CHAPTER 6

General discussion and perspectives
Building a toolbox to systematically investigate HSPH, HSPA and DNAJ functions.

HSP70/HSPA proteins are among the most conserved proteins in evolution which is highly appreciated by molecular taxonomists (1). For this reason, there has been a large interest in HSP70 sequences. After sequencing of whole genomes, it was surprising to find that organisms have such a large number of HSP encoding genes. Currently, evidence on the functional divergence of different HSPs is slowly emerging, mainly coincidently with the use of genetic screens. Only recently, people have started to systematically analyze some of the different HSP members (2-4). However, tools to investigate all of the different members were merely lacking. Currently, common strategies to investigate protein function inside cellular systems are based on either enhancing or decreasing protein levels. Although RNAi technology has been developed to a level which makes it possible to systematically knockdown individual genes, we encountered large technical difficulties to use it on a large scale in mammalian cells. First of all, some HSPs were found to be essential. Second, HSPA members are extremely homologous making specific knockdown challenging. Thirdly, many of these proteins are very stable and expression only decreases by dilution during cell division. Finally, our EST analysis and qPCR approaches showed that the majority of HSPs in a single cell type are either very low or not expressed. Another commonly used method to investigate protein function is to increase the protein levels in the cell via the transfection of expression plasmids harboring the desired gene. Although convenient in practice, some disadvantages have been reported for enhancing the protein level using strong promoters and a high copy number of plasmids in transient transfection protocols. Therefore, we have chosen a system which allows for modulating the expression level and integration at a single locus in the genome. Using this system as a starting point, we have cloned the HSPH, the majority of the HSPA, the DNAJA and the majority of the DNAJB family (Chapter 2). Different fusion tags were employed to use the library in a flexible manner for different purposes such as life cell imaging (GFP), immunoprecipitation, Western blotting, slot blotting (V5) and purification from crude cell extracts (His). This library is an initial effort intended for a comprehensive library including all human chaperones.

Although, we have retrieved the majority of the human HSPA and DNAJ members using NCBI gene, we have found, by using BLAT analysis (5) that some HSP paralogs are pseudogenes scattered throughout the genome. It should be noted that discriminating pseudogenes from true genes using the current computational molecular annotation tools is far from perfect and therefore our set of identified HSPH, HSPA and DNAJ members should be used as a working draft model which is likely to change over time and is ultimately intended to contain all members. As far as we know, this is the most comprehensive available library of chaperones. Using this library of constructs, we were able to study the functional differences between members of the HSPH, HSPA and DNAJ members as described below and in Chapter 4 and 5 and further discussed below.

Organellar stress resistance: Chaperones and an unfolded protein response system in peroxisomes?

Apart from the above described library, we constructed a library of GFP-tagged luciferase constructs targeted to different cellular compartments which can be used to compare differences in organellar stress resistance. In addition, this library can be used to study organellar thermotolerance, a transient condition of enhanced resistance to heat which is primed by a former heat shock and correlated to the enhanced expression of various heat shock proteins and described in detail below.
Using this library, we found that the nucleus is the most heat sensitive compartment. However, we found that cells could develop a high level of thermotolerance in the nucleus and under these conditions, the nucleus was only slightly more sensitive than the cytosol. Many HSPs migrate to the nucleus upon heat stress (6-8). As this takes time (approximately 15 minutes for HSPA8 (7)), most of the protein damage has already occurred during the nuclear migration. In contrast, a priming heat shock triggers, besides an induction of HSPs, also the migration of HSPs to the nuclear compartment providing immediate protection during the second heat shock. The absence of HSPs from the nucleus under normal growth conditions indicates that HSPs are relatively karyophobic in nature (6) and suggests that high concentrations of HSPs in the cells nucleus may be detrimental for the cell. Currently, to the best of our knowledge, no data exist to speculate why chaperones are at lower levels in the nucleus. Nevertheless, the lower levels of chaperones may put this compartment at risk for a proteomeostatic imbalance and indeed various studies show that the nucleus is more susceptible for the toxic effects of non-foldable proteins (9;10). In contrast to the nuclear compartment, we were surprised to find that the peroxisomal compartment was as resistant to heat as the cytosol. This result indicates that peroxisomes are equipped with (proteinous) factors to deal with heat induced protein unfolding. So far, no peroxisomal chaperones have been found in mammalian cells.

