.

In the second accepted mechanism of aggregation - a nucleated conformational conversion - an equilibrium exists between monomers and structurally heterogeneous oligomers that are generally more stable than the monomers, but this does not always have to be the case (Fig. 2B)[63]. Over time, the oligomers are converted into a nucleus and then into amyloid fibrils. The fibrils could conformationally convert the proximal monomers into amyloid fibers, but this does not appear to occur with all proteins.

A third mechanism of protein aggregation posits that category 1 proteins sometimes aggregate by a downhill mechanism (Fig. 2C), a process that is not seedable[68]. In the downhill polymerization scenario, formation of the aggregation-prone misfolded monomer from a natively folded protein is the rate-limiting step (a step not shown in Fig. 2C). Partially folded monomeric proteins aggregate to afford thermodynamically more stable higher order aggregates including amyloid fibrils, thus high energy nucleus formation is not required for efficient aggregation[68]. Subsequent addition of misfolded monomers to the growing polymer is always energetically favorable in this mechanism of aggregation. Transthyretin appears to form amyloid fibrils and other aggregate structures in parallel by a pathway analogous to that shown in Fig. 2C, wherein tetramer dissociation is rate limiting.

While these models of protein aggregation provide a framework for our understanding, it seems likely that conditions in living organisms are more complex and it is possible that several different aggregate structures may coexist, e.g., due in part to unsuccessful lysosomal or proteasomal degradation. Different cellular environments (e.g., lysosomes vs. endoplasmic reticulum vs. cytoplasm vs. the extracellular space) provide distinct conditions for the formation and sustainment of different aggregate structures, likely influencing the ensemble of aggregate structures present. Efforts are underway to improve detection and quantification of aggregate structures in addition to cross-β-sheet amyloid. The availability of these probes is expected to greatly advance our understanding of structure-proteotoxicity relationships.

## Amyloid versus the Aggregation Process as the Toxicity Driver

The extracellular and/or intracellular cross-β-sheet amyloid fibril deposits in tissues of patients afflicted with amyloid diseases are recognized as the histopathological hallmarks, and still serve as the basis for a definitive diagnosis of the systemic amyloidoses and diagnostic confirmation of brain disorders such as AD postmortem (Box 1)[7]. While the protein deposits in human amyloid diseases often consist of aggregates of diverse structure, the focus has been on the cross β-sheet amyloid fibrils because of their well-defined structure, and thus ease of detection[6]. Amyloid fibrils bind to Thioflavin T, Congo red, and other aromatic chromophores that exhibit fluorescence and/or birefringence. These attributes help pathologists identify amyloid in tissue. Amyloid fibrils are typically covered by or co-

deposit with glycosaminoglycans, the amyloid P glycoprotein, and other cellular and extracellular macromolecules in humans.

It remains unclear to what extent amyloid fibrils are by themselves the toxic entities, or if other forms of misassembled proteins, e.g., non-uniformly structured oligomers, are contributors to cytotoxicity or are the primary drivers of proteotoxicity. Whether this cytotoxicity derives from intracellular and/or extracellular aggregates is unknown. It also remains unclear whether the sequestration of the smaller non cross-β aggregates into cross-β-sheet amyloid fibrils would possibly mediate some protection (Box 1). Amyloid load often does not correlate with disease status, calling into question whether amyloid fibrils are the main driver of neurodegeneration, a topic that has been discussed intensively in the AD field[69]. It has been suggested that amyloid formation may even be protective, the hypothesis being that the more toxic, structurally diverse aggregates are converted into less toxic cross-β-sheet amyloid fibrils, minimizing exposure of hydrophobic surface area and open hydrogen bond valencies that mediate aberrant interactions with proteins, nucleic acids and carbohydrates[70]. Experimental evidence suggests that neuronal survival is increased in cultured cells when mutant huntingtin is sequestered into inclusion bodies, concomitant with a decrease in the more diffuse aggregated forms of cellular mutant huntingtin[71]. Moreover, amyloid clearance occurs in some, but not in the majority of patients benefiting from disease-modifying therapies on the timescale of the clinical response. Amyloid clearance needs to be studied more systematically, especially in the peripheral amyloidoses where disease-modifying therapies exist so that clear conclusions can be drawn[30, 72, 73]. Numerous laboratories have hypothesized that smaller diffusible aggregates of poorly characterized structure, often called oligomers or protofibrils, that often escape histological detection, are the toxic species driving post-mitotic tissue degeneration in the amyloidoses[29, 58]. Acute cellular toxicity assessments *in vitro* and *in vivo* also indicate that smaller soluble and diffusible aggregates are notably more toxic than amyloid fibrils[74-76], while conversion of toxic oligomers to amyloid fibrils can reduce toxicity[75, 77]. Our understanding of structure-proteotoxicity relationships is currently incomplete, largely due to technical limitations; thus we urgently need better technologies to specifically detect and quantify amyloid and non-amyloid aggregate types, which could also be useful as much needed biomarkers for diagnosing and monitoring response to therapy in the amyloidoses.

There is mounting evidence that the process of protein aggregation or amyloidogenesis is an important driver of neurodegeneration. Amyloidogenesis is dynamic, i.e., there is a constant flux of newly synthesized proteins aggregating into a spectrum of aggregates that are transient structures[78, 79]. These fleeting and structurally heterogeneous aggregates evolve into progressively larger aggregates[80], and if the conditions are right, into amyloid fibrils. Presumably, most of the non-fibrillar aggregate structures (Fig. 1) are intrinsically unstable owing to exposed hydrophobic surfaces and/or the presence of unsatisfied hydrogen bonds, and can engage in aberrant molecular interactions. Amyloid fibrils are less able to engage in inappropriate molecular interactions because of the more extensive burial of hydrophobic residues and the formation of intermolecular hydrogen bonds[6]. It is now clear that nearly all proteins capable of forming amyloid are also capable of forming a spectrum of aggregates of

diverse structure that appear to be more toxic than amyloid fibrils to cultured cells, but whether this is the case in humans remains unclear.

Autosomal dominantly inherited familial amyloid diseases are caused by mutations that can destabilize the native structure of category 1 proteins, enabling their partial unfolding and rendering them aggregation prone[33, 34, 36]. Similarly, mutations in intrinsically disordered category 2 proteins can facilitate aggregation and cause familial forms of amyloidosis[81, 82]. An increased concentration of category 2 proteins, e.g., of α-synuclein through gene duplication in Parkinson's disease and of APP and Aβ in Down's Syndrome-linked Alzheimer's disease, or variations within promoter or regulatory regions can lead to aggregation-associated degenerative pathologies[83-85].

## Strategies to Target The Process of Protein Aggregation

### Protein reduction strategy

The first successful treatment of a human amyloid disease not caused by an underlying pathology (such as a cancer or inflammation) occurred in the context of the transthyretin (TTR) amyloid disease, familial amyloid polyneuropathy (Box 2). This strategy involved surgically-mediated gene therapy[86, 87](Table 1). Familial TTR amyloid diseases are caused by the aggregation of predominantly mutant TTR and, to a lesser extent, wild type (WT) TTR[88]. Since the liver secretes 95% of WT and mutant TTR into the bloodstream, replacing the liver of a heterozygous polyneuropathy patient secreting V30M and WT TTR with a WT / WT TTR secreting liver from a normal subject by liver transplantation abrogates the secretion of mutant TTR into the blood. This resulted in increased serum concentrations of the more kinetically stable, less aggregation-prone WT TTR tetramers (Fig. 3A), which slowed the progression of peripheral and autonomic neuropathy and significantly extended the lifespan of familial amyloid polyneuropathy patients.[89, 90]. Amyloid clearance appears to occur slowly in transplanted patients. The newly available Positron Emission Tomography amyloid imaging agents would enable the rate of TTR amyloid clearance to be studied more rigorously at multiple centers and this should be done[91].

Since amyloid clearance does not appear to correlate with clinical benefit, amyloid fibrils *per se* are unlikely to directly cause the neuronal dysfunction and death associated with this malady[92-94]. However, the fragmentation of amyloid fibrils or incomplete catabolism could contribute to the spectrum of oligomers formed during the process of aggregation (Fig.1), which appear to be more proteotoxic[76]. Liver transplantation might also be useful to treat other familial amyloid diseases wherein the amyloidogenic protein is predominantly secreted by the liver, e.g., in hereditary fibrinogen amyloidosis[95].

While the pioneering liver transplant strategy has improved the health, and prolonged the lives of thousands of familial amyloid polyneuropathy patients and demonstrated that intervention was possible after the start of neuropathy, it is not without its challenges. These include the 10% mortality due to surgical complications, limited organ availability, the risks of infection associated with life-long immunosuppression, and the emergence of WT TTR aggregation-associated cardiomyopathy upon aging[96]. In addition, because liver transplant-mediated gene therapy does not alter mutant TTR secretion by the choroid plexus into the

CSF or by the retinal pigment epithelium cells into the vitreous of the eye, mutant TTR aggregation in the eye[97] and in meningeal vessels[98] appears to lead to ophthalmological and neurological symptoms in the patients treated by liver transplantation. These challenges warrant the development of alternative treatments discussed below.

The “protein reduction” strategy has also been used to successfully treat light chain (LC) amyloidosis (AL)[27, 99]. AL results when a proliferating plasma cell (a cancer cell similar to that causing multiple myeloma) secretes an immunoglobulin LC protein that is aggregation prone owing to its destabilization and misfolding and misassembly proclivity[27, 34, 100]. The misfolding and/or aggregation of LC within the circulatory system leads to multiple organ system dysfunction, including renal dysfunction and cardiomyopathy[72, 101, 102]. The concentration of LC is reduced by eliminating the clonal plasma cells secreting LC into the blood using a variety of chemotherapy agents, including proteasome inhibitors (Table 1)[27, 103]. This therapeutic strategy (Fig. 3B) leads to a dramatic and rapid improvement in patient health, especially in cardiac function, even though there is overwhelming evidence that the amyloid fibrils are not cleared from the hearts of these patients on this timescale[72]. Although the presence of amyloid in the heart is deleterious , it appears that it is the process of active LC aggregation - including the apparent uptake of proteotoxic misfolded LC monomers or oligomers by cardiomyocytes and other relevant cells - that causes the severe multiple organ system toxicity and pathology[71, 95, 96, 98]. Unfortunately, approximately 30% of the LC amyloidosis patients presenting with severe cardiomyopathy are too sick to tolerate the chemotherapy regimen[99]. Thus, other strategies to ameliorate aggregation-associated LC proteotoxicity are still needed.

