Chapter 1

Introduction and aim of the thesis

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1.1 Protein homeostasis & protein quality control

After their production at the ribosomes as amino acid chains, proteins have to fold and acquire their functional “native” three-dimensional state. For most proteins, this is a very complex process and misfolding can increase the risk of exposing interactive surfaces, like hydrophobic regions, which normally would be buried in the native state (Dyson et al., 2006). These surfaces can engage into non-functional interactions with other proteins, leading to protein aggregation. This is a constant challenge for the cells since not only normal newly synthesized proteins are facing the risk of aggregation. For instance, there are proteins or protein regions that remain unstructured (intrinsically disordered) at their functional state and that are thus constantly susceptible to aggregation. Moreover, native proteins under denaturing stress conditions can become (partially) unfolded. Finally, genetic mutations can result in production of abnormal proteins that either cannot acquire a normal native state and remain misfolded or contain excessive regions with increased aggregation potential, both of which scenarios can generate aggregating protein species. Protein aggregation can have various negative consequences in a cell; for example, it can induce co-aggregation of other metastable/ aggregation-prone proteins (Olzscha et al., 2011), disrupt organelles (Hashimoto et al., 2003; Lin and Beal, 2006; Xie et al., 2010) and membranes (Bäuerlein et al., 2017; Lashuel and Lansbury, 2006; Liu et al., 2015), interfere with protein transport (Woerner et al., 2016; Dormann et al., 2010; Chou et al., 2018) or degradation (Bence et al., 2001; Bennett et al., 2005; Duennwald and Lindquist, 2008) and many more. Therefore, the cells have developed several protein quality control (PQC) mechanisms to minimize the risk of aggregation and ensure a functional proteome by maintaining protein homeostasis in the cell.

Protein homeostasis, often referred to as proteostasis, describes the regulation of protein production, folding, remodelling, transport and degradation in order for them to retain a functional conformation, concentration and localization according to the needs of the cell (Balch et al., 2008). Despite the “-stasis” suffix in the term proteostasis, protein homeostasis is not a static condition but rather a very dynamic one as the requirements of the protein content are changing constantly and depend on the cell type, age, status etc. That is why the PQC machineries, by refolding, unfolding, disaggregating, degrading or disposing proteins, play a major role in managing the constantly and dynamically changing requirements of protein production and remodelling. PQC primarily relies on a network of molecular chaperones that are involved in almost every aspect of protein maintenance; they assist folding of nascent proteins, they prevent aggregation by holding aggregation-prone proteins, they aid protein trafficking towards their final destination, they
guide damaged/ misfolded/ aggregating proteins towards disposal by the degradation machineries via the ubiquitin-proteasome system (UPS) or autophagy, they disentangle already formed aggregates (disaggregation) or they accompany aggregates into specific “deposit” cellular compartments (Hartl et al., 2011; Bukau et al., 2006; Chen et al., 2011).

1.2 Protein homeostasis disruption and neurodegeneration

Protein aggregation is a common hallmark of many, otherwise non-related, neurodegenerative diseases, like Huntington’s disease (HD), different types of spinocerebellar ataxias (SCAs), Parkinson’s disease (PD), Alzheimer’s disease (AD), amyotrophic lateral sclerosis (ALS) (Soto, 2003). Some types of these diseases are inheritable and a result of genetic mutations that lead to abnormal, aggregation-prone, protein production. However, most cases, especially of AD, ALS and PD, are sporadic, although some of them have been linked with genetic mutations (de novo or previously undetected hereditary mutations) (Soto, 2003; Ajroud-Driss and Siddique, 2015). There are also many cases of unidentified causes, although some of these could also be the result of so far undetected genetic mutations (Mitsui and Tsuji, 2014). Different proteins are found in aggregates in the brains and/or other parts of the central nervous system of the patients for each disease for example polyglutamine huntingtin (polyQ-Htt) in HD, polyglutamine ataxins (polyQ-ATX) in spinocerebellar ataxias (SCAs) (Shao and Diamond, 2007). For some of these diseases, there is also variability in the proteins involved within the disease; in ALS, for instance, different protein aggregates like SOD1, FUS, TDP43, C9orf72 and more, have been implicated in the pathology of the disease (Blokhuis et al., 2013).