An unfolded protein response in all cellular compartments?

In principle, two fundamentally different mechanisms can underlie the development of thermotolerance in a certain compartment. Firstly, the priming heat shock activates or translocates pre-existing heat shock proteins or other stress protective molecules to the relevant compartment. Secondly, the priming heat shock induces a transcriptional upregulation of genes encoding HSP or stress protective proteins resulting in enhanced chaperone protein expression. The latter response is also referred to as the heat shock response (cytosol) or the Unfolded Protein Response (ER).

The heat shock response was discovered more than four and a half decades ago as heat specific puffing patterns in *Drosophila* salivary gland chromosomes (11). Much later, it was found that such loci encode for heat inducible proteins (12) and that the expression of such proteins positively correlates with a transient resistance to heat-induced cell death, referred to as cellular thermotolerance (13-15). However, it was not until the discovery of the transcription factor HSF-1 (16-18) before a specific transcriptional activation route was elucidated which enhances the pool of heat shock proteins in cells, providing them with a transient state of enhanced proteotoxic resistance. HSF-1 was originally found to be activated by heat. Its name is misleading as it has been shown to be activated by various cellular stressors (19;20) and is actually triggered by the presence of unfolded proteins (21). Therefore, unfolded protein response (UPR) would be a better description. At present, the unfolded protein response is assigned specifically to the ER stress response but in principle each individual organelle could have its own UPR and therefore we will name it according to the organelle involved (e.g. UPR (cyt/nuc), UPR (ER), etc.) Due to the spatial separation of cellular organelles and the nuclear transcriptional machinery, organellar UPR systems must consist of tightly regulated signal transduction routes that involves the detection of unfolded polypeptides within an organelle, followed by the perpetuation of the signal over membranes and subsequent nuclear entry and transcriptional activation. Only for the cytosolic/nuclear UPR or UPR (cyt/nuc), transduction of the signal over membranes is not required.

HSF-1 is normally HSP90 bound and upon stress, HSF-1 is released from HSP90 and forms homo-trimers (22) which translocate to the nucleus (23-25) and binds to five-nucleotide repeat units consisting of nGAAAn in head-to-tail orientation (26) and induces transcription (Figure
The system is believed to be subsequently attenuated by an auto regulatory feedback mechanism whereby HSP70 and HSP40 interact with the transactivation domain of HSF-1 causing the reversion of the HSF-1 trimeric complex to inert monomers (27;28). Interestingly, HSF-1 enhances mainly the transcription of cytosolic and nuclear chaperone genes (29;30). In agreement with this phenomenon, an endoplasmic reticulum specific unfolded protein response, UPR (ER), has been identified (31;31;32) which triggers the transcription of ER specific chaperones such as HSPA5/BIP/GRP78, DNAJB9/ErdJ4 and DNAJB11/ErdJ3 (Figure 1B) (33;34).

Accumulation of unfolded proteins in the ER causes the activation of three different routes mediated by the three different ER stress transducers IRE1, ATF6 and PERK (35) which are believed to be negatively controlled by HSPA5/BIP/GRP78 that bind and lock IRE1 and PERK in an inactive state (36;37). Upon activation, the cytosolic domain of PERK phosphorylates eIF2alpha, inhibiting general translation (38). However, PERK-mediated eIF2alpha phosphorylation also contributes to transcriptional activation of many UPR genes that involve the transcription factors ATF4 (39) and NF-k-B (40;41) but the details remain to be elucidated. The second route of UPR (ER) is mediated via IRE1. The activated cytosolic domain of IRE1

