Hsp70 machinery vs protein aggregation

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Chapter 5

General discussion and future perspectives
The Hsp70 machinery is a highly conserved and central chaperone system involved in many different protein homeostasis related processes and, in humans, it consists of different families with several members each (Radons, 2016). Compared to prokaryotic cells or unicellular eukaryotes like yeast, there is increased complexity of the human chaperone systems (Table 1), with not only multiple members that can lead to multiple combinations but also different expression levels in different cell types which can favour certain combinations.

Table 1. Evolutionary expansion of the Hsp70 machinery

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5.1 Different functionalities between Hsp70 family members

Hsp70s are highly conserved in sequence (Table 2). That is also why it has been assumed by many people in the field that most Hsp70s have a similar function and can be used interchangeably. And to a certain extent, based on in vitro studies, most of the different Hsp70s tested can perform the basic functions associated with them, like protein folding, holding and disaggregation (Daugaard et al., 2007; Radons, 2016; Vos et al., 2008; Mayer and Bukau, 2005; Mayer, 2013).

However, functionalities amongst members can differ substantially. In yeast, it has been shown that different members of the Hsp70 family (containing SSA and SSB proteins) have distinct functions in de novo folding (SSBs) or stress response (SSAs) (Albanèse et al., 2006; Yam et al., 2005). Differences between SSA and SSB proteins have been previously dissociated with their ability to interact with substrates but rather associated with differences in their NBDs (James et al., 1997). Even regarding human Hsp70s, there are a few reports that implicate only specific members with certain functions like involvement in viral replication (Taguwa et al., 2019, 2015) or refolding/aggregation suppression (Hageman et al., 2011; Kakkar et al., 2016a; Hageman et al., 2010).

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<tr>
<th>HSPA1A</th>
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*Percentages were calculated using Clustal Omega protein sequence alignment program (EMBL-EBI) (https://www.ebi.ac.uk/Tools/msa/clustalo/).
In Chapter 2, we show that the within the human Hsp70 family of chaperones, there is a great variability in handling substrates, here in particular mutant SOD1. Retrospective analysis of other studies actually hinted towards these suggestions but never truly emphasized such differences. For example, different Hsp70s showed different effects on aggregation of various proteins, including polyglutamine proteins (Hageman et al., 2010), mutant parkin (Kakkar et al., 2016a) or heat denatured luciferase (Hageman et al., 2011). In most of these cases, overexpression of most Hsp70 family members did not alleviate aggregation of the mutant protein. However, other studies have shown that certain Hsp70 family members can have some anti-aggregating effects (Aprile et al., 2017a; Kundel et al., 2018; Kakkar et al., 2014).

In addition, there are more cases of conflicting results for Hsp70 functionality, for example in immune response (rev. in Borges et al., 2012), aggregation and neuroprotection (rev. in (Turturici et al., 2011)) or ion channel misfolding and trafficking (rev in (Young, 2014)). These conflicts in literature may have been due to the fact that different members were used for these studies, as this was not specified in many cases. Alternatively, cell type dependent differences (e.g. in endogenous Hsp70 or co-chaperone levels) may lead to different outcomes in such experiments. However, in our studies, modulating expression of the different Hsp70 members for the same substrate using the same cellular background, led to a differential outcome; and this was confirmed for several different substrates like luciferase, polyglutamine, mutant Parkin and SOD1 (Chapter 2, (Hageman et al., 2011, 2010; Kakkar et al., 2016a). Therefore, we conclude that the Hsp70 family members are not functionally identical. Hence, the general term “Hsp70” for human members should be avoided and the specific protein nomenclature should be used instead.

5.1.1 Functional protein domains
But why can proteins that are so similar in sequence differ so much in function and how is this determined? Specific protein domains are coupled with function and thus protein or domain conservation is largely conceived as a major determinant for a protein’s function. Especially for Hsp70, where family conservation is particularly high with most Hsp70s sharing > 50% residue identity.

The substrate binding domain (SBD) of Hsp70s is highly conserved and it is considered to be functionally similar in the different Hsp70s, all recognizing hydrophobic protein patches (Mayer and Gierasch, 2018; Clerico et al., 2015), although some specificity in substrate binding has been suggested by some studies (Fourie et al., 1994). However, there is a large overlap in these binding sequences and it was not clear whether the differences would truly transfer to altered functionality in vivo. This was why Hsp70s had been largely
considered as functionally similar, mainly serving as the “horsepower” of the Hsp70 machinery and were largely ignored as determining factors for the fate of substrates that enter the cycle. However, in Chapter 2, we show that Hsp70s do play a role in the decision-making process and fate of substrates. However, this was unrelated to their SBD but rather related to differences in their nucleotide binding domains (NBD) that was further found to differentiate the Hsp70 interaction with co-chaperones. In fact, we show that only a few amino acid changes in the very conserved NBD domain can have a major impact on the protein’s functionality.

