Chaperone Disaggregation Machinery in Mammalian System and the Role of DNAJB1

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Abstract

The protein aggregates can be toxic to the cells. A number of neurodegenerative diseases result from the accumulation of misfolded protein aggregates, in some cases caused by mutations. Therefore, it is important for the cells to have a system protein homeostasis, or proteostasis, that repairs or removes misfolded protein, to maintain viability. The key proteins that manage the misfolded proteins in the cells are the molecular chaperone Hsp70 and its co-chaperones. Hsp70 mediates protein refolding and also directs proteins into degradation, either by the ubiquitin-proteasome system (UPS) or autophagy. However, disaggregation of proteins in mammalian cells is not well understood. For a long time, it was thought that animal cells lack disaggregation activity, but Hsp70 is now thought to mediate disaggregation. In our study, we aim to demonstrate disaggregation activity in mammalian cells and to elucidate the mechanism of the core disaggregation machinery. Experiments using heat shocked luciferase and amyotrophic lateral sclerosis (ALS) related SOD1 mutant G85R both show that there is disaggregation in mammalian cells. In the heat shock response, we have found that the DNAJ protein, DNAJB1, and the small heat shock protein, HspB1, are the most induced in heat stress, suggesting they have an important role in maintaining the proteostasis in cells. Overexpression of DNAJB1 in heat shocked cells, surprisingly, resulted in suppression of protein folding as well as disaggregation. However, DNAJB1 knock out shows that DNAJB1 is an important part of disaggregation. Knocking down HspB1 did not affect disaggregation significantly. Chaperones and co-chaperones were tested for co-localization with aggregates, and a subset was found to associate, and therefore may be involved with disaggregation. These included Hsc70, Hsp70, DNAJA, DNAJA2, DNAJB1, and HspB1.

Abstract (Français)

Les agrégats de protéines peuvent être toxiques pour les cellules. Un certain nombre de maladies neurodégénératives résultent de l'accumulation d'agrégats de protéines mal repliées, dans certains cas causés par des mutations. Il est donc important que les cellules disposent d'un système d'homéostasie des protéines, ou protéostasie, qui répare ou élimine les protéines mal repliées, afin de maintenir leur viabilité. Les protéines clés qui gèrent les protéines mal repliées dans les cellules sont le chaperon moléculaire Hsp70 et ses co-chaperones. Hsp70 assure la médiation du repliement des protéines et dirige également les protéines vers la dégradation, soit par le système ubiquitine-protéasome (UPS), soit par l'autophagie. Cependant, la désagrégation des protéines dans les cellules de mammifères n'est pas bien comprise. Pendant longtemps, on a pensé que les cellules animales n'avaient pas d'activité de désagrégation, mais on pense maintenant que Hsp70 est le médiateur de la désagrégation. Dans notre étude, nous visons à démontrer l'activité de désagrégation dans les cellules de mammifères et à élucider le mécanisme de la machinerie centrale de désagrégation. Des expériences utilisant de la luciférase ayant subi un choc thermique et le mutant G85R de SOD1 lié à la sclérose latérale amyotrophique (SLA) montrent qu'il existe une désagrégation dans les cellules de mammifères. Dans la réponse au choc thermique, nous avons constaté que la protéine DNAJ, DNAJB1, et la petite protéine de choc thermique, HspB1, sont les plus induites en cas de stress thermique, ce qui suggère qu'elles jouent un rôle important dans le maintien de la protéostase des cellules. La surexpression de DNAJB1 dans les cellules soumises à un choc thermique a, de manière surprenante, entraîné la suppression du repliement des protéines ainsi que leur désagrégation. Cependant, l'élimination de DNAJB1 montre que cette dernière joue un rôle important dans la désagrégation. Le knocking down de HspB1 n'a pas affecté la désagrégation de manière significative. Les chaperons et les

co-chaperons ont été testés pour la co-localisation avec les agrégats, et un sous-ensemble a été trouvé pour s'associer, et donc peut être impliqué dans la désagrégation. Il s'agit de Hsc70, Hsp70, DNAJA, DNAJA2, DNAJB1 et HspB1.

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Introduction

1. The chaperone system

1.1 Proteostasis

Proteostasis, protein homeostasis, maintains the balance within the cellular proteome via regulating various processes that determine the states of proteins. Functional proteins are in the native state, folded into the correct three-dimensional structure dictated by the amino acid sequences. However, proteins can lose their native conformation due to inherent genetic mutations or various stress conditions such as oxidation or heat. Since the non-native proteins are dysfunctional, keeping the conformational integrity is a crucial matter for the cell's well-being. Thus, proteostasis includes mechanisms that promote the folding of proteins, or the removal of damaged proteins (*1*)

Protein Folding Energy Landscapes

In early days, researchers thought that proteins are folded in well-defined sequential steps to reach the final native conformation. However, it was found that unfolded and partially folded proteins are conformationally heterogeneous. Therefore, the protein folding energy landscape was introduced to explain how polypeptides search for the native state on a rugged energy surface until the energetically favorable native state is achieved. As the number of native contacts increases, the number of available conformations decreases, eventually driving the polypeptides into the native form (*2*). Thus, different states of proteins can be described using the protein folding energy landscapes (*3*). The unfolded polypeptides progress to the native state by searching the funnel-shaped potential energy surfaces downhill (**Figure 1.1**). However, the energy surface is often rugged, which means that polypeptides must overcome several kinetic

barriers to reach the native state. Some proteins have higher kinetic barriers compared to the others. Such proteins are often heavily dependent on specialized proteins called molecular chaperones, which aid them to go over the kinetic barriers toward the native conformation (*4*).

Without molecular chaperones, the population of misfolded or partially folded proteins may increase. These polypeptides often have exposed hydrophobic surfaces which are prone to interact with each other intermolecularly, eventually leading to protein aggregation (*3, 5*). Protein aggregation is often toxic to the cell in two ways: the loss of function and the gain of toxicity. Misfolded, aggregated proteins cannot perform their intended functions. Not only that, they take away other proteins from cellular processes by abnormal interaction and co-aggregation. In serious conditions, toxic aggregates may result in cell death (*6, 7*). Protein aggregation will be further discussed in section 3.1.

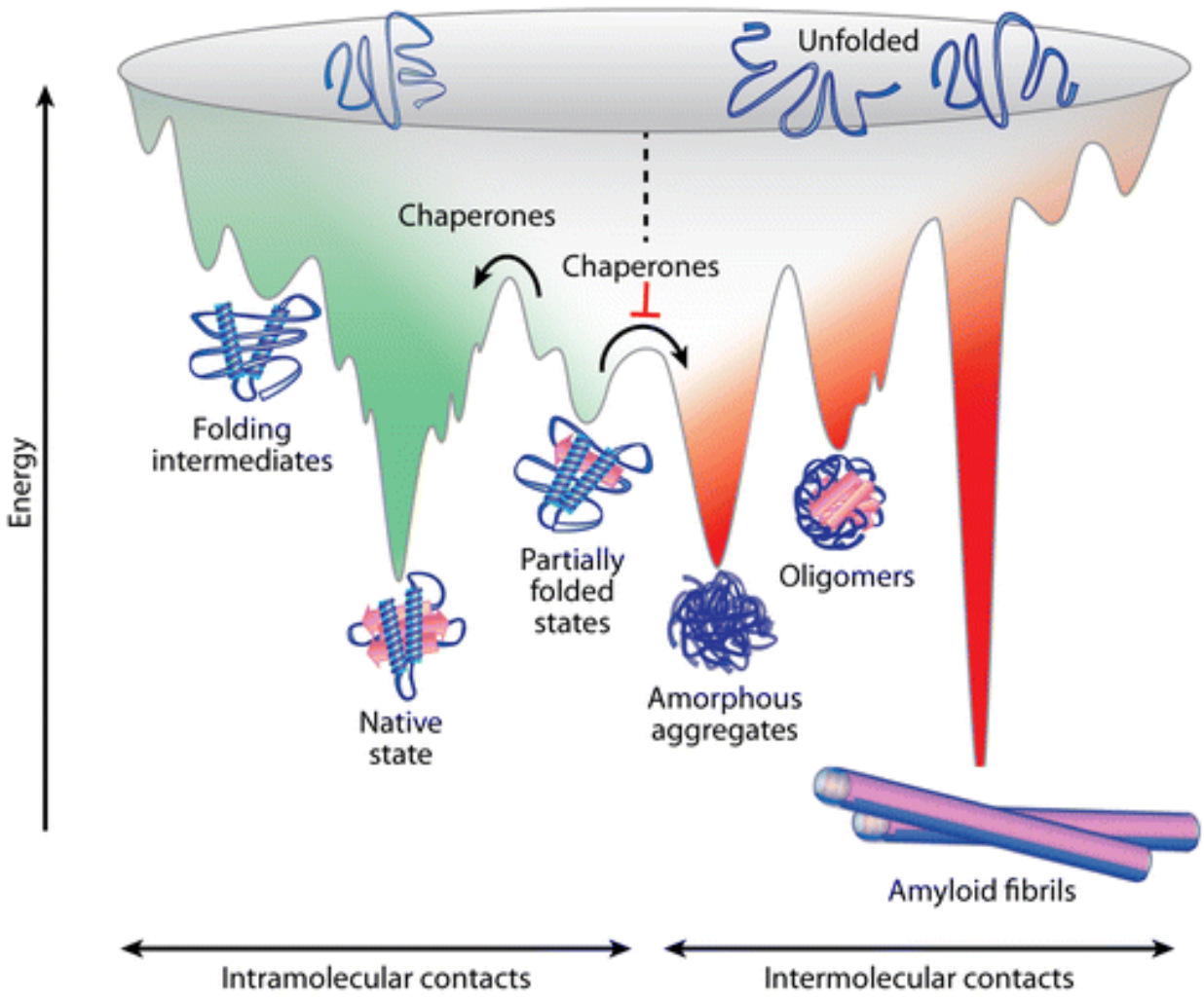


Figure 1.1 Protein Folding Energy Landscapes

Proteins exist in many different states. The unfolded polypeptides search rugged energy surfaces and go through energy barriers to reach the energetically favorable native state. Polypeptides may be trapped in partially folded states or folding intermediates which can lead to aggregation via intermolecular contacts (*3*).

Protein Quality Control Network

To protect cells from proteotoxicity, cells have developed the protein quality control network. The protein quality control network consists of molecular chaperones and co-chaperones, and the degradation machineries within the cell. Molecular chaperones are proteins that assist other proteins to fold and to assemble correctly, and they may also carry out other functions. Co-chaperones are proteins that regulate the activity of the chaperones. When proteins are misfolded, chaperones can unfold and refold the misfolded proteins. When proteins are aggregated, chaperones can disaggregate the proteins. When proteins are not recoverable, chaperones help to clear them via degradation. Overall, chaperones and co-chaperones ensure that proteostasis is maintained by regulating the well-being of the proteins (**Figure 1.2**).

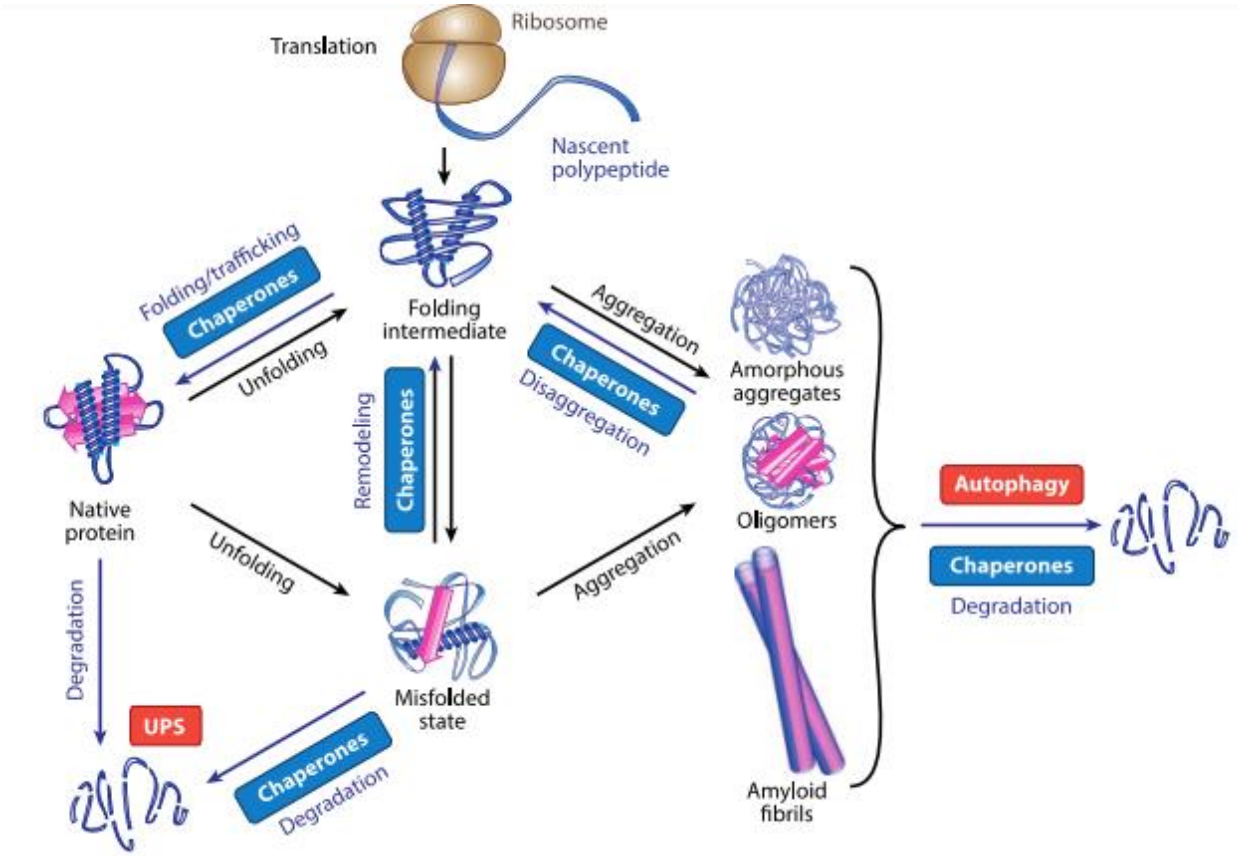


Figure 1.2 Protein quality control network

Protein quality control network consists of chaperones and co-chaperones which guide nascent polypeptides to the native proteins. Chaperones are also involved in refolding of misfolded proteins, disaggregation of protein aggregates and degradation of aberrant proteins (*3*).

Heat Shock Response

When the cells are stressed, they increase the number of molecular chaperones and co-chaperones to fight against the expected proteotoxicity in a process called the heat shock response (HSR). HSR is regulated by the transcription factor heat shock factor 1 (HSF1). In non-stress conditions, HSF1 is sequestered by molecular chaperones. Specifically, Hsp70 and DNAJB1 are shown to bind HSF1 (*8, 9*). When bound to chaperones, HSF1 is in the monomeric form which is inactive. Inactive HSF1 is in monomer-dimer equilibrium in both nucleus and cytosol (*10, 11*). When stressed, HSF1 is phosphorylated at different serine sites by various protein kinases. For example, serine 307 is phosphorylated by MAPK and serine 303 by GSK3. This causes HSF1 to trimerize and accumulate in the nucleus. Trimerized HSF1 has increased affinity for heat shock elements (HSEs) which are the promoter regions of different molecular chaperones. Once bound to HSE, HSF1 recruits transcriptional machinery and activates transcription (*12, 13*). Once transcription is activated, HSF1 becomes SUMOylated by the SUMOylation machinery. SUMOylated HSF1 has repressed activity due to the decreased interaction with transcriptional machinery (*8*). Once an excess population of chaperones are made, they bind and monomerize HSF1, mediating its dissociation from DNA and stopping transcription of chaperones. Thus, HSF1 cycle is under a feedback regulation (**Figure 3.3**). Thanks to HSF1-mediated HSR, the increased number of chaperones enables the cells to survive the proteotoxicity. However, when the proteotoxicity is too great and/or when the chaperone capacity is insufficient, the cells can lose function or die.

