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Role of J-domain proteins in regulation of Hsp70-mediated protein disaggregation

Rola białek z domeną J w regulacji dezagregacji agregatów białkowych przez system Hsp70

A Ph. D. dissertation in the field of Natural Sciences specialization Biotechnology presented to The Scientific Council of Biotechnology Sciences University of Gdańsk

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1. Streszczenie

Homeostaza białek komórkowych jest nieustannie zagrożona wystąpieniem warunków stresowych. Nagłe zmiany w środowisku komórki, mutacje czy błędy podczas translacji mogą prowadzić do zaburzenia struktury natywnej białek. Nagromadzenie źle sfałdowanych białek może prowadzić do utworzenia amorficznych struktur nazywanych agregatami białkowymi.

By przeciwdziałać temu procesowi, komórki wytworzyły wiele mechanizmów obrony. Jednym z nich jest aktywność systemu białek opiekuńczych Hsp70-Hsp100, który oddziałuje z agregatami, ostatecznie prowadząc do odzyskania białek w nich uwięzionych i przywrócenie im natywnej konformacji. Głównymi regulatorami aktywności systemu Hsp70 są białka z domeną J (*ang.* J-domain proteins). Ich aktywność polega na rozpoznaniu i związaniu agregatu, a następnie zadokowaniu do agregatu białka Hsp70. Z uwagi na to, że aktywność dezagregacyjna może być promowana przez więcej niż jedno białko J, głównym założeniem mojej pracy było ustalenie funkcjonalnych różnic w aktywności systemu Hsp70 w procesie dezagregacji w zależności od zaangażowanego białka J.

W moich badaniach użyłem modelu drożdżowych, cytozolowych białek opiekuńczych z domeną J, białka klasy A, Ydj1 oraz białka klasy B, Sis1. Używając pomiarów biochemicznych w czasie rzeczywistym, badałem, jak aktywność systemu Hsp70 w procesie dezagregacji zależy od zaangażowanego białka z domeną J. Pokazałem, że Ydj1 jest zdolny do wiązania agregatu, co następnie pozwala na dokowanie białka Hsp70. Z kolei Sis1 wymaga jednoczesnej obecności Hsp70, by oddziaływać z agregatem, ale jego aktywność pozwala na znacznie efektywniejsze dokowanie białek Hsp70 do agregatu niż w przypadku Ydj1. Duża ilość oddziałujących z agregatami cząsteczek Hsp70 może wzmagać efekt entropowy, prowadząc do globalnego rozluźnienia struktury agregatu, co odsłania nowe potencjalne miejsca wiązania dla białka Hsp70. Ten cykl zdarzeń może ostatecznie prowadzić do pełnej dezagregacji i odzyskania zagregowanych białek, nadając im z powrotem strukturę natywną. Z drugiej strony, z uwagi na możliwość samodzielnego oddziaływania z substratem, Ydj1 może wiązać uwolnione przez Sis1-Ssa1 polipeptydy, przeciwdziałając ich reagregacji. Te komplementarne aktywności w dezagregacji mogą stanowić podstawę efektywnego odzyskiwania białek, utraconych w wyniku agregacji.

Jako że u Metazoa, w przeciwieństwie do drożdży, nie występuje dezagregaza z rodziny Hsp100, jest możliwe, że taka dywersyfikacja aktywności systemu Hsp70 może grać główną rolę w procesie dezagregacji u tych organizmów. Używając ludzkich ortologów białka Hsp70 oraz białek J klasy A i B, zaobserwowałem podobne trendy jak dla systemu drożdżowego, w których białko J klasy B, w odróżnieniu od białka klasy A, promuje wydajniejszą dezagregację oraz pozwala na budowanie większego kompleksu białek opiekuńczych na agregacie. To implikuje ewolucyjne znaczenie wzajemnie komplementarnych aktywności systemu Hsp70 zależnych od białek J w dezagregacji i ponownym fałdowaniu białek.

2. Abstract

Cells are at constant risk of stress affecting protein homeostasis. Environmental factors as well as mutations and translational errors could lead to protein misfolding. The inability to attain native conformation by multiple proteins leads to formation of protein aggregates. To fight their detrimental influence cells developed multiple pathways of their clearance. One of them is the Hsp70-Hsp100 chaperone system, which can act on protein aggregates ultimately leading to recovery of proteins in their native state. Main regulators of the Hsp70 system's activity are J-domain proteins (JDPs). Their main objective is to recognize and bind substrates and eventually recruit Hsp70 protein to them.

Considering the fact that the promotion of protein disaggregation through Hsp70 is not limited to a single J-domain protein, I examined what are the functional differences in protein disaggregation imposed by J-domain proteins of different classes. As a model, I used the yeast cytosolic Hsp70 system, which involves Class A Ydj1 or Class B Sis1 JDPs.

Using real-time biochemical methods, I studied how the activity of the Hsp70 system changes due to the employed JDP. I found that Class A Ydj1 is superior in aggregate binding, which then promotes Hsp70 loading onto the substrate. In turn, Sis1 requires simultaneous presence of Hsp70 to interact with an aggregate but yields more abundant loading of Hsp70. High level of Hsp70 on the aggregate potentiates the entropic effect, which can lead to overall relaxation of the aggregate, which uncovers more binding sites for Hsp70. This cycle of events can ultimately lead to aggregate dissolution and recovery of native proteins. In turn, Class A Ydj1, due to its autonomous substrate binding ability, can bind previously released polypeptides, preventing their reaggregation, to allow them to await Hsp70-assisted folding. Taken together, these complementary activities in disaggregation could be the driver of efficient protein recovery.

Since Metazoa, unlike yeast, lack the Hsp100 disaggregase, such diversification of Hsp70 system activity could play a key role in disaggregation and refolding in these organisms. Using human orthologues of Class A and B JDPs, I observed similar trends as for the yeast proteins. Class B JDP, contrary to Class A JDP, can promote higher level of protein recovery during disaggregation and lead to more abundant chaperone complex formation

on the substrate. This implicates the evolutionary relevance of the J-domain driven complementing Hsp70 system activities in protein disaggregation and refolding.

3. Abbreviations

AAA+ ─ ATPases associated with various cellular activities

ATP ─ Adenosine triphosphate

ADP – Adenosine diphosphate

ATPase ─ Enzyme which hydrolyses ATP

BLI ─ Bio-Layer Interferometry

CTD – C-terminal domain

DLS – dynamic light scattering

DTT ─ Dithiothreitol

ER – endoplasmic reticulum

E. coli ─ *Escherichia coli*

EDTA ─ Ethylenediaminetetraacetic acid

JDP – J-domain protein

GFP – green fluorescent protein

HSP ─ heat shock protein

HEPES - N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid

IPTG ─ Isopropyl β-D-1-thiogalactopyranoside

LB ─ Luria broth

LA – Luria broth with agar

Luc ─ Luciferase

NBD ─ Nucleotide-binding domain

NEF ─ Nucleotide exchange factor

PIKK - Phosphatidylinositol 3-kinase-related kinase

RPM ─ Rotations per minute

RT – room temperature

S. cerevisiae – *Saccharomyces cerevisiae*

SBD ─ Substrate-binding domain

SDS ─ Sodium dodecyl sulphate

SDS-PAGE ─ SDS polyacrylamide gel electrophoresis

TRIS ─ Tris(hydroxymethyl)aminomethane

ZFLR – zinc-finger like region

4. Introduction

Proteins, fundamental molecules of every living cell, perform a plethora of biological activities that define life as we know it. These irreplaceable and complex structures are stabilized by vast networks of intra- and intermolecular interactions. Maintenance of proteins is one of the great challenges of every living cell.

Proteostasis (homeostasis of the proteome) is the term defining the ability of the cells to regulate the level and structural well-being of proteins, which need to be guarded throughout many stages of the protein life cycle. It begins at the level of transcriptional regulation of expression, through tightly regulated translation and folding, which ultimately delivers functional protein. However, the complexity of the process and its protein products render it susceptible to errors and dangers, especially under stress conditions. Their common consequence is protein misfolding and aggregation.

4.1 The mechanism behind protein aggregation

Polypeptides, which self-organize to form native protein structures, are stabilized through a vast net of intra- and intermolecular interactions. Even though native conformation must be maintained, it also needs to allow a certain level of structural plasticity to perform biological functions, which are specific for every protein - for example enzymatic activity. This equilibrium needs to be kept all throughout the protein life cycle until its eventual degradation. Protein stability depends on energy of the folded state: the lower the energy of the folded state, the more resilient the protein is to the structure-destabilizing conditions. Proteins have evolved to be at balance between the aforementioned plasticity and the level of stability that prevents spontaneous loss of fold. Nevertheless, excess disruption of this equilibrium due to elevated temperature, increased osmotic pressure, alcohol content, high concentration of heavy metals or oxidative stress can lead to protein unfolding events across the cell, leading to the formation of multi-polypeptide amorphous assemblies, called protein aggregates (Fig. 1).

What predominantly drives protein aggregation is the exposure of hydrophobic amino acids at the surface. Normally, native states of proteins determine the assembly of hydrophobic amino acids into regions, named hydrophobic cores (Munson et al. 1996), shielding them from thermodynamically unfavorable polar environment. Disruption of these cores might lead to polypeptide unfolding, as long as environmental conditions do

not favor spontaneous or assisted regaining of the native state. Local concentration of misfolded polypeptides could lead to intermolecular interactions between hydrophobic residues reaching energetically favored local minimum. These interaction drives oligomerization (Onuchic, Luthey-Schulten, Wolynes 1997), what leads to aggregate assembly.

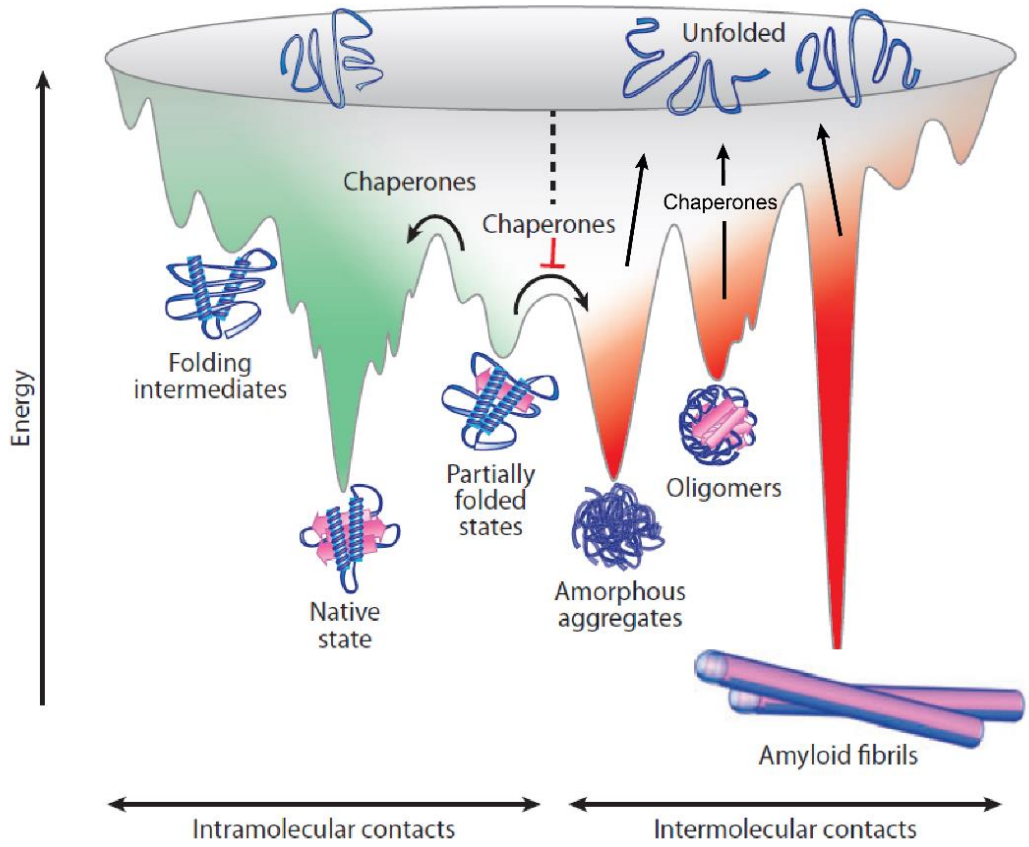

Figure 1. ***Energy landscape of protein folding.*** *Folding polypeptide samples various conformations in folding energy landscape. It progresses through energetically favorable states, until reaching local energetic minima. The range of possible conformations leading to the native state are marked in green. The conformational states that can lead to misfolding and aggregation are marked in red. Chaperones can promote folding of kinetically trapped partially folded polypeptides or prevent intermolecular interactions that lead to aggregation. Chaperone systems with high disaggregation and refolding ability can resolubilize protein aggregates returning the recovered polypeptides to correct folding pathways. Adapted from Kim et al, 2013.*

Specific form of protein aggregation is the highly ordered assembly of polypeptides into amyloid fibrils, predominantly assembled at favorable conditions from particular intrinsically disordered proteins (Scollo and La Rosa, 2020). They assemble highly ordered beta-sheet rich oligomers, which are stabilized by vast contact zones (Chiti and Dobson 2006). Due to that, they present very high level of stability (Gilliam and Macphee 2013).

Protein unfolding and aggregation have detrimental effects on the cell, as it may impair biochemical pathways, destabilize cellular membranes, induce DNA damage, interfere with lysosomal degradation and many others (Wen et al. 2023). What is more, misfolded polypeptides can directly interact with and promote destabilization of other functional proteins or folding intermediates. All in all, protein aggregation and its relevance for cell viability, as well as potential mechanisms of cytotoxicity are still a topic of debates and research (Stefani and Dobson 2003; Zhou and Xu 2014, Saarikangas and Barral, 2016, Fassler et al, 2021).

4.2 Molecular chaperones in counteracting protein misfolding

During evolution, cells to act against protein misfolding and aggregation developed multiple ways to fight it. Across all living organisms, one can find systems involved in various pathways specialized in protein homeostasis maintenance. Special subgroup of proteins called molecular chaperones combat events of protein aggregation (Fig. 1). Specifically participating in these processes is Hsp70 with its co-chaperones: J-domain proteins and nucleotide exchange factors as well as Hsp100 disaggregases. Their cooperation allows for rescue of polypeptides trapped in protein aggregates (Fig. 2) (Liberek et al, 2008; Saibil 2013; Mogk et al, 2018; Rosenzweig et al, 2019). Detailed involvement of specific chaperones in this process is described in the following subchapters.

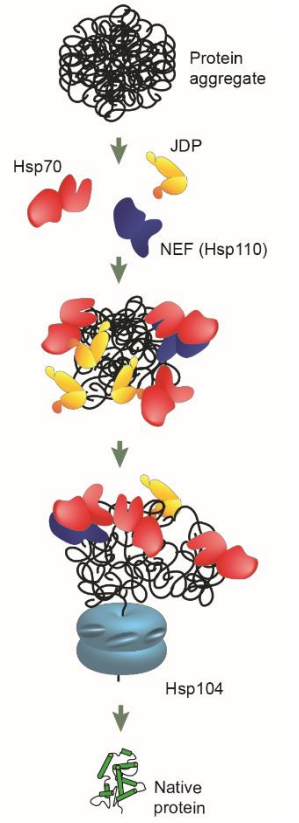

Figure 2. ***Scheme of protein disaggregation.*** *Protein aggregate is bound and processed by Hsp70 system. Next, the Hsp100 disaggregase can be recruited by the Hsp70. The activity of chaperones rescues polypeptides trapped in aggregates and allows their refolding to native state.*

4.3 Hsp70

Hsp70 is a versatile chaperone involved in various protein maintenance activities (Rosenzweig et al, 2019). It is involved in folding of newly synthesized polypeptides (Nelson et al. 1992), translocation of proteins through membrane channels (Young, Hoogenraad, Hartl 2003; Craig 2018), assembly and disassembly of protein regulatory complexes (Liberek, Georgopoulos, Żylicz 1988; Hwang, Crooke, Kornberg 1990), protein disaggregation (Hartl 1996) and targeting proteins for degradation (Shiber et al, 2014). This 70-kDa protein consists of two domains:

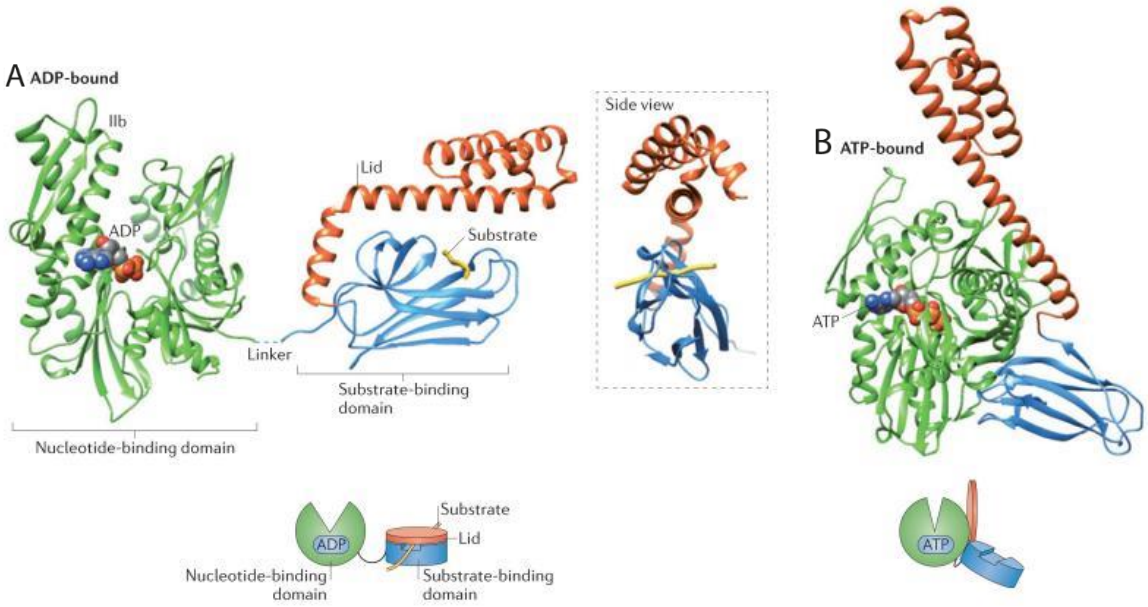

Figure 3. ***Structure and conformational states of Hsp70 protein.*** *(A) ADP bound state, the nucleotide binding domain (NBD) (green, PDB code: 3HSC) is separated front substrate binding domain (SBD) (blue, PDB code: 1DKZ) by inter domain linker. Peptide substrate marked in yellow is locked onto the SBD by the lid domain in orange. Side view of the SBD is shown on the right. Cartoon representation of the state is depicted below. (B) The ATP bound state, NBD is docked to SBD with its lid domain bound to NBD, what exposes the substrate binding domain (PDB code: 4B9Q). Adapted from Saibil 2013.*

Nucleotide Binding Domain (NBD) and Substrate Binding Domain (SBD) (Vogel et al, 2006), which are separated with disordered, flexible linker, which mediates allosteric communication between the two domains (Fig. 3) (Jiang et al, 2007; Kityk et al, 2015). Substrate Binding Domain can be subdivided into SBDβ, which harbors a beta-sandwich with a peptide binding cleft and SBDα, which assembles into an α-helical lid, which can close on the SBDβ substrate binding pocket, stabilizing Hsp70 interaction with a substrate (Fig. 3) (Zhu et al. 1996; Flaherty et al, 1994, Bertelsen et al. 2009).

Hsp70 predominantly binds short hydrophobic stretches of amino acids and its interaction with substrates is directly connected with its ATPase activity (Russel, Jordan, McMacken 1998). Bound nucleotide determines the domain arrangement within the Hsp70 protein. In the ATP-bound state, SBD is wrapped around the NBD domain, opening the substrate binding cleft. ATP hydrolysis triggers the separation of the domains and closure of the α-helical lid over the substrate binding pocket (Rosenzweig et al, 2019). Shifting between these two states is tightly regulated by Hsp70 co-chaperones: J-domain proteins and nucleotide exchange factors. Subsequent interactions between Hsp70, its partners and substrate, assemble a cycle of ordered steps being the core of Hsp70 system activity, termed Hsp70 ATPase cycle (Kityk, Kopp, Mayer et al, 2018). The cycle is prompted by substrate-interacting J-domain protein, which promotes binding of the Hsp70 protein.