An analogous “protein reduction” strategy has been employed to successfully treat systemic amyloid A, or AA amyloidosis, which arises in a subset of individuals facing chronic inflammatory disease, (e.g., rheumatoid arthritis, Crohn's disease, and recurring infections)[104]. The SAA protein is an acute phase reactant protein; therefore sustained high concentrations occur as a consequence of chronic inflammation. While high serum amyloid A (SAA) concentrations appear to be the prominent risk factor for the onset and progression of AA amyloidosis[105], they are insufficient to cause AA. Apparently, other factors are needed to contribute to the development of AA, including endoproteolysis of the SAA protein, which generates an intrinsically disordered amyloidogenic SAA fragment[106]. Kidney damage appears to result from the aggregation of SAA protein[3]. Treating the underlying inflammatory disease with anti-inflammatory drugs (Table 1) lowers the concentration of SAA and its amyloidogenic fragments (Fig. 3C), ameliorating renal dysfunction, providing strong evidence that SAA fragment aggregation causes AA amyloidosis[107-112].

Seeding of SAA fragment aggregation by injecting amyloid fibrils comprising an SAA fragment hastens the onset of AA amyloidosis in a rodent model, providing further compelling evidence that the process of aggregation is causing the characteristic renal dysfunction[66]. Seeded protein aggregation by a prion-like mechanism is now considered a major driver for propagation and intra-organismal spreading of protein aggregation for many amyloid diseases (discussed in more detail below)[8, 17]. Not surprisingly, disease severity is

also influenced by the exact sequence of the SAA protein that is transcriptionally upregulated by inflammation and generated through aberrant endoproteolytic processing[113].

The “protein reduction” strategy is also being pursued very aggressively in AD .One approach is to inhibit or modulate the activity of the β- and/or γ-secretases - endoproteases that cut Aβ out of the large amyloid precursor protein - in an effort to lower the Aβ concentration (Fig. 3D)[40, 114, 115]. After long development phases due to several challenges, including achieving effective blood-brain barrier penetration, a new generation of small molecule β-secretase (BACE) inhibitors are being evaluated in clinical trials (Table 1)[116-119]. BACE1 (β-site APP cleaving enzyme 1) is the major β-secretase enzyme in the brain initiating Aβ generation (Fig. 3D). Promising BACE inhibitors are emerging in phase I clinical trials, raising hope that a good safety profile, good pharmacokinetics, and significant Aβ lowering by BACE1 targeting is achievable, which will hopefully translate into an AD modifying strategy. Other compounds exhibiting BACE1 selectivity over BACE2, a homolog with low neuronal expression, are being developed by several companies and are starting to emerge in the patent literature. Complete inhibition of BACE1 will likely not be necessary to ameliorate AD pathogenesis, owing to the striking concentration dependence of Aβ aggregation, although BACE knockouts exhibit mild phenotypes in rodents[120].

γ-Secretase is a multi-protein complex with four essential subunits conducting intramembrane cleavage (Fig. 3D) of numerous substrates[121]. The nicastrin and PEN-2 (presenilin enhancer 2) subunits are identical in every γ-secretase complex, whereas the APH-1 (anterior-pharynx defective) subunit comes in two varieties APH-1A or APH-1B, analogous to the catalytic subunit, which is either presenilin 1 (PSEN1) or presenilin 2 (PSEN2). Challenges arose when it was discovered that γ-secretase cleaves not only APP to afford the Aβ peptides, but also >100 protein substrates, one of which is Notch, an important cellular signaling protein[122]. Studies and clinical trials undertaken so far suggest that direct γ-secretase inhibition may be problematic as a long-term pharmacologic strategy for AD, due to the strong mechanism-based toxicity observed (Table 1). Current efforts focus on modulating γ-secretase substrate specificity using small molecules that function by an allosteric mechanism (Table 1), shifting Aβ generation towards shorter, non-amyloidogenic fragments without compromising the cleavage of other important γ-secretase substrates[122, 123]. Emerging knowledge about the assembly, regulation, and specificity of the γ-secretase complexes is expected to guide these endeavors and has been reviewed elsewhere[121].

Another “protein reduction” approach is to inject antibodies (passive immunization) into AD patients that bind to Aβ peptides, promoting their clearance and thus inhibiting Aβ aggregation [124] (Table 1). Based on clinical trial data so far, this appears to be a promising approach for reducing amyloid load and continues to be pursued intensively. An active immunization strategy (vaccination) using the appropriate antigen could ultimately produce these “protein reduction” antibodies[125]. For a more detailed discussion of the immunization strategies for amyloid disorders, please see below.

Another “protein reduction” strategy being actively pursued involves gene silencing by RNA interference (RNAi) or antisense oligonucleotide technology (Fig. 3E). The reduction

of both WT and mutant TTR protein levels in the blood stream has been successfully achieved by applying either of these technologies in the liver[126, 127] (Table 1). TTR familial amyloid polyneuropathy clinical trials are currently being enrolled to test TTR RNAi (Alnylam) and to evaluate the efficacy of an antisense TTR oligonucleotide approach (Isis) (Fig. 3E). Analogous mRNA reduction approaches are also being explored in tissue culture and animal models for other amyloidoses, including AL[128, 129].

## Protein Stabilization Strategy

A different approach to preventing the detrimental effects of misfolding and aggregation of category 1 proteins is the “protein stabilization” strategy or “kinetic stabilization” strategy. Portuguese family members carrying the familial amyloid polyneuropathy V30M TTR mutation, but who exhibited a very mild polyneuropathy phenotype or no obvious pathology[130], were found to be compound heterozygotes, expressing V30M TTR from one allele and T119M TTR from the other allele, which results in the secretion of TTR heterotetramers comprising both V30M and T119M subunits in the expected stoichiometries[25]. Biophysical studies revealed that T119M TTR subunit incorporation into tetramers otherwise composed of the disease-associated TTR subunits proportionately reduces the rate of tetramer dissociation and the rate of aggregation[25, 26], by destabilizing the dissociative TTR transition state and thus increasing the activation energy for tetramer dissociation[131, 132]. This kinetic stabilization mechanism provides strong evidence that the process of aggregation or amyloidogenesis is the cause of these maladies, i.e., the V30M subunits within the context of natively folded tetramers are not in themselves proteotoxic[33, 132]. We hypothesize that V30M TTR aggregates expose hydrophobic surface area and unsatisfied hydrogen bonds that enable aberrant interactions with proteins, lipids, nucleic acids, and carbohydrates, rationalizing how the process of TTR aggregation becomes proteotoxic. While the TTR concentration is not reduced in this strategy, the concentration of TTR that can dissociate, misfold and aggregate is dramatically reduced by this interallelic trans-suppression kinetic stabilization mechanism[133].

The data outlined directly above demonstrate that TTR aggregation is suppressed and a variety of pathologies are ameliorated via the “kinetic stabilization approach”. This human genetic evidence motivated efforts to discover small molecules that could prevent TTR aggregation through an analogous kinetic stabilization of the TTR tetramer[26, 131, 132, 134, 135]. Initial *in vitro* studies revealed that when thyroxine ($T_4$) bound to the $T_4$-binding sites within TTR, the tetramer was stabilized and TTR amyloidogenesis was inhibited[134]. This finding led to a screening[136] and structure-based drug design effort[137, 138] to discover small molecule ligands that selectively and avidly bind to the TTR tetramer over the dissociative transition state, kinetically stabilizing the native, non-amyloidogenic tetramer, slowing the rate-limiting step of amyloidogenesis[26, 131, 132].

Over 1000 small molecules exhibiting structural complementarity to the $T_4$-binding sites within TTR have been synthesized over the last 20 years and over 100 ligand$_2$·TTR crystal structures have been solved, enabling the synthesis of very potent kinetic stabilizers[131, 135, 137, 138]. One of these, tafamidis,[31, 133] was evaluated in a placebo-controlled clinical trial and in a follow-on open-label study[22, 23]. Both primary endpoints

were met in the “efficacy-evaluable” population (n=87) and were just missed in the “intent-to-treat” population (n=125), apparently due to more patients than expected in the intent-to-treat population being selected for liver transplantation during the course of the trial, not as a consequence of treatment failure. However, these patients were classified as treatment failures in the conservative analysis used[22, 23]. Tafamidis (Vyndaqel® 20 mg, once daily) is now approved for the treatment of early stage transthyretin-related hereditary amyloidosis (polyneuropathy; all mutations) by the regulatory agencies of Europe, Japan, Mexico, and Argentina. Tafamidis is currently being considered for approval by the neurological division of the United States Food and Drug Administration (FDA) (Table 1).

In the process of searching for TTR kinetic stabilizers, we demonstrated that the non-steroidal anti-inflammatory drug (NSAID) diflunisal binds to and kinetically stabilizes the TTR tetramer[139, 140]. An international, randomized, double-blind, placebo-controlled clinical trial showed the ability of diflunisal to reduce the rate of progressive neurological impairment and preserve quality of life in patients suffering from familial amyloid polyneuropathy, thus enabling diflunisal to be repurposed as a TTR kinetic stabilizer (Table 1)[24]. Even though diflunisal is not very potent as a TTR kinetic stabilizer, it efficiently kinetically stabilizes TTR at a dose of 250 mg twice a day because of its very high oral bioavailability and its correspondingly very high plasma concentration (≈ 300 μM–1 mM). Since diflunisal is an NSAID, it slows renal blood flow, which is a contraindication for those patients exhibiting cardiomyopathy or renal compromise.

Collectively, the tafamidis and diflunisal clinical trial results provide compelling pharmacologic evidence that the process of TTR amyloidogenesis causes neurodegeneration, and that slowing tetramer dissociation and amyloidogenesis after symptom presentation by small molecule kinetic stabilization dramatically slows and in up to 60% of the patients stops the progression of neurological impairment. These data very strongly support the validity of the amyloid hypothesis, the notion that the process of aggregation causes post-mitotic tissue loss[132]. These data motivated the tafamidis cardiomyopathy clinical trial that is currently enrolling WT and familial patients (placebo *vs.* 20 mg *vs.* 80 mg tafamidis once daily).

