On protein quality control, myofibrillar myopathies, and neurodegeneration
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CHAPTER 1

From Chaperones to Proteinopathies;
Protein Folding, Protein Aggregation, and Protein Degradation

Melanie Meister-Broekema
In the crowded environment of the cell, polypeptides continuously face the risk of attaining conformations that prevent them from functioning properly. Misfolded proteins tend to aggregate, which may result in their loss of function (LOF) or in their toxic gain of function (TGF). It becomes increasingly evident that protein aggregates may result from mutations in multiple genes and are a hallmark for nearly all known neurodegenerative and muscular diseases.

To ensure adequate protein homeostasis, cells are equipped with an elaborate protein quality control (PQC) network. The core of the PQC is comprised of chaperones that assist proteins in intermediate folded states to cross (intra-molecular) energy hubs and prevent off-pathway intermolecular interactions (aggregate prevention). If protein folding fails, chaperones may sequester misfolded proteins or target them to the cells degradation systems.

This thesis will address different aspects of protein folding, degradation, and human diseases associated with impaired protein homeostasis. After providing a short overview of protein folding and protein quality control, this Chapter will concentrate on the Hsp70-machine, which serves as a central hub in the PQC network, and the regulation of Hsp70-client delivery and release by cofactors. Furthermore, this Chapter will give a short introduction into protein misfolding, neurodegenerative (proteinopathies), and muscular diseases (e.g. chaperonopathies).

1.1 Proteins; Folding is Key
The cell’s crowded environment evokes continuous protein interactions. Aberrant protein interactions may endanger the folding state of proteins and impair their designated function. As part of PQC, molecular chaperones guide the conformation of proteins throughout their lifetime; in this function they may:

- Assist in folding of nascent proteins into functional 3-dimensional structures
- Assist in assembly and disassembly of mature proteins into functional protein complexes
- Associate with interactive surfaces in proteins to prevent protein aggregation
- Assist in targeting of misfolded proteins for degradation if folding is impossible
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Typical conditions under which polypeptides are especially prone to aberrant protein interactions include:

1. *Newly formed nascent chains emerging from ribosomes*[^1]:[^8]
   Due to their unfolded state, the hydrophobic interactive surfaces of nascent amino acid chains are exposed to the intracellular environment. To facilitate folding and to prevent unwanted protein-protein interactions, dedicated chaperones bind to nascent chains and target them for degradation if folding is unsuccessful (e.g. mutant proteins).

2. *Folded protein species that become unfolded upon stress (e.g. heat)*[^2]:[^3]:[^9]:[^10]
   Many stresses may lead to the unfolding or fragmentation of mature proteins, thereby rendering them aggregation-prone. On a molecular level, stress (like heat shock) may lead to the exposure of hydrophobic surfaces and prion-like domains. Whereas the exposure of hydrophobic surfaces can be managed by canonical chaperones, prion-like domains could form β-hairpin structures based on H-bonding, which may eventually result in amyloid-like aggregates and impose different needs on the chaperone network, as will be discussed later in this Chapter.

3. *Mature, folded protein species that need to be remodeled to fulfill their function*[^5]
   In order to form or disperse functional protein complexes, mature, folded proteins need to be partially unfolded and remodeled. Chaperones, such as certain Hsp70s and DNAJs, assist proteins in this function by providing “pulling forces”.

4. *Mature, folded protein species that need to be transported across membranes to fulfill their function*[^11]:[^17]
   The translocation of proteins across membranes (e.g. endoplasmic reticulum [ER], mitochondria, peroxisomes) requires sophisticated protein translocation machines, consisting of a core channel and accessory subunits. The translocation process often requires proteins to be in a soluble state, which may be facilitated by chaperones. Furthermore, chaperones may actively drive protein translocation by serving as motors fuelled by ATP hydrolysis.
The vast network of molecular chaperones engages a variety of clients associated with a variety of client fates. Next to functioning in client folding and degradation, HSPs may also be active following protein aggregation. Several chaperones have been shown to engage in aggregate sequestration, protein disaggregation (e.g. HSPH), and aggregate disposal (e.g. DNAJB2, DNAJB6, HSPB7).

1.2 Protein Quality Control; a Tightly Regulated Network of Proteins
To ensure adequate protein folding, sequestration, and degradation, the cell has developed an elaborate PQC network with tightly regulated components. Heat shock (HS) experiments lead to the initial discovery of Heat shock proteins (HSPs), which are up-regulated by a phenomenon termed “heat shock response (HSR)”.

The HSR is regulated by the transcription factor Heat shock factor 1 (HSF-1), which is known to be up-regulated by many stresses, including pathophysiological states and normal cellular processes. All known stress conditions that are associated with the accumulation of un- or misfolded protein species or increased protein translation induce the HSR, which led to the believe that the HSF-1-mediated increase in HSPs is a molecular reaction to an increased cellular need for chaperone activity.

1.3 Nomenclature for HSPs
Since the annotation of the human genome, the naming of HSPs in the literature was erratic; whereas some gene products were given up to 10 different names, almost identical names were given to various different gene products. Therefore, this thesis will refer to the members of the HSP-families based on their systematic gene symbols assigned by the HUGO Gene Nomenclature Committee (HGNC) and primary identifiers in databases, such as Entrez Gene and Ensemble. The individual members of HSP-families will be referred to as: HSPA1-9 (Hsp70s), HSPB1-10 (small HSPs), HSPC1-4 (Hsp90s), HSPD1 and 2 (Hsp60/GroEL), HSPE1 (Hsp10, GroES), HSPH1-4 (Hsp110s), CCT1-9 (chaperonins), DNAJA1-4, DNAJB1-12, and DNAJC1-30 (Hsp40s) (see Table 1).
1.4 The Hsp70-Machines; Central Hubs for Protein Quality Control

Hsp70s are central to protein folding and degradation. The interactions between Hsp70s and their clients depend on the cycling of Hsp70s between low affinity ATP- and high affinity ADP-bound states (see Figure 1). Hsp70s themselves have a very promiscuous binding capacity that enables them to function as central hubs in the PQC network. In this function, Hsp70s can function protein folding, degradation, translocation, and remodeling. Several factors affect the ATP/ADP-cycle and are thought to play a role in the crucial functional specification –or “fine-tuning”- of the Hsp70 machines. In vitro experiments indicate that a “minimal” HSP70 machine consists of at least one DNAJ, one nucleotide exchange factor (NEF), and one HSPA. The family of DNAJs (over 50 members in humans) is thought to provide client specificity to the Hsp70 machine. DNAJs have various client-interaction motifs that facilitate binding to (specific) clients and a J-domain through which they can bind ATP-bound Hsp70. Combined, these domains may allow DNAJs to target specific clients onto Hsp70s. Simultaneous binding of DNAJ and the client to HSPA is thought to stimulate ATP-hydrolysis, which facilitates client-transfer from DNAJ to HSPA. Subsequently, DNAJ releases and HSPA undergoes a conformational change, resulting in a higher client affinity state that is thought to protect the client from protein interactions. Client-release requires HSPA to regain its low-affinity state, which is stimulated by the recruitment of NEFs. Following release, the client may be folded completely and fulfill its cellular function or need further folding, in which case it may re-enter the Hsp70 cycle (see Figure 1). Alternatively, clients may also be transferred to degradation systems, depending on the state of folding and the particular ensemble of DNAJs and NEFs that were engaged in the reaction cycle.

