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HSP70/ HSP40 Chaperones, involved in the Protein Quality Control System, suppressing Toxic Protein Aggregation

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Abstract

Many neurodegenerative diseases are characterized by the accumulation of misfolded proteins, which results in loss of function of the particular proteins and may lead to neuronal cell death. Chaperones (HSPs) are highly involved in the protein quality control system and maintain protein homeostasis in the cell. HSP70 and its co-factor HSP40 play a major role in protein folding, refolding and degradation. The HSP70/HSP40 complex protects nascent proteins from misfolding and aggregation as well. HSP40 stimulates ATPase activity of HSP70 by increasing the rate of ATP hydrolysis. Several studies showed that overexpression of HSP70 with HSP40, in models transfected with a plasmid coding for a neurodegenerative disease, suppresses aggregate formation *in vitro* (Evans et al., 2006, Kobayahi et al., 2000) and *in vivo* (Cummings et al., 2001, Chan et al., 2000). Overexpression of HSP40 family members alone show more or less anti-aggregation activity dependent on the protein model or which family member of HSP40 was overexpressed. DNAJB6 (MRJ), a family member of HSP40, showed anti-aggregation activity in several *in vitro* studies (Chuang et al., 2002). DNAJB6 suppresses toxicity in *in vivo* studies also (Fayazi et al., 2006). DNAJB6 together with DNAJB8 show anti-aggregation activity independent of HSP70 (Hageman et al., 2010). The anti-aggregation activity of these chaperones gives expectations for the use of chaperones for the protection against aggregation in protein misfolding disorders. This study explores the role of HSP70 and HSP40 in the protein quality control system and their anti-aggregation function in different cell models.

Contents

Introduction

Every cell in our body is challenged to maintain an intracellular protein balance. To keep this balance, the cell contains a quality control system which promotes correct protein folding and refolding or degradation of misfolded proteins. Correct protein folding in cells is important for proper function of a particular protein and is highly regulated by proteins called chaperones. Misfolding of proteins occurs when proteins are not able to achieve their native state due to a mutation in their amino acid sequence or because of an error in the folding process.

An imbalance in the protein homeostasis happens when the protein quality control system is not able to degrade or refold the misfolded proteins. This failure of the quality control system can have several reasons. One reason may be shortage of the capacity because, for example mutations in proteins or stress can result in too many misfolded proteins in the cell. Another reason can be an error in the protein quality control system itself. Misfolded proteins can aggregate with each other and/or with healthy proteins, which may have toxic influence on cells. (Kubota et al., 2009). Aggregation of proteins is seen in several protein-misfolding disorders.

Many neurodegenerative disorders are associated with protein misfolding. Most of the misfolded proteins are not degraded or refold by the control system and start to accumulate. The formed aggregates lead to neuronal dysfunction and/or cell death (Outeiro et al.,2007). Cell death can result in degeneration of the brain, for example in Alzheimer disease. Other diseases which are characterized by aggregates in cells are Parkinson and Polyglutamine (polyQ) diseases. Misfolded proteins formed in polyQ diseases are caused by a CAG repeat expansion in the genomic sequences of proteins. PolyQ disease is a collective noun for Huntington disease, several SCA (Spinocerebellar ataxia) diseases, Spinobulbar muscular atrophy (SBMA) and Dentatorubropallidoluysian atrophy (DRPLA).

First this study gives general information about the working mechanism of chaperones which are involved in protein folding and degradation of the protein quality control system. Further some causes of protein misfolding will be discussed. Chaperones contain a large protein family. Special attention will be paid to the HSP70 machinery and co-chaperone HSP40 because of their major role in maintaining protein homeostasis. Since many neurodegenerative diseases are associated with forming aggregates, this study will give insight whether chaperones HSP70 and HSP40 may have the ability to suppress aggregation. This section will be split up in anti-aggregation activity of the HSP70 machinery and anti-aggregation activity of HSP40 family members. The aim of this study is to discuss the role of HSP70 and HSP40 in the protein quality control system and their ability of suppressing the formation of aggregates in protein misfolding disorders.

Introduction into chaperones and their folding capacity

Protein folding is an important process in all living cells because nascent proteins have non-functional linear structures. Proteins become functionally active when they are folded in their own unique three-dimensional structure. However, protein folding not only generates a functional active protein, it also prevents degradation and aggregation of non-native structures. The question that seems to develop is how a non-native protein reaches its native state. The cell contains a crowded intracellular environment, which lead to aggregation when unfolded nascent proteins try to fold on their own. The native state of a protein is encoded in its amino-acid sequence but because of the crowded environment unfolded proteins need complex machinery for protection. This complex machinery contains several proteins called chaperones. Besides the protection against aggregation, the chaperone machinery also guides proteins to their correct functional three-dimensional structure. (Frydman et al., 2001).

Nomenclature chaperones

Molecular chaperones are a large protein family and the majority of the chaperone families are up-regulated under various conditions of stress. In a stress situation, like high temperatures, proteins unfold and have a greater chance to become misfolded and are unable to reach their correct native state. The up regulated chaperones take care of degradation or refolding of misfolded proteins. Due to the discovery of chaperones induced under stress conditions these proteins are called heat shock proteins (HSPs). Shortly after the discovery of heat shock responsive proteins, it became clear that these proteins are also constitutively expressed. These constitutively expressed proteins are called the Hsc members. (Kampinga et al., 2008). The classification of HSPs into families is based on molecular mass (Table. 1)(Sghaier et al., 2004).

Table 1: **Classification of HSPs.** Adapted from Sghaier et al. (2004).

Name	Mass (kDa)
Small HSPs (sHSPs)	< 35
HSP40s	35-54
HSP60s	55-64
HSP70s	65-80
HSP90s	81-99
Heavy HSPs (high molecular mass, HSP100)	100 or higher

Chaperones involved in protein folding

The several chaperone families present in the cell differ in their involvement in protein folding. One group of chaperones present in the cell are small HSPs. The exact role of small HSPs in protein folding is unclear. Known is that small HSPs bind tightly with unfolded proteins to prevent aggregation. But the strong interaction results in a slow or no release of the unfolded protein. Folding of the protein takes place when the unfolded protein is released from the chaperone, so the tight interaction between small HSPs and an unfolded protein gives reason to assume that small HSPs are not highly involved in protein folding. (Fink, 1999). Another chaperone, the HSP40, is familiar in the role as co-chaperone for HSP70. But less is known about the folding capacity of this chaperone separately. The affinity of HSP40 members with nascent proteins from the ribosome gives evidence that these chaperones are involved in early protein folding and are active on their own (Fink, 1999).