## Protein Quality Control Strategy

Aging is the most significant risk factor for the development of either sporadic or inherited amyloidoses, for reasons that are still not fully understood[141]. One explanation for this is that the capacity of the cellular and/or extracellular protein homeostasis networks declines with aging, a hypothesis gaining experimental support[142]. Another non-mutually exclusive possibility is that the capacity to activate the stress-responsive signaling pathways regulating these proteostasis network functions declines with aging[142-145]. The classic view of amyloid disease etiology is that the aggregation of a specific protein causes the aggregation-associated degenerative phenotype through a gain-of-toxic-function type mechanism (Fig. 1). However, there is mounting evidence that the intracytosolic aggregation of proteins linked to specific amyloid diseases compromises the folding of the endogenous proteome by sequestering chaperones and chaperonins—resulting in the aggregation of proteins required for critical cellular functions such as controlling transcription[142, 146]. While there is no

doubt that the conformational integrity of one protein is compromised in specific amyloid diseases, it now appears that the native conformations, and thus function, of many additional proteins could be compromised due to consumption of proteostasis network capacity, suggesting that a breakdown in the ability to maintain the proteome is part of degenerative disease etiology, if not the cause[57, 142, 146].

Thus, a “protein quality control strategy” could be beneficial for ameliorating the amyloidoses. This approach focuses on adapting the intracellular and/or extracellular proteostasis pathways involved in protein folding, trafficking and/or degradation to reduce the aggregation of disease-associated proteins and possibly additional cellular proteins[147-153]. Adaptation of these cellular proteostasis pathways alters the intracellular interactions between destabilized, aggregation-prone proteins and the proteostasis network components composing these pathways, providing the opportunity to directly or indirectly attenuate proteotoxic aggregation. This “protein quality control strategy” has been primarily pursued through the activation of organelle-selective stress-responsive signaling pathways such as the heat shock response (HSR) or the unfolded protein response (UPR) that transcriptionally regulate proteostasis capacity in specific intracellular environments, such as the cytosol or the secretory pathway, respectively[154-158]. Thus, genetic and small molecule stress-independent activators of individual stress-responsive signaling pathways are now being developed (Table 1)[147, 159, 160].

Celastrol activation of the HSR, which increases the cytosolic proteostasis network capacity, has been used in the context of pre-clinical Huntington's disease models to validate the “protein quality control” therapeutic strategy[161]. This molecule primarily functions through the activation of heat shock factor 1 (HSF1; the predominant transcription factor responsible for stress-responsive upregulation of cytosolic proteostasis network components via the HSR)[155, 156]. These small molecules (Table 1) exhibit considerable promise for ameliorating pathologic intracellular protein aggregation, although not all reports agree on whether HSR activation is beneficial[162].

Another strategy to induce the HSR is to inhibit the cytosolic ATP-dependent Hsp90 chaperone. Hsp90 binds to and negatively regulates HSF1. Inhibition of Hsp90 may prevent its binding to HSF1 and also leads to the misfolding and misassembly of many cytosolic proteins that in turn activate HSF1, leading to transcriptional reprogramming of the cytosolic proteostasis network via the HSR. Hsp90 inhibition ameliorates aggregation and proteotoxicity in several experimental models of aggregation-associated degenerative diseases, including Huntington's, Parkinson's and Alzheimer's diseases[163, 164]. HSF1 can also be activated indirectly by reducing insulin / insulin growth factor 1-like signaling (IIS), which provides significant benefits in Alzheimer's and Huntington's disease models, with likely contribution from the FOXO transcriptional program(s)[75, 165]. Chemical-genetic and pharmacologic approaches (Table 1) have recently become available to activate HSF1 in the absence of stress or IIS inhibition[159, 160].

Increasing cytosolic proteostasis network capacity through the overexpression of the molecular chaperone Hsp70 was also found to significantly reduce proteotoxicity and neurodegeneration in models of Huntington's[166] and Parkinson's diseases[167]. However,

other studies did not observe the same effect[168], possibly due to different relative stoichiometry of the proteostasis network components owing to differences in Hsp70 overexpression. Binding of Hsp70 to α-synuclein aggregates was shown to reduce toxicity[169] and may even act extracellularly on oligomer formation and protect from trans-synaptic spreading of aggregates[170].

While the Hsp70-Hsp40-nucleotide exchange factor pathway is often considered to be a pro-folding pathway, there is abundant evidence that this pathway makes critical folding versus degradation or quality control decisions[153, 171-174]. Thus, small molecule modulators of this pathway could be used to enhance the degradation of certain aggregation-prone client proteins[175-177]. This approach also falls into the “protein reduction” therapeutic strategy category. There is evidence that Hsc70 (the constitutive chaperone) stabilizes tau against degradation in the brain[152], whereas Hsc72 recruits the co-chaperone ubiquitin ligase CHIP, which is known to ubiquitinate tau and facilitate its degradation[177]. Thus, inhibitors of Hsc70 should partition tau in the brain into the Hsc72-CHIP pathway, facilitating its degradation. The allosteric small molecule Hsc70 inhibitors, YM-01 and YM-08 (Table 1), both decreased tau levels in a variety of preclinical brain-relevant model systems, rendering this therapeutic strategy very promising[176].

Extracellular protein aggregation can also be attenuated by activating the unfolded protein response (UPR), which functions to regulate proteostasis (quality control) in the endoplasmic reticulum (ER) and in downstream compartments of the secretory pathway[157, 158], including in the extracellular space[178]. The UPR signaling pathway comprises three arms activated downstream of the ER transmembrane stress-sensor proteins, IRE1, ATF6, and PERK. Activation of these arms adapts ER proteostasis through both transient attenuation of new protein synthesis (downstream of PERK) and the activation of stress-responsive transcription factors including XBP1s (downstream of IRE1), ATF6 (a cleaved product of full-length ATF6), and ATF4 (downstream of PERK). These transcription factors upregulate ER protein folding, trafficking and degradation pathways comprising the ER proteostasis network.

Arm-selective activation of the UPR appears to be a very promising strategy for increasing the scrutiny of protein quality control in the secretory pathway of human cells by selectively reducing secretion of destabilized, amyloidogenic proteins[147, 148]. The hypothesis underlying this strategy is that UPR transcriptional remodeling of the ER proteostasis network could lead to increased partitioning of amyloidogenic mutant proteins (e.g., V30M TTR) to ER degradation pathways, including ER-associated degradation (ERAD) or autophagy, while still allowing efficient secretion of the more stable WT protein (e.g., WT TTR) at normal levels[147]. To date, preclinical results from this approach are very encouraging. For example, selective activation of the ATF6 arm of the UPR leads to an ≈ 40% reduction in the secretion of a destabilized A25T TTR variant, while permitting efficient secretion of WT TTR[147]. Importantly, the preferential reduction in destabilized TTR secretion afforded by ATF6 activation corresponds with increased partitioning of the destabilized TTR variants to ER degradation pathways, indicating that the reduced secretion of destabilized TTR does not lead to the intracellular accumulation of destabilized, aggregation-prone TTR that could aggregate in the ER. The ATF6-dependent reduction in

destabilized TTR secretion lowers the extracellular concentration of destabilized TTR available for concentration-dependent aggregation [147]. Both the selective degradation of an energetically compromised TTR variant and the global reduction in extracellular concentrations of destabilized TTR will reduce the TTR aggregation propensity in the extracellular space. Moreover, ATF6 activation results in the secretion of an Hsp40 chaperone, ERdj3, which further reduces aggregation of amyloidogenic proteins in the extracellular space[178]. Stress-independent activation of UPR-associated transcription factors has also recently been shown to reduce the secretion of a destabilized amyloidogenic LC without affecting the secretion of an energetically normal LC[148].

Such a dual “quality control and protein reduction strategy” is also a very attractive approach for enhancing the degradation capacity of the cytosolic proteostasis network, which is currently being investigated in patients with degenerative diseases[179, 180]. For example, the concentration of α-synuclein or tau can be reduced by diminishing the negative regulation of the proteasome[172,180]. The deubiquitination enzymes, or DUBs, are proteasome-associated enzymes that inhibit ubiquitin-mediated proteasomal degradation by removing the ubiquitin from the client protein. Thus, DUB inhibitors are being developed by several companies to enhance the degradation of client proteins, including α-synuclein and tau to treat neurodegenerative diseases [172,180]. Small molecules that inhibit USP14 (ubiquitin-specific peptidase 14, a DUB) (Table 1) are being actively pursued by more than one company to enhance clearance of aggregation-prone proteins.

### Proteolysis Strategy

Proteases such as the Aβ-degrading enzyme, neprilysin, were shown to degrade monomeric Aβ, and possibly also low n oligomeric Aβ[181]— an observation which may potentially be exploited therapeutically [182, 183]. A concern is that Aβ amyloid fibrils and possibly also late stage oligomers are no longer enzymatically degradable owing to their kinetic stability. However, it is not inconceivable that depletion of the monomer pool could not only prevent aggregate formation, but also promote fibril dissociation owing to the change in equilibrium concentrations (see ‘Targeting Amyloid Fibrils’ below). Consistent with this hypothesis, upregulation of neprilysin levels can delay Aβ deposition in APP transgenic mice[182]. Such an approach may be most useful as a preventive treatment or in the early stages of AD[184].

Catalytic antibodies that bind and proteolytically degrade amyloidogenic proteins are being explored as possible biologic drugs. If subsets of such “catabodies” could recognize a toxic/amyloidogenic epitope of a protein not exposed in the natively folded form, i.e., differentiating the native form from the toxic form, then such catabodies could hold promise for specific degradation of toxic protein structures. Importantly, degradation by catabodies should not elicit an inflammatory response. A recent study reported physiologic IgM class catabodies that degrade misfolded soluble and particulate forms of transthyretin (Table 1), but not the physiological form, suggesting a protective function of such catabodies against TTR amyloidogenesis[185]. The same lab recently engineered catabodies capable of degrading aggregated Aβ[186] (Table 1).

## Strategies that Target Amyloid Fibrils

In addition to therapeutic strategies that aim to prevent protein misfolding, and thereby the initiation and propagation of aggregation, approaches to remove or inactivate amyloid fibrils and other higher order aggregates are emerging. Due to the long presymptomatic phase of amyloid diseases, initiation of treatment may only be feasible after substantial protein deposition has occurred, especially in sporadic cases. Removal of existing aggregates is advisable, since these aggregates can themselves be toxic. Moreover, amyloid fibrils are known to dissociate[187-189], and thus it is possible that amyloid fibril dissociation to diffusible more toxic structures could continue to drive pathology in some patients treated by a “protein reduction strategy”, “kinetic stabilizer”, or “protein quality control” strategy. It might therefore be necessary to combine one of the aforementioned approaches with strategies that target the amyloid fibrils themselves, such as amyloid crosslinking/ remodeling, removal or disaggregation coupled to degradation. Elimination or inactivation of the existing amyloid load should also stop the self-sustaining cascade of seeded or prion-like misfolding hypothesized to play a significant role in driving the propagation of many neurodegenerative disorders (see below).