Figure 1: Transcriptional induction of organelle specific Unfolded Protein Response pathways. (A) Cytosol/Nucleus, (B) Endoplasmic Reticulum, (C) Mitochondria, (D) Peroxisomes. The projection of a peroxisomal UPR system is entirely speculative.
cleaves the 252bp intron from its substrate XBP1 mRNA, enabling its translation leading to the expression of the transcription factor XBP1 (42;43). XBP1 subsequently causes the transcriptional activation of many UPR (ER) target genes (42). The third route of UPR (ER) involves the Activated Transcription Factor 6 (ATF6). Upon activation, ATF6 translocates to the Golgi and is cleaved by proteases to form an active 50 kDa fragment (ATF6 p50) that induces UPR (ER) target gene transcription (44). The UPR (ER) is strongly responsive to inhibition of glycosylation induced by tunicamycin (45) and to a lesser extend to heat stress (Lubsen N.H., personal communication) indicating that different organelles require different unfolded protein response pathways.

In line with the UPR (ER), a mitochondrial unfolded protein response has been recently discovered using C. elegans as a model organism (Figure 1C). Protein unfolding is somehow sensed and transmitted via ClpP, a mitochondrial matrix homologue (46). Next, the signal is transmitted to the nucleus via a yet to determined mechanism where it activates the homeodomain-containing transcription factor DVE-1. This transcription factor drives the transcription of the small ubiquitin-like protein UBL-5 which is needed for a full response (47). In the current model, both DVE-1 and UBL-5 form a complex that drives the expression of UPR (mt) target genes (Figure 1C). As ClpP is known to function in the bacterial heat-shock response, these findings suggest that mitochondria still utilize components from the proto-mitochondrial symbiont to signal the UPR (mt). Identification of this mitochondrial UPR pathway supports our view that maybe most other organelles utilize different UPR systems for their specific needs. However, it could be the case that these pathways do partially overlap. For instance it has been found that heat induces the UPR (mt) as the UPR (mt) marker ubl-5::gfp is activated by heat (48). As the HSP60 promoter contains HSF-1 elements (49) this could mean that HSP60 is activated by both the UPR (cyt/nuc) and the UPR (mt). It remains to be elucidated which stressors induce the UPR (mt) and to what extent the pathways overlap.

Until now, there is no evidence for the existence of a stress inducible chaperone system in peroxisomes. Indirectly, our studies in chapter 3 for the first time provide support for the view that peroxisomes not only contain (specific) chaperones, but also may have an UPR that may regulate their expression. We found that luciferase targeted to peroxisomes could be reactivated after heat shock and cells could build up thermotolerance in peroxisomes as peroxisomal luciferase reactivation was enhanced in primed cells. In search for peroxisome specific chaperones, one could try to isolate peroxisomes from pre-stressed cells to boost their abundance, followed by MASS-SPEC identification of the complete heat-induced peroxisomal proteome and compare that to the proteome of peroxisomes from non stressed cells. Regarding the signaling mechanism underlying the UPR (Per) (Figure 1D) one can only speculate at this stage. Interestingly, peroxisomes contain an abundant AAA ATPase protease which is called LonP (50;51). In analogy to the mitochondrial protease Clp, LonP, could be involved to initiate the UPR (per) response. In search for the existence of a UPR (per), one could use the promoter sequences from peroxisomal chaperones (provided that they exist). These promoter sequences could then be used in promoter reporter assays in a similar manner as performed for the UPR (mit) (46). These proposed experiments could give a clear answer to the intriguing question whether peroxisomes contain a sophisticated protein quality control system and possibly even a peroxisome specific unfolded protein response providing a machinery to repair damaged peroxisomal proteins instead of the turnover by pexophagy only.
Higher eukaryotic cells contain two distinct pathways to deal with protein refolding and aggregation suppression

In chapter 4, we used two substrates with fundamentally different biochemical properties to study the activity of different HSPA and DNAJ members inside higher eukaryotic cells. Luciferase, a substrate that can reach its active state after being heat-denatured, was used to examine the activity of different over-expressed HSPA and DNAJ chaperones on the stimulation of refolding. In addition, a non foldable polyglutamine substrate was used to study the ability of different HSPA and DNAJ members to suppress its aggregation. Using this strategy, we identified a group of chaperones that, when overexpressed, stimulates heat denatured luciferase to regain its activity and therefore likely its native state. In addition, a separate group of chaperones was identified that, at a given level of ectopic overexpression, was ineffective to enhance luciferase refolding but clearly effective in suppressing polyglutamine aggregation. This was not related to client specificity per se, as the polyglutamine aggregation inhibitors also suppressed heat-induced aggregation of luciferase. These results therefore point towards the existence of (at least) two functionally different sets of chaperones in higher eukaryotic cells.