HSP60 is a chaperone by which the binding and release of proteins is ATP regulated. Non-native proteins are captured into the central ring of HSP60 where they are protected from aggregation. Besides the protective function, HSP60 also plays a role in the folding and refolding of proteins in the open formation of HSP60 (Hartl et al., 2002). The next chaperone, HSP90, is one of the most abundant chaperones in the cell but little is known about its role in folding of nascent proteins. A mutant in yeast has been used to get more knowledge about this chaperone. Results showed that HSP90 is not required for the *de novo* folding but HSP90 is important for correct folding of proteins that have greater difficulty reaching their native state. (Nathan et al., 1997). In this report the focus is mainly on HSP70 and its co-factor HSP40 therefore the role of the HSP70 machinery is discussed separately.

The HSP70 machinery

Characteristics of the HSP70 machinery are the ability to prevent misfolding and aggregation of unfolded polypeptides. Misfolding is prevented through the HSP70 machinery because it is assisting nascent proteins to fold into their correct three-dimensional structure (Morimoto et al., 2008). Activity of the HSP70 depends on the ATP or ADP bound state. Several co-factors within the HSP70 machinery influence this activity of HSP70. Co-factors that play a role in this process are HSP40, BAG-1, HIP and CHIP (Hageman, 2008).

HSP70 and HSP40 interaction

HSP40 is one of the co-factors that has an important function by stimulating the ATPase activity in the HSP70 machinery and helping HSP70 by the folding of proteins. HSP70 itself has a very weak ATPase activity, so HSP40 is necessary to stimulate the ATPase activity of this chaperone. HSP40 protein can bind a nascent protein at the C-terminal and its J domain promotes ATP hydrolysis of HSP70 (figure 1). After the interaction of HSP70 with HSP40 the transfer of the substrate from HSP40 to HSP70 takes place (Qiu et al., 2006). HSP70 recognizes and bind nascent peptides by itself also. The substrate binding domain of HSP70 is located near the C-terminal (figure 1). The ATP bound state of the HSP70 is called the open conformation state. HSP40 hydrolyze ATP, which results in a closed conformation of HSP70. In the closed conformation the peptide has a higher affinity with the complex than in the open conformation. The difference between the open and closed conformation is the frequency of opening and closing of the substrate binding domain. The frequency is much lower in the ADP bound state in comparison with the ATP bound state (Mayer et al., 2000). The

protein is free to fold to their native state when it is released from HSP70. The ADP bound state of HSP70 release proteins slowly, because of the low frequency, which results in folding but also in preventing aggregation (Hartl et al., 2002). When the binding domain is closed, the substrate is trapped within the complex, this is called holding. Holding non-native polypeptides in the HSP70 complex prevents them from misfolding and aggregation (Mayer et al,. 2000). The ATPase domain of the HSP70 is located on the N-terminal. HSP40 binds the ATPase domain and the C-terminal of HSP70 (figure 1) (Hageman, 2008).

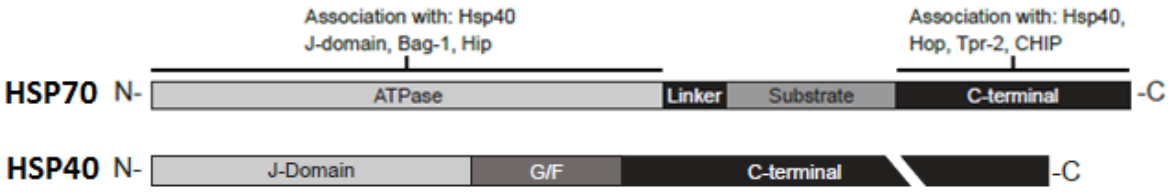


Figure 1. **Structure of HSP70 and HSP40.** Adapted from Hageman (2008).

Other co-factors stimulating the HSP70 machinery

There are several other co-factors besides HSP40 that play a role in the ATPase activity of the HSP70 machinery. BAG-1 is one of these co-factors. The action of BAG-1 is, like HSP40, stimulating the hydrolysis of ATP. BAG-1 binds also at the ATPase domain of HSP70 (figure 1). A co-chaperone that plays a role in stabilizing the ADP state of HSP70 is HIP. HIP takes care of forming a stable complex of HSP70 with its substrate protein (Fink, 1999). Another co-factor CHIP inhibits the ATP hydrolysis. Inhibiting of ATP hydrolysis takes care of keeping HSP70 in the open conformation state. Interestingly, all co-factors except BAG-1 have the capability to bind unfolded proteins. This may play a role in giving specificity to the HSP70 machinery. (Hageman, 2008)

This chapter made clear that HSP70 and its co-chaperone HSP40 are involved in protein folding, but in the same time preventing unfolded proteins from misfolding and aggregation. Next chapter will discuss the degradation system of the protein quality control system in the cell when misfolding of proteins occurs.

Degradation of misfolded proteins

A certain level of protein misfolding occurs in cells despite the presence of chaperones. When the amount of misfolded proteins exceeds the chaperone capacity they tend to aggregate, which can directly damage essential cellular systems. The cell contains a protein quality control system which is capable in removing and refolding these misfolded proteins. Chaperones have the ability to refold misfolded proteins but also play an important role in removing misfolded proteins from the cytosol. Chaperones recognize misfolded proteins and transfer them to the ubiquitin-proteasome system and chaperone-mediated autophagy.

Ubiquitin-proteasome system

The ubiquitin-proteasome system is a major system in the cell that takes care of the degradation of misfolded proteins. The HSP70/HSP40 complex recognizes misfolded proteins and tries to refold the misfolded protein into the correct structure. When refolding fails, degradation of a misfolded protein via the ubiquitin-proteasome pathway starts. To degrade misfolded proteins, the HSP70/HSP40 complex interacts with co-factors that have degradation capacities. One of these co-factors is CHIP, a dependent E3-protein. E-proteins are ubiquitin-carrier proteins. The interaction of CHIP with HSP70/HSP40 complex results in ubiquitination of the substrate. Finally the ubiquinated substrate will be recognized and degraded by the proteasome (figure 2) (Cyr et al., 2002) (Goldberg, 2003).

