Chapter 2

Barcoding Heat Shock Proteins to Human Diseases

Looking Beyond the Heat Shock Response

Vaishali Kakkar*, Melanie Meister-Broekema*, Melania Minoia*, Serena Carra, Harm H Kampinga

* equal contribution

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Vaishali Kakkar*, Melanie Meister-Broekema*, Melania Minoia*, Serena Carra,
Harm H Kampinga.
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ABSTRACT

There are numerous human diseases that are associated with protein misfolding and the formation of toxic protein aggregates. Activating the heat shock response (HSR) - and thus generally restoring the disturbed protein homeostasis associated with such diseases - has often been suggested as a therapeutic strategy. However, most data on activating the HSR or its downstream targets in mouse models of diseases associated with aggregate-formation have been rather disappointing. The human chaperonome consists of many more heat shock proteins (HSPs) that are not regulated by the HSR, however, and researchers are now focusing on these as potential therapeutic targets. Here, we summarize the existing literature on a set of aggregation diseases and propose that each of them can be characterized or 'barcoded' by a different set of HSPs that can rescue specific types of aggregation. Some of these 'non-canonical' HSPs have demonstrated effectiveness \textit{in vivo}, in mouse models of protein aggregation disease. Interestingly, several of these HSPs also cause diseases when mutated - so-called chaperonopathies - which are also discussed in this chapter. This chapter aims at understanding the role of HSPs under chronic and acute stress conditions. Further, the aim and outline of this thesis is discussed.
INTRODUCTION

Many heat shock protein (HSP) family members are known to function as molecular chaperones, meaning that they stabilize and assist in the correct folding of nascent polypeptides (1). In addition to their role in de novo protein folding, HSPs are involved in various aspects of proteome maintenance, including macromolecular complex assembly, protein transport and degradation, as well as aggregate dissociation and refolding of stress-denatured proteins. Under normal cellular conditions, HSP levels match the overall level of protein synthesis. Under stress-induced conditions, mature proteins unfold and exceed the capacity of chaperone networks to prevent aggregation. This type of acute proteotoxic stress induces a regulated response resulting in increased expression of some HSPs, which helps to rebalance protein homeostasis.

The human genome encodes more than 100 different HSPs that are grouped into 7 different families: HSPH (Hsp110), HSPC (Hsp90), HSPA (Hsp70), DNAJ (Hsp40), HSPB (small Hsp), the human chaperonins HSPD/E (HSP60/HSP10) and CCT (TRiC), plus several regulatory co-factors (2). In terms of their regulation, the HSP family members can also be categorized into three groups: i) constitutively expressed, but not induced by stress; ii) constitutively expressed and induced upon stress; and iii) induced only upon stress (3). In addition to their differential regulation, the various HSPs also show a large degree of functional diversity with respect to client specificity and client processing (4). These functional differences could be very important when investigating their potential relevance for diseases in which cells are chronically exposed to proteins that are prone to form toxic protein aggregates. Examples of such diseases are polyglutamine diseases, Parkinson’s disease, amyotrophic lateral sclerosis and Alzheimer’s disease. This review discusses how these diseases can be labeled or ‘barcoded’ by specific sets of HSPs that can rescue their disease-specific aggregations.

The cellular functions of HSPs

HSPs and de novo protein folding

The general organization of co-translational folding is highly conserved throughout evolution. Ribosome-binding chaperones (e.g., specialized Hsp70/HSPAs) first interact with the nascent polypeptide, followed by a second set of HSPs that do not have a direct affinity for the ribosome (the classical Hsp70/HSPA system). The Hsp70/HSPA family is the central component of the cellular network of molecular chaperones and folding catalysts (Fig. 1A). Hsp70/HSPA proteins are involved in a wide range of protein quality control (PQC) functions, including de novo protein folding, refolding of stress-denatured proteins, protein transport, membrane translocation and protein degradation. Hsp70/HSPAs never function alone; they require Hsp40/DNAJ proteins and nucleotide exchange factors (NEFs) as partners. DNAJ
proteins bind and deliver client proteins to the Hsp70/HSPA system, upon which the client protein and DNAJ function together to stimulate HSPA to hydrolyze ATP, leading to high substrate affinity of HSPA. Following ATP-hydrolysis, NEFs such as BAG-1, HSPBP1 and HSPH, bind HSPA and induce ADP-ATP exchange, leading to substrate release. DNAJs thus mainly confer client specificity to the Hsp70/HSPA machine, but can also affect the fate of HSPA clients, whereas NEFs seem to be mainly involved in client fate (4–6) (Fig. 1A). The DNAJ/HSPA system might also receive clients from small Hsp/HSPB proteins. HSPB chaperone activity does not need ATP. However, direct interaction with ATP-dependent chaperones like HSPA promotes the release of the bound substrate and subsequent refolding (7,8).

Proteins that cannot be completely folded by Hsp70/HSPA machines are transferred to, or handled independently by, the chaperonins or the Hsp90/HSPC system (9,10) (Fig. 1A). Substrate transfer to Hsp90/HSPC protein is mediated by the HSP-organizing protein (HOP), which uses multiple tetratricopeptide repeat domains to form a bridge between HSPA and HSPC (9,11). The handoff from Hsp70 to chaperonins remains unclear in mammals; however, work in the prokaryotic system has begun to reveal some interesting possibilities. For example, it has been shown that Hsp70/DnaK binds the M domain of ClpB to recruit DnaK-bound substrates to the chaperonin (12).

HSPs and acute proteotoxic stress conditions
Cells are constantly challenged by changes in their environment. Acute stress conditions, such as heat shock, cause many proteins to become unfolded. The accumulation of stress-denatured proteins increases the risk of aggregate formation. In addition to their role in co-translational folding, the constitutively expressed HSP members might also assist in aggregate protection and refolding of stress-unfolded proteins (Fig. 1A). However, it has been shown in yeast that the stress-inducible cytosolic members of the families, which are strongly upregulated by the transcription factor heat shock factor-1 (HSF-1), become more important under such conditions (13). Next to this transcriptional response, HSPB proteins represent an even more rapid response to environmental stresses (Fig. 1A). Several HSPB-members are rapidly and transiently phosphorylated, whereby their oligomeric state is dynamically altered and their protective activities are activated. These protective activities include prevention of cytoskeletal collapse and chaperoning of soluble proteins, which can enhance protein refolding or support client degradation (8).