Interestingly, the group of Frydman recently identified two chaperone networks in yeast cells using system biology approaches (4). One network deals with protein biogenesis (CLIPS) while the second is specialized to work under stress conditions post-translationally (HSPs). It is not yet clear whether the different sets of chaperones identified in our study serve in both these networks or not. It is tempting to speculate that e.g. those chaperones that support refolding are overrepresented in the CLIPS network or whilst those that suppress aggregation predominantly belong to the stress network (Figure 2). However, this remains to be tested.

Interestingly, we found that chaperones with activity on folding stimulation (HSPA1A) or aggregation suppression (DNAJB1) are known components of the same HSP70 heteromeric multi-protein complex (52). So how can overexpression of 2 proteins from the same complex lead to such drastic phenotypic differences in chaperone-like activities? Part of the answer may lie in the stoichiometric balance of endogenous expressed members, the binding dynamics of the multimeric HSP machine and the differences of the different chaperones in substrate binding affinities. As shown in Chapter 2 and 4, HSP70 machine components such as HSPA and DNAJ members are expressed in a highly complex tissue dependent manner which is also dependent on various (stress-induced) signal transduction routes. Sudden changes in the concentrations of HSPA and DNAJ members may affect the molecular architecture of cellular HSPA machines. Likewise, the specific overexpression of HSPA or DNAJ members is likely to shift the molecular constitution of HSP machines. As DNAJ members bind HSPA members with low affinity (53), changes in the expression of DNAJ members might result in a pool of free DNAJ complexes. For some DNAJ members it has been shown that they form stable V-shaped molecular clamps with a relative large exposed surface area with affinity for its substrate (54). Enhanced expression of DNAJ members might thus result in an increase of stable chaperones with high affinity for substrates and a very low on/off rate. Such complexes may sequester non-foldable substrates, suppress aggregation and keep them competent for

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Figure 2: Higher eukaryotic cells contain two chaperone networks with distinct molecular function.
(proteasomal) degradation. On the contrary, enhanced expression of HSPA members with low substrate affinity and a high substrate on/off rate may result in an enhanced refolding stimulatory capacity. Although these results are currently only based on observations by the specific overexpression of different HSPA and DNAJ members, it is likely that cells do specifically change their plethora of chaperones to the need of enhanced folding (in the case of foldable substrates) or aggregation suppression (in the case of non-foldable proteins).

An evolutionary conserved group of DNAJB members to cope with aggregation prone proteins.

The capacity of many HSPs to bind hydrophobic patches have been long suggested to suppress toxic polyglutamine aggregation. Indeed, several chaperones have been shown in previous studies to suppress polyglutamine aggregation. However, due to the lack of sophisticated research tools, this has been largely focused on the most established components of the HSP70 chaperone machine such as HSPA1A and DNAJB1 (55-59). In chapter 5, we present data on a systematic reverse genetic over-expression screen for suppressors of aggregation. We have identified an evolutionary conserved group of homologous proteins, in particular DNAJB6 and DNAJB8, that are very effective in the suppression of polyglutamine aggregation. Although very active in the suppression of polyglutamine aggregation, the precise biological function of this homologous subfamily is, at this stage, unclear.