1.4.1 DNAJs and HSPBs: Client Specificity and Delivery to Hsp70

DNAJs recognize and transfer clients to Hsp70s. The family of DNAJs is defined by the presence of a J-domain, which consists of four alpha helices and an accessible loop containing a Histidine Proline Aspartate (HPD) motif that is crucial for binding to the HSPA-ATPase domain. The DNAJ-family of proteins is divided into three classes based on their domain structure: DNAJAs, DNAJBs, and
Whereas several J-proteins are believed to have promiscuous client binding capacities, others display high substrate specificity (e.g. many of the DNAJCs).\textsuperscript{5,28}

In addition to DNAJs, several members of the HSPB-family are thought to capture and transfer misfolded clients to HSPAs for processing.\textsuperscript{29,30} Except for a C-terminal alpha-crystallin domain, HSPBs share little sequence homology.\textsuperscript{31,32} Whereas several purified HSPBs display ATP-independent substrate holding activity in vitro, ATP-dependent chaperones, like Hsp70s, are thought to be required for substrate release.\textsuperscript{32,33} Furthermore, some HSPBs can interact, directly or indirectly, with the proteasome (HSPB1, HSPB5, HSPB10) or the autophagy-lysosome system (HSPB8).\textsuperscript{32,34-36}

**Figure 1: The Hsp70-cycle.** Mis- or un-folded proteins are recognized by DNAJs or HSPBs (not displayed in this figure) and transferred to ATP-bound Hsp70. Hsp70 facilitates client (re-)folding. Following the conversion of ATP to ADP and the dissociation of DNAJ from Hsp70, NEFs bind ADP-bound Hsp70. NEFs, such as BAGs, facilitate release of the client. Whereas properly folded clients will fulfil their cellular functions, un-folded, mis-folded, or un-foldable proteins will either enter another Hsp70-cycle or be directed to cellular degradation machines or to protein storage.
1.4.2 Nucleotide Exchange Factors; Client Release and Client Fate

Human cells contain three very different types of NEFs: “Pure” NEFs (HSPBP1, BAP), HSPA-like NEFs (Hsp110/HSPH), and the BAG-family of NEFs. The only homology between these protein-families is their NEF-function, although the mechanism by which they destabilize HSPA-nucleotide binding differs drastically.37–39

1. The only known function of pure NEFs is the exchange of nucleotides on HSPAs. The cytosolic HSPBP1 and the ER-resident BAP (BiP-associated protein) exclusively consist of domains involved in Hsp70-nucleotide release and are thought to support Hsp70-mediated protein folding and translocation.40,41

2. The family of HSPHs (Hsp110s) consists of 4 members, one of which is stress-inducible (HSPH1). HSPHs contain an N-terminal ATPase and a C-terminal peptide-binding domain, which are connected by a flexible linker. The strong homologies between HSPHs and HSPAs in sequence (30-35%), as well as in function (binding of misfolded clients), led to their initial co-categorization.42 Next to their function as NEFs, HSPHs act as ‘holdases’ that always require the ‘foldase’ HSPA, which may independently employ nucleotide-dependent peptide-release cycles, to achieve client folding.37

3. Bcl2-associated anthogenes (BAGs) form the most complex family of NEFs, in number as well as in diversity. All 6 members of this structurally diverse protein-family share a BAG-domain, which is required for binding to HSPAs,43–47 whereas domains specific to the individual family members are thought to provide functional specificity.47 Until now, 5 cytoplasmic and one ER-associated BAG have been identified, all of which have been associated with the refolding (BAG2) and degradation of Hsp70-clients via the ubiquitin-proteasome system (BAG1, BAG5, BAG6) or the autophagy-lysosome system (BAG3), as will be discussed in section 1.6 of this Chapter.48

1.4.3 Interaction of Hsp70 with Other Chaperone Machines

Although Hsp70 is a central hub for protein folding; folding of many clients, such as newly synthesized or large multi-domain proteins, requires further processing
by other chaperone machines, including chaperonins (GroEL/ES in mitochondria and CCT/TRiC in the cytosol) and the Hsp90 machine.\textsuperscript{1,3}

Chaperonins, such as the cytosolic TRiC (TCP1 ring complex), are not induced by stress but are transcriptionally and functionally linked to protein synthesis.\textsuperscript{49} TRiC is a large complex composed of multiple homologous subunits that each contain an ATPase domain.\textsuperscript{50} Following initial stabilization of unfolded substrates by the protein prefoldin or the Hsp70 machine, about 5-10\% of newly synthesized proteins are transferred to the TRiC-cage for optimal, unimpaired folding.\textsuperscript{1,3}

The final maturation of another subset of Hsp70 clients (e.g. steroid hormone receptors, several key transcription factors) demands additional folding by the Hsp90-machine. Similar to the Hsp70, Hsp90 engages in nucleotide-dependent peptide binding and release cycles, meaning that classic Hsp90 clients (e.g. steroid receptor) continuously cycle into and out of hetero-complex with Hsp90.\textsuperscript{51}

Several co-factors facilitate interaction between the Hsp70- and Hsp90-machines, amongst which HOP (Hsp70-Hsp90 organizing protein), HIP (Hsc70-interacting protein), and CHIP (C-terminus of Hsc70 stress protein-interacting protein).\textsuperscript{49} HOP can simultaneously bind Hsp70s and Hsp90s and thereby facilitate binding of specific ligands, whereas HIP may stabilize Hsp70 in its ADP-bound state by competing with BAGs and thereby slow down the nucleotide cycle and facilitate the formation of Hsp70-Hsp90-complexes.\textsuperscript{52,53} CHIP may function following destabilization of Hsp90-client interaction and redirect Hsp90-clients to Hsp70s. Furthermore, CHIP may interact with E3-ligases through its U-box domain and re-direct proteins to the proteasome for degradation.\textsuperscript{54} Additionally, CHIP has been suggested to inhibit DNAJ-mediated Hsp70-functions by promoting the proteasomal degradation of Hsp70 clients.\textsuperscript{54}