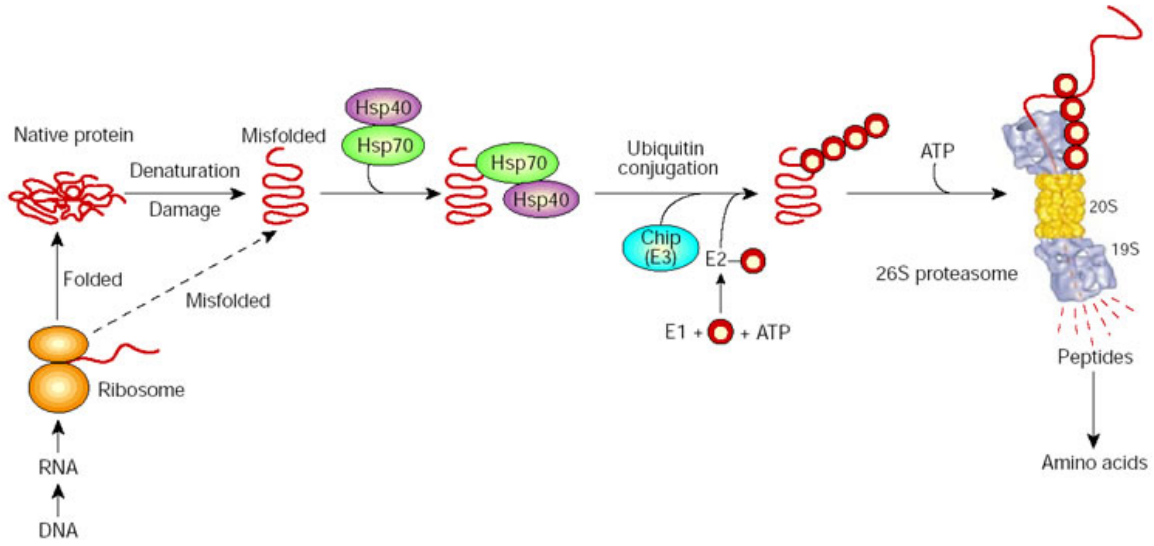


Figure 2. **The Ubiquitin-Proteasome Pathway.** Adapted from Goldberg (2003).

Chaperone mediated autophagy

Another degradation system is chaperone mediated autophagy (CMA), which degrade misfolded proteins in lysosomes. Lysosomes are organelles with an internal acidic environment. Lysosomes contain enzymes that are able to break down misfolded proteins. Molecular chaperone, Hsc70, present in the cytosol recognizes misfolded because they are containing a pattern consistent of five amino-acids; KFERQ. The interaction between the misfolded protein and Hsc70, with modulating co-chaperones, leads to the delivery of this complex to the lysosomal membrane. The complex interacts with a protein called lamp2a in the lysosomal membrane, which lead to uptake of the substrate. Translocation through the membrane is escorted by the presence of lysosomal chaperone Hsc70. Finally, the protein will be degraded within the intracellular environment of the lysosome. (Figure 3) (Massey et al., 2004).

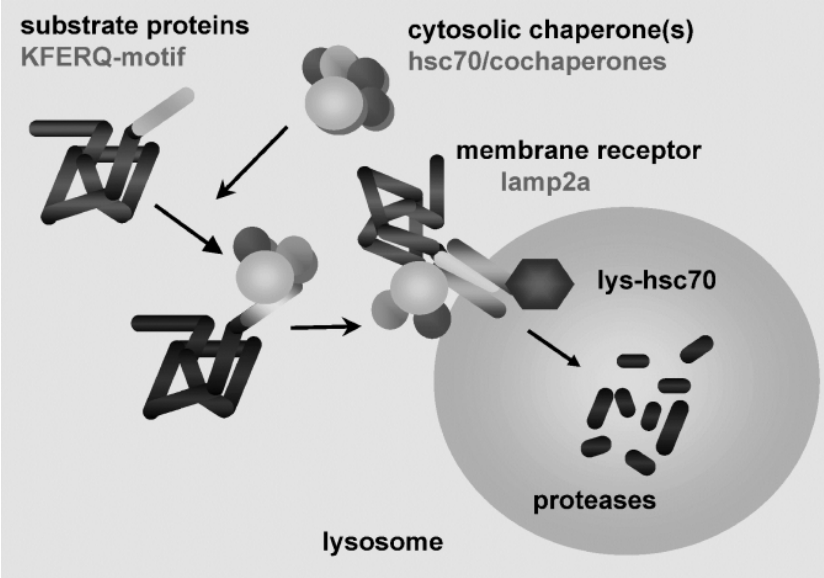


Figure 3. **Model of Chaperone Mediated Autophagy.** Adapted from Massey et al. (2004).

Causes for an elevation in the amount of misfolded proteins

Degradation systems are active in the cell because a certain level of protein misfolding occurs, but a higher efficiency of these degradation systems is necessary when there is an elevated amount of misfolded proteins. There are some influences that can cause an elevation in the level of misfolded proteins, which results in an imbalance in the protein homeostasis. (Morimoto et al., 2009). The number of misfolded proteins is elevated under stress conditions. Healthy cells are capable to respond to this elevation and restore the protein homeostasis. A constant imbalance of protein homeostasis is seen in aging and in several neurodegenerative diseases. Some of the neurodegenerative diseases, including Alzheimer, Parkinson and Poly-Q disease, are expressed in a later stage of life. Therefore it is possible to remark that aging is involved in the onset of these diseases.

Cell stress response

In stress conditions misfolded proteins are significantly increased and many chaperone genes are induced to protect the cell against the toxicity of misfolded proteins. This response is called the heat shock response. It is important to understand that this response is activated under several stress conditions and not only due to an elevated temperature. The major transcription factor that mediates the heat shock response is heat shock factor 1 (HSF1). This transcription factor binds to the heat shock response element (HSE) in the promoter region of stress-responsive genes (Kubota et al., 2009). The interaction of HSF1 with HSE causes an up-regulation of several stress-responsive chaperones which degrade or refolds the misfolded and damaged proteins.

In non-stressed conditions HSF1 is present in non-DNA binding state and interacts with chaperones including HSP90, HSP70 and HSP40. The interaction between HSF1 and chaperones suppresses transcription of chaperones genes. HSF1 forms an active DNA-binding factor when stress conditions are induced. The primary signal for the heat shock response is a flux of proteins that is detected as misfolded or damaged. The protein equilibrium shift activates HSF1 for binding the promoter region of stress-responsive chaperone genes. (Morimoto et al., 2008).

Aging

Aging is characterized by an accumulation of protein damage mainly induced by reactive oxygen species. Due to the oxidation of proteins there is a higher chance of misfolding which can result in aggregation. It is interesting to notice the association with aging and protein conformational diseases. There are several reasons that indicate why chaperone function is less during aging and aggregation of misfolded proteins occurs in a later onset of life. (Arslan et al., 2006)

It has been proposed that during aging the heat shock response is declining. Consequently there is a reduced level of HSPs and a loss of protein quality control during stress conditions. The exact mechanism of the reduced heat shock response is not yet identified but the elevated concentrations of chaperones during aging can have some influence. In aging elevated concentrations of chaperones are present in the cells due to increased oxidative stress. The interaction between chaperones and the HSF1 in the heat shock response was set out previously. The elevated concentration of chaperones in aging inhibits more HSF1 activity which results in a reduced heat shock response. (Tower., 2009).