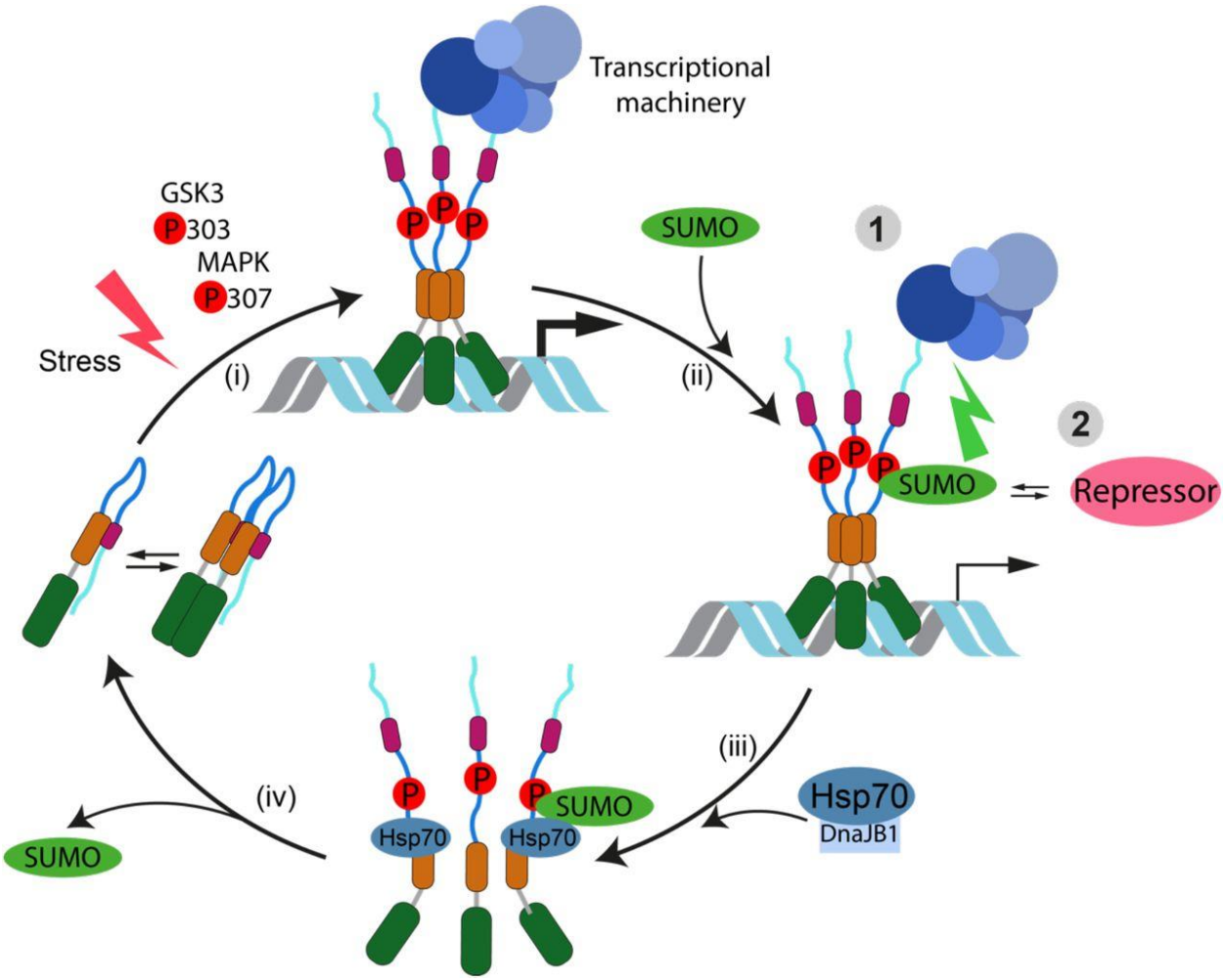


Figure 1.3 HSF1 is under feedback regulation by chaperones and co-chaperones

When stressed, HSF1 is phosphorylated and trimerizes. Trimerized HSF1 binds DNA and promotes transcription of chaperones and co-chaperones. Once SUMOylated, HSF1 loses its interaction with transcriptional machinery. Chaperones, especially Hsp70 and DNAJB1, bind and inactivate HSF1, decreasing the amount of chaperone production (*8*).

Neurodegenerative Diseases

Decreased protein quality control capacity has been implicated in many diseases. For example, the increased proteotoxicity due to low chaperone activities are the hallmark of the aging-related neurodegenerative diseases. With age, the protective roles of chaperones deteriorate and as result, protein aggregates accumulate in cells. Accumulation of toxic protein aggregates in neurons eventually leads to the neuronal death. Consequently, neurogenerative diseases are associated with the aggregation of misfolded proteins. Some aggregated proteins in neurodegeneration include amyloid β (Aβ) and tau protein in Alzheimer's Disease (AD), α-synuclein (α-syn) in Parkinson's Disease (PD), polyglutamine (polyQ) expansion on huntingtin (Htt) in Huntington's Disease (HD), and superoxide dismutase 1 (SOD1) in Amyotrophic lateral sclerosis (ALS) (**Table 1.1**) (*14*, *15*). With the rising number of aging populations in the world, the importance understanding proteostasis is escalating.

Table 1.1 Neurodegenerative diseases and the major aggregating protein

Neurodegenerative Diseases	Major Aggregating Protein
Alzheimer's disease	amyloidβ tau
Amyotrophic lateral sclerosis	SOD1 TDP-43 FUS
Huntington's disease	Htt (glutamine expansion)
Parkinson's disease	α-synuclein

1.2 Hsp70

The 70 kDa heat shock protein (Hsp70) is at the centre of the protein quality control network. The molecular chaperones of the Hsp70 family are involved in protein folding, refolding of misfolded proteins, disaggregation of protein aggregates, and assembly of newly folded proteins. Not only that, Hsp70s also play a role in protein translocation and degradation (*16-18*). Hsp70's diverse functions in protein quality control network come from its interaction with various co-chaperones and other co-factors. (**Figure 1.4**). The most important co-chaperones of Hsp70 include the J domain proteins, DNAJs, and the nucleotide exchange factors, NEFs. Co-chaperones will be addressed in more detail in section 1.3.

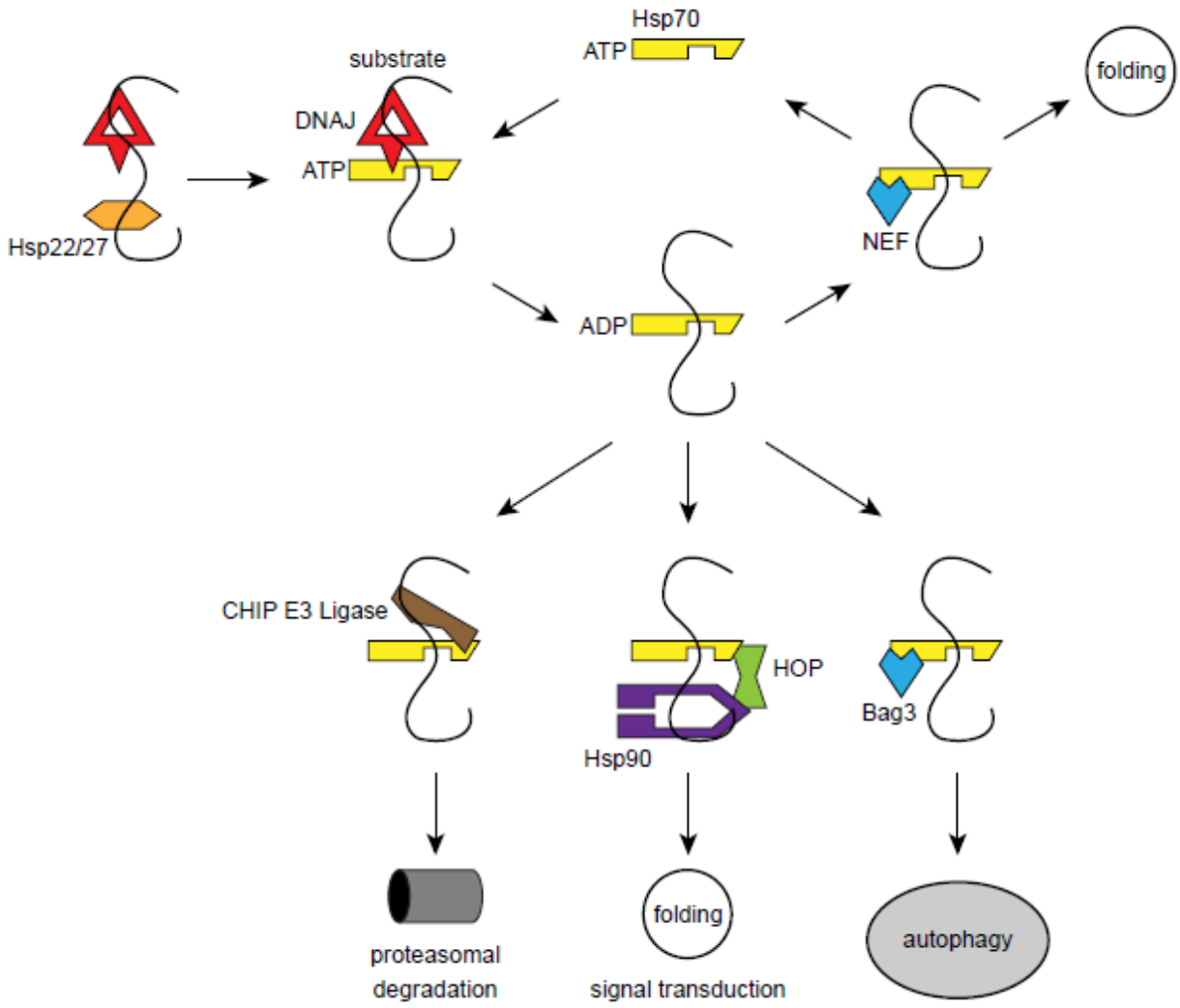


Figure 1.4 Hsp70 interacts with a variety of co-chaperones and is involved in many different cellular pathways

Hsp70's ATPase cycle is accelerated by DNAJs and NEFs, promoting protein folding. Hsp70 interacts with the E3 ubiquitin ligase CHIP to lead proteins to proteasomal degradation. Hsp70 forms a complex with Hsp90 via HOP and mediate the folding of many proteins important for signal transduction. Hsp70 also interacts with Bag3 to bring the proteins to autophagy (*19*).

Hsp70 vs Hsc70

In the cytosol, the major Hsp70 family chaperones are the constitutively expressed Hsc70 and the stress-induced Hsp70. Hsc70 and Hsp70 share 94% homology. They were first thought to be redundant in terms of their functions. However, more recent evidence has shown that they are not identical in their cellular roles. It was shown in mouse that the deletion of Hsc70 is lethal while the deletion of Hsp70 is not (*20*). A study in yeast has also shown that the presence of Hsc70 alone supports the growth of yeast while Hsp70 does not (*21*). Despite the differences, their major roles in proteostasis mostly remain the same.

Hsp70 ATPase Cycle and Two Conformations

Hsp70 functions by alternating between two different conformations: open and closed. The conformational change is coupled with Hsp70's ATPase cycle. Hsp70s consist of two domains, the N-terminal nucleotide-binding domain (NBD) and the C-terminal substrate-binding domain (SBD), connected by a linker (**Figure 1.5A**). The NBD has a cleft where ATP/ADP can bind (*22, 23*). SBD is further divided into SBDβ, which binds substrates, and SBDα, which functions as a lid (*24, 25*). When ATP binds the NBD, a conformational change occurs and causes the SBD to open. In this open state, SBDα is away from SBDβ, allowing substrates to bind to SBDβ. At the same time, the linker binds to NBD which brings SBDβ closer to NBD and therefore Hsp70 becomes compact (*26*). Once a substrate is bound, ATP is hydrolysed to ADP which results in another conformational change. Upon ATP hydrolysis, the SBDα closes onto SBDβ, clamping the substrate in a closed conformation. The linker is extended, pushing the NBD away from the SBD, causing Hsp70 to be elongated. Once ADP is released, another ATP binds the NBD, cycling back to the open conformation and therefore releasing the substrate (**Figure 1.5B**) (*27, 28*). The repeated binding and release of substrate is thought to be the key to

the Hsp70 protein folding mechanism. Therefore, Hsp70 ATPase cycle coupled with the conformational changes are essential for its roles in proteostasis.

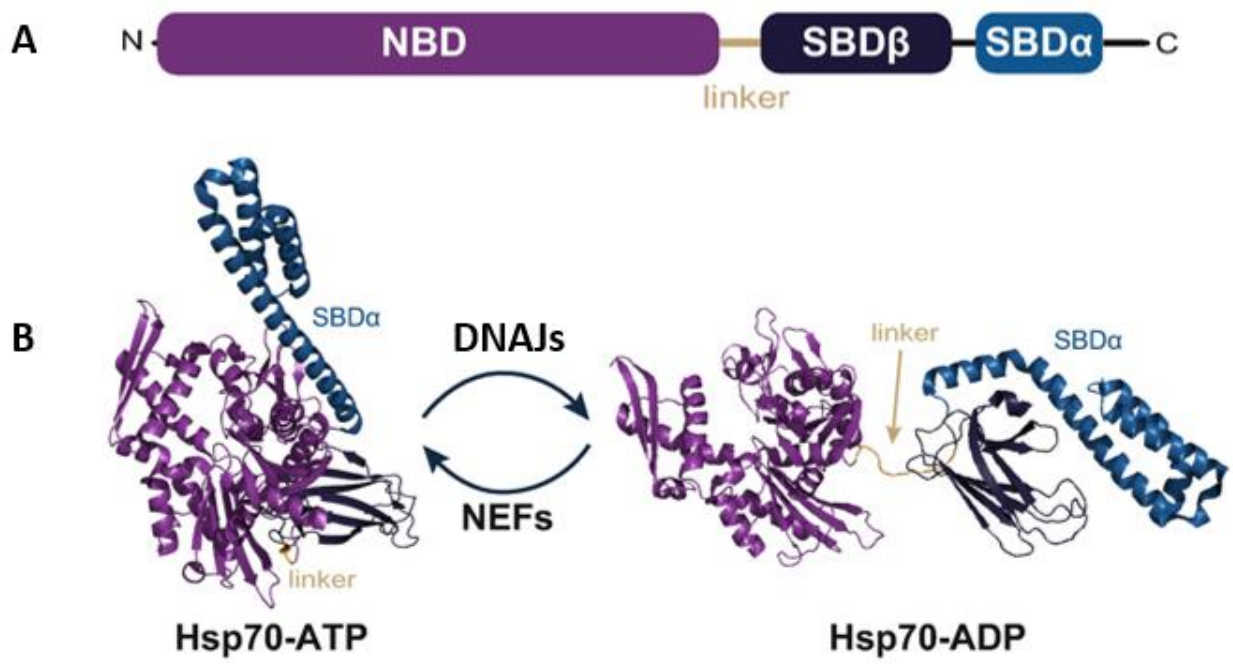


Figure 1.5 Hsp70 structure and two different conformations

Hsp70 is consisted of NBD, linker, SBDβ and SBDα. Hsp70 has two conformations: ATP-bound open, compact conformation and ADP-bound closed, elongated conformation. The cycle between two different conformations is promoted by the co-chaperones, DNAJs and NEFs. (*29*)

1.3 Hsp70 co-chaperones

By itself, an Hsp70 chaperone's intrinsic ATPase activity is very low. In most cases, Hsp70 interacts with two major groups of co-chaperones that promote its ATPase cycle, namely, DNAJs and NEFs.

DNAJs

DNAJ family proteins have diverse structures but they all share one conserved domain called the J domain. Some DNAJs have a C-terminal polypeptide-binding domain that can bind unfolded polypeptide substrates. The N-terminal J domain, on the other hand, binds the NBD of Hsp70 and recruits its substrate to the SBD of Hsp70. Once the J domain binds the NBD, it accelerates ATP hydrolysis by Hsp70 which causes the allosteric change in Hsp70 to clamp onto the substrate. In the J domain, there is a conserved His-Pro-Asp (HPD) motif which is essential for the stimulation of ATPase activity (*30*). DNAJ proteins will be addressed in more detail in section 2.

NEFs

Nucleotide exchange factors (NEFs) promote the Hsp70 ATPase cycle by assisting in ADP replacement. They bind to the NBD of Hsp70 and cause conformational change in the ATP binding site, resulting in the release of ADP and the exchange for ATP (*31*-*33*). There are two main groups of NEFs for Hsp70: the heat shock protein 110 kDa family (Hsp110s) and Bcl-2-associated athanogene family (Bags).

Hsp110 family proteins

Hsp110 family proteins includes Hsp110, APG1 and APG2 in the mammalian cytosol (*34*). The Hsp110 family is structurally related to the Hsp70 family and sequence similarity between members may be as high as 70% (*35, 36*). Accordingly, the overall structure is also very similar, with N-terminal NBD and C-terminal SBD. Hsp110 needs ATP to work as an NEF but whether it has its own ATPase cycle is unclear. The SBD shows some evidence that it can bind polypeptides (*37, 38*). Despite the sequence and structural resemblance, Hsp110 cannot replace Hsp70 functionally. Rather, it works as a regulatory co-chaperone protein for Hsp70.

Hsp110 assists Hsp70 in protein folding. In yeast, lack of Sse1, the yeast Hsp110, impairs folding of nascent polypeptides (*39, 40*). A pure protein study also shows that adding Hsp110 to Hsc70-DNAJA2 increases protein folding (*41*). Hsp110 is also a part of the mammalian disaggregase system along with Hsp70 and DNAJB1 (*42-44*). Consequently, various studies have indicated the importance of Hsp110 in different neurodegenerative diseases. In mouse studies, lack of Hsp110 increases the age-dependent accumulation of tau proteins (*45*) and mutant SOD1 (*46*). In cells, Hsp110 prevents the aggregation of polyQ-Huntingtin protein along with DNAJB1 (*47*). Hsp110 is also involved in protein degradation in complex with Hsp70 by delivering the substrates to proteasome (*48*).

Bag family proteins

Bag proteins are a group of proteins sharing a conserved BAG domain, which interacts with Hsp70's NBD. Bag proteins also interact with many other proteins and therefore have a wide range of cellular functions from apoptosis to proliferation (*49, 50*). In humans, there are 6 different Bag Proteins: Bag1 to Bag6, all in the cytosol (*51*). (figure (*52, 53*)) Among them, Bag1

is the most studied and it plays a role in the regulation of proteasomal degradation (*54*). Bag2 and Bag3 are the most abundant Bag proteins in humans (*55*). Contrary to Bag 1, Bag2 inhibits ubiquitin ligase activity related to the C-terminus of Hsp70 interacting protein (CHIP). CHIP is an E3 ubiquitin ligase that mediates the attachment of a ubiquitin chain to Hsp70 client proteins. It therefore targets misfolded proteins to proteasomal degradation (*56*). Bag2 inhibits CHIP by disrupting its interaction with ubiquitin-conjugating E2 enzymes. By inhibiting CHIP-mediated degradation, Bag2 allows maturation of certain proteins such as CFTR (*31*, *57*). Bag3 interacts with a wide range of proteins via several domains and motifs. It has 2 Isoleucine-Proline-Valine (IPV) motifs which bind the small heat shock protein chaperones HspB8 as well as HspB6 (*58-60*). A Proline-x-x-Proline (PxxP) motif allows Bag3 to bind the motor protein dynein which consequently brings Bag3 and its substrate to the aggresome for autophagy (*61*, *62*). Bag3 mediates autophagic degradation with the help of Hsp70 and small heat shock proteins, mainly HspB8 (*61*, *63*). Proteasomal and autophagic degradation will be discussed further in section 3.3.