1.4.4 Regulation of the Hsp70-Machine; a Delicate Balance
The eventual fate of Hsp70-client proteins is believed to depend on the delicate interplay between chaperones involved in client delivery (DNAJs, HSPBs), release (NEFs), and other regulators (e.g. HIP, HOP, CHIP).\textsuperscript{5} Expression levels of these chaperones are tightly regulated and the overabundance and lack of Hsp70-co-factors have been associated with decreased cellular chaperone capacity.\textsuperscript{5} An imbalance in the delicate regulation of chaperone expression levels may change
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client fate. For instance, a substoichiometric DNAJ:HSPA ratio (10-20x less DNAJ) has been associated with optimal client refolding in vitro; tilting this balance in favour of DNAJs decreases folding capacity. Likewise, the overstimulation of Hsp70-ATPase activity and the over-abundance of NEFs have been associated with improper client folding.\(^5\)

The mechanisms underlying the decision as to whether Hsp70-clients are folded or degraded still mostly remains an enigma. A stochastic model predicts that the fate is mainly determined by the client itself: clients are either refolded during multiple cycles of Hsp70 binding and release or they are recognized as UPS substrates and redirected for degradation upon release from Hsp70s.\(^5\) An alternative or even parallel operating model assumes that chaperones and degradation systems are intimately connected: distinct sets of co-chaperones may specifically recognize certain Hsp70 clients and target them either for folding or degradation.\(^28,55,56\) To date, more than 50 DNAJs, 10 HSPBs, 11 HSPAxs, and 13 NEFs have been identified in humans, enabling many possible Hsp70-combinations. Depending on the chaperone combination, the same client may either be rapidly refolded (DNAJB1 + HSPA1A) or degraded (DNAJB2 + HSPA1A + BAG1).\(^19,28\) Recently, our group proposed a competitive operating model, in which co-chaperones determine whether degradation prone Hsp70 clients are targeted to the proteasome (HspA + BAG1) or autophagy (HSPA+BAG3) (see section 1.6.1, see Figure 2).\(^57\)

1.5 Hsp70 and Protein degradation

Misfolded proteins may be efficiently removed by the ubiquitin proteasome system (UPS), microautophagy, chaperone mediated autophagy (CMA), and macroautophagy (autophagy).\(^38,39\) Generally, as a first line of defense, soluble misfolded proteins are thought to be targeted to the UPS for degradation or to CMA, if misfolded proteins expose a KFERQ degradation signal.\(^60-62\) Misfolded proteins that either escape degradation by the UPS and CMA or tend to form aggregates can be targeted to the bulk degradation system autophagy.\(^58,61,63\)

1.5.1 Hsp70 and the Proteasome

The proteasome consists of two subunits (26S, 19S) and can quickly and selectively degrade ubiquitinated substrates into peptides.\(^58,64\) Whereas ubiquitinated proteins with mild tertiary structure perturbations generally remain
soluble, terminally misfolded ubiquitinated proteins (e.g. prion like proteins) generally suffer from an extensive loss of secondary structure, which may impede on their solubility and increase their tendency to aggregate. Usually, a large and heterogeneous number of highly selective E3-ligases mediate ubiquitin-conjugation to degradation-prone proteins, thereby facilitating their delivery to the proteasome. It has since become clear that the majority E3 ligase complexes require the activity of Hsp70s, which may partially be due to the its holdase activity that may keep proteasomal substrates soluble and accessible for the UPS. For instance, some E3-ligases, such as CHIP, may simultaneously bind HSPAs and the proteasome. Accordingly, Hsp70 was shown to be an integral part of the CHIP-E3 ligase complex and escorts CHIP-substrates to the proteasome. Interestingly, the presence of HSPAs seems to be a prerequisite for the degradation of terminally misfolded proteins, but redundant for the degradation of partially misfolded proteins.

Next to ensuring solubility of proteasomal substrates, HSPs may also escort partially or terminally misfolded proteins to the UPS. For instance, HSPA-ubiquitylation was shown to facilitate proteasome-substrate interactions by enabling direct binding of HSPAs to ubiquitin binding domains (UBDs) on 19S-proteasome receptors, thereby facilitating proteasomal docking of the Hsp70-CHIP E3 ligase complex. In line, the HSPA co-factor DNAJB1 has been found to facilitate nuclear degeneration of partially folded proteins by continuously shuttling between the cytosol and the nucleus, whereas DNAJB2 was discovered to be involved in an escort pathway that facilitates proteasomal degradation of CHIP-substrates like CFTR, SOD1, and mutated ataxin 3. By binding to poly-ubiquitin chains on proteasomal substrates through its ubiquitin interaction motif (UIM) and facilitating substrate loading onto Hsp70 through its J-domain, DNAJB2 is thought to prevent the trimming of ubiquitin chains and aggregation of specific ubiquitinated substrates. Although substrates may be delivered to the proteasome by multiple routes, proteasomal substrate range is limited to individual soluble ubiquitinated proteins because proteins need to be recognized by the 19S regulatory subunit, de-ubiquitinated, and –at least partially- unfolded in order to enter the narrow active catalytic core formed by the 20S subunit.
1.5.2 Hsp70 and Autophagy

A cellular degradation system with a broader substrate range is the autophagy-lysosome system, which delivers a wide variety of (misfolded) proteins, aggregates, pathogens, and organelles to lysosomes for degradation.58,59 There are three main forms of autophagy: CMA, microautophagy, and macroautophagy.58

1. HSPA8 may recognize soluble misfolded proteins that expose the KFERQ degradation signal and mediate their degradation via CMA or microautophagy.69 In CMA, HSPA8 is bound to LAMP-2A (lysosomal membrane-associated protein 2A) and may help to unfold and translocate soluble misfolded proteins directly into the lysosomal lumen for degradation.62,70

2. In microautophagy, HSPA8 helps to translocate soluble misfolded proteins into multivesicular bodies (MVBs) or late endosomes by a mechanism similar to CMA. The components of the molecular pathways that facilitate the degradation of vesicular content by endosomal/MVB microautophagy are also engaged in endocytic (ESCRT complexes) and autophagic pathways (ATG-core machine).69 During starvation, microautophagy recycles nutrients, just as macroautophagy.59,69

3. Hsp70 clients, aggregates, or organelles that cannot be degraded by the UPS, microautophagy, or CMA may be selectively delivered to macroautophagy via ubiquitin-dependent and ubiquitin-independent pathways.71 Autophagic clients may be recognized by the adaptor protein p62 (also known as SQST1) and HDAC6.63,72–74 By specifically interacting with LC3II (lipidated light chain 3), cargo-loaded p62 is delivered and anchored into the membranes of autophagic vacuoles, which effectively traps LC3-bound degradation prone proteins in phagophores. Once misfolded proteins are loaded into phagophores, the autophagic membrane structures are merged, grow into autophagosomes, and fuse with lysosomes. Acidic lysosomal content is released inside the newly formed autophagolysosomes and its content degraded.63,75 While starvation results in the aspecific degradation of cytoplasmic content, various cellular stresses lead to the
selective targeting and degradation of specific cellular content (e.g. mitophagy, xenophagy).\textsuperscript{59,76}