Another reason for a loss of chaperone function in aging is chaperone overload. Chaperone overload is present when the number of misfolded proteins may exceed the chaperone capacity (Soti et al., 2006). More than half of the proteins in aged organisms are oxidized, which results in a large amount of misfolded proteins. In an overload situation misfolded proteins that should be degraded can now aggregate and hidden mutations can now appear in the phenotype of the cell. Previous hidden mutations can be a reason why some protein misfolding disorders start in a later stage of life.

An elevated amount of misfolded proteins and formed aggregates are seen in several neurodegenerative disorders. Previously this study set out the working mechanism of folding and degradation of proteins and the involvement of HSP70/HSP40. Next thing is to look whether the HSP70 machinery is able to suppress the formation of aggregates, which are associated with several misfolding disorders.

Anti-aggregation activity of the HSP70 machinery

The question remains whether HSP70 machinery is able to protect cells from misfolding and aggregation in cells with a protein misfolding disorder. This chapter will discuss the anti-aggregation activity of the HSP70 machinery in disease cell models *in vitro* and suppressing neurodegeneration by HSP70 machinery *in vivo*.

HSP70 / HSP40 suppress aggregate formation

The first reason to assume that HSP70 and HSP40 influence the formation of aggregates is co-localization of HSP70 and HSP40 with aggregates in cultured neuronal cell models of Spinal and bulbar muscular atrophy (SBMA). (Kobayahi et al., 2000) SBMA is a neurodegenerative disease caused by expansion of a CAG repeat which leads to inclusion formation in neuronal cells. Figure 4 shows the co-localization of endogenous HSP70 and HSP40 with the poly-Q formed aggregates.

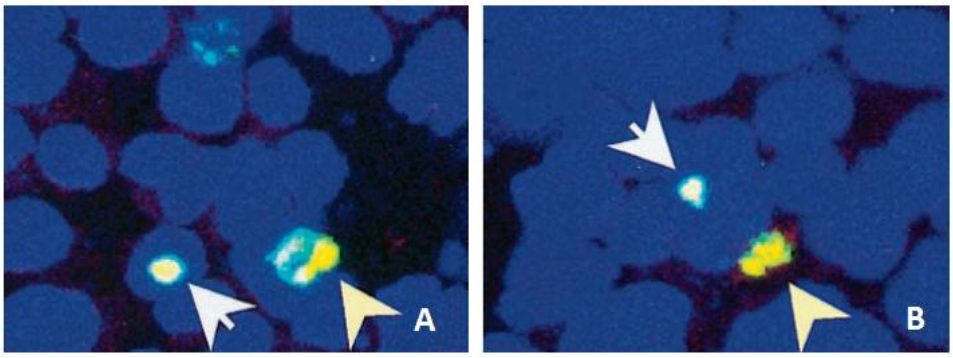


Figure 4. **Co-localization of endogenous chaperones with polyglutamine formed aggregates.** Images taken by confocal microscopy. Arrowheads indicate the cytoplasmic aggregates and arrows point to the nuclear aggregates. HSP70 (a) and HSP40 (b) colocalized to the nuclear aggregates (white) and cytoplasmic aggregates (yellow), respectively. Adapted from Kobayahi et al.(200).

Besides the co-localization of endogenous HSP70 and HSP40 there was an increase in expression levels (Kobayahi et al., 2000). The increased amount of HSP70 and HSP40 by itself is not enough for protection of aggregates formed in the cellular model of SBMA. Overexpression of the chaperones HSP70 and HSP40 in the neuronal cell models of SBMA showed that this combination has a strong anti-aggregation function. (Kobayahi et al., 2000).

Another *in vitro* study showed also reduction in aggregation of Amyloid β in cells with overexpression of HSP70 and HSP40. Aggregates of amyloid β is a characteristic of Alzheimer disease. Besides this, the study showed that the combination of HSP70 and HSP40 is more effective against aggregate formation than HSP70 alone (figure 5) (Evans et al., 2006). In conclusion, the HSP70 machinery has the ability to suppress aggregation *in vitro*.

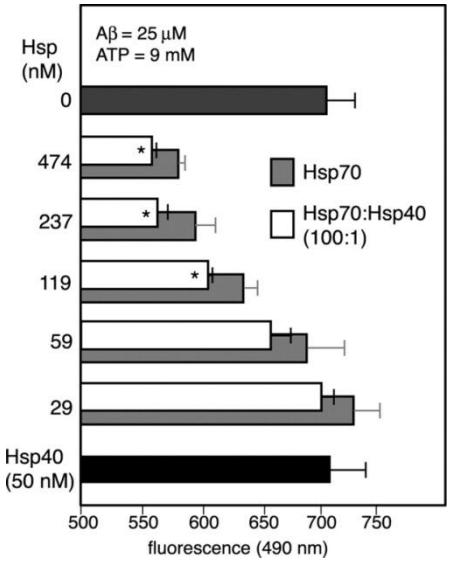


Figure 5. **Anti-aggregation activity of Hsp70 is enhanced by Hsp40.** Amyloid β treated with Hsp70, Hsp40 and the combination of Hsp70/Hsp40. Hsp70 alone blocks aggregation but addition of Hsp40 leads to enhancement of the anti-aggregation activity. Hsp40 failed to block aggregation. Adapted from Evans et al. (2006)

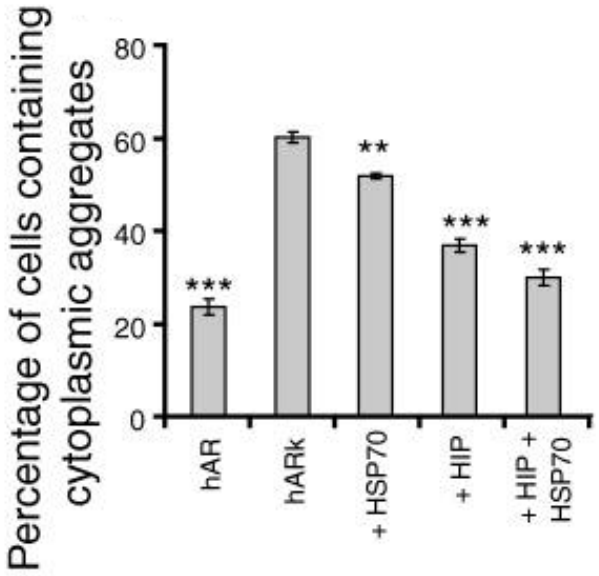
What the molecular mechanism is for the reduction of aggregates is still not completely resolved. Both studies give several suggestions for mechanisms that are active in the anti-aggregation system. One of the possibilities is that overexpression of chaperone is enhancing the function of the ubiquitin-proteasome system (Kobayahi et al., 2000). This enhancement results in more degradation of misfolded proteins and less aggregation. Two other mechanisms are holding and refolding of misfolded proteins by HSP70. An increase in the amount of HSP70 means holding more misfolded proteins and a better protection against aggregation (Evans et al., 2006).