Poly-glutamine disorders are relatively rare and we have found DNAJB6 like sequences so far in all sequenced metazoa including nemathoda and urochordata. Interestingly, urochordata fossil records have been traced back as far as the early Cambrian period (60). In addition, recent molecular evidence indicates that nemathodes originated around 1200 million years ago in the pre-Cambrian era (61). Providing that no lateral gene transfer has occurred, this suggests that DNAJB6-like proteins are over 1200 million years old and have been originated before the (debated) Cambrian explosion of complex organisms (62). It is unlikely that DNAJB6-like proteins have exclusively evolved to combat rare disease like poly-Q or other (heritable) protein folding disease. Moreover, for many animals with a relatively short life span such as mice, no poly-glutamine disorders have been described so far. So what could be the natural function of this highly homologous subfamily? DNAJB6 has been shown to interact with keratin 18 (63) and recently it has been shown that DNAJB6 mediates keratin turnover and prevents its toxic aggregation in chorionic trophoblast cells during chorioallantoic attachment in placental development (64). Various mouse models have shown that the correct formation of a keratin network in trophoblast cells is a key event in the chorioallantoic attachment (65). DNAJB6 like proteins are clearly not limited to placental mammals. Indeed, the chorioallantoic attachment is not restricted to placental mammals as the vascular fetal membrane that consists of the fused chorion and allantois is also found adjacent to the eggshell in reptiles and birds (66) meaning that DNAJB6 may serve in this process in all amniotes. This example shows the need for chaperones with the capacity for the toxic aggregation of regular proteins. Consistent with this, we found that DNAJB6-like proteins were rather non-specific for substrates as they suppressed the aggregation of mutant Huntingtin, SCA3, SBMA and heated luciferase (Chapter 5). Interestingly, it has been proposed recently that the formation of aggregates is a small but significant propensity of all proteins during the folding of compact globular structures (67). Therefore, it is likely that powerful suppressors of protein aggregation were developed in most life forms, especially those that live long enough for a high propensity of fibrillar protein accumulation. However, why DNAJB6 like proteins are restricted to metazoans is at this stage unclear.
How do DNAJB6 and DNAJB8 function in molecular detail? Although the molecular action on the function of DNAJB1 has been sorted out to a reasonable detail during the last decade, little is known about DNAJB6 and DNAJB8. First of all, the short isoform of DNAJB6 (DNAJB6b) and DNAJB8 are much smaller compared to DNAJB1 and their C-terminus, beside a G/F rich region, shows little homology. In addition, we found that DNAJB6 and DNAJB8 form high molecular complexes in cells unlike DNAJB1 which forms dimers (68). Interestingly, DNAJB6 has been implicated as a nuclear shuttling factor (69;70) and it has been shown to mediate transcriptional repression through class II histone deacetylase recruitment (71). We have also found that DNAJB6 and DNAJB8 bind to some histone deacetylases such as class II HDACs (HDAC4, HDAC6) and a class III HDAC (SirT2). In addition, we have found that this association is mediated through a serine rich domain and that this domain makes contact with the catalytic site of the HDAC4 molecule (72). The deletion of this domain not only dissociated the HDAC interaction but also interfered with the activity of DNAJB8 in poly-Q aggregation suppression.

Although not completely clear at this stage, our data indicate that DNAJB6 and DNAJB8 behave partially like HSPB chaperones. They not only form oligomeric complexes, but the activity of these complexes is dependent on the activity of post-translational modifications. Instead of phosphorylation/dephosphorylation events for HSPB complexes, DNAJB6 and DNAJB8 oligomeric complexes are activated via acetylation/deacetylation events. Although we can not rule out a role for phosphorylation/dephosphorylation events at this stage, acetylation/deacetylation affect the active status of the DNAJB8 oligomeric complex via a yet to be defined mechanism. Such active molecules are subsequently blocked either the fibril formation or the oligomeric aggregate formation of misfolded proteins during the natural lifetime of the protein. As such, the non-foldable polypeptides are kept in a state that is accessible and compatible with proteasomal degradation. Proteasomal degradation of unfoldable proteins via the HSP70 machine and CHIP has been demonstrated (73;74) and indeed we found that DNAJB6 and DNAJB8 do bind HSPA members. Deletion of this HSPA interacting domain or mutating a single amino acid crucial for HSPA interaction showed a clear reduction of polyglutamine expression suggesting that the coupling with the HSPA machine is important for degradation of the non-foldable substrate.

