# Balanced Interplay Between Hsp110, Hsp70 and Class B J-Domain Protein Improves Aggregate Disassembly

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**Abstract**

**Hsp70 is a key cellular system counteracting protein misfolding and aggregation, associated with stress, ageing and disease. Hsp70 solubilizes aggregates and aids protein refolding through substrate binding and release cycles regulated by co-chaperones: J-domain proteins (JDPs) and Nucleotide Exchange Factors (NEFs). Here, we elucidate the collaborative impact of Hsp110 NEFs and different JDP classes throughout Hsp70-dependent aggregate processing. We show that Hsp110 plays a major role at initial stages of disaggregation, determining its final efficacy. The NEF catalyses the recruitment of thick Hsp70 assemblies onto aggregate surface, which modifies aggregates into smaller species more readily processed by chaperones. The stimulation is much limited with class A JDPs and requires the auxiliary interaction between class B JDP and the Hsp70 EEVD motif. Furthermore, we demonstrate for the first time that Hsp110 disrupts the JDP-Hsp70 interaction. We propose that the limited destabilisation of the chaperone complex improves disaggregation, but also leads to the inhibition above the substoichiometric Hsp110 optimum. This suggests that the tuned proportion between the co-chaperones of Hsp70 is critical to reach its disaggregating potential.**

## Introduction

During stress, protein homeostasis is perturbed by protein misfolding and aggregation. Accumulation of protein aggregates disrupts cellular functions and contributes to ageing and is a hallmark of many neurodegenerative diseases (Hartl *et al*, 2011; Morimoto, 2008). A protein quality control system, relying on a network of cooperating chaperones, has evolved to moderate stress-induced proteotoxicity and rebalance proteostasis.

To refold proteins from aggregates into the native state, Eukaryotes use a system comprising Hsp70, an ATP-dependent chaperone, and its co-chaperones: an Hsp110 nucleotide exchange factor (NEF) and a J-domain protein (JDP/Hsp40), together with an Hsp100 disaggregase (Glover & Lindquist, 1998). The process is initiated by a JDP, which delivers Hsp70 to an aggregated substrate and whose J-domain induces ATP hydrolysis in Hsp70, resulting in conformational changes that stabilise the interaction with aggregates (Rohland *et al*, 2022). Next, Hsp100 interacts with the Hsp70-aggregate complex and the aggregate-trapped polypeptides are translocated through the Hsp100 hexamer. The disentangled and released polypeptides can fold back into their native conformation, alone or with further aid of chaperones (Lum *et al*, 2004; Schaupp *et al*, 2007; Seyffer *et al*, 2012; Weibezahn *et al*, 2004; Zietkiewicz *et al*, 2004).

Hsp110 co-chaperone boosts the Hsp70 activity by stimulating nucleotide exchange and substrate release (Dragovic *et al*, 2006; Raviol *et al*, 2006; Shaner *et al*, 2005). It belongs to the Hsp70 superfamily, with identical domain organisation to Hsp70 but distinct size and arrangement of the nucleotide-binding domain (NBD) and substrate-binding domain (SBD) (Easton *et al*, 2000; Liu & Hendrickson, 2007). In contrast to Hsp70, Hsp110 is unable to refold denatured proteins, yet it may bind to and prevent aggregation of certain misfolding substrates (Garcia *et al*, 2017; Goeckeler *et al*, 2008; Goeckeler *et al*, 2002; Oh *et al*, 1997; Oh *et al*, 1999; Polier *et al*, 2010; Xu *et al*, 2012). In its well-established NEF function, SBD of Hsp110 interacts with the ADP form of Hsp70, which mediates the release of the nucleotide. Subsequent ATP binding to NBD of Hsp70 prompts the opening of the SBD domain and substrate dissociation, resetting Hsp70 for another round of the cycle (Andreasson *et al*, 2008a; Laufen *et al*, 1999; Liberek *et al*, 1991; Mayer & Bukau, 2005; Mayer & Gierasch, 2019).

*In vivo* studies on yeast chaperones demonstrated that the Hsp110 co-chaperone Sse1 plays a key role in Hsp70 recruitment to aggregates and is essential for protein disaggregation (Kaimal *et al*, 2017). Hsp110 is a major cytoplasmic NEF of Hsp70 and the deletion of its both paralogs, *SSE1* and *SSE2*, is lethal and the growth can only be supported, albeit with less efficacy, by overexpression of *FES1,* another cytoplasmic NEF from an unrelated armadillo family (Abrams *et al*, 2014; Raviol *et al.*, 2006).