After demonstrating anti-aggregation activity of the HSP70 machinery *in vitro*, the next step is to look whether same results are reached in *in vivo* studies. One *in* vivo study illustrates that overexpression of HSP70 lead to a reduction of polyglutamine induced neurodegeneration in a mouse model of SCA1 (Cummings et al., 2001). Another study with Drosophila M. showed that interaction between a subfamily of HSP40 and HSP70 suppresses toxic polyglutamine proteins (Chan et al., 2000). *In vitro* and *in vivo* studies demonstrate suppression in the accumulation of misfolded proteins in several diseases by overexpression the combination of HSP70 and HSP40.

Co-factors of HSP70 machinery and their role in anti-aggregation activity

Previous studies showed anti-aggregation function by overexpressing HSP70 in combination with HSP40. But the HSP70 machinery includes, besides HSP40, other co-factors as BAG-1, CHIP and HIP, who are regulating the activity of HSP70. The effect of overexpression BAG-1 and CHIP is observed in cells transfected with constructs containing exon-1 of the Huntington Disease (HD) gene with an expansion of a CAG repeat. The results showed no decrease of aggregation when overexpression of BAG-1 and CHIP alone or in combination with HSP70 was generated (Rujano et al., 2007).

Contrary to the non-anti-aggregation activity of BAG-1 and CHIP, is the reduced inclusion formation in SBMA cell models by overexpressing HIP in combination with HSP70. In this study the cells were transfected with vectors encoding for the human Androgen Receptor construct plus 20 CAG repeats (hAR) and 51 CAG repeats (hARk). The results showed that a decrease of aggregate formation was larger in transfected cells with HSP70 and HIP than in cells with HSP70 alone. Also a decrease of aggregates was shown in overexpression of HIP alone (figure 6) (Howarth et al., 2009). The mechanism of action is not known yet, but possibilities are that HIP leads to more refolding of misfolded proteins by chaperones, holds proteins to prevent them from aggregation or increases the degradation of misfolded proteins.



Figuur 6. **HIP leads to a reduction of aggregation in SBMA transfected cells.** The results are presented as the percentage of cells containing aggregates relative to the untreated control. Adapted from Howarth et al. (2009).

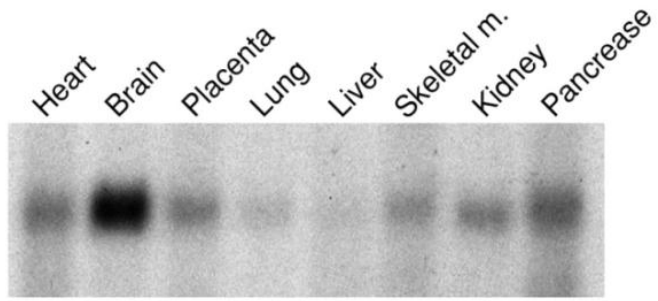
The anti-aggregation activity of DNAJ protein MRJ

Previous chapter showed a significant reduction of aggregates by overexpression HSP70 in combination with HSP40. The anti-aggregation activity of the HSP40 chaperone alone differs among the members of the DNAJ (HSP40) family and differs from in which cell models it is overexpressed. Jana et al. (2000) showed that HDJ-1 (HSP40 family member) suppresses aggregation in Huntington disease models. Contrary to this is the observation of increased aggregation by overexpression of HSP40 family member HDJ-2 in Huntington disease models as well (Wyttenbach et al., 2000). However observation of the HDJ-2 in SBMA transfected cells did show a reduction of aggregates (Stenoien et al., 1998). In this chapter we will discuss anti-aggregation activity of a DNAJ family protein, MRJ (Mammalian Relative of DNAJ; DNAJB6), in different cell models *in vitro* and *in vivo*.

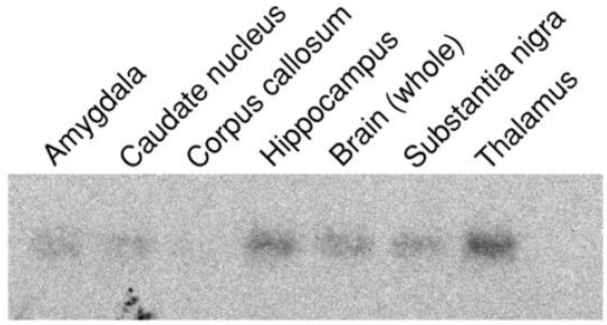
A Chaperone of the DNAJ protein family: MRJ

MRJ chaperone is one of the DNAJ-like family proteins. It contains a J-domain at the N-terminus and a C-terminus sequence for binding a substrate. It also contains the characterizing Glycine/Phenalynine rich domain. Chuang et al. (2002) showed that besides the J domain of MRJ, as set out before, also the C-terminal has the ability to enhance the HSP70 ATPase activity.

The tissue distribution of MRJ showed to be highest in the brain and lower levels of expression are present in other tissues (figure 7). Within the brain, different expression levels are detected as well (figure 8) (Chuang et al., 2002). Because of the expression in the brain of MRJ, it is interesting to examine whether this protein can reduce protein aggregate formation in several neurodegenerative diseases.



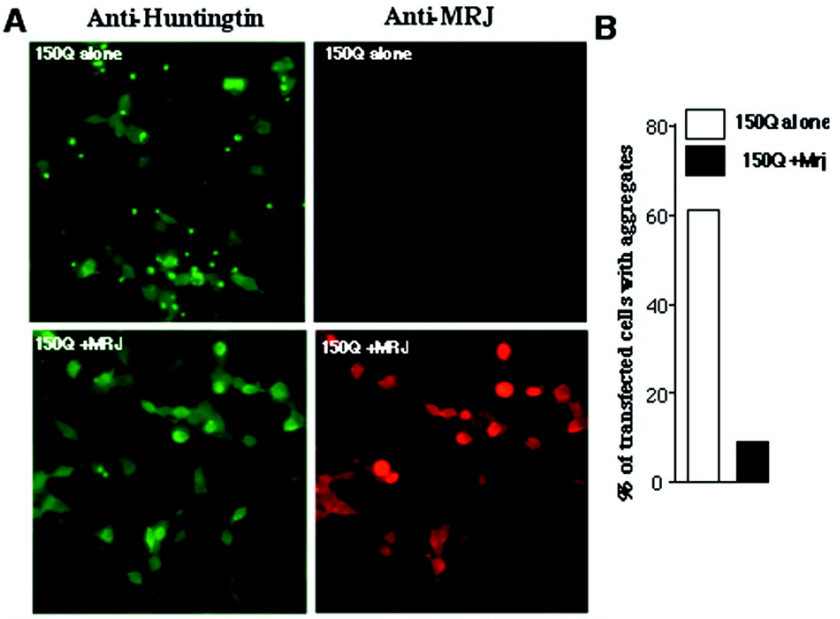
Figuur 7. **Expression patterns of MRJ in human tissues determined by Northern Blotting.** Human tissue probed with radiolabeled human MRJ cDNA fragment. Adapted from Chuang et al. (2002)