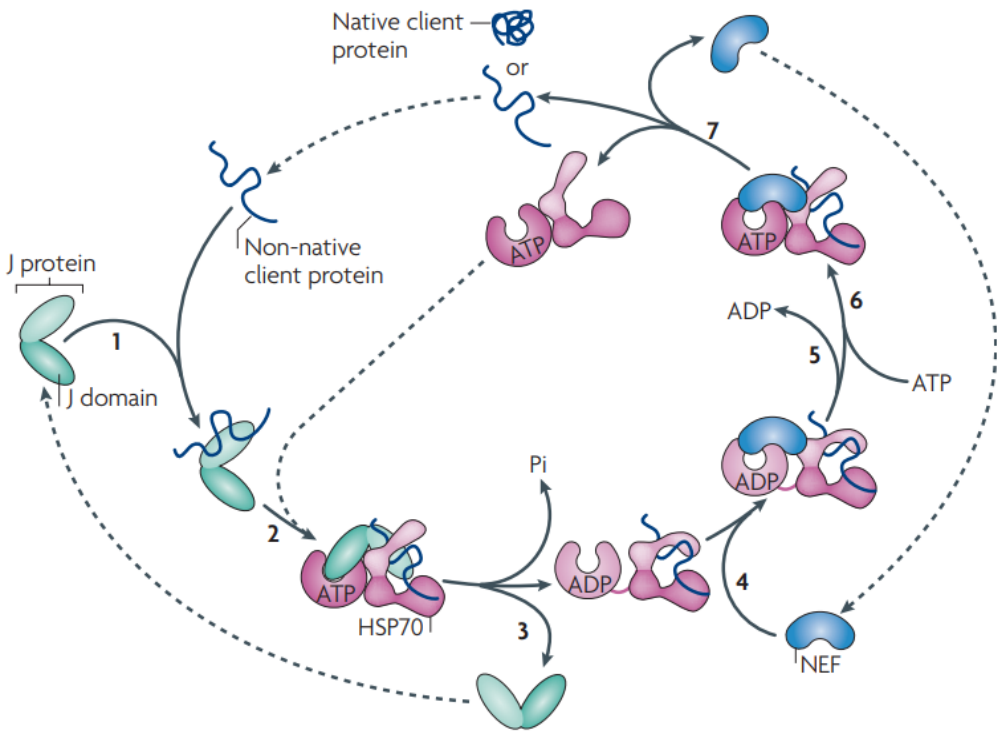

Figure 4. ***Hsp70 ATPase cycle.*** *(1) J-domain protein binds client polypeptide, (2) stimulation of Hsp70 ATPase via J-domain interaction, (3) The client polypeptide binds to the SBD of Hsp70, while due to ATP hydrolysis, the Hsp70 changes conformationally and stabilizes its interaction with the polypeptide, J-domain leaves the complex, (4) Nucleotide exchange factor interacts with Hsp70, lowering its affinity for ADP. ADP dissociates (5), which is followed by subsequent binding of ATP by Hsp70 (6), which results in client polypeptide dissociation (7). Adapted from Kampinga and Craig, 2010.*

Through the stimulation of nucleotide hydrolysis by JDP, Hsp70 changes its conformation, substantially increasing affinity for its substrate. Subsequent substrate release is mediated by Nucleotide Exchange Factor by prompting nucleotide exchange and reassuming the open conformation by Hsp70 (Fig. 4) (Packschies et al, 1997; Silberg and Vickery 2000). How Hsp70 activity leads to polypeptide folding has sprung many theories. Current understanding of the Hsp70 folding ability was named: the entropic pulling mechanism (De los Rios and Goloubinoff, 2006). In protein disaggregation, Hsp70, which interacts with unfolded polypeptide, seeks to increase its freedom of movement due to the Brownian motions, which applies force to the polypeptide allowing it to pursue a correct folding pathway, or in case of disaggregation, leads to its disentanglement from the protein aggregate. It is further fueled by restricting the freedom of movement of the Hsp70 molecule, due to the close vicinity of the aggregate and other aggregate-bound Hsp70 molecules (Fig. 5) (De los Rios and Goloubinoff, 2006). A net of interacting Hsp70 molecules on the aggregate can lead to global relaxation of the aggregate, by partial or complete liberating of polypeptides reoccurring until aggregate dissolution (De los Rios and Goloubinoff, 2007).

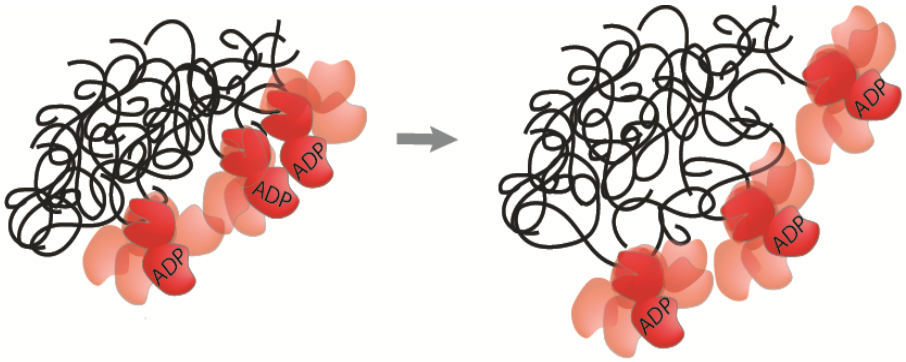

Figure 5. ***Effect of Hsp70 entropic pulling on the protein aggregate.*** *Movement of Hsp70 (red) near the chaperone binding site leads to loosening of the polypeptides trapped in aggregate. Faded depictions represent the available spaces for exploration by the same Hsp70 molecule.*

4.4 Nucleotide exchange factors

The Hsp70 system activity depends on conformational cycling of Hsp70 protein dictated by the bound nucleotide (Rozenzweig et al, 2019). To commence exchange of ADP to ATP, which promotes substrate release and regain of productive conformation for another cycle of activity, the ADP molecule must dissociate. This change can occur spontaneously at very low rates (Packschies et al, 1997, Silberg and Vickery, 2000). To boost it, the system utilizes Nucleotide Exchange Factors (NEFs), which are co-chaperones, which promote ADP dissociation from Hsp70 by direct interaction with the chaperone (Fig. 4) (Blatch et al, 2007). Immediate binding of another ATP is conditioned by high cellular concentration of ATP in respect to ADP. There are multiple, independently evolved families of NEFs, which perform similar function. The one taking part in protein disaggregation is termed Hsp110 family (Bracher and Verghese, 2015).

4.5 Hsp104 disaggregase

Battling protein aggregation in severe stress might not be manageable by the sole Hsp70 system (JDP-Hsp70-NEF). Especially, in the case of monocellular organisms like bacteria and yeast, which lack any global regulation of their temperature, and plants, whose ability to escape unfavorable conditions is very limited. Changes in the surrounding environment, like prolonged rise in the temperature can lead to severe protein aggregation. To handle such conditions, the abovementioned organisms utilize another chaperone family, which empower the Hsp70 machinery – Hsp100 disaggregates (Fig. 2).

Hsp100 disaggregases belong to the AAA+ family. They assemble into hexameric oligomers, forming central channel (Erzberger and Berger, 2006). The monomer of the protein is composed of N-terminal domain, which is associated with substrate recognition (Doyle et al. 2013; Rosenzweig et al. 2013). It is followed by two Nucleotide Binding Domains NBD1 and NBD2 (Lee et al, 2013; DeSantis et al, 2014), M-domain involved in disaggregase regulation (protruding from NBD1) (Fig. 6) (Mogk et al, 2015) and ending with C-terminal domain, which takes part in disaggregase hexamerization (Mackay et al. 2008). Hsp100 hexamer, fueled by energy from ATP-hydrolysis, coordinates a cascade of motions leading to translocation of the polypeptide substrate through its central channel (Yokom et al. 2016; Yu et al. 2018). In consequence, the released polypeptide can attempt to regain its native state or await Hsp70 for folding assistance.

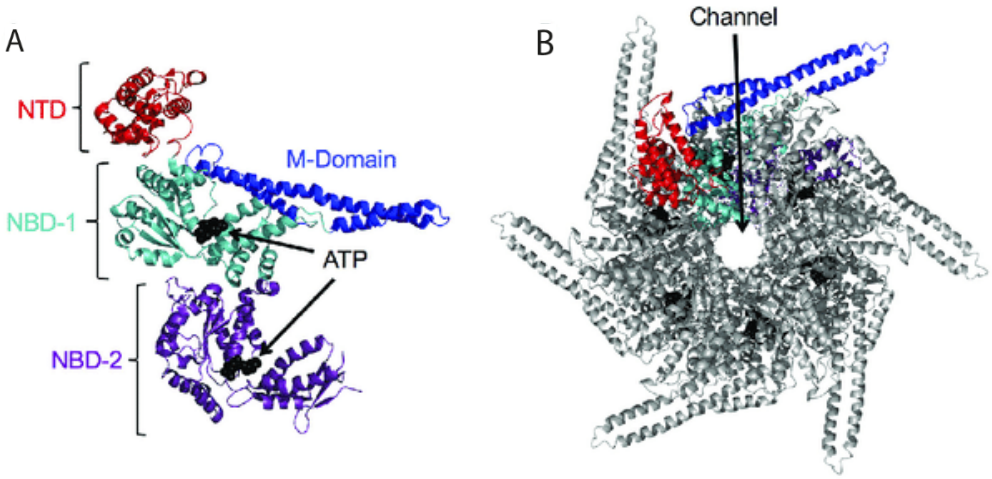

Figure 6. ***Ribbon representation of Hsp100 family disaggregase.*** *(A) Monomer of Hsp100. N-terminal domain (NTD) is depicted in red. Nucleotide binding domain-1 (NBD-1) is marked in green, with the M-domain in blue. Nucleotide binding domain-2 (NBD-2) is marked in purple (PDB code: 1QVR). (B) View from the top of the hexamer of Hsp100. Adapted from Johnston et al. 2017.*

The activity of the Hsp100 disaggregase is tightly regulated, as the employment of Hsp100 in the disaggregation process depends on the direct recruitment by the Hsp70 chaperone (Winkler et al. 2012). Hsp70 recruits Hsp100 to the substrate and promotes its translocation by interacting with M-domain (Mogk et al, 2015). Dysregulation of this control by mutations in the M-domain, can render the disaggregase hyperactive (Haslberger et al, 2007; Gates et al, 2017), meaning that it can spontaneously recognize and translocate both unfolded and properly folded proteins containing intrinsically disordered regions (Tessarz et al, 2009). Uncontrolled unfolding of proteins can be toxic to the cell. Hsp70 modulates the substrate preference by targeting the disaggregase to

abnormal protein aggregates, swaying the disaggregase from intrinsically disordered proteins (Chamera et al, 2019). Another level of regulation is connected with the cellular level of ATP/ADP. Due to its low affinity for ATP, the Hsp100 is highly repressed by ADP concentration, which can be overcome by tight cooperation with Hsp70 (Kłosowska et al. 2016). Summarizing, the functional cooperation between Hsp70 system and Hsp100 constitutes the full bacterial, plant, yeast, and other single cell eukaryotes disaggregation machinery (Fig. 2).

4.6 J-domain proteins

4.6.1 Overview and history

J-domain proteins form the largest family of proteins among molecular chaperones (Nillegoda et al. 2023). For example, humans have 41 J-domain proteins, *Arabidopsis thaliana* – 116, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Saccharomyces cerevisiae* contain 22 J-domain proteins (Walsh et al, 2004; Rajan and D'Silva, 2009; Qiu et al, 2006; Jungkunz et al, 2011; Kampinga and Craig, 2010). They are involved in a broad variety of protein homeostasis processes, which are: folding of newly synthesized polypeptides, protein transport through membranes, assembly and disassembly of multiprotein complexes, regulation of the stability and activity of proteins through misfolding and aggregation prevention and degradation of unfolded or obsolete proteins (Bukau and Horwich, 1998; Mayer et al. 2001; Mayer and Bukau, 2005; Kampinga and Craig, 2010).

Their principal feature, from which their name originated, is the heavily conserved ~70 amino acid-long domain with substantial similarity to the N-terminal domain of *Escherichia coli* DnaJ protein (Pellecchia et al. 1996). It is composed of four alfa-helices (helices 1-4) with a loop region between helix 2 and 3, which harbors extremely conserved tripeptide of histidine, proline and aspartic acid, referred to as the HPD motif (Fig. 7A) (Hill et al. 1995, Qian et al. 1996, Laufen et al. 1999). Specifically, this part of the J-domain is established to be the principal interaction site between a JDP and its Hsp70 partner (Corsi and Schekman 1997, Landry 2003, Wittung-Stafshede et al. 2003). Transient contact between the HPD motif and Hsp70's NBD domain triggers ATP hydrolysis (Misselwitz et al. 1999; Suh et al. 1999; Jaing et al. 2007). This interplay between J-domain protein and its Hsp70 partner is the basis of Hsp70 ATPase cycle, which results in 1000-fold stimulation of the ATPase activity (Jordan and McMacken,

1995). Apart from the J-domain, JDPs possess a variety of different domains, which together promote targeting of their Hsp70 partner to specific cellular activities. These may include: extended regulation of interaction with Hsp70 partner, substrate selection and promoting their transfer to Hsp70, acting as an scaffold for multiprotein complexes assembly in junction with Hsp70 and targeting Hsp70 to cellular compartments (Kampinga and Craig, 2010, Maliverni et al, 2022, Dekker et al, 2015).

Structural differences of J-domain proteins were the basis of their classification. In homology to DnaJ, Class A/Type I JDPs feature: the N-terminally localized J-domain, an unstructured region rich in glycines and phenylalanines (G/F-rich region), followed by conserved C-terminal domain (CTD, subdivided to CTDI and CTDII), which contains zinc-finger like region (ZFLR) and alfa-helical dimerization domain at the C-terminus. Class B/Type II J-domain proteins present analogical domain composition and arrangement, however the differentiating feature is the lack of the zinc-finger like region (ZFLR) (Fig. 7B). Lastly, Class C/Type III characterizes proteins featuring a J-domain, yet its localization and overall domain composition and arrangement are not homological to DnaJ (Cheetham and Caplan, 1998). However, this historical classification does not specify biochemical function or activity of the members. What is more, the ability of binding protein substrates, which is thought to be the primary function, is also not taken into consideration. When comparing some Class B JDPs (e.g., yeast Sis1), which exhibit substrate binding domains like Class A JDPs (e.g., yeast Ydj1), other members of Class B JDPs might not have similar substrate binding domains (e.g., human DNAJB6, DNAJB8) or the substrate binding region is totally missing. All at the same time, the majority of Class C contain substrate binding domains, which could bind one or a few specific substrates (e.g., yeast Jac1). Therefore, the proper classification of the J-domain proteins is still a matter of discussions in the field (Kampinga and Craig, 2010; Craig and Marszałek, 2017). To simplify the nomenclature, I will refer to groups of JDPs with their classical names.

Most prominent, especially in the beginning, was research of *E. coli* Class A protein DnaJ, which is to this day referred to as a standard of comparison with other J-domain proteins. First reports implicated DnaJ to be necessary for bacteriophage lambda and host DNA replication in *E. coli* (Yochem et al. 1978; Żylicz and Georgopoulos, 1984; Żylicz et al. 1985; Liberek et al. 1988; Żylicz et al. 1989). Next, it was associated with heat shock response and DnaK (bacterial Hsp70 partner), as their expression at heat shock conditions

was elevated (Bardwell et al. 1986). Soon after, the chaperones were speculated to bind hydrophobic regions of unfolded or denaturing proteins and facilitate their folding or recovery to functional, native form (Pelham, 1986; Skowyra et al. 1990). In light of these discoveries, in parallel, DnaJ was extensively studied structurally. Following the above-mentioned J-domain, DnaJ features G/F region associated with proper positioning of the J-domain during the ATPase stimulation in the Hsp70 partner (Huang et al. 1999; Kampinga and Craig, 2006; Rajan and D'Silva, 2009). What is the hallmark of DnaJ-like Class A group is the zinc-finger like region (ZFLR), which contains two metal ion binding centers. Center I plays a role in substrate binding, while Center II facilitates interaction of bound substrate with the Hsp70 partner (Linke et al. 2003, Fan et al. 2005). Further part of the protein contains C-terminal domain, which harbors two double β-sheets followed by a short α-helix, substrate binding regions, which predominantly bind peptides enriched in aromatic and hydrophobic residues (Rüdiger, Schneider-Mergener, Bukau, 2001).

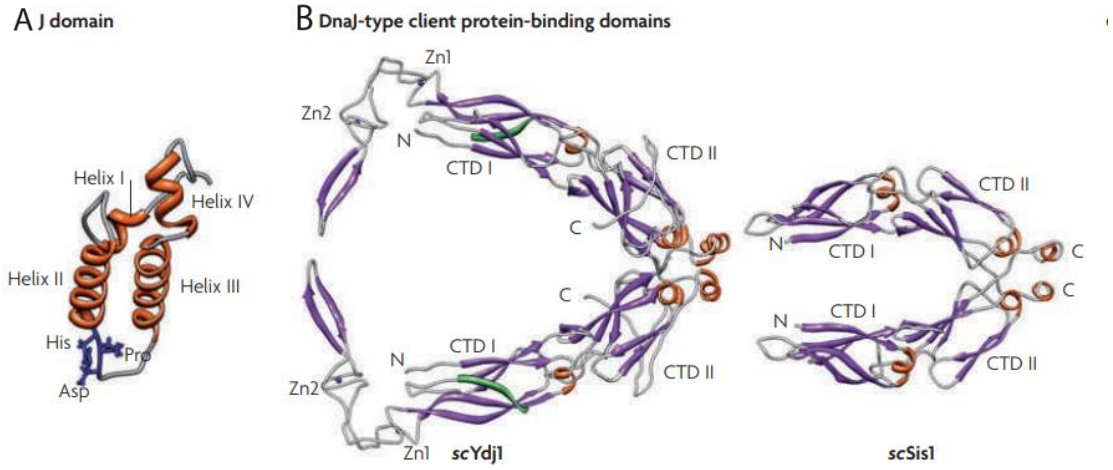

Figure 7. ***Structure of J-domain and Saccharomyces cerevisiae Class A Ydj1 (scYdj1) and Class B Sis1 (scSis1) J-domain proteins.*** *(A) Ribbon representation of the J-domain. It contains four alpha-helices, in which two middle ones from coiled-coil around the hydrophobic core of the domain. The extremely conserved tripeptide His-Pro-Asp (shown in blue) is located between the helix II and III. (PDB code: 1XBL) (B) Structure of Ydj1 (residues 102-350, PDB code: 1NLT) and Sis1 (residues 180-343, PDB code: 1C3G). J-domain proteins from Saccharomyces cerevisiae with truncated J-domains. Both proteins exhibit N-terminal localization of J-domains, followed by G/F rich region and similar peptide binding domains (CTD I and CTD II), ended with C-terminal dimerization motifs. Ydj1 also contains zinc-finger like domain, which extends from CTD I. Adapted from Kampinga and Craig, 2010.*

Protein folding seemed to be the major function of the bacterial JDP-regulated Hsp70 system (DnaJ and DnaK), either during assisting nascent polypeptides or refolding in the cytosol (Deuerling et al, 1999; Teter et al, 1999; Schlieker, Bukau, Mogk, 2002). Study of DnaJ led to the discovery of orthologues in possibly every organism and species (Kominek et al. 2013; Powers and Balch, 2013, Hip et al, 2019). For example, both highly

homologic yeast Class A Ydj1 and Class B Sis1 cooperate with Ssa1, which is the yeast orthologue of bacterial Hsp70, DnaK (Lu and Cyr, 1998; Kim et al. 1998; Aron et al. 2005). However, protein folding events by JDP-Hsp70 are not limited to the yeast cytosol. There were also reports of JDP Mdj1, which works with Ssc1 Hsp70-protein in the mitochondrial matrix (Rowley et al. 1994; Krzewska et al. 2001; Hermann et al, 1994). Likewise, Scj1 JDP cooperates with Kar2 Hsp70-protein in the endoplasmic reticulum (ER) (Schlenstedt et al. 1995). Analogical Hsp70 systems have been found in mammalian cells. Examples include: Class A DNAJA2 or Class B DNAJB1 that cooperate with Hsc70 in protein disaggregation in the cytosol (Wentink et al. 2020, Irwin et al. 2021) and Class A DNAJA3/Tid1, which works with HSPA9/mortalin Hsp70-protein in mitochondria (Havalova et al. 2021).

4.6.2 JDP regulators of protein disaggregation in yeast cytosol

In cytosol, yeast utilize two J-domain proteins, which harness Hsp70 to protein folding activities (Kampinga and Craig, 2010; Nillegoda et al, 2017; Fan et al, 2003). Both of them have been extensively studied, however their contributions to the disaggregation process still pose some mystery. J-domain proteins that participate in protein disaggregation in yeast cytosol are: Class A Ydj1 and Class B Sis1.

Over time, there were multiple studies attempting to discriminate between functions of either JDP in yeast cell. *SIS1* gene was found to be essential for yeast cell viability, while deletion of *YDJ1* allowed for growth, yet very poor at physiological temperature (30 degrees Celsius) and the growth phenotype became thermosensitive (Caplan and Douglas, 1991). Overexpression of *YDJ1* does not rescue a strain with deleted *SIS1*. Interestingly, it has been found that only truncated J-domain with G/F rich region of Sis1 is sufficient to rescue Δ*SIS1* strain (Yan and Craig, 1999). This phenomenon spurred speculations that Sis1 can mediate a process, which that cannot be fulfilled by Ydj1. However, it was reported that a point mutation in Ydj1 can compensate for the lack of *SIS1* gene (Schilke et al, 2017). Later, it has been reported that Sis1 takes part in maintenance of the PIKKs, most likely through involvement in folding of Tti1 (chaperone of PIKKs), as a substitution of single amino acid in Tti1, which hypothetically can influence its folding, removes the requirement for *SIS1* (Schilke and Craig, 2022). Ydj1 and Sis1 share some overlapping functions, which is, for example, transport of nascent polypeptides to cellular compartments, like ER and mitochondria (Jores et al, 2018; Cho et al, 2021). However, only Sis1 is involved in targeting proteins for degradation (Shiber

et al, 2013; Summers et al, 2013; Prasad et al, 2018) and prion maintenance (Sondheimer et al, 2001; Kirkland et al, 2011).