The importance of Hsp110 is also manifested *in vitro* in a low disaggregation activity of the yeast Hsp70 system without Hsp110 and the complete interdependence between their human orthologs (Glover &

Lindquist, 1998; Mattoo *et al*, 2013; Nillegoda *et al*, 2015; Rampelt *et al*, 2012). Recent studies on the human system revealed that the Hsp110 function in disaggregation of amyloid fibrils is not limited to its NEF activity but it may also affect the architecture of chaperone complexes, inducing Hsp70 clustering, although it is not clear whether similar effects occur during disaggregation of non-fibrillar, stress-associated aggregates. The effect is not well understood, but it presumably increases the entropic pulling of aggregated polypeptides, ultimately leading to their disentanglement by the Hsp70 system, efficient even without the Hsp100 disaggregase. Developing an Hsp70 system that is self-sufficient in disaggregation could have compensated for the loss of the Hsp100 disaggregase in a common metazoan ancestor (Mattoo *et al.*, 2013; Shorter, 2011).

Another factor that potentiates the Hsp70 system is the diversity of JDP paralogs, assigned to cytoplasmic classes A and B, which differently regulate Hsp70 (Lu & Cyr, 1998; Nillegoda *et al.*, 2015). The main distinction between class A and B JDPs is an auxiliary interaction site between CTD1 domain of class B JDPs and the C-terminal EEVD motif of Hsp70 (Yu *et al*, 2015). Class B JDPs are additionally regulated by an autoinhibitory mechanism, in which Hsp70 binding by the J-domain is restricted by a neighbouring helix. Upon binding to the C-terminal EEVD motif of Hsp70, the J-domain is released and can interact with the NBD of Hsp70 (Faust *et al*, 2020; Wentink *et al*, 2020). We have recently shown that the yeast class A JDP Ydj1 and class B JDP Sis1 exhibit diverse mechanisms during Hsp70 binding to aggregated substrates. Ydj1, in accordance with the classical model of Hsp70 ATPase cycle, binds misfolded polypeptides and loads Hsp70 onto aggregates. Unlike that, Sis1 only weakly binds protein substrates but due to a the more complex interaction with Hsp70, it loads more Hsp70 molecules onto aggregates, which results in more efficient disaggregation (Wyszkowski *et al*, 2021).

Despite the vast knowledge on how Hsp110 serves as a regulator of the ATPase cycle of Hsp70 (Andreasson *et al.*, 2008a; Andreasson *et al*, 2008b; Dragovic *et al.*, 2006; Raviol *et al.*, 2006; Shaner *et al*, 2006) little is known about the function of Hsp110 considering the mechanism of Hsp70 interaction with different JDP classes.

Here, we demonstrate that the interplay between Hsp110 and Hsp70 in disaggregation strictly relies on the class of a JDP and unravel the critical role of the B-class specific interaction with the EEVD motif of Hsp70 for the Hsp110-dependent stimulation. Furthermore, we elucidate differential contribution of the NEF across different phases of protein recovery from aggregates. We employ Bio-Layer Interferometry to investigate the Hsp110 impact on the formation of chaperone complexes at the aggregate surface and we assess changes in aggregates properties associated with the abundant chaperone binding. Finally, we address a question of the competition between the NEF and JDP co-chaperones. Our findings shed new light on the mechanisms behind the potentiation and inhibition of the disaggregation activity of Hsp70 by its co-chaperones.

## Results

### Stimulation of the Hsp70 disaggregation activity by Hsp110 depends on the class of JDP

Our recent studies showed that during the recovery of aggregated proteins, the Hsp70 chaperone system exhibits different mechanisms of action with class A and class B JDP co-chaperones (Wyszkowski *et al.*, 2021). To better understand the interplay within the Hsp70 chaperone network, we addressed how Hsp110 affects Hsp70 with members of different JDP classes. First, we tested the effect of Sse1, the most abundant yeast NEF, on the recovery of a model protein substrate, aggregated luciferase, by Hsp70 (Ssa1) and class A (Ydj1) or class B (Sis1) JDPs. Compared with Ssa1-Sis1, which exhibits delayed start of disaggregation characteristic for class B JDPs, the initial luciferase recovery was significantly faster, with higher overall output, in the presence of Sse1 (Fig. 1A). In contrast, Sse1 slightly decreased the disaggregation efficacy in the case of Ssa1-Ydj1 (Fig. 1A). With another aggregated substrate, GFP, Sse1 improved the disaggregation activity with either of the JDPs, albeit for Ssa1-Sis1, the stimulation was almost 4 times higher than with Ydj1 (Fig. S1A).

Addition of the Hsp104 disaggregase to the system increased the total disaggregation efficacy and enhanced the positive effects of Sse1 in the case of either JDP and either aggregated substrate (Fig. S1B,C). The chaperone system comprising Hsp104-Ssa1-Sis1 with Sse1 yielded approximately 3 times higher luciferase and GFP recovery rates than without the NEF, while in the case of Hsp104-Ssa1-Ydj1, the simulation by Sse1 was much weaker, from marginal to twofold, depending on the protein substrate (Fig. S1B,C). Thus, the positive effect of the Hsp110 nucleotide exchange factor on protein recovery from aggregates is substantially more pronounced when the Hsp70 chaperone system comprises a class B JDP.