Figuur 8. **Expression patterns of MRJ in different departments of the brain determined by Northern Blotting.** Different human brain regions probed with radiolabeled human MRJ cDNA fragment. Adapted from Chuang et al. (2002)

MRJ prevents aggregation in several diseases

One of the studies in MRJ anti-aggregation function showed reduction of aggregates in cells models of Huntington disease. Co-expression of MRJ decreased the amount of aggregates in the disease cell models (figure 9) (Chuang et al., 2002). MRJ proteins without the J domain showed similar ability in suppressing aggregation also. But involvement of HSP70 in anti-aggregation activity cannot be ruled out yet because the C-terminal of MRJ stimulates the ATPase activity of HSP70 as well (Chuang et al., 2002). The reduction of aggregates in the presence of MRJ *in vitro* may indicate a preventing mechanism for aggregation and suppression of polyglutamine toxicity *in vivo*.



Figuur 9. **MRJ inhibits aggregation in a huntingtin disease model.** Cells were transfected with 150 glutamine (150Q) or 150Q and MRJ. (A) The transfected cells were stained with mouse antibody to huntingtin and rabbit antibody to MRJ.(B) 61.8% of the cells which were transfected with plasmin encoding polyQ, contained aggregates and only 11.2% of cells transfected with both a plasmid coding for huntingtin and MRJ contain aggregates. Adapted from Chuang et al. (2002)

A study *in vivo* showed suppression of polyglutamine toxicity in the presence of MRJ in *Drosophila Melongaster* and co-localization of MRJ with the inclusions (Fayazi et al., 2006). The co-localization shows that the reduced polyglutamine toxicity can result from interaction of MRJ with the inclusions.

Another *in vivo* study demonstrates the role of MRJ in preventing the accumulation of proteins. MRJ is expressed in adult mice but also in development of the embryo. This study set out that MRJ-deficient mice lack a normal keratin cytoskeleton and contains large keratin aggregates in contrast with mice that did contain MRJ. (Watson et al., 2007). These *in vivo* studies show that MRJ is important for preventing and suppressing the formation of aggregates.

Interestingly in the study of Watson et al. (2007) is that keratin aggregates formed in cells with inhibited proteasomes were similar to those in MRJ-deficient cells. These results indicate that MRJ is required for keratin degradation by the proteasome. These results may give some more information about the mechanism of preventing aggregation in diseases where accumulation of proteins is present.

DNAJB6 and DNAJB8 suppress toxic protein aggregation

The MRJ (DNAJB6) chaperone has anti-aggregation activity and suppresses toxicity in several diseases. Recently the first study demonstrated higher expression levels of DNAJB6 present in astrocytes in Parkinson Disease models (Durrenberger et al., 2009). This higher expression levels may indicate a protective mechanism for the aggregates formed in this disease. This last chapter demonstrates, besides DNAJB6, anti-aggregation activity of one other DNAJ protein and gives some more insight in the anti-aggregation working mechanism.

DNAJB8 suppresses formation of aggregates as well

In the search for more DNAJ proteins involved in suppressing formation of aggregates, cells were transfected with a plasmid coding for exon-1 of the huntingtin gene containing a repeat of 119 glutamines. The results of this experiment indicate suppression of polyglutamine aggregation by DNAJB6b and DNAJB8 (figure 10) (Hageman et al., 2010). The other proteins of the DNAJ family showed much less or no anti-aggregation activity. The induced expression of DNAJB6b and DNAJB8 did only suppress increase of aggregation and did not reduce pre-existing aggregates (Hageman et al., 2010). This result may indicate that DNAJB8 and DNAJB6b do not interact with formed large aggregates but are interacting with misfolded proteins and during the formation of aggregates.

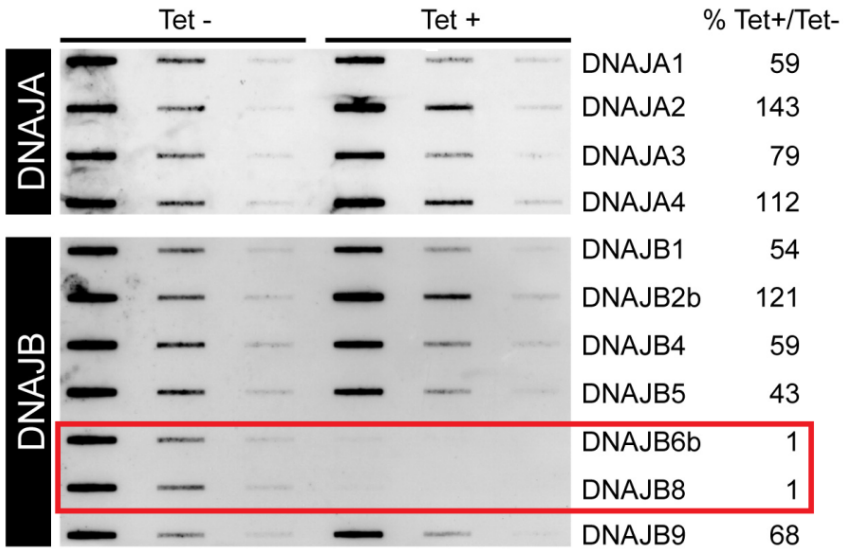
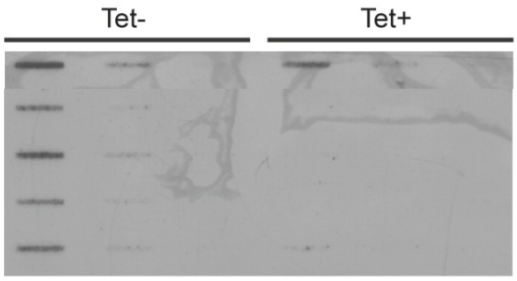


Figure 10. **DNAJB6b and DNAJB8 suppress polyglutamine aggregation.** Filter trap assay of cells with and without an overexpression of DNAJ chaperones. The numbers represent the percentage of aggregation in cells with exogenous chaperones (+Tet) compared to cells without exogenous chaperones (-Tet). Adapted from Hageman et al. (2010)

Set out before is the high expression level of DNAJB6 in the human brain. Different is the expression of DNAJB8, because this chaperone is only expressed in testis tissue (Hageman et al., 2009). Expression patterns of DNAJB6b and DNAJB8 are similar in the cell because they are both present in the nucleus and the cytosol. The longer isoform of DNAJB6, DNAJB6a is only present in the nucleus and it was as effective as DNAJB6b when using a nuclear-targeted poly-Q protein. (Hageman., 2010).