Whether hereditary or sporadic, all these diseases, show an age-related onset of the disease, implying that ageing is a contributing factor to the development of the disease and that the underlying defect can be tolerated from several years to even several decades of a person’s life. This has led to the protein homeostasis collapse model as a possible aetiology of neurodegenerative diseases (Balch et al., 2008; Douglas and Dillin, 2010; Hipp et al., 2014; Kakkar et al., 2012; Kampinga and Bergink, 2016). According to this hypothetical model, during aging, there is increasing imbalance in the protein homeostasis within the organism due to at least two parallel processes. Firstly, several lines of evidence show an age-related decrease in the capacity of the PQC system, like the heat shock response, the degradation machineries (ubiquitin-proteasome and autophagy systems) or the chaperone capacity (Koga et al., 2011; Saez and Vilchez, 2014; Yang et al., 2014a; Hamatani et al., 2004; Ben-Zvi et al., 2009). Secondly, aging is more and more associated with an increase in production of aggregation-prone proteins (David et al., 2010; Walther
et al., 2015; David, 2012), likely as a result of accumulated somatic mutations, increased molecular mis-readings (transcriptional or translational errors) or accumulated damage due to exposure to various cellular stresses (e.g. heat stress, oxidative stress, infections) (Fig 1). As a consequence, the (increased) amount of ‘clients’ overwhelm the capacity of the (declining) PQC machineries of the cell leading to a collapse of the system and initiation of the disease. In the cases of hereditary genetic mutations, the protein homeostasis model further predicts that the burden is already increased since birth and it is initially manageable but the collapse of the system appears at an earlier age, which is consistent with the earlier age of onset of the hereditary forms of neurodegenerative diseases.

These two parallel pathways may furthermore be intertwined and self-perpetuating as entrapment of crucial PQC components by the misfolded or aggregated proteins can lead to a further shortage of the available pool of PQC components and a progressive decline in the total capacity of the system. Indeed, many PQC components like chaperones and proteasomal components have been found associated with aggregates (Wang et al., 2009; Wigley et al., 1999; Kim et al., 2016; Suhr et al., 2001; Wang et al., 2007; Olzscha et al., 2015; David, 2012).

Figure 1. Proposed protein homeostasis model. During aging, metastable protein levels increase due to accumulated mutations and proteotoxic stress (black line). On parallel, the protein quality control capacity of the cell declines with aging (red line), eventually leading to a collapse of the system and onset of sporadic neurodegenerative diseases (meeting point of the red and black line). In the case of genetic mutations affecting disease-associated proteins, there is an increased burden of metastable protein production already since birth (blue line), which leads to an earlier collapse of the system (meeting point of blue and red line) and heritable disease onset. Interindividual differences in any of these factors may further contribute to the variability of the disease onset (dotted lines). Figure adapted from Kakkar et al., 2012.
This sequestering of chaperones was also demonstrated to directly interfere with chaperone-related functions like protein folding, degradation or complex disassembly (Hinault et al., 2010; Park et al., 2013; Yu et al., 2014). Another forward feedback loop contributing to the proteostasis imbalance is the induction of aggregation of other aggregation-prone proteins due to co-aggregation, as it has been shown that amyloid aggregates can sequester various metastable proteins with essential functions (Olzscha et al., 2011).

The protein homeostasis model was firstly supported by experiments in C. elegans which showed that polyQ and temperature-sensitive mutants can increase each other’s aggregation (Gidalevitz et al., 2006). Following that, temperature sensitive mutants have been shown to increase mutant SOD1 aggregation in C. elegans (Gidalevitz et al., 2009) and polyQ can lead to aggregation of thermosensitive luciferase mutants (Gupta et al., 2011). These findings with thermosensitive mutant proteins do point towards defects in protein homeostasis induced by aggregation, but the use of an increase in temperature in order to activate misfolding and aggregation of the mutant proteins does entail some potential confounders as the temperature shift for the lines adapted to grow at the permissive temperature is a heat stress and can elicit more global changes in protein homeostasis as well as activate the heat shock response. Moreover, a common general collapse of the system cannot explain why the disease manifestation is so different, depending on the aggregating protein present. It is known that PQC needs are likely different for each protein as, for example, different chaperones are involved in processing different aggregating proteins (Kakkar et al., 2014). Therefore, it can be hypothesized that the impact of each aggregating protein on the protein homeostasis can vary significantly. For example, trapping specific chaperones or other PQC components initially involved in the aggregating protein’s processing and inducing shortage of these certain components. An alternative would be that each aggregating protein can interact, sequester and ultimately induce aggregation of only certain proteins, leading to a shortage of these specific factors. In any of these cases, aggregation subsequently leads to specific cellular defects eventually causing a disease. Moreover, each disease-related protein may be differently expressed and differently modified or regulated in different brain areas, changing its aggregation-propensity. Indeed, it has been shown that e.g. post-translational modifications and proteolytic processing are important factors for the aggregation process of disease-related proteins (Park et al., 2018; Kuiper et al., 2017; Rhoads et al., 2018; Greenberg et al., 1990; Zhang et al., 2009; Sambataro and Pennuto, 2017). In Chapter 3, we further dissect the differences between the different impact of aggregating proteins on protein homeostasis using polyQ and mutant SOD1 as model aggregating proteins.
Finally, hereditary genetic mutations in chaperones or other PQC components also cause age-related aggregation diseases (Macario and De Macario, 2007), again suggesting that in these cases PQC is already impaired at birth and collapses at an early age (Fig 1). Taken together, all these results point towards protein homeostasis collapse as being the common underlying cause of a general cellular dysfunction that subsequently leads to many different degenerative diseases in neurons, but also skeletal and cardiac muscles.