The NBD of Hsp70s is a multi-factor binding domain; besides nucleotides, many chaperone co-factors interact with this part of Hsp70 proteins. Via the NBD, these co-factors regulate ATP hydrolysis and nucleotide cycling, which require proximity to the nucleotide interaction site. The fact that all these different proteins interact with the same domain means that the interaction sites are not always available and that antagonism between them likely exists. For example, all DNAJs interact with a conserved J-domain with Hsp70s, which means that they are likely competing with each other for Hsp70 binding. The same applies for example to BAG family of NEFs, which all interact with Hsp70 via their conserved BAG-domain. And, although for example different NEF families (Hsp110, BAG, HSPBP1 etc) have different binding sites on Hsp70-NBD, these are substantially overlapping and therefore also possibly compete with each other. But how do all these proteins ultimately form complexes? Is there a preference based on affinity, avidity and/or availability of chaperones?

These questions still remained largely unanswered. In vitro, so far, no differences between different Hsp70 family members and NEF binding has been reported, at least between HSPA1A and HSPA8 that are the most studied and compared Hsp70s (Rauch and Gestwicki, 2014; Rampelt et al., 2012). Even between HSPA1A and HSPA1L, interaction with Hsp110 NEFs is similar in vitro (unpublished data) although our data show that there is a clear preference between Hsp70s and Hsp110 interactions. This suggests that unknown factors regulate these interactions in vivo.

Finally, the NBD of the Hsp70s -via co-factor interactions- is likely a member-specifying determinant. The NBD has been also previously suggested as function-determining domain for refolding and anti-aggregation capacity differences between HSPA1A and HSPA6 (Hageman et al., 2011). In Chapter 2, we went one step further showing how this is related to the co-factor binding to ultimately determine substrate fate.
5.1.2 Differential chaperone expression affects functional interactions

Our findings of functional differences between Hsp70 depending on (co)chaperone interactions, have multiple implications for evaluating the literature on this subject. There is a large variety in (co)chaperone expression levels in different tissues, cell types, and even subcellular compartments. This can significantly alter the partnering possibilities between these proteins. This can subsequently affect the outcome of results when manipulating their levels across different cell lines experimentally or when cells are naturally exposed to external stresses, deal with genetic mutations or undergo differentiation under physiological conditions.

The functionality of chaperones is not only determined by the expression levels but could also be affected by the occupancy of chaperones by certain substrates. For example, if certain chaperones are occupied or trapped by aggregating or misfolded proteins (Chapter 3), those specific ones will be unavailable from the general pool for this cell. This can then not only lead to a decreased ability of these chaperones to deal with other problematic proteins, but also alter their functionality, as stoichiometric ratios between Hsp70s and the different co-chaperones may have been changed. This model could, to some degree, explain tissue or cell-specific defects when genetic mutations lead to increased production of aggregation-prone proteins, although several other factors can also contribute (like different expression pattern of the mutated protein and the presence of other sensitive proteins). The fact that different protein aggregates lead to different diseases that affect different tissues may also, in part, be due to cell type-related availability of the optimal set of (co)chaperones needed to handle this mutant substrate.

As stated above, differential co-chaperone expression between cell types may also affect how manipulations would have functional impact. For example, in Chapter 2 we show that overexpression of Hsp70 family members in different cell lines can have a different effect on SOD1 aggregation and this could be related to the different endogenous co-chaperone levels in these different cell types. As another example, we show that Hsp110s interaction is important for Hsp70-mediated suppression of SOD1 aggregation. However, overexpression of Hsp110s in our cell model could not alleviate mutant SOD1 aggregation (Chapter 2), although Hsp110 family members have been reported to reduce mutant SOD1 aggregation or toxicity in cell and animal models (Nagy et al., 2016; Song et al., 2013; Yamashita et al., 2007).

5.1.3 Co-chaperone interactions impact functionality

As discussed previously, Hsp70 is involved in many different processes for protein handling like folding, degradation, disaggregation, transport (holding) etc. But what determines the
fate of a substrate entering the Hsp70s cycle? It was recently proposed that for client specificity especially DNAJ proteins play a role (Rüdiger et al., 2001; Kampinga and Craig, 2010; Fan et al., 2003; Laufen et al., 1999; Hennessy et al., 2005; Vembar et al., 2010), whilst NEFs have been especially implicated in decision making regarding the fate of an Hsp70-substrate (Mandal et al., 2010; Gowda et al., 2013; Kandasamy and Andréasson, 2018b; Gamerdinger et al., 2009; Minoia et al., 2014; Rauch and Gestwicki, 2014). This model fits the order of events that is long proposed for the Hsp70 cycle, which is that the NEFs are coming into action at the substrate release step of the Hsp70 cycle. We are the first to show, however that NEFs also play, at least a co-determining and crucial role in substrate entry into the Hsp70 machine (Chapter 2). However, this specific role could be limited to the Hsp110 NEFs. Hsp110s are distinctive compared to other NEFs as they are bona fide substrate interacting chaperones containing an SBD domain (Dragovic et al., 2006; Shaner et al., 2004; Raviol et al., 2006). Moreover, their ability to directly interact with substrates via their SBD might provide them with an advantage for specific functions, like disaggregation for example; in fact, it has been shown that they are way more efficient than BAG proteins in protein disaggregation (Rampelt et al., 2012).