Theoretically, the stimulation by Sse1 might occur at one or many stages of protein disaggregation – starting from the association of the Hsp70 system with an aggregate surface, through polypeptide disentanglement, to the final folding of the released substrate. Previous studies show that Ssa1-Ydj1 is much more effective than Ssa1-Sis1 in restoring the native structure of unfolded luciferase (Lu & Cyr, 1998), which could represent the final step of disaggregation – the polypeptide folding after translocation by Hsp104. To test how Sse1 contributes to the protein folding by the Hsp70 system, we measured the recovery of denatured, non-aggregated luciferase (Imamoglu *et al*, 2020). Curiously, neither of the systems: Ssa1-Sis1 nor Ssa1-Ydj1, was stimulated in luciferase folding by Sse1 (Fig. S1D), suggesting that the NEF plays an important role at earlier stages of disaggregation.

We recently showed that JDP co-chaperones determine the association kinetics and the size of chaperone complexes formed at the aggregate surface, which contributes to the total effectiveness of disaggregation (Wyszkowski *et al.*, 2021). To gain insight into the influence of the Hso110 on the assembly of the protein

disaggregation complex, we analysed the binding of the Hsp70 system to the heat-aggregated luciferase using bio-layer interferometry (BLI). The presence of Sse1 increased the rate of Ssa1-Sis1 association with aggregate-covered biosensor approximately 2 times and resulted in a 50% thicker protein layer (Fig. 1B). In contrast, Sse1 did not substantially influence the binding level of Ssa1-Ydj1 (Fig. 1B). In both cases, the binding of chaperones required ATP (Fig.1B), which is consistent with ATP-dependence of Hsp70 (Fig. 1B). Similar effects were observed with heat-aggregated GFP or heat-aggregated yeast lysate proteins immobilised on the sensor, indicating that they are not substrate-specific (Fig. S1E,F).

We also assessed the evolutionary conservation of the observed trends and applied BLI to investigate how Hsp105, a human Hsp110, affects aggregate binding by the Hsp70 system. Similarly as the yeast system, Hsc70-DNAJB4 (orthologous to Ssa1-Sis1) exhibited delayed binding and the addition of Hsp105 resulted in much faster association and a 6-fold increase in the binding level (Fig. 1C). In the case of Hsc70-DNAJA2 (orthologous to Ssa1-Ydj1), the binding also increased upon Hsp105 addition, however the stimulation was three times less pronounced than with DNAJB4 (Fig. 1C). In contrast to the yeast proteins, the stimulation occurred with either JDP class, which is in line with the stronger dependence of the human Hsp70 system on the NEF in protein disaggregation (Fig. S1G).

The BLI results suggest that the Hsp110 nucleotide exchange factor greatly improves Hsp70 binding to aggregates, especially with class B JDPs. What could be the basis for this specificity? A major discrimination factor between the two JDP classes is the stable interaction between class B JDPs and the EEVD-motif of Hsp70 (Yu *et al.*, 2015). Perturbation of this interaction restricts the J-domain-dependent Hsp70 activation, which can be restored through E50A mutation in Sis1 (Yu *et al.*, 2015) or the mutation in DNAJB4 Helix 5 (Faust *et al.*, 2020). Previous studies showed that $Ssa1^{\Delta EEVD}$-$Sis1^{E50A}$ resembles Ssa1-Ydj1 in aggregate binding and disassembly (Wyszkowski *et al.*, 2021), therefore we asked what effect this disruption has on the stimulation by Sse1. We observed that the Sis1 E50A-Ssa1 ΔEEVD system is strongly inhibited by Sse1 in protein disaggregation (Fig. S1H) and the level of aggregate binding is also negatively affected (Fig. S1I). This suggests that the Hsp70-JDP stimulation by Hsp110 is functionally linked to the Sis1-Ssa1 EEVD interaction.

We next asked whether Hsp110 contributes to the extra chaperone layer at the aggregate surface in a way that it comprises more Hsp70 molecules. To evaluate the amount of bound Hsp70, we carried out an analogous BLI experiment with fluorescently-labelled Ssa1 (Ssa1*A488). After protein dissociation to the basal level, the total dissociated Ssa1*A488 was quantified based on fluorescence. In the presence of Sis1 and Sse1, the amount of Ssa1*A488 was nearly 2 times higher than in the presence of Sis1 only, whereas Sse1 did not influence the amount Ssa1*A488 bound to the biosensor when Ydj1 was applied (Fig. S2A). The increase in the fluorescence signal corresponded to that of BLI (Fig. S2A), suggesting a major contribution of Ssa1 to the thickness of the chaperone complex forming on the aggregate surface.