1.3 Molecular chaperones & the Hsp70 system

Molecular chaperones play a central role in many PQC-related processes like protein folding, aggregation prevention, disaggregation, degradation, transport, complex assembly/disassembly and more (Hartl et al., 2011; Bukau et al., 2006). The largest and most generic class of molecular chaperones are the families of heat shock proteins (HSP). Heat shock proteins received their name from their discovery as proteins up-regulated by heat shock (Ritossa, 1962, 1963, 1996; Tissières et al., 1974), referred to as the heat-shock response (HSR), which is transcriptionally activated by the heat shock factor-1 (HSF-1) (Richter et al., 2010). In eukaryotes, there are several different families of chaperones, each comprising of multiple members, which are organised in different systems: the Hsp70 system, the Hsp90 system, Hsp60 system, CCT/TRiC and the small HSPs (Saibil, 2013; Garrido et al., 2012) (Fig 2). Interplay between these different systems that form an extended network of chaperones ensures proper substrate processing to avoid aberrant protein production. Except small HSPs that act in an ATP-independent manner, each of these systems, or molecular machineries, process a variety of substrates in an ATP-dependent binding and release cycle. While each of these families contains members that are regulated by the classical HSR, which

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**Figure 2. Chaperone networks.** At the various stages of protein handling (thin black arrows), different chaperone machineries are involved. In humans, they are comprised of the Hsp70 system, the Hsp90 system, the Hsp60 system, CCT/TRiC and the small HSPs. Some of these chaperone systems can communicate with each other (thick green/red arrows) in the different processes.
can be activated by many more proteotoxic stresses than heat shock (Morimoto, 1998), many HSP family members are either not at all HSR-regulated, or (also) constitutively synthesized, or regulated by other stress pathways like the unfolded protein responses in the ER or mitochondria (Hageman et al., 2011; Harding et al., 2002; Patil and Walter, 2001; Pellegrino et al., 2013).

A central system involved in almost all PQC-related processes is the Hsp70 machinery (Fig 3). The minimal requirements for the system are one Hsp70 (HSPA) family protein, one DNAJ (Hsp40) family protein and one nucleotide exchange factor (NEF). The Hsp70 protein confers an ATPase activity which powers a binding and release cycling of the substrates, causing HSP70 to alternate between a high-affinity ADP-bound state and low-affinity ADP-bound state. This cycle is aided by the co-chaperones DNAJs for stimulating ATP hydrolysis (usually together with the substrate) and NEFs for the ADP/ATP exchange (Mayer and Bukau, 2005). Hsp70, DNAJ and NEF families of the human Hsp70 system consist of several members each (Table 1), hereby increasing the flexibility of the system to serve multiple clients and to connect to multiple other PQC components and hence serve in many cell biological processes.

DNAJs are divided into three subfamilies: DNAJAs, DNAJBs and DNAJCs (Kampinga and Craig, 2010). DNAJAs and DNAJBs apart from the J-domain also contain a Gly/Phe-rich region of unknown function and a variable C-terminus that contains a substrate binding region. DNAJAs additionally contain a zinc finger-like region, thus deviating from DNAJBs. All other proteins with a J-domain that do not fall under these two categories, are classified as DNAJCs. DNAJAs and DNAJBs