Surprisingly, whilst some DNAJs did and others did not suppress SOD1 aggregation (Chapter 2), the DNAJs did not seem to play a deciding role in the differential actions of HSPA1A and HSPA1L. All DNAJ interactions tested were found to be similar for HSPA1A and HSPA1L. In fact, this was also true for the above mentioned functional differences between HSPA1A and HSPA6 that were shown to bind with equal affinity to the same plethora of DNAJs (Hageman et al., 2011).

In addition to the above, it is not known yet what is the involvement of the substrate itself in the outcome of the Hsp70 cycle. The nature of the substrate could influence its own fate by steering the Hsp70 machinery members towards specific partnerships. That could be achieved for example by a DNAJ “sensor” for specific substrate conformations that leads substrates to specific Hsp70s coupled with specific NEFs for folding, degradation or disaggregation. Alternatively, DNAJs could find the most available Hsp70 at this time and place and deliver the substrate to it. That would suggest, however, that substrates could activate another type of “sensor” that changes availability of Hsp70s (coupled with certain NEFs for a certain activity) via DNA, RNA, protein or PTM changes that regulate Hsp70/NEF expression. This “sensor” could be for example the time that a substrate spends into the Hsp70 cycle or the rate of Hsp70 occupancy.

Together, these data suggest that the Hsp70 cycle may have more (or other) modes of action than previously assumed. But many questions still remain unanswered. Does a
DNAJ always interact first with the substrate and does it always dissociate when a NEF binds? Can all three components form a complex with the substrate at the same time? If yes, is it possible that DNAJs play a role in selecting the appropriate NEF and together determine substrate fate. If not, that means that Hsp70-NEF affinities alone can have such independent, substrate determining, roles as well.

To further elucidate the precise mechanism and timing of substrate-chaperone complex formation, it will be needed to perform in vitro sequence-of-addition and competition assays with different combinations of these 3 partners as this could reveal their importance in substrate fate determination. Emerging single molecule techniques (like single molecule FRET or molecular tweezers for example) would be ideal methods to study this, as they can provide the time resolution needed as well as the conformational changes of proteins at a single molecule level. For instance, it could be studied if in the context of Hsp70-NEF partnership, addition of a DNAJ would play a role for substrate folding or disaggregation. Or the other way around, if addition of a NEF would play a role in Hsp70-DNAJ partnership. And this could be done combining different types of DNAJs and NEFs. Moreover, so far most of the in vitro studies have been done with the bacterial or yeast proteins of the Hsp70 system using only single family members. It would be interesting to study different combinations of the human chaperones and understand if there are indeed preferred combinations for certain activities. In this line of thought, it has been shown in vitro that different Hsp70-DNAJ-NEF combinations (and even ratios) can be more efficient than others for certain functions like disaggregation (Rampelt et al., 2012) or (re)folding (Rauch and Gestwicki, 2014). However, a combined in vitro and in vivo approach would be ideal for future studies since in vitro studies can give a closer look into the mechanistic aspect of the system and in vivo studies can confirm whether these are biologically relevant.

5.1.4 How is differential Hsp70-NEF interaction established?

How is then the partnership within the Hsp70 cycle regulated, since there are only few Hsp70s available for multiple DNAJs and multiple NEFs? In cells, we found different binding (co-IP) between HSPH2 and either HSPA1A or HSPA1L. However, in vitro data using HSPA1A and HSPA1L suggest that purified proteins have very similar functioning and Hsp110 dependence (unpublished data - not shown). This suggest that factors other than affinity between these proteins must play a role in the interaction and functional differences observed in cells. Such factors could include post translational modifications (PTMs), intracellular-proximity, competition with other Hsp70s, partnering with other proteins or substrate interaction, which all could change the preference of Hsp70s towards partnering with certain co-chaperones. Indeed, several post translational modifications for Hsp70s have been reported (Cloutier and Coulombe, 2013). However, most were done in
high through-put studies largely without follow-up for functional relevance. Interestingly though, it has been discovered that C-terminal phosphorylation of HSPA1A regulates CHIP or HOP co-chaperone binding and thus the substrate fate towards degradation or folding (Müller et al., 2013), showing one example of in vivo regulation of functional partnerships.

For our major finding that HSPA1L shows decreased interaction with HSP110s, PTMs may also be implicated: Hsp110 binding sites might be “masked” by PTMs that -on the other hand- do not affect binding with the other NEFs (BAG and HSPBP1), for which we found no differential co-IP for HSPA1A or HSPA1L. Indeed, although they all interact with the NBD of the Hsp70s, the mode of actions and binding of these different NEFs to Hsp70 are different (Bracher and Verghese, 2015; Sondermann et al., 2001; Schuermann et al., 2008; Arakawa et al., 2010; Polier et al., 2008; Shomura et al., 2005; Xu et al., 2008).