Since the presence of Sse1 substantially increased the association of Ssa1 with aggregates, we asked if the problem limiting Hsp70 binding that is overcome by Hsp110 is the insufficient availability of Hsp70 molecules targeting the aggregate. To address that, we tested whether a similar effect to the Sse1-induced stimulation of Ssa1 binding can be obtained by increasing Ssa1 concentration itself. Sse1 stimulated Ssa1 binding much above the level that could be achieved for the saturating concentration of Ssa1 in the absence of Sse1 (Fig. S2B). This indicates that the mechanism of stimulation by Hsp110 is more complex than an enrichment of the pool of Hsp70 molecules capable of substrate binding and might involve generating more Hsp70-binding sites.

To explore whether the observed effects are unique to Hsp110, we examined another cytosolic NEF that belongs to the Armadillo type family, Fes1. It is structurally distinct from Hsp110, with a C-terminal armadillo domain that triggers nucleotide exchange in Hsp70 through a different mechanism (Gowda *et al*, 2018). Fes1 has weaker affinity for Hsp70 and lower nucleotide exchange activity than Sse1 (Dragovic *et al.*, 2006). Unlike Sse1, Fes1 does not directly bind protein substrates, but its N-terminal RD domain is involved in substrate release from Hsp70 (Gowda *et al.*, 2018). When we carried out luciferase disaggregation by Ssa1-Sis1, the level of stimulation achieved by Sse1 required 10 times more Fes1 (Fig. S3A). Similarly, 1 μM Fes1 stimulated fluorescent Ssa1 (Ssa1*A488) binding to luciferase aggregates on the BLI sensor (Fig. S3B) to the level achieved with 0.1 μM Sse1 (~3 x $10^5$ a. u.) (Fig. S3C, S2A). This result suggests that the substrate-binding activity specific to Hsp110 is not necessary to increase the Sis1-Ssa1 binding to aggregates and that the effective NEF concentration is negatively correlated with its affinity for Hsp70.

**Sse1 improves aggregate modification by Hsp70**

Recently, we have reported that larger Hsp70-JDP assemblies at the aggregate surface, dependent on Sis1 and its interaction with EEVD, can modify aggregates into misfolded protein species that are more amenable to disaggregation (Wyszkowski *et al.*, 2021). To assess whether Hsp110 further stimulates such aggregate-remodelling activity, and this way contributes to more efficient protein recovery, we used a variant of Hsp104 with abrogated interaction with Hsp70, D484K F508A ($Hsp104^{mut}$). $Hsp104^{mut}$ does not require Hsp70 for allosteric activation and aggregate binding and it can serve as an indicator of Hsp70-dependent aggregate modification by facilitating final folding of its products (Chamera *et al*, 2019). When heat-aggregated GFP was initially incubated with Ssa1-Sis1-Sse1, which yielded very low protein recovery, we added $Hsp104^{mut}$ and observed much faster and more effective GFP reactivation than when the substrate was first incubated with Ssa1-Sis1 only (Fig. 2A). In an analogous experiment with Ydj1, we also observed stimulation by Sse1, yet not as strong as in the presence of Sis1 (Fig. 2A).

Aggregate remodelling by Hsp70-JDP, improved by Hsp110, might induce changes limited to the aggregate surface, such as partial polypeptide disentanglement that uncovers additional chaperone-

binding sites, or also lead to global rearrangements, changing aggregate size and total exposed surface area, e. g. through partial aggregate dissolution and fragmentation. To shed light on this, we visualised the aggregates of luciferase C-terminally fused with GFP using fluorescence microscopy. After heat denaturation, approximately 80% of Luciferase-GFP aggregates had the size of 2 μm or more (Fig. 2B). Incubation with Ssa1-Sis1 for 1 h decreased the fraction of aggregates larger than 2 μm to 25,8%, whereas the addition of Sse1 reduced it to 12,5% (Fig. 2B). The decrease depended on ATP and the aggregates size was only moderately affected by Ssa1-Ydj1, irrespectively of the presence of Sse1 (Fig. 2B).

We also measured the size of aggregates using dynamic light scattering (DLS). Heat-denatured luciferase formed aggregates with hydrodynamic diameter of approximately 2000 nm (Fig. S4), while the DLS signal of approximately 10 nm corresponded to the native luciferase and chaperones, consistent with their theoretical size (Fig. S4). Incubation with Ssa1-Sis1 for 1 h slightly reduced the 2000 nm peak and produced small-size aggregates of approximately 30 nm (Fig. S4). The presence of Sse1 increased the amount of 30 nm aggregates more than 2-fold and significantly reduced the signal at 2000 nm (Fig. S4). The change in aggregate size was not observed unless ATP was present (Fig. S4). In agreement with the microscopy data, Ssa1-Ydj1 with or without Sse1 did not have any substantial effect on the size of the aggregates (Fig. S4).

Taken together, Sse1 boosts the aggregate-remodelling activity of Ssa1, specifically with the class B JDP Sis1, causing major reduction in aggregate size.