**Figure 3. The Hsp70 cycle.** DNAJ proteins recruit non-native substrates through their variable substrate-binding domain (1) and interact with ATP-bound Hsp70s through their conserved J-domain (2). The substrate together with the DNAJ stimulate ATP hydrolysis that leads to a conformational change of the Hsp70 and the subsequent “locking” of the substrate (which has increased affinity for ADP-Hsp70) and release of the DNAJ (3). Following that, a nucleotide exchange factor (NEF) binds to ADP-Hsp70 (4) to aid ADP dissociation (5) and ATP binding (6). Since ATP-Hsp70 has low affinity for both the substrate and the NEF, they are both released from the Hsp70 (7). If the substrate is in a non-native state after being released by the Hsp70, it can re-enter the cycle. Figure adapted from Kampinga and Craig, 2010.
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are considered as rather “promiscuous”, i.e. interacting with a wide range of substrates, while several DNAJCs are considered as chaperones for specific proteins, whilst others have yet unknown substrate binding capacity or no such capacity at all but may only serve to tether Hsp70 (Kampinga and Craig, 2010). Whereas the interaction of DNAJs with Hsp70s is thought to be mediated by the J-domain, which is common in DNAJ family members, the role of the different DNAJs in regulating the function of Hsp70s is something that still remains unclear. In yeast, different DNAJs have been shown to form preferred combinations for certain activities (Lu and Cyr, 1998). In humans, there are 13 Hsp70s and around 50 DNAJ proteins (Table 1). Therefore, a preference in the combinations of DNAJ and Hsp70 couples that can be formed, in general or under certain conditions, is very likely to exist.

The Hsp70 family is among the most conserved families of proteins through evolution, from bacteria to humans (Hunt and Morimoto, 1985; Gupta and Golding, 1993). In humans, 13 Hsp70 proteins have been identified with the different family members exhibiting high sequence homology (Radons, 2016). Hsp70 proteins contain three distinct structural domains: an ATPase or nucleotide binding domain (NBD), a substrate binding domain (SBD) and a C-terminal domain (CTD) forming a lid that stabilizes the substrates upon binding (Mayer and Bukau, 2005). They interact with substrates via their SBD and have a generic recognition motif that consists of hydrophobic-rich stretches flanked by positively charged amino acids (Rüdiger et al., 1997). Such stretches can be found in virtually all proteins but are usually buried within the core of proteins and only become exposed when they are unfolded or misfolded. Substrate interaction can allosterically stimulate hydrolysis of ATP, which is bound to their NBD (McCarty et al., 1995; Flynn et al., 1991). Hsp70 NBD is divided into 4 subdomains (lobe IA, IB, IIA and IIB) forming a V-shaped structure (Fig 4). The

**Figure 4. Hsp70 nucleotide binding domain (NBD).**

Hsp70 NBD forms a V-shaped structure and consists of two lobes that are divided into two subdomains each: IA (purple), IB (yellow), IIA (green) and IIB (red). Between the two lobes is the nucleotide (here ADP, orange) binding site. Figure generated with Pymol using HSPA1A-NBD structural data from Wisniewska et al., 2010 (PDB ID: 3JXU).
cleft between the four domains serves as the binding domain for the nucleotides (ATP/ADP). Upon ATP hydrolysis, there is a conformational change that leads to an increase in substrate affinity, thus trapping the substrate in the ADP-bound Hsp70; therefore, a subsequent ADP/ATP exchange is required for substrates to be released (Mayer, 2013). However, the Hsp70 alone has a low-efficiency ATPase that requires assistance by co-chaperones for its efficient cycling; a DNAJ family member binds to Hsp70, stimulates ATP hydrolysis and promotes substrate binding while a nucleotide exchange factor (NEF) catalyses ADP/ATP exchange thereby promoting substrate release (Mayer, 2013). This is achieved through the interaction of the co-factors DNAJs and NEFs with the NBD of the Hsp70s.

DNAJ proteins, besides stimulating Hsp70 ATPase activity, are thought to be the recruiters of the substrates that are entering the Hsp70 cycle. They are a large family of proteins (more than 50 members in humans) with great variability among the different members (Kampinga and Craig, 2010). The family is characterized by a conserved J-domain, that serves as the interacting region with the NBD and (a part of) the SBD of the Hsp70s (Kityk et al., 2018) (Fig 5) and is required for Hsp70 ATPase activity stimulation (Wall et al., 1994; Karzai and McMacken, 1996). Upon J domain interaction, substrate-mediated ATPase activity is highly increased and after a conformational change of the Hsp70 domains, the substrate is efficiently trapped by Hsp70.