Besides PTMs, competitive binding with other proteins, like the co-chaperone Hip for example, may also play a role. Hip keeps Hsp70s in the ADP-bound conformation and therefore attenuates Hsp70 cycle (Höfeld et al., 1995; Li et al., 2013). Hip has been previously found to compete with NEFs like BAGs for Hsp70 binding (Nollen et al., 2001) and also has some overlapping binding sites with Hsp110 (Li et al., 2013).

Finally, collaborations with other chaperones machines, like the Hsp90 machine (via the linking chaperone HOP), may explain differences between HSPA1L and HSPA1A. Hsp90 machinery has been suggested to act together with Hsp70 system to promote protein handling, with the Hsp90 acting after the substrates have exited the Hsp70 cycle (Karagöz and Rüdiger, 2015). Taken this into account, some Hsp70s might transfer certain substrates to the Hsp90 machinery for further processing while others not. And since the NEF is acting at the release step of the Hsp70 cycle, it might facilitate (or not, depending on the NEF) this transfer. It would be interesting to perform in vitro or in vivo binding assays with different members of the Hsp70 family and the Hsp90 family and investigate if there are specific Hsp70s that work with Hsp90s for substrate processing.

5.2 Mechanism of SOD1 handling by the HSPA1A cycle

We show in Chapter 2 that HSPA1A, at least DNAJB1 (and possibly other DNAJs) and HSPH2 are involved in mutant SOD1 aggregation suppression. But how does HSPA1A cycle lead to a decrease in mutant SOD1 aggregation?
Chapter 5

5.2.1 Accelerated degradation

One possibility of how HSPA1A leads to decrease in SOD1 aggregation would be through degradation. This could be achieved either by degrading misfolded monomeric SOD1 or by disaggregation of SOD1 aggregates and subsequent degradation. A role for degradation is supported by the fact that total SOD1 expression levels are also lower in the presence of HSPA1A (Chapter 2). HSPA1A has been previously suggested to suppress aggregation by promoting degradation of SOD1 mutants (Urushitani et al., 2004) or other aggregating proteins like polyglutamine proteins (Wang et al., 2013; Bailey et al., 2002). Moreover, Hsp110s in yeast were shown to directly interact with the proteasome and target Hsp70 substrates for degradation (Kandasamy and Andréasson, 2018b).

Other partners, like E3-ligases, are most likely involved as well in the degradation pathway after the Hsp70 cycle. For example, the decision for degradation vs refolding could involve specific E3-ligases, that might interact anyway with Hsp70s but only be able to ubiquitinate specific misfolded substrates; alternatively, ubiquitination by E3-ligases might happen only when the substrates are handled by specific Hsp70 family members. One candidate that could be involved in SOD1 handling here, which is possibly associated with degradation, is CHIP, an Hsp70 interacting E3-ligase (Murata et al., 2001). CHIP has been previously reported as a protein involved in SOD1 aggregation suppression together with Hsp70 (Urushitani et al., 2004). Although CHIP interaction site is at the C-terminus of Hsp70 (Zhang et al., 2015) and this region is conserved between HSPA1A and HSPA1L, allosteric communication between the different Hsp70 domains might also play a role in this interaction in vivo. HSPA1A is not the only chaperone working with CHIP. HSPA1A and HSPA8 both work with CHIP to promote degradation of substrates; however, HSP70 has higher affinity to CHIP (Ballinger et al., 1999) and also gets co-degraded by CHIP as well while HSPA8 not (Qian et al., 2006). Since HSPA1A expression levels are increased with heat stress, it can be a preferred solution for fast degradation of substrates under these conditions, beyond the normal "housekeeping" degradation of physiological substrates that could be taken care by constitutively expressed chaperones like HSPA8. It would be interesting to check whether HSPA1L is also involved in protein degradation and whether it interacts differently with CHIP or other E3-ligases compared to HSPA1A.

5.2.2 Co-translational processing

Another explanation for decreased total SOD1 levels in the presence of HSPA1A (but not HSPA1L) may be via specific actions at the level of the ribosome to decrease translation of the mutant protein or aid to its immediate co-translational degradation. Possible collaboration of HSPA1A with ribosome associated E3-ligases like Listerin or others (Wang et al., 2015; Gandin and Topisirovic, 2014), could lead to a more efficient ubiquitination
and degradation of newly synthesized misfolded SOD1. However, the co-translational degradation of misfolded proteins and the factors involved in it is something that is in still not well understood, especially in human cells. Therefore, more research is needed in order to establish whether HSPA1A is somehow involved in this process.