Whereas CMA and microautophagy are largely coupled to metabolic processes during cell growth and are regulated by mTOR (mammalian target of rapamycin), macroautophagy can also be induced by various additional cellular stress pathways.\textsuperscript{59,75}

In this thesis, we focus on aggrephagy, which is the removal of aggregated protein species. In aggrephagy, HDAC6 (histone deacetylase 6) and other molecular chaperones bind freely floating ubiquitinated misfolded or damaged proteins that accumulate beyond cellular protein quality control capacity and deliver them via microtubules to aggresomes at a location that minimizes their toxicity until they can be degraded by the UPS or macroautophagy.\textsuperscript{77} In Chapter 2, we investigated the question as to whether the Hsp70-machine locates to polyglutamine aggregates by analyzing the post-mortem brain tissue of patients with Spinocerebellar Ataxia type 3 (SCA3) by using immunohistochemical analyses.\textsuperscript{7}

To summarize, as opposed to the fast and specific degradation by the UPS, autophagy is a relatively slow process that degrades proteins and organelles in bulk. Unfortunately, neither system seems to be sufficient to manage the toxic effects of aggregation prone proteins associated with neurodegenerative diseases.

1.6 BAGs; More than Nucleotide Exchange Factors

The BAG family contains six members that can bind and modulate Hsp70s through their BAG domains.\textsuperscript{48} In their function as NEFs, BAGs may not only release Hsp70-clients by dissociating ADP from Hsp70, but may also determine the fate of Hsp70-clients by promoting either refolding (BAG2) or directing them to the UPS (BAG1, BAG5, BAG6) or autophagy (BAG3).\textsuperscript{48}

It has been suggested that competing NEFs with opposing functions may determine the fate of Hsp70-clients by promoting refolding or directing them towards degradation pathways by generating functionally distinct protein complexes.\textsuperscript{79} Initial research showed that BAG1 may compete with Hsp\textsuperscript{80} and catalyze nucleotide exchange on HSPA1A and HSPA8,\textsuperscript{81} thereby negatively regulating Hsp70-activity.\textsuperscript{82} Furthermore, BAG1 was shown to contain an ubiquitin-like (UBL) domain that may sort and associate BAG1 to the
proteasome.\textsuperscript{47} In line, BAG1 was associated with the proteasomal degradation of Hsp70 clients,\textsuperscript{83} which is most likely facilitated by its cooperation with CHIP.\textsuperscript{84} Similar to BAG1, BAG6 contains an UBL-domain and was associated with the proteasomal degradation of Hsp70-clients.\textsuperscript{47,85} Of note, whereas BAG1 is active in the cytoplasm, BAG6 is active in the endoplasmic reticulum.\textsuperscript{83,85} Interestingly, BAG2 has been identified to favor substrate renaturation by specifically inhibiting the chaperone-associated ubiquitin ligase CHIP on the one hand and assisting in the clearance of phosphorylated tau by competing with BAG1 on the other hand.\textsuperscript{86–88} BAG5 was identified as Hsp70-independent inhibitor of parkin, an E3-ligase that is associated with genetic forms of Parkinsons disease (PD) and may serve as link between the chaperone system and the UPS by inhibiting Hsp70.\textsuperscript{89,90} To date, little is known about the chaperone functions of BAG4; current research is mainly conducted in the field of oncology and focuses on its anti-apoptotic functions.\textsuperscript{45} In vitro, there seems to be a distinct hierarchy in the affinity of HSPA1A for NEFs; The affinity for BAG3 is strongest, followed by BAG1, Hsp105, and BAG2.\textsuperscript{79} These data further indicate that the abundance of chaperones may determine the functional Hsp70 complex, thereby influencing client fate.

This thesis will focus on BAG3, which has been implicated in the delivery of ubiquitinated Hsp70 clients to the autophagy lysosome system\textsuperscript{35,57} and may inhibit proteasomal degradation of HSP90 clients.\textsuperscript{47} BAG3-expression seems to be regulated by multiple cellular pathways, including proteasomal inhibition,\textsuperscript{91} heat stress,\textsuperscript{92} and other protein denaturing conditions.\textsuperscript{93} By cooperating with the HSPAs, HSPB8 (HSP22), and SQSTM1, BAG3 may lead to the selective autophagic degradation of disease-associated proteins, like mutated huntingtin (Htt) and superoxide dismutase 1 (SOD1).\textsuperscript{35,57}

1.6.1 BIPASS; Balance is the Key to Protein Quality Control

It has been long noticed that a decline of proteasomal functions usually coincides with the up-regulation of autophagy, presumably in a cellular attempt to dispose of protein waste.\textsuperscript{75,91,94} This phenomenon may not only be observed upon chemical inhibition of the proteasome, but also seems to occur during ageing.\textsuperscript{95} Interestingly, ageing and various forms of stresses that impair or overload the proteasome are also associated with a decline in cellular BAG1-levels and therefore the cells ability to deliver clients to the proteasome. Usually, this decline in BAG1 levels is paralleled by an increase in cellular BAG3 levels.\textsuperscript{95} This
interesting association resulted in the hypothesis that changes in the BAG1:BAG3 ratios may serve as a switch for cellular re-routing of proteasomal Hsp70-clients to autophagy.

Recently, our research group confirmed this hypothesis and showed that multiple stress pathways that result in proteasomal overload also induce the up-regulation of BAG3, thereby enabling cells to redirect HSPA1A-associated proteasomal clients to the autophagy pathway.\(^{57}\) In this process, termed BAG-instructed proteasome to autophagy switch and sorting (BiPASS), ubiquitinated HSPA1A-clients are sequestered into BAG3-associated p62/LC3-positive punctae and delivered to autophagosomes for degradation (see Figure 2). Surprisingly, instead of being involved in BiPASS, proteasomal impairment leads to the relocation of the BAG3-partner HSPB8 to stress-granules, which suggests that it plays a role in translational inhibition and/or the rescue of mRNA under conditions of cellular stress.\(^{57}\)

This interesting phenomenon seems to be especially apparent in the brain, where long-lived cells, such as thermally differentiated neurons, display a ‘switch’ in the BAG1:BAG3 ratio, whereas short-lived cells, such as astrocytes, do not display such a switch.\(^{95}\) Combined, these results further indicate that BAG proteins are modulators of PQC and may have role in neurodegenerative diseases.\(^{95}\)