Figure 5. Hsp70-DNAJ interaction. DNAJs interact with the NBD and the SBD of Hsp70s through a conserved HPD motif on their J-domain. Shown above are: Hsp70-NBD lobe I (dark teal) and lobe II (cyan), Hsp70-linker (yellow), Hsp70-SBD (dark red and orange), DNAJ-J-domain (purple) and DNAJ-HPD motif (green spheres). Figure depicting bacterial Hsp70 (DnaK) in complex with the J-domain of bacterial DnaJ, adapted from Kityk et al., 2018.
While DNAJ proteins act at the entry of the substrates into the Hsp70 cycle, NEFs act at their exit point and aid substrate release. There are four structurally different families of proteins that can act as NEFs for human Hsp70s: the BAG family with 5 members, the Hsp110/Grp170 (HSPH) family with 4 members, the HspBP1/Sil1 family with just 2 members and the GrpE family with 2 members (Bracher and Verghese, 2015). These different types of NEFs have completely different structures and they use different mechanisms to catalyse ADP/ATP exchange by interacting with the NBD of the Hsp70s at different, partially overlapping sites (Polier et al., 2008; Schuermann et al., 2008; Sondermann et al., 2001; Arakawa et al., 2010; Xu et al., 2008; Shomura et al., 2005). GrpE type, which originated from bacterial NEFs, are only present in mitochondria and act as NEFs for the mitochondrial Hsp70 (HSPA9). Hsp110s and Grp170 (ER-specific NEF for HSPA5, the ER Hsp70) are structurally similar to the Hsp70s, with an N-terminal NBD followed by an SBD. Although they have ATPase activity, they do not possess the same mechanism as Hsp70s with a substrate binding and release cycle and primarily act as NEFs for Hsp70s (Dragovic et al., 2006; Raviol et al., 2006; Shaner et al., 2004). The HspBP1/Sil1 type of NEFs are characterized by an Armadillo-repeat domain, which is also the interacting region with the Hsp70-NBD (Shomura et al., 2005). Finally, the BAG family is the largest family of NEFs, with a common conserved BAG domain that contains the Hsp70-interaction site (Sondermann et al., 2001; Arakawa et al., 2010; Xu et al., 2008). While NEF activity is important for efficient cycling of Hsp70, they are only needed in substoichiometric ratios to Hsp70s and excessive numbers speed up ADP dissociation and negatively affect the cycle efficiency (Rauch and Gestwicki, 2014; Nollen et al., 2000; Dragovic et al., 2006; Tzankov et al., 2008; Yamagishi et al., 2000).

In addition to DNAJs and NEFs, an Hsc70-interacting protein (Hip) has been identified as an indirect regulator of the Hsp70 cycle by antagonizing NEF binding and thus attenuating ADP dissociation and delaying substrate release by the Hsp70s (Kanelakis et al., 2000; Li et al., 2013; Höfeld et al., 1995). Under certain conditions, this may increase the dwelling time of substrate on Hsp70 enhancing their likelihood of folding (Nollen et al., 2001). Finally, a series of tetratricopeptide repeat (TPR) domain-containing cochaperones of Hsp70 exists, like Hop and CHIP, that regulate interactions of the Hsp70 machinery with other main chaperone systems like the Hsp90 (Hop) (Johnson et al., 1998; Alvira et al., 2014) or that connect the Hsp70 machine with the UPS degradation machinery (CHIP) (Mcdonough and Patterson, 2003; Zhang et al., 2015).

As evident from the above, Hsp70 machines may exist in many different assemblies consisting not only of different Hsp70 members, but each recombining with different DNAJs and NEF couples together with other co-factors. It is far from clear yet whether and how such specific combinations are favoured under certain conditions and whether
any preferred assemblies are made between different core Hsp70 members. Competition between BAG and Hsp110 members for NEF activity for Hsp70s has been previously reported (Rauch and Gestwicki, 2014) and different assemblies may be driven by differential specific affinities and post-translational effects (Assimon et al., 2015; Rauch and Gestwicki, 2014). Moreover, combinations of gene expression regulatory pathways controlling total and local (intra)cellular concentration depending on the client or cellular condition may affect chaperone complex formation as well. A clear example of such regulation is the specific induction of BAG3 under conditions of stress that impair or overload the proteasomal capacity (Rapino et al., 2014; Du et al., 2009). BAG3 then outcompetes BAG1, which links Hsp70 to UPS (Lüders et al., 2000), in binding Hsp70 loaded with ubiquitylated proteins which enables the cells to switch from UPS to autophagy for degradation of the HSP70-bound client (Minoia et al., 2014).