5.2.3 SOD1 refolding
Finally, there is the possibility that HSPA1A specifically enables mutant SOD1 (partial) refolding. HSPA1A has been shown to be more effective than any other Hsp70 family member tested in refolding of heat-denatured luciferase (Hageman et al., 2011). And since refolding of a mutant protein like SOD1 might be even more challenging than heat-denatured unfolded proteins like luciferase, HSPA1A cycle might be more efficient for this process. Restoration of (partial) folding for mutant SOD1 could lead to an increased SOD1 dimer formation, possibly together with endogenous wild type SOD1, leading to an increased solubility and less exposed, aggregation-prone, misfolded monomers. This idea could be tested by checking, for example, SOD1 activity levels in the presence of the different Hsp70 family members, as more refolded SOD1 should lead to an increased SOD1 activity.

5.3 What is the physiological function of HSPA1L?
Since our data showed that HSPA1L lacks functions like aggregation suppression, which are normally attributed to Hsp70s like HSPA1A (Chapter 2, (Hageman et al., 2011; Kakkar et al., 2016a; Hageman et al., 2010)), one wonders what its normal function would be. HSPA1L is mainly expressed in testis and is strongly stress-inducible in other cells, whilst HSPA1A is low but present in most human cell types and very stress-inducible as well (Hageman et al., 2011).

Up-regulation of HSPA1L (like that of HSPA1A) can increase the ability of cells to refold heat denatured luciferase (Hageman et al., 2011; Takahashi et al., 2017), suggesting that it can function in protein (re)folding. Moreover, it was shown to be able to stabilize cellular prion protein (PrP<sup>C</sup>), a prion protein mainly expressed in the nervous system, by inhibiting GP-78 E3-ligase binding and subsequent ubiquitination (Lee et al., 2017), suggesting it could also play a role as a “holdase”. Our results of HSPA1L on mutant SOD1 (Chapter 2) may also be explained by a blockage of SOD1 ubiquitination or degradation, which in this case is an unfavourable outcome. However, for proteins like prion proteins, interaction with HSPA1L might have a protective role, protecting them from both degradation and aggregation and possibly assisting their intracellular transport to their functioning location. As an example,
in testis, that HSPA1L is highly expressed, there are prion proteins like Doppel (PRND) (Allais-Bonnet and Pailhoux, 2014) which might serve as HSPA1L’s chaperone clients.

5.4 Protein aggregation and protein homeostasis: a vicious self-perpetuating disaster?

Aggregation generally refers to the formation on non-native, non-functional protein assemblies. It clearly is a very generic terms and many different types of aggregates can be found in cells like amorphous, disordered or porous aggregates and dense amyloids. Amyloids in some cases may even be functional assemblies (Fowler et al., 2007) and the condensation of proteins into membrane-free compartments (phase separated liquid droplets (Alberti, 2017; Strzyz, 2015)) also should be clearly distinguished from non-functional aggregates, although proteins inside such liquid droplets may easily convert from functional (fluid) to pathogenic (solid/crystals) (Molliex et al., 2015). In terms of structural properties, amyloids generally consist of ordered cross beta sheets that form a dense core that cannot be taken apart even by strong denaturing detergents. On the other hand, amorphous aggregates are mostly disordered and not so dense and can be dissociated by strong detergents such as SDS. Hydrophobic interactions play a role in both types of aggregate formation but there are other factors like h-bond formations that likely contribute to the formation of a stronger and more structured amyloid type (Vetri and Foderà, 2015; Chiti and Dobson, 2017; Dobson, 2003).

The fact that there are different types of aggregates already suggests that aggregation is not a uniform process and therefore impose different challenges upon the protein quality control systems of the cells and inflict pathogenic damage via different routes. In turn, different aggregates may have a different impact of the PQC system, and as such have a different impact on the cellular protein homeostasis. In fact, in Chapter 3, we show that the impact of polyglutamine aggregates has direct consequences for the ability of the cells to handle mutant SOD1 aggregation, whilst SOD1 aggregates do not affect the capacity of PQC system to handle polyglutamine proteins. This suggests that different PQC systems or chaperone modules can function as independent units to some extent.

Indeed, various chaperones may be crucial for suppression of aggregation of different proteins (Kakkar et al., 2014) but these not necessarily overlap and may have different requirements in terms of critical availability of their individual constituents. For example, both polyQ and SOD1 aggregates can trap chaperones like Hsp70 that are crucial and rate-limiting for handling SOD1 and many other proteins. For polyQ on the other hand, levels of
certain DNAJ proteins are more rate-limiting and those are potentially not trapped in SOD1 aggregates, possibly explaining the absence of an effect of SOD1 on polyQ aggregates. This is only one aspect of why one cannot simply translate findings (like toxicity) for one aggregating protein to findings for another aggregating protein.

In any case, however, reduced chaperone availability due to aggregating proteins, will most likely impede on the PQC systems in a way that it will progress into a vicious, self-perpetuating, disaster, the collapse of protein homeostasis. Normal protein folding and transport could become affected or/and cells might become more sensitive to proteotoxic stress and be unable to deal with conditions that would normally be tolerated. Therefore, heat stress in neurons due to fever, or oxidative stress in muscle cells due to increased physical activity, could have a greater impact in cells that already carry an aggregation-prone protein and actually become a trigger for disease progression.