2. DNAJ proteins and DNAJB1

2.1 J domain

DNAJs are essential proteins for Hsp70 chaperone activity involving the ATPase cycle. DNAJs are characterized by a J domain which promotes the ATPase cycle of Hsp70. The J domain is about 70 residues and was first discovered in *E. coli* DnaJ (*64*) J domain is composed of four α-helices: helix I, II, III and IV. Between helices II and III lies a conserved HPD motif which is required for the stimulation of the NBD of Hsp70. HPD was first discovered in the yeast DNAJ, Ydj1. When the HPD motif was mutated, Ydj1 was not able to stimulate the Hsp70 cycle. On the other hand, the HPD mutation did not affect the substrate binding of Ydj1 (*30*). Upon the interaction with the NBD of Hsp70, helix II of DNAJ bends which causes conformational change in the flexible II-III loop and helix III (*65*). This conformational change is required for DNAJ to activate the NBD of Hsp70. Recent studies have also shown that the J domain not only interacts with the NBD but also with SBDβ. The crystal structure of the bacterial DnaJ with bacterial Hsp70 DnaK show that the J domain sits at the NBD/SBDβ/linker interface. The helices II and III form polar and hydrophobic interactions with both the NBD and SBDβ of Hsp70 (**Figure 1.6**) (*66*). By forming DNAJ-Hsp70 intermolecular interactions, DNAJ interrupts Hsp70's intramolecular interactions, which eventually activate the structural changes in Hsp70.

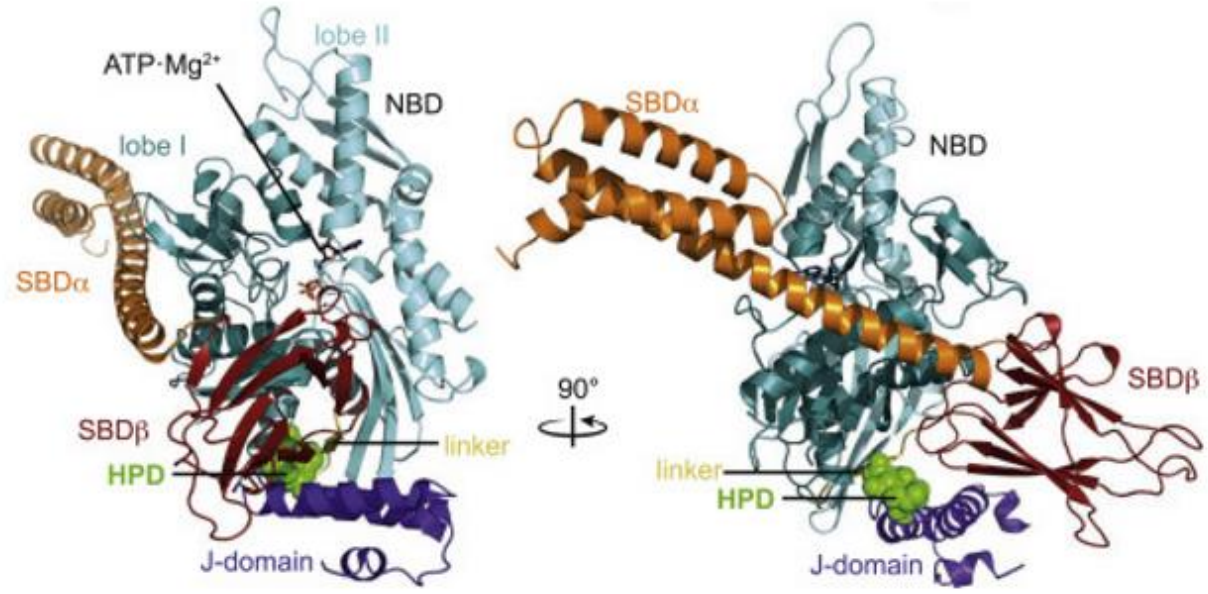


Figure 1.6 Crystal structure of DnaK-J domain binding

The bacterial Hsp70 DnaK makes contact with J domain at the surface of the NBD and SBDβ as well as with the linker. Especially, the interaction with the HPD motif in the J domain is important in the stimulation of the ATPase activity of Hsp70 (*66*).

2.2 DNAJ classification

The DNAJ family is well-conserved across different species. DNAJ proteins are very heterogeneous, with molecular weights ranging from 10 to 250kDa (*67*). There are more than 50 DNAJs but only 11 Hsp70s in human (*68*). A large variety of DNAJs allow Hsp70s to bind a wide range of substrates and to perform diverse functions as required in the cell. DNAJs in human are divided into 3 groups depending on their structure (**Figure 1.7**). Class A has the same structure as the bacterial DnaJ, with an N-terminal J domain, followed by a glycine-phenylalanine (G/F) rich region, a zinc-finger region, 2 C-terminal substrate binding regions (CTDI and II), and a dimerization domain (DD) at the end. The zinc-finger is inserted in the first C-terminal substrate binding domain. Class B proteins have a similar structure as class A but they lack the zinc-finger. Class C is less defined in terms of structure. The only common region amongst class C is the presence of a J domain (*68*). Instead, class C proteins are often localized in a certain area of the cell and have domains which bind specific substrates.

The structural diversity allows DNAJs to bind many different substrates and to perform a wide range of functions (*68*). For example, DNAJA2 primarily binds to small aggregates while DNAJB1 prefers larger aggregates (*44*). While DNAJA1 promotes the folding of immature CFTR, DNAJA2 leads immature CFTR to degradation (*69*). As can be seen, the substrate specificity and function vary not only between different classes but also within each class.

The class A and B proteins mostly exist in homodimer forms via the dimerization domain at the C-terminus. Interestingly, the J domains are located differently in the quaternary structure of DNAJA and B. In DNAJAs, the J domains are facing each other, close to the axis of symmetry. In DNAJBs, the J domains are far from each other, sticking outward from the axis of symmetry (*70, 71*).

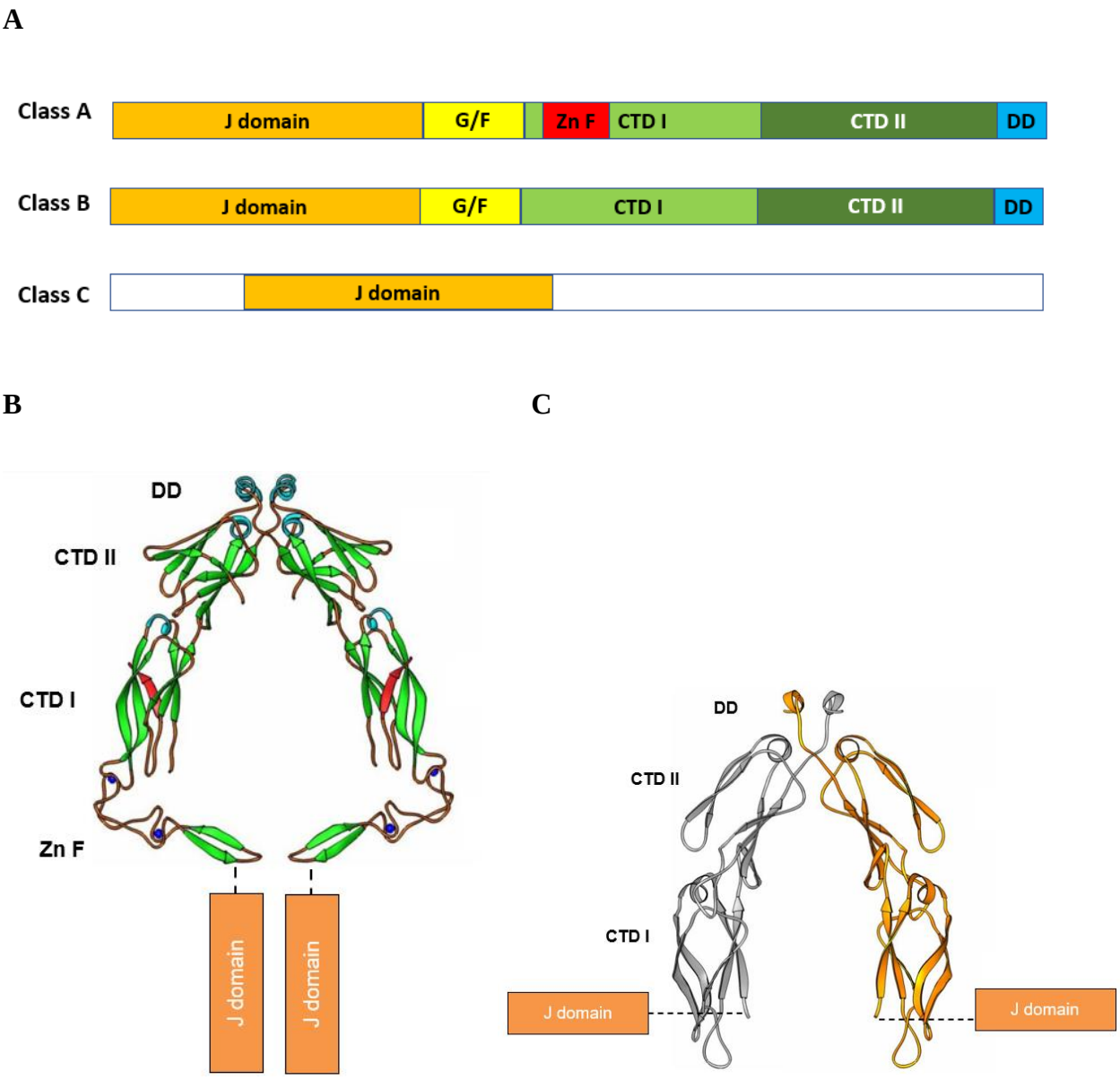


Figure 1.7 Structures of DNAJA, B and C

DNAJs are divided into three different classes depending on their structures. G/F: glycine-phenylalanine rich region, Zn F: zinc finger, CTD: C-terminal polypeptide-binding domain, DD: dimerization domain. A) Schematic diagram of class A, B, and C DNAJs. B) The ribbon

structure of DNAJA with the proposed position of J domains. (Modified from (*72*)) C) The ribbon structure of DNAJB with the proposed position of J domains. (Modified from (*72*))

Protein binding of DNAJs

Class A and B DNAJs bind to unfolded and nascent polypeptides. Thus, they not only promote the ATPase activity of Hsp70 but also recruit the polypeptides to Hsp70 (*73*). Since these DNAJs can bind substrates, whether they have an intrinsic chaperoning activity was a question. Studies with bacterial and human proteins have shown that they can suppress protein aggregation independently from Hsp70. Since DNAJs can bind substrates, they hold onto the exposed hydrophobic patches of polypeptides, preventing them from aggregation (*74, 75*).

Interestingly, despite the structural similarity, class A and B DNAJs bind polypeptides differently. Class A proteins bind the polypeptides at three different domains: the zinc finger, CTD I and II. By default, the binding site at CTD II is auto-inhibited by its own C-tail. However, the C-tail may be replaced by the polypeptide as needed. A polypeptide can bind at all three binding sites simultaneously. It is also possible that a polypeptide only binds the class A DNAJs at one site. On the other hand, class B proteins bind the polypeptides only at the CTD I (*76*).

The CTD I domain of DNAJs not only bind the substrates but also the C-terminal EEVD motif of Hsp70. The EEVD motif of Hsp70 is involved in binding various co-chaperones and other chaperones (*77*). The negatively charged residues of the EEVD motif interact antiparallelly with the positively charged lysine residues of CTD I (*78*). The class A DNAJs can bind the EEVD motif of Hsp70 and the polypeptide at the same time. When both are bound, DNAJAs bind the substrate at the zinc finger region and the EEVD motif of Hsp70 at the CTD I. The CTD II is auto-inhibited by the C-tails. In contrast, DNAJBs are not able to bind the substrate and the Hsp70 simultaneously. Since they both bind to the CTD I of DNAJBs, they displace each other (**Figure 1.8**) (*76*).

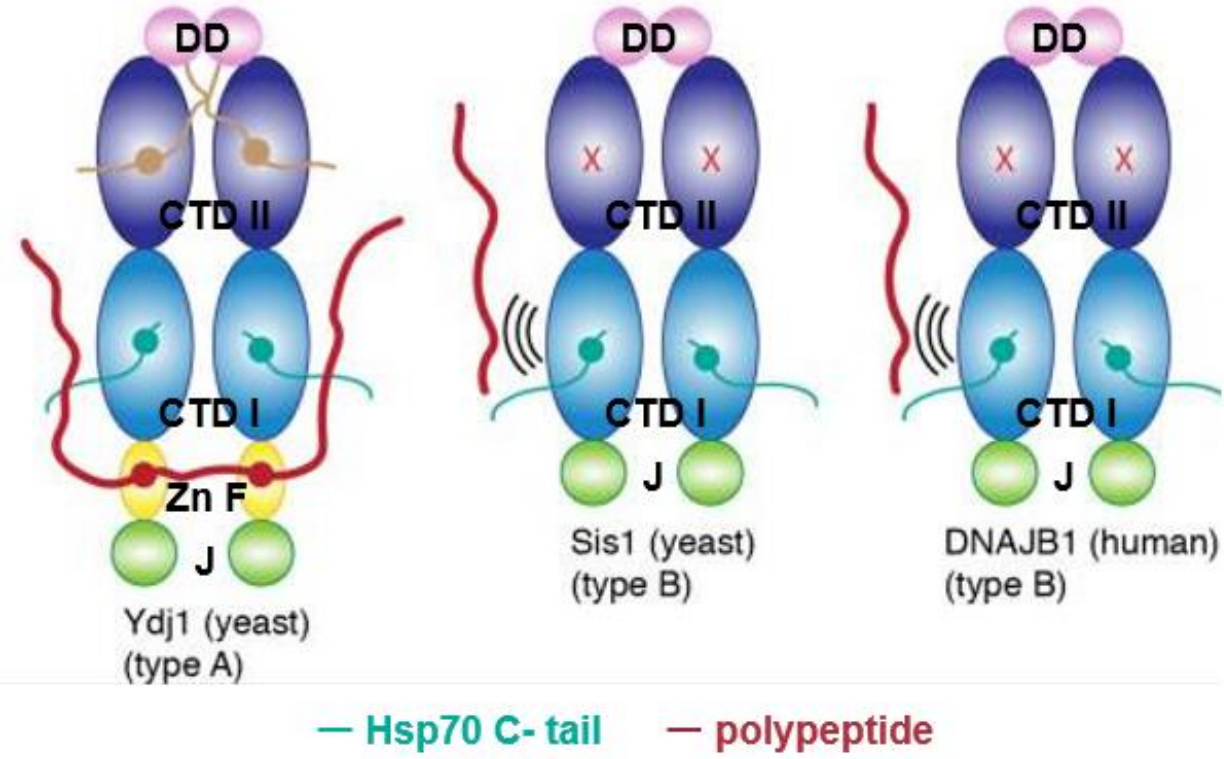


Figure 1.8 DNAJA and B bind the polypeptides and the Hsp70 C-tails differently

DNAJA protein can bind the polypeptide and the EEVD motif of Hsp70 simultaneously by binding the polypeptide with the zinc finger and the Hsp70 with CTD I. DNAJB protein cannot bind the two at the same time as it binds both at CTD I. (*76*)

Certain DNAJs are also involved in protein translation by interacting with a specialized ribosome-associated complex (RAC). DNAJC2, for example, recruits Hsc70 to the ribosome-bound nascent chains. It allows Hsc70 to bind not only the newly synthetized polypeptides but also polypeptides that are still being synthesized by ribosomes (*79, 80*). The yeast DNAJB protein Sis1 is also associated with and required for the initiation of translation (*81*)

DNAJs and Neurodegenerative Diseases

Since DNAJs are involved in the Hsp70-mediated protein folding and disaggregation, their roles in neurodegenerative diseases have also been studied. DNAJA1 co-localizes with α-synuclein (α-syn) in the brain tissue of PD patients and overexpressing DNAJA1 in a cell culture decreases α-syn fibril formation (*82*). Overexpression of DNAJA1 in cells also decreases AD-associated tau toxicity (*83*). Interestingly, DNAJA1 increases aggregation of mutant huntingtin (Htt) proteins when overexpressed (*84*). Like DNAJA1, DNAJA2 is protective against tau aggregation and the level of DNAJA2 increases with the level of tau in human brain (*85*). Amongst class B proteins, DNAJB6 and B8 have been associated with the suppression of polyglutamine (polyQ) aggregation in Huntington's Disease (*86, 87*). DNAJB1 also shows anti-aggregation activity with Htt, α-syn and tau. DNAJB1 not only suppresses the formation of these disease-associated proteins but also reverses the aggregation by disaggregation activity (*88-90*). DNAJB1 will be discussed further in section 2.3 and 3.2. Several DNAJCs have also been associated with neurodegenerative diseases. Notably, DNAJC7 inhibits tau aggregation and loss of function mutations in DNAJC7 have been found in ALS patients (*91, 92*). Earlier studies also show that overexpression of DNAJC10 in *C. elegans* decreases Aβ and α-syn aggregation (*93, 94*).

2.3 DNAJB1

DNAJB1 is the most abundant amongst class B proteins. It is greatly induced by environmental stresses, suggesting it plays an important role in keeping proteostasis during stress conditions (*95*). DNAJB1 is mainly localized in the cytoplasm. But in stress conditions, a subset of DNAJB1 translocates into the nuclei along with Hsp70. During recovery phase, DNAJB1 re-enters the cytoplasm with Hsp70 (*96*). The co-localization of DNAJB1 with Hsp70 suggests that DNAJB1 is an important co-chaperone for Hsp70. DNAJB1, along with Hsp70, exhibit chaperone activities in both the cytoplasm and the nucleus. When mutant firefly luciferases are directed to the nucleus, DNAJB1 with Hsp70 could still act on them, although at a lesser degree (*97*).