In parallel to the HSF-1 regulated heat shock response (HSR) in the cytosol, interconnected pathways in different cellular compartments also respond to acute cellular stress, including the unfolded protein response (UPR) in the endoplasmic reticulum and the mitochondria (14–16). Each pathway not only induces the transcriptional up-regulation of genes that enhance...
refolding capacity, but also the expression of HSP members that assist in degradation of unfolded proteins through the proteasome and lysosome-mediated pathways, together protecting cells from stress (14–17).

**HSPs and chronic stress conditions**

Protein aggregation hallmarks a high number of chronic diseases (18) that can either be loss-of-function or toxic gain-of-function disorders. Loss-of-function diseases, including cystic fibrosis and Gaucher's disease, are typically caused by recessive mutations leading to inefficient folding of the mutated proteins and their consequent degradation or dysfunction (19) (Fig. 1B). Of note, in recessive diseases, the HSF-1-regulated HSPs can promote some refolding of (metastable) mutant proteins, thereby displaying disease-rescuing potential (20). In addition, chaperone inhibition, resulting in less recognition of the mutant peptides and their degradation, has been shown to be protective in such diseases (21). Toxic gain-of-function diseases, on the other hand, usually manifest with the formation of intracellular and/or extracellular deposits of aggregated proteins, as will be further discussed below (3,18,22).

These aggregates are often fundamentally different from those formed during acute stress as they initially are formed without being sensed by the (acute) stress responses in the cells. Moreover, unlike in response to acute stress where proteins are unfolded, proteins in chronic stress are intrinsically misfolded and can generally not be refolded but must be disposed of (Fig. 1B). This could imply that different HSPs might be crucial –or rate-limiting– to provide protection in chronic protein aggregation diseases than for acute stress. Below, we will focus on toxic gain-of-function diseases and provide an overview of the literature on HSPs that could prevent aggregation or/and toxicity of the disease-associated proteins. Because we aim to identify HSPs that might be rate-limiting factors for aggregate prevention and thus targets for intervention in these diseases, we will mainly discuss effects of HSP-overexpression and not include studies on down-regulation of HSPs. Interestingly, HSP down-regulation is often associated with toxicity and lethality and can result in disease itself (Table 1). Therefore, this review will furthermore provide an overview of aggregation diseases, known as chaperonopathies that are caused by mutations in HSPs. In this way, we aim to recapitulate the role of HSPs in chronic aggregation diseases from two angles: the prevention of toxic gain-of-function diseases and their role in causing disease themselves.

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Figure 1. Model of actions and interactions of the HSP network required for normal protein folding and refolding upon (A) acute stress or (B) during chronic stress

HSP families constitute a large group of chaperones that interact with non-native proteins, assisting their correct protein folding. HSPs are constitutively expressed, but their expression level can increase under conditions of stress. They are mainly divided into groups: HSPBs, HSPAs, HSPCs, and members of the chaperonin family (see main text for details).

(A) During de novo protein folding and for the refolding of acute stress-denatured, unfolded proteins, the functional cooperation of different HSPs is primarily aimed at the structural stabilization of native proteins for (re)folding. However, in case of failure of protein folding HSPs can also assist client degradation through the ubiquitin-proteasome system (UPS) or the autophagy-lysosome. The central component of the chaperone network and folding catalysts is the Hsp70/HSPA family. DNAJs hydrolyze ATP (bound to HSP70/HSPA) to ADP increasing the affinity of its substrate-binding domain for unfolded proteins. Nucleotide exchange factor (NEF) proteins remove ADP and substitute ATP, reducing HSP70/HSPA's substrate binding affinity, allowing release of the folded protein. Proteins that are unable to utilize HSPAs for complete folding are transferred to the chaperonin or the HSPC system. HSPC proteins require HOP as a co-chaperone in order to transfer substrates from HSPA to HSPC. Under acute stress conditions, HSPB oligomers dissociate into dimers to bind unfolded proteins.
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**HSPs and proteinopathies**

There are numerous human diseases that are associated with the aggregation of a single dominant peptide or protein. Examples of such diseases, known as proteinopathies, include polyglutamine diseases (polyQ), Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS), and Alzheimer’s disease (AD). The monogenic forms of neurodegenerative proteinopathies are rare, and are generally histopathologically indistinguishable from their corresponding sporadic forms, making it likely that both forms share a final common pathway. Protein aggregates are either found inside neurons (e.g. tau tangles in AD) or outside neurons, in the extracellular space (e.g. amyloid-β-plaques in AD). Aggregates are generated when proteins become destabilized, either by mutations changing their native state (e.g. SOD1 in ALS) or quantity (e.g. α-synuclein in PD), by the elongation of a certain domain (e.g. huntingtin in Huntington’s disease, HD), or by domain truncations (e.g. TDP-43 in ALS). Aggregates range from extremely dense amyloidogenic aggregates with β-sheet cores (huntingtin, ataxin-3, amyloid-β) to more amorphous aggregates (α-synuclein, SOD1, TDP-43). Although it is still debated whether the small oligomers or the large inclusions are more toxic, the overall evidence from model systems strongly suggests that aggregate prevention generally results in disease-amelioration. Therefore, this review focuses on aggregate prevention by HSPs and will reveal that each of these proteinopathies is associated with a different pattern or ‘barcode’ of rescue depending either on the HSR or individual HSPs. The elucidation of these barcodes provides a platform for a rational design of disease-specific therapeutic strategies. For each disease, evidence was categorized into 4 levels (Fig. 2): *in vitro* (lowest level),
cell studies, non-mammalian model systems and mammals (highest level). Furthermore, evidence in Fig. 2 was graded according to their specific effects: prevention of aggregate formation (black), buffering of toxic effects caused by diseased protein (gray), and absence of protective effects (white).