**Biphasic effects of Sse1 on the Hsp70 disaggregation activity**

Contrary to the strong stimulation by Hsp110 of the Hsp70 system comprising class B JDP, its impact on Hsp70 with class A JDP strongly varied with an experimental setup (Fig. 1A,B, S1A,B,C). Knowing that the effects of Sse1 on disaggregation by Ssa1-Ydj1 are concentration-dependent, with effective substoichiometric amount of Sse1 (Dragovic *et al.*, 2006), we asked to what degree the optimum Hsp110 level depends on the class of a JDP. When we titrated the Hsp70 system comprising either Sis1 or Ydj1, we observed an inhibition of luciferase disaggregation at increased Sse1 concentrations, yet the system with Ydj1 was much more sensitive to Sse1 (IC50 = 0.1 μM), while Ssa1-Sis1 was still stimulated at 0.3 μM Sse1 (molar ratio Ssa1:Sse1 1:0.3) (Fig. 3A). When we included the Hsp104 disaggregase, both Hsp104-Ssa1-Ydj1 and Hsp104-Ssa1-Sis1 systems were stimulated at low and inhibited at high Sse1 levels, with the highest yield at 0.05 μM and 0.2 μM of Sse1, respectively (Fig. S5A). The hormesis was also observed for human orthologs, with Hsc70-DNAJB4 tolerating higher Hsp105 concentration than Hsc70-DNAJA2 (Fig. 3B).

In contrast to protein disaggregation, the reactivation of denatured, non-aggregated luciferase by Ssa1 with either Sis1 or Ydj1 was only negatively affected across a range of Sse1 concentrations (Fig. 3C), suggesting that the positive contribution of Hsp110 takes place before polypeptides get released from the

aggregate. The negative effect of Sse1 on protein folding could potentially mask a positive effect of saturating Sse1 on aggregate modification, e. g. through entropic pulling. We thus analysed the size of aggregates after their incubation with Ssa1-Sis1 and 1 μM Sse1, however it was unaffected (Fig. S5B,C), suggesting that higher Sse1 levels also limit the aggregate-remodelling activity of Hsp70. Consistently, when we measured Hsp70 association with luciferase aggregated on the BLI sensor across Sse1 concentrations, we observed the biphasic effect for Ssa1-Ydj1 and Ssa1-Sis1, with the maximum binding at 0.05 μM and 0.2 μM of Sse1, respectively (Fig. 3D). Thus, Hsp110 promotes Hsp70 assembly at aggregates and their modification only at sub-stoichiometric concentrations, with peak performance at higher levels of Sse1 in the presence of class B than class A JDPs.

To establish whether the inhibition that occurs at higher Sse1 concentrations is directly associated with the interaction between Sse1 and Ssa1, or rather between Sse1 and a substrate, we used the previously characterised Sse1-2 variant (N572Y E575A), with disrupted interaction with the A300 residue of Ssa1, which reduces Hsp70 binding to 20% and nucleotide exchange to 5% of that of the wild type (Polier *et al*, 2008). The variant has been reported to partially compensate for the *SSE1/SSE2* double deletion (Polier *et al.*, 2008). Consistently with the weak Ssa1 binding affinity of Sse1-2 (Fig. S5D), Ssa1-Sis1 required 20-times higher concentration of the Sse1-2 variant than Sse1 WT to reach the same GFP disaggregation activity and no inhibition was observed up to 2 μM of Sse1-2 (Fig. 3E). Also, the Sse1-2 variant had a much weaker impact on luciferase disaggregation (Fig. S5E). Nonetheless, 1 μM Sse1-2 was sufficient to obtain a similar effect as 0.1 μM WT Sse1 in stimulating Ssa1-Sis1 binding to the aggregated luciferase in the BLI assay (Fig. S5F).

This suggests that it is the high-affinity interaction between Hsp70 and the NEF that enables strong stimulation already at very low Hsp110 levels. On the other hand, such strong interaction is associated with the inhibition of the disaggregation activity when Hsp110 concentration exceeds sub-stoichiometric proportion to Hsp70.

**Hsp110 limits JDP interaction with Hsp70**

Since all NEFs induce polypeptide release from Hsp70, excessive dissociation from the substrate seems the most apparent explanation of Hsp70 inhibition by Hsp110. However, the Sse1-2 variant is nearly as effective as WT in triggering peptide release from Ssa1 (Polier *et al.*, 2008) and yet, no inhibition by Sse1-2 was observed at much higher concentrations (Fig. 3E). This prompted us to search for other potential mechanisms behind the low Hsp70 chaperone activity under high Hsp110 concentration.

Knowing that the sensitivity to Sse1 (Fig. 3A,D) is negatively correlated with the strength of the Hsp70-JDP interaction, higher in the case class B JDP due to its binding of EEVD motif of Hsp70 (Wyszkowski *et al.*, 2021), we explored the possibility that Hsp110 restricts the formation of the Hsp70-JDP complex.