Ydj1 and Sis1 have been often used interchangeably as a model JDP in protein disaggregation in yeast (Cashikar et al, 2005; Franzmann et al, 2011; Tessarz et al, 2008; Żwirowski et al, 2017; Chamera et al, 2019). However, there were differences in protein refolding with Hsp70, which could implicate distinct mechanisms of action leading to functional discrepancies (Lu et al, 1998). The studies showed that both Ydj1 and Sis1 are capable of binding peptides (Li et al, 2004; Lee et al, 2002). Both of them are able to stimulate Hsp70's ATPase activity (Lu and Cyr, 1998). However, only Ydj1 was reported to bind unfolding polypeptides, preventing their aggregation (so-called holdase function), while Sis1 required Hsp70 to present such activity. Presence of both allows for protein refolding by JDP-Hsp70 so lack of aggregate formation cannot be regarded as only holdase activity (Lu et al, 1998).

The distinctions in protein folding and disaggregation are not limited to functional outcomes. Class A Ydj1 and Class B Sis1 are both able to stimulate ATPase of Hsp70 and share high level of structural similarity. Some Class B JDPs, including Sis1, utilize additional level of regulation in interaction with Hsp70, which tunes the availability of J-domain for Hsp70 ATPase stimulation. Yeast Class B Sis1 has been reported to form additional interaction between its J-domain and adjacent G/F rich region resulting in J-domain autoinhibition, which functionally conditions its cooperation with Hsp70 (Qian et al. 2002, Yu et al. 2015, Faust et al. 2020). It has been described to require binding of EEVD motif, located in the extreme C-terminus of Hsp70, in the JDP CTDI region (Li et al, 2006). In consequence of the binding, the autoinhibition is alleviated and the J-domain is available for interaction with NBD of Hsp70 (Faust et al. 2020). Naturally, as the stimulation of Hsp70 ATPase by J-domain is the key step in activity, this additional requirement has been reported to be crucial in protein disaggregation and refolding of amorphous aggregates (Yu et al. 2015) and in the case of Sis1 human orthologue DNAJB1 – of amyloid fibrils (Wentink et al. 2020). This additional level of regulation has been shown to be a critical factor in forming high local concentration of Hsp70 molecules on the substrate, named Hsp70 clusters. Hsp70 crowding on the aggregate surface potentiates the entropic effect and allows for disaggregation of even such stable structures as amyloid fibrils (Wentink et al, 2020). However, if the latter trait, meaning Hsp70 clustering, is

common among Class B J-domain proteins participating in protein disaggregation was not investigated.

Class A and Class B present many functional differences in the process of protein disaggregation. Due to that, using Class A Ydj1 and Class B Sis1, in this study, I wanted to systematically examine how they regulate this process.

5. Aim of the project

The aim of my doctoral project was to assess the functional differences in the activity of the Hsp70 system dictated by Class A and B J-domain proteins in protein disaggregation. I wanted to assess how the Hsp70 assembles on the aggregated substrate - what are the principles dictating the speed and abundance of chaperone complex formation. What is more, how these traits determine the efficacy of protein recovery during disaggregation. This analysis was done biochemically, with the focus on real-time observation of chaperone interactions with protein aggregate, while almost all previous analyses reported have used equilibrium (“static”) methods.

6. Materials

6.1 Bacterial strains

Escherichia coli DH5α supE44 _lacU169 (φ80 lacZ_M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1

Escherichia coli ArcticExpress (DE3) B F– ompT hsdS(r – m –) dcm+ Tetr gal λ(DE3) endA Hte [cpn10 BB cpn60 Gentr]

Escherichia coli BL21(DE3) codon+ F– ompT hsdS(rB – mB –) dcm+ Tetr gal endA Hte [argU ileY leuW] (CmR)

6.2 Yeast strains

Saccharomyces cerevisiae W303 *MATa/MATα {leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15} [phi+]*

6.3 Plasmids

pCA533 used for overproduction of His_6-SUMO-Ssa1 and its variants, KanR, T7 lac promoter, induction - IPTG

pET21a used for overproduction of Ydj1, AmpR, T7 lac promoter, induction – IPTG

pPROEX used for overproduction of His_6-TEV-Sis1 and its variants, AmpR, trc (trp-lac) promoter, induction - IPTG (Shorter et al. 2004)

pCA528 vector used for overproduction of His_6-SUMO-DNAJA2, His_6-SUMO-DNAJB4 and His_6-SUMO-Hsc70, KanR, T7 lac promoter, IPTG – induced (Nillegoda et al. 2015)

pPROEX vector used for overproduction of His_6-TEV-Hsp105, AmpR, trc (trp-lac) promoter, IPTG – induced (Nillegoda et al. 2015)

6.4 Proteins

Chaperones

Sis1, Ydj1, Ssa1, Sis1 E50A, Sis1 F201H, Ssa1 ΔEEVD, Ssa1 T201A V435F, DNAJA2, DNAJB4, Hsc70, Hsp105 – this work

Hsp104 D484K F508A was from laboratory's collection (Chamera et al. 2019)

Chaperone substrate proteins

Luciferase (Luc) from *P. pyralis*, recombinant (Promega)

Luciferase-His from *P. pyralis*, recombinant (Chamera et al. 2019)

GFP from *A. victoria*, recombinant (Ziętkiewicz et al. 2004)

6.5 Antibodies

Rabbit anti-sera specific for:

- **Ydj1**
- **Sis1**
- **Ssa1**
- **Hsp104**
- **Luciferase**

HRP (horse radish peroxidase) conjugated anti-rabbit IgG secondary antibodies (Bio-Rad)

6.6 Culture broths

LA 1% peptone, 0,5% yeast extract, 1% NaCl, 1.5% agar

LB 1% peptone, 0,5% yeast extract, 1% NaCl

YPD 2% peptone, 1% yeast extract, 2 % glucose

6.7 Antibiotics

Ampicillin (100 μg ml^{-1})

Kanamycin (50 μg ml^{-1})

6.8 Oligonucleotides

Name	Sequence 5’ -> 3’	Description
SisForE50A	GTTTAAGGAGATATCAGCGC	Forward, site specific mutagenesis primer for introduction of E50A in Sis1

SisRevE50A	CAAAGGCCGCTGATATCTCCT	Reverse, site specific mutagenesis primer for introduction of E50A in Sis1
Sis1FF201H	GTTGGTAAAAAGAAGTCACATAAAATTGGA	Forward, site specific mutagenesis primer for introduction of F201H in Sis1
Sis1RF201H	CTTCCAATTTTGTATGACTTCTTTTTACCA	Reverse, site specific mutagenesis primer for introduction of F201H in Sis1
EEVDfor	GAGGCTGAAGCTCCATAAGTTGAAGAAGTTGATTAA	Forward, site specific mutagenesis primer to introduce TAA stop codon to delete C-terminal EEVD motif of Ssa1
EEVDrev	TTAATCAACTTCTTCAACTTATGGACCTTCAGCCTC	Reverse, site specific mutagenesis primer to introduce TAA stop codon to delete C-terminal EEVD motif in Ssa1
Ssa1_T201A_for	TGCGTTCGATGTCTCTTTGTTGTCCATTG	Forward, site specific mutagenesis primer for introduction of T201A in Ssa1
Ssa1_T201A_ref	CATCGAACGCACCACCACCCAAGTCGAA	Reverse, site specific mutagenesis primer for introduction of T201A in Ssa1
Ssa1_V435F_for	CAGGTTTTTTGATTCAAGTCTTTGAAGGTGAAAG	Forward, site specific mutagenesis primer for introduction of V435F in Ssa1
Ssa1_V435F_rev	TCAATTTACCTGGTTGGTTATCAGCATAA	Reverse, site specific mutagenesis primer for introduction of V435F in Ssa1

7. Methods

7.1 Preparation and transformation of *E. coli* competent cells

E. coli competent cells preparation and transformation was done using Mix and Go! *E. coli* Transformation Kit (Zymogen), according to the enclosed protocol using selected *E. coli* strain and plates with LA medium and appropriate antibiotic.

7.2 Isolation of plasmid DNA

Plasmid DNA from overnight *E. coli* culture was isolated using DNA isolation kit (A&A Biotech), according to the enclosed protocol.

7.3 PCR-based site-directed mutagenesis

Primers for mutagenesis had approximately 25-35 nt, with 1 or 2 nucleotide mismatch. Primers were synthesized by oligo.pl, genomed.pl or Invitrogen. 50 µl PCR reaction mixture contained 2,5 U of Pfu Ultra II polymerase, 5 µl PFU Ultra II buffer, 50-200 ng of DNA matrix, 125 ng of Forward and Reverse primer, 1 mM dNTPs (250 µM of all four). PCR was performed using Bio-Rad C1000 Thermal PCR Machine. The conditions of the PCR were optimized for each mutagenesis. The basis consisted of hot-start in 95 degrees Celsius, initial denaturation for 10 min in 95 degrees Celsius, 17 cycles of denaturation in 95 degrees Celsius for 30 seconds, annealing for 60 seconds, elongation in 68 degrees Celsius for 60 seconds per 1 kbp, ending with final elongation for 10 minutes in 68 degrees. The PCR reaction was incubated with DpnI enzyme (2 hours in 37 degrees Celsius) to remove DNA matrix, and was then used for transformation of competent cells.

7.4 Protein purification

7.4.1 Sis1 and its variants

BL21 (DE3) codon+ strain was transformed with pPROEX plasmid harboring *SIS1* gene with N-terminally fused His-tag and cleavage sequence recognized by TEV protease. 6

liters of LB broth supplemented with 100 μg/ml ampicillin was inoculated with overnight culture of prepared transformants (1 ml of o/n culture per 20 ml of LB broth) and grown in 37 degrees Celsius with 160 rpm shaking. When the culture reached OD_{600}= ~0,5, the expression was induced by adding IPTG to final concentration of 1 mM and carried for 3h in 30 degrees Celsius. The cells were then pelleted using centrifugation in 5000 rpm for 7 min using rotor JLA 10.500 (Beckman Coulter). Pellet was frozen and stored until protein purification. To purify, the pellet was suspended in buffer for lysis (50 mM HEPES-KOH pH 8, 750 mM KCl, 5 mM β-mercaptoethanol, 10% glycerol), which was done by French Press (2 rounds at 1000 PSIG). To remove the soluble fraction, the lysate was centrifuged for 30 min at 25000 rpm using rotor JA 30.50 (Beckman Coulter). As His-TEV-Sis1 remains in the soluble fraction, the supernatant was incubated with buffer-equilibrated Ni-NTA resin for 3 hours with slight shaking. Next, the resin was washed with the same buffer, then with second buffer (50mM HEPES-KOH pH 8, 500 mM KCl, 5 mM β-mercaptoethanol, 10% glycerol, 50 mM imidazole). The protein was eluted with similar buffer, however the concentration of imidazole was increased to 500 mM. The collected protein fraction was dialyzed against buffer for TEV proteolysis (50 mM HEPES-KOH pH 8, 150 mM KCl) for 30 minutes. During this time, the protein solution should precipitate. Then EDTA was added to final concentration of 2 mM along with TEV protease, centrifuged again in 25000 rpm for 30 min, to remove the precipitated fraction and the supernatant was left for overnight dialysis. The protein preparation was then incubated with Ni-NTA resin to select for cleaved Sis1. Lastly, the protein preparation was supplemented with glycerol to final concentration of 10%, flash frozen and stored in -80 degrees Celsius.

7.4.2 Ydj1

BL21 (DE3) codon+ strain was transformed with pET21a vector with *YDJ1* gene. 6 liters of LB broth supplemented with 100 μg/ml ampicillin was inoculated with overnight culture of acquired transformants (1 ml of o/n culture per 20 ml of LB broth) and cultivated in 37 degrees Celsius with 160 rpm shaking. When the culture reached OD_{600}= ~0,5, the expression was started by adding IPTG to final concentration of 1 mM and carried out for 3h in 30 degrees Celsius. The cells were pelleted by centrifugation in 5000 rpm for 7 min. The pellet was flash frozen and stored in -80 degrees Celsius. The purification was started by thawing the pellet in an ice bath and resuspending it in buffer for lysis (40 mM HEPES-KOH pH 7,5, 80 mM KCl, 5 mM β-mercaptoethanol, 10 %

glycerol). The lysis was done using French Press by 2 round treatment of the bacterial suspension at 1000 PSIG. The lysate was centrifuged at 25000 rpm for 30 min in 4 degrees Celsius. The Ydj1 remained in the soluble fraction, which was then incubated with Q-Sepharose resin. It was then washed with same buffer, the lysate was in. The elution of the proteins was done by generating gradient between the previous buffer and analogical with increased salt to 300 mM KCl. The selected fractions were dialyzed against buffer for hydroxyapatite resin (25 mM KPi pH 7,0, 50 mM KCl, 10% glycerol). After the washing the resin was washed with the same buffer and the elution was done by generating gradient to buffer with higher phosphate concentration (400 mM KPi pH 7,0, 50 mM KCl, 10% glycerol). The pooled fractions were then dialyzed against buffer for purification on Heparin resin (25 mM HEPES-KOH pH 7,5, 50 mM KCl, 10% glycerol). The resin was washed with the same buffer and gradient to higher salt buffer was generated to elute the bound protein (25 mM HEPES-KOH pH 7,5, 400mM KCl, 10% glycerol). The purified protein was dialyzed against final buffer (50 mM HEPES-KOH pH 7,5, 150 mM KCl, 10% glycerol), aliquoted, flash frozen and stored in -80 degrees Celsius.

7.4.3 Ssa1 and its variants

BL21 (DE3) codon+ strain was transformed with pCA533 vector with *SSA1* gene fused with N-terminal His-tag and SUMO. 12 liters of LB broth supplemented with 50 μg/ml kanamycin was inoculated with overnight culture of acquired transformants (1 ml of o/n culture per 20 ml of LB broth) and cultivated in 37 degrees Celsius with 160 rpm shaking. When the culture reached OD_{600}= ~1, the expression was started by adding IPTG to final concentration of 1 mM and carried out for 3h in 30 degrees Celsius. The cells were pelleted by centrifugation in 5000 rpm for 7 min. The pellet was immediately resuspended in buffer for lysis (20 mM HEPES pH 8,0, 500 mM KCl, 50 mM imidazole, 5 mM β-mercaptoethanol, 10 % glycerol). The lysis was done using French Press (2 rounds at 1000 PSIG). The lysate was fractionated by centrifugation at 25000 rpm for 30 minutes. The Ssa1 remained in the soluble fraction, which was immediately incubated with Ni-NTA resin. Next, it was washed with same buffer and, then it was washed with buffer containing 50 mM imidazole and finally eluted with buffer with imidazole at 500 mM concentration (The other components of the buffer remained the same.). The acquired protein preparation was then dialyzed against buffer for Ulp1 cleavage (50 mM HEPES-KOH pH 8, 150 mM KCl, 5 mM β-mercaptoethanol) for 1 hour, and then the Ulp1

protease was added and the whole solution was left for dialysis overnight. The solution was then selected for cleaved Ssa1 using NiNTA resin and dialyzed against the buffer for selection of enzymatically active molecules on the 5'-ATP Agarose resin (20 mM HEPES-KOH pH 8, 50 mM KCl, 10 mM $Mg(OAc)_2$, 5 mM β-mercaptoethanol, 10 % glycerol). The protein preparation was then incubated with the resin, washed with the same buffer and the elution was done with the buffer supplemented with 10 mM ATP. The most concentrated fractions were selected, pooled together and dialyzed against final buffer (50 mM HEPES-KOH pH 8, 100 mM KCl, 5 mM $Mg(OAc)_2$, 10 % glycerol). The purified protein was aliquoted, flash frozen and stored in -80 degrees Celsius.

7.4.4 Hsc70

BL21 (DE3) codon+ strain was transformed with pCA528 vector with *HSC70* gene fused with N-terminal His-tag and SUMO. 12 liters of LB broth supplemented with 50 μg/ml kanamycin was inoculated with overnight culture of acquired transformants (1 ml of o/n culture per 20 ml of LB broth) and cultivated in 37 degrees Celsius with 160 rpm shaking. When the culture reached OD_{600}=~0,5, the overproduction was initiated by adding IPTG to final concentration of 1 mM. It was carried out for 3 hours at 28 degrees Celsius. The culture was pelleted by centrifugation in 5000 rpm at 4 degrees Celsius. The bacterial pellet was collected, flash frozen and stored at -80 degrees Celsius until purification. To purify the protein, the bacteria were suspended in buffer for lysis (50 mM HEPES-KOH pH 7,5, 750 mM KCl, 5 mM $MgCl_2$, 10% glycerol). The lysis was done using French Press by subjecting the bacterial suspension to 2 rounds at 1000 PSIG. The lysate was then separated by centrifugation in 25000 rpm at 4 degrees Celsius for 30 minutes. The Hsc70 remained in the soluble fraction and the protein preparation was immediately incubated with NiNTA resin. It was washed with the buffer for lysis and then washed again with buffer with low imidazole concentration (50 mM HEPES-KOH pH 7,5, 500 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 50 mM imidazole). The elution was done using the same buffer with high concentration of imidazole buffer (50 mM HEPES-KOH pH 7,5, 500 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 300 mM imidazole). The preparation was then dialyzed against buffer I for Ulp1 cleavage (50 mM HEPES-KOH pH 7,5, 300 mM KCl) for 30 minutes, then the buffer was changed to buffer II for Ulp1 cleavage (50 mM HEPES-KOH pH 7,5, 150 mM KCl) for 1 hour and then Ulp1 protease was added, and the dialysis was left overnight. The protein preparation was then selected for cleaved molecules of Hsc70 using NiNTA resin. The cleaved Hsc70 preparation was then

dialyzed against buffer for 5'-ATP agarose resin purification (20 mM HEPES-KOH pH 8,5, 50 mM KCl, 10 mM $Mg(OAc)_2$, 10% glycerol). The protein solution was incubated with the resin, washed with the same buffer, and eluted with addition of 10 mM ATP. The most concentrated fractions were pooled, dialyzed against final buffer (50 mM HEPES-KOH pH 7,5, 100 mM KCl, 5 mM $Mg(OAc)_2$, 10% glycerol), aliquoted, flash frozen and stored in -80 degrees Celsius.

7.4.5 DNAJA2 and DNAJB4

BL21 (DE3) codon+ strain was transformed with pCA528 vector with *DNAJA2* gene or with *DNAJB4* gene fused with N-terminal His-tag and SUMO. 12 liters of LB broth supplemented with 50 μg/ml kanamycin was inoculated with overnight culture of acquired transformants (1 ml of o/n culture per 20 ml of LB broth) and cultivated in 37 degrees Celsius with 160 rpm shaking. When the culture reached OD_{600}=~0,5, the overproduction was initiated by adding IPTG to final concentration of 1 mM. It was carried out for 3 hours at 28 degrees Celsius. The culture was pelleted by centrifugation in 5000 rpm at 4 degrees Celsius. The bacterial pellet was collected, flash frozen and stored at -80 degrees Celsius until purification. The pellet was resuspended in buffer for lysis (50 mM HEPES-KOH pH 7,5, 750 mM KCl, 10% glycerol) supplemented with 2 mM PMSF. The lysis was carried out using French Press by 2-time pushing through the bacterial suspension at 1000 PSIG. The lysate was fractioned by centrifugation in 25000 rpm, at 4 degrees Celsius. Since the JDPs remained in the soluble fraction, it was incubated with NiNTA resin. Firstly, the resin was washed in the same buffer, then with the buffer with reduced salt and added imidazole (50 mM HEPES-KOH pH 7,5, 500 mM KCl, 50 mM imidazole, 10% glycerol). The elution was done by increasing the imidazole concentration in the buffer to 300 mM. The fractions were dialyzed against buffer for Ulp1 cleavage (50 mM HEPES-KOH pH 7,5, 100 mM KCl, 10% glycerol) for 30 min, the Ulp1 protease was added, and the dialysis was left overnight. The cleaved protein was selected using NiNTA resin. The protein preparation was then incubated with Q-Sepharose resin, then it was washed with the previous buffer. The elution was done by generating gradient to buffer with higher salt concentration (50 mM HEPES-KOH pH 7,5, 500 mM KCl, 10% glycerol). The fractions containing the protein of interest were pooled and dialyzed against final buffer (50 mM HEPES-KOH pH 7,5, 150 mM KCl, 10% glycerol), aliquoted, flash frozen and stored at -80 degrees Celsius.

7.4.6 Hsp105

ArcticExpress (DE3) strain was transformed with pPROEX vector with *HSP105* gene fused with N-terminal His-tag and TEV cleavage sequence. 6 liters of LB broth was inoculated with overnight culture of acquired transformants (1 ml of o/n culture per 20 ml of LB broth) and cultivated in 37 degrees Celsius with 160 rpm shaking. When the culture reached OD_{600}=~0,5, the overproduction was initiated by adding IPTG to final concentration of 1 mM. It was carried out for 12 hours at 14 degrees Celsius. The culture was pelleted by centrifugation in 5000 rpm at 4 degrees Celsius. The bacterial pellet was collected, flash frozen and stored at -80 degrees Celsius until purification. Purification was started with resuspending the cells in buffer for lysis (50 mM HEPES-KOH pH 7,5, 750 mM KCl, 10% glycerol). The cells were lysed using French Press (2 rounds at 1000 PSIG). The lysate was fractioned using centrifugation in 25000 rpm, at 4 degrees Celsius for 30 minutes. The Hsp105 protein remained in the soluble fraction, which was then incubated with NiNTA resin. The resin was washed with the same buffer as the lysate was in and then washed again with second buffer (50 mM HEPES-KOH pH 7,5, 500 mM KCl, 10% glycerol, 50 mM imidazole) and finally eluted with buffer with high concentration of imidazole (50 mM HEPES-KOH pH 7,5, 500 mM KCl, 10% glycerol, 500 mM imidazole). The elution was then dialyzed against buffer for Ulp1 cleavage (50mM HEPES-KOH pH 7,5, 150 mM KCl, 1 mM ATP). The dialyzed protein was incubated with Ulp1 protease and then selected for cleaved Hsp105 molecules using NiNTA resin. It was then dialyzed against final buffer (50 mM HEPES-KOH pH 7,5, 150 mM KCl, 5 mM $Mg(OAc)_2$, 5 mM β-mercaptoethanol, 10% glycerol), aliquoted, flash frozen and stored at -80 degrees Celsius.