## Amyloid Remodeling

A recent observational report suggests that epigallocatechin-3-gallate (EGCG), the principal polyphenol present in green tea and an amyloid fibril binder and crosslinker, is effective at slowing the progression of cardiac transthyretin amyloidosis[190]. EGCG (Table 1) is able to remodel protein aggregates, including amyloid fibrils and oligomers, comprising different amyloidogenic proteins, although the mechanistic underpinnings of this process are not entirely clear[191-198]. Ample evidence shows that the EGCG amyloid remodeling activity *in vitro* is dependent on auto-oxidation of the EGCG molecule. The amyloid remodeling observed by EGCG treatment appears to be driven by hydrophobic binding of oxidized EGCG molecules to amyloid fibrils, followed by the oxidized EGCG reacting with free amines in the amyloid fibril, which cross-links the fibrils[194, 197]. Amyloid remodeling appears to stabilize the aggregates and prevent the formation of toxic oligomers, possibly explaining the reduction in proteotoxicity in the patients taking EGCG who have experienced clinical benefit[190]. A recent study suggests that EGCG can also reduce toxicity of α-synuclein oligomers, presumably by binding to the oligomers and thereby moderately reducing their toxic interaction with membranes[199]. It was also shown that EGCG is able to direct α-synuclein aggregation towards the formation of off-pathway, nontoxic oligomers, to prevent seeding of α-synuclein aggregation, and to remodel α-synuclein fibrils to smaller, nontoxic aggregates [191, 192].

## Amyloid Removal by Immunization

Another approach to rid the tissue of amyloid deposits is passive or active immunization against the amyloidogenic protein. Antibody binding to the monomeric protein, to oligomers, and/or to fibrils can direct the degradation of these structures by phagocytic cells, resulting in their clearance. This strategy is being widely explored in the context of Alzheimer's disease. Indeed, in APP transgenic mice, immunization against Aβ peptides elicits an anti-Aβ immune response and is effective at removing β-amyloid deposits[200].

Importantly, active immunization was also effective in preventing Aβ deposition when initiated during the predepositing stage (before amyloid deposition had occurred). Additional studies confirmed these findings, found improved behavioral/cognitive performance[201, 202], and suggested that this approach was suitable for removal of Aβ deposits in such mice[32]. Unfortunately, an Aβ active immunization clinical trial in late-stage AD patients revealed serious adverse events in approximately 6% of the patients, specifically aseptic meningoencephalitis, forcing Elan Pharmaceuticals to halt the trial in 2002[203]. Postmortem analysis of the brains of patients who participated in this trial confirmed lower Aβ load and evidence that amyloid was removed by immunotherapy, showing that the biological outcome was met[204, 205]. Closer examination suggests that extracellular plaques are cleared first, while vascular Aβ deposits remain longer or may even increase due to clearance of solubilized Aβ along perivascular drainage pathways[206, 207].

Passive immunization trials with monoclonal antibodies against Aβ also demonstrate that the Aβ plaque burden in mice is cleared[124, 208]. Hopes remain high that the passive immunization approach will be a safe alternative, as it does not elicit an uncontrolled autoimmune response (reviewed in [209]). Even though the AD passive immunization trials completed so far have demonstrated removal of Aβ deposits, neither significant improvement of cognitive performance, nor slower progression of the disorder, nor improved survival was observed in these later stage AD patients[205, 209]. However, recent data from a Phase Ib study has shown that Aducanumab (Biogen) treatment resulted in statistically significant reductions in amyloid load and a reduction in cognitive decline in patients with a prodromal or mild AD, providing hope for the use of this strategy. Thus, new passive immunization trials are focusing on early treatment of patients carrying AD risk genes[210].

Similar immunization strategies are also being developed for intracellular amyloid disorders such as tauopathies and synucleinopathies. Promising results were obtained in transgenic rodent models and these therapies are progressing toward clinical trials [211-216]. A Phase 1 study targeting tau by immunotherapy is ongoing (AADvac1, Axon Neuroscience SE) with results expected in 2015. With regard to α-synuclein immunization, AFFiRis (Austria) conducted a small Phase I trial and reported in a recent press release that the immunization regimen (vaccine PD01A) appears to be well tolerated, safe, and that most participants developed anti-α-synuclein antibodies. As the development of immunization strategies against tau and α-synuclein are in early stages, it is currently unknown if there is a disease-modifying effect in humans.

## Macromolecular Disaggregation of Amyloid Fibrils

Enzymatic aggregate disaggregation linked to degradation may be a possible strategy to clear amyloid deposits from tissue. The proteostasis networks of yeast and bacteria have established disaggregases, Hsp104 and ClpB, respectively[217]. Despite intensive efforts, the mammalian equivalent of these disaggregases has not yet been identified and may not exist. However, it may still be possible to engineer yeast Hsp104 or bacterial ClpB to disaggregate certain amyloids and use these variants as injectable protein drugs. For example, Jackrel et

al. showed that “potentiated” variants of Hsp104 (Table 1) discovered by screening were able to reduce TDP-43, FUS, and α-synuclein proteotoxicity in model organisms[218, 219].

Similarly, it was recently demonstrated that α-synuclein aggregates could be disassembled by the Hsp70-40-nucleotide exchange factor pathway; however, the more kinetically stable Aβ amyloid fibrils could not be disassembled[220]. It may be the case that multicellular organisms abandoned a disaggregation strategy as the primary detoxifier, instead utilizing autophagy or other pathways ending in lysosomal degradation to purge aggregates because of the risk of dissociating amyloid fibrils to more toxic structures. Strategies to use autophagy enhancement therapeutically are currently being explored, but are presently limited by our incomplete understanding of this process[221, 222].

## Inhibition of Amyloid Seeding and Aggregate Cell-to-Cell Spreading

For several amyloidogenic proteins, the transition from their native structures to amyloid appears to follow a nucleation-dependent polymerization process[61, 65, 223]. In this mechanistic paradigm, the formation of the nucleus (a putative β-sheet-rich species) is energetically unfavorable and its formation is the rate-limiting step for efficient aggregation (Fig. 2A). The formation of such a nucleus may not occur during the lifetime of a healthy individual and if nucleus formation does occur, the nucleus should typically be identified by and neutralized via one of the multiple mechanisms used by the intracellular or extracellular proteostasis networks[149]. However, if the amyloid nucleus escapes neutralization or degradation in one rogue cell or in one extracellular location within the body, it can act as a template for the further aggregation of soluble homotypic proteins (Fig. 2A)[224]. The resulting higher order aggregates are referred to as “seeds” since their addition to a monomeric solution immediately initiates aggregation of the homotypic protein without a requirement for nucleus formation. This templated or seeded aggregation is relatively efficient, relentlessly progressive and initiates a fatal cascade of progressive aggregation that also appears to drive proteotoxicity and tissue degeneration (Fig. 4).

This concept of seeded aggregation was first hypothesized to explain the pathogenesis of prion diseases[62, 225]. It is now widely accepted that prion disorders arise from misfolded and misassembled prion protein ($PrP^{Sc}$) that is sufficient to act as a seed, thus promoting sustained aggregation of the normal, cellular prion protein ($PrP^{C}$)[225]. In sporadic prion disorders, e.g., Creutzfeld Jacob Disease (CJD) in humans, $PrP^{Sc}$ nucleus and subsequent seed formation occurs spontaneously, followed by aggregate spreading from cell to cell by a seeding mechanism. Prion seeds ($PrP^{Sc}$) are also known to be transmissible between individuals, as seen in very rare instances of surgical instrument contamination that occurred in the past before adequate testing and precautions were implemented, or by funerary cannibalism as in Kuru, or more commonly by uptake of prions from the environment in bovine spongiform encephalopathy (BSE, mad cow disease), and in scrapie in sheep[225].

Several human amyloid diseases are now hypothesized to spread within the affected tissue by a seeded aggregation or prion-like mechanism, however the seeds do not appear to be transmissible between humans in contrast to the very rare transmissible prion disorders[226]. The evidence for seeded spreading of Aβ, tau, and α-synuclein aggregates comes mainly from studies *in vitro* and from animal models. However, the pathology observed during the

course of the disease in human brain provides additional support for this hypothesis, as pathology seem to spread via neurons known to be connected to one another.

Multiple research groups have now demonstrated that injecting a tissue homogenate from a human or mouse harboring an amyloid burden into an amyloid-free mouse can dramatically accelerate amyloid deposition of the homotypic protein in the recipient mouse. This seeding phenomenon for non-prion diseases was first shown *in vivo* in animal models of AA amyloidosis[227]. More recently, this approach of injecting homotypic aggregates has been shown to accelerate Aβ[228, 229], tau[230], and α-synuclein[20, 231] aggregation. The idea that aggregation can begin in one rogue cell or extracellular area and then spread within an organism is also under discussion for several other amyloid diseases[232, 233].

The experimental seeded induction of amyloidogenesis by injection of seeds is first evident within the injected brain area; however, the induced pathology subsequently spreads to neighboring and interconnected regions along neuronal pathways with increasing incubation time[234-238]. This is especially interesting and important because spreading of amyloid seeds along neuroanatomical routes would also explain why and how neurodegenerative diseases target neuronal networks[239]. Although the mechanisms of cellular release and uptake of seeds, as well as of intra- and extracellular spreading are poorly understood, it appears that seeded active aggregation on cell membranes can compromise membrane integrity, rendering the cells quite permeable, even for fibrillar aggregates[240-242]. Recent studies also show that injection of amyloid in the periphery, i.e., intraperitoneal injections of brain extracts containing Aβ or tau aggregates, can induce progressive aggregation in the brains of transgenic mice[17, 243, 244].

The aggregation-inducing seed is almost certainly the aggregated protein (e.g., Aβ) itself, in a conformation generated most effectively in the living brain[229, 245]. However, amyloid seeds can also be generated *in vitro* from synthetic or recombinant proteins[237, 238, 246-249]. Convincing evidence for seeded spreading comes from recent studies showing strain-like phenomena for several amyloidogenic proteins (Fig. 4B). Strains or specific misassembled conformations of a protein were first defined in prion disorders and were found to correlate with distinct biochemical, biophysical and pathological characteristics[250-254]. Recent studies showed similar strain-like phenomena for seeded Aβ[255-257], tau[258, 259] and α-synuclein[260, 261].