**Polyglutamine diseases (polyQ)**

In polyQ diseases, the polyglutamine tract is elongated beyond a certain threshold. The transcribed polyQ peptide fragments are thought to be the initiators of amyloid fibrils and have a strong propensity to assemble into highly ordered polymers that are extremely rich in β-sheet structure, thereby creating SDS-insoluble aggregates (22,23). PolyQ expansions in huntingtin (Htt), ataxins, and in the androgen receptor have been associated with dominant, late onset, toxic gain-of-function diseases; HD, spinocerebellar ataxias (SCA) and spinal bulbar muscular atrophy (SBMA), respectively. All these diseases are associated with severe motor problems and/or muscle atrophy (22,24,25). Both age of onset and protein aggregation propensity are strongly associated with the length of the polyQ expansion, further suggesting that aggregate formation forms the basis of disease (23,26).

In cells and non-mammalian model organisms, activation of the acute HSR pathways has been shown to reduce the extent of polyQ aggregation. Overexpression of HSF-1 led to fewer but larger polyQ aggregates in cells (27). In agreement with this finding, chemical up-regulation of the HSR in cells and non-mammalian animal organisms reduced a number of dysfunctions caused by polyQ overexpression (see Fig. 2 for associated references). Although overexpression of HSF-1 in muscle tissue of the R6/2 mouse model for HD increased life span, there were only small effects on aggregates (28), implying that effects were compensatory and did not affect the underlying toxic gain-of-function. Chemical up-regulation of the HSR by the use of Hsp90/HSPC-inhibitors in the R6/2 mouse model led to transient beneficial effects, which disappeared during disease progression (29). Moreover, Hsp90/HSPC-inhibition led to accelerated degradation of soluble polyQ-Htt which was apparently independent of HSR activation; however, this was most likely due to pleiotropic effects associated with the inhibition of Hsp90/HSPC instead (9,10,30).

Whereas injection of HSF-1 into an SBMA-mouse model resulted in only small effects in neurons, Hsp90/HSPC inhibition by 17-AAG, GGA and geldanamycin (GA) not only substantially increased life span, but also diminished aggregates (Fig. 2). Hsp90s/HSPCs are required for the degradation, regulation, ligand-binding affinity and stabilization of the androgen receptor, as well as for its trafficking (31). The strong effects of Hsp90/HSPC-inhibitors on SBMA -but not HD- suggest that HSF-1-activation and the resulting up-regulation of the HSR is insufficient to modulate polyQ diseases in general. Protective effects of Hsp90/HSPC-inhibitors, if found, are therefore most likely due to HSF-1 unrelated effects (30).
Up-regulation of individual members from HSF-1-regulated HSP families (e.g. HSPA1A, DNAJB1, HSPB1) was effective in preventing polyQ aggregation or the associated toxicity in vitro and in cellular models (Fig. 2). However, in comparative screens involving larger polyQ expansions, the HSR-regulated HSPs were usually rather ineffective compared to non-canonical HSPs (32–34). Some effects of Hsp70/HSPA overexpression on polyQ toxicity were reported in Drosophila melanogaster (Fig. 2). However, these effects were not associated with aggregate reduction, suggesting that the observed protection was due to compensatory effects downstream of aggregate formation; for instance, the loss of normal protein quality control functions due to entrapment of key chaperones, such as DNAJB1 (35). Yet, this loss of protein quality control is apparently not at the heart of disease in mammals, as restoration of protein quality control by HSP70 overexpression did not delay disease progression in the R6/2 HD mouse model (36,37). The same is true for the canonical small Hsp HSPB1. Opposing an earlier report which suggested that HSPB1 overexpression led to a small delay of Htt-toxicity in rats (38), studies in a mouse model for HD and cells proved HSPB1 to be rather inefficient in delaying polyQ-aggregation (39); Fig. 2. Combined these results suggest that HSPB1 might have some compensatory effects that initially slightly delay disease but are not affecting aggregates directly, thereby eventually proving to be insufficient to rescue the disease in mammals (Fig. 2).

In dedicated screens for members of the HSP families that might be better suppressors of polyQ aggregation, a number of very effective HSPs were identified, including DNAJB2, DNAJB6, DNAJB8, HSPB6, HSPB7, HSPB8, HSPB9. Interestingly, most of them were not, or were only marginally regulated by HSF-1 and were not effective in stimulating substrate-refolding after acute stress (32–34); Chapter 3. Instead, these HSPs were associated with degradation of clients through the proteasomal and autophagic degradation routes. Moreover, whereas DNAJB6, HSPB7, and HSPB8 delayed aggregation in Drosophila, DNAJB2 was the first HSP that demonstrated a protective effect on aggregate formation, functional endpoints, and survival in mice (Fig. 2). Interestingly, our data regarding transgenic overexpression of DNAJB6 indicate even larger protective effects in the R6/2 mice (Chapter 5). The effectiveness of these non-canonical HSPs in cells, non-mammalian model organisms and mice might be related to their ability to prevent initiation of aggregate formation or to their ability to assist aggregate clearance through autophagy, a finding that would be consistent with the important role autophagy plays in proteinopathies (7,32,40–42).

In a nutshell, the HSR and individual HSF-1-regulated HSP members have marginal and mainly compensatory effects in polyQ diseases. In contrast, other members of the HSP families that can prevent aggregate initiation or dispose of aggregates might have potential as targets for therapy in polyQ diseases.
Parkinson’s disease (PD)

About 5-10% of PD cases are monogenic and are caused by either loss-of-function or toxic gain-of-function mutations. The most commonly occurring disease-causing mutations are in the mitochondria-associated genes Parkin (PARK2), PINK1 (PARK6), and DJ-1 (PARK7) (43-46). Mutations in these genes are recessively inherited and usually result in a loss-of-function effect, mainly impeding mitochondrial function and turnover. By contrast, a toxic gain-of-function phenotype resulting in PD is caused by rare dominantly inherited mutations and multiplications in the SNCA (PARK1, PARK4) and LRRK2 (PARK8) (43,44,46). This review will focus on these rare toxic gain-of-function mutations.