To test this, we immobilised Sis1 on the BLI sensor and monitored Ssa1 binding across a range of Sse1 concentrations. The incubation with 0.1 μM Sse1 was almost inert, however, at 1:1 Ssa1:Sse1 ratio, the binding greatly diminished (Fig. 4A). The reduced affinity of Sse1-2 for Ssa1 resulted in its milder negative effect on Ssa1 binding to Sis1 (Fig. S6A), in agreement with its impact on disaggregation (Fig. 3E). The degree of inhibition by the two Sse1 variants (Fig. S6B) correlated with their capacity to exchange nucleotides (Dragovic *et al.*, 2006; Polier *et al.*, 2008).

In an analogous way, we analysed the interaction between the human Hsc70 and sensor-bound DNAJB4. With an increasing Hsp105 concentration, the binding signal declined to reach 85% at 1:1 Hsc70:Hsp105 ratio (Fig. S6C), indicating that the negative Hsp110 impact on the Hsp70-JDP interaction exhibits a similar pattern across the Fungi/Metazoa group.

Since Sse1 inhibits Ssa1 binding to Sis1, we asked whether the disaggregation rate limitation imposed by Sse1 depends on the level of Sis1. To verify this, we incubated GFP aggregates with the Hsp70 system comprising various Sse1 and Sis1 concentrations (Fig. 4B, S6D). The susceptibility of the Hsp70 system to elevated Sse1 concentration significantly decreased with increasing Sis1 concentration and at 2 μM Sis1, the stimulation of the initial disaggregation rate was still observed at 2 μM Sse1 (Fig. S6D). Interestingly, in the absence of Sse1, the disaggregation rate dropped with increasing Sis1 concentrations and the trend was reversed at higher Sse1 levels, with an inflection point at approximately 0.5 μM of Sse1 (Fig. S6E).

Thus, Sse1 and Sis1 exhibit an apparent competition for Ssa1 binding. These results indicate that the balance between the JDPs and NEF co-chaperones is critical for the performance of the Hsp70 chaperone system in protein disaggregation.

## Discussion

Our results uncover a comprehensive picture of the role of Hsp110 co-chaperones in aggregate processing by the Hsp70 system. Potentiation of the Hsp70 activity by the NEF strongly depends on the class of a J-domain protein and the preference for class B, conserved in yeast and human orthologs, relies on Hsp70 binding through the EEVD motif. Furthermore, we demonstrate that Hsp110-dependent stimulation of disaggregation is limited to its initial stages: chaperone recruitment to aggregates and their disassembly, but not final protein folding (Fig. 5). The initial Hsp110-dependent loading of more Hsp70 onto aggregates correlates with their remodelling into smaller aggregated species, which improves their recognition by the Hsp104 disaggregase. We also gained insight into the biphasic impact of Hsp110: with increasing Sse1 level, the stimulation is overshadowed by inhibition and the contribution of each trend depends on the phase of disaggregation and the composition of the disaggregating system, with a crucial role of the NEF's

affinity for Hsp70. Finally, we show that the disaggregation inhibition by Hsp110 involves disruption of the Hsp70-JDP interaction, suggesting competition between JDP and Hsp110 co-chaperones.

The balance between the partners within the Hsp70 system is key for efficient disaggregation, as an individual co-chaperone with a critical function at one step may inhibit another. To investigate this differential contribution, we used biochemical assays reflecting individual stages of protein disaggregation. The results of the BLI experiments show that Hsp110 with class B JDP ($JDP^B$) greatly increases Hsp70 aggregate binding (Fig. 1B,C, S1E,F, S2A), which correlates with the final reactivation yield (Fig. 1A, S1G), implying that the initiation phase determines the overall disaggregation efficiency. On the other hand, the final folding of a soluble substrate by Ssa1 relies much more on the class A JDP ($JDP^A$) Ydj1 than on Sis1 (Fig. S1D) (Lu & Cyr, 1998), and not on Sse1 altogether (Fig. S1D), which is in line with previous reports that its human ortholog, Hsp105 inhibits luciferase folding (Rauch & Gestwicki, 2014).

The higher degree of stimulation with class B JDP has been previously observed, albeit without broader insight, for Hsp110 and other NEF BAG (Rampelt *et al.*, 2012; Rauch & Gestwicki, 2014). We show that the distinguishing feature fundamental for this specificity is the auxiliary interaction between the CTD domain of Sis1 and the EEVD motif of Hsp70. The Sis1 E50A variant, featuring derepressed J-domain (Yu *et al.*, 2015), together with Ssa1 ΔEEVD exhibited a similar aggregate binding pattern with or without Sse1 to the system with Ydj1 (Fig. 1B, S1E,F,I). However, while Ydj1 can bind misfolded proteins and prevent aggregation, Sis1 cannot (Lu & Cyr, 1998). When Sis1 is deprived of Ssa1 binding through EEVD, which has a major contribution to the stability of their interaction (Wyszkowski *et al.*, 2021), the effect of Sse1 on disaggregation is detrimental (Fig. S1H). This underlines the importance of the Hsp70-JDP complex stability to enable the stimulation of substrate binding and disaggregation by Sse1.