The availability of the soluble, homotypic protein for incorporation into the growing aggregate is important, as is the seed concentration, in determining the efficiency of propagation of protein aggregation[229, 236, 243]. Small soluble protein aggregates are the most potent inducers of cerebral Aβ-amyloidosis; however, seeds range in size and also comprise large, insoluble, amyloid fibrils[262263]. Importantly, seeded protein aggregation in these animal models induces the typical pathological signature that also arises from spontaneous aggregation of the respective protein in animals and humans. This suggests that the process of seeded spreading initiates the typical ensemble of aggregate structures, ranging from relatively disordered to cross-β-sheet amyloid fibrils. This process of aggregation generates proteotoxicity[241] and induces the typical tissue response, including inflammation and eventually tissue dysfunction and degeneration.

These exciting findings raise many questions, perhaps the most important being, in addition to amyloid fibrils, which other types of aggregates are formed? That the field cannot answer this question reflects how much remains to be learned. We need tools to discern whether other aggregate structures are present in these mice and in human patients in addition to cross-β-sheet amyloid structures that appear to be acting as seeds. A related question that also needs to be answered by the field is, if there are non-cross-β-sheet aggregates present, how are they formed? Is seeded growth also possible for non-cross-β-sheet aggregates or are they formed in parallel with cross-β-sheets? It is possible that incomplete degradation of amyloid fibrils or fibril fragmentation could yield the diffusible aggregate structures. A complete inventory of aggregates formed as a function of aging in non-seeded and seeded animal models of amyloid disease, as well as in human amyloidosis patients, should provide the much needed structure-proteotoxicity relationships required to understand how the process of aggregation causes post-mitotic tissue loss.

Taken together, the findings of seeded propagation of aggregates and pathology suggest therapeutic opportunities for the treatment and the prevention of these degenerative diseases (Fig. 4A). Since the formation of proteopathic seeds is a self-sustaining process, neutralizing or removing existing seeds should represent promising therapeutic strategies. Since seeded protein aggregation is concentration dependent, the formation of seeds could be prevented by reducing the concentration of the native amyloidogenic protein by the various “protein reduction” strategies discussed above. For well-folded category 1 proteins, the “protein stabilizer” strategy should prevent seed formation as well as the progression of aggregation by reducing the concentration of aggregation-competent protein. The “amyloid removal” strategies should reduce the efficiency of seeded spreading, and potentially the neurotoxic effects of protein aggregates if applied early. Immunotherapy seems to be especially promising for removing extracellular protein seeds and was shown to be effective in reducing seeded spreading of Aβ and tau in animal models[10, 229, 243]. In addition, stabilizing or remodeling fibrils to prevent fragmentation and/or seeding may slow the progression of prion-like spreading. Progression of the disease should be further prevented or restrained by inhibiting aggregate spreading within and between cells and tissues. An effective approach towards this end will likely require a better understanding of how aggregates enter cells, how they move along axons and how they are secreted from one neuron and taken up by others. All of these strategies will likely be most effective if applied early, before widespread protein aggregation and tissue damage has occurred, hence the importance of early disease detection, which is currently difficult.

## Perspective

While the exact mechanism(s) by which protein aggregation causes loss of post-mitotic tissues remains unclear, the genetic and pharmacologic evidence summarized above makes a compelling case for the hypothesis that the process of protein aggregation drives degenerative disease pathology.

Which of the therapeutic strategies discussed above will be most appropriate, most easily applicable and most effective will likely depend on the nature of the target protein, i.e., intrinsically disordered versus natively folded, extracellular versus intracellular amyloid,

nucleation-dependent polymerization versus downhill polymerization. The disease stage is also an important factor in discerning which strategy will be most effective. We envision that drugs functioning to reduce aggregation through distinct mechanisms of action will be used together in the future as combination therapies to more effectively treat amyloid diseases (Fig. 5). For example, we envision small molecule TTR kinetic stabilizers[131, 132] will be used in combination with drugs that enhance the capacity of the proteostasis network to achieve proteome maintenance[149-151] or possibly with drugs that lower the concentration of the amyloidogenic protein of interest[126, 127, 153, 160, 180, 264] (Fig. 5).

A major challenge in assessing successful therapeutic strategies for the amelioration of human amyloid disease is the lack of biomarkers for early diagnosis and for monitoring response to candidate therapies in human clinical trials. We envision that these biomarkers will be identified through basic research efforts focused on detecting and quantifying all the aggregate structures present before and after clinical symptom presentation. We also expect such biomarkers to emerge from much needed natural history studies on individual amyloid diseases. Natural history or disease progression studies facilitate the design of better clinical trials, ideally utilizing patients with early stage pathology.

Currently, we also lack an understanding of how protein aggregates induce cell toxicity and tissue degeneration, the prevention of which remains the ultimate goal of any therapeutic strategy or combination strategy. Understanding the structures of the aggregates mediating proteotoxicity and elucidating their mechanism(s) of tissue degeneration will undoubtedly provide new insights into therapeutic strategies to ameliorate these maladies, which as a group represent a significant economic burden to modern society and are therefore arguably the most urgent unmet medical need.

## Acknowledgments

The authors would like to thank the reviewers for their critical and insightful comments that shaped our thinking and helped improve the manuscript. YSE expresses particular thanks to Mathias Jucker, Tübingen, Germany, for helpful discussions and for his mentorship. We appreciate NIH support from DK46335 (JWK), AG46495 (JWK) and GM101644 (ETP). SEE is supported by an Ellison Medical Foundation New Scholar in Aging Award. YSE is supported by a postdoctoral fellowship from the German Academic Exchange Service (DAAD). JWK and ETP discovered tafamidis and JWK founded FoldRx to commercialize it. FoldRx is now owned by Pfizer, which both JWK and ETP have a financial interest in. We apologize to our colleagues whose work we were unable to include due to space limitations.

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## Glossary

**Amyloid fibrils** — Lateral assemblies of protein aggregates adopting a cross-β-sheet structure. These aggregates bind Congo red and thioflavin T and analogous fluorophores

**Amyloidogenesis** — The process of protein aggregation in a multicellular organism wherein physical chemical forces and biological modifiers together influence the aggregate structural ensembles afforded

**Nucleus** — An energetically unfavorable, sparsely populated, typically oligomeric species that is thought to be rich in β-sheet structure. Nucleus formation is typically the rate-limiting step for efficient aggregation: a step that is followed by rapid monomer addition, affording a seed

**Proteostasis Network** — The macromolecular machinery that generates, folds, moves, and degrades the proteome. Proteostasis network components include

chaperones, the proteasome, trafficking machinery, and a variety of enzymes that act on the proteome, such as disulfide isomerases

**Seed** A stable aggregate resulting from the addition of monomers to a nucleus. Seeds can also arise from the fragmentation of fibrils. Seeds enable homotypic protein aggregation without a requirement for nucleus formation, as seeded aggregation bypasses the requirement for a nucleation step

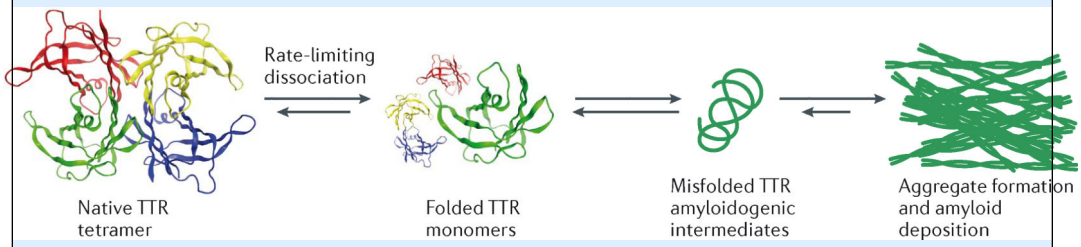
## Box 1: The amyloid hypothesis

The amyloid cascade hypothesis was first postulated in the context of systemic amyloidosis in the 1960s[265] and as a framework for Alzheimer's disease (AD) in the early 1990s[266-268]. AD is characterized by the aggregation of two proteins, namely Aβ, depositing in the form of extracellular amyloid plaques, and tau, becoming hyperphosphorylated and aggregating intracellularly to form neurofibrillary tangles[269]. The amyloid cascade hypothesis for AD, which incorporates genetic, biochemical and histological evidence, posits that the deposition of Aβ in the brain due to an imbalance between its production and clearance initiates a sequence of events that ultimately lead to AD dementia. It was proposed that the sequence of events is similar for the rare familial forms as well as for the common sporadic forms of AD. While it was originally thought that amyloid plaques containing fibrillar forms of Aβ were the pathogenic entities[268], some findings appear to contradict this. For example, reports that the amount of amyloid does not correlate with disease status[270]. Moreover, clinically normal individuals can have a substantial amyloid load, similar in amount to severely symptomatic patients[271]. More recent evidence points to a stronger neurotoxic effect from smaller, diffusible protein aggregates, often referred to as oligomers, and suggests that they may represent the disease-driving force[272, 273]. It could be that oligomers and amyloid fibrils are part of a dynamic structure-proteotoxicity relationship and that numerous structures contribute to AD pathogenesis. The amyloid cascade hypothesis for AD proposes that Aβ aggregation is the primary event and that aggregation of tau, inflammation and other changes observed in AD brains are downstream[272, 274]. If correct, prohibiting the formation of Aβ aggregates, as well as reducing or removing them should be therapeutically useful. However, so far, clinical trials targeting Aβ have not exhibited the ability to slow AD progression. This may be due to several reasons, including that advanced patients too far along in the progression of AD have been enrolled in clinical trials and/or that drugs targeting the wrong Aβ species are being pursued. These results have led investigators to question whether Aβ is the right primary target, in addition to questioning the validity of the amyloid cascade hypothesis for AD. While the amyloid cascade hypothesis was postulated to explain the temporal relationship of Aβ and tau aggregation for AD, the amyloid hypothesis in a broader sense posits that the conversion of natively folded proteins into non-native protein aggregates is the primary event in amyloid pathogenesis. Moreover, this hypothesis states that aggregation results in alterations in the cellular microenvironment occur that lead to pathological changes and eventually cellular malfunction and degeneration. In this broader sense, the amyloid hypothesis provides a framework for all amyloid disorders. Recent advances in the treatment of familial amyloid polyneuropathy, a transthyretin amyloid disease (Box 2), supports the notion that indeed protein misfolding and aggregation is the primary culprit and that dramatically slowing the process of active aggregation is disease modifying.