When overexpressed, DNAJB1 is localized in both the cytoplasm and the nucleus even in non-stress conditions. Interestingly, overexpressing DNAJB1 without Hsp70 reduces the cell's chaperoning capacity. In contrast, when DNAJB1 is overexpressed with Hsp70, there is an increase in the chaperoning capacity in both the cytoplasm and the nucleus (*98*). This shows that the balance between DNAJB1 and Hsp70 is important to keep the proteostasis. It is not clearly known why DNAJB1 overexpression has a negative effect on Hsp70. One possible reason might be that overexpressing DNAJB1 saturates the Hsp70, which in result disrupts the Hsp70's binding to the other DNAJ proteins. In support of this idea, it was shown that expressing DNAJB1 in the presence of auxilin, the DNAJ protein that is required for uncoating clathrin baskets, inhibited the activity of auxilin in clathrin uncoating (*99*).

DNAJB1 was the first discovered DNAJ protein in humans and in earlier studies, it was mainly associated with protein folding, along with Hsp70 (*100*). The Hsp70-DNAJB1 combination was often used to show the refolding activity of Hsp70, using substrates such as β-

galactosidase and firefly luciferase (reference?). However, DNAJA1 and DNAJA2 were later found to promote refolding much more efficiently than DNAJB1 (*101, 102*). Recently, more evidence indicates that DNAJB1 is involved in protein disaggregation, rather than folding, in humans. Pure protein studies showed that human Hsp70, DNAJB1 and Hsp110 can disaggregate proteins *in vitro* with the aid of a yeast small heat shock protein (*42, 43*). A later study shows that DNAJB1 forms heterodimers with DNAJA2 to disaggregate proteins (*44*). More about the disaggregation machinery will be discussed in the section 3.2.

DNAJB1's protective role in neurodegenerative diseases has also been indicated by various studies. *In vitro*, the fibrilization of Htt was suppressed by Hsp70 and DNAJB1 and the same was seen in yeast (*103*). A later study *in vivo* also confirmed that the Hsp70-Hsp110-DNAJB1 combination suppresses Htt fibril formation and resolubilizes fibrils (*90*). Similarly, Hsp70-Hsp110-DNAJB1 disassembles α-synuclein amyloid fibrils in *vitro (88).* Another recent study using microfluidic diffusional sizing has shown that Hsp70-Apg2-DNAJB1 reverses α-synuclein aggregates to monomeric forms (*104*). DNAJB1 was also shown to prevent tau aggregation by recognizing and binding both small and large tau fibrils (*105*).

3. Disaggregation System

3.1 Protein Aggregation

When proteins are misfolded or unfolded, the hydrophobic residues, which are usually buried inside, become exposed on the outside. The exposed hydrophobic surfaces of separate polypeptides can then interact abnormally with each other, resulting in protein aggregates (*106, 107*). Protein aggregates can be divided into two different groups: amorphous aggregates and amyloid fibrils. Amorphous aggregates are unstructured and disorganized. Proteins in amorphous aggregates are held together by the intermolecular hydrophobic forces. On the other hand, the amyloids are structured and self-assemble in β-sheets. They not only depend on the hydrophobic contacts but also on the ionic interactions (*108*).

Amyloid Fibrils

Amyloid formation is often dependent on the sequence. Certain mutations in the sequence may cause amyloid formation by disrupting the native state of the protein and establishing non-native contacts to form β-sheets. On the other hand, the presence of certain charged residues in a stretch of hydrophobic chain may prevent the formation of amyloid formation (*109, 110*). For example, glutamine-rich polypeptides can form hydrogen bonding between carbonyls and nitrogens of the backbone and side chains, which may result in the β-sheet conformation in certain diseases such as Huntington's disease. Amyloid formation is also affected by various factors such as the protein concentration, pH, and temperature.

Amyloids propagate through the polymerization of monomeric proteins into long fibers. Amyloid polymerization is dependent on the nucleation, which is the formation of the smallest structural unit that can propagate the fibril growth. A nucleus is usually a monomer or a small oligomer. The nucleation is the slow step in the formation of amyloid fibrils. Once the

nucleus is formed, the amyloid grows fast in the elongation step (**Figure 1.9**) (*111, 112*). Interestingly, if there is a fibril growing already, the formation of the second nucleus happens much more easily. In many amyloid diseases, fibril fragmentation is observed. When fibrils are fragmented, fibrils get shorter and the number of fibrils increases. Since fragmented fibrils skip the nucleation step, they accelerate amyloid formation and cause the increased cytotoxicity (*113, 114*).

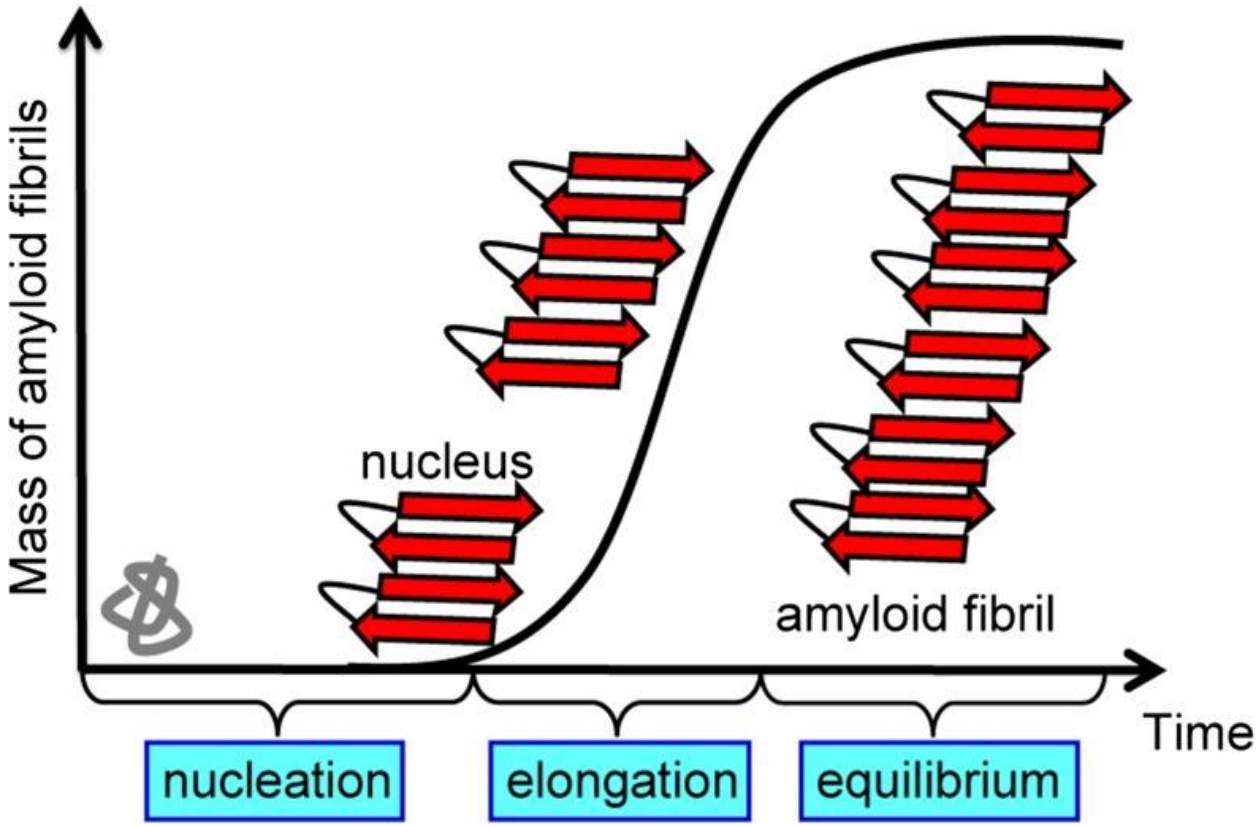


Figure 1.9 Kinetics of Amyloid Fibril Formation

The amyloid formation in nucleation-dependent model follows three steps: nucleation, elongation and equilibrium. The kinetics of amyloid fibrilization takes a sigmoidal shape, where the nucleation is lagged and the elongation is exponential. (*115*)

Amorphous Aggregates

The amorphous aggregation is not as well understood as the amyloid formation. While amyloid fibrilization is a one-dimensional process where monomers add onto the fibril in one direction, amorphous aggregation is a disordered three-dimensional process, where monomers are added from any directions (**Figure 1.10**) (*116*). There are a few experimental methods to monitor amorphous aggregation. Dynamic Light Scattering (DLS) can measure the size of aggregates. Size Exclusion Chromatography (SEC) can also determine the size and the fraction of differently sized aggregates. More recently, Förster Resonance Energy Transfer (FRET) and fluorescence self-quenching have been used to analyze the intra- and intermolecular interactions in aggregates. A study has suggested that the polypeptides in amorphous aggregation go into condensation phase first, where polypeptides are close to each other but not aggregated (*117*).

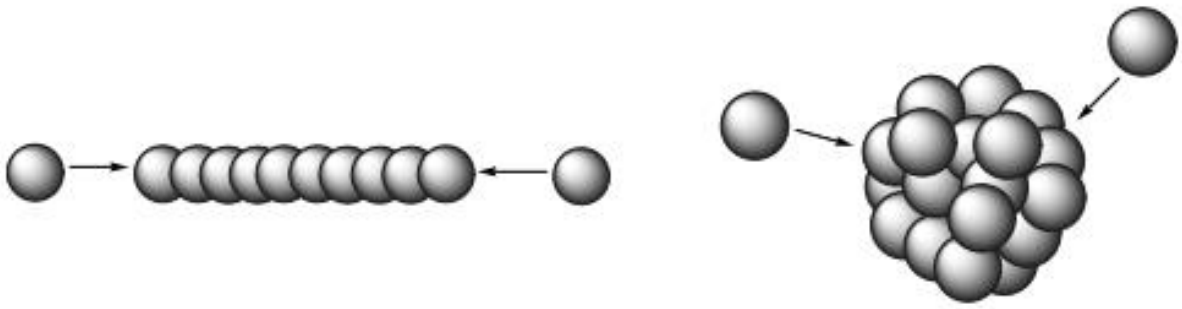


Figure 1.10 Amyloid fibrilization versus amorphous aggregation

While amyloid grows linearly, one monomer at a time, amorphous aggregate grows randomly and monomers are added from any directions (*116*).

Cytotoxicity of Protein Aggregates

Research has shown that there are several reasons why protein aggregates are toxic to the cell. The most obvious reason is the loss of function of the aggregated protein. Cellular pathways are heavily dependent on proteins and therefore loss of proteins is critical in cells. Making things worse, the aggregated proteins can cause other normally folded proteins to aggregate also. Proteins work by interacting with other proteins. In some cases, the protein aggregates can still interact with other proteins, trapping them in the aggregates. For example, huntingtin causes transcription factors such as CREB binding protein to be recruited to the aggregates (*118*). Aggregates also take the chaperones and co-chaperones away from their cellular functions. Chaperones are involved in many different cellular processes that are crucial to the cell. Without sufficient amount of chaperones, the proteotoxicity in the cell increases with the accumulation of misfolded proteins and lack of the clearance of aberrant proteins. Some aggregates also disturb the cell membrane. For example, β-amyloid and α-synuclein form amyloid pores in cell membrane (*119*). Amyloid pores cause inappropriate permeabilization which may eventually lead to cell dysfunction or even cell death.

Protein Aggregates and Diseases

Protein aggregates have been associated with various diseases. Amorphous aggregates are the cause of the development of cataract in eyes (*120*). Amorphous immunoglobulin aggregation induces renal failure (*121*). Amorphous aggregates are also observed in Alzheimer's and Parkinson's along with amyloid fibrils (*122, 123*). However, the current focus lies with amyloidogenic aggregates, especially in neurodegenerative diseases. Neurodegenerative diseases are discussed in section 1.1. Other diseases with amyloids include systemic amyloidosis and type II diabetes. In systemic amyloidosis, the amyloid fibrils accumulate in skeletal tissue and joints.

In some cases, they are also found in heart and kidney (*124*). In type II diabetes, a peptide hormone called amylin forms amyloid fibrils. Interestingly, type II diabetes may contribute to the development of Alzheimer's disease (*125*).

3.2 Disaggregation system

In bacteria and yeast, the disaggregation machinery involves Hsp70s and AAA family proteins, ClpB and Hsp104 respectively. AAA family proteins are ATPases which form hexameric rings. The inside ring is lined with aromatic residues and binds polypeptide termini or surface loops sticking out from the aggregates. It then pulls the polypeptide towards itself until the polypeptide is disengaged from the aggregate (*126-128*). Hsp70 is also involved in this process by activating the AAA proteins and remodeling the surface of the aggregates to make polypeptide available for AAA proteins to bind (*129, 130*). However, human cells do not have orthologs of these AAA family proteins. Therefore for a long time, mammalian disaggregation was thought not to exist (*128*). Contrary to this belief, some recent pure protein studies have shown disaggregation activity using purified human chaperones.

Human Hsp70, DNAJB1 and Hsp110 can disaggregate proteins *in vitro* with the help of a yeast small heat shock protein Hsp26 (*42, 43*). Hsp70 and DNAJB1 were enough to show some disaggregation activity but the addition of Hsp110 family protein synergized Hsp70-DNAJB1 mediated disaggregation. Hsp110 as well as Apg2 were tested and Apg2 showed a greater amount of disaggregation. Disaggregation required Hsp110's NEF activity but not an ATPase-dependent chaperone activity (*43*).

More recently, another report has shown that not only DNAJB1 but also DNAJA2 are involved in the disaggregation activity *in vitro.* This report shows that DNAJA2 and B1 homodimers transiently bind each other to optimize the disaggregation. DNAJA2 and B1 bind in an antiparallel direction. The J domains of DNAJA2 bind the CTD domains of DNAJB1 and the CTD domains of DNAJA2 binds the J domains of DNAJB1 (*44*). It was previously known that DNAJ homodimers can interact with two independent Hsp70s via two individual J domains (*131*). Therefore, the heterocomplex of DNAJA2 and B1 homodimers can recruit four Hsp70 to the aggregates. Increased recruitment of Hsp70 near aggregates potentially increases the pulling force on polypeptides, accelerating the rate of disaggregation.

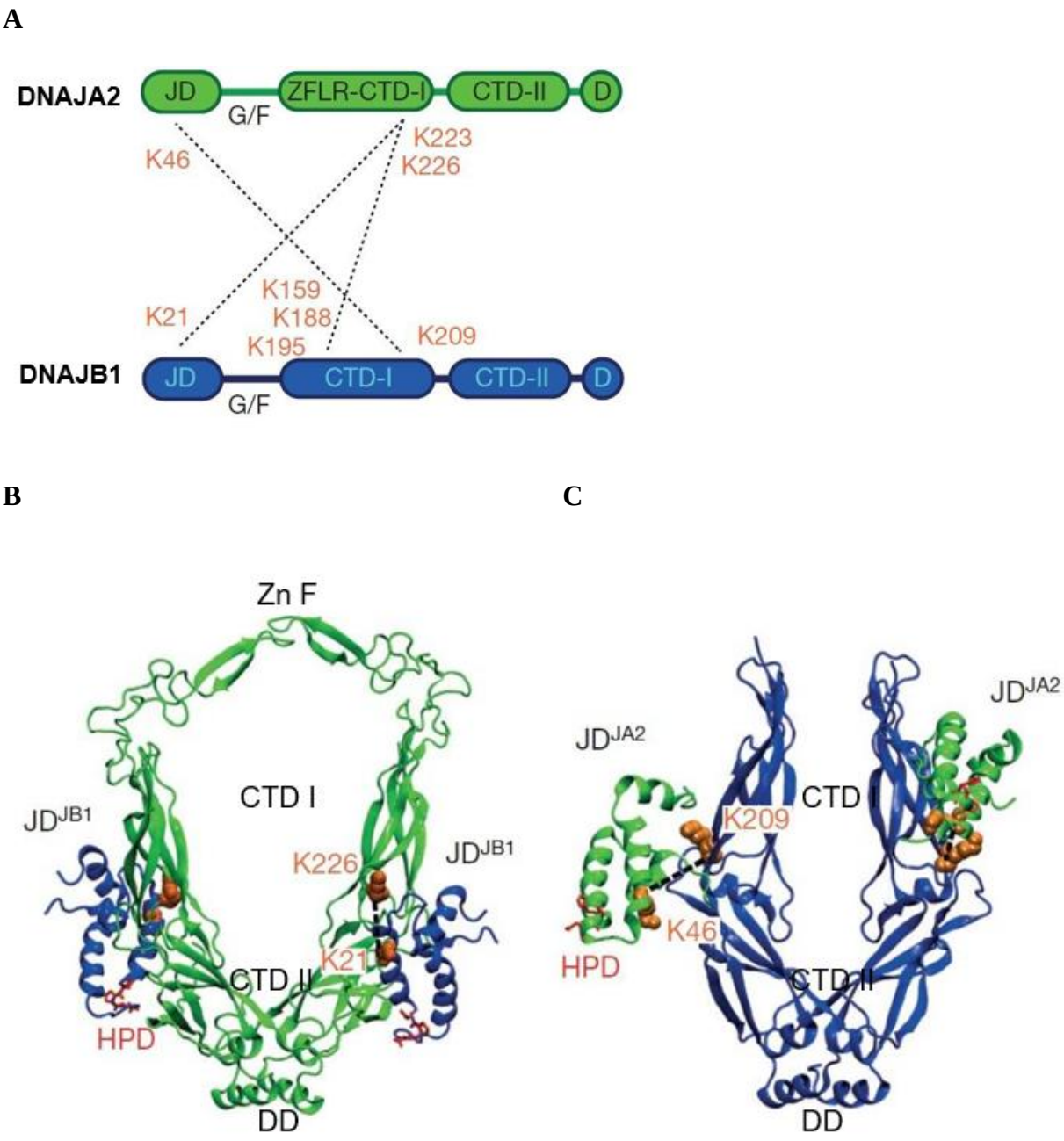


Figure 1.11 DNAJA2 and DNAJB1 transiently bind each other antiparallelly

DNAJA2's J domain binds CTD domain of DNAJB1 and DNAJB1's J domain binds CTD domain of DNAJA2. A) Schematic diagram of DNAJA2 and B1 and their contact sites revealed by the intermolecular cross-linking. B) Ribbon representation of DNAJA2 binding the J domain of DNAJB1. C) Ribbon representation of DNAJB1 binding the J domain of DNAJA2 (*44*).