Mutations in/or multiplications of SNCA lead to increased oligomerization of the gene product α-synuclein, which is an intrinsically disordered protein. This enhanced oligomerization increases the tendency of α-synuclein to form β-sheet structures and eventually fibrous amyloidogenic inclusions, called Lewy bodies and Lewy neurites (43,45,47). LRRK2 is a kinase that is involved in the phosphorylation of α-synuclein. Mutations in LRRK2 are thought to promote α-synuclein expression, aggregation, and toxicity, thereby increasing the propensity of α-synuclein to self-aggregate (44,45). As in polyQ diseases, genetic or chemical activation of HSF-1 can temporarily compensate for LRRK2 and α-synuclein toxicity in cells and Drosophila (Fig. 2).

Although individual HSPs such as DNAJA1, DNAJB2, HSPB2/HSPB3, HSPB6 and HSPB8 inhibited α-synuclein aggregation in vitro, none of them have proven to be effective in cells thus far (Fig. 2). HSPB1 and HSPB5 were effective in preventing α-synuclein aggregation in vitro, in cells and in Drosophila; however, there is currently no evidence of success in mouse models. Overexpression of HSPA1 was also shown to be able to inhibit α-synuclein aggregation in vitro, and decrease α-synuclein toxicity in cells and in Drosophila. Moreover, HSPA1 overexpression in mice did show some protective effects, although the data are still disputed (48,49) (Fig. 2). As is the case for polyQ diseases, neither Hsp70/HSPA1, nor any other canonical HSP could prevent aggregate formation or reduce aggregate size and quantity.

These data taken together would suggest that compensation for loss of normal protein quality control by sequestration of HSPs into aggregates plays a more important role in PD than it does in polyQ diseases. In line with this notion, a study of α-synuclein in mice showed that transgenic overexpression of HSPA5 delayed disease onset without affecting cytosolic protein aggregation. Because HSPA5 is an ER-resident Hsp70/HSPA and is not expressed in the cytoplasm of the cell, its mode of action must be indirect. Instead of directly affecting aggregate formation, HSPA5 most likely compensates for downstream consequences of aggregation and thereby delays disease onset (50).
To conclude, the biophysical nature and intracellular localization of α-synuclein aggregates are clearly different from aggregates in polyQ diseases (51). Expression of (mutant) α-synuclein rapidly activates HSF-1, whereas polyQ expression either does not activate HSF-1 at all, or only transiently and very late in disease (25,51). The potential HSP suppressors of PD thus seem to differ from that of polyQ diseases, thereby resulting in a different HSP barcode of potential treatment targets (Fig. 2). We further tested this idea, with specific emphasis on DNAJ proteins, in Chapter 7.

Amyotrophic lateral sclerosis (ALS)

Until recently, about 5% of ALS cases were categorized as dominant monogenic ALS; the most commonly occurring mutations being in SOD1, TDP-43, and FUS (52,53). In addition, the identification of a hexanucleotide repeat GGGGCC expansion was recently identified in the open reading frame 72 on chromosome 9 (C9orf72) as the most common known genetic cause of familial ALS, familial frontotemporal dementia (FTD) and ALS-FTD that has completely shook the ALS field (209, 210). This C9orf72 mutation is more than twice as common as mutations in the SOD1 gene as a cause of familial ALS, and more than three times as common as mutations in TDP-43, FUS, and other genes combined together. Mechanistically, how C90RF72 mutation causes disease is still poorly understood. However, it is known that it leads to repeat-associated non-ATG (RAN) translation, where C9RAN proteins lead to nuclear inclusions throughout the CNS of the diseased patients (211). The role of HSPs in such cases is still not investigated and hence here in this review, we will only summarize the existing literature on HSPs involved in SOD1 or TDP-43 inclusions.

Clinically, sporadic and monogenic ALS are nearly indistinguishable, as SOD1 and TDP-43 positive inclusions are present in both forms of disease, thereby implying a final common pathway (54). About 166 mutations in SOD1 have been associated with monogenic ALS. Although SOD1 mutations were initially thought to cause disease via a loss of wild-type SOD1 function, SOD1-knockout mice displayed no phenotype (55). Instead, the overexpression of mutant SOD1 leads to disease, implying that the mutant gained a toxic function (52,56). Mutations in SOD1 indeed structurally destabilize the protein, thereby increasing its aggregation propensity, which eventually results in amyloid fibril-formation (57). Mutations in TDP-43, a RNA-processing protein that usually shuttles between the nucleus and cytoplasm of the cell, render the protein aggregation-prone, which leads to the formation of dense round or filamentous aggregates in the cytoplasm alongside stress granules (52,53,57). Mutations in FUS, another protein involved in RNA metabolism, also result in large globular and elongated cytoplasmic inclusions (52,53). Nevertheless, FUS-related ALS is defined as an atypical form because TDP-43 positive aggregates are not part of the pathology, which is why this review will not discuss this FUS-related ALS.
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Treatment of Drosophila with the Hsp90/HSPC-inhibitor 17-AAG reduced the characteristic ALS eye-degeneration-phenotype in a TDP-43-model (58). In addition, treatment of the SOD1-G93A mouse model with 17-AAG not only delayed age of symptom onset, but also increased lifespan (59–61). However, these protective effects were not reproducible in the SOD1-G37R or the SOD1-G85R mouse model (22,62). These contradictory results indicate that protein aggregation and toxicity mechanisms might depend on the exact kind of mutation, and therefore result in a different barcode of HSPs for each SOD1-mutation.

Regarding the effects of individual HSPs on SOD1 aggregation and toxicity, data in cell lines expressing mutant SOD1 suggest protective effects of HSPA1, DNAJB1, DNAJB2, HSPB1 and HSPB8 (Fig. 2). Furthermore, HSPB8 alleviated TDP-43 aggregation and toxicity in cells and HSPA1A reduced TDP43-associated eye degeneration in Drosophila (Fig. 2). The intracranial injection of SOD1-G93A mice with HSPA1 was also protective, whereas long-term effects of HSPB1 overexpression in mice were absent, although this awaits further investigation (62–65).

To conclude, except for the aforementioned Hsp90/HSPC-inhibitors, none of the discussed HSPs resulted in long-term rescue or had direct effects on SOD1-aggregates (Fig. 2). Moreover, it is not clear whether the effects of the Hsp90/HSPC-inhibitors are due to the elevation of HSF-1-regulated HSPs, or whether they are due to the broad effects these inhibitors exert on cell homeostasis. In summary, the barcode of HSPs that protect against ALS is still very limited.