1.4 Chaperone specificity

Chaperones of the Hsp70 system are involved in almost all different processes of the protein quality control including protein folding, aggregation prevention, disaggregation and degradation by the ubiquitin-proteasome or autophagy systems (Hartl et al., 2011; Bukau et al., 2006). In human, the Hsp70 system comprises many members for each of the different families (Table 1). The existence of multiple Hsp70, DNAJ and NEFs points towards specialization in the necessary chaperones needed for different activities or for different substrates. In vitro, certain Hsp70-DNAJ-NEF combinations have been found to be more efficient for certain activities (e.g. folding) and even ratios between the components are influencing the system efficiency (Tzankov et al., 2008; Rauch and Gestwicki, 2014). However, it is still unclear if and how those combinations are formed in living cells and if and how different combinations are required for different activities.

1.4.1 Spatial diversity of chaperones: same function in different compartments

Despite the existence of multiple members for each chaperone family, not all of these members are expressed at the same time or in the same compartment in each cell (Hageman and Kampinga, 2009; Hageman et al., 2011; Kampinga and Craig, 2010; Kampinga and Bergink, 2016), which limits the chaperone combinations as well as the substrates involved in the different cellular compartments. Moreover, there is enormous variability in chaperone expression at different tissues or cell types (Hageman and Kampinga, 2009; Hageman et al., 2011), which suggests that the needs of each chaperone differ per cell. Although increasing the system complexity, such spatiotemporal limitations in chaperone availability might reflect the presence of specific type of substrates under different cellular
conditions and increase efficiency in substrate processing.

1.4.2 Functional diversity of chaperones

As stated above, Hsp70s are highly homologues and their substrate recognition has been suggested to be quite general (Rüdiger et al., 1997) and most of them perform functions like folding or aggregation suppression efficiently in vitro (Freeman and Morimoto, 1996; Gassler et al., 2001; Hendershot et al., 1996; Fink, 1999). These observations led to a popular belief that Hsp70s just provide a generic ATPase activity and are therefore largely interchangeable (Warrick et al., 1999; Fernandez-Funez et al., 2016; Shukla et al., 2014; Auluck et al., 2002, 2005; Chan et al., 2000; Wong et al., 2008; McLear et al., 2008). However, especially in cells and/or animal models, not all Hsp70s behave in the same way (Kampinga and Craig, 2010; Kakkar et al., 2014; Kampinga and Bergink, 2016; Clerico et al., 2015). For example, yeast Hsp70s have been found to be differentially capable to perform certain functions (James et al., 1997; Sharma and Masison, 2011). Moreover, different human Hsp70s have been found to have different efficiency in in vitro lysosomal proteolysis (Terlecky et al., 1992) and show differences in the ability to support the cellular capacity to reactivate heat-denatured luciferase (Hageman et al., 2011) or to suppress the aggregation of mutant polyQ (Hageman et al., 2011, 2010) or mutant parkin (Kakkar et al., 2016a) in cells. Despite the accumulating evidence for such a diversity between the different Hsp70s, the reason behind it remains unknown.