7.4.7 Measurement of purified protein concentration

Concentration of purified proteins was assessed using densitometry using BSA standard curve (Sigma-Aldrich) and ImageLab software (Bio-Rad).

7.5 Biochemical assays

7.5.1 Luciferase refolding assay

Luciferase (Promega) in the concentration of 30,24 μM was chemically denatured by 15-minute incubation in 25 degrees Celsius, in the buffer containing high concentration of urea (25 mM HEPES-KOH pH 8, 75 mM KCl, 15 mM $MgCl_2$, 6M Urea), then it was transferred to 48 degrees Celsius for 10-minute incubation. Next, it was rapidly diluted 25 times (25 mM HEPES-KOH pH 8, 75 mM KCl, 15 mM $MgCl_2$). The reactivation of luciferase was initiated by adding the above prepared aggregates to final concentration of 0,2 μM to the mixture containing chaperones in the same buffer with 5 mM ATP. The chaperones were used at 1 μM concentration, unless specified differently, except for the Hsp104 D484K F508A (104mut), which was used at 0,5 μM concentration. In human disaggregation by the human Hsp70 system the DNAJA2 and DNAJB4 were used at 1 μM concentration, same for Hsc70 and 0,1 μM Hsp105. The concentration of NEF was selected according to Reactivated luciferase activity was measured by hand, by taking an aliquot of the reaction, mixing it with Luciferase Assay Kit (Promega) and assessing the activity using Sirius Luminometer (Berthold).

7.5.2 GFP reactivation assay

GFP (2 mg/ml) was thermally aggregated by incubation in 85 degrees Celsius in buffer containing 40 mM Tris-HCl pH 7,5, 150 mM NaCl, 10% glycerol. The final concentration of GFP in the disaggregation was 0,5 μM. The disaggregation was performed in buffer containing 28 mM Tris-HCl pH 7,8, 60 mM potassium glutamate, 7 mM DTT, 10 mM ATP, 7 % glycerol. All chaperones were used at 1 μM concentration, except for Hsp104 D484K F508A, which was at 0,15 μM. In the aggregate remodeling experiment, the sodium chloride concentration was 120 mM. Fluorescent signal of renatured GFP was measured using JASCO FP-8000 Fluorescence Spectrometer or Beckman Coulter DTX 880 Plate Reader.

7.5.3 Bio-layer interferometry

The experiments were done using BLItz and Octet K2 instruments.

7.5.3.1 Direct protein-protein interactions

The Dip and Read® NiNTA biosensor was hydrated in buffer A containing: 25 mM HEPES-KOH pH 8, 75 mM KCl, 15 mM MgCl2 for 10 minutes. The 30s baseline was

also performed in the buffer A, then the sensor was immersed in the tube containing buffer A with specified protein for 10 minutes (0,5 μM His-SUMO-Ssa1; 0,5 μM His-SUMO-Ssa1 ΔEEVD; 1 μM His-SUMO-Ssa1 T201A V435F; 0,4 μM His-Sis1; 0,9 μM His-SUMO). Saturation level was different for each protein: ~6 nm for His-SUMO-Ssa1 and its variants, ~12 nm for His-Sis1 and ~3 nm for His-SUMO. Next, the sensor was washed with buffer A with addition of 2 mM DTT and 5 mM ATP and then immersed in the same buffer with specified chaperone at 1 μM concentration, unless specified differently. The dissociation step was performed in the same buffer.

7.5.3.2 Chaperone binding to luciferase aggregates generated on the BLI biosensor

The Dip and Read® (Sartorius) NiNTA sensor was hydrated in buffer A for ten minutes. It was then immersed in the same buffer supplemented with 6M of Urea and 8,2 μM His-tagged luciferase. This generated an increase of binding signal to about 6 nm. Next, the sensor was washed with buffer A for 5 min, then transferred to a tube containing 1,6 μM native His-tagged luciferase in buffer A and incubated in 44 degrees Celsius for 10 minutes. This resulted in the increase of bio-layer thickness up to about 16 nm. The sensor was then equilibrated for 10 minutes in the buffer A supplemented with 2 mM DTT and 5 mM ATP.

To generate cross-linked aggregates on the sensor, the sensor prepared as specified above, was additionally incubated in buffer A with 0,1% glutaraldehyde for 5 min, which was followed with a 5 min wash in buffer A.

All steps of binding, meaning baseline, chaperone binding, dissociation were performed in buffer A with 2 mM DTT and 5 mM ATP, unless specified differently. Chaperones were used at 1 μM concentration (unless specified differently). In human Hsp70 system binding Hsp105 protein was used at 0,1 μM concentration.

7.5.3.3 Chaperone binding to yeast lysate aggregates generated on the BLI biosensor

Preparation of the yeast lysate: W303 yeast cells cultured for 72h in the YPD medium were pelleted by centrifugation in the buffer A (25 mM HEPES-KOH pH 8, 75 mM KCl, 15 mM MgCl2) with addition of zymolyase (2 mg were added per 1g of the yeast cell pellet) and incubated for 30 minutes in 37 degrees Celsius. Next, the cells were lyzed using French Press (2 rounds at 1500 PSIG) and centrifuged at 30000 rpm at 4 degrees Celsius for 30 minutes. Protein concentration was assessed by standard Bradford assay.

Sensor preparation and binding: The Dip and Read® NiNTA sensor was hydrated in buffer A for 10 minutes and then transferred to the tube containing 6M Urea and 8,2 μM His-tagged luciferase for 10-minute incubation. The binding of luciferase would result in ~6nm thickness of bio-layer. Next, the sensor was washed with buffer A for 5 minutes and then transferred to the buffer A containing soluble yeast proteins (5 mg/ml concentration in buffer A) and incubated for 10 minutes in 55 degrees Celsius. This temperature was selected according to Jarzab, Kurosawa et al. 2020, which exceeds the melting point of the majority of yeast proteome. The incubation would result in the assembly of ~30 nm aggregated protein bio-layer thickness. Last step was the 5-minute equilibration of the prepared sensor in buffer A supplemented with 2 mM DTT and 5 mM ATP. Chaperone binding experiment was performed in the same buffer at 1 μM concentrations.

7.5.3.4 Chaperone binding to GFP aggregates generated on the BLI biosensor

The Dip and Read® NiNTA biosensor was hydrated in buffer A containing: 25 mM HEPES-KOH pH 8, 75 mM KCl, 15 mM MgCl2 for 10 minutes. The 30s baseline was also performed in the buffer A. 0,5 mg/ml His-tagged GFP was incubated in 8 M Urea in 85 degrees Celsius for 15 minutes and then used for assembling basal layer of protein on the sensor giving signal of about ~10 nm. Next, the sensor was washed with buffer A for 5 minutes and then transferred to tube containing 0,1 mg/ml native His-tagged GFP and incubated in 85 degrees Celsius for 15 minutes. This would increase the layer of bound proteins to about ~25 nm. Lastly, it was washed for 5 minutes in buffer A with addition of 2 mM DTT and 5 mM ATP. Such prepared sensor was then incubated with chaperones at 1 μM concentration.

7.5.4 Western Blot

Level of chaperone binding to the aggregate-saturated sensor was examined with Western Blot in the following scheme: instead of dissociation step, the sensor was removed from the device and incubated for 10 min in 100 degrees Celsius in the Laemmli buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0,004% bromophenol blue, 125 mM Tris-HCl, pH 6,8) supplemented with 50 mM EDTA. Polyacrylamide gel electrophoresis in denaturing conditions (SDS-PAGE) and Western Blot were carried out according to standard procedures. Rabbit anti-sera, which was specific to specified chaperones was used as primary antibodies. HRP (horse radish peroxidase) conjugated anti-rabbit

antibodies were used as secondary. Blots were visualized using SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific), then scanned using ChemiDoc MP Imagining System (Bio-Rad).

7.5.5 Sedimentation analysis of aggregate remodeling

The 30 μM luciferase in buffer A with 6M Urea was incubated in 25 degrees Celsius for 15 minutes. Next, it was transferred to 48 degrees Celsius for 10 minutes, rapidly diluted in buffer A and incubated in 25 degrees Celsius for 15 minutes. This preparation was 4-times diluted into mixture of specified proteins. Final concentration of luciferase was 0,3 μM, either aggregated or native, and indicated chaperones at 1 μM concentration in buffer A with 2 mM DTT and 5 mM ATP. Assembled reactions were incubated for 1h in 25 degrees Celsius and applied onto 3,5 ml 10%-60% (v/v) glycerol gradient in the same buffer. The gradients were centrifuged at 4 degrees Celsius using Beckman SW60 rotor for 15 hours at 35000 rpm. The gradients were fractioned, subjected to SDS-PAGE, and analyzed with Western Blot according to standard procedures with antibodies specific for luciferase.

7.5.6 Statistical analysis

Statistical analysis of acquired results was performed using GraphPrism software.

8. Results

Protein disaggregation chaperone system in yeast consists of Class A Ydj1, Class B Sis1 J-domain proteins, Hsp70 protein – Ssa1 and Hsp100 disaggregase – Hsp104. This disaggregation machinery is functional, with either of the employed J-domain protein. Although, intensively studied, yeast Hsp70 system activity was predominantly examined using one of these J-domain proteins as a model protein. Here, I employ biochemical approach to comparatively analyze, what are the dynamics of the Hsp70 system when the particular J-domain protein: Ydj1 or Sis1 is involved.

8.1. Ydj1 and Sis1 have distinct influence on protein disaggregation

In literature, both JDPs Ydj1 and Sis1 proteins promote the protein disaggregation (Nillegoda et al, 2017; Lu et al, 1998). Firstly, to examine what are the dynamics of Hsp70 system in protein disaggregation, which are imposed by the J-domain protein, I performed protein disaggregation experiment with the yeast Hsp70 system, supplemented with Hsp104 disaggregase, using model protein substrates: luciferase and GFP. In luciferase disaggregation and refolding, the system containing Sis1 exhibited about ~7 min delay before the start of the recovery of the substrate, the overall efficiency of the

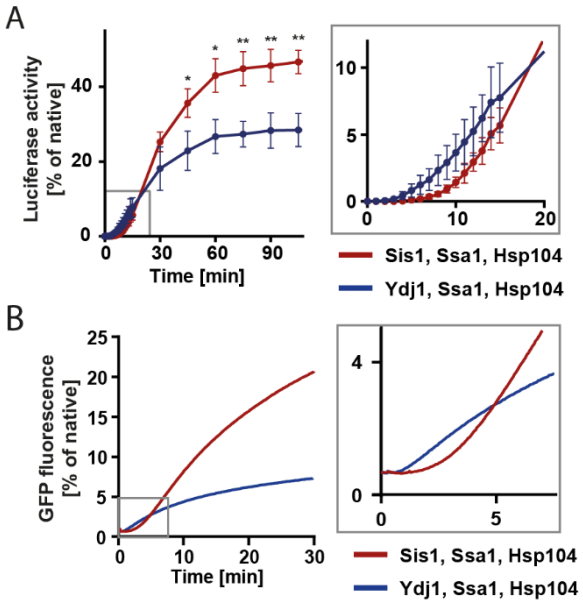

Figure 8. ***Disaggregation by Hsp70-Hsp100 system presents differences imposed by J-domain protein.*** *(A) Reactivation of aggregated luciferase by Ssa1/Hsp104 with Sis1 (red) or Ydj1 (blue). Error bars show SD from three experiments. Luciferase activity was normalized to the activity of native luciferase in the same concentration. The significance was calculated using Two-tailed t test: **P <_0,01 and *P<_0,05. Grey inset shows magnification of the first 20 minutes of the experiment. (B) Aggregated GFP renaturation by Ssa1/Hsp104 with Sis1 or Ydj1. Grey inset shows magnification of the first 8 minutes for GFP renaturation. GFP fluorescence was normalized to the fluorescence of native GFP in the same concentration. The experiments were conducted according to Methods 7.5.1 and 7.5.2.*

refolding was much higher (~50%) than for the system containing Ydj1 (~25%) (Fig. 8A). However, given that protein aggregation is an unregulated and chaotic process, using more than one model substrate can mitigate the problem that observed trends could be substrate specific. To expand the analysis, I used aggregated GFP in an analogical experiment and observed correlating results. The Hsp70-Hsp100 system containing Sis1 promoted higher level of substrate recovery (~20% Sis1-Ssa1-Hsp104; ~7% Ydj1-Ssa1-Hsp104) and exhibited delayed start of the disaggregation (~3 minute lag) (Fig. 8B).

Given that the JDP seemed to be the main influencer of observed course of disaggregation, I limited the experiment to JDP-Hsp70 proteins. Firefly luciferase recovery without Hsp104 is greatly limited, however it also presented the same recovery trends as observed in the previous experiments. The Hsp70 system containing Sis1 protein refolded up to ~6 times more (~3%) luciferase than with Ydj1 (~0,5%) (Fig. 9).

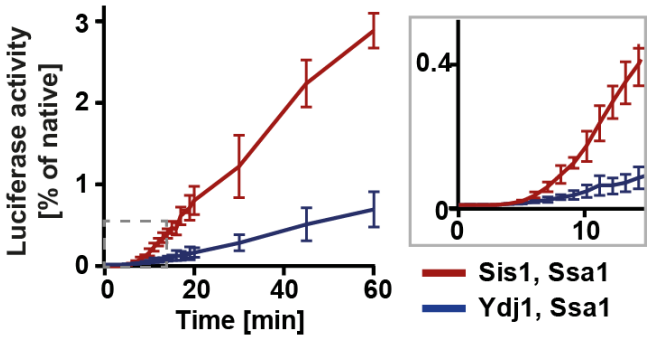

Figure 9. ***Hsp70 system refolding efficacy depends on the employed JDP.*** *Reactivation of aggregated luciferase by Ssa1 with Sis1 or Ydj1. Luciferase activity was normalized to the activity of native luciferase in the same concentration. Inset in grey shows the first 14 minutes of the experiment. Error bars show SD from three experiments. The experiment was done according to Methods 7.5.1.*

Summarizing the obtained results, both JDPs are able to promote protein disaggregation when coupled with Hsp70 and Hsp104, however Sis1 promotes higher level of substrate recovery when compared to Ydj1, as well as it presents delayed start of disaggregation. These results gave rise to questions:

- What is the reason behind differences in substrate recovery levels?
- What dictates the kinetics of the disaggregation?

8.2 Hsp70 complex assembly on the aggregate is modulated differently by Ydj1 or Sis1

Since the observed differences in protein disaggregation are connected with the modulation of the Hsp70 system activity by the employed J-domain protein, I wanted to examine the first step of the disaggregation process, being the JDP-Hsp70 chaperone complex assembly on the substrate. To do so, I employed bio-layer interferometry (BLI) based approach. This optical method takes advantage of light interference generated by molecules bound to the BLI sensor, which the instrument interprets as the thickness of molecule layer. Finding a way to generate amorphous aggregates on the BLI sensor allowed me to observe the assembly of disaggregation complex on the aggregated substrate in real time (Chamera et al, 2019).

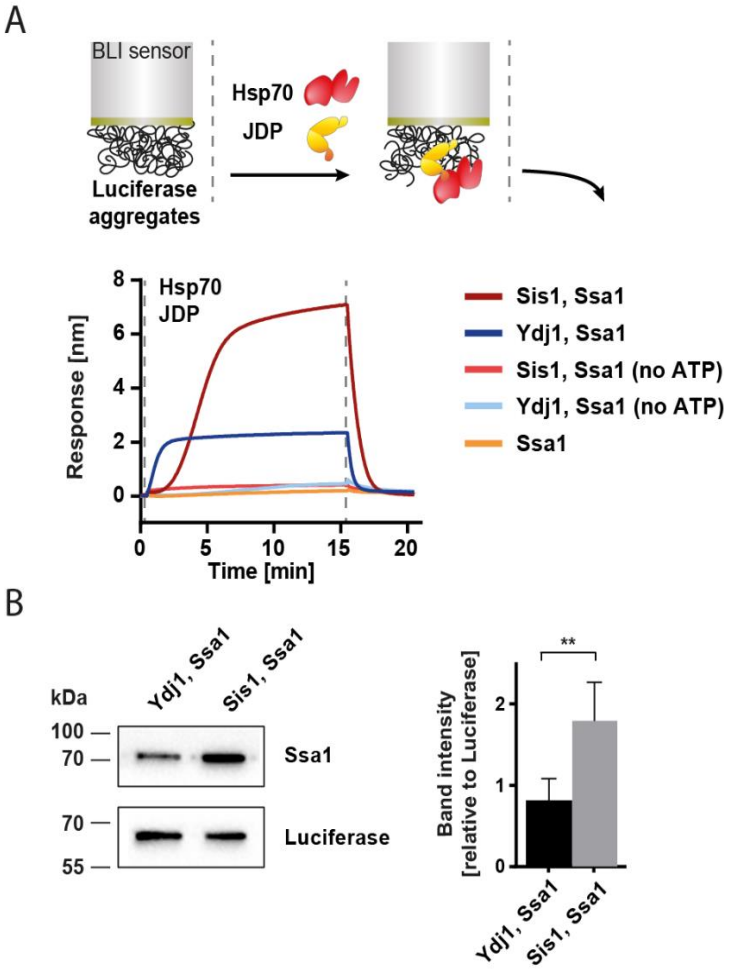

Figure 10. ***Hsp70 system shows differences in aggregate binding with Sis1 or Ydj1.***

(A) The sensor with luciferase aggregate was incubated with indicated chaperones. Dashed lines indicate the moment of chaperone introduction and dissociation steps.

*(B) Western blot analysis of aggregate-interacting chaperones. The BLI binding was performed in the scheme as above, however just before the dissociation step, the sensor was removed and boiled in Laemmli buffer and subjected to Western Blot analysis using anti-Ssa1 and anti-luciferase antibodies. Band intensities were quantified. Error bars are from three independent experiments. Two tailed t-test was performed: **P<_0,01.*

The experiments were done according to Methods 7.5.3.2 and 7.5.4.

Western blots were performed by Wiktoria Sztangierska.

First, I aggregated firefly luciferase on the biosensor and incubated the biosensor with Ydj1-Ssa1 or Sis1-Ssa1. In agreement with the disaggregation kinetics, Hsp70 system with Sis1 displayed delayed binding to the aggregate (~30 second lag before signal increase), reaching approximately ~6 nm of protein thickness. Hsp70 system with Ydj1 bound rapidly, reaching about ~2 nm of protein thickness. In compliance with the Hsp70 ATPase cycle (Fig. 4), ATP is required for Hsp70 system to interact with substrates, therefore in the absence of ATP, I observed no binding (Fig. 10A).

What is more, to examine the specificity of the Hsp70 system interaction with the aggregate, I used Ssa1 T201A V435F, which is, for one, unable to hydrolyse ATP, due to mutation in catalytic site in NBD domain, secondly, it cannot bind substrates due to mutation in its hydrophobic pocket in SBD (McCarty et al, 1994; Pfund et al, 2001). Coupling this Ssa1 variant with JDPs, presented no binding, meaning that observed binding requires active participation of Ssa1 in the Hsp70 ATPase cycle to assemble chaperone complex on the aggregate (Fig. 11).

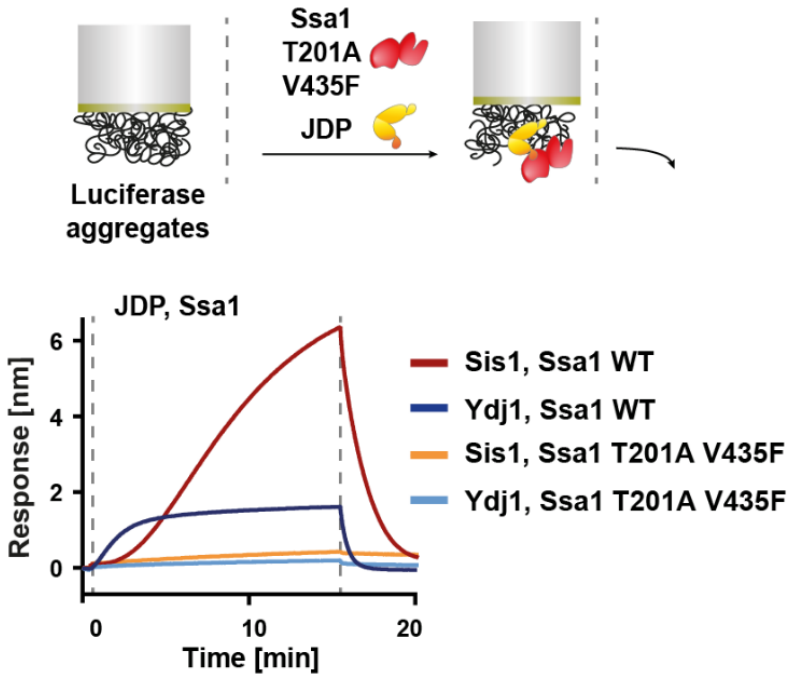

Figure 11. ***Substrate binding ability and ATPase activity of Hsp70 are required for binding to aggregated substrate with Sis1 and Ydj1.*** *Dashed lines separate the association and dissociations steps. Scheme of experiment is depicted above the graph. The experiment was done according to Methods 7.5.3.2.*

Moreover, to eliminate the possibility, that observed discrepancies are only limited to luciferase as a model of aggregated substrate, I extended the study by using another model substrate – GFP (Fig. 12A). Additionally, I tried to mimic the native substrate of yeast chaperones by aggregating proteins present in yeast lysate (Fig. 12B). I observed analogical trends in binding for both systems. Maximal binding levels were different for each substrate, however the relation between Sis1 and Ydj1 containing systems remained the same: Sis1-Ssa1 was binding to higher level, than Ydj1-Ssa1, with delayed binding.