DNAJB6b and DNAJB8 work independently of HSP70

The J domain of DNAJ proteins is important in the interaction with HSP70. To investigate whether the interaction of these two chaperones is important for anti-aggregation function of DNAJB6b and DNAJB8, the cells were co-expressed with HSP70 family members. None of the HSP70 members increased the anti-aggregation activity of these DNAJBs. To rule out the interaction with Hsc70, mutants of DNAJB8 and DNAJB6b with J domains (H31Q), incapable of binding with Hsc70, are overexpressed. The mutants are still able to suppress aggregation but they do have less anti-aggregation activity (figure 11). This indicates that the J domain is not crucial for suppressing aggregation and HSP40 has anti-aggregation activity separately from HSP70. In contrast to the J-domain, the C-terminus is important for the anti-aggregation activity of DNAJB6b and DNAJB8. (Hageman et al.,2010).



	% Tet+/Tet-
No HSP	55
DNAJB6b	0
DNAJB6b H31Q	1
DNAJB8	0
DNAJB8 H31Q	12

Figure11. **J domain not necessary for suppressing polyglutamine aggregates formation.** Filter trap assay of cells with DNAJB6 and DNAJB8 and mutants of these DNAJBs. Mutants of the DNAJBs lack the ability to bind with Hsc70. Adapted from Hageman et al (2010)

Post-translational modification plays a role in the anti-aggregation activity

Post-translational modification is the last step of protein synthesis and takes place after translation of the protein. Acetylation is the covalent binding of an acetyl group to lysine residues catalyzed by histone acetyltransferase (HAT), Histone Deacetylase (HDAC) removes the acetyl group (Pennuto et al., 2009). Co-localization takes place between HDAC4 and MRJ (figure 12) (Dai et al., 2005), which indicates that there may be a relation between acetylation and the activity of MRJ.

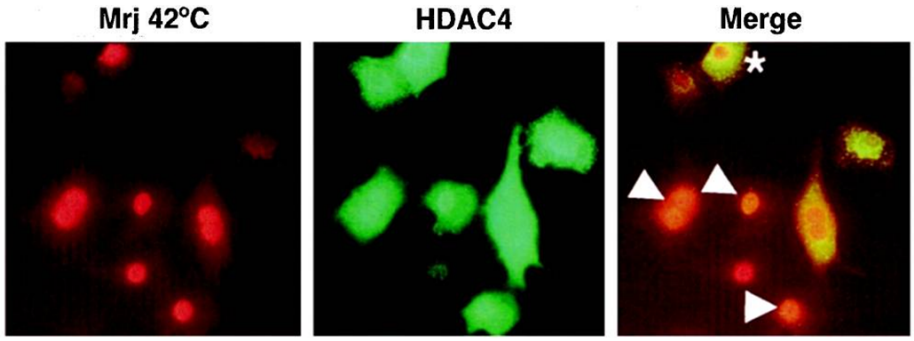


Figure 12. **Co-localization of MRJ with HDAC4.** The arrowheads indicate co-localization, the asterisk show a cell which fails in co-localization. Adapted from Dai et al. (2005).

The interaction between MRJ and HDAC4 found in the study of Dai et al. takes place at the serine rich region in the C-terminus, the SSF-SST region, of DNAJB8. Hageman et al. (2010) showed indeed that the DNAJB8 binds HDAC4, 6 and SIRT2 whereas DNAJB8 without the SSF-SST region did not. Further research made clear that de-acetylation of DNAJB8 and DNAJB6b by HDAC4 is essential for the anti-aggregation activity of these DNAJBs. The importance of HDAC4 is illustrated in several ways. First inhibition of HDAC4 has been accomplished by treating the cells with TSA, an inhibitor of HDAC4. Results showed that this led to an inhibition of the anti-aggregation activity of DNAJB6b and DNAJB8. Subsequently HDAC4 was down-regulated with siRNA which resulted in no longer suppressing activity of DNAJB8 in cells. The last experiment shows the importance of acetylated/deacetylated lysines in DNAJB6b and DNAJB8. The lysines at positions K216 and K223 are highly conserved in DNAJB6b and DNAJB8 and are present in the C-terminal. Remember that deletion of the C-terminal led to a loss of anti-aggregation activity. Substitution of the lysine K216 for alanine (mimic the acetylated state) leads to a substantial loss of activity of DNAJB8. DNAJB8 also has a lysine at position K223 in the C-terminal. Substitution of this lysine for an alanine did not demonstrate a great loss of anti-aggregation activity. Substitution of both lysines for alanines showed the most loss of activity (figure 13). (Hageman et al., 2010). Substitution of lysine by argenine (mimicking the de-acetylated state) showed no difference in anti-aggregation function compared with the DNAJB8 wild type. The de-acetylation of these two C-terminal lysines in DNAJB8 do play a major role in the anti-aggregation activity.

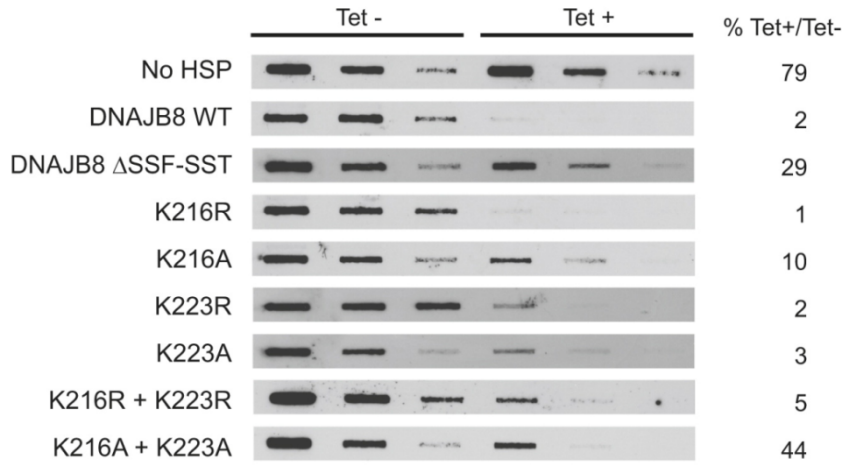


Figure 13. **DNAJB8 activity depends on the presence of lysines which can be acetylated/de-acetylated at the C-terminal.** Filter trap assay of DNAJB8 and DNAJB8 mutants. The DNAJB8 mutants K216R and K223R have substitution of the lysine for arginine to mimic the de-acetylated state. The DNAJB8 mutant K216A and K223A mimic the acetylated state. DNAJB8 ΔSSF-SST is DNAJB8 with a deleted SSF-SST region. Adapted from Hageman et al. (2010)

The protective mechanism of another HDAC, HDAC6, against the toxicity of protein aggregate formation is demonstrated in another study as well (Boyault et al., 2007). Because the anti-aggregation activity of DNAJB8 and DNAJB6b depends on the presence of HDAC4 there may be a role for HDAC in the quality control system of the cell.