Since the recognition of substrates by Hsp70 is quite generic and since there is a lot more variability in co-chaperones, especially DNAJs, the latter have been suggested as the ones that confer specificity to the Hsp70 system (Kampinga and Craig, 2010; Vembar et al., 2010) in terms of recruiting it to specific substrates. But whether different Hsp70s have preferences for certain DNAJ proteins has remained elusive so far. On the other hand, the multiple NEF families and family members points toward them as potential regulators for the output of the Hsp70 cycle. For example, Hsp110 NEFs have been specifically identified as crucial members of a human disaggregation machine, together with Hsp70s and DNAJs, while BAG type of NEFs could not support such function (Rampelt et al., 2012; Shorter, 2011; Mattoo et al., 2013; Nillegoda et al., 2015; Nillegoda and Bukau, 2015). Furthermore, whereas Hsp110 proteins have been implicated in both refolding and degradation of proteins, BAG proteins are mostly associated with degradation (Bracher and Verghese, 2015; Kandasamy and Andréasson, 2018b; Mattoo et al., 2013; Kriegenburg et al., 2012; Carra et al., 2009; Lüders et al., 2000; Gamerdinger et al., 2009). And as stated above, different ratios of BAG proteins have been shown to switch between different types of degradation, proteasomal degradation and autophagy (Minoia et al., 2014). But as mentioned for DNAJs, it is yet unclear whether different Hsp70s have preferences for certain NEFs or not.
The specificity of (co)chaperones or chaperone combinations in the context of Hsp70 machines has also been particularly addressed in their ability to suppress protein aggregation of disease-causing mutant proteins. Members of the Hsp70 system were found to be potent suppressors of aggregation of proteins associated with neurodegenerative diseases but, interestingly, different (co)chaperones seem to be needed for aggregation suppression of different disease-associated proteins (Kakkar et al., 2014; Kampinga and Bergink, 2016). For example, overexpression screens in HEK293 cells of individual Hsp70 machinery members revealed that polyglutamine (polyQ), heat denatured luciferase or mutant parkin (parkin<sup>C289G</sup>) aggregates require different chaperones for aggregation suppression (Hageman et al., 2010; Kakkar et al., 2016a; Hageman et al., 2011). This is connected with the fact that aggregates of different proteins are not the same and therefore probably require different processing by chaperones. Structurally, some proteins like polyglutamine expansion-proteins form beta-sheet structured amyloid fibrils while others like mutant SOD1 or TDP43 form unstructured, amorphous aggregates (Johnson et al., 2009; Capitini et al., 2014; Matsumoto et al., 2006). Besides structure, also cellular localization of aggregates can vary as different aggregates can be sequestered into different compartments in the cell (Kaganovich et al., 2008; Miller et al., 2015a; Weisberg et al., 2012; Kamhi-Nesher et al., 2001; Roy et al., 2015; Escusa-Toret et al., 2013), which might also influence the chaperone availability for each of these aggregates.

All the above-mentioned studies suggest that co-chaperones DNAJs and NEFs are key to regulating the specificity and functionality of Hsp70 cycle. By regulating different expression levels of the (co)chaperones in the different cell types or via PTMS, specific combinations of Hsp70 machine may direct the handling of specific clients. In Chapter 2, we experimentally address the specificity of the different Hsp70 chaperones focussing on two closely related Hsp70 family members (HSPA1A and HSPA1L).

### 1.5 Chaperonopathies

Mutations in several chaperone genes have been identified as causal to a number of diseases, together often referred to as chaperonopathies. Interestingly most of them affect tissues that (in adulthood) are mostly post-mitotic, causing either neuropathies, (cardio) myopathies or cataract (Behl, 2016; Kakkar et al., 2014; Macario and de Macario, 2005; Macario and De Macario, 2007; Macario and Conway de Macario, 2007; Kakkar et al., 2012). Mutations in the different chaperones generally lead to diseases affecting different tissues or same tissue but with different phenotypes, further accentuating that each chaperone is involved in different PQC processes and/or processing different types of substrates.
However, irrespective of the type of tissue that is affected the pathology, many of them are hallmarked by the presence of protein aggregates in the affected tissues, for example mutations in DNAJs (Harms et al., 2012; Nosková et al., 2011; Sanchez et al., 2016), HSPBs (Ackerley et al., 2006; Andley et al., 2002, 2008; Mackay et al., 2003; Bova et al., 1999; Hayes et al., 2008; Perng et al., 2004; Vicart et al., 1998; Ghaoui et al., 2016) or BAGs (Fang et al., 2017; Selcen et al., 2009) (Fig 6).

The chaperone mutations identified so far that can cause a pathological condition are DNAJs, BAGs, small HSPs (HSPBs), Hsp60 (HSPDs) and CCT/TRiC (Fig 6). Strikingly, there are no disease-associated mutations in members of the Hsp70, Hsp110 or Hsp90 genes, which suggests that either all these chaperone functions are essential for life or that there are overlapping functions between the members of each of these families.