### 1.7. Neurodegenerative Diseases;
#### Proteinopathies Affecting the Brain
Proteinopathies, such as Alzheimers disease (AD), Parkinsons disease (PD), Amyotrophic lateral sclerosis (ALS), and Huntington’s disease (HD), are progressive neurodegenerative disorders characterized by the -initially selective- loss of neurons in the Entorhinal cortex (AD), the substantia nigra (PD), the striatum (HD, or the upper and lower motor neurons in the cortex and spinal
Figure 2: BAG-induced switch and sorting (BiPASS). Following cellular stress, BAG3 is released from HSPB8 and competes with BAG1 for the binding of ADP-Hsp70 ubiquitinated client complexes. Whereas binding of BAG1 to the complex targets ubiquitinated Hsp70-clients to the proteasome for degradation, BAG3 binding targets ubiquitinated Hsp70-clients to the autophagy-lysosome system. Because BAG3-Hsp70 binding affinity is stronger than that of BAG1-Hsp70 binding affinity, increased cellular expression levels of BAG3 lead to the re-direction of ubiquitinated proteasomal Hsp70-clients to the autophagy lysosome systems under conditions of stress in a process termed BiPASS.

cord (ALS) that progresses through the brain via disease-stereotypic routes. It is now widely accepted that proteinaceous aggregates due to sporadic or inherited mutations in genes, including amyloid-β (Aβ), tau, α-synuclein, mutant huntingtin (mHtt), TDP43, and SOD1 are at the heart of this progressive neuronal degeneration and the associated symptoms.
1.7.1 Astrocytes; Another Player in Neurodegeneration

Many genes underlying neurodegenerative diseases are expressed ubiquitously and proteinopathy-associated aggregates can be observed in neurons as well as in other cells of the brain, called glial cells. Glial cells, including microglia, oligodendrocytes, NG2 cells, and astrocytes, engage in a dynamic range of functions that are essential for the development and physiology of the brain. Astrocytes are the most abundant cell type in the brain (up to 50%) and exist in various forms, which is defined by their morphology and localization within the brain, and fulfill distinct functions, including supplying metabolites to neurons, regulating the blood flow and the blood-brain-barrier, controlling extracellular concentrations of ions, neurotransmitters, and fluids, and releasing gliotransmitters to influence synaptic activity (tripartite synapse). Astrocytic aggregates have been observed in humans and in mouse models of PD, AD, ALS, SCA, and HD.

The distinct morphology and physiology of different neurons and astrocytes may cause differences in the reliance of various types of neurons on astrocytes specific to each brain area, making some neuronal types especially vulnerable to astrocytic (dys-)function and disease-specific patterns of neurodegeneration.

1.7.2 Neurodegeneration; from Off-Pathway Conformers to Protein Aggregation

Protein aggregates have been associated with both acute and chronic cellular stress. Unlike protein accumulation in acute cellular stress, disease-associated aggregates are usually initiated by a single (sometimes mutant) protein. These disease-associated proteins are not necessarily misfolded or (intrinsically) expose hydrophobic patches and their aggregation may require an additional “trigger” event in many instances.

If disease-associated mutant proteins are misfolded and hydrophobic stretches are exposed, they may be recognized and degraded by the PQC system, which may ultimately result in their loss-of-function (LOF) due to haploinsufficiency (e.g. cystic fibrosis, Gaucher’s disease). In contrast, disease-associated proteins that are not misfolded and/or expose hydrophobic surfaces (e.g. most neurodegenerative diseases) are generally not recognized by the
canonical PQC, which may result in dominant negative effects on the protein complexes the disease-associated protein functions in and/or in a toxic-gain-of-function (TGF) associated with the aggregation of the disease-associated protein (e.g. Aβ₁₋₄₂ in AD, mHtt in HD). In general, monogenic forms of neurodegenerative diseases are rare and histopathologically indistinguishable from the corresponding sporadic forms, indicating that the final disease pathway is common to both forms of disease. Although age and the specific disease-associated mutation seem to influence the age of onset in neurodegenerative diseases, disease-associated proteins most likely do not aggregate spontaneously; instead, aggregation initiation most likely requires additional processing, such as poorly understood external “trigger” factors. The following paragraph will elaborate on the aggregation process and aggregation-associated toxicity.

### 1.7.3 Proteinopathies; Aggregate Initiation and Toxicity

Aggregation is the conversion of specific proteins from their soluble and functional states into non-functional aggregates (see Figure 3). In vitro data indicates that the aggregation of aberrantly folded or imperfectly degraded proteins most likely progresses via ‘nucleated growth’, which consists of at least 3 different phases: Primary nucleation, secondary nucleation, and elongation. Research suggests that initial nucleation is followed by a lag-phase, during which nuclei are formed that is followed by the rapid exponential growth of fibrils. This process may result in the formation of off-pathway conformers that initially aggregate into oligomers, which consist of diffusible, non-fibrillar proteins. In the literature, the term “aggregate” is used ambiguously and usually defined by detergent solubility or the appearance of protein dense materials in immunohistochemical or immunofluorescent dye stainings. However, there are different forms of aggregates; they can organize into highly organized, β-sheet-rich, amyloidogenic structures or into more amorphous and porous aggregates and may occur intra- or extra-cellularly.

Some data suggest that off-pathway intermediate aggregate species, including soluble monomeric, fibrillar, and oligomeric protein species, are the primary toxic agents underlying most proteinopathies, whereas fibrils may be considered inert or even cytoprotective. However, as most of these arguments are based on experiments that study the effects of extra-cellularly
applied aggregates on dividing, non-neuronal cells with cell death or toxicity as endpoint (e.g. Mtt assays, dye uptake, caspase activation), these arguments may not apply or be less relevant to long-lived, non-dividing neurons in which aggregates arise intra-cellularly. Evidence from animal studies indicates that -at least in polyglutamine diseases- inclusion pathology and most symptoms may be observed before the onset of neuronal cell loss,115,116 further supporting the notion that cell death parameters as used in in vitro-experiments may not be a good proxy for assessing toxicity in neurodegeneration. To assess the relative (potential) neuronal toxicity of aggregates, it is important to also consider the secondary loss of function that may be caused by aggregates by entrapping other cellular proteins, enzymes, chaperones, and factors.105 For instance, the entrapment of transcription factors in aggregates may lead to the indirect inhibition of cellular signaling pathways and thereby impair regular cellular functions.