## Box 2: Transthyretin Amyloidogenesis

Transthyretin (TTR) is a natively folded, β-sheet-rich, tetrameric category 1 protein secreted into the blood by the liver, into the vitreous by the retinal pigment epithelial cells, and into the cerebrospinal fluid (CSF) by the choroid plexus[275-277]. In most people, TTR tetramers are made up of four identical subunits; however, the TTR tetramers in heterozygotic amyloidosis patients comprise wild type (WT) and mutant subunits[278]. The established function of TTR in the bloodstream is to transport ≈ 0.5 equivalents of retinol binding protein bound to vitamin A[279]. Transthyretin carries a substantial quantity of thyroxine ($T_4$) in the CSF, but in the blood, the two $T_4$-binding sites of TTR are 99% unoccupied owing to the presence of additional $T_4$ carriers[280]. Substantial biophysical data suggest that in the case of the 125+ distinct mutations linked to the familial TTR amyloidoses[281] (http://amyloidosismutations.com/mut-attr.php), incorporation of mutant TTR subunits into a TTR tetramer otherwise composed of WT subunits destabilizes the heterotetramer—leading to faster TTR tetramer dissociation kinetics (kinetic destabilization), an increased population of misfolded aggregation-prone TTR monomer (thermodynamic destabilization), or both[282, 283]. Kinetic destabilization is especially relevant for aggregation and likely for disease progression, since TTR tetramer dissociation is the rate-limiting step in the amyloidogenesis cascade (see figure)[25, 26]. The subsequent monomer misfolding required for TTR aggregation is much faster than tetramer dissociation[33, 68, 283-286]. Misfolded TTR monomers efficiently aggregate by a downhill polymerization mechanism (Figure 2C). Thus, TTR aggregation is not susceptible to seeding[68].

Depending on the destabilized mutant inherited in the familial TTR amyloidoses, TTR aggregation can compromise the function of the autonomic and/or peripheral nervous systems (familial amyloid polyneuropathy)[1, 287], and/or the heart (familial amyloid cardiomyopathy)[288-291], and/or the eyes[97], and/or the meningocerebrovascular system in the brain[292]. The recently renamed WT TTR Amyloidosis—formally called senile systemic amyloidosis (SSA)—results in a cardiomyopathy that affects as much as 15% of the population exceeding 80 years of age[288, 289, 293, 294]. It was recently reported that WT TTR aggregation in and around the vasculature may be a more prominent cause of vascular diseases than previously thought[295].



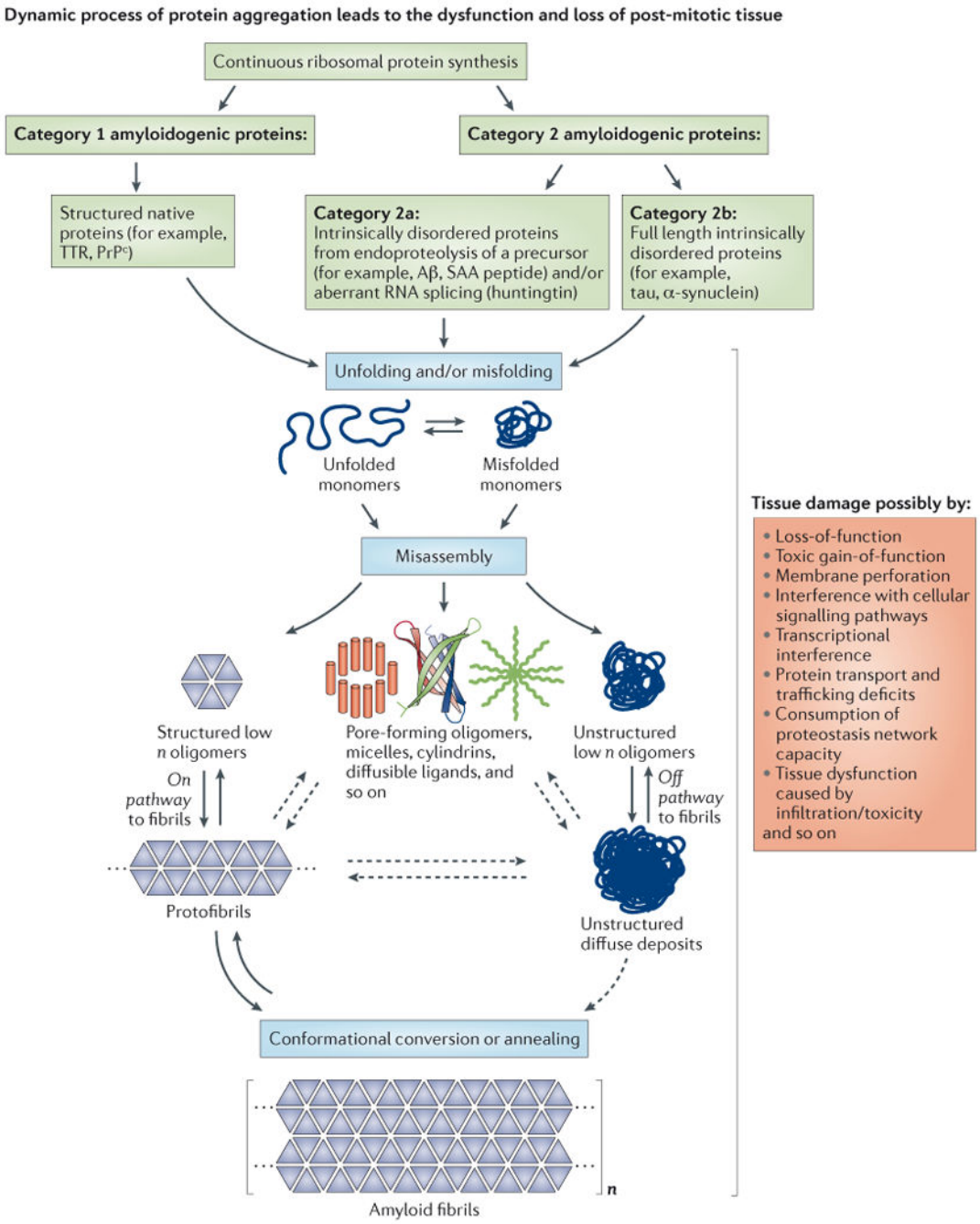



**Figure 1. Amyloidogenesis—a process of aggregation influenced by the physical chemistry of the protein as well as cellular and extracellular components**

Amyloidogenic proteins associated with degenerative disorders can be subdivided into two categories based on their native structure. Category 1 proteins, such as transthyretin (TTR) and the prion protein ($PrP^C$), exhibit a well-defined native state three-dimensional structure, whereas category 2 proteins are intrinsically disordered. Both, intrinsically disordered polypeptides generated by endoproteolytic processing of a precursor protein (category 2a), such as Aβ generated by cleavage of the amyloid precursor protein (APP), as well as full-length intrinsically disordered proteins (category 2b), such as tau and α-synuclein, can be

amyloidogenic. The critical step in amyloidogenesis is misfolding and aggregation of category 1 proteins or misassembly of category 2 proteins into a spectrum of aggregate structures, including β-sheet-rich structures and amyloid fibrils. The structures associated with the amyloid cascade are depicted along with their hypothesized mechanisms of proteotoxicity (shown on the far right). The ensemble of structures is likely influenced and some may be generated by the biology of the organism, e.g., incomplete degradation of amyloid could afford novel structures, or aggregation on cell membranes could afford aggregate structures that can only form in the presence of certain lipids and/or carbohydrates.

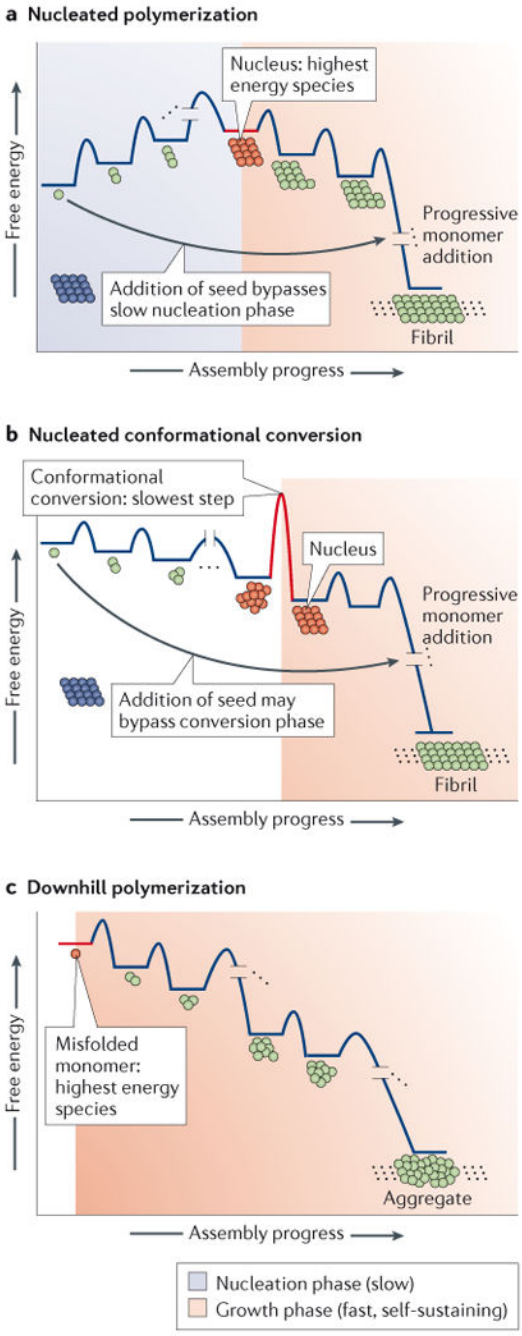



**Figure 2. Mechanisms of Protein Aggregation**

**(A)** In a nucleation-dependent polymerization, the initiating step of aggregation involves the formation of a nucleus, a sparsely populated, high energy species that has a conformation that differs from that of the soluble protein. The nucleus is typically rich in β-sheet structure and presumably is oligomeric, although monomeric species have been implicated. Once the nucleus is formed, monomers are added rapidly to the growing non-covalent polymer to generate thermodynamically more stable aggregates that can also act as seeds. Monomer aggregation can proceed very rapidly by the addition of preformed aggregates, or seeds,

because nucleation is no longer required in a “seeded” aggregation reaction. **(B)** In a nucleated conformational conversion, an equilibrium exists between monomers and structurally heterogeneous oligomers, which are typically (but not always) more stable than monomers. Over time, the oligomers are converted into a nucleus. This nucleus then initiates conformational conversion of neighboring monomers comprising the oligomer into amyloid fibers. Seeding can bypass the requirement for a slow nucleated conformational conversion step. **(C)** In a downhill polymerization, formation of the aggregation-prone misfolded monomer from a natively folded protein is the rate limiting step (not shown). Subsequent addition of monomers to the growing polymer is energetically favorable and leads to amorphous aggregate formation, as well as the generation of cross-β-sheet amyloid fibrils. Seeding does not accelerate the rate of aggregation in a downhill polymerization, at least in transthyretin amyloidogenesis studied largely in vitro.