chaperonopathies

Figure 6. Chaperonopathies with aggregation pathologies. Many mutations in chaperone genes lead to different types of diseases (chaperonopathies) which are hallmarked by protein aggregation pathologies. Genes associated with dominantly inherited diseases are in bold letters.
Some of these chaperonopathies are dominantly inherited while some others are recessive (Kakkar et al., 2014; Macario and Conway de Macario, 2007) (Fig 6). For the dominantly inherited, the underlying cause of the disease could be a toxic gain of function, a (partial) loss of function via haploinsufficiency or a dominant negative effect of the mutant. Haploinsufficiency has been proposed to be the causative factor for mutations in mtHsp60 (Hansen et al., 2002; Bross et al., 2008), DNAJC5 (Nosková et al., 2011), HSPB1 (Lewis et al., 1999; Boncoraglio et al., 2012). On the other hand, most mutations in small HSPs have been associated with the presence of protein aggregates (Ackerley et al., 2006; Andley et al., 2002, 2008; Mackay et al., 2003; Bova et al., 1999; Hayes et al., 2008; Perng et al., 2004; Vicart et al., 1998; Ghaoui et al., 2016), suggesting that they either directly aggregate themselves (toxic gain of function) or they indirectly cause aggregation of their substrates by loss of function. BAG3 mutations are also of particular interest as they cause a very severe and early onset muscle disease and it has been recently suggested that a combined loss of function and dominant negative effect on the Hsp70 machinery is the underlying mechanism of the disease (Meister-Broekema et al., 2018). Of particular interest are also several DNAJB6 mutations that cause limb-girdle muscular dystrophy type 1D (LGMD1D). Although the exact mechanism that leads to the disease is not yet understood, it has been suggested that the mutants may display reduced chaperone function (Palmio et al., 2015; Sarparanta et al., 2012; Stein et al., 2014; Tsai et al., 2017), which would imply that the full activity of this chaperone in critical to muscle tissue with a minor loss of function being sufficient to cause disease. However, the disease-associated mutants are located in a region that was initially thought to be less critical for DNAJB6 function (Hageman et al., 2010), creating a controversy in the field. For that reason, in Chapter 4, we study those mutants of DNAJB6 and their effect on chaperone activity.
Introduction

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Chapter 1

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Introduction


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Aim and outline of the thesis

As explained above, protein homeostasis imbalances could lead to protein aggregation, which has been associated with a variety of diseases. Therefore, understanding how the protein quality control mechanisms of the cell work is of great importance as it could be the key to prevent some of these diseases. Here, we aim to understand the mechanism by which protein aggregation can lead to a protein homeostasis imbalance, unravel the role of chaperones of the Hsp70 machinery in handling protein aggregation and shed some light into their mechanism of action.

In Chapter 2, we study how different members of the Hsp70 machinery can modulate ALS-associated mutant SOD1 aggregation, with particular focus on Hsp70 family members. Using cellular models of mutant SOD1 aggregation, we screened for positive or negative modulators and discovered substantial differences between different Hsp70 family members. This was quite surprising as all Hsp70s had been suggested to have very comparable substrate binding characteristics. To identify the reason behind these differences, we compared in more detail two highly conserved Hsp70 family members, HSPA1A and HSPA1L, that were found to have opposing effects on SOD1 aggregation. We discovered that these differences are not associated with their substrate binding capacity, but are attributed to minor amino acid changes in their NBD that alters their interaction pattern with NEFs. This chapter highlights the delicate interplay between Hsp70 machinery members and their regulators to ensure proper substrate handling.

We mentioned earlier that protein aggregation can impair protein homeostasis but it is not clear how different aggregating proteins affect protein homeostasis. Therefore, in Chapter 3 we examine if different disease-associated aggregating proteins, like polyglutamine proteins (polyQ) and mutant SOD1, can indirectly influence each other’s aggregation. These two proteins can aggregate independently in the cells and they rely on a different set of chaperones for their aggregation suppression, making them ideal to study indirect effects of protein aggregation on protein quality control mechanisms. We show that polyQ aggregation enhances mutant SOD1 aggregation but not vice versa, suggesting that each aggregating protein generates a specific defect on protein homeostasis and not a generic system collapse.

Another way to understand the importance of chaperones in suppressing protein aggregation is by looking at mutations that lead to diseases and the underlying cause. In Chapter 4, we study DNAJB6, a co-chaperone of the HSP70 system that can suppress
aggregation of various aggregation prone proteins, like polyQ, alpha-synuclein, amyloid beta, prions and mutant parkin. Several mutations in a conserved G/F-rich region of DNAJB6 gene have been associated with limb-girdle muscular dystrophy type 1D (LGMD1D), an autosomal dominant myopathy. Previous studies reported conflicting results regarding the functionality of this region and thus the impact that these mutations may have on the function of DNAJB6. Here, we found mutants of DNAJB6b, the cytoplasmic/nuclear isoform, show only minor loss of activity against polyQ or mutant parkin aggregation. However, we found that a LGMD1D-associated mutant of DNAJB6a, the exclusively nuclear DNAJB6 isoform, exhibited better aggregation suppressing activity in the cytoplasm than the wild type DNAJB6a, possibly suggesting a different function for the affected region between the different isoforms.

In Chapter 5, we summarize the results presented here and we discuss the implications for the chaperone roles in protein aggregation and the future perspectives.