Several internal (e.g. mutations) and external (e.g. chemical or environmental stress) factors favor off-pathway folding, which may be counter-acted by molecular chaperones.108 For instance, the lag-phase required for protein nucleation is often regarded as rate-limiting for protein aggregation because proteins may still be targeted for refolding or degradation by the PQC system. The lag-phase may be shortened by certain protein characteristics, such as hydrophobic charge, secondary structure, amino acid sequence, and the presence of unfolded regions, thereby increasing the proteins propensity to aggregate;4 paragraph 1.9 and Chapter 3 will discuss the potential effects of chaperones on proteinopathy-associated proteins in more detail. Accumulating evidence suggests that aggregation in many proteinopathies, including neurodegenerative diseases, may be due to nucleated polymerization, whereas the age-related decline in protein quality control systems may determine the onset of these diseases.108 Alternatively -or additionally- an age-related change in external ‘trigger factors’ may lead to the sequestration of chaperones that may prime an imbalance in protein homeostasis.105

Taken together, current studies on aggregate toxicity mainly focus on the effects of extra-cellularly applied aggregates on cells and do not consider the fact that intermediate species, such as proto- and pre-fibrillar oligomers, may actually confer more direct toxicity through their ability to elongate and seed further aggregation of their soluble counter parts (e.g. nucleated polymerization) than fully formed aggregates.108
1.7.4 Inclusion Bodies; Organized Aggregate Storage

It has been noted that aggregated proteins, fibrils, and aggregate intermediates may be targeted into organized structures, called inclusion bodies, which are generally located at distinct cellular locations.\(^{61,117,118}\) Interpretations of studies on the cellular ability to store aggregates into inclusion bodies have often been confounded by discussions surrounding aggregate toxicity (see 1.7.1) and its potential to serve as a driver of aggregation ("aggregases"; i.e. active promotion of proteins towards aggregation). This reasoning is -at least partly- incorrect; inclusion body formation may rather represent an organized redirection of pre-existing, partially immobilized entities that are microscopically visible and must thus already exist in larger complexes (aggregates). We therefore refer to the re-organization of these complexes into an even larger structure at a defined position as a form of (temporal) aggregate storage.

The first evidence for the organized storage of aggregates was provided by experiments showing that misfolded proteins accumulate into so-called aggresomes near the microtubule organizing centre (MTOC).\(^{118}\) Further evidence indicated that heat-induced unfolded nuclear proteins temporarily accumulate at the periphery of nucleoli until they can be refolded by the Hsp70-machine once the cell returns to physiological temperatures.\(^{119}\) Later on, studies in yeast described the compartmentalization of aggregated proteins into IPODs and JUNQs.\(^{61,117}\)

A short description of these cellular "compartments" is provided below:

- **Aggresome**
  Ubiquitinated misfolded or aggregated proteins are directed to aggresomes through retrograde motor proteins on microtubules. Aggresomes are located at the MTOC next to the nucleus and co-localize with γ-tubulin, components of the proteasome, HSPs, and mitochondria. Vimentin and other filament protein form a cage around the core of this structure, which is most likely eventually cleared via autophagy.\(^{117,118,120,121}\) Recently, evidence of a transient localization of soluble proteins to an aggresome-like induced structure (ALIS) that co-localizes with ubiquitin and p62 aggresome-like is accumulating. This structure was observed following immune activation or stress conditions and is usually degraded by the UPS or autophagy.\(^{61,117}\)
• **Juxtanuclear quality control (JUNQ) or intranuclear quality control (INQs)**

   Generally thought to be located next to the nucleus, the JUNQ contains chaperones and proteasomal subunits and is thought to serve as (temporary) storage for soluble misfolded proteins that are targeted to the UPS for degradation or for chaperone refolding.\(^61,117\) Recent data have shown that this structure, now referred to as INQ, is located inside the nucleus, where it serves as temporary storage for cytoplasmic and nuclear proteins under conditions of stress.\(^122\)

• **Insoluble protein deposit (IPOD)**

   Insoluble aggregated proteins, such as disease-associated Htt, may be targeted to this cytoplasmic structure,\(^61\) which co-localizes with autophagy-associated proteins, suggesting that its constituents may eventually be degraded by autophagy.\(^61,117\)

• **Stress Granules (SGs)**

   The assembly of RNA binding proteins into transient SGs is mediated through prion-like glyicine-rich domains and serves to adapt protein expression levels to the stress response by sequestering, silencing, or degrading RNA transcripts. Furthermore, it was suggested that disease-associated proteins, such as mutant TDP43, FUS, and tau could perturb SG dynamics, thereby lengthening their cytoplasmic persistence. This can result in pathological SGs that initiate the formation of aggregates associated with dementia and Amyotrophic Lateral Sclerosis (ALS).\(^123,124\)

Irrespective of the type of ‘aggregate’ and its assumed toxicity, the storage of aggregates into inclusion bodies is regarded as a cellular attempt to diminish the toxicity of potentially harmful proteins, and hence considered to be cyto-protective.\(^61,117,125\) However, in order to fully assess the relative protectiveness or toxicity of aggregates, the toxicity of aggregate components as well as their localization and secondary effects need to be assessed.\(^105\) The intracellular storage of potentially large physical structures may confer multiple problems. Whereas nuclear aggregates may physically interfere with DNA and RNA transactions and therefore affect processes such as transcription or RNA processing, cytoplasmic inclusions may interrupt membrane integrity, interfere with ERAD, or impair intracellular trafficking (e.g. autophagy, stress granule dynamics).\(^105\) Neurons might be particularly
sensitive to the aberrant effects of aggregate localization, as aggregates may interfere with axonal vesicle transport and affect neuronal signaling, eventually resulting in Wallerian degeneration and neuronal death.\textsuperscript{126,127}

1.7.5 Aggregate Toxicity in Astrocytes

As stated before, astrocytic aggregates have been observed adjacent to aggregate-containing neurons as well as in brain areas that did not display neuronal aggregates.\textsuperscript{96} Such astrocytic aggregates may perturb regular astrocytic functions and/or make astrocytes reactive, which may contribute to neurodegeneration in AD, PD, ALS, and HD, as is exemplified below:\textsuperscript{96}

- **Functional impairment of astrocytes**
  Amongst others, astrocytic mHtt-expression was found to lead to a decrease in glutamate transport and enhanced calcium-dependent glutamate release in mice, thereby hypothetically increasing the likelihood of glutamate-mediated neuronal excitotoxicity cell non-autonomously.\textsuperscript{128-131} It has been suggested that mHtt-induced astrocytic and neuronal changes synergize to cause the HD-stereotypic degeneration of the striatum in a polyQ-length dependent manner.\textsuperscript{96}

- **Reactive astrocytes**
  An insult or injury to the neurons can cause astrocytes to become reactive, which is a graded astrocytic, immune-like, response that may range from hypertrophy, proliferation, migration, to the formation of a glial scar.\textsuperscript{96} Next to the release of neuroprotective substances (e.g. inflammatory modulators, chemokines, cytokines, neurotrophic factor), reactive astrocytes can also lead to the release of a variety of neurotoxic factors that may contribute to or exacerbate disease progression. The context, duration, and nature of the causative stimulus determine the exact nature and extent of this astrocytic immune response, which has been observed to varying degrees in the brains of AD-, PD-, HD-, and SOD1-mediated ALS-patients.\textsuperscript{96} As example, reactive astrocytes that surround $\text{A}_\beta\text{1-42}$ plaques have been shown to produce high levels of pro-inflammatory cytokines that are thought to exacerbate neuronal damage in AD.\textsuperscript{96,132,133} Similar observations have been made for PD, where alpha-synuclein containing astrocytes have even been detected in brain areas without neuronal Lewy bodies.
1.9. The Prion Hypothesis

In recent years, evidence has accumulated that indicates proteinopathy-associated proteins, including Aβ₁-42, tau, alpha synuclein, SOD1, and Htt, may behave like prions and convert their native, non-aggregation prone, counter parts into aggregated forms that can propagate from one neuron to the next, thereby progressively spreading neurodegenerative diseases across different brain areas.