Such scenarios are likely exacerbated during normal aging where the PQC capacity of the cells is reduced even more (Douglas and Dillin, 2010; Higuchi-Sanabria et al., 2018; Ben-Zvi et al., 2009), either via epigenetic effects leading to lower expression of these PQC components (Yang et al., 2014b; Naidoo et al., 2008) or/and due to an increase in the PQC need, e.g., because of accumulated mutations, molecular misreading or protein oxidation products over time. Such may have an even greater impact on post-mitotic tissues, as differentiated cells have lower PQC capacities than (proliferating) stem cells (Buckley et al., 2012; Vilchez et al., 2012; Saretzki et al., 2004; Battersby et al., 2007) and lack the ability to rejuvenate via asymmetric segregation of aggregates during cell division (Lindner et al., 2008; Rujano et al., 2006; Moore and Jessberger, 2016; Ogrodnik et al., 2014; Bufalino and van der Kooy, 2014). Indeed, most protein aggregation diseases and chaperonopathies mainly affect tissues with mostly post-mitotic cells, like neurons or muscles (Behl, 2016; Kakkar et al., 2012; Macario and De Macario, 2007; Macario and Conway de Macario, 2007; Macario and de Macario, 2005).

### 5.5 Chaperonopathies

As stated earlier, an increasing number of (rare) diseases are being related to mutations in molecular chaperones (chaperonopathies). Both dominant and recessive chaperonopathies have been described (Kakkar et al., 2014; Macario and Conway de Macario, 2007) and a majority of them is also associated with protein aggregation, emphasizing again protein aggregation as being a disease-causing event and not a mere epi-phenomenon.
The phenotype (and tissue) specificity of the recessive chaperonopathies (involving mostly co-chaperones DNAJs or HSPBs) and the related loss of function highlights again that many of these co-chaperones are functionally distinct and have selected numbers of (tissue-specific) targets. The dominant diseases can have different underlying mechanisms: a) toxic gain of function, for example protein aggregation of the mutant proteins, b) (partial) loss of function leading to haploinsufficiency, and c) dominant negative effect of the mutant on other protein quality control proteins or systems.

For some chaperonopathies, as for example in the case of mtHsp60 (Hansen et al., 2002; Bross et al., 2008), DNAJC5 (Nosková et al., 2011) and (recessive) HSPB1 mutations (Lewis et al., 1999; Boncoraglio et al., 2012), a loss of function of the protein leads to the disease. In other cases, however, the mechanism leading to the disease is more complicated. For instance, our lab has demonstrated that BAG3 mutations lead to a combination of loss of function and dominant negative effects of the mutant proteins on the Hsp70 cycle which then actually results in a gain-of-toxicity causing a collapse of other protein quality control systems (Meister-Broekema et al., 2018).

5.5.1 DNAJB6 mutations leading to LGMD1D
DNAJB6 mutations leading to the dominantly inherited disease LGMD1D are likely an exception to the above-mentioned rule and likely cause the disease via a minor loss of function (partial haplo-insufficiency). So far, all LGMD1D-causing DNAJB6 mutation have been identified in the G/F-rich region of the protein. Earlier data (Hageman et al., 2010) had revealed that this G/F region as such was largely dispensable for DNAJB6 action on polyQ aggregation and that this activity rather required a S/T-rich region of the protein (Hageman et al., 2010; Kakkar et al., 2016b). However, it has been suggested by others that these LGMD1D-associated mutants may display reduced chaperone function (Palmio et al., 2015; Sarparanta et al., 2012; Stein et al., 2014; Tsai et al., 2017). We now confirm in Chapter 4 that these mutations of DNAJB6 show indeed some, but mostly mild, loss of function, which is only statistically significant in the case of the most severely aggregating polyQ client. Ongoing work in the lab, further supports significant, but minor loss of function for these and other DNAJB6 mutants as well. This would be consistent with the genetic data suggesting that full loss of function of the mutant allele may not be tolerated. It also suggests that a minor change in DNAJB6 levels and activity for a prolonged period can have devastating degenerative effects, highlighting the important physiological role of this co-chaperone, especially for muscle cells.

5.5.2 Molecular mechanism of loss of function of DNAJB6 G/F mutations
As stated above, deletion of the G/F region has only limited effect on the anti-aggregation
function of DNAJB6. So, the question remains: how do the G/F region mutations affect DNAJB6 function? This region can be important for interaction with other partners or flexibility of the protein as it is a flexible disordered region (Pellecchia et al., 1996). One option is that the mutations allosterically affects the substrate-biding/stabilizing region, interfering with either substrate interaction and/or the DNAJB6-Hsp70 communication. For example, J domain and CTD of DNAJs have been shown to be crucial interaction sites between DNAJAs and DNAJBs in a disaggregation machinery involving HSP70s, DNAJs and HSP110s (Nillegoda et al., 2015). In this proposed machine, a J domain from one subfamily (DNAJA or DNAJB) interacts with a CTD of the other subfamily. Since between these two crucial domains lies the flexible G/F-rich region, this part could be critical to keep the DNAJ structure flexible in order to adapt these complex conformations. So far only DNAJB1 from the DNAJB subfamily has been shown to be part of such a complex. However, it cannot be excluded that DNAJB6 can also be involved in a similar setup for different substrates and possibly these G/F-rich region mutations decrease the flexibility and functionality of the complex.