It is not clear how DNAJs are recruited to the aggregates. One possibility is that they recognize the exposed polypeptide stretches sticking out of the aggregates. Interestingly, DNAJA2 and DNAJB1 solubilize aggregates of different sizes. DNAJB1 homodimers preferentially recognize larger aggregates (~700 to >5000kDa) while DNAJA2 homodimers recognize smaller aggregates (~200 to 700kDa). It is possible that DNAJB1 and A2 bind the less preferred size of aggregates but in that case, their disaggregation efficiency drops. When DNAJB1 and A2 are combined, they are able to work on a wide size range of aggregates. Not only that, they are also able to disaggregate much more efficiently, regardless of the size of aggregates (*44*).

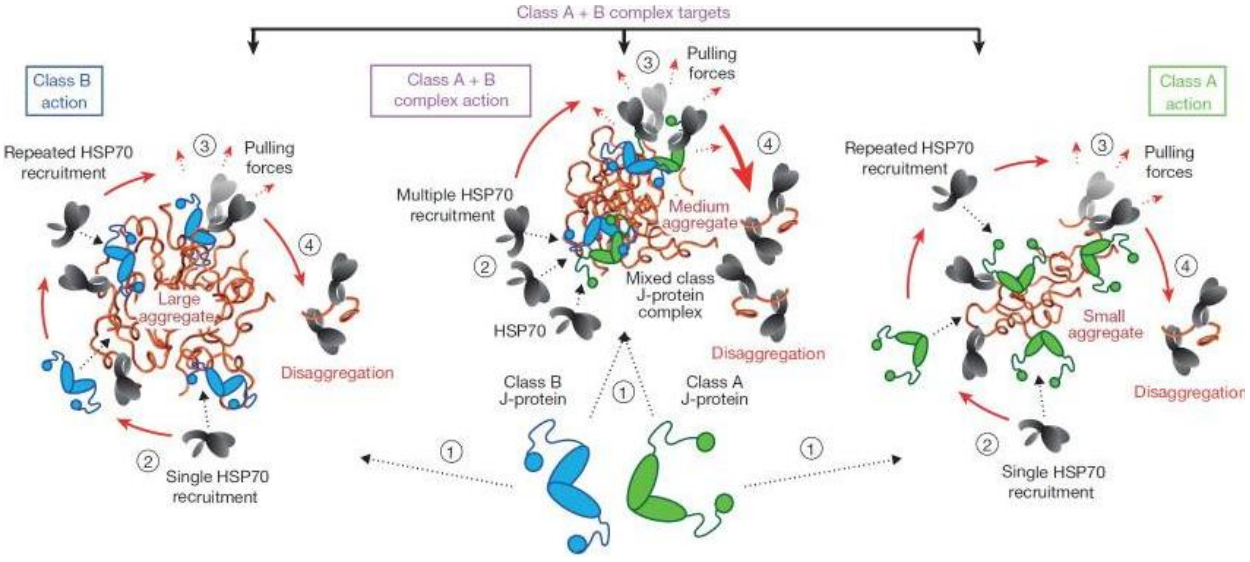


Figure 1.12 DNAJB1 and A2 complex with each other for the optimal disaggregation

When DNAJB1 and A2 work together, the disaggregation machinery is able to work on a wide range of aggregate sizes and is the most efficient.

Small Heat Shock Proteins

The small heat shock (sHsp) protein chaperones are another component of disaggregation. Humans have 10 sHsps which form homodimers that in some cases can form larger oligomers. The IPV motif in the C terminal region of sHsps is responsible for oligomerization (*132*). This oligomerization state is disturbed upon stress. When stressed, the sHsp gets phosphorylated at certain sites, which in turn causes the dissociation of oligomers into dimers (*133*). The major sHsps are HspB1, 5, and 8. HspB1 and 5 are ubiquitous and 8 is most abundant in muscles and brains. The rest are mostly tissue-specific (*134*). HspB1 and 5 are stress-inducible while 8 is somewhat inducible (*135*). None of the other sHsps are stress-inducible. HspB1, 4, and 5 are found to suppress aggregation. They are thought to bind misfolded/unfolded proteins, preventing the accumulation of aggregates (*136*). A recent study also showed that sHsps hold onto misfolded proteins in near-native conformation for protection and efficient refolding (*137*).

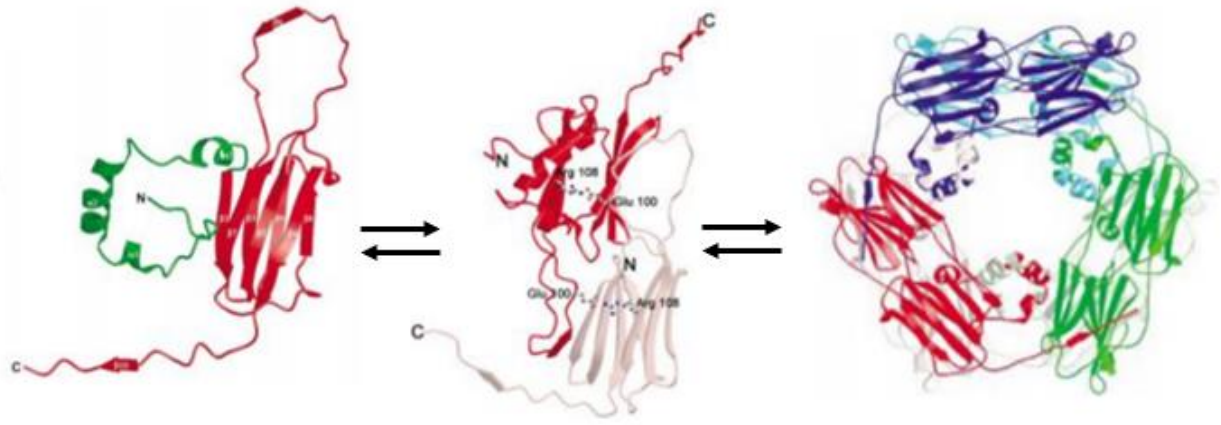


Figure 1.13 Monomer, dimer, and oligomer states of sHsp

sHsp bind each other and form dimers. The dimers oligomerize via IPV motif in the C-terminal region. (Modified from (*138*))

HspB1

HspB1 is one of the most induced proteins in stress along with DNAJB1. Therefore, it must play an important role in maintaining proteostasis during stress conditions. When stress-induced, HspB1 is phosphorylated at three different sites, S15, S78, and S82. This phosphorylation causes dissociation of oligomers into dimers. The dimers are the active form of HspB1 as they can bind polypeptides (*139*).

Earlier studies have shown that HspB1 prevents aggregation. But recently, it was shown that HspB1 is not only involved with the prevention of aggregation but also with disaggregation (*140*). Earlier studies have shown that yeast sHsp, Hsp26, co-aggregates with the aggregated proteins. Hsp26 then changes the characteristics of the aggregates so that the aggregates are more susceptible to disaggregation (*42*). Recently, the human ortholog HspB1 has shown similar behaviours as Hsp26 in terms of disaggregation. An *in vitro* study with human proteins has shown that HspB1 does co-aggregate with protein aggregates. Not only that, the aggregates are smaller in the presence of HspB1 than in its absence. Furthermore, the disaggregation machinery could not disaggregate when the substrates are not co-aggregated with HspB1 (*140*). Therefore, HspB1 is an important part of mammalian disaggregation system along with Hsp70, DNAJA2 and DNAJB1, and Hsp110/Apg2.

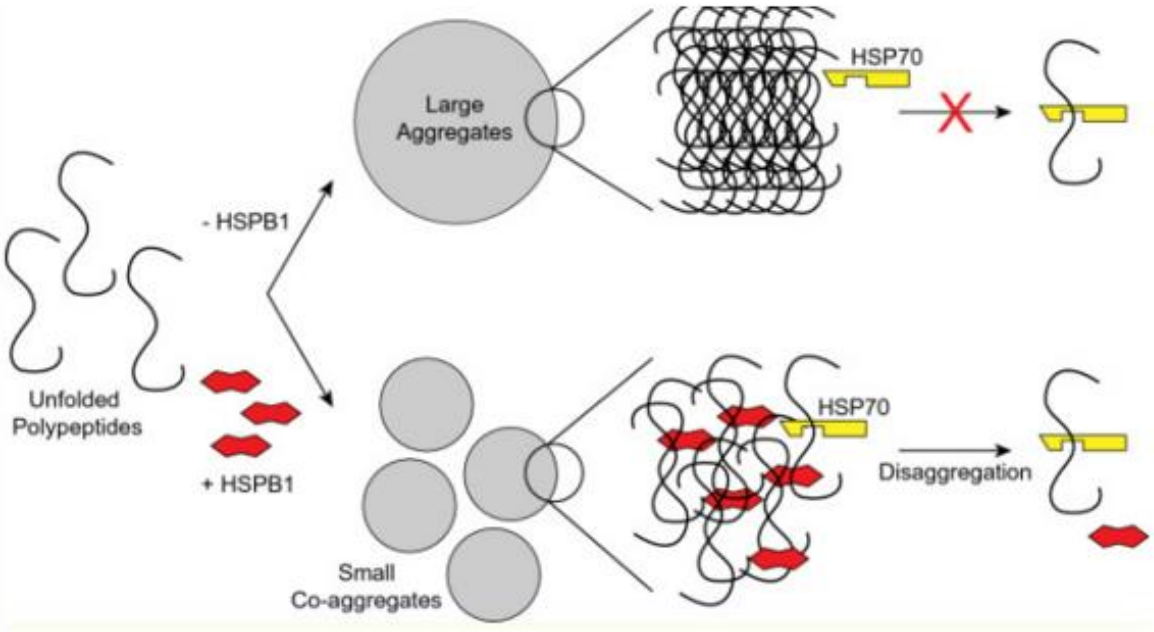


Figure 1.14 HspB1 is an essential part of mammalian disaggregation

HspB1 binds to unfolded polypeptide and prevent the formation of large aggregates. The co-aggregation of HspB1 with substrates causes the smaller, looser aggregates which can be disaggregated by the Hsp70 disaggregation machinery (*140*).

3.3 Degradation and autophagy

The other side of protein quality control involves getting rid of the damaged proteins. The two major pathways for protein clearance are proteasomal degradation and autophagy.

Proteasomal degradation

Proteasomal degradation is the primary way to degrade the unwanted proteins. Proteasomes are protein complexes which have proteolytic activities. The mammalian proteasomes are called 26S and they are composed of a barrel-shaped 20S core and two regulatory 19S cap subunits at both ends of the barrel. The 20S core has the proteolytic activity while 19S has ubiquitin binding sites which recognizes polyubiquitinated proteins and ATPase activity which provides the energy for the substrates to enter the 20S core (*141*). Most proteins targeted to proteasomes must be ubiquitinated. There are three types of enzymes involved in ubiquitination: ubiquitin-activating E1, ubiquitin-conjugating E2, and ubiquitin ligase E3. As the name suggests, E1 activates ubiquitin so that it can become conjugated onto E2, and E3 transfers the ubiquitin from E2 to the targeted protein (*142*). There are many different E3 ligases in human cells, which target native and misfolded/unfolded proteins to degradation (reference: cite a review article on E3 ligases). The E3 ligase associated with Hsp70 is CHIP. The tetratricopeptide repeat (TPR) domain of CHIP binds the C-terminal EEVD motif of Hsp70. Then, CHIP is able to ubiquitinate Hsp70's substrate (*143*). Therefore, the proteasomal degradation pathway is also called the ubiquitin-proteasome system (UPS).

Autophagy

Autophagy removes dysfunctional components of the cell through a lysosome-dependent mechanism. Lysosomes are membrane-bound organelles which contain materials which degrade

and recycle the unwanted parts of the cell. There are different forms of autophagy, the most common being macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (*144*). Macroautophagy is the primary pathway where cells form an autophagosome around the unneeded cellular waste. Then, autophagosome fuses with lysosome and the cellular waste inside of autophagosome is degraded by the lysosomal hydrolase (*145*). Microautophagy skips the formation of autophagosome and the cellular material is engulfed directly by the lysosome (*146*). CMA is very selective as chaperones target specific proteins to lysosomes. The proteins targeted by chaperones have a common targeting motif that are recognized by Hsc70 (*147*). Hsp70 complexed with Bag3, HspB8 and CHIP is often observed in aggresomes, the accumulation site of aggregates near the microtubule organizing centre. This complex interacts with the autophagy adaptor p62/SQSTM1 and brings the aggregated proteins into autophagosomes (*148*).

Degradation of aggregates

The degradation of aggregates in human cells is not well understood, but may be by either the UPS, autophagy or both. Upon proteasome inhibition, the resolubilization of certain protein aggregates is prevented, suggesting proteasome works on resolubilized proteins from the aggregates (*149*). For example, the mutant α-synuclein aggregate is degraded by the proteasome (*150*). On the other hand, there are some studies that show autophagic clearance of certain aggregates. Mutant huntingtin and tau aggregates are cleared by autophagy (*151, 152*). Soluble mutant SOD1 G85R and G93A are degraded by the proteasome, and aggregates are cleared by autophagy (*153*).

The proteasome can only degrade soluble monomeric polypeptides. Therefore, aggregates degraded by the proteasome must be disaggregated or resolubilized first. This may be by the disaggregation machinery composed of Hsp70, DNAJB1, DNAJA2 and Hsp110.

However, most disaggregation studies were in vitro and disaggregation in cells is not yet well understood.

Results

The mammalian cells have a disaggregation system

To observe disaggregation in mammalian cells, we started with an assay for protein misfolding and refolding from previous work (*41*). When firefly luciferase was transfected in HEK293 cells and subjected to heat shock to induce misfolding, luciferase enzymatic activity was greatly decreased, but increased again when cells recovered at 37°C in the presence of cycloheximide to prevent new synthesis, indicating luciferase refolding. Soluble luciferase also decreased after heat shock and increased during recovery, and insoluble luciferase increased after heat shock. However, the soluble and insoluble amounts were not quantified (*41*). Therefore, we now analyzed soluble and aggregated luciferase more systematically.

Firefly luciferase was transfected in HEK293 cells, and heat shocked at 45°C for 1 h to induce misfolding and aggregation. The insoluble fraction of HEK293 cell lysate was observed by analyzing the pellets. The insoluble fraction was extracted from pellets by first dissolving in 8M urea and 0.5% SDS then sonicating with ultrasonic sonicator. Equivalent amounts of the soluble and insoluble fractions were observed by immunoblot for HA-tagged luciferase and quantified relative to total luciferase before heat shock. Upon heat shock, luciferase aggregated and about 60% of the initial amount of soluble luciferase ended up in the pellet. Over 2 hours of recovery in the presence of cycloheximide to prevent new synthesis, the amount of luciferase in pellet decreased (**Figure 3.1A, B**), demonstrating the presence of the disaggregation system in mammalian cells.

In separate experiments, aggregates in the total cell lysate were collected by vacuum microfiltration on porous membranes, and luciferase detected by immunoblot of the filters. These filter trap assays have been used to analyze large neurotoxic aggregates such as those of SOD1 mutants that cause ALS (*154*). The filter trap experiments also showed the formation of luciferase aggregates upon heat shock and the clearance of aggregates over 2 hours (**Figure 3.1C, D**).

The formation and clearance of aggregates were also seen with the ALS-related mutant of SOD1, G85R in filter trap experiments. Cells transfected with SOD1 G85R were treated with 5μM MG132 for 16 hours to accumulate aggregates of the mutant (*154*). After 16 hours, cycloheximide chase was carried out for 10 hours. During the 10-hour chase, about 40% of G85R aggregates cleared (**Figure 3.1E, F**). Therefore, experiments with both luciferase and mutant SOD1 showed evidence of disaggregation in mammalian cells.