Alzheimer’s disease (AD)

The most commonly occurring form of AD is sporadic late onset AD. In contrast, only about 1-2% of AD cases display an early onset and are due to autosomal dominant mutations in amyloid precursor protein (APP), presenilin 1 (PSEN1), or presenilin 2 (PSEN2) (Guerreiro et al., 2012). Although intracellular tangles, consisting of hyperphosphorylated tau and extracellular amyloid-β (Aβ) plaques are present in both sporadic and monogenic AD, it is still unclear how toxicity in AD proceeds. It is disputed as to whether amyloid-β aggregation leads to cellular stress and results in tau-hyperphosphorylation and aggregation (described as the amyloid cascade hypothesis), or tau-hyperphosphorylation and aggregation precede amyloid-β accumulation (described as the tau-axis-hypothesis) (66). Here, we will provide an unbiased summary on the effects of HSPs on both, Aβ- and tau-related aggregation.

Amyloid-β peptides are the result of APP-cleavage via one of two pathways: a non-amyloidogenic pathway that leads to the generation of the most common isoform, Aβ40, or an amyloidogenic pathway that results in the generation of Aβ42 (67). AD-related mutations in APP usually affect the ratio or properties of these different amyloid-β species
Similarly, mutations in PSEN1 and PSEN2, which are rate-limiting components of the 
_-secretase complex in the amyloidogenic pathway, result in increased generation of the 
more fibrillogenic Aβ42 (66,67). HSF-1-injection into an APP rat model increased neuronal 
health and reduced Aβ plaque-load (68). Similarly, in another study, genetic overexpression 
of HSF-1 in APP mice diminished soluble Aβ-levels (69). In line with these findings, treatment 
of APP-mice with the HSF-1 activator celastrol slightly decreased Aβ-plaque load (Fig. 2).

*In vitro*, it was shown that HSPA1 and HSPA5, HSPC1, HSPA1/DNAJB1, as well as HSPB1, 
HSPB5, HSPB6 and HSPB8 slow down Aβ-aggregation (Fig. 2). Furthermore, purified DNAJB1, 
HSPB1, HSPB5 and HSPB8 protect cells from extracellular Aβ-toxicity when co-incubated 
(70,71). However, considering that HSPs are intracellular proteins, whereas Aβ-plaques are 
generally considered to be extracellular, the relevance of such findings could be debated. 
Interestingly, evidence stating that intracellular Aβ-aggregation might precede extracellular 
plaque formation is accumulating, which increases the relevance of findings indicating that 
HSPs are able to prevent the initiation of aggregation in cells (72,73). Although there is 
no cellular data available yet, these findings might explain why transgenic overexpression 
of HSPA1 and HSPB1 had protective effects in mouse models for Aβ (74,75). However, it 
is questionable whether Aβ-aggregation was directly affected by HSPA1 overexpression 
in transgenic mice, or whether the observed protective effects were due to more general 
compensatory effects of HSPA1 (74).

Another protein that has been associated with neuronal death in AD is tau. Tau is an 
unstructured and dynamic protein that is normally involved in stabilization of microtubules, 
but becomes hyperphosphorylated and detaches from microtubules under stress conditions. 
This detachment results in microtubular collapse and in the aggregation of tau into well- 
ordered and periodic protein deposits (66,76). In cells, the HSF-1 activator HSF1A reduced 
tau aggregation by increasing proteasomal degradation of tau (77). Likewise, Hsp90/HSPC- 
directed drugs, such as geldanamycin, enhanced clearance of tau from cells, thereby 
reducing its toxicity (Fig. 2). Moreover, the HSF-1 activator radicicol and the GA derivative 
17-AAG alleviated tau toxicity in *Drosophila* larvae (Fig. 2). Multiple HSPs alleviated tau 
toxicity in cells, including HSPA8, HSPA1, Hsp90s/HSPC, DNAJA1 and HSPB1 (Fig. 2). In 
addition, HSPB1 rescued behavioral defects in a mouse model for tauopathy (78). However, 
there are no studies investigating the role of the other HSPs in mammals available yet.

To conclude, interpreting the data about the effects of HSPs on AD needs to be done with 
great caution for two main reasons: Firstly, it is not yet known whether AD is initiated by 
intracellular (tau or Aβ) or extracellular (Aβ) aggregates. Secondly, although extracellularly 
added or leaked HSPs might affect toxicity of Aβ-plaques, it is still unclear how intracellular 
overexpression of HSPs can have direct effects on toxicity in AD, thereby limiting proper
interpretation of the barcode of HSPs for AD.

**Different aggregation diseases have a different HSP barcode**

Although all diseases discussed here are toxic gain-of-function aggregation diseases, the barcode of HSPs with protective potential clearly differs depending on the disease (Fig. 2). This variability strongly suggests that the neurodegenerative proteinopathies discussed in this review are biochemically and biologically distinct. Because all proteinopathies presumably impede on overall protein homeostasis, simply rescuing the overall folding capacity by activation of the complete (acute) HSR (Fig. 1A), or expression of individual components thereof might lead to some protective effects. However, these effects are generally small and transient, and do not actually affect aggregate formation of the specific diseased proteins themselves. Instead, the effects of the HSR might compensate for entrapment of chaperones and/or other components into aggregates (e.g. certain crucial transcription factors). The underlying toxicity of the aggregates themselves is likely to go beyond these effects on protein homeostasis and will furthermore directly impair other functions, such as axonal transport, organelle dynamics (physical obstruction or cytoskeletal collapse) and membrane integrity. Assuming that aggregation indeed is the reason for toxicity in all these diseases, each proteinopathy requires specific HSPs that either directly prevent aggregation (initiation) or that recognize early aggregate intermediates and target these for degradation. These HSPs are likely to be found amongst the “non-canonical HSPs”, many of which have not been fully explored yet for each of these diseases.

**Chaperonopathy: the case of “sick” HSPs**

So far we have highlighted how HSPs might act as a first line of defense in preventing proteinopathies. Sometimes, however, HSPs themselves are mutated, leading to several pathological conditions, termed chaperonopathies (79–81). Although the term chaperonopathy was initially used to include any putative alteration in the expression, post-translational modifications or localization of chaperones, this review will only discuss genetically inherited mutations in the HSPs that are the direct causative factor to a specific disease (Table 1).