5.5.3 Consequences of the G/F mutants in DNAJB6-related functions

DNAJB6b has been found to co-localize and/or being associated with RNA-binding proteins like hnRNPA1, hnRNPA2/B1, TIA1 and TDP43 and suggested to play a role in stress granule formation and clearance after stress, hereby preventing these phase separations to progress from the fluid (physiological) to the (solid, aggregated and pathological) state (Stein et al., 2014; Bengoechea et al., 2015; Li et al., 2016). LGMD1D-associated mutants did not interact and could not recover accumulation of RNA-binding proteins after stress in the cytoplasm (Bengoechea et al., 2015) or the nucleus (Stein et al., 2014). Moreover, in a LGMD1D mouse model with the DNAJB6<sup>F93L</sup> mutation, RNA-binding proteins were accumulating in muscle cells and co-localizing with mutant DNAJB6 (Li et al., 2016). Overall, these data suggest that DNAJB6 might be a component of the stress granule dynamics or the dynamics of stress granule-associated proteins (e.g. nucleo-cytoplasmic shuttling). The latter would be consistent with DNAJB6 being a protein that is shuttling between cytoplasm and nucleus, where it might be involved in chaperoning RNA-binding proteins.

Finally, our finding that G/F mutants of the nuclear DNAJB6a actually lead to a decreased (rather than increased) cytoplasmic polyglutamine aggregation remains puzzling. Whereas we did not detect it, this suggests that there has been a subtle increase in cytoplasmic to nuclear ratio of the mutant DNAJB6a. Given the low stoichiometry needed for DNAJB6 to suppress polyQ aggregation (Månsson et al., 2014b) and the minor loss of function of the mutant, traces of DNAJB6a leaking to the cytosol may indeed suffice to lead to suppression
of aggregation of polyQ. This finding is also in line with previous studies showing that DNAJB6a mutations do not contribute to the disease phenotype (Sarparanta et al., 2012; Bengoechea et al., 2015). That could be explained, for example, if DNAJB6b and DNAJB6a have overlapping functions and only differ in their localization. In that case, if DNAJB6a is mutated, DNAJ6b could still compensate for DNAJB6a function in the nucleus, since it is present in both cytoplasm and nucleus. However, the other way around is probably not possible due to the strictly nuclear expression of DNAJB6a. That would also suggest that the cytoplasmic functions of DNAJB6b are of critical importance.

It would be important to investigate what the relationship between DNAJB6a and DNAJB6b is. Do they collaborate with each other, with DNAJB6a transferring/receiving substrates to/from DNAJB6b that is shuttling between cytoplasm and nucleus? Or are they acting independently on (different) substrates (at different locations)? It would be interesting to express both proteins with different tags and track them in the same cell to see if they colocalize and check with immunoprecipitation if they interact (or at least be part of the same complex).

5.5.4 Specific effect of DNAJB6 mutants on muscle cells
As previously discussed, an interesting fact is that chaperonopathies often affect only specific tissues, generally neurons or muscle cells (Kakkar et al., 2014; Macario and Conway de Macario, 2007). This could be related to their different expression in the different tissues, in order to achieve different combinations of chaperones for specific functions. This expression may also reflect the abundance and the type of substrates present in each cell type/tissue that need these specific chaperones for their maintenance.

In Chapter 4 we discuss about mutations of DNAJB6 that cause a muscular dystrophy, suggesting that, although the mutations are present in all cell types, there is a specific vulnerability of the muscle cells for these mutants. One explanation for this is that DNAJB6 is crucial for muscle cell homeostasis, possibly because it is involved in chaperoning specific proteins crucial for muscle cell function. Another explanation could be that DNAJB6 is involved in specific processes that are of high importance in these cell types, for example stress granule formation. Stress granule regulation can be important in a post-mitotic cell that undergoes increased levels of oxidative and possibly other type of stresses. Hsp70 is also involved in stress granule formation (Alberti et al., 2017) and mutation in one of the Hsp70 machinery components (like DNAJB6 in our case) could cause an imbalance in Hsp70 complexes, and possibly a dominant negative effect as seen in the case of BAG3 mutations (Meister-Broekema et al., 2018).
Interestingly, mutations in other chaperone genes also cause muscle-associated diseases, like small HSPs (e.g. HSPB8) and BAG family of NEFs (Kakkar et al., 2014; Macario and Conway de Macario, 2007; Lupo et al., 2016). This suggests that there might be a connection between the defects caused by the mutations in these different genes. Interestingly, HSPB8, BAG3 and DNAJB6 have all been implicated in stress granule homeostasis (Alberti et al., 2017), which makes this an interesting hypothesis to investigate further. It would be interesting to experimentally combine different mutant chaperones (like DNAJB6 and BAG3 for example) and investigate their combined effect in stress granule formation. A combination of mutants would reveal if they act together in the same pathway (in which case a combined mutant would be as bad as the single mutants) or if they are involved in different pathways (in which case the combination would create a larger effect). Finally, since muscles seem to be mostly affected by these mutations, it would be interesting to establish a muscle cell model to execute the above-mentioned experiments. This could potentially reveal a tissue-specific function for these chaperones.