Although future attempts are necessarily to unravel the molecular details of DNAJB6-family members in aggregation suppression, it is clear that they form specialized complexes to deal with non foldable proteins and are as such superior to all other identified chaperone complexes so far. Thus, searching for pharmacological inducers of DNAJB6-like proteins may lead to the discovery of disease ameliorating compounds. Hereto, we already have generated promoter reporter constructs for screening compound libraries. Alternatively, agents that stimulate activity (e.g. via HDAC related stimulation of deacetylation) may provide lead compounds for therapies to suppress the progression of poly-glutamine disorders. Modifications of the entire heat shock response (via HSE/HSF modifications) has also been reported to substantially affect poly-Q progression, but may have a down side as this also affects tumorigenesis (75) which, at least in part, may be due to affecting HSP70 levels that when elevated alone, also promote tumor growth (2). As the DNAJB6-family members that we identified do not require endogenous up-regulation of HSP70, modification in DNAJB6-like protein levels could be effective without such cancer related risks. Indeed, we found no effects on cell growth and cell morphology in cells chronically overexpressing DNAJB6 or DNAJB8. Also, no developmental or growth related problems were associated with long-term in vivo expression in tadpoles. However, further studies in mouse models are required to test whether chronic manipulation in the level of DNAJB6 members are tumor promoting or not.
DNAJ proteins: HSPA co-factors or sophisticated chaperone machines?

DNAJ proteins were originally discovered as an essential factor for bacteriophage lambda replication (76). Later it was found that DNAJ accelerates DNAK ATPase activity and thereby regulates the binding and release cycle of the DNAK machine. As such, DNAJ members are often described in literature as co-chaperones implying that they regulate the activity of HSPA proteins but that they cannot act as chaperones independently (77). The common factor of DNAJ proteins, the J-domain, is very conserved among the different members. This domain is able and required for DNAJ proteins to enhance the HSPA ATPase activity and forms the docking site on HSPA proteins. However, the J-domain is not reported to function in substrate binding and apart from the J domain, DNAJ members are extremely diverse in the remaining part of the protein. Nevertheless, some DNAJ proteins have been found to bind substrates independent from interactions with HSPA proteins (78-80). Although DNAJ-like proteins alone do not facilitate protein refolding in the absence of HSP70 (81), various DNAJ members have been shown to prevent protein aggregation in vitro (78;82;83). As those were nearly all type A J proteins, containing a Zn finger domain, it has been proposed that such activity might be limited to type A DNAJ proteins (84). More recent structural data on the DNAJ members showed that due to the dimerization of some DNAJ members, two relatively short peptide binding domains may form a relatively large peptide binding surface projection (54) and it may be speculated that this could significantly enhance the binding affinity for substrates. In our studies, we were surprised to find large effects on aggregation suppression by the over-expression of individual DNAJB, but not DNAJA members. Although the C-terminal domain architecture of DNAJB6 and DNAJB8 is currently unknown, we found putative repeats of beta–sheets, commonly found in chaperone substrate binding domains (54) in the region required for the anti-aggregation effects of DNAJB6 and DNAJB8 using the Pyre web server (data not shown). We also found that DNAJB8 was present in high molecular weight protein complexes. If DNAJB8 forms high (homo) oligomers, this could drastically enhance the surface projection and the affinity towards unfolded substrates. Although entirely speculative at this stage, in this view, some DNAJ proteins could be sophisticated relatively independent chaperone machines. The J-domain could serve only to recruit and hand over the unfoldable substrates for proteasomal dependent degradation.

Given the extreme diversity in the primary sequence of DNAJ proteins in the region outside the J-domain, especially DNAJC proteins, it will be best to view them as proteins with individual and unrelated functions. The J-domain may serve only as a temporal physical link to a HSPA machine. Although it is now reasonably established that some members do bind unfolded substrates and show chaperone-like properties themselves, many other members might not do so and have other, yet to be identified, molecular functions.

References


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