Genetic chaperonopathies described so far only concern members of the HSPB, DNAJ and chaperonin families, as well as some chaperone co-factors (Table 1). No genetic chaperonopathies are associated with the Hsp70/HSPA or Hsp90/HSPC family members, either because of functional redundancy within these families or because they are crucial to the central chaperone machinery such that functional mutations are incompatible with life.

Disease-wise, genetic chaperonopathies can be categorized into neuropathies (hereditary spastic paraplegia, motor neuropathy, distal hereditary motor neuropathy), myopathies
Fig. 2: Heat shock protein barcodes associated with diverse proteinopathies

Summary of literature pertaining to the effects of activating either the cytosolic heat shock response (HSR/HSF-1), using HSF-1 activators or HSP90 inhibitors, or overexpressing specific HSPs from the different families (HSPC, HSPA, HSPD/CCT, DNAJ, or HSPB) on proteinopathies. For each disease, evidence was categorized into 4 levels according to the system/organism in which the effect was examined: *In vitro* (A), cell studies (B), non-mammalian model systems (C) and mammals (D). Evidence was further graded according to the specific effects of the heat shock protein(s) on the disease: prevention of aggregate formation (black), buffering of toxic effects caused by diseased protein (gray), and absence of effects (white). See main text for further explanation.

Abbreviations: ref – articles with general information used for the figure; polyQ – polyglutamine diseases; Htt – huntingtin; SCA – spinocerebellar ataxia; AR – androgen receptor; PD – Parkinson’s disease; α-syn – α-synuclein; ALS – amyotrophic lateral sclerosis; AD – Alzheimer’s disease; Aβ – Amyloid-β; 990 – HSP990; AMCL – arimoclomol; GA – geldanamycin; CLST – celastrol; RA – radicicol; PU – PU-H71.

References (Fig.2): 1- (137); 2- (138); 3- (139); 4- (140); 5- (141); 6- (36); 7- (37); 8- (142); 9- (143); 10- (144); 11- (145); 12- (146); 13- (147); 14- (34); 15- (148); 16- (33); 17- Kakkar et al., unpublished; 18- (31); 19- (65); 20- (180); 21- (181); 22- (182); 23- (183); 24- (184); 25- (185); 26- (186); 27- (187); 28- (28); 29- (27); 30- (2); 31- (153); 32- (155); 33- (154); 34- (156); 35- (157); 36- (158); 37- (159); 38- (160); 39- (112); 40- (161); 41- (162); 42- (163); 43- (164); 44- (165); 45- (166); 46- (167); 47- (168); 48- (169); 49- (170); 50- (171); 51- (172); 52- (173); 53- (174); 54- (175); 55- (176); 56- (50); 57- (177); 58- (178); 59- (179); 60- (180); 61- (48); 62- (49); 63- (181); 64- (182); 65- (183); 66- (184); 67- (185); 68- (186); 69- (187); 70- (63); 71- (62); 72- (188); 73- (189); 74- (190); 75- (86); 76- (65); 77- (64); 78- (191); 79- (192); 80- (60); 81- (61); 82- (59); 83- (193); 84- (58); 85- (194); 86- (195); 87- (196); 88- (74); 89- (197); 90- (71); 91- (75); 92- (70); 93- (198); 94- (68); 95- (69); 96- (199); 97- (200); 98- (201); 99- (202); 100- (78); 101- (77); 102- (203); 103- (204); 104- (205); 105- (206); 106- (207); 107- (208).
(dilated cardiomyopathy leukodystrophy, desmin related myopathy, mitochondrial myopathy, muscular dystrophy), or retina and eye lens related diseases (congenital cataracts) (80). Although some chaperonopathies are recessive (and thus probably related to loss-of-function of the chaperone), most were found to be dominant, as is especially the case for the HSPBs (Table 1). We have labeled or “barcoded” these HSP-associated chaperonopathies depending on their disease type and inheritance (Fig. 3).

**Hsp60/HSPD and TRiC/CCT-related chaperonopathies**

A mutation in the Hsp60/HSPD chaperone system has been linked to an autosomal dominant disease known as hereditary spastic paraplegia 13 (SPG13). The disease is characterized by spasticity of lower limbs due to massive degeneration of distal ends of long axons in the spinal cord. The mutation leads to reduced chaperonin activity, which has been attributed to haploid insufficiency due to incorporation of functionally deficient Hsp60/HSPD subunits (82,83). Another chaperonopathy involving Hsp60/HSPD is the recessive mitCHAP-60 disease, associated with psychomotor developmental delay, where mutations lead to entropic destabilization of the Hsp60/HSPD oligomer and cause its premature disassembly. This renders Hsp60/HSPD incapable of fulfilling its normal function and thus results in disease (84).

The Hsp60/HSPD complex resides in mitochondria; however, a comparable eukaryotic chaperonin system known as TRiC/CCT is present in the cytosol and is mainly involved in tubulin and actin folding. Mutations in TRiC/CCT subunits might affect its complex formation and thereby its ability to bind and fold tubulin and actin. As cytoskeletal integrity is crucial in axonal transport, this might explain why such mutants primarily affect functionality of long axons thus leading to sensory neuropathies (85).

**DNAJ related chaperonopathies**

There are four recessive chaperonopathies associated with members of the DNAJ family (Table 1). The first one involves a DNAJB2 (splice) mutation that causes distal hereditary motor neuropathy (dHMN) due to progressive degeneration of motor neurons in the spinal cord characterized by muscle weakness of the extremities (86). DNAJB2 has several clients and possesses degradation-related functions (87,88). The DNAJB2 mutant is unable to handle its natural clients, which therefore eventually aggregate and form intracellular inclusions (86).