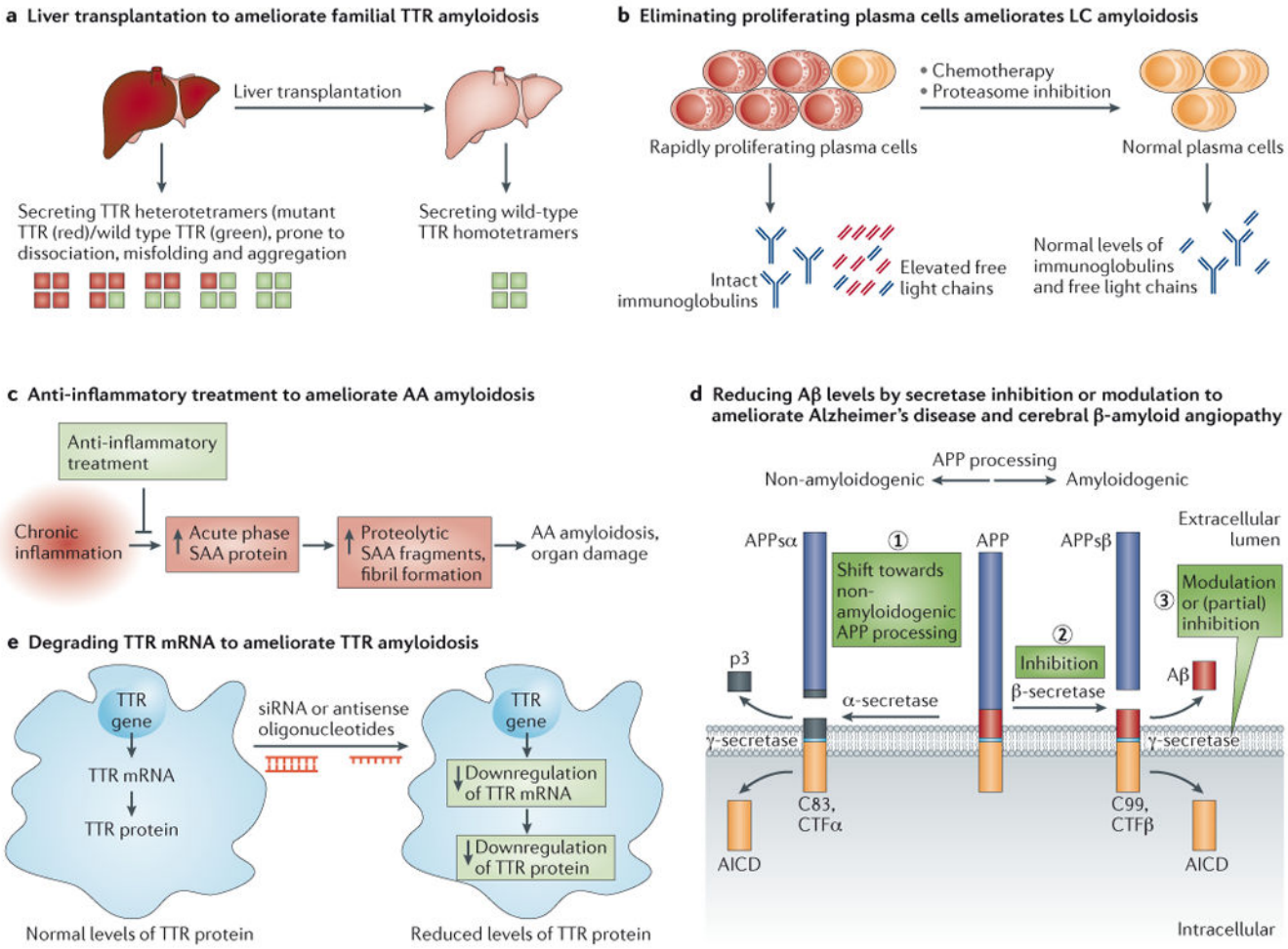



**Figure 3. Therapeutic Strategies to Ameliorate Amyloidosis**

**A. Gene therapy by liver transplantation to treat Familial Amyloid Polyneuropathy (FAP)**. Since TTR is largely produced by the liver, liver transplantation was introduced in the 1990s as a therapy for early stage patients with FAP. Wild type (WT) TTR tetramers (depicted with green squares) produced by the donor liver are much less amyloidogenic than the mutant TTR/WT TTR heterodimers (depicted with red and green squares, respectively) produced by the patient's liver before transplant. This approach successfully slows the progression of FAP and extends life span, but requires organ availability, life-long immunosuppression, and is associated with 10% mortality due to the transplant procedure.

**B. Protein reduction by chemotherapy to treat Light Chain Amyloidosis (AL)**. The amyloidogenic protein in AL amyloidosis is an immunoglobulin light chain or a fragment thereof overproduced by a plasma cell dyscrasia. Removal of the proliferating cancerous plasma cells producing the amyloidogenic light chain by chemotherapy regimens, with or without autologous stem cell transplantation, is used for patients without major cardiac involvement that are not too sick to tolerate these aggressive regimens, which leads to apparently durable disease remission.

**C. Anti-inflammatory treatment dramatically lowers acute phase serum amyloid A (SAA) protein production for the treatment of Amyloid A Amyloidosis (AA**

**amyloidosis)**. Systemic amyloid A amyloidosis is a long-term complication in some patients suffering from chronic infection or chronic inflammation (e.g., chronic inflammatory arthritis). The amyloidogenic protein is derived by proteolysis of SAA, an acute-phase reactant protein transcriptionally upregulated during inflammation. Persistent high concentrations of SAA fragments in plasma above a critical threshold for aggregation can trigger AA deposition. Treatment of the underlying infectious or inflammatory trigger can reverse this type of amyloidosis, if diagnosed early.

**D. Reduction of pathogenic Aβ levels by secretase inhibition or modulation has therapeutic potential for Alzheimer's disease (AD) and Cerebral β-Amyloid Angiopathy**. The amyloid precursor protein (APP) is a transmembrane protein that is constitutively cleaved by enzymes called secretases. Endoproteolytic processing via the non-amyloidogenic pathway, i.e., α-secretase cleavage followed by γ-secretase cleavage (shown on left) generates non-amyloidogenic APP fragments. In contrast, processing by β-secretase and subsequent γ-secretase cleavage produces amyloidogenic Aβ peptides (shown in red on right). Aggregation of Aβ peptides into extracellular amyloid fibrils or plaques is a histopathological hallmark and a diagnostic criterion for Alzheimer's disease. Reduction of Aβ peptide concentration can be achieved by (1) shifting APP processing towards non-amyloidogenic endoproteolysis, or (2) inhibition of β-secretase , or (3) γ-secretase inhibition or modulation. Inhibition of γ-secretase was shown to be feasible experimentally, however, side effects are a concern due to its many substrates. Interestingly, γ-secretase cleavage generates Aβ peptides of various lengths—as a rule of thumb, the longer the peptide, the more amyloidogenic. Modulation of γ-secretase processing of APP to generate shorter, less amyloidogenic Aβ peptides is currently being investigated.

**E. Gene silencing by siRNA or antisense oligonucleotides for the treatment of Familial Amyloid Polyneuropathy (FAP)**. Recently published early-stage, small, placebo-controlled clinical trial data has identified small interfering RNAs (siRNA, also RNAi) that lower mutant and WT transthyretin (TTR) protein production in patients with FAP. Analogous clinical data on antisense oligonucleotides that degrade *TTR* mRNA and thus lower TTR production and secretion into the blood from the liver are promising.