Prion-diseases, such as Creutzfeld-Jakob disease, scrapie, or bovine encephalopathy, are proteinopathies that may be genetic, sporadic, or contagious (e.g. consumption of infected meat). Prion-diseases cause progressive neurodegeneration, which is tightly associated with the conversion of the normally folded PrP proteins into an abnormal, infectious, aggregated prion form (‘template assistance’ model, see Figure 3).

Figure 3: Protein aggregation and Prion-like seeding. Aggregation is the conversion of specific proteins from their soluble, functional states into non-functional aggregates. ‘Nucleated growth’ of aberrantly folded or imperfectly degraded proteins may lead to the rapid exponential growth of pre-fibrils and the formation of toxic off-pathway conformers. These toxic conformers may initially aggregate into oligomers consisting of diffusible, non-fibrillar proteins and grow into native fibrils that can grow indefinitely, eventually entrapping the components of the PQC. The exact sequence of events is yet unclear and may depend on multiple factors. Aβ₁-42, tau, alpha synuclein, SOD1, and Htt, may behave prion-like and convert their native, non-aggregation prone counter parts into aggregated forms that can propagate from one neuron to the next and seed aggregation (‘template assistance’ model). It has yet to be determined which of the (pre-)aggregate species confers this prion-like properties.
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Aggregated prion-oligomers tend to be unstable and grow by incorporating prion proteins in similar conformational states until a stable nucleus is formed. Stable prion aggregates are thought capable of growing indefinitely and break into smaller fragments that may act as nuclei for new prion aggregates in adjacent cells (‘seeded polymerization’ model). Furthermore, PrP aggregates were found to progress from one cell to another via tunneling nanotubes, which are 50-200nm diameter actin-rich hollow filaments that interconnect cells in culture. Other potential mechanisms of transmission are active PrP release via vesicle-mediated exocytic processes (e.g. incomplete autophagocytosis, lysosomal exocytosis) and passive PrP release (e.g. local rupture of the plasma membrane, cell lysis); PrP is most likely internalized via endocytosis or by directly crossing the cellular membrane of adjacent cells.

1.9.1 Proteinopathies; Prion-Like Propagation of Neurodegeneration

The classification of a protein as prion-like encompasses several criteria, including

- irreversibility of non-native protein assemblies
- efficiency of cell-to-cell transfer, inherent to aggregates
- infectiousness of disease-associated protein
- transmittance of disease-associated proteins

Although it is still debated whether the diseased proteins underlying neurodegenerative diseases may indeed be classified as Prion-like this definition might explain the (seemingly stereotypic) progression of neurodegenerative diseases beyond the neurons or brain areas of aggregate initiation.

Accumulating evidence indeed indicates that most neurodegenerative proteinopathies may have prion-like properties:

- As discussed above, all proteinopathy-associated aggregates discussed in this chapter are either largely resistant to the cellular PQC

- Originally, it was observed that some healthy human fetal grafts that were transplanted into the brains of patients with PD contained Lewy bodies and Lewy neurites after a time period of 10 to 14 years. It has since been shown that the extracellular application of proteinopathy-associated proteins results in the cellular entrance of aggregates in vitro. Although these in
vitro results were frequently considered to be artifacts, the localized injection, over-expression, and transplantation of artificially generated peptides and of human brain homogenates confirmed that disease-associated Aβ, tau, and α-synuclein may propagate to originally non-affected cells in rodent-brains according to a stereotypic propagation pattern.\textsuperscript{147} Although similar mammalian studies have yet to be performed for PolyQ disease- and ALS-associated proteins, recent findings from the Kopito group provide initial evidence for the cell-cell propagation of disease-associated Htt in D. melanogaster.\textsuperscript{148} This idea is further explored in Chapter 4.

- A self-propagating agent is considered to be infectious if it is capable of generating identical copies of itself through various mechanisms that may or may not require exogenous agents.\textsuperscript{149} The ability of proteinopathy-associated mutant proteins to seed the aggregation of native conformers was proven in cells (of polyQ, tau, α-synuclein, SOD1) and in mice (tau, α-synuclein).\textsuperscript{108,150} Although little evidence exists for the Aβ-mediated seeding of intra-cellular aggregates, a study in mice indicates that different Aβ-strains govern the type of aggregates.\textsuperscript{151} Similar evidence exists for α-synuclein, which indicate that some -but not all- α-synuclein-strains seed morphologically distinct aggregates.\textsuperscript{152}

- Findings that Aβ, as well as α-synuclein, can be transported across the blood brain barrier, and may thereby leave and enter the brain, raised debates on the possible prion-like transmittance -and thus contagiousness- of these proteinopathies.\textsuperscript{153,154} These debates were fueled by a recent study that analyzed post-mortem brain tissue of patients with Creutzfeldt-Jakob disease due to injections of growth hormone derived from human cadavers who had died in their 40s and 50s. Although neither of these patients had any AD risk factors, some brain samples not only contained PrP\textsubscript{Sc}-aggregates but also Aβ-plaques reminiscent of early stage AD.\textsuperscript{155} Although it has yet to be established whether these Aβ-plaques originated from the patients’ growth hormone injections, the study fueled debates on the possible transmittance of proteinacious (Aβ) aggregates.\textsuperscript{156}

Taken together, these studies support the hypothesis that the cell-to-cell transmission of aggregate (precursors) and their seeding of native conformers may be a generic phenomenon at the origin of most neurodegenerative diseases.
and govern the disease-specific stereotypic propagation of neurodegeneration through the brain. Whether these proteinacious aggregates can also be transmitted between humans –or even species- needs yet to be established.