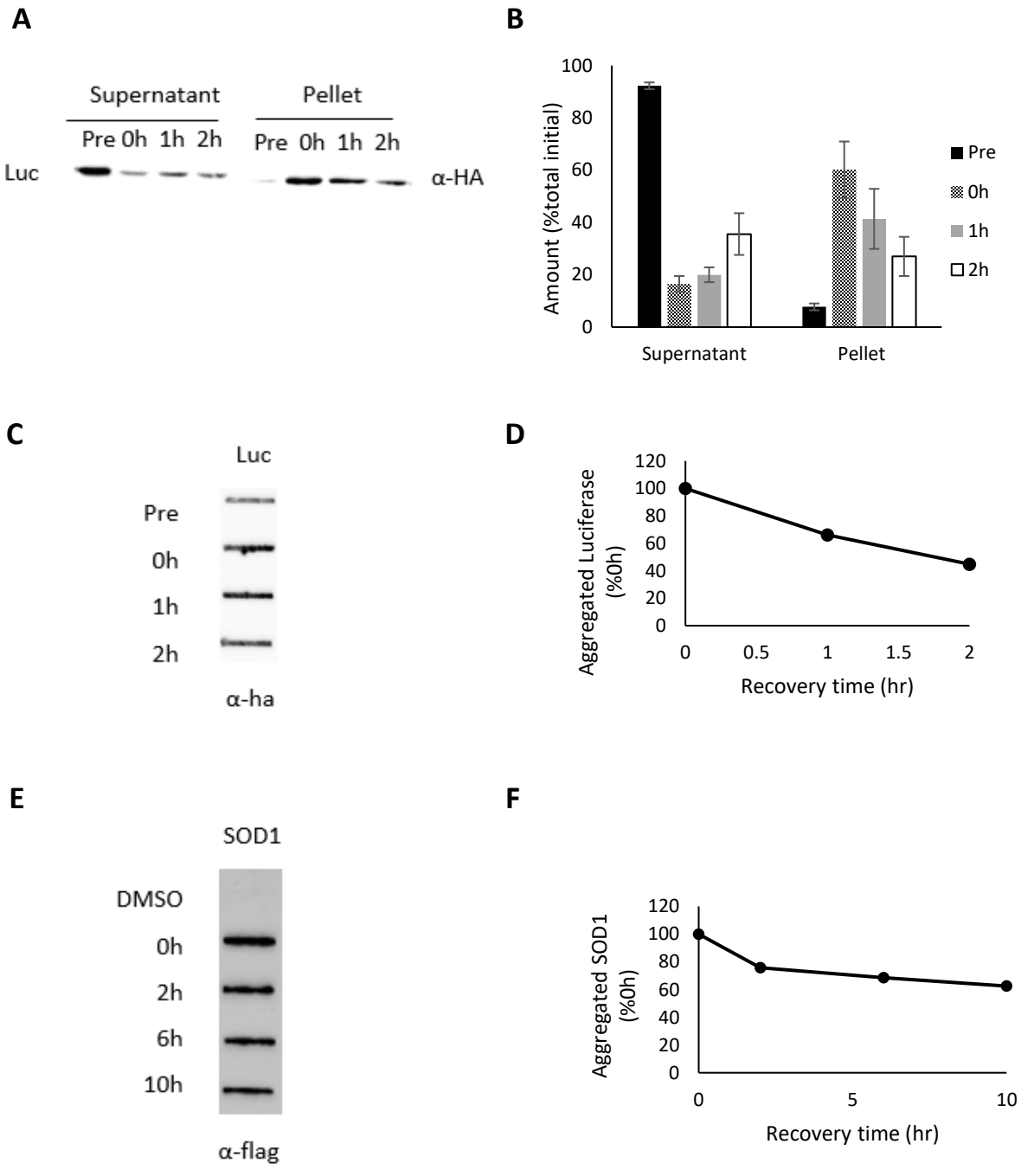

A
Supernatant
Pellet
Pre 0h 1h 2h
Pre 0h 1h 2h
Luc
α-HA
B
Amount (%total initial)
100
80
60
40
20
0
Pre
0h
1h
2h
Supernatant
Pellet
C
Luc
Pre
0h
1h
2h
α-ha
D
Aggregated Luciferase (%0h)
120
100
80
60
40
20
0
0
0.5
1
1.5
2
Recovery time (hr)
E
SOD1
DMSO
0h
2h
6h
10h
α-flag
F
Aggregated SOD1 (%0h)
120
100
80
60
40
20
0
0
5
10
Recovery time (hr)

Figure 3.1. Disaggregation is observed in HEK293 cells

(A-D) HEK293 cells were transfected with HA tagged luciferase and then heat shocked at 45°C for 1 hour. The recovery was observed over 2 hours in the presence of cycloheximide. (A, B) The supernatant and pellet were separated at 20000xg for 5min. The pellet were dissolved in 8M urea, 0.5% SDS and sonicated at 20% for 15 seconds, at 5-second intervals with 5-second breaks in between. The supernatant and sonicated pellet were immunoblotted with antibody against HA. (C, D) The 1000xg supernatant was passed through a filter trap using a microfiltration apparatus to collect insoluble aggregates, and immunoblotted against HA. (E, F) HEK293 cells were transfected with FLAG tagged SOD1 G85R and were treated with 5μM MG132 for 16 hours. Then, the cells were chased with 50ug/ml cycloheximide for 10 hours. The 1000xg supernatant was separated by filter trap slot blot, and immunoblotted against FLAG.

Heat shock response helps maintain proteostasis by increasing the expression of certain chaperones and co-chaperones

To find out the possible chaperones and co-chaperones involved in disaggregation, the heat shock response was induced in HEK293 cells to observe the level of increase in these proteins. Cells were transfected with luciferase and then heat-stressed for 1 hour at 45 °C to induce the heat shock response. 16 hours later, cells were heat-shocked for 1 hour at 45 °C to unfold and aggregate proteins. Then, the soluble, active amount of luciferase was observed over 2 hours of recovery time in the presence of cycloheximide.

The result shows that the heat-stressed cells are able to maintain higher amount of soluble, active luciferase than control cells. Upon heat shock, cells that induced a heat shock response maintained about 40% of soluble, active luciferase compared to the control cells which could only maintain about 15% of soluble, 5% of active luciferase (**Figure 3.2A-C**). Over the course of 2 hours recovery, cells in heat shock response maintained high amount of soluble luciferase and showed 20% increase in active luciferase (**Figure 3.2A-C**).

The fold increase of chaperones and co-chaperones were also observed. 16 hours after heat shock response was initiated, Hsp70, Hsp90α, DNAJB1, Hsp110, APG2, Bag3, and HspB1 were induced. Among those, DNAJB1 and HspB1 showed the most substantial increase. This is interesting since they are not required parts of the protein folding machinery for luciferase (*16*). The fact that cells highly increase the amount of DNAJB1 and HspB1 upon stress tells us that they have important roles in proteostasis, apart from protein folding/refolding.

Hsc70, Hsp90β, DNAJA1, and DNAJA2 did not get induced upon heat shock (**Figure 3.2D, E**). It is interesting to note the difference in response to stress between Hsp70 and Hsc70,

as well as between Hsp90α and Hsp90β, albeit their similarity in structure. DNAJA1 and A2 are not induced although they are thought to be the DNAJ proteins involved in Hsc70/Hsp70 mediated protein folding, for luciferase and other proteins (*16*).

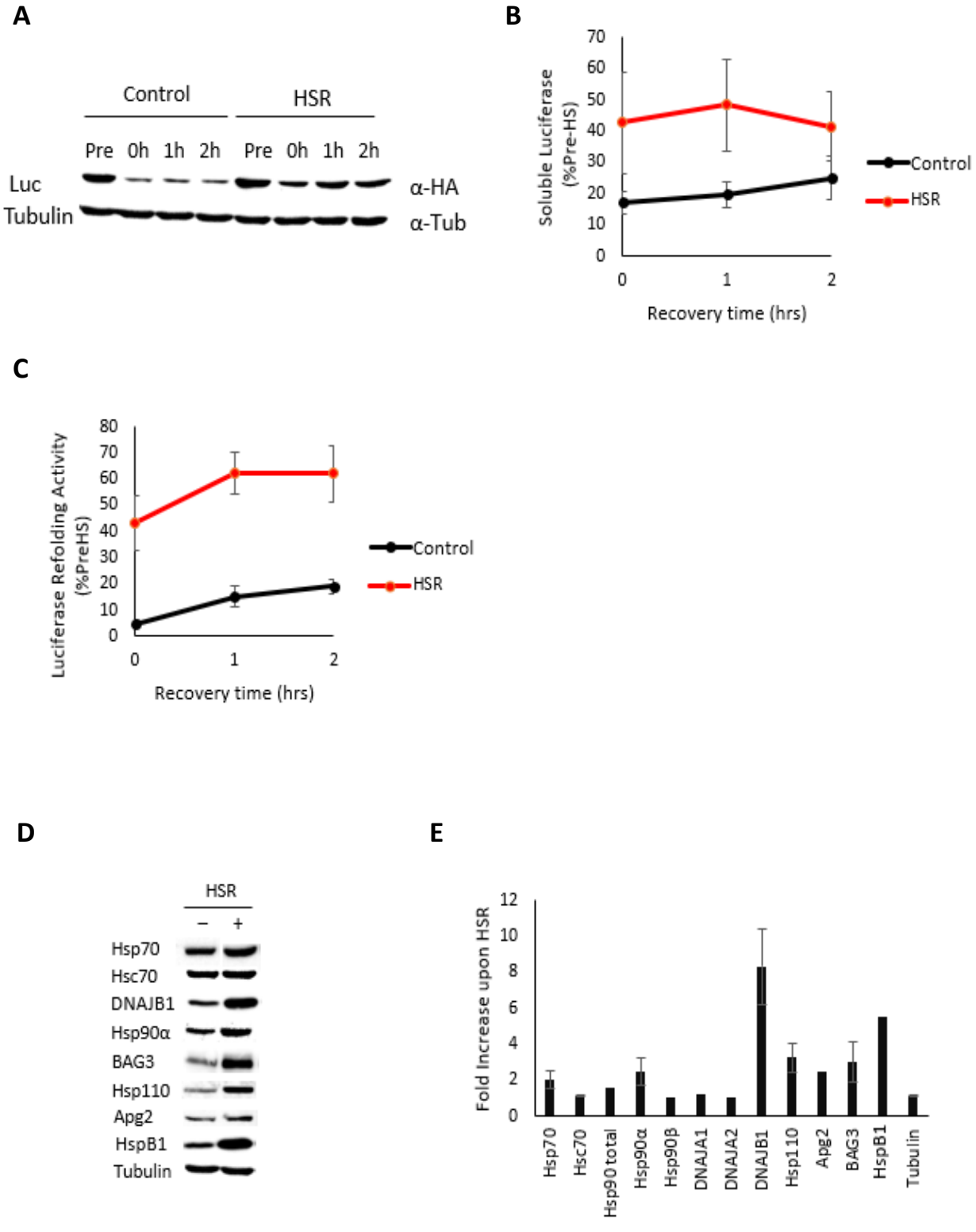

A
Control
HSR
Pre 0h 1h 2h Pre 0h 1h 2h
Luc
Tubulin
α-HA
α-Tub
B
Soluble Luciferase (%Pre-HS)
Recovery time (hrs)
Control
HSR
C
Luciferase Refolding Activity (%PreHS)
Recovery time (hrs)
Control
HSR
D
HSR
– +
Hsp70
Hsc70
DNAJB1
Hsp90α
BAG3
Hsp110
Apg2
HspB1
Tubulin
E
Fold Increase upon HSR
Hsp70
Hsc70
Hsp90 total
Hsp90α
Hsp90β
DNAJA1
DNAJA2
DNAJB1
Hsp110
Apg2
BAG3
HspB1
Tubulin

Figure 3.2. Heat shock response keeps luciferase soluble and active and increases the level of some chaperones and co-chaperones

HEK293 cells transfected with HA tagged luciferase were heat-shocked at 45°C for 1 hour to induce the heat shock response, while control cells were left unstressed. 16 hours later, heat shock response (HSR) and control cells were subjected to an additional heat shock at 45°C for 1 hour. (A, C) The soluble lysate was immunoblotted against HAover 2-hour recovery. (B) Luciferase enzymatic activity in soluble lysates was measured over 2-hour recovery, using luciferin as substrate in a luminometer. (D, E) The soluble lysates of control and HSR cells were immunoblotted against antibodies specific to each chaperone and co-chaperone.

Excessive amount of DNAJB1 is detrimental to protein homeostasis in cells

Since DNAJB1 is highly induced upon heat shock, it might be an important part of the disaggregation machinery. To observe the role of DNAJB1, DNAJB1 was overexpressed in HEK293 cells and heat shock experiments were carried out as in Figure 3.1. Interestingly, DNAJB1-overexpressing cells maintained less soluble and active luciferase upon heat shock (**Figure 3.3A-C**). Furthermore, the cells were not able to increase the solubility or activity of luciferase over time in the presence of excessive DNAJB1 (**Figure 3.3A-C**). This suggests that excessive amounts of DNAJB1 interferes with protein quality control, possibly by competing with other DNAJ proteins for Hsp70.

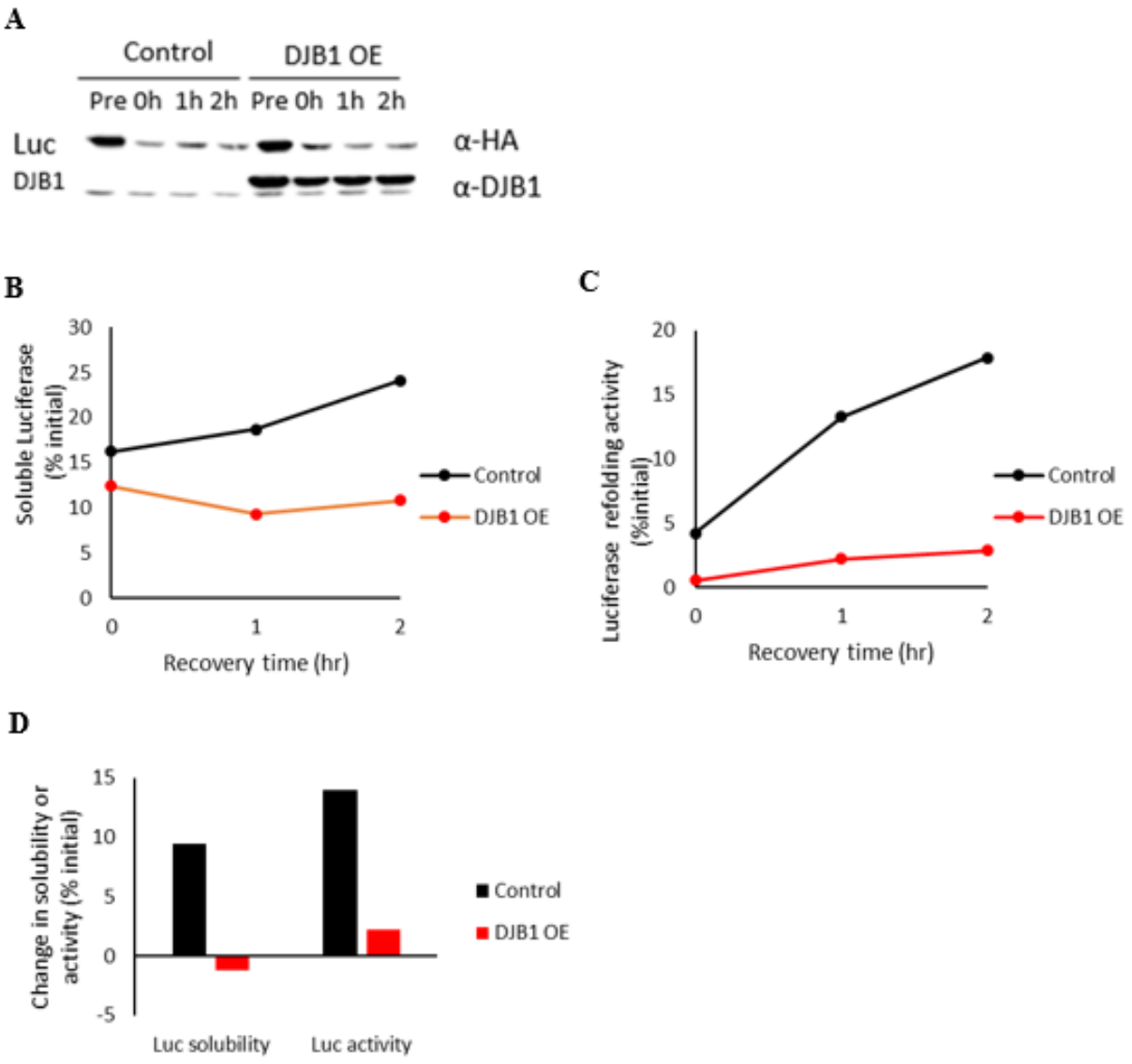


Figure 3.3. DNAJB1 overexpression inhibits protein refolding and solubilization

HEK293 cells were transfected with HA tagged luciferase and pcDNA3.1 (control) or DNAJB1. Then, they were heat shocked at 45°C for 1 hour. The recovery was observed over 2 hours. (A, D) The soluble lysate was immunoblotted against HA over the 2-hour recovery. (B) Luciferase activity was measured over the 2-hour recovery. (C) The amount of recovery was measured by comparing the amount of soluble and active luciferase at the beginning and the end of recovery period.

DNAJB1 is important in disaggregation system

Since overexpressing DNAJB1 did not reveal a role in protein homeostasis, DNAJB1 knockout cells were generated using CRISPR/Cas9. We also intended to rescue the knockout by re-expression of DNAJB1.

We first knocked out DNAJB1 in HEK293 FlpIn cells. However, re-expressing DNAJB1 transiently at the endogenous level was a challenge **(Figure 3.4**). Also, the transfection was not uniform throughout the cells. Therefore, we moved onto HEK293 FlpIn TREX cells, which allow the stable transfection and the control of the expression of the transfected protein of interest.

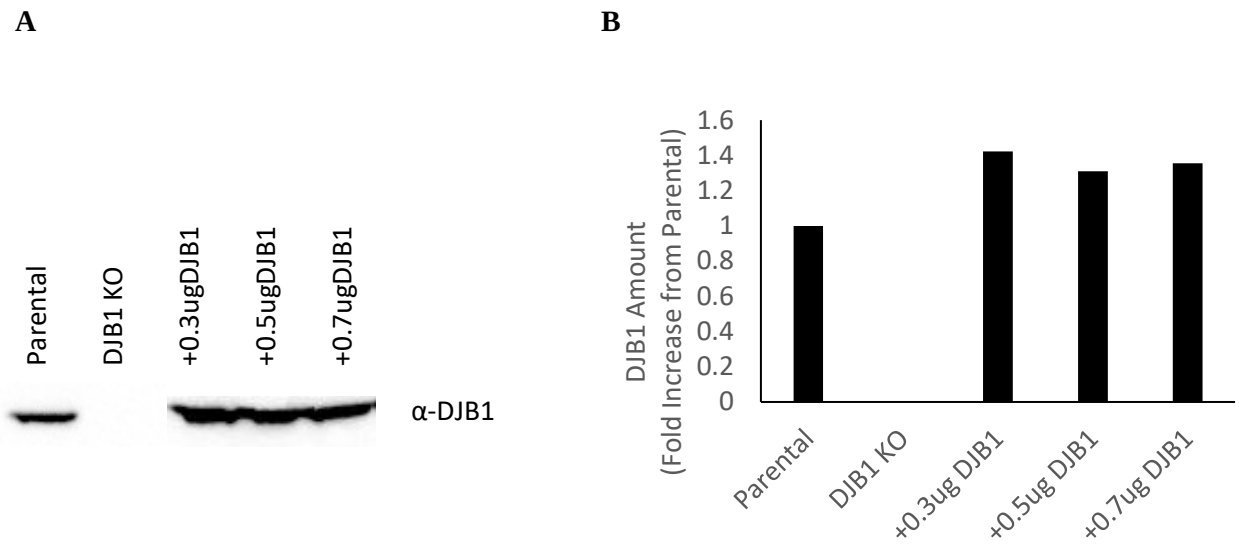


Figure 3.4. Re-expressed DNAJB1 levels in HEK293 FlpIn DNAJB1 KO cells are higher than the endogenous DNAJB1 level

Various amount of DNAJB1 was transiently transfected in DNAJB1 KO cells for 5 hours using Lipofectamine 2000.