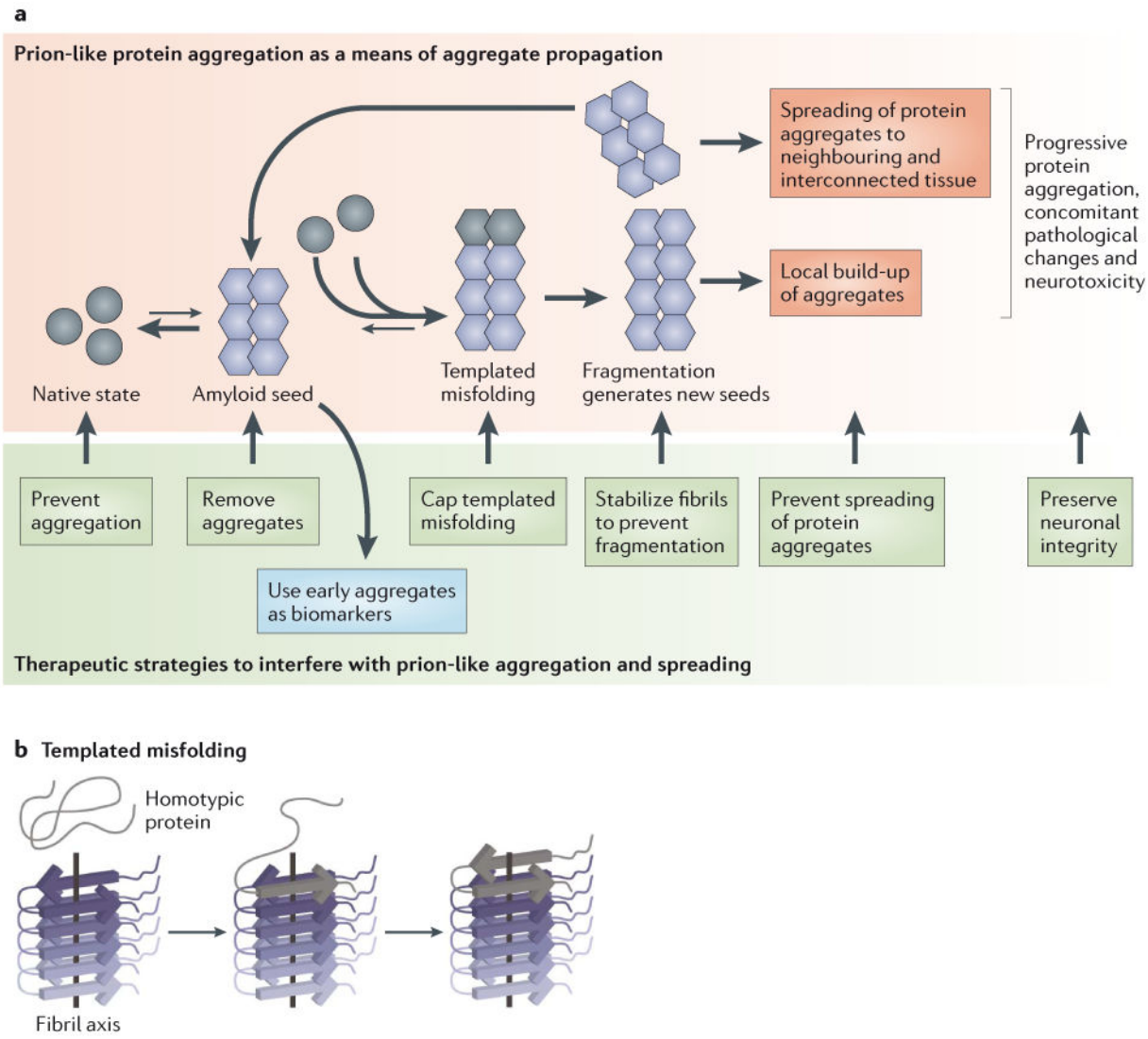



**Figure 4. Seeded or Prion-like Protein Aggregate Spreading: Disease Initiation, Progression and Therapeutic Strategies**

(A) Recent advances in our understanding of protein aggregation disorders have revealed that several amyloidogenic proteins once aggregated into amyloid fibrils can seed or catalyze the misfolding and/or misassembly of homotypic monomers, leading to accelerated aggregate formation and aggregate spreading from tissue to tissue. This type of mechanism was first proposed for prion disorders. Aggregate spreading by this mechanism is currently termed “templated misfolding” or “seeded aggregation” (see Figure 2). Once an amyloid seed is formed, it can catalyze the misfolding and/or misassembly of a monomeric homotypic protein into growing aggregates. Amyloid fibril fragmentation generates new seeds, and the vicious cycle of template misfolding/aggregation leads to the spreading of amyloid to neighboring and interconnected tissue, assisted by cellular aggregate uptake, which can result in subsequent template misfolding followed by aggregate secretion. This mechanistic paradigm has important therapeutic implications. Most importantly, it argues for an early intervention or preventive treatment before the amyloid spreading cascade

progresses out of control. Several therapeutic strategies are possible (shown in the green box), ranging from stopping active aggregation, to removing aggregates, to inhibiting templated misfolding, to stabilizing seeds against fragmentation and/or altering their structure, to preventing the cellular uptake or secretion of aggregates. Early detection of amyloid diseases, which is still problematic, is important for these therapeutic strategies to have maximal impact (B) Amyloid fibrils often adopt an in-register β-sheet arrangement, wherein the strands are oriented perpendicular to the fibril axis. The amyloid fibril serves as a seed or template to incorporate the homotypic monomer into the growing fibril. The same protein can adopt distinct amyloid structures or strains, which can be reliably propagated by template misfolding, and which appear to be associated with different pathogenic phenotypes. Figure adapted from ref 224.

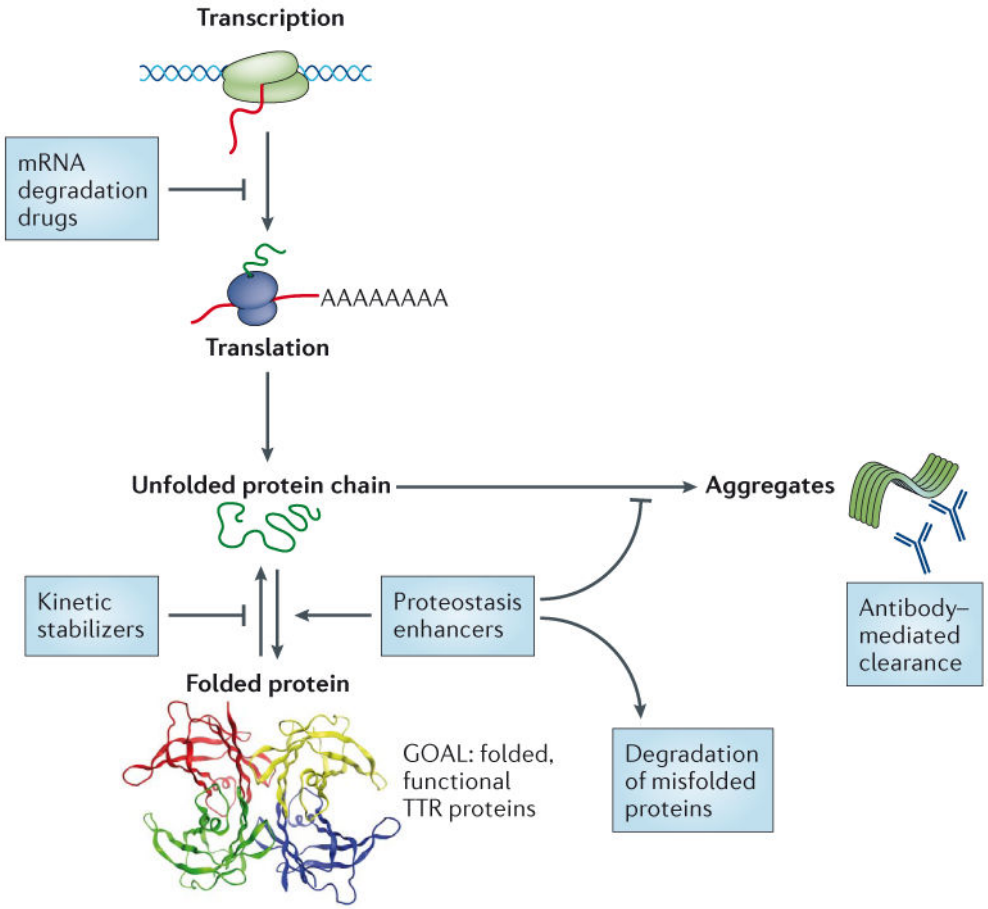



**Figure 5. Combining Therapeutic Strategies to Ameliorate Protein-misfolding / Aggregation Diseases**

We envision that amyloid diseases featuring degenerative phenotypes will be treated in the future using combinations of drugs exhibiting distinct mechanisms of action. For example, we envision treating the TTR amyloidoses with kinetic stabilizers (tafamidis; already in clinical use) and TTR messenger RNA degradation drugs (currently in clinical trials), or using a kinetic stabilizer in combination with drugs that enhance the capacity of the proteostasis network (currently being developed) to achieve proteome maintenance. Given the myriad proteins that lead to aggregation-associated degenerative diseases, strategies such as proteostasis network adaptation that could be useful for multiple maladies are particularly appealing. The goal is to achieve a fully functional proteome without pathogenic amyloidogenicity.

## Table 1

Emerging, potentially disease-modifying, therapeutic strategies.

| Therapeutic strategy / agent | Disease | State of development | References |
|---|---|---|---|
| *Protein Reduction* | | | |
| Anti-inflammatory drugs | AA amyloidosis | Regulatory-agency approved | 107-112, 296, 297 |
| mRNA reduction | AA amyloidosis | Preclinical | 128, 129 |
| β-Secretase inhibitors/modulators; Especially Ligand Pharmaceuticals / Merck-8931 | Alzheimer's Disease | Clinical trials | 118, 119 |
| γ-Secretase modulators | Alzheimer's disease | | 123, 298 |
| Chemotherapy | Light chain amyloidosis | Regulatory-agency approved | 27, 103, 299, 300 |
| Proteasome inhibitors | Light chain amyloidosis | Regulatory-agency approved | 27, 103, 299, 300 |
| mRNA reduction | Light chain amyloidosis | Preclinical | 128 |
| Liver transplantation mediated gene therapy | TTR amyloidoses Hereditary fibrinogen amyloidosis | Regulatory-agency approved | 86, 87, 301, 95 |
| RNAi or antisense/RNAse | TTR amyloidoses | Clinical trials | 126, 127, 302 |
| *Protein Stabilization* | | | |
| Kinetic stabilizers: tafamidis, diflunisal | TTR amyloidoses | Regulatory-agency approved | 22-24 |
| *Protein Quality Control* | | | |
| Small molecule activators of the heat shock response | Huntington's disease / other degenerative disorders | Preclinical | 160, 161 |
| Small molecule activators of the unfolded protein response | TTR amyloidoses<br>Light chain amyloidoses | Preclinical | 147<br>148, 178 |
| Hsp 90 inhibition | Alzheimer's Disease<br>Huntington's disease<br>Parkinson's disease | Preclinical | 303<br>163, 304<br>164 |
| *Protein reduction and quality control* | | | |
| Small molecule Hsc70 inhibitors | Alzheimer's Disease | Preclinical | 175-177 |
| Deubiquitination enzyme inhibitors | Alzheimer's disease<br>Parkinson's disease | Preclinical | 179, 180<br>179, 180 |
| *Amyloid Remodeling* | | | |
| EGCG | Different amyloidogenic proteins<br>Transthyretin cardiomyopathy<br>Parkinson's disease | Preclinical<br>Clinical report<br>Preclinical | 191-197, 305<br>190<br>199 |
| *Amyloid removal* | | | |
| Variants of Hsp104 disaggregase | Parkinson's disease<br>Amyotropic lateral sclerosis<br>Frontotemporal dementia | Preclinical | 218, 219<br>218, 219 |
| Catalytic antibodies | TTR amyloidoses<br>Alzheimer's disease | Preclinical | 185<br>186 |
| *Protein Reduction and Amyloid Removal* | | | |
| Passive immunization, Aβ and tau | Alzheimer's Disease | Clinical trials | 205, 209, 211-214, 306, 307 |
| Active Immunization | Alzheimer's Disease | Clinical trial halted | 203-207 |
| Passive immunization | Parkinson's disease | Preclinical | 211-216 |