In our previous work we reported sigmoidal aggregate binding kinetics characteristic for the Ssa1-Sis1 system, suggesting that the binding gradually generates more chaperone binding sites (Wyszkowski *et al.*, 2021). Now, we show that this apparent cooperativity is conserved in yeast and human, implying mechanistic similarities between the systems (Fig. 1B,C). While the s-shaped Ssa1-Sis1 binding to the aggregate results in the association of more Ssa1 than with Ydj1 (Fig. S2A), Hsp110 shortens the initial lag phase and boosts the binding efficacy, loading a thicker layer of Hsp70 molecules onto aggregates (Fig. 1B,C, S2A). This corresponds with Hsc70 clustering on amyloid in the presence of Hsp110, reported by Wentink and co-workers (2020). Clustered, densely packed Hsp70 has been proposed to generate entropic pulling effect that leads to fibril fragmentation (Goloubinoff & De Los Rios, 2007; Sousa & Lafer, 2019; Wentink *et al.*, 2020). Likewise, we observed remodelling of amorphous aggregates into smaller aggregated species, which resembles amyloid disassembly in terms of the dependence on the NEF activity of Hsp110, the class B JDP and its interaction with the Hsp70 EEVD motif (Fig. 2B, S4) (Beton *et al*, 2022). We propose that the local extraction of polypeptides by Hsp70-$JDP^B$-Hsp110, which penetrates the surface and takes apart amorphous aggregates, although not enough for full protein recovery, could successfully

generate more manageable substrates for chaperones, as we demonstrated by applying the derepressed Hsp104 variant (Fig. 2A, 5). This Hsp70 activity, albeit yielding low protein reactivation, might be relevant for the fragmentation of a thinner, linear amyloid structure. Such clustering-augmented remodelling could also explain the importance of Hsp110 in aggregate clearance in the cell (Kaimal *et al.*, 2017).

Above a certain Hsp110 level, disaggregation is inhibited. The susceptibility depends on the phase of the process and the composition of the Hsp70 system (Fig. 3A,B,C,D, S5A), which may explain discrepancies among different studies of Hsp110 impact on disaggregation (Dragovic *et al.*, 2006; Polier *et al.*, 2008; Raviol *et al.*, 2006; Shorter, 2011) As well-established, the most effective is substoichiometric Hsp110 proportion to Hsp70 (Gao *et al*, 2015; Polier *et al.*, 2008; Wentink *et al.*, 2020) which corresponds with the physiological conditions, where the ratio between all cytoplasmic Hsp70 and Hsp110 paralogs involved in disaggregation ranges from approximately 10 : 1.7 in yeast (Brownridge *et al*, 2013) to 10 : 2.4 in humans (Finka & Goloubinoff, 2013). According to our data, the optimum, at which the stimulation and inhibition curves intersect, is shifted to higher levels: a) during initiation versus final protein folding (Fig. 1A, S1D), (b) with class B JDP, in comparison with class A (Fig. 1A), c) with Hsp104, comparing with the system without the disaggregase (Fig. 3A, S5A) and d) for the human Hsp70 system, comparing with the yeast chaperones (Fig. 3A, S1G). It is tempting to speculate that the human system, lacking Hsp104, has evolved towards better tolerance for Hsp110 to boost the disaggregation activity of the Hsp70 system alone, e.g. to counteract amyloid toxicity.

Excessive dissociation of Hsp70 from a substrate, promoted by nucleotide exchange, may explain the system's susceptibility to Hsp110. The fact that NEF and JDP binding occurs at mutually exclusive nucleotide states of Hsp70 has raised a question of competition between the two co-chaperones (Sousa & Lafer, 2019), which, to our knowledge, has never been addressed before. Our results clearly demonstrate that Hsp110 disrupts the class B JDP complex with Hsp70 (Fig. 4A, S6C), providing an additional mechanism behind the inhibition. Class B JDP goes beyond the classical model of the Hsp70 cycle due to the auxiliary interaction with the EEVD motif (Wyszkowski *et al.*, 2021; Yu *et al.*, 2015), which theoretically might not be exclusive with Hsp110 binding. Nonetheless, the $JDP^B$-Hsp70 interaction is highly sensitive to Hsp110, analogously as aggregate binding and disaggregation (Fig. 3D, 4A,B).

The apparent competition between the co-chaperones is reflected in the fact that the inhibition by Hsp110 is moderated at increasing Sis1 level (Fig. 4C). Interestingly, the class B JDP also exerts a biphasic effect on disaggregation, and optimal concentrations of the two co-chaperones are strongly interdependent (Fig. 4B,C, S6D,E). The highest disaggregation yield occurs at Ssa1 : Sis1 : Sse1 ratio closest to the average reported in the cytosol, 10 : 0.3 : 1.7 (Fig. 4C) (Brownridge *et al.*, 2013), pointing to a possibility that the proportions between the co-chaperones have undergone evolutionary fine-tuning to develop higher tolerance to stress.