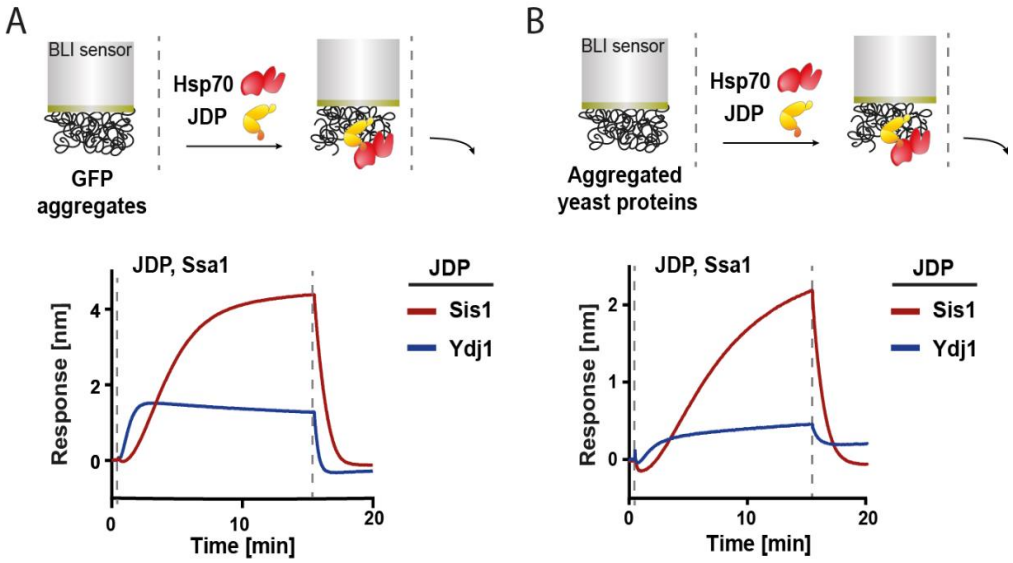

Figure 12. ***Hsp70 system binding trends with Sis1 and Ydj1 are not limited to used substrate.*** *(A) Binding of Hsp70 with Sis1 or Ydj1 to sensor saturated with GFP aggregates. (B) Binding of Hsp70 with Sis1 or Ydj1 to aggregated yeast proteins (aggregates formed by proteins present in soluble fraction of yeast lysate). The experiments were done according to Methods 7.5.3.4 and 7.5.3.3.*

The overall thickness of the protein layer interacting with the aggregate could be constituted by both JDP and Hsp70. I observed nearly no increase of protein layer when Ssa1 was present alone, which is expected when there is no JDP involved (Fig. 10A, 11). To probe the contribution of JDPs to binding level, I performed an analogous experiment for sole JDPs and observed that Ydj1 can bind the aggregated substrate, what was contrary to Sis1, which exhibited only residual binding (Fig. 13A). To test the specificity, I used Sis1 with introduced mutation in its CTDI domain that impairs its ability to bind substrates (Fan et al, 2004). This variant exhibited no binding, which proved residual interaction of wild-type Sis1 to be specific (Fig. 13B).

As the Hsp70 ATPase cycle includes the introduction of the JDP-substrate complex to the Hsp70, I mimicked this course of action by incubating the aggregated substrate with JDP and then, I added Hsp70. Although, Ydj1 bound the substrate, and then promoted

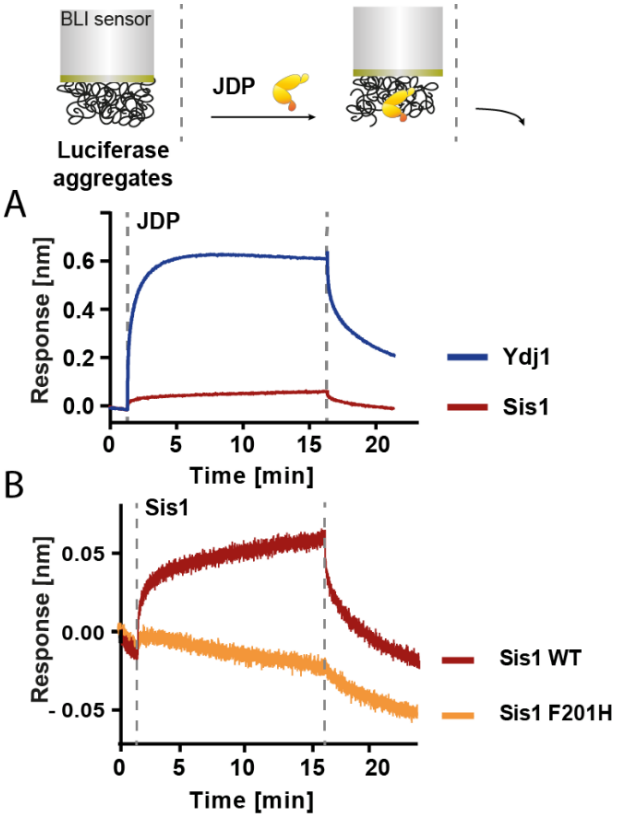

Figure 13. ***Ydj1 and Sis1 show distinct ability to interact with aggregated substrate.***

(A) The sensor covered with luciferase aggregates was incubated with Sis1 or Ydj1.

(B) Experiment analogical to A, using Sis1 or Sis1F201H.

Dashed lines indicate start of association and dissociation step.

The experiment was done according to Methods 7.5.3.2 using Octet K2 instrument.

binding of Ssa1. Surprisingly, prior incubation of the substrate with Sis1 didn't promote binding of Ssa1. This could mean that in case of Sis1, the simultaneous presence of JDP with Hsp70 is crucial to assemble the disaggregation complex (Fig. 14). To conclude, Ydj1 binding to the substrate is stable, while Sis1 binds weakly and easily dissociates so its binding is insufficient to promote latter Hsp70 system assembly on the substrate. Furthermore, as there are substantial differences in the overall thickness of protein layer in the Hsp70 system assembly and J-domain proteins pose distinct contribution to the signal, I proposed that the level of binding translates to the amount of loaded Hsp70 onto the substrate. To confirm, Western Blot analysis of the Hsp70 interacting with the sensor-bound aggregate was performed. As previously done, the JDP-Hsp70 pair was incubated with sensor bound aggregate. Upon reaching plateau, the sensor was removed from the instrument and analysed. The result of this comparative analysis is: Sis1 promotes nearly two times higher abundance of Ssa1 protein on the aggregate (Fig. 10B).

Interaction of JDP-Hsp70 system with protein aggregates, regarding Ydj1 and Sis1 differs in terms of kinetics and binding level. Sis1-Ssa1 exhibits delayed binding, however, promotes loading of more Hsp70 molecules. The same trend was observed for every used model substrate. What is surprising, the JDPs alone exhibit disparate ability to interact with the aggregated substrate.

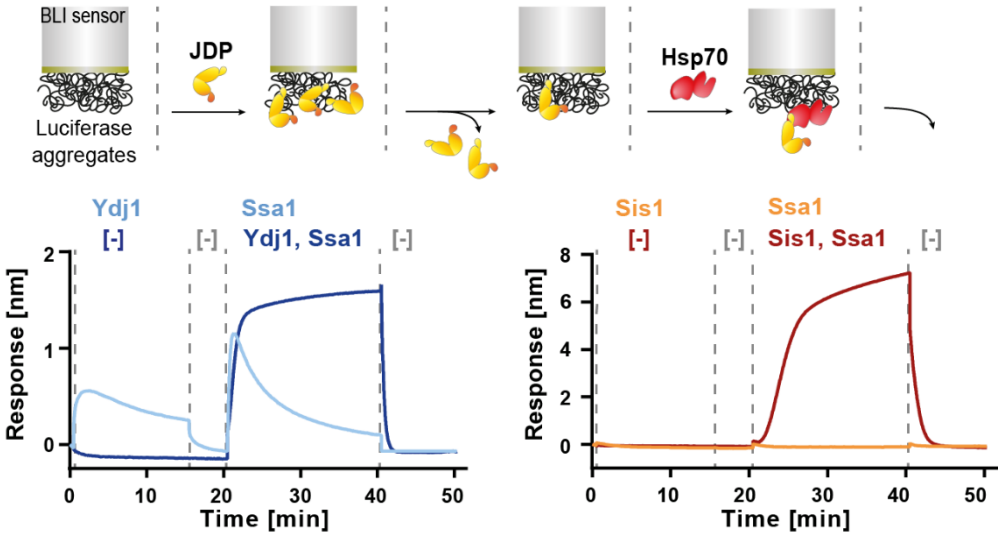

Figure 14. ***Sis1 and Ydj1 drive disparate modes of Ssa1 loading onto the aggregated substrate.*** *BLI sensor covered with aggregates was incubated with or without Ydj1 or Sis1, and then washed. Next, the sensor was incubated with Ssa1 or Ydj1-Ssa1 or Sis1-Ssa1, as indicated. Dashed lines represent start of association and dissociation steps. The scheme of experiment for both systems is depicted at the top. The experiment was done according to Methods 7.5.3.2.*

8.3 Differences in amount of loaded Ssa1 directly influences the amount of docked Hsp104

The yeast disaggregation machinery consists of the Hsp70 system, which cooperates with the Hsp104 disaggregase. Its interaction with substrates and disaggregation activity is controlled by Hsp70. Hsp70-Hsp104 collaboration in protein disaggregation involves docking of the disaggregase to protein aggregates and disaggregase activation by the Hsp70 interaction with Hsp104's M-domain (Mogk et al, 2015). In principle, the saturation of a protein aggregate with Hsp70 creates an abundance of possible binding sites for Hsp104 disaggregase. Coming from that, the amount of docked Hsp104 should be in direct proportion to the interacting Hsp70. To test this hypothesis, I extended the aggregate binding experiment by the step of Hsp104 addition. As I established before, Sis1 promotes substantially higher abundance of Ssa1 on the aggregate. The addition of Hsp104 to the interacting Sis1-Ssa1 system generated increase of protein layer by ~2 nm. In the experiment done analogically for the system Ydj1-Ssa1, the increase was only about ~0,5 nm (Fig. 15A). I confirmed with Western Blot that the increase in the signal corresponds with the amount of interacting Hsp104 (Fig. 15B).

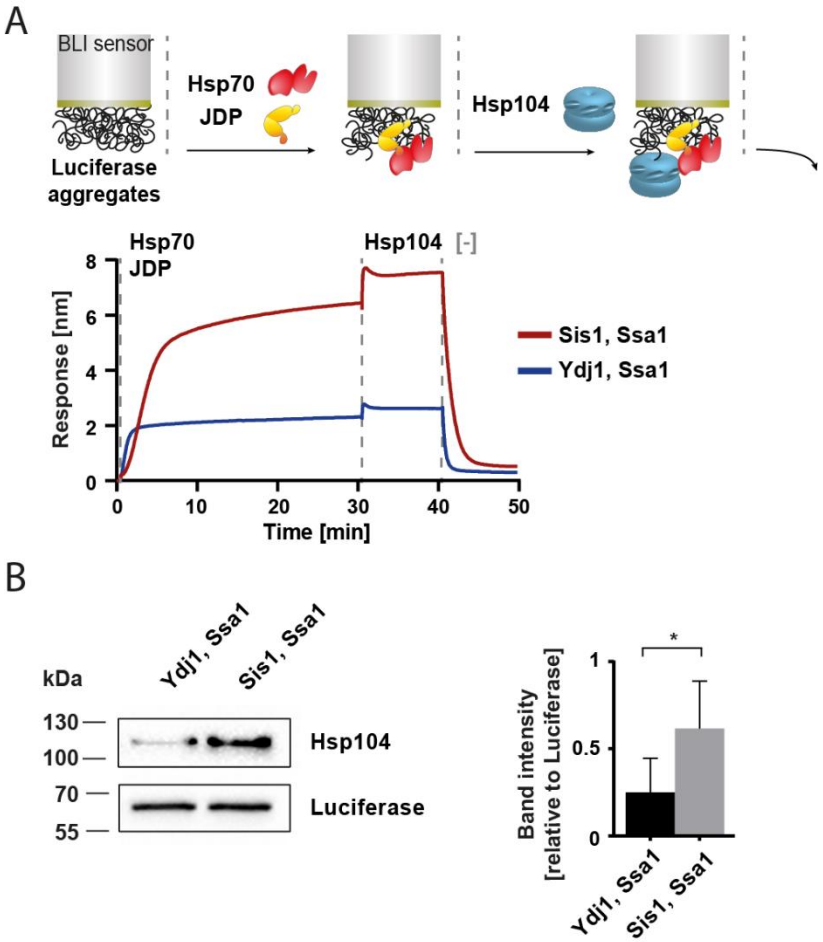

Figure 15. ***Level of JDP/Hsp70 interacting with aggregated substrate influences the level of Hsp104 docking.*** *(A) Aggregate bound by Sis1-Ssa1 or Ydj1-Ssa1 was incubated with Hsp104. Dashed lines represent start of association and dissociation steps. (B) Western Blot analysis of interacting Hsp104 in the presence of Sis1-Ssa1 or Ydj1-Ssa1. The experiment was performed as in A, however just before the dissociation step the sensor was removed and boiled in Laemmli buffer and then subjected to Western Blot analysis using anti-Hsp104 and anti-luciferase antibodies. The band intensities were quantified. Error bars show SD from three experiments. The statistical analysis was done using two tailed t-test: *P<_0,05. Western blots were performed by Wiktoria Sztangierska. The experiments were conducted according to Methods 7.5.3.2 and 7.5.4.*

Summarizing, Sis1 promotes more abundant loading of Hsp70 protein, which allows for more Hsp104 molecules to interact with the substrate, potentiating the disaggregation capability. This fact could be one of the factors behind the higher protein substrate recovery in case of Sis1 containing disaggregation system (Figs. 8, 9 ,10 and 15).

8.4 Differences of kinetics in disaggregation and aggregate binding by Hsp70 system due to employed J-domain protein

Hsp70 loading by J-domain proteins displays different kinetics of disaggregation and binding and Sis1 drives higher abundance of Ssa1 molecules on the aggregated substrate, I suspected two scenarios. First being, that Sis1 to promote Hsp70-mediated protein disaggregation and refolding, needs to interact with Hsp70, either via its J-domain or by binding Hsp70's EEVD motif. If this interaction has to happen prior to aggregate interaction, slow association rate between the two proteins could explain the delay before aggregate binding and disaggregation. To examine this idea, I incubated the two proteins together before subjecting them to binding, yet there was no change in the kinetics (Fig. 16).

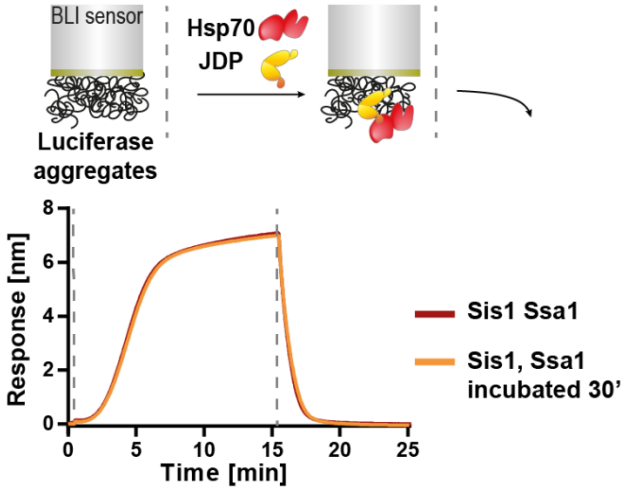

Figure 16. ***Incubation of Sis1 and Ssa1 prior to aggregate binding does not influence the mode of binding.*** *Scheme of experiment is presented above. the graph. Dashed lines indicate the start of association or dissociation steps. The experiment was done according to Methods 7.5.3.2.*

Next, I immobilized the N-terminally His_6-tagged Ssa1 and added Sis1 to examine direct interaction between the proteins. The Sis1 binding to Ssa1 was rapid and quickly reached equilibrium. At the same time, I performed an analogical experiment with Ydj1 and observed no binding, which was surprising as the J-domain of Ydj1 would be available for interaction with Ssa1. The interaction may be undetectable due to its transient nature or due to N-terminal localization of the tag on Ssa1, which facing the biosensor's resin, can render the interaction site unavailable. Basing on that fact, the observed signal due to Sis1 binding to Ssa1 might be through Sis1's CTDI and Ssa1's EEVD motif (Fig. 17). Since, the binding of the proteins was fast and quickly reaching saturation, I dismissed the slow-association between Sis1-Ssa1 as the delaying factor in aggregate binding.

The second hypothesis is that the aggregated substrate is being pre-conditioned by the Hsp70 system activity to allow for such gradual and abundant assembly of chaperones on it. The sigmoidal shape of the Sis1-Ssa1 binding might be a result of subsequent

association of Ssa1 molecules, of which every single one has an entropic effect on the substrate (Fig. 5), which uncovers more possible binding sites for Ssa1 association, which potentiates further modification. This cycle of action ultimately results in abundant association of Ssa1 molecules.

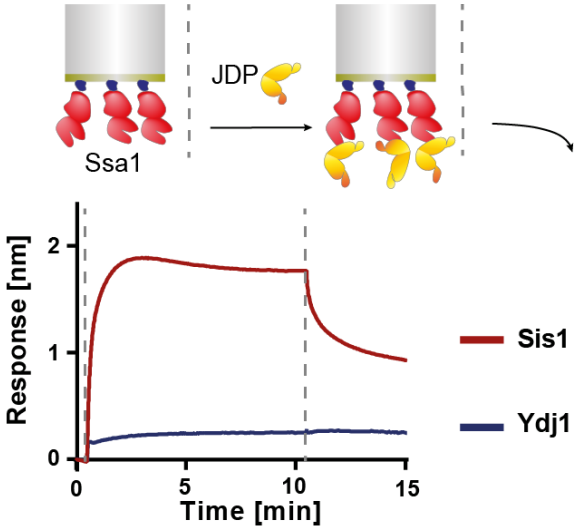

Fig. 17 ***Sis1 binds rapidly to Ssa1.*** *Incubation of sensor bound His-SUMO-Ssa1 with Sis1 (red) and Ydj1 (blue). Scheme of experiment is depicted at the upper part. Dashed lines indicate the start of association or dissociation steps. The experiment was done according to Methods 7.5.3.1.*

To test this hypothesis, I extended the aggregate binding experiment. After the first association of Sis1-Ssa1 system, the aggregate would already be modified, so after the dissociation of the system, the second association of the Sis1-Ssa1 would theoretically present faster kinetics. As expected, when the aggregated substrate was firstly bound by Sis-Ssa1, after dissociation step, another round of chaperones binding presented parabolic

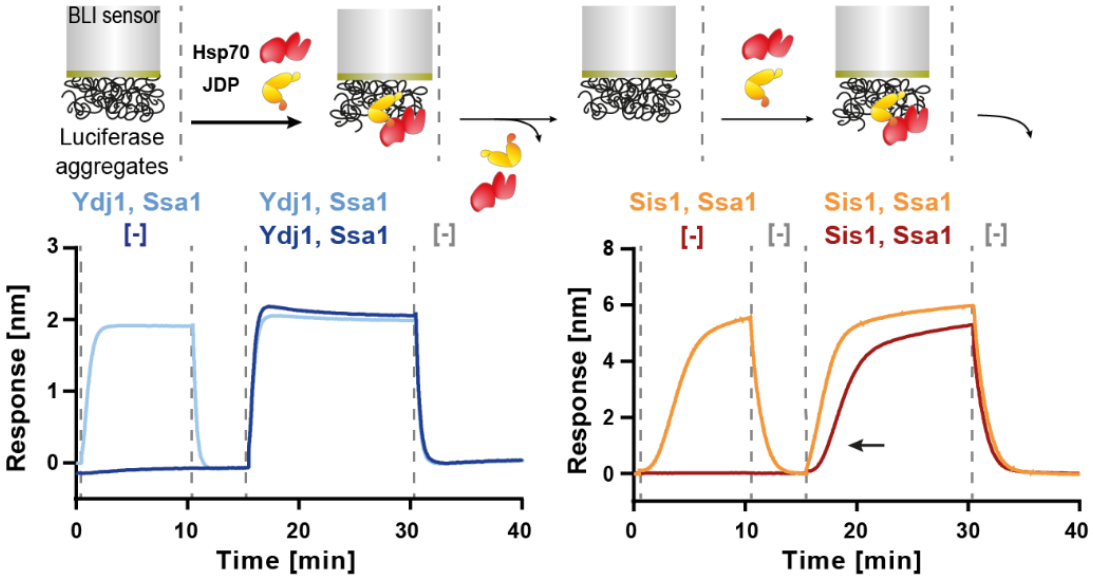

Figure 18. ***Sequential incubation of aggregate-covered sensor with JDP/Hsp70.*** *As depicted on the experiment scheme, the aggregate-covered sensor was incubated in the buffer with or without chaperones, then washed and incubated with chaperones again. Dashed lines separate washing, association and dissociation steps. The experiments were done according to Methods 7.5.3.2.*

kinetics, which equilibrated and the same level as achieved in the first step of Sis1-Ssa1 association. In the analogical experiment done for Ydj1-Ssa1, the first binding did not affect the second, suggesting that aggregate modification is, at least, not as pronounced or Ydj1-Ssa binding does not depend on it (Fig. 18).