DNAJB1 was knocked out in HEK293 FlpIn Trex cells. DNAJB1 knockout did not alter the expression level of other chaperones (**Figure 3.5A**) In heat shock-recovery experiments, compared to the control, the DNAJB1 knockout cells did not show the increase of soluble luciferase and had less increase in the active luciferase over time (**Figure 3.5B-E**).

When the insoluble fraction was analyzed, DNAJB1 knockout showed less increase in soluble luciferase, and somewhat less clearance of aggregated luciferase (**Figure 3.6A-D**). Filter trap assay with SOD1 G85R further showed that lack of DNAJB1 inhibited the disaggregation of proteins (**Figure 3.6E-F**). Not only that, DNAJB1 knockout cells had much more accumulated aggregated material at the start of the chase, compared to the control (**Figure 3.6E**). These results suggest that DNAJB1 is important for protein disaggregation in cells.

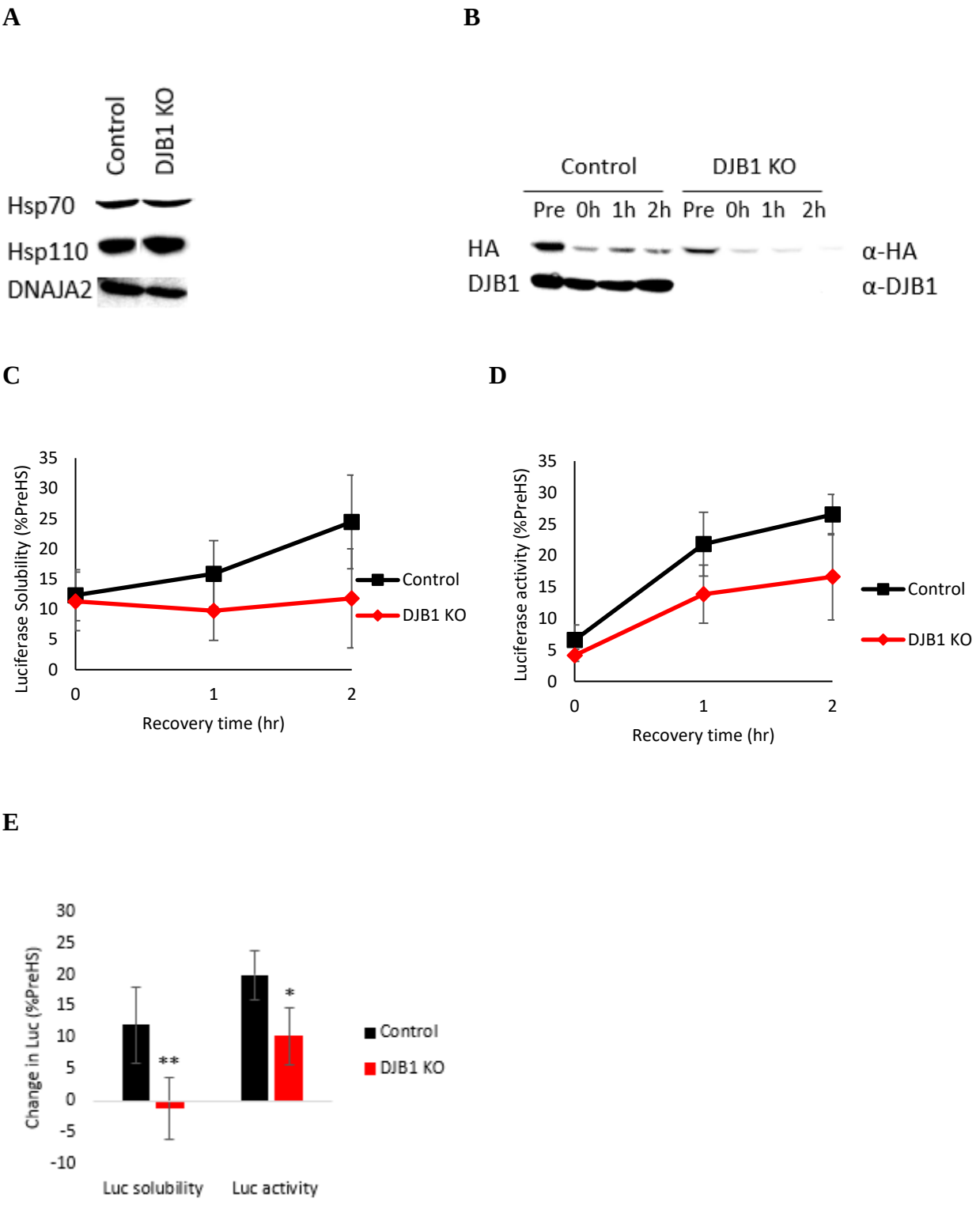


Figure 3.5. DNAJB1 knockout decreases luciferase refolding and solubilization

DNAJB1 was knocked out (KO) in HEK293 using CRISPR/Cas9. HEK293 cells and DNAJB1 KO cells were transfected with HA tagged luciferase. Then, they were heat shocked at 45°C for 1 hour. The recovery was observed over 2 hours. (A) Control and DNAJB1 KO cells were immunoblotted for Hsp70, Hsp110 and DNAJA2. (B-C) The soluble lysate was immunoblotted against HA over 2-hour recovery. (D) Luciferase activity was measured over 2-hour recovery. (E) The amount of recovery was measured by comparing the amount of soluble and active luciferase at the beginning and the end of recovery period.

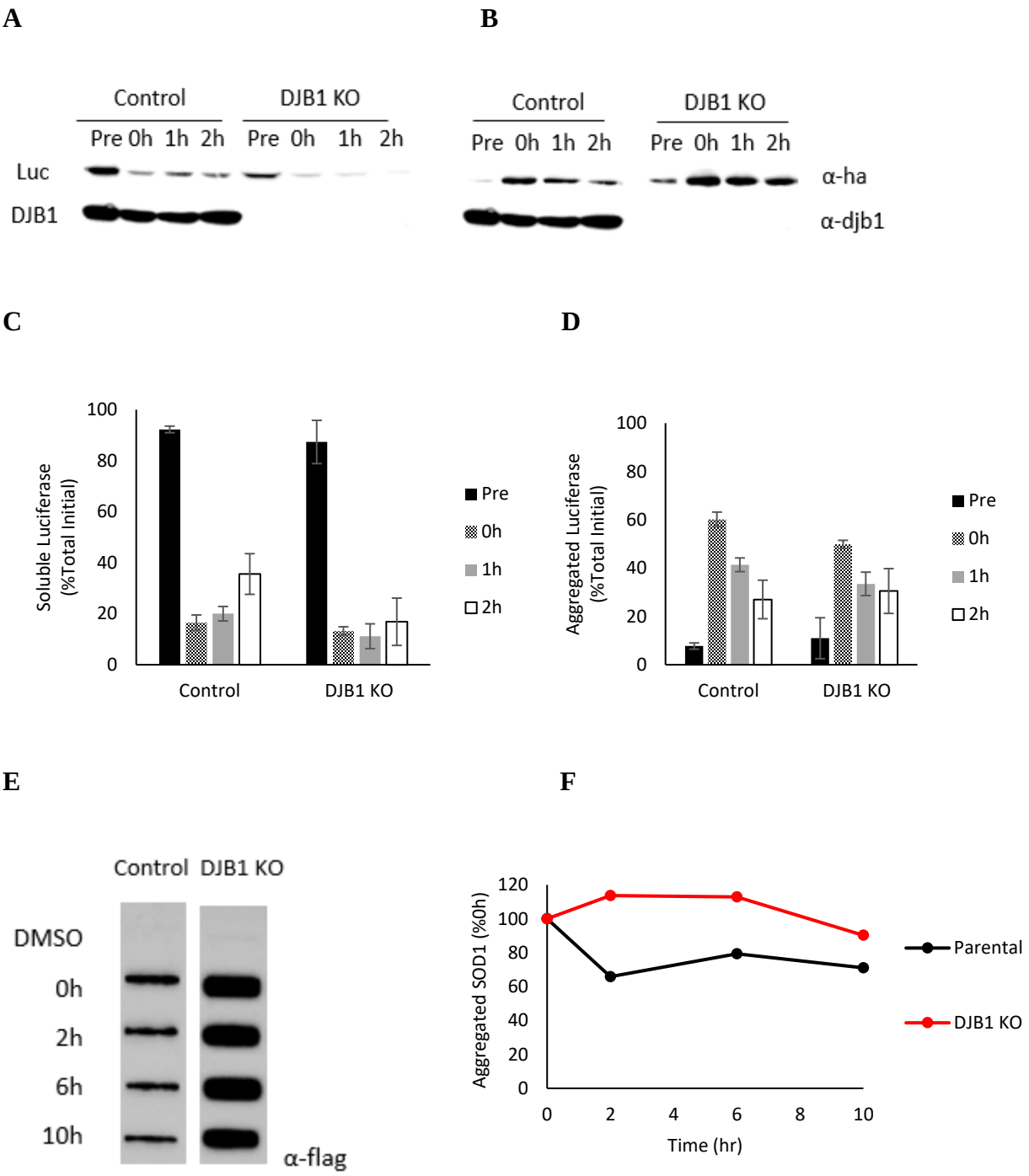


Figure 3.6. DNAJB1 knockout decreases the level of luciferase and SOD1 disaggregation

(A-D) HEK293 or DNAJB1 KO cells were transfected with HA tagged luciferase and then heat shocked at 45°C for 1 hour. The recovery was observed over 2 hours. The supernatant (A, C) and pellet (B, D) were separated at 20000xg for 5min. The pellet was dissolved in 8M urea, 0.25% SDS and sonicated. Then, they were immunoblotted against HA. (E, F) Parental HEK293 or DNAJB1 KO cells were transfected with FLAG tagged SOD1 G85R and were treated with 5μM MG132 for 16 hours. Then, the cells were chased with 50ug/ml cycloheximide for 10 hours. The 1000xg supernatant was analyzed by filter trap as in Figure # and immunblotted against FLAG.

DNAJB1 was re-expressed in HEK293 FlpIn Trex cells

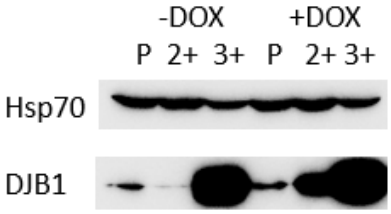
To perform rescue experiments with DNAJB1, DNAJB1 was re-expressed in the knock out cells. DNAJB1 was stably integrated in knockout cells using FlpIn system. Due to the Trex system, the re-expressed DNAJB1 was inducible using doxycycline. Out of 3 clones, clone 1 did not have a sufficient growth rate and therefore discarded. Clone 3 also had a problem where DNAJB1 was expressed even before the induction by doxycycline (**Figure 3.7B**). Therefore, the experiment proceeded with clone 2, which lacked DNAJB1 in the default non-induced condition and successfully induced DNAJB1 upon the addition of doxycycline (**Figure 3.7A,B**).

To express the endogenous level of DNAJB1 in clone 2, a titration experiment was done with a range of duration and concentration of doxycycline. The result showed that 8-hour induction with 0.01-1ug/ml doxycycline brought the level of re-expressed DNAJB1 to the endogenous level (**Figure 3.7C,D**).

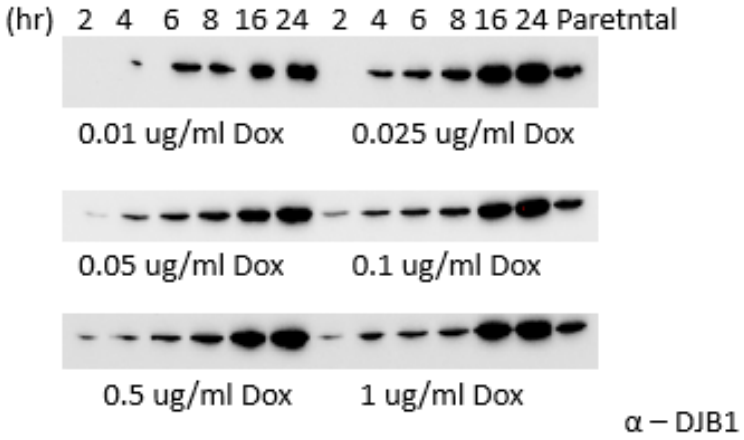
A


1+ 2+ 3+ Parental
α-DJB1

B


-DOX +DOX
P 2+ 3+ P 2+ 3+
Hsp70
DJB1

C


(hr) 2 4 6 8 16 24 2 4 6 8 16 24 Paretntal
0.01 ug/ml Dox
0.025 ug/ml Dox
0.05 ug/ml Dox
0.1 ug/ml Dox
0.5 ug/ml Dox
1 ug/ml Dox
α – DJB1

D

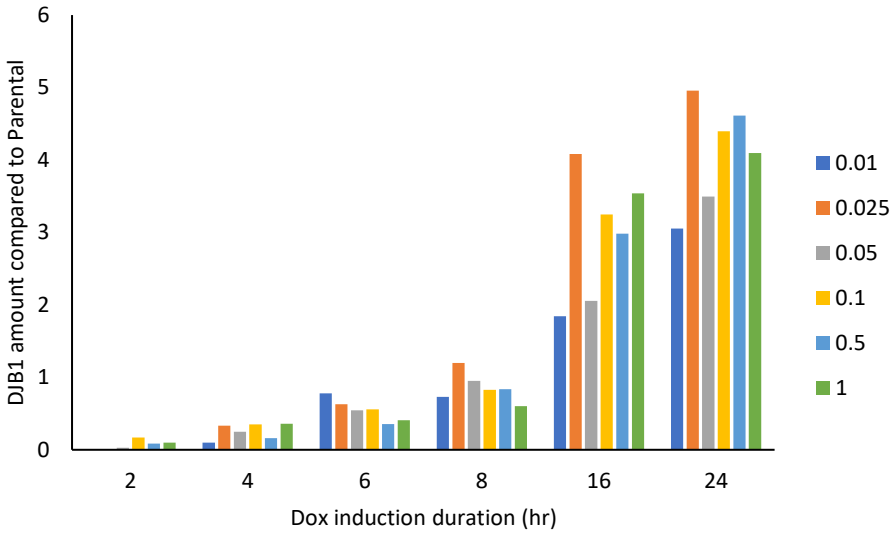

DJB1 amount compared to Parental
0
1
2
3
4
5
6
2
4
6
8
16
24
Dox induction duration (hr)
0.01
0.025
0.05
0.1
0.5
1

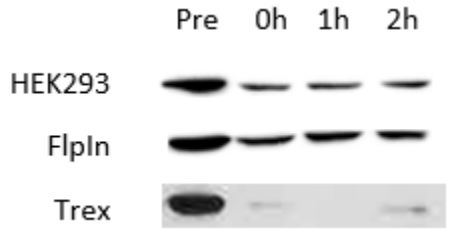
Figure 3.7. DNAJB1 was re-expressed in DNAJB1 knockout cells

(A) DNAJB1 was knocked out in HEK293 FlpIn Trex cells using CRISPR/Cas9. The lysates of clone 1, 2, and 3 along with the parental cell were immunoblotted against DNAJB1 (DJB1). (B) Clone 2 and 3 along with the parental cells were induced by doxycycline for 24 hours at 1ug/ml. (C, D) DNAJB1 was induced with 0.01-1ug/ml of doxycycline for 2-24 hours. The amount of DNAJB1 was compared to that of parental cells.

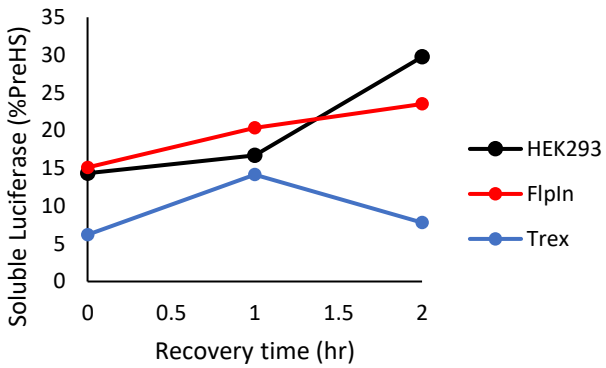
Different HEK293 cells behave differently

We next compared the parental and DNAJB1 knock out cell lines in the aggregation of firefly luciferase. Three different cell lines of HEK293 have been used in our experiments: HEK293, HEK293 FlpIn, and HEK293 FlpIn Trex. Although they are all HEK293 cells, their behaviours in heat shock experiments were surprisingly different. HEK293 FlpIn cells behaved similarly to HEK293 cells as soluble luciferase decreased after heat shock and increased during, but the amount of increase was less (**Figure 3.8**). HEK293 FlpIn Trex cells however were very different from the other two. After heat shock, the amount of soluble luciferase was so minimal it was difficult to detect and analyse on immunoblots. Also, they did not show the increase in soluble luciferase over time. Since DNAJB1 rescue experiment needed to be done in HEK293 FlpIn Trex cells to match the level of DNAJB1 to the endogenous level, this made it difficult to proceed further with experiments.