The second recessive disease caused by mutations in DNAJ involves DNAJC29. Mutations in DNAJC29 lead to cerebellar ataxia with peripheral neuropathy, which is referred to as ARSACS. The disease is characterized by dysarthria, distal muscle wasting, foot deformities and truncal ataxia, including absence of sensory evoked potentials in the lower limbs (89).
Although the normal function of DNAJC29 is not well understood, roles in mitochondrial dynamics and in recruitment of HSPA for the mediation of ataxin-1 degradation have been suggested (90,91). In turn, a recently found ARSACS causing mutation (T3702A) resides in the ubiquitin-binding domain of this protein (92). However, mutations outside this domain can also lead to disease, and it remains unclear if/how this could lead to loss-of-function.

Mutations in DNAJC19 have been identified to cause an autosomal recessive cardiomyopathy (93,94). DNAJC19 normally plays a crucial role in mitochondrial import (95), implying that deficiency causes disease via mitochondrial defects. More recently, mutations in DNAJC6 were found to be associated with juvenile-onset Parkinsonism (96,97). DNAJC6/Auxilin is a neuron-specific protein that assists Hsc70/HSPA8 in mediating clathrin-coated vesicle disassembly and thus plays a role in synaptic vesicle recycling (98,99). Mutations in DNAJC6 are predicted to lead to a truncated version of the protein, which fails to support Hsc70/HSPA8 in its normal function.

Next to these recessive diseases, two DNAJ-related chaperonopathies are dominantly inherited (Table 1) and could either cause disease through haploinsufficiency, by dominant-negative effects or via a toxic gain-of-function. Mutations in DNAJB6, all of which are found in the glycine-phenylalanine region (G/F), are associated with limb-girdle muscular dystrophy type 1D (LGMD1D). The disease leads to progressive muscle weakness and muscle atrophy. The molecular mechanism underlying the disease has been suggested to involve loss-of-function, resulting in protein accumulations and autophagic pathology in muscle fibers (100,101). This reduced chaperone function might be due to haploinsufficiency, but as DNAJB6 is present in cells as polydispersed complexes, mutants might also exert dominant negative effects on the wild-type protein.

Mutations in DNAJC5 cause an autosomal dominant neurodegenerative disease, named Kuf’s disease or adult onset neuronal ceroid lipofuscinoisis. Clinical symptoms include a dementia, ataxia and speech disorder which worsens over time. Normally, DNAJC5 is found in synaptic vesicles and involved in polymerization of dynamin (121). Dysfunction of DNAJC5 due to mutations in the crucial lysine position leads to its reduced palmitoylation and hence abnormal sorting and localization of DNAJC5 with ER and Golgi markers (107). This leads to decreased levels of DNAJC5 in the brain of diseased individuals, meaning that the disease is most likely caused by haplo-insufficiency.

**HSPB related chaperonopathies**

Mutations in several members of the HSPB family, irrespective of the member involved, are found in highly conserved amino acid residues or in the α-crystallin domain, which is a characteristic feature of this family of HSPs (7). The α-crystallin domain is required
for intra/inter-molecular interactions and the stabilization of homo- and hetero-oligomer formations of the HSPB members. As HSPBs are highly expressed in muscles and have a role in cytoskeleton stability (122,123), mutations usually affect the cells’ axonal transport (neurological and sensory disorders) and contractile functions (muscular disorders).

The presence of many of the dominant HSPB mutants in protein aggregates implies that they might have acquired a toxic gain-of-function similar to the proteins in proteinopathies (Fig. 2). There is indeed biochemical evidence that some mutants, such as the P182L mutant of HSPB1 (124), R49C and R116C of HSPB4 (125,126), and R120G, Q151X and 464delCt of HSPB5 (127–129) are intrinsically unstable and might thus cause disease by forming aggregates (toxic gain-of-function). However, it must be noted that the presence of HSPBs in aggregates could also be due to a loss-of-function, reflecting their failed attempt to handle a client with whom they subsequently co-aggregate.

Evidence for haplo-insufficiency, at least for HSPB1 mutations, is suggested by findings implying that reduced levels of HSPB1 lead to damage in sensory and motor neurons, that can be rescued by ectopic expression of HSPB1 (7,130). Partial evidence for haplo-insufficiency has also been provided for HSPB8 mutants, which have lost the HSPB8 chaperone-like activity to deal with aggregation-prone polyQ proteins and cause Charcot Marie Tooth disease, characterized by distal muscle weakness and atrophy and sensory loss (131).

Moreover, considering that HSPBs are known to form oligomers with other members of the same family, it is possible that HSPB mutants could affect the function of other HSPBs via dominant negative effects. HSPB8 mutants for example have an abnormally high affinity for endogenous HSPB1, thus potentially impairing HSPB1 or HSPB1-HSPB8 complex function (118). Similarly, certain HSPB1 mutants affect endogenous HSPB8, leading to loss of HSPB8-HSPB1 complex formation (132). Furthermore, abnormal interaction of the R116C-HSPB4 mutant with HSPB5 and HSPB1 has been reported (133).

Therefore, not only a toxic gain-of-function (aggregation) might be responsible for HSPB-related chaperonopathies, but also a loss-of-function, which could either be direct, due to the mutation, or indirect, due to sequestration of wild-type HSPBs by the mutated HSPB forms. In addition, alteration of HSPB-oligomerization properties and interactions with other HSPB members and/or haploinsufficiency might play a role in HSPB-related chaperonopathies.

**Chaperone intervention to rescue chaperonopathies**

Different HSPs have a role in anti-aggregation of various proteinopathies (Fig. 2). However, whether other HSPs might be able to rescue chaperonopathies has been scarcely studied.
Table I: Chaperonopathies