In conclusion, DNAJB6b and DNAJB8, family members of HSP40, have anti-aggregation function in Huntington disease cell models. Their mechanism of action is independent of HSP70 and depends on the de-acetylation of two lysines in the SSF-SST region.

Discussion

The HSP70 machinery is important in maintaining protein homeostasis in the cell. To maintain this balance the HSP70 complex performs several functions in the protein quality control system. One of the functions of HSP70 and his co-factors is to protect nascent proteins from misfolding and guide them to their correct three-dimensional functional structure. Another function of the HSP70 complex in maintaining protein homeostasis is the recognition and refolding or degradation of misfolded proteins. Co-factor HSP40 stimulates the HSP70 ATPase activity by increasing the rate of ATP hydrolysis.

The protein quality control system fails in maintaining balance of the protein homeostasis in several diseases and aging. Aged organisms have more misfolded proteins, which exceed the capacity of the protein quality control system. This results in an imbalance of the protein homeostasis. Misfolded proteins are more vulnerable to aggregation. Aggregates are toxic to cells and can lead to cell death. Several neurodegenerative diseases show accumulation of misfolded proteins. Misfolded proteins in neurodegenerative disease are caused by mutations and/or failure of the protein quality control system.

The HSP70 machinery plays a role in preventing aggregation of misfolded proteins by refolding and degradation of these misfolded proteins. In neurodegenerative diseases misfolded proteins are able to aggregate and damaging the cells due to an imbalance of protein homeostasis. An *in vitro* study of Kobayahi et al. (2000) showed an increase in expression levels of HSP70 and HSP40 in SBMA diseased cell models. The higher levels of HSP70 and HSP40 in cells containing aggregates may indicate that the chaperones try to protect the cell from misfolded proteins. This protective mechanism might be enough at the onset of the disease, however it has not enough capacity when the amount of misfolded proteins increases. Results of several *in vitro* and *in vivo* studies indicate that overexpression of HSP70 and HSP40 lead to a protective mechanism against the formation of aggregates. An increased suppression of aggregates was seen in overexpressing the combination of HSP70 and HSP40 rather than HSP70 alone. The anti-aggregation working mechanism of these chaperones is still unknown however a better anti-aggregation function of HSP70 in combination with HSP40 may indicate that processes of refolding, degradation or protection of unfolded proteins are involved. The higher anti-aggregation activity of HSP70 by overexpressing HIP can give some more information about the working mechanism in the feature as well. Further studies in cell and animal models are required to examine the mechanism of anti-aggregation function of the combination HSP70 and HSP40.

Because the involvement of HSP40 in the anti-aggregation activity of HSP70 it may be interesting to notice that HSP40 has anti-aggregation activity of itself. One chaperone of the HSP40 protein family, DNAJB6 / MRJ, showed anti-aggregation activity in several cell models and the presence of MRJ prevented aggregation of proteins in the embryonic state. Interesting is the observation of Watson et al. (2007) that proteasome inhibition had the same amount of keratin aggregates as MRJ-deficient cells. This indicates that degradation by the proteasome is important for the anti-aggregation activity. The study of Hageman et al. (2010) showed that another HSP40 family member, DNAJB8, had the same anti-aggregation activity as DNAJB6. A major difference between these proteins is the distribution in human tissue. DNAJB6 is most expressed in brain tissue and DNAJB8 is only expressed in testis tissue. Because many diseases caused by misfolding are

neurodegenerative diseases, it is interesting to study whether up-regulation of DNAJB6 in the human brain may have a protective effect on the toxicity of aggregates in neurodegenerative diseases. The reason for the expression of DNAJB8 only in testis tissue is not yet known. In the feature more studies should investigate whether other DNAJ family members have the same anti-aggregation activity as DNAJB6 and DNAJB8.

Hageman et al. (2010) ruled out interaction of DNAJB6/DNAJB8 with HSP70 by investigating co-expression of DNAJB6/DNAJB8 with HSP70. The interaction of DNAJB6 and DNAJB8 with Hsc70 was ruled out by using DNAJB8 and DNAJB6 mutant that had J domains who were not capable of binding with Hsc70. Results showed a little less anti-aggregation activity of DNAJB6/DNAJB8 but they were still able to suppress aggregate formation. Chuang et al. showed before that the C-terminal of DNAJB6 also capable is to enhance the ATPase activity of HSP70. To rule out the complete interaction of DNAJB8/DNAJB6 with Hsc70 the stimulating ATPase activity of the C-terminal of DNAJB6/DNAJB8 should be investigated. Hsc70 may play a role in the anti-aggregation function of DNAJB6/DNAJB8 because the involvement of this chaperone in the "chaperone mediated autophagy" degradation system. Investigating the role of the C-terminal will be difficult because deletion of the C-terminal of DNAJB6/DNAJB8 results in a complete loss of activity, due to the importance of two lysines at the C-terminal. Next studies should demonstrate the suppression of toxicity by DNAJB8 or DNAJB6 in models with a neurodegenerative disease *in vivo* also.

In the cell models used in the study of Hageman et al. (2010) HDAC4 was required to maintain the anti-aggregation function of DNAJB6 and DNAJB8. The interaction between these DNAJBs and HDAC4 may indicate an important role for HDAC4 in the protein quality control system. Due to this discovery there is a clearer view about the anti-aggregation mechanism of DNAJB8.

In summary, this paper showed anti-aggregation activity of HSP70 and HSP40 family members in diseased models *in vitro* and suppression of toxicity *in vivo*. There was an elevated level of chaperones in diseased cells but this was not enough to protect cells for the large number of misfolded proteins. But this elevation may indicate a first line of defense against misfolded proteins in the early stages of neurodegenerative diseases. Up-regulation of HSP70 in combination with HSP40 or DNAJB6/DNAJB8 alone suppresses the formation of aggregation in protein folding diseases. More studies are required *in vivo* and *in vitro* to understand the anti-aggregation working mechanism of these chaperones and for the therapeutically use of chaperones in protein folding diseases in the feature.

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