A



B



C

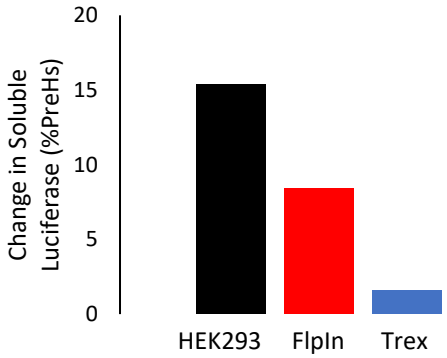


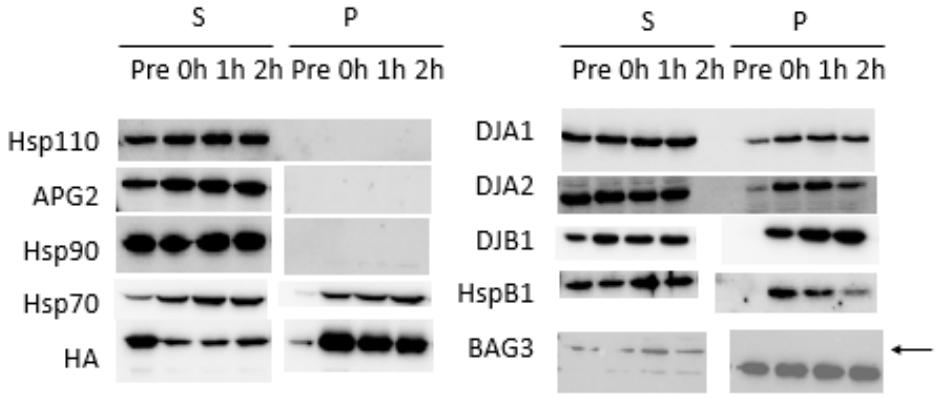
Figure 3.8. HEK293, HEK293 FlpIn, and HEK293 FlpIn Trex cells all behave differently in heat shock experiment

(A-B) HEK293, HEK293 FlpIn or HEK293 FlpIn Trex cells were subjected to 45 °C heat shock for 1 hour and then their recovery was observed over 2 hours. The cell lysates were immunoblotted against HA. (C) The amount of recovery was measured by comparing the amount of soluble luciferase at the beginning and the end of recovery period.

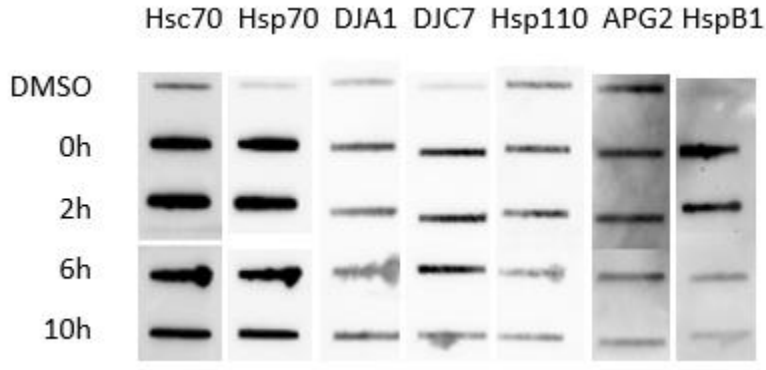
Various chaperones and co-chaperones co-aggregate with proteins

Finding chaperones and co-chaperones where the aggregates are could mean that they play a role in disaggregation. To see which chaperones and co-chaperones co-localize with aggregated proteins, various chaperones were detected by immunoblot in soluble and insoluble fractions. Upon 1 hour heat shock at 45°C, Hsp70, DNAJA1, DNAJA2, DNAJB1, and HspB1 co-localized with aggregates. On the other hand, Hsp110, APG2, Hsp90 and Bag3 did not co-localize with aggregates upon heat shock (**Figure 3.9A**). Filter trap experiments with SOD1 G85R were also carried out. Cells were transfected with mutant SOD1 G85R and then treated with MG132 for 16 hours. The recovery over 10 hour showed that Hsc70, Hsp70,DNAJA1, DNAJC7, HspB1 and DJB1 co-localized with aggregates (**Figure 3.9B**). Interestingly, unlike in heat shock experiment, DNAJA2 did not co-localize with aggregates (**Figure 3.9C**).

A



B



C

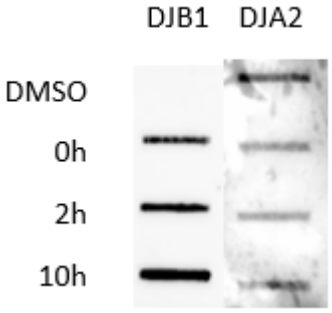


Figure 3.9. Certain chaperones and co-chaperones co-localize with aggregates

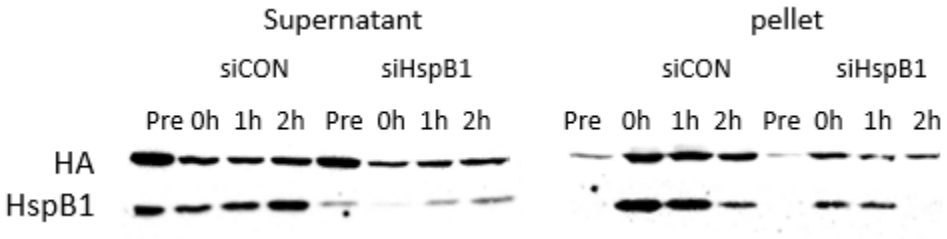
(A) HEK293 cells were treated as in Fig 3.6A-D and immunoblotted against different chaperones and co-chaperones. (B) HEK293 cells were treated as in Fig 3.6E-F and immunoblotted against different chaperones and co-chaperones.

HspB1 does not promote disaggregation in cells

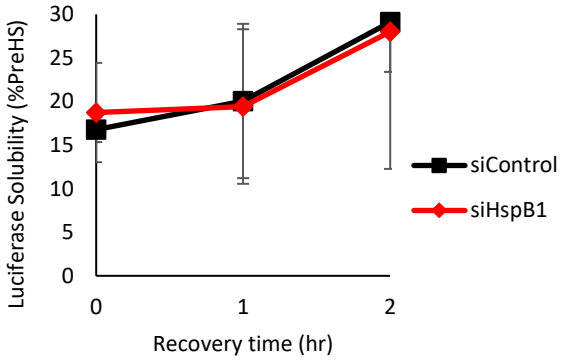
Earlier literature and other data from our laboratory suggested that in vitro, solubilization of aggregates takes place in two steps: first, misfolded proteins co-aggregate with a chaperone that affects aggregate properties, and second, the disaggregation machinery including Hsp70, DJB1 and other co-chaperones solubilize the aggregates. Moreover, there was evidence in vitro that HspB1 was important in the first co-aggregation step (*140*). In cells, HspB1 was induced substantially in the heat shock response (**Figure 3.2D,E**). Also, HspB1 appeared with aggregates in the insoluble fraction after heat shock or with SOD1 G85R after MG132 treatment (**Figure 3.9**).

To see if HspB1 has a role in disaggregation, HspB1 was knocked down in HEK293 cells using siRNA. Luciferase was transfected in HspB1 knock down cells and heat shock experiments were carried out as above. HspB1 entered the insoluble fraction upon heat shock with aggregated luciferase (**Figure 3.10A**). This suggested that HspB1 co-localizes with aggregated proteins. The amount of gain in soluble luciferase over 2 h of recovery did not differ between control and knock down cells. Surprisingly, knocking down HspB1 increased the amount of active luciferase after 2 hours of recovery. The amount of aggregates as well as the clearance of aggregate did not differ significantly in HspB1 knock down cells compared to the control. Although there was no difference in the relative recovery of soluble material, HspB1 knock down cells maintained less soluble luciferase than the control in the initial pre-heat shock condition, for reasons which are unclear.

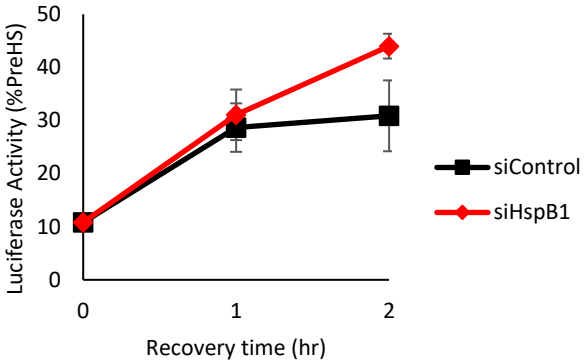
A


Supernatant
pellet
siCON
siHspB1
siCON
siHspB1
Pre 0h 1h 2h Pre 0h 1h 2h
Pre 0h 1h 2h Pre 0h 1h 2h
HA
HspB1

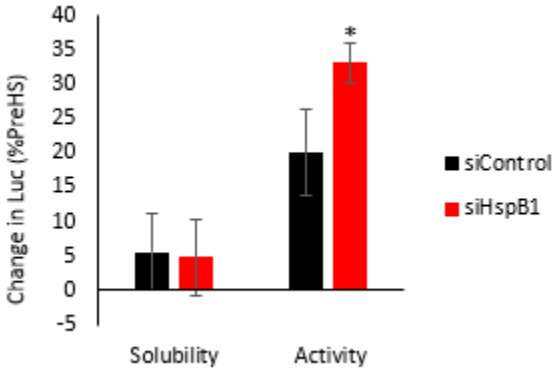
B


Luciferase Solubility (%PreHS)
30
25
20
15
10
5
0
0
1
2
Recovery time (hr)
siControl
siHspB1

C


Luciferase Activity (%PreHS)
50
40
30
20
10
0
0
1
2
Recovery time (hr)
siControl
siHspB1

D


Change in Luc (%PreHS)
40
35
30
25
20
15
10
5
0
-5
*
Solubility
Activity
siControl
siHspB1

D

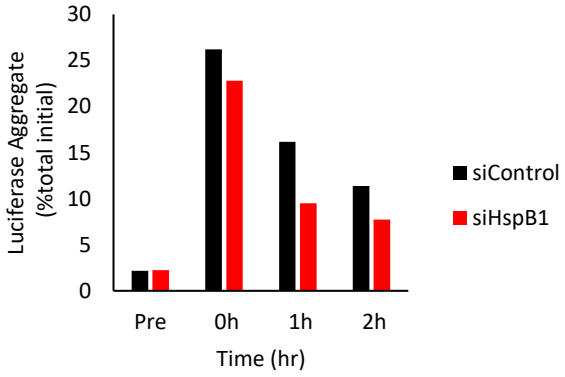

Luciferase Aggregate (%total initial)
30
25
20
15
10
5
0
Pre
0h
1h
2h
Time (hr)
siControl
siHspB1

Figure 3.10 HspB1 knockdown does not affect luciferase disaggregation

HEK293 cells treated with HspB1 siRNA or siControl were transfected with luciferase. Then, cells were heat shocked at 45°C for 1 hour. The recovery was observed over 2 hours. (A, B) The soluble lysates were immunoblotted against HA as The soluble luciferase was measured as in Fig 3.2. (C) Luciferase activity was measured as in Fig 3.2. using cell lysates and luciferin as the substrate in luminometer. (D) The amount of recovery was measured as in Fig 3.3. (E) The insoluble fraction was extracted as in Fig 3.6.

Discussions

Protein misfolding and the consequential aggregation of proteins are the hallmark of protein conformational diseases, including neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and ALS. Therefore, it is crucial for the cells to successfully get rid of misfolded and aggregated proteins for their well-being.

For a long time, toxic protein aggregates in mammals were thought to be cleared by degradation only since mammalian disaggregation was believed to be non-existent (*128*). However, recent studies have shown evidence of protein disaggregation in mammals. Numerous *in vitro* studies have shown that Hsp70, DNAJA2, DNAJB1, Hsp110 and HspB1 are involved in the mammalian disaggregation machinery (*42-44, 140*). I have confirmed that there is a protein disaggregation system in mammalian cells (**Figure 3.1**). Upon heat stress, cells accumulate protein aggregates and the amount of aggregates decreases over time as cells recover from the stress. As the amount of aggregates decreases, the soluble amount of protein increases which proves the presence of protein disaggregation in cells.

Cells recover from proteotoxicity caused by various stresses thanks to the heat shock response. During heat shock response, the expressions of certain chaperones and co-chaperones are increased. The most increased chaperones are DNAJB1 and HspB1 (**Figure 3.2**) which are the components of the disaggregation machinery. This shows that as proteotoxicity increases, cells put more effort into disaggregation than refolding.

As hinted from the heat shock response, DNAJB1 is the key factor of mammalian protein disaggregation. The absence of DNAJB1 in cells resulted in the decrease of protein refolding, solubilization and disaggregation (**Figure 3.5-6**). Interestingly, even though DNAJB1 is the

crucial part of disaggregation machinery, having excessive amount of DNAJB1 does not help but rather hinder protein refolding and solubilization (**Figure 3.3**). This may be due to the sequestration of Hsp70. As the high level of DNAJB1 takes Hsp70 away from other DNAJ co-chaperones, the cells will have difficulty performing the fundamental functions to keep the proteostasis.

On the other hand, the partial absence of HspB1 did not affect protein disaggregation in cells (**Figure 3.10**). It is possible that, in the absence of HspB1, other small heat shock proteins such as HspB5 and HspB8 substitute for the role of HspB1. As the presence of HspB1 is required for the disaggregation *in vitro* (*140*), more studies need to be done to clarify the role of HspB1 regarding disaggregation in cells.

Interestingly, while Hsp70, DNAJB1, and HspB1 are co-localized with protein aggregates, the other disaggregation components, Hsp110, is not (**Figure 3.9**). This suggests that there are multiple steps in disaggregation and that Hsp110 is likely involved in the early stage of disaggregation. DNAJA2 is detected with luciferase aggregates but not with SOD1 mutant aggregates. This suggests that while DNAJB1 is the universal handler of all protein aggregates, DNAJA2 is substrate-specific. The fact that the other DNAJ proteins including DNAJA1 and DNAJC7 are also co-localized with the aggregates also suggests that the cells use different DNAJ proteins for different protein aggregates.

The results from this study confirm the presence of mammalian disaggregation machinery and its key component DNAJB1. For further studies on the mechanism of DNAJB1, rescue experiments using DNAJB1 mutants with abolished interaction with other components of disaggregation machinery should be conducted. These findings may not only elucidate the

mechanism of disaggregation but also shine some light on the possible treatment of the neurodegenerative diseases.

Materials and Methods

Plasmids

Hsc70, Hsp70, Hsp90, DNAJA1, DNAJA2, DNAJB1, DNAJC7, HspB1, Hsp110, APG2, and Bag3 were encoded to pProEx-HTa (Clontech) expression vector as previously described (*41*). FLAG-SOD1, both mutant and wild type, were gifts from the lab of Dr. Heather Durham.

Cell lines

HEK293, HEK293 FlpIn (Thermo Fisher), HEK293 FlpIn TREX (Thermo Fisher) were cultured in Dulbecco's modified Eagle medium 61 (DMEM) high glucose, glutamine and sodium pyrubate (Gibc0-Invitrogen), supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were cultured at 37°C in 5% CO_2. HEK293 FlpIn TREX was a gift for the lab of Dr. David Thomas.

Antibodies

Table 5.1 Antibodies

Company	TARGET
Stressmarq	Hsc70, Hsp70, Hsp90, Hsp110, HspB1, DNAJB1, DNAJC7
Millipore	Bag3, HA
Santa Cruz	APG2
Home-made	DNAJA1, DNAJA2

siRNA duplexes

HspB1 siRNA duplexes were ON-TARGETplus SMARTpool from Theromo Fisher. siRNA duplexes were added using Lipofectamin 2000 (Invitrogen) and then incubated for 5 hours.

Sonication

Cells were collected in PBS and lysed in PBS, 1% Triton X-100. Lysates were centrifuged at 20,000 x g for 5 minutes and the supernatants were collected as soluble material. The pellets were sonicated in PBS, 8M Urea and 0.5% SDS. Then, they were sonicated at 20% for 15 seconds, at 5-second intervals with 5-second breaks in between. They were briefly centrifuged and then the supernatants were collected as insoluble material.

Western blots

Once soluble and insoluble materials were collected from cells, the protein amounts were measured using the BCA Protein Assay (Pierce). The equal amounts of total lysates were separated by SDS-PAGE and transferred to nitrocellulose using Semi-Dry Transfer Cell (Bio Rad). Chemiluminescence was detected by ECL or ECL prime (GE Healthcare) on ChemiDoc Imaging System (Bio Rad) for quantification.

Luciferase solubility and refolding assays

Cells were transfected with HA-tagged luciferase as previously described (*41*). Cells were transfected for 48 hours and then were treated with 50 μg/ml of cycloheximide. Cycloheximide-treated cells were then heat-shocked at 45°C for 1 hour and brought back to 37°C. Cells were collected before heat shock (preHS), 0h, 1h, and 2h after heat shock. The enzymatic activity of folded luciferase was assayed by the Luciferase Reporter Assay Kit (Promega) and normalized to the total lysate amounts. The soluble amount of luciferase was quantified using Western blot.

Filter Trap

Cells transfected with SOD1 wild type or G85R mutant were treated with 5 µM MG132 for 16 hours. After 16 hours, cycloheximide chase was done for 10 hours and cells were collected at 0, 2, 6, and 10 hour marks. Cells were harvested in PBS, 1% Triton X-100 and centrifuged at 1000 x g for 5 minutes to remove large debris. 100 µg of protein were diluted to 1ml. The cellulose acetate membrane was equilibrated in PBS, 1% Triton X-100 and then assembled into the Bio-Dot SF microfiltration apparatus (Bio Rad) with two sheets of filter paper underneath the membrane. The membrane was washed with buffer twice before applying the samples. The blots were detected by western blot.

Generation of HEK293 FlpIn and HEK293 FlpIn TREX stable cell lines

DNAJB1 was cloned into pcDNA5/FRT/TO vector (Thermo Fisher). Cells were transfected with 10:1 ratio of pOG44 (Thermo Fisher) and pcDNA5/FRT/TO DNAJB1. 4 days after transfection, cells were selected with 200 µg/ml hygromycin B. The cells which survived the selective media were collected as the stable cell line.

CRISPR knock out

Cells were transfected with 500ng CRISPR/Cas9 plasmids (Santa Cruz). 48 hours later, cells were selected with 1 µg/ml puromycin. After 72 hours of puromycin incubation, puromycin was removed and colonies were grown. Once visible under microscope, individual colonies were picked with P10 pipette.

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