8.5 Sis1-Ssa1 activity promotes superior protein aggregate modification

Since aggregate modification could be a significant part of Sis1-Ssa1 binding, to prove it, I wanted to structurally confine the aggregate structure by using a unspecific cross-linker to hypothetically hinder this activity. Glutaraldehyde interacts with lysine residues creating net of stabilizing interactions between aggregated polypeptides, which would reduce the ability of Hsp70 system to modify the aggregate. The prepared sensor with bound protein aggregate was additionally incubated in glutaraldehyde and then used to assess chaperone binding. Sis1-Ssa1 binding was diminished by ~65% (comparing highest association level in the binding time period) when compared to non-cross-linked aggregate control, which signifies the importance of aggregate modification in the chaperone assembly on the aggregated substrate. Ydj1-Ssa1 was nearly not affected (Fig. 19), which corroborates conclusion from the previous experiment (Fig. 18).

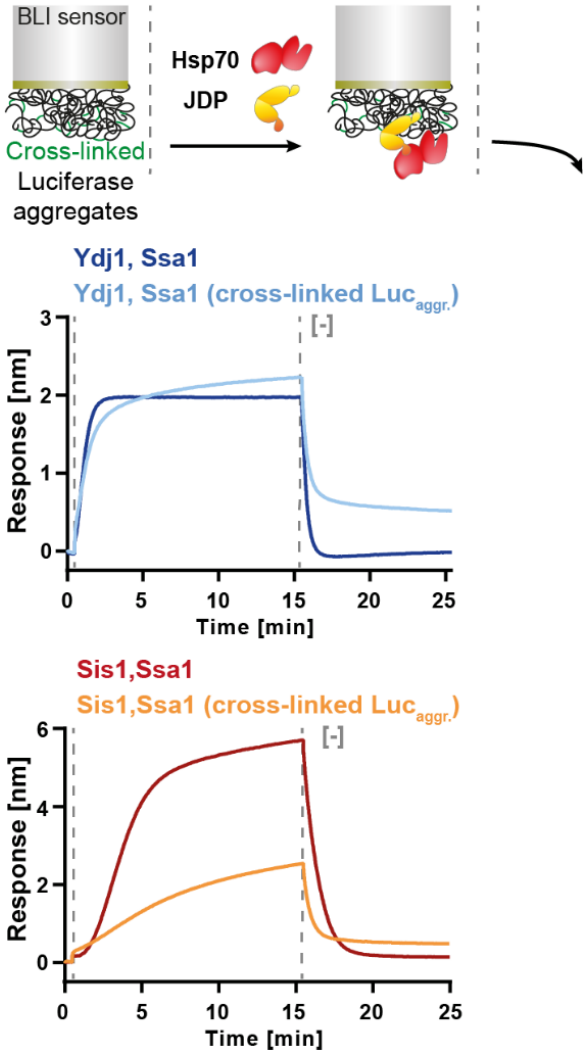

Figure 19. ***Structural confinement of aggregate hinders binding of Sis1-Ssa1.*** *Cross-linked aggregates or untreated were incubated with JDP/Hsp70 system as indicated. Scheme of experiment is depicted above the graph. Dashed lines indicate the start of association of dissociation steps. The experiment was done according to Methods 7.5.3.2.*

Efficient aggregate modification could be the crucial factor when it comes to protein disaggregation. This activity could be especially manifested with higher level of overall recovery of the substrate by the system with Sis1 (Figs.

8, 9). I wanted to probe how this aggregate-modification activity impacts overall disaggregation efficiency. To do this, I employed a variant of Hsp104 disaggregase (Hsp104 D484K F508A), which possesses two key characteristics for this experiment. First, it can bind to aggregates and translocate substrates independently of Hsp70, which is due to mutation D484K, which makes the disaggregase constantly derepressed (Lipińska et al, 2013). Secondly, the disaggregase activity is not stimulated by Hsp70 due to mutation F508A in the M-domain, which is the key residue for the interaction between Hsp70 and Hsp104. (Chamera et al, 2019). When Hsp104 harbors these two mutations, it is completely independent of Hsp70. It also features low refolding capability (Chamera et al, 2019), down to ~5% of firefly luciferase recovery, when compared to the wild type JDP/Hsp70/Hsp100 system (Fig. 8 and 20). If Hsp70 aggregate modification impacts the possible binding sites for further processing, the Hsp104 D484K F508A (104mut) could have its substrate pool increased, which would manifest in higher efficiency of substrate reactivation. In the experiment, I used aggregated firefly luciferase, which was incubated with JDP/Hsp70 proteins for 60 minutes, which was followed by addition of the Hsp104 D484K F508A. The recovery of luciferase by the sole Hsp70 system in that time was low, about ~7% for Sis1-Ssa1 and about ~0,5% for Ydj1-Ssa1. However, when the disaggregase mutant was added, the recovery largely increased, up to ~50%, when the aggregates were first incubated with Sis1-Ssa1 and to ~20%, when the aggregates were first incubated with Ydj1-Ssa1. Such recovery level was not achieved when I added the JDP/Hsp70 together with Hsp104 mutant, which underlines the effect of initial incubation of the aggregates with the Hsp70 system (Fig. 20). To exclude that the phenomenon is substrate-specific, an analogical experiment was performed using aggregated GFP. In a similar scheme, GFP aggregates were incubated with JDP/Hsp70 and then, at the 60-minute time point, the Hsp104 D484K F508A was added. Hsp104 D484K F508A can refold about ~35% of the aggregated GFP on its own, however the prior incubation with Sis1-Ssa1 increases this level to ~70%. In the case of Ydj1-Ssa1, the level increases to ~55% (Fig. 21). Interestingly, the Hsp70 system activity, in the case of aggregated GFP, does not exhibit detectable recovery of the substrate, however it has large influence on the refolding by the hyperactive, independent disaggregase variant. This manifests that Hsp70 system activity can influence the aggregates, creating a better substrate for the disaggregase, while it does not produce refolded protein.

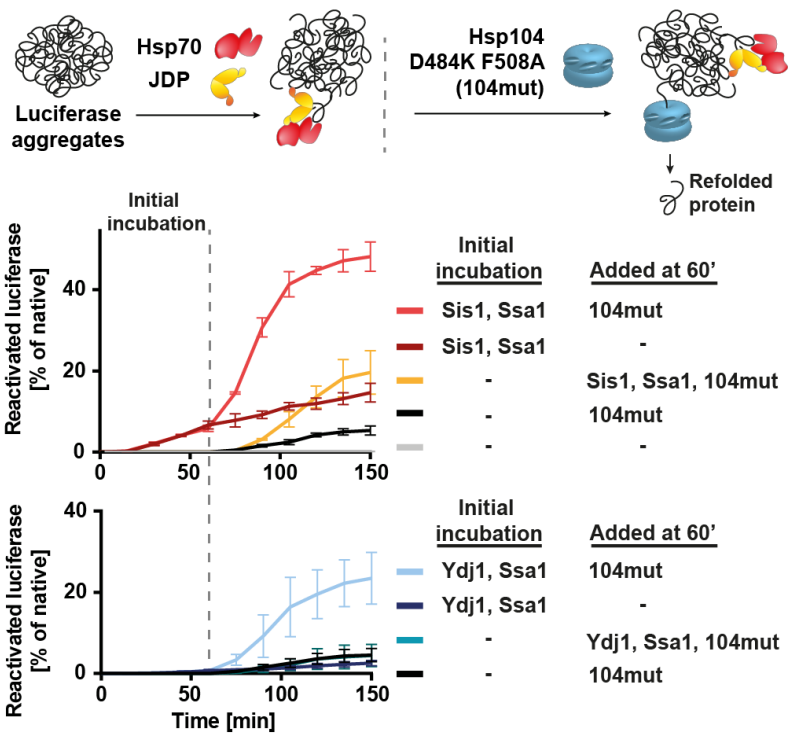

Figure 20. ***Hsp70 system facilitates Hsp104 D484K F508A - driven protein disaggregation.*** *Impact of initial incubation of luciferase aggregates with JDP/Hsp70 followed by addition of Hsp104 D484K F508A (labeled as 104mut). Luciferase activity was normalized to the native protein in the same concentration. Error bars show SD from three independent experiments. Dashed lines represent the end of initial incubation and addition of Hsp104 D484K F508A.*

The experiments were done according to Methods 7.5.1.

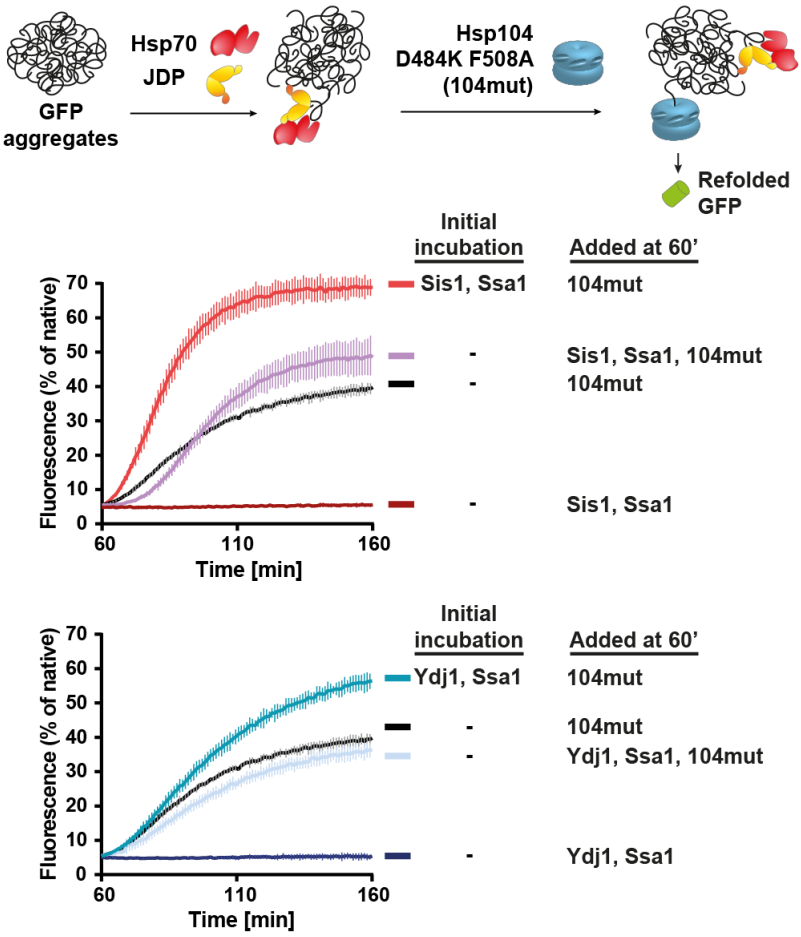

Figure 21. ***Impact of initial incubation with Hsp70 system facilitates Hsp104 D484K F508A -mediated protein refolding.***

Initial incubation of GFP aggregates was carried out with indicated JDP/Hsp70 system, the start of the graph indicates the addition of Hsp104 D484K F508A (104mut) and start of the measurement.

The GFP fluorescence was normalized to the fluorescence of native GFP in the same concentration. Shade over the lines represents SD for every measurement point from three experiments. Experiments were done by dr Agnieszka Kłosowska according to Methods 7.5.2.

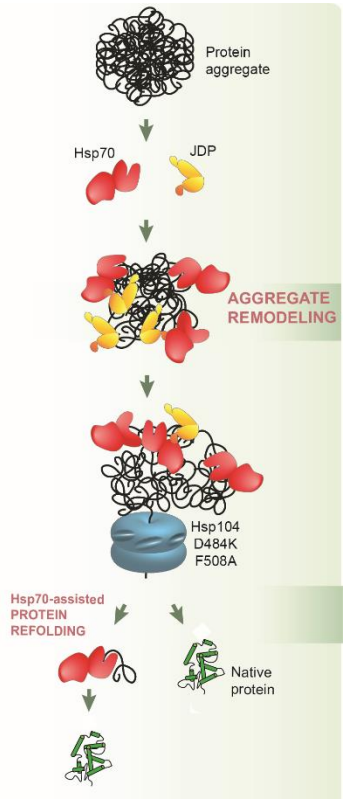

Figure 22. ***Protein disaggregation scheme depicting indirect cooperation between Hsp70 system activity and Hsp104 D484K F508A disaggregase.*** *Due to Hsp70 activity more possible binding sites are exposed for hyperactive disaggregase. After the translocation the released polypeptide can fold spontaneously or can be refolded with the help of Hsp70.*

Substrates translocated by the disaggregase can be also assisted in their folding by Hsp70 system (Fig. 22). To see if the observed effects are due to aggregate remodeling activity, which happens prior to polypeptide translocation, we decided to abolish Hsp70 activity post-incubation with the aggregates, before the addition of the disaggregase variant. We took advantage of the fact that Ssa1 activity depends on potassium ions (Lopez-Buesa, Pfund, Craig 1998), meaning that if they would be outcompeted by adding high amount of sodium ions, the activity of Hsp70 would be lost. This characteristic allows for inhibition of the Hsp70 system after the initial incubation, before adding Hsp104 D484K F508A, to examine the contribution to the substrate recovery by the Hsp70-assisted folding after the translocation. For this experiment, we selected Sis1-Ssa1, being the Hsp70 system of a more pronounced effect. At the 60-minute time point, the Hsp70 activity was

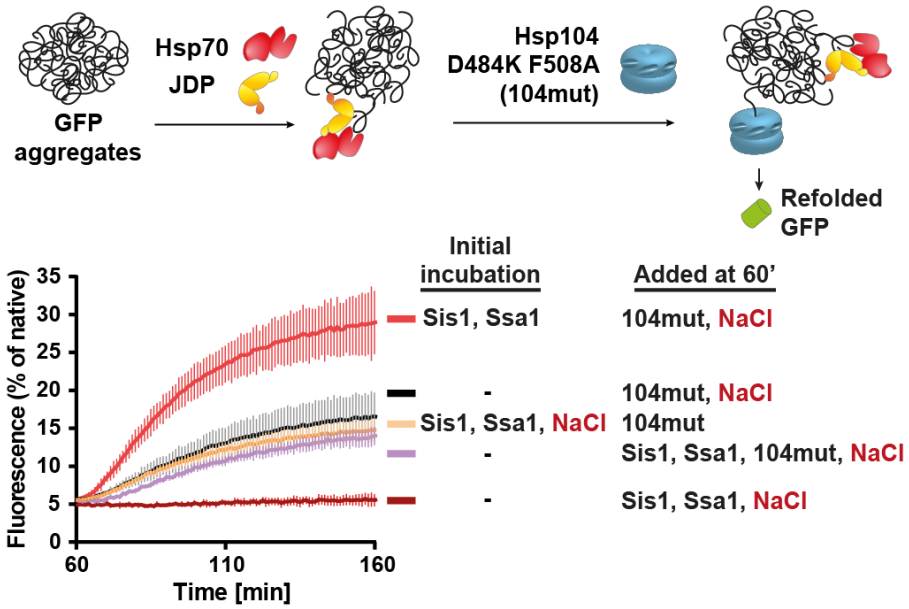

Figure 23. ***Activity of Sis1-Ssa1 prior to translocation by the disaggregase increases the refolding efficacy.*** *The beginning of the graph indicates the addition of Hsp104 D484K F508A (104mut) and the start of measurement. The GFP fluorescence was normalized to the fluorescence of native GFP in the same concentration. Shade over the lines represents SD from three independent experiment. Experiment was done by dr Agnieszka Kłosowska according to Methods 7.5.2.*

halted by the addition of sodium chloride and then Hsp104 D484K F508A was added. However, we still observed high stimulation of the disaggregase refolding, from about 15% to 30% of the native substrate fluorescence. As expected, when the Hsp70 system was incubated with the aggregates in the presence of sodium, the stimulation was lost, meaning that the recovery was at the level of the sole disaggregase (Fig. 23).

The experiments described above present the contribution of the Hsp70 system aggregate modification. Both Sis1 and Ydj1 show a beneficial effect, yet Sis1 exhibits superiority.

Already having assessed the effect on the recovery of aggregated substrates, I used glycerol gradient centrifugation to visualize what physically happens to the protein aggregates during Hsp70-mediated remodeling. The aggregate remodeling activity could influence the size, mass, or shape of the aggregates, which would impact their sedimentation pattern. Upon 60-minute incubation of the luciferase aggregates with the Hsp70 systems – Ydj1-Ssa1 or Sis1-Ssa1, the mixtures were subjected to sedimentation and then the fractions were analyzed with Western Blot (Fig. 24).

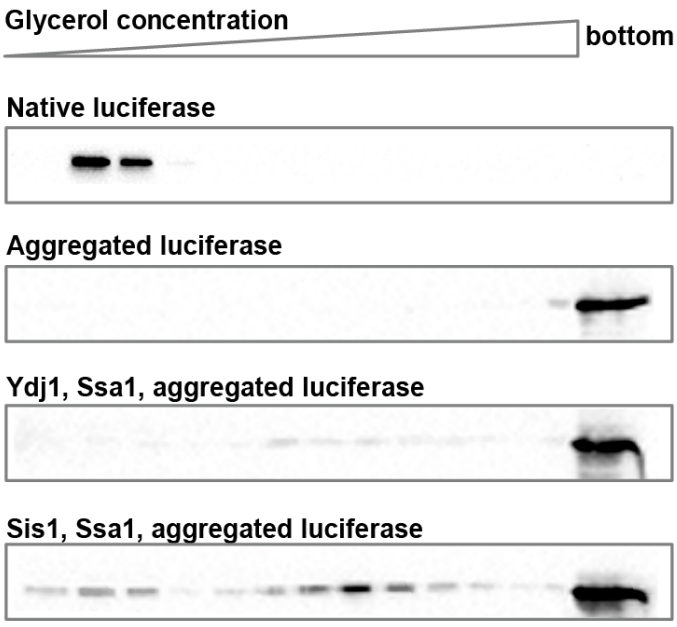

Figure 24. ***JDP/Hsp70 activity changes the sedimentation profile of luciferase aggregates.*** *The fractionated gradient was visualized using Western Blot technique with anti-luciferase antibodies. Experiment was done by Wiktoria Sztangierska according to Methods 7.5.5.*

The Sis1-Ssa1 sample contained several protein species scattered all across the gradient, with small amount of luciferase present at the top, which is in compliance with the localization of native luciferase. The Ydj1-Ssa1 sample featured some barely detectable protein species in the middle of the gradient, with no luciferase in the top fractions.

This further emphasizes the Sis1-Ssa1 ability to modify aggregates into species of different qualities, which facilitates further aggregate processing, ultimately leading to protein recovery.

8.6 Interaction of Sis1 CTDI with EEVD-motif of Ssa1 allows for aggregate modification

The distinct level of Ssa1 loaded onto the substrate by Ydj1 or Sis1 is influenced by the efficiency of aggregate modification. However, what allows for such chaperone assembly could be connected with additional requirement of Sis1 for its cooperation with Ssa1. Sis1 has been previously reported to require interaction with the C-terminal EEVD motif of Ssa1 to cooperate in protein disaggregation. What is more, this requirement can be alleviated due to the single mutation E50A, which disrupts the salt bridge between the J-domain and G/F rich region, which allows the Sis1 J-domain to be accessible for Hsp70 interaction. If the mutation is introduced to Sis1, it no longer requires to interact with EEVD-motif to promote protein disaggregation (Yu et al, 2015). First, I examined if this interaction is required also for aggregate binding. In the BLI-based approach, I incubated the JDPs with Ssa1 and its ΔEEVD variant. Sis1 together with Ssa1 ΔEEVD showed no increase in the binding signal, however the deletion of the EEVD motif seemed to not affect Hsp70 system assembly with Ydj1 (Fig. 25).

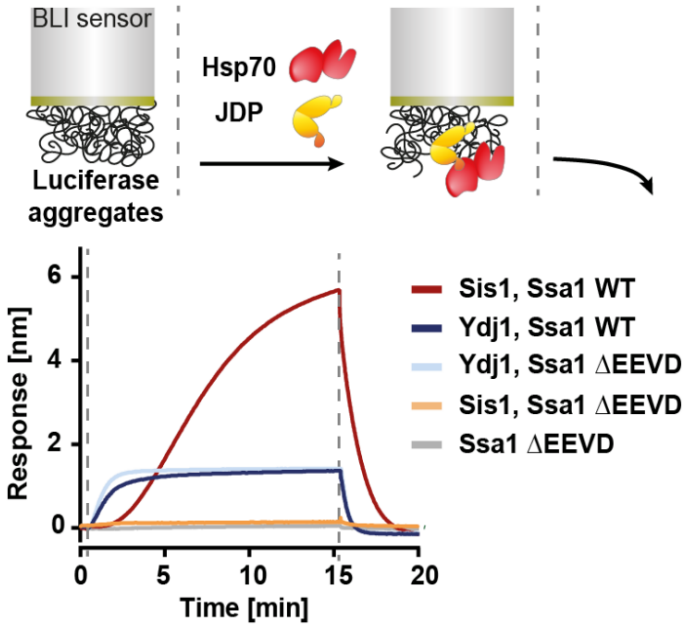

Figure 25. ***Sis1-Ssa1 cooperation in aggregate binding is dependent on the CTDI-EEVD interaction.*** *Scheme of the experiment is depicted above the graph. Dashed lines represent the beginning of association and dissociation steps. The experiment was conducted according to Methods 7.5.3.2.*

The introduction of the E50A mutation to Sis1 allowed it to promote the aggregate binding with Ssa1 ΔEEVD, however it equilibrated at about ~3 nm, which is half of what is achieved by wild-type proteins. It also featured rapid kinetics of binding (Fig. 26).