1.9.2 Astrocytes; Possible Role in Prion Propagation
Astrocytes are a particularly interesting cell type as their intricate connections with -and close proximity to- neurons (e.g. nutrient exchange, tripartite synapse) might enable them to ameliorate the prion-like neuron-to-neuron transmission of aggregates in neurodegenerative diseases. Next to the already described astrocytic functions in maintaining neuronal homeostasis and their role in the brains immune response, astrocytes have also been shown capable of internalizing (pre-)aggregated protein species, including Aβ1-42, α-synuclein, and mHtt. Although the mode of uptake is still under investigation and most likely depends on the target protein itself, the ability of astrocytes to restrict or slow down the prion-like propagation of proteinacious aggregates from one neuron to the next by internalizing extra-cellular aggregates and hereby protect the progression of neurodegeneration would open up intriguing alternative possibilities for disease interventions.

Taken together, specific characteristics of the disease-associated proteins in neurons and glial cells (α-synuclein in PD, Htt in HD, Aβ1-42 in AD, SOD1 in ALS), their localization, secondary intra-cellular effects and their propensity to act as prion-like seed in adjacent cells, thereby spreading disease to adjacent brain cells, most likely contribute to the disease-associated molecular toxicity.

1.10 Chaperones; Aggregate Prevention and Disposal
As stated above, most canonical heat shock proteins can bind client proteins that expose hydrophobic interactive surfaces. However, in general, the initiation of amyloidogenic aggregates is not mediated through hydrophobic interactions but through H-bonding, which may prevent their detection by the canonical chaperone system. Interestingly, some non-canonical members of the HSP families have been found capable of recognizing these amyloidogenic cores in proteins, prevent the initiation of aggregation, and target early intermediate species for degradation. Although chaperones are generally not able to disaggregate ‘naked’ amyloids, they may bind hydrophobic sides chains of growing amyloids, thereby slowing down the elongation-phase (e.g. allowing for
aggrephagy before they grow to larger entities), preventing the sequestration of other proteins into the aggregates, and processing aggregated proteins into inclusion bodies. In addition such side-chains may form anchors for chaperones for disaggregation.

Besides the above-mentioned general chaperone-effects on aggregation-processes, pathways leading to protein aggregation inherent to various disease-associated proteins may differ. This not only pertains to the (final) aggregates formed by individual proteins, but also to the routes leading to the final (often morphologically similar) amyloid fiber. To answer the question whether specific chaperones are need to handle different toxic protein species, we investigated the existing literature on the activity of mammalian chaperones against multiple toxic protein species in various model organisms in chapter 3.

1.10.1 Chaperones; potential functions in prion-like aggregate transmission
Another potential function for chaperones may lie in restricting the prion-like propagation of proteinopathy-associated ‘infectious’ proteins by: \(^{108, 137}\)
- Interfering with aggregate-release; e.g. by promoting exocytosis
- Interfering with aggregate-uptake by (adjacent) neurons (e.g. reduced neuronal uptake or by enhancing uptake by astrocytes); e.g. several chaperones have been associated with the endocytic machines
- Interfering with aggregate-seeding in otherwise healthy neurons following aggregate uptake; e.g. by inhibiting secondary nucleation or elongation
- Protecting negative consequences of seeding aggregation in recipient cells; e.g. preventing aggregate toxicity

In Chapter 4, we investigated the question whether and how the over-expression of the chaperone DNAJB6b in astrocyte-like cells can ameliorate disease progression in a DM-model for HD in which mHtt is specifically expressed in neurons.

1.11 Chaperonopathies; the crucial role of the PQC in muscle formation and maintenance
Interestingly, a number of protein aggregation diseases have recently been linked to mutations in HSPs. These so-called chaperonopathies are responsible for some
myopathies, cardiopathies, neuropathies, and retina-related diseases.\textsuperscript{6,167–169} Several chaperonopathies are dominantly inherited\textsuperscript{168,169} and it is not clear whether these are TGF diseases similar to classic aggregation disease or whether they are due to a LOF of chaperone activity (haploinsufficiency) or whether they have dominant negative (DN) effects on the chaperone complex they function in. The muscle cells in heart and skeletal tissues are subject to continuous mechanical stress, suggesting that they may highly depend on maximal chaperone activity. Accordingly, studies over the past decade have shown that the active regulation of chaperones plays an essential role in myofibrillogenesis, the maintenance of muscle cell structure, and in in the maintenance of protein homeostasis under conditions of stress.\textsuperscript{170,171} For instance, the Hsp70-machine may protect against cellular damage and dysfunction (e.g. following eccentric exercise or injury), HSPBs may support muscle cell structure, and proteins involved in autophagy and the UPS may mediate cellular remodeling and remove abnormal or damaged proteins and organelles.\textsuperscript{170,172–174} Subsequently, deficiencies and over-activity of various PQC components, including HSPBs, proteasomal, and autophagosomal components, have been associated with muscular dystrophies, myopathies, cardiomyopathies, and neuropathies through various mechanisms.\textsuperscript{5,170,172–174} While chaperonopathies are extensively reviewed in Chapter 3 (BARcoding heat shock proteins to human disease)\textsuperscript{6}, this introduction shortly introduce chaperonopathies caused by mutations in BAG3, which was the central focus of Chapter 5 in this PhD thesis.

\subsection*{1.11.1 BAG3 Mutations; PQC Out of Balance}
As stated above, BAG3 is a NEF for Hsp70 machines that has been associated with a process called ‘BIPASS’, i.e. the rerouting of Hsp70-bound proteasomal clients (see 1.6).\textsuperscript{5,7} Via its two IPV-domains, BAG3 forms stable complexes with HspBs and HSPB8 in particular\textsuperscript{35}, which disperse upon proteotoxic or cellular stress following which BAG3 functions in BIPASS and HSPB8 in stress granules (unpublished data).

Multiple dominant mutations in BAG3 have been causatively associated with a variety of human muscular and cardiac diseases. Most of these BAG3 mutations are located in the BAG-domain (Hsp70-binding) or in or in the vicinity of the IPV-domain (HspB-binding); the specific location of the mutation determines the
disease type (neuropathy, cardiopathy, and/or myopathy), the severity (mild vs. fatal), and the age of onset (childhood vs. late adulthood).\textsuperscript{175-183}

One BAG3 mutation, BAG3 P209L, is located in the second IPV-motif and has been associated with a particularly devastating pathology that starts early in life and results in a fulminant myofibrillar myopathy accompanied by a cardiopathy, and respiratory complications.\textsuperscript{175-183} Observational studies indicate that the BAG3 P209L mutation is associated with the accumulation of several Z-disc associated proteins and the aggregation of BAG3 P209L in-between myofibrils, a thickening of the Z-disc, and myofibrillar and muscular disintegration.\textsuperscript{175,184} Interestingly, BAG3 knock-out mice also develop Z-disk defects that eventually result in non-inflammatory fiber-degeneration and the lethal failure of intercostal muscles, suggesting LOF as possible mechanism.\textsuperscript{185} To further elucidate whether the BAG3 P209L-mutation causes disease by LOF mechanism or not, we studied its effects in detailed cellular experiments (Chapter 5).
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