5.6 The process of aggregation drives degenerative diseases

While many neuro- or muscle- degenerative diseases are associated with protein aggregates, there is still a debate whether aggregates are actually the cause of the disease or they are protective for the cells (Balah, 2011; Todd and Lim, 2013; Takalo et al., 2013).

Much of the confusion about this is related to inaccurate usage of nomenclature and definitions. As stated previously (5.4), aggregation generally refers to the formation on non-native, non-functional protein assemblies. These are distinct from a) functional amyloids (Fowler et al., 2007) b) phase separation of proteins into membrane-free compartments (Alberti, 2017; Strzyz, 2015) c) other types of inclusions in which aggregates can be actively sequestered (Weisberg et al., 2012; Kaganovich et al., 2008; Miller et al., 2015a; Sontag et al., 2014; Alberti, 2012; Miller et al., 2015b; Roy et al., 2015; Kamhi-Nesher et al., 2001) and may have pro-survival effects in dividing cell populations, e.g. by supporting asymmetrical aggregate segregation during cell division (Lindner et al., 2008; Rujano et al., 2006; Ogrodnik et al., 2014; Spokoini et al., 2012; Zhou et al., 2014; Sontag et al., 2014).

Moreover, many different types of aggregates exist (amorphous, disordered or porous aggregates and dense amyloids), all with different properties of interaction with biomolecules, which implies that they could affect cell function via different mechanisms. In addition, effects of protein aggregates on toxicity are often only evaluated by external addition of aggregates on cells, which may be highly distinct from the way the aggregates
would act when generated inside the cells. Finally, clonogenic or apoptotic cell death assays - often used to measure toxicity - may be a wrong endpoint as they can be irrelevant for differentiated cells (clonogenic assays) and/or because they are actually only a late manifestation of the cellular adaptions happening in response to the actual functional deficit aggregation can cause.

To sum up, there is a myriad of data strongly arguing that protein aggregation and loss of protein homeostasis is the driving force in many of the late-onset degenerative diseases in post-mitotic tissues (Chapter 1 and Fig 1). This evidence includes the observations that a) in all aggregation diseases, mutants (substrates) that generate an earlier onset of the disease are aggregation-prone, for example Huntingtin, SOD1, ataxin, α-synuclein and many more (Soto, 2003), b) chaperone modulations can delay the onset of these diseases, for example overexpression of DNAJB6 in Huntington’s disease animal models (Kakkar et al., 2016b; Bason et al., 2019), and c) chaperone mutations cause aggregation diseases (earlier collapse) (Kakkar et al., 2014; Macario and Conway de Macario, 2007; Lupo et al., 2016).

The data from this thesis have further contributed to this evidence and thus argues that a better understanding of the PQC systems that control protein homeostasis may facilitate its specific and more effective use as a therapeutic or preventive approach for many degenerative diseases. It also denotes that a more careful approach should be used as the human PQC system is often complicated and specialized but with limited resources that are efficiently combined in different ways to serve different purposes. A great example of this is the use of chaperones

**Figure 1. Adapted protein homeostasis model.** During aging, protein damage levels increase due to accumulated mutations and proteotoxic stress (red line). On parallel, the protein quality control capacity of the cell declines with aging (green line), eventually leading to a collapse of the system and onset of sporadic neurodegenerative diseases (meeting point of the red and green line). In the case of genetic mutations of the disease-associated proteins (substrates-upper blue line) or the PQC components (e.g. chaperones-yellow line), there is an increased burden of protein damage already since birth, which leads to an earlier collapse of the system (meeting point of blue & green line and yellow & red line) and disease onset. Intervention with supplemental PQC components (e.g. chaperones) can eventually lead to a delay of the disease onset (lower blue line).
of the Hsp70 system in preventing or treating these diseases (Fig 1). However, even the Hsp70 chaperone machinery, which is only a part of the whole PQC system, is a complex system involved in many functions and the exact mechanism of action in these different functions must be well understood in order to optimally use this therapeutically against a disease. Further studies are required to decipher the exact way this system works and how a functional interplay with other chaperone systems and protein quality control components, like degradation machineries, is achieved in order to maintain a functional protein homeostasis under different circumstances.

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General discussion and future perspectives