The interaction between Sis1 and the C-terminus of Ssa1 conditions the sigmoidal shape of binding and allows for such abundant loading of Ssa1 molecules. Surprisingly, Sis1 E50A, when coupled with Ssa1, still maintained the sigmoidal shape of binding, indicating that the J-domain inhibition does not influence this characteristic, however the level of binding was highly reduced, comparable to Sis1 E50A-Ssa1 ΔEEVD.

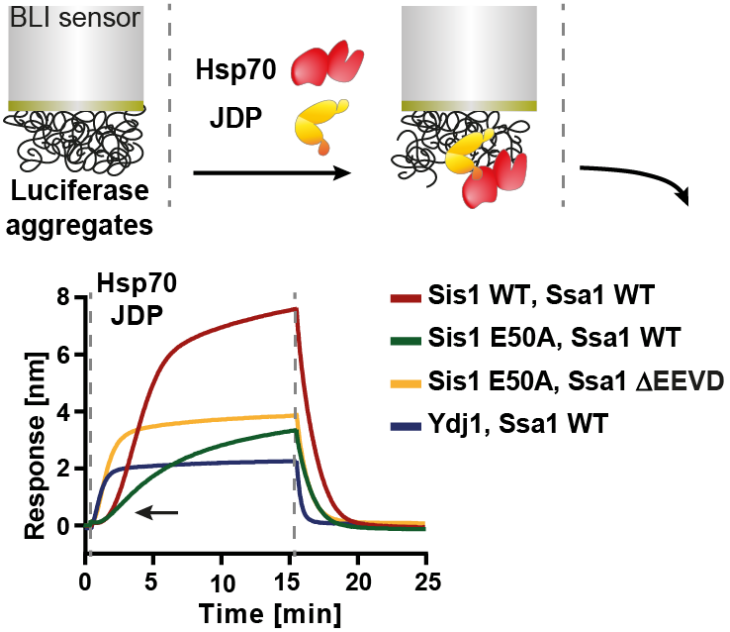

Figure 26. ***Disruption of Sis1 J-domain autoinhibition allows for cooperation with Ssa1 ΔEEVD in aggregate binding.*** *Dashed lines represent start of association and dissociation steps. The aggregate-covered sensor was incubated with indicated chaperones, as depicted in the scheme above the graph. The experiment was conducted according to Methods 7.5.3.2.*

Lower level of binding to the aggregate corresponds with the level of interacting Hsp70 molecules, which might reflect lower ability to remodel protein aggregates. Given that, Sis1 E50A-Ssa1 ΔEEVD equilibrated at much lower level than wild-type proteins, the aggregate modification can be less pronounced. To test this, I incubated the variants in BLI-based aggregate binding where the aggregate was additionally cross-linked. While the structural confinement of aggregate with a cross-linker hindered the ability of Sis1 - Ssa1 to bind (Fig. 19), the binding of mutants was not decreased (Fig. 27).

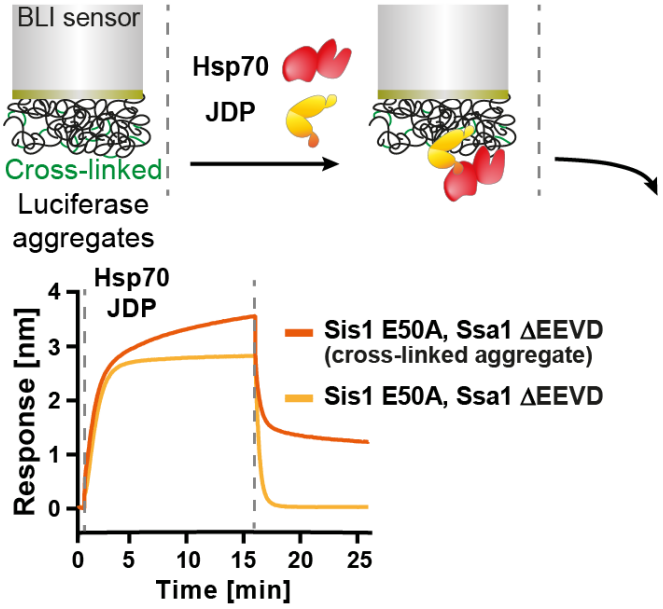

Figure 27. ***Binding of Sis1 E50A – Ssa1 ΔEEVD is hardly affected by structural confinement of the sensor-bound aggregate.*** *The scheme of experiment is depicted above the graph. The dashed lines represent start of association and dissociation steps. The experiment was conducted according to Methods 7.5.3.2.*

Furthermore, I checked the ability of this system to augment protein refolding by Hsp104 D484K F508A, by incubating the aggregates with Sis1 E50A-Ssa1 ΔEEVD prior to addition of Hsp104 variant. Initial incubation of the aggregates with Sis1 E50A – Ssa1 ΔEEVD increased the recovery level by the disaggregase only to about ~15%, which is much lower when compared with the effect of incubation with wild-type proteins.

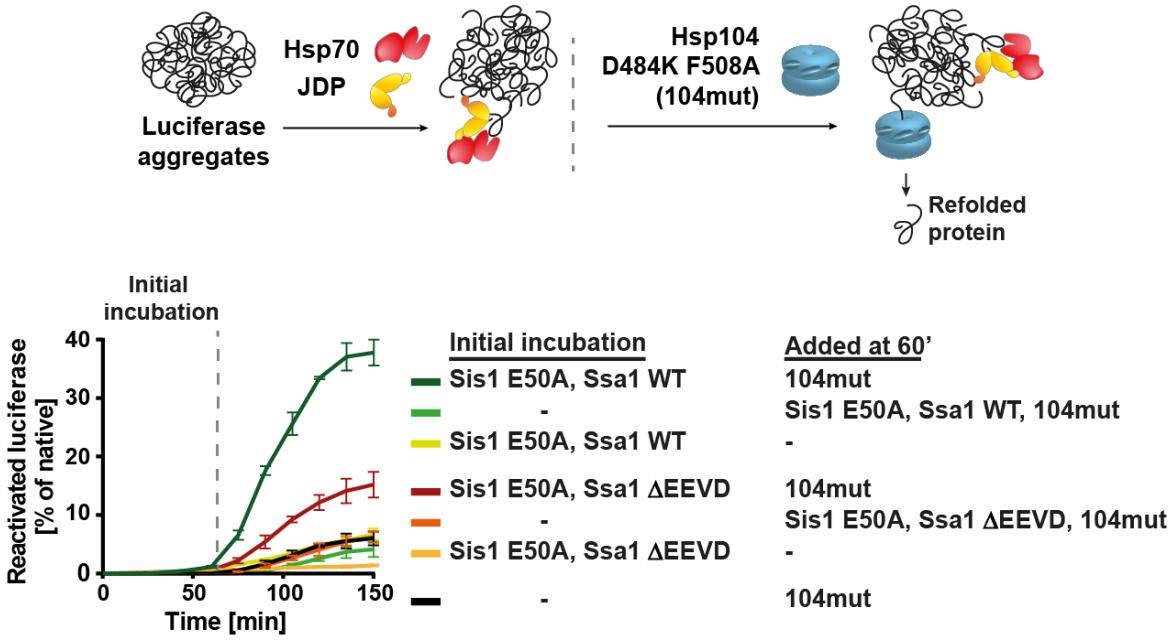

Figure 28. ***Sis1 (CTDI) – Ssa1 (EEVD) interaction is crucial for the aggregate-remodeling activity.*** *(A) Luciferase aggregates were incubated with indicated chaperones, which was followed by addition of Hsp104 D484K F508A (104mut). Dashed line represents the end of the initial incubation and the addition of the disaggregase. The luciferase activity was normalized to the activity of native luciferase in the same concentration. The experiment was conducted according to Methods 7.5.1.*

Interestingly, initial incubation with Sis1 E50A with wild-type Ssa1 allowed to increase the refolding to ~40%, similarly to the wild type Sis1 - Ssa1 (Fig. 28).

Similar trends were observed for analogical experiment using GFP aggregates: initial incubation with Sis1 E50A-Ssa1 ΔEEVD showed no increase in the refolding efficiency, while the Sis1 E50A-Ssa1 influence was exact as that of the Sis1 - Ssa1 (Fig. 29).

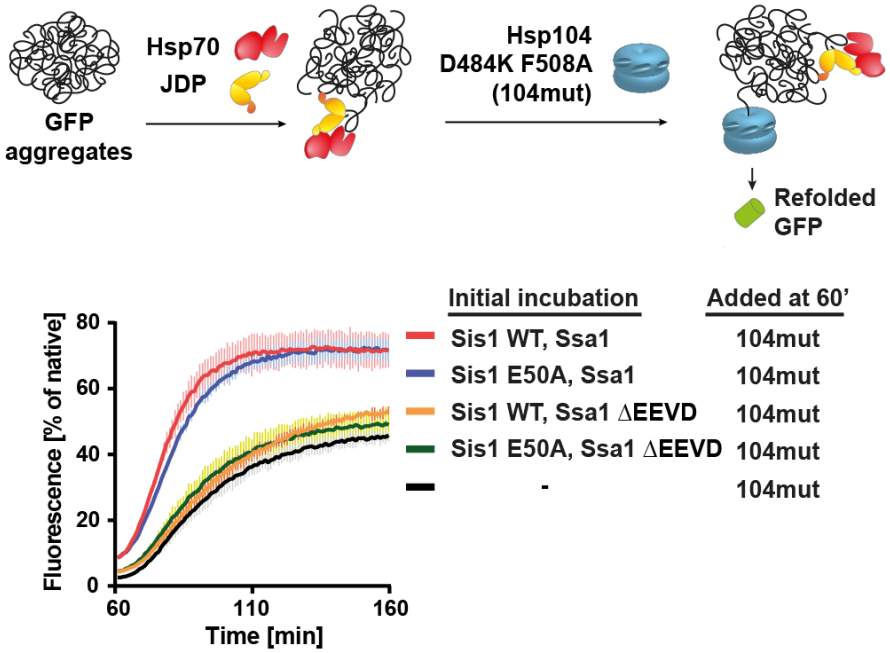

Figure 29. ***The relevance of the Sis1 (CTDI) – Ssa1 (EEVD) interaction in aggregate remodeling activity.*** *GFP aggregates were incubated with indicated chaperones, which was followed by addition of Hsp104 D484K F508A (104mut). The measurement was started after addition of the disaggregase. The GFP fluorescence was normalized to fluorescence of native GFP in the same concentration. The experiment was done by dr Agnieszka Kłosowska according to Methods 7.5.2.*

Taken together, Sis1 allows for abundant Ssa1 loading onto the substrate, which results in protein aggregates modification, which ultimately impacts protein recovery. This ability is directly associated with the interaction between Sis1 CTDI and EEVD, the C-terminal motif of Ssa1.

8.7 Aggregate binding by human system follows the trends observed for yeast system

Aggregate modification might play a relevant part in substrate recovery, especially in the activity of human disaggregation machinery, as it lacks the homolog of Hsp100 disaggregase (King et al, 2008; Putnam et al, 2007). Due to that, the human Hsp70 system activity in disaggregation is highly dependent on nucleotide exchange factors (Raviol, Bukau, Mayer 2006). To examine if the trends observed for yeast proteins also

characterize the human Hsp70 system, I chose orthologues J-domain proteins: Class A DNAJA2 for Ydj1 and Class B DNAJB4 for Sis1, together with human Hsp70 protein – Hsc70 and nucleotide exchange factor Hsp105, a member of Hsp110 family. Both of the JDPs were characterized to be active in protein disaggregation (Nillegoda et al. 2015).

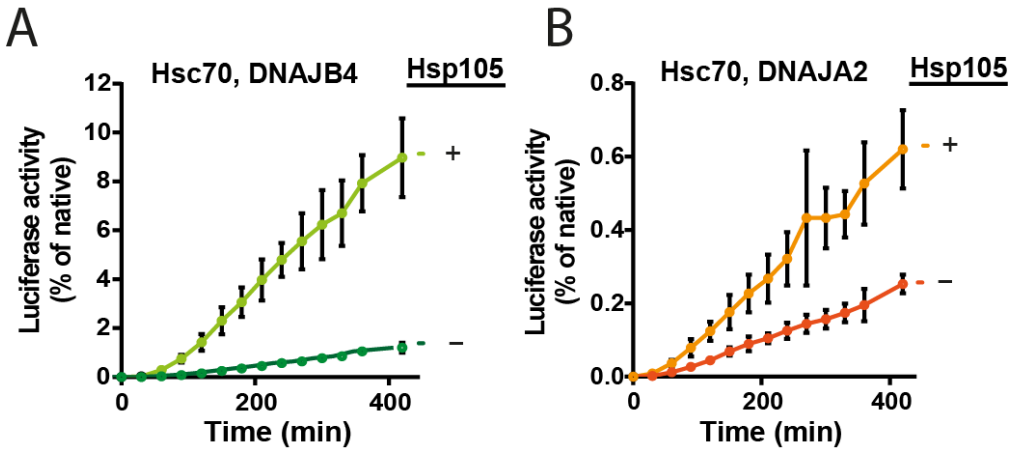

Figure 30. ***Class B JDP-Hsp70-dependent refolding yields more recovered substrate.*** *Luciferase aggregates were incubated with chaperones as indicated. Error bars indicate SD from three independent experiments. (A) Refolding of luciferase aggregates by DNAJB4, Hsc70 with or without Hsp105. (B) Refolding of luciferase aggregates by DNAJA2, Hsc70 with or without Hsp105. Luciferase activity was normalized to the activity of native luciferase in the same concentration. The experiments were done according to Methods 7.5.1.*

Similarly, as for yeast proteins, the Hsp70 system containing Class B J-domain protein promoted a higher level of substrate recovery, which was even more pronounced when supplemented with Hsp105. DNAJB4-Hsc70 refolded about ~1%, with Hsp105 up to ~9% (Fig. 30A). The Hsp70 system activity with Class A J-domain protein, DNAJA2-Hsc70 was low, similar to Ydj1-Ssa1, about ~0,2%, with Hsp105, ~0,6% (Fig. 30B).

Additionally, I tested aggregate binding by the human system. Interestingly, DNAJB4-Hsc70 bound slowly, while DNAJA2-Hsc70 bound rapidly. Both of them reached a similar level of binding. About ~2 nm for DNAJB4-Hsc70 and ~3 nm for DNAJA2-Hsc70. However, when the systems were supplemented with Hsp105, DNAJB4-Hsc70 featured slower binding, reaching about ~6 nm, as observed for Sis1-Ssa1 (Fig. 10), yet only slight increase was present in case of DNAJA2-Hsc70 (Fig. 31).

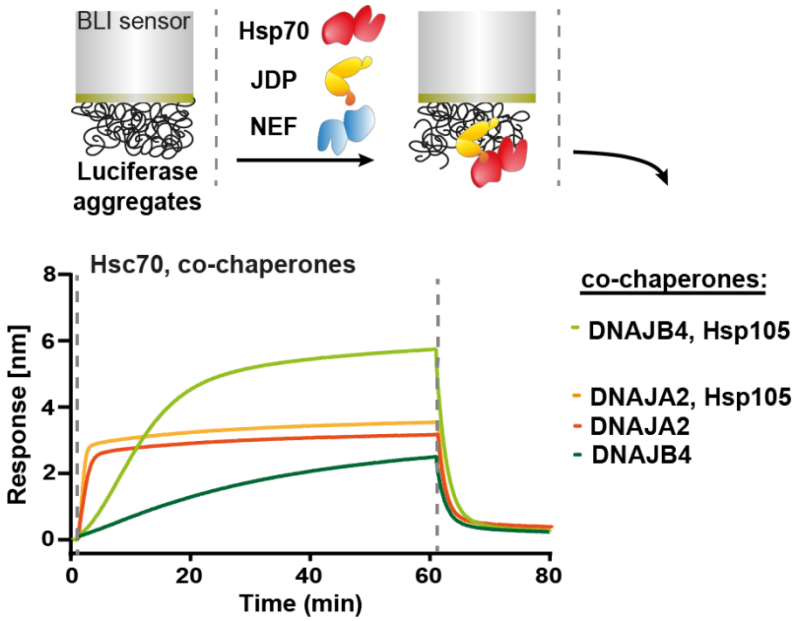

Figure 31. ***Binding of human Hsp70 system: JDP/Hsp70/Hsp110 follows the trends observed for yeast Hsp70 system.*** *The sensor covered with aggregates was incubated with indicated chaperones. The dashed lines indicate the start of association and dissociation steps. The scheme of experiment is placed above the graph. The experiment was done according to Methods 7.5.3.2.*

Summarizing, obtained results suggest similar dynamics in human disaggregation machinery, however, substrate recovery and aggregate binding were more pronounced with the presence of the nucleotide exchange factor.

Most of data, which was presented in this chapter was published in "Class-specific interactions between Sis1 J-domain protein and Hsp70 chaperone potentiate disaggregation of misfolded proteins" (Wyszkowski H, Janta A, Sztangierska W, Obuchowski I, Chamera T, Kłosowska A, Liberek K. Class-specific interactions between Sis1 J-domain protein and Hsp70 chaperone potentiate disaggregation of misfolded proteins. (2021). Proc Natl Acad Sci U S A ;118(49):e2108163118. doi: 10.1073/pnas.2108163118.).

9. Discussion

9.1 Distinctive mechanisms of J-domain mediated Hsp70 loading

JDP - Hsp70 system is the main driver of protein disaggregation and folding. Obtained results show that J-domain proteins Ydj1 (Class A) and Sis1 (Class B) have distinctive effects on protein disaggregation. Both of them are able to promote protein refolding (Figs. 8A, B; 9), however they display substantial differences already in aggregate interaction (Fig. 10). Sis1 yields higher abundance of Hsp70 molecules on the aggregate than Ydj1 (Fig. 10B). This allows for activity, which leads to aggregate modification, what makes the aggregate more amenable for further disaggregation and ultimate dissolution. What is more, it allows for more pronounced Hsp104 docking (Fig. 15A, B). These potentiating factors increase overall effectiveness of disaggregation, which is manifested in higher substrate recovery levels.

Ample amount of Ssa1 present on the aggregate could have at least two possible outcomes. First of all, every Hsp70 possesses a docking site for Hsp104 disaggregase and close proximity between Hsp70 molecules supports avid interactions with multiple subunits of Hsp104 hexamers, what is required for Hsp70-Hsp104 cooperation (Chamera et al, 2019). Secondly, Hsp70 abundance on the aggregate potentiates entropic pulling of the aggregate-trapped polypeptides, which leads to aggregate modification. The modification could involve partial or complete polypeptide disentanglement, leading to local and/or global relaxation of the aggregate assembly.

Another thing to consider is the functional aspect of such modifications. To probe them, I employed Hsp104 disaggregase variant that autonomously solubilizes substrates independently of Hsp70. Initial incubation of aggregates with the JDP-Hsp70 system enhances the refolding capability of this variant, which could indicate that misfolded polypeptide chains become more approachable or easier to extract, or both. Sis1-Ssa1 significantly enhanced the refolding, while Ydj1-Ssa1 also had a positive impact (Fig. 18), although much smaller, which can be associated with lesser entropic pulling due to lower amount of loaded Hsp70 (Fig. 10B). High local concentrations of Hsp70 were shown to be critical for amyloid fibril disaggregation, which can be only mediated by Class B DNAJB1 JDP (human homolog of Sis1) (Wentink et al, 2020). In this work, I

show that this can be also true for Sis1, in terms of high amounts of Hsp70 loaded onto the aggregate, yet distribution of Hsp70 on it, is still an object of my studies.

The exact nature of how protein aggregates are changed by Hsp70 system is not clear, however the aggregate processing ability is making the pool of substrates more manageable. Generation of such substrates can precondition them to be also suitable for other pathways of their utilization, like proteasomal degradation or autophagy (Lu et al, 2017; Mogk et al, 2018). This can especially be vital in case of cellular proteostasis of organisms (non-metazoan eukaryotes and some archaea), which lost its Hsp104 homolog and therefore protein refolding can be limited.

Experiments done in this work are also in compliance with *in vivo* studies. Recent study described that overexpression of Sis1 results in recruitment for Ssa1 to luciferase aggregates and inclusions of polyglutamine tracts. That can subsequently allow the chaperones to penetrate these structures, which could be a result of global relaxation with new binding site exposition (Klaips et al, 2020), which corroborates acquired *in vitro* data.

9.2 Interaction between Sis1 CTDI and EEVD-motif of Ssa1 is crucial for aggregate remodelling activity

The main distinction between the two J-domain proteins is the requirement of Sis1 to bind the EEVD motif of Ssa1 within its CTDI domain. This additional interface of interaction could alter the scheme of Hsp70 loading onto the substrate, which might result in such abundant assembly of Hsp70 on the substrate. Analyzing the possible sites of interaction with Hsp70 within the two J-domain proteins, one can assume that Ydj1 homodimer can bind two Hsp70 molecules, while Sis1, taking into account the additional interface, could bind two Ssa1 molecules per monomer, four in total in the homodimer. The affinity between the CTDI and EEVD seems to be stronger than between the J-domain and NBD of Ssa1, neither is sensitive to the type or presence of nucleotide (Supplementary Fig. S2). Assuming that each site is structurally available to interact with Ssa1, this additional net of interactions could increase the effective local concentration of J-domains, which would facilitate Hsp70 interaction with the protein aggregate.