| Family | Mutation | Inheritance | Disease | Chaperone Mediated rescue | References |
|--------|----------|-------------|---------|---------------------------|------------|
| Hsp60/HSPD | V72I in SPG13, Chromosome 2p33.1; c.292G > A p.V96I | Dominant | Hereditary Spastic Paraplegia | NV | (82,83) |
|         | D29G     | Recessive   | Hypomyelinating leukodystrophies (HMLs); MICHAP-60 disease | NV | (102) |
| CCT/TRIC | C5012 in MKKS gene, H84Y, A242S | Recessive | McKusick-Kaufman Syndrome (MKS) | NV | (103) |
|         | MKKS locus BBS6, BBS10, BBS12 | Recessive | Bardet-Biedel Syndrome (BBS) | NV | (103–106) |
|         | C450Y in CCT delta | Recessive | Hereditary Sensory Neuropathy (HSN); Charcot-Marie-Tooth (CMT), Hereditary Motor & sensory neuropathy (HMSN) | NV | (85) |
| Hsp40/DNAJ | Splice mutation in HSJ1 gene | Recessive | Distal Hereditary Motor Neuropathy (dHMN) | NV | (86) |
| DNAJB6 (HSJ1) | F39L, F89L, P96R | Dominant | Limb-Girdle Muscular Dystrophy type 1D (LGMD1D) | NV | (100,101) |
| DNAJC5 | c.346_348 delCTC, c.344T>G; p.Leu115del, p.Leu115Arg | Dominant | Autosomal dominant adult-onset neuronal ceroid lipofuscinosis (ANCL) or Kufs disease | NV | (107–109) |
| DNAJC6 | p.G734X; c.801-2 A>G | Recessive | juvenile Parkinsonism | NV | (96,97) |
| DNAJC19 | p.E1162X; c.11,707C>T, p.R182L in SACS; T3702A | Recessive | Spastic ataxia of Charlevoix-Saguenay (ARSACS) | NV | (89,92,110,111) |
| HspB1 | P96R, G34R, E41K, G84R, L99M, R127W, S135F, R136W, R140G, K141Q, T151I, S156Y, T164A, T180I, P182L, R188W, 476_477delCT, p.Gln175X | Dominant; Recessive (L99M) | Williams Syndrome; Charcot-Marie-Tooth Disease 2 (CMT2), Distal Hereditary Motor Neuropathy (dHMN) | HspB8 mediated rescue for P182L mutation* | *(7,112,113) |
| HspB3 | R75    | Recessive | Motor Neuropathy (MN) | NV | (114) |
| HspB4 | W9X, R12C, R21L, R49C, R54C, F71L, G98R, R116C | Dominant; Recessive (W9X) | Autosomal dominant Congenital Cataract (ADCC) | NV | (7) |
| HspB5 | R11H, P20S, 450delA, R69C, D109H, D140N, A171T, R69W, c.343delT, R120G, Q151X, G154S, R157H, 464delCT | Dominant; Recessive (R56W) | Congenital Cataract, myofibrillar myopathy, Dilated cardiomyopathy, Desmin related myopathy | HSPB1, BAG3, HSPB8 mediated rescue** | **(115–117) |
| HspB8 | K141E, K141N, K141T | Dominant | Distal Hereditary Motor Neuropathy (dHMN), Charcot-Marie-Tooth Disease 2 (CMT2) | NV | (118–120) |

NV: Not verified

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**Table notes:**
- HspB8 mediated rescue for P182L mutation:
- HSPB1, BAG3, HSPB8 mediated rescue:
- NV: Not verified
A few reports suggest this could indeed be possible: Firstly, aggregation caused by some HSPB5 mutants could be prevented by overexpression of wild-type HSPB1 (115, 117), BAG3 (116) and wild-type HSPB8 (134). Secondly, aggregation associated with the expression of the P182L-HSPB1 mutant in cell models was significantly reduced by the overexpression of wild-type HSPB8 (112). Whether such rescues are due to prevention of the formation of toxic aggregates containing mutant HSPB or whether they reflect a compensation for loss of (redundant) HSPB functions remains to be elucidated.

CONCLUSION AND FUTURE PERSPECTIVE

The existence of different ‘barcodes’ for the rescue of specific aggregation diseases suggests that, although loss of protein homeostasis with aging might contribute to disease initiation (e.g., by HSF-1 abrogation, restoring general protein homeostasis or components thereof), boosting HSF-1 activity is usually insufficient for long-term protection in most dominantly inherited proteinopathies. Chronic expression of these aggregation-prone proteins in fact often does not trigger activation of the HSR until late in disease. By then, aggregates might have already sequestered chaperones and thereby disturbed normal protein homeostasis, resulting in cell death. In earlier stages of disease, protein aggregates could already affect neuronal and muscular cell function (even without causing cell death) by altering functions such as axonal transport, organelle dynamics and plasma membrane (receptor) function, without directly impairing protein homeostasis. It has been shown in several mouse models for HD that reversible functional impairments precede neuronal cell loss (135). However, in cellular and simple animal models, such functional defects could be missed and cell death-related effects (including disturbances in protein homeostasis) might prevail, which would explain the observed rescue by the activation of the HSR or by the overexpression of its individual components. However, these HSR-related effects usually do not coincide with aggregate prevention and therefore do not lead to significant, long-term, effects in mammalian animal models.

The human genome encodes many HSP members that are not regulated by the acute HSR. Although not yet studied intensively, our review clearly shows that some of these “non-canonical” members can specifically rescue aggregation caused by the distinct proteinopathies, some of which have now also been demonstrated to be effective in mouse models, including DNAJB6 (Chapter 5). Interestingly though, several of these non-canonical HSPs also cause chaperonopathies if mutated (DNAJB2, DNAJB6, HSPB8). This not only indicates that these HSPs have essential PQC functions, but furthermore suggests that their effects on proteinopathies might not be an artifact of their overexpression, but rather reflect an augmentation of their natural function.
A potential worry in all HSP overexpression or boosting studies is that it leads to network adaptations (which would annihilate long-term effectiveness) or to multiple side effects, including increasing carcinogenesis, as was demonstrated for the manipulation of HSF-1 activity (136). Although network adaptations are to be expected upon manipulation of the driving forces of chaperone machinery (e.g. Hsp90/HSPC or Hsp70/HSPA), such effects might be less likely for those components that only steer the specificity of these machines (e.g. HSPBs or DNAJs). Although we found no evidence for DNAJB6 effects on the chaperone network (Chapter 3 & Chapter 5), it remains important to further investigate whether (long-term) overexpression of DNAJB6 or other proteinopathy-rescuing HSPs might have side effects.

Finally, only limited comparative data on the potential rescue of the non-HSR regulated HSPs and the various proteinopathies or chaperonopathies are available. There still might be many novel suppressors of specific diseases to be uncovered, which would further barcode these diseases. This would not only help to find therapeutic targets for intervention, but would also help with understanding differences and similarities between the toxic mechanisms underlying the various proteinopathies.
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