Sigmoidal shape of the curve during binding of Sis1-Ssa1 to the aggregated substrate indicates a cooperativity. However, the mechanism of Sis1-Ssa1 chaperone complex formation on the substrate is not clear, it seems to be intertwined with aggregate

modification. Gradually loaded Hsp70s exert force onto the substrate exposing new possible binding sites, accelerating the binding, eventually leading to saturation of the aggregate with Hsp70 molecules. Such effect is especially underlined by hindered Sis1-Ssa1 binding to the aggregates structurally confined by cross-linking (Fig. 18). Decreased substrate plasticity forbids uncovering of new binding sites for chaperones, therefore limits binding. Furthermore, Sis1 E50A, which is the variant with disrupted J-domain autoinhibition, together with Ssa1 ΔEEVD, presented weakened aggregate-remodeling activity. If the sigmoidal shape is associated with aggregate remodeling, lack of thereof corroborates this result (Fig. 26). I also observed minor effect in binding to cross-linked aggregate and weaker stimulation of Hsp104 D484K F508A variant (Figs. 27, 28 and 29). The described characteristics are shared with Ydj1-Ssa1, as both systems are only able to interact through J-domain, and they do not utilize the additional high affinity interaction site. Removing the ability of the CTDI-EEVD interaction possibly limits the Sis1-Ssa1 cooperation in aggregate binding to only the J-domain stimulated loading of Ssa1 onto the aggregate, as seen for Ydj1-Ssa1. How this interaction contributes to Hsp70 system interaction with protein aggregate is the subject of my current studies.

9.3 Complementary roles of Class A and Class B JDPs in protein disaggregation

Whereas Sis1 seems to be superior in protein disaggregation due to its ability to promote abundant binding of Ssa1 onto aggregated substrates, Ydj1 has been described to be an efficient holdase, meaning that it is able to bind misfolding polypeptides and prevent their further aggregation (Lu and Cyr, 1998). In compliance with my results, Ydj1 is able to bind aggregates rapidly and stably, which could be particularly advantageous under stress conditions, as it can limit or prevent aggregation of misfolding polypeptides. Sis1 has also been reported to bind misfolded substrates, yet with low affinity (Lu et al, 1998; Fan et al, 2004), which agrees with the observed marginal interaction with the aggregate (Fig. 13 A, B). Coming from the described Hsp70 ATPase cycle, Ydj1 follows the classical scheme, meaning that it binds aggregated substrates and facilitates its loading to Hsp70. Sis1, on the other hand, does not form stable interactions with the aggregate and requires Hsp70 to be present simultaneously to promote chaperone complex formation (Figs. 13, 14). In agreement, aggregation inhibition can only be achieved when Sis1 is coupled with Ssa1 (Lu and Cyr, 1998; Faust et al, 2020). What is also worth mentioning, Ydj1 is more effective in refolding of misfolded non-aggregated substrates (Supplementary Fig. S3),

which is conserved across the evolution of Class A and Class B JDPs (Nillegoda et al, 2015; Nillegoda et al, 2017; Lu and Cyr, 1998). These diverse activities comply with the acquired results. Sis1 activity in protein disaggregation seems to be hugely dependent on cooperation with Hsp70, while Ydj1 also can utilize its substrate binding ability to prevent further misfolding or aggregation. Summarizing, these functions can complement each other in event of protein aggregation during stress.

Sis1 promotes more abundant loading of Ssa1 across tested aggregated substrates, which might imply higher ability of substrate recovery. However, upon comparison of the disaggregation efficiency of GFP and luciferase aggregates, the recovery was much more effective with Sis1 than with Ydj1, especially in the case of GFP aggregates (Fig. 8A, B). Interestingly, in literature, these trends were switched and can hugely depend on the used model substates and protein aggregate preparation (Lu and Cyr, 1998; Nillegoda et al. 2017). The observed activities of Hsp70 systems are only pronounced if the Hsp70 system can act on the aggregates without crucial, at this condition, pulling force of Hsp104 disaggregase. The substrates used in this study are predominantly protein aggregates, which are exposed to chaperones only after their aggregation, what limits the holdase activity of Ydj1. Sis1, which mediates more effective disaggregation in this case, can be roughly estimated to be more suited to tackle amorphous aggregates, as the ability to accumulate high amounts of Hsp70 can influence appropriately sizeable aggregates. What is more, these discrepancies might come from the different requirement for Hsp70 assistance in achieving the native structure by the model substrate. For example, GFP, in contrast to luciferase, can fold spontaneously without chaperones, so when the protein refolding is only limited by disaggregation, Sis1 proves to be more effective, yet when additional folding of polypeptide is required, this could be fulfilled by Ydj1.

Summarizing, the distinct abilities of the J-domain proteins Sis1 and Ydj1 could be required at different stages of protein disaggregation. Abundant Hsp70 presence on the aggregated substrate, mediated by Sis1, would lead to further predisposition of the aggregate for gradual loading of Hsp70 molecules. Each of them could be a possible binding site for Hsp104 disaggregase. Upon peptide disentanglement and release, Ydj1 could capture the released polypeptides and keep them as folding intermediates to await Hsp70-assisted folding, which would ultimately allow them to regain native structure.

These complementary functions of Class A and B J-domain proteins could be especially significant as the human disaggregation machinery lacks the homolog of Hsp104 disaggregase. Same as in the yeast system, Class B JDP promoted higher level of folded luciferase than the Hsp70 system with Class A JDP (Fig. 30A and B). Aggregate-binding BLI experiments showed that DNAJB4-Hsc70 binds more slowly and to a lower level than DNAJA2-Hsc70, binding of which was rapid, but both equilibrated at similar level (Fig. 31). Similar level for both systems seems to be contradictory to the trends observed for the yeast proteins. However, since nucleotide exchange factor appears to be a vital part in the protein disaggregation by the human Hsp70 system (Raviol, Bukau, Mayer 2006), I decided to implement it in both experiments: protein aggregate binding and protein disaggregation. The addition of Hsp105 greatly increased refolding in case of DNAJB4-Hsc70, however only slightly for DNAJA2-Hsc70, when considering the overall efficiency of protein disaggregation (Fig. 30). Only aggregate binding by Class B JDP was strongly stimulated by Hsp105, and now appears to resemble binding of Sis1-Ssa1. Binding of DNAJA2-Hsc70 was slightly enhanced, however still resembled Ydj1-Sis1 (Figs. 10 and 31). Obtained results could mean that observed cooperation between JDP and Hsp70 can be evolutionary relevant, yet in this case it is only observable in the presence of nucleotide exchange factor. In case of amyloid fibril disaggregation, Class B JDP DNAJB1 promoted assembly of Hsp70 clusters, which were more numerous or denser in the presence of Hsp110 nucleotide exchange factor (Faust et al, 2020; Wentink et al, 2020; Beton et al, 2022). This can also be the case in human Hsp70 interaction with amorphous aggregates. In the cell, given that metazoan Hsp70-driven protein disaggregation and refolding cannot be enhanced by Hsp104, the processivity comes from strict cooperation between JDP-Hsp70-Hsp110.

9.4 Perspectives

The described work still leaves open questions. To start, Ydj1 can bind protein aggregates and then attract Hsp70 to the substrate, whilst Sis1 to promote chaperone complex formation on the substrate requires simultaneous presence of Hsp70. While it can be explained by low affinity of JDP to substrates, it is not in agreement with low concentration requirement to achieve nearly full level of system activity in disaggregation and chaperone complex assembly on the substrate. Ydj1 to promote full activity needs to be present in nearly equimolar concentration to Ssa1, however Sis1 is promoting nearly full activity in substoichiometric concentration to Ssa1 (0,1:1) (Supplementary Fig. S1).

It hints different mechanism of JDP-Hsp70 interaction with the substrate and raises several questions. What are the requirements of Sis1 to stabilize it on the substrate? Is Hsp70 loaded onto the substrate in agreement with canonical Hsp70 ATPase cycle? At which point of chaperone system assembly on the substrate Sis1 CTDI-Ssa1 EEVD interaction is relevant? Ideas to answer these questions are part of the project, which I later conducted.

The nature of aggregate modification remains to be elucidated. Due to amorphous and chaotic assembly of misfolded polypeptides, which a protein aggregate is, it serves a challenge to visualize how it is changed. In the project, being the consequence of this work, to determine how Hsp70 system modifies protein aggregates: I) we observed how hydrodynamic radius of protein aggregates changes due to Hsp70 activity using dynamic light scattering (DLS). We took advantage of the fact, that DLS is predominantly blinded by the biggest molecules in the solution, so we could observe how the aggregates are converted into smaller species, while JDP-Hsp70 remained invisible. 2) Given that DLS is a crude method in such application, we also tried different approach. We used luciferase fused with GFP (Luc-GFP). Given that, in a condition, in which luciferase undergoes aggregation, GFP remains folded, by applying denaturing agent, we can acquire luciferase aggregates, which are fused with non-aggregated GFP. To trace the changes, we employed confocal fluorescence microscopy to see how, over time, Luc-GFP aggregates are being converted to much smaller species due to Hsp70 system activity. What still remains an open question in this case is: is the aggregate modification a straightforward detachment of whole “chunks” of polypeptides from protein aggregates? Another explanation would be that only single polypeptides or low-number oligomeric assemblies are being freed, which by interacting with each other, unable to spontaneously fold, form smaller aggregate species. Both scenarios can be true and even both can happen at the same time. To examine this, filter trap assay can be employed to quantify how much of aggregated protein remains captured and how much is soluble. Another idea is to employ GroEL chaperonine variant (D87K) termed GroEL-TRAP (Ziętkiewicz et al, 2006), which would bind and hold single misfolded polypeptides during Hsp70 activity on the protein aggregates. If the amount of smaller species would be decreased, it could mean that majority of polypeptides is held by GroEL-TRAP preventing their reaggregation. To further evaluate the solution could be applied to glycerol gradient centrifugation to determine how big of a fraction of substrate is being held by GroEL-TRAP.

Ydj1 and Sis1 sway Hsp70 towards different activities and it is unknown how the cell regulates employment of each. It seems that Sis1 promotes more efficient aggregate processing, whereas Ydj1 employs Hsp70 to efficient protein folding. Looking at these specializations, it may give rise to an idea of subsequent action of these Hsp70 systems during stress in cells. Ydj1 and Sis1 have been reported to subsequently colocalize with stress granules *in vivo* (Walters et al, 2015). I would propose a similar experiment in vivo, with labelled Ydj1, Sis1, Hsp70 and luciferase, in which I could observe colocalization of JDPs with luciferase aggregate puncta over time, and, hopefully, what is the consequence of their activity for the protein aggregate.

Yeast Hsp70 system, even though is active in JDP-Hsp70 setup, it comprises Hsp110 nucleotide exchange factors (NEF). It is then curious to examine how NEF influences Hsp70 activities presented in this work. This aspect was investigated in later study I was a part of. We found that, the yeast Hsp110 – Sse1, differentially influences Class A (Ydj1 or DNAJA2) and Class B JDP (Sis1 or DNAJB4) containing Hsp70 system. While, the activity of Class B JDP - Hsp70 was greatly stimulated in activity, the stimulation of Class A JDP – Hsp70 was minor or even absent. Sis1-Ssa1-Sse1 system, when compared to Sis1-Ssa1, was more efficient in protein disaggregation, the level of binding to protein aggregates was also increased, which we connected with the amount of loaded Hsp70 onto the substrate. The higher amount of Hsp70 also improved aggregate remodeling. This corroborates previously published data for human Hsp70 system (Wentink et al, 2020; Beton et al, 2022). However, Ydj1-Ssa1-Sse1 activity was not stimulated in protein disaggregation, as well as, aggregate binding. Human Hsp70 system presents similar trends. DNAJB4 – Hsc70 was stimulated due to the presence of Hsp105, yet DNAJA2 – Hsc70 stimulation was minor.

Trends observed for the yeast Hsp70 system could be also true for the human proteins. What remains to be checked is, whether such specialization of JDPs also remains in this case or the basis for Hsp70 activity is comprised differently. How each Hsp70 system influences protein aggregates and if the level of binding translates to the amount of Hsp70 loaded onto the substrate. What is more, why human Hsp70 system is more dependent on NEF, and why its effect is different for each class of JDP in Hsp70 system activity. Human Hsp70 system, as it cannot be supplemented by a homolog of Hsp104, functional cooperation of Hsp70 and JDPs of different classes can be crucial in protein disaggregation. Taking into consideration the obtained results for the yeast proteins, the

activities of either JDP-Hsp70 system can complement each other and by employing JDPs of multiple classes efficient disaggregation machines can be generated. This cooperation was previously reported in literature (Nillegoda et al, 2015; Nillegoda et al, 2017). All of these aspects would be easily testable using similar approaches as used in this work.

9.5 Final remarks

Different contributions of Hsp70 system activity in protein disaggregation driven by the employed J-domain protein are potentially a key to understand how misfolded or aggregated substrates are handled in cell during stress and later recovery. This is especially important in case of amyloid fibrils, which formation in neuronal cells is the hallmark of neurodegenerative diseases. Understanding the mechanisms of their handling by Hsp70 system can give rise to new targeted therapies, through boosting or inhibiting certain pathways within Hsp70 system activity. My studies corroborate and extend knowledge how specifically Class B JDPs are able to efficiently promote solubilization of complex assemblies of misfolded proteins: amorphous aggregates or amyloid fibrils.

10. References

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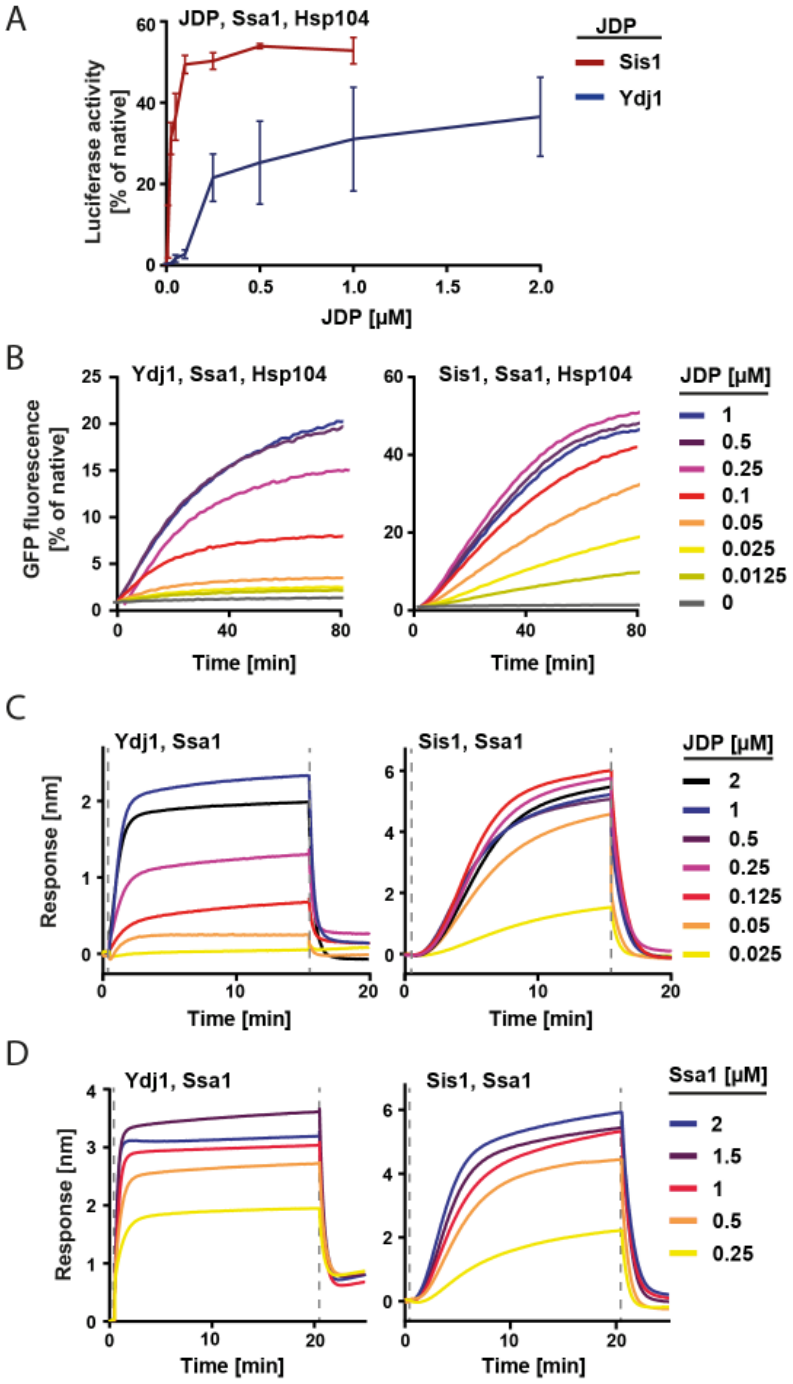
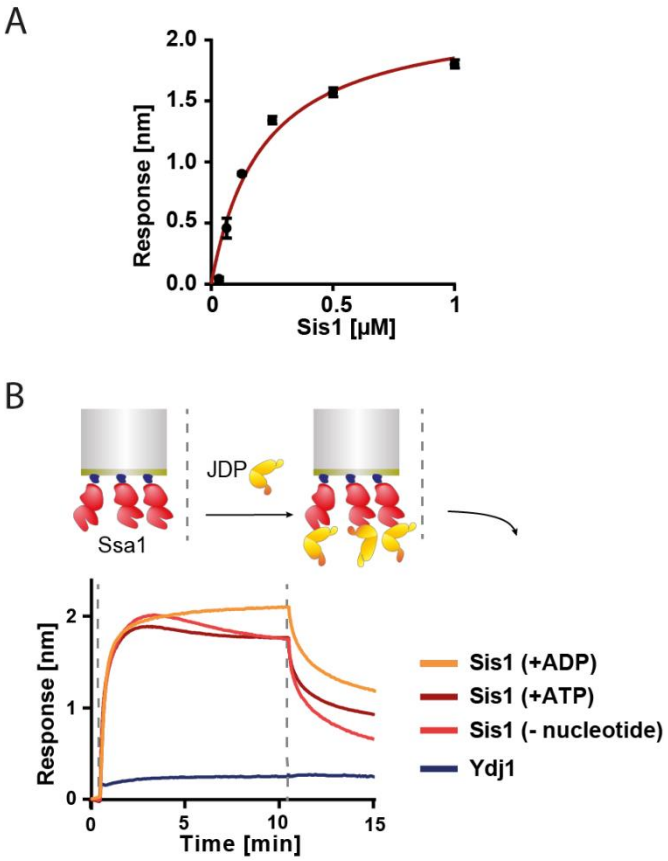
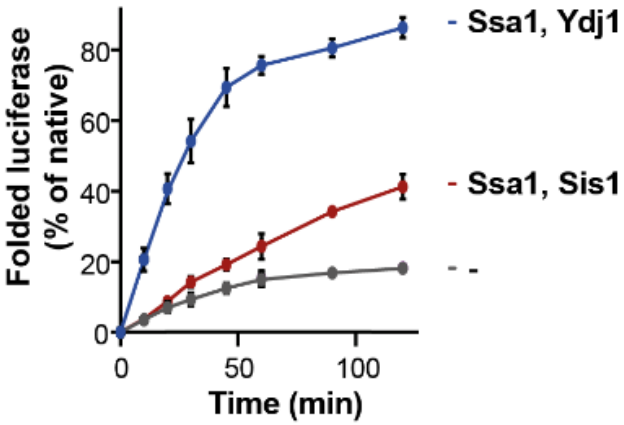
11. Supplementary figures



Supplementary Figure S1. (A) Aggregated luciferase reactivation level after 90 minutes by Ssa1, Hsp104 and indicated concentration of JDP. Error bars indicate SD from three experiments. (B) Disaggregation of heat-aggregated by Ssa1, Hsp104 and indicated JDP concentration. (C) Binding of the Hsp70 system consisting of JDP at indicated concentration and Ssa1 to heat-aggregated luciferase on the biosensor. Left panel -Ydj1. Right panel- Sis1. (D) Binding of the Hsp70 system consisting of JDP and indicated concentrations of Ssa1 to sensor-bound luciferase aggregates. The experiment was done as described in Methods 7.5.1 (A), 7.5.2 (B), 7.5.3.2 (C, D).



Supplementary figure S2. (A) Apparent dissociation constant determined based on steady state equilibrium levels of Sis1 binding to His_6-SUMO-Ssa1 immobilized on the BLI sensor (K_D^{app} 210 ± 32 nM, B_{MAX}^{app} = 2.24 ± 0.13 nm). Sis1 was used at concentrations: 31,25 nM, 62,5 nM, 125 nM, 250 nM, 500 nM, 1000 nM. Error bars indicate SD from three independent experiments. The One site- specific binding, $Y=B_{MAX}*X/(K_D+X)$ model (solid red line) was fitted using Least Squares approximation (GraphPrism). (B) Binding of JDPs to sensor bound His_6-SUMO-Ssa1in the presence (5 mM ATP or 5 mM ADP) or absence of nucleotides as indicated. The experiment was done as described in Methods 7.5.3.1.



Supplementary figure S3. Spontaneous or assisted folding of non-aggregated luciferase diluted from 5 M GuHCl into buffer containing indicated chaperones. The error bars represent SD from 3 experiments. The experiment was done by Wiktoria Sztangierska according to Imamoglu et al, 2020. 10 μM luciferase was denatured by incubation in 5M GuHCl and 10 mM DTT for 1h in 25 degrees Celsius. The experiment was initiated by diluting the luciferase to final 100 nM concentration in buffer (25 mM HEPES-KOH pH 7,5, 100 mM KCl, 10 mM $Mg(OAc)_2$, 2 mM DTT and 0,05% Tween-20) containing indicated chaperones at 1 μM concentration. Luminescence was measured using Glomax 20/20 (Promega) by hand, through taking aliquot of the mixture and mixing it with Luciferase Assay Kit (Promega) at indicated timepoints.