The sub-stoichiometric optimum is unique to Hsp110 (Gao *et al.*, 2015; Wentink *et al.*, 2020), while other NEF, Fes1, requires 10 times higher level to reach the same degree of stimulation, similarly as the Sse1-2 variant with reduced affinity for Hsp70. Fes1 ability to improve Hsp70 loading onto aggregate is surprising, regarding findings of Wentink et al., who demonstrated, using Hsp110 and BAG1 variants, that Hsp70 clustering on amyloid requires a bulky NEF. Although Fes1 is only 33 kDa, the stretch of its armadillo repeats and different mode of interaction with NBD could possibly lead to similar excluded volume effects as Hsp110. Nevertheless, Fes1 is present in a cell at approximately one-fifth the level of Sse1, and even its 8.4-fold overexpression from 2 plasmids, shown by Kaimal et al. as insufficient to complement the thermosensitive phenotype of *sse1-200 sse2*Δ (Kaimal *et al.*, 2017), was 6 times too low to reach the level effective in disaggregation *in vitro* (Fig. 3SA). It is worth noting that Fes1 has been reported to target substrates to degradation (Gowda *et al.*, 2018; Gowda *et al*, 2013) and apparently its role in Hsp70 recruitment to disaggregation is minor.

An abundant association of Hsp70-JDP$^{B}$ with aggregate surface, although generating strong pulling effect to disentangle polypeptides, could also mask access of chaperones to the newly emerging sites adjacent to and buried beneath the complex. We demonstrate that Hsp110 could theoretically uncover such sites not only through Hsp70 dissociation from the substrate, but also by disrupting the JDP$^{B}$-Hsp70 interaction (Fig. 5). Four Hsp70 binding sites in a JDP$^{B}$ dimer allow to form an extensive network of interactions (Wyszkowski *et al.*, 2021). We speculate that Hsp110 introduces plasticity into this network, enabling the chaperone complex to infiltrate cavities emerging within the aggregate in a fluid-like manner, binding to the uncovered chaperone-binding sites and pulling up to the aggregate fragmentation (Fig. 5). Such behaviour would require only a small destabilising effect of the NEF that would not dissociate the complex completely (Fig. 4A), similarly as observed at the Hsp110 concentration that supports the most effective disaggregation (Fig. 1A). Further experimental verification of this scenario and future studies of the dynamics within the Hsp70 chaperone system will be critical to understand and combat stress- and disease- related protein aggregation.

## Materials and methods

### Proteins

Sse1 (Andreasson *et al.*, 2008a), Ssa1 (Andreasson *et al.*, 2008a), Sis1 (Wyszkowski *et al.*, 2021), Ydj1 (Lipinska *et al*, 2013), Hsp104 (Lipinska *et al.*, 2013) His-tagged luciferase (Chamera *et al.*, 2019) and GFP (Zietkiewicz *et al.*, 2004) were purified using published protocols. The same protocol as for Sse1 was used for Fes1 (Andreasson *et al.*, 2008a). Sse1 N572Y N575A was constructed by introduction of point

mutations using PCR-specific mutagenesis (Qiagen) and confirmed with sequencing. Untagged luciferase was purchased from Promega (E1702).

Heat-aggregated luciferase refolding

Reactivation of luciferase from aggregates was performed as previously described (Wyszkowski *et al.*, 2021). Chaperones were added at the following concentrations: 1 μM Ssa1, 1 μM Sis1, 1 μM Ydj1, 0.1 μM Sse1, 1 μM Fes1, 3 μM Hsc70, 1 μM DNAJB4, 1 μM DNAJA2 and 0.1 μM Hsp105, if not stated otherwise.

Heat-aggregated GFP refolding

GFP was subjected to disaggregation as previously described (Wyszkowski *et al.*, 2021). Fluorescence was measured using Beckman Coulter DTX 880 Plate Reader. IC50 was calculated by fitting the *[Inhibitor] versus response* model to the data from three experiments using the *GraphPrism* Software.

BLI

Binding experiments were performed as previously described using the BLItz and Octet K2 instruments (Wyszkowski *et al.*, 2021). To prepare the sensor with GFP aggregates, after the initial hydration in buffer A for 10 min, it was immersed in the buffer A with 9 M urea, containing 12.5 μM GFP and incubated at 85 °C for 15 min. After washing with buffer A for 5 min, it was immersed in the buffer A containing 4.2 μM GFP and incubated at 85 °C. Next, the sensor was washed for 5 min. The aggregate layer thickness was ~40 nm.

BLI with fluorescently labelled protein

Ssa1 was incubated with 10x molar excess of Alexa Fluor 488 C5-maleimide (Invitrogen) for 2 h at 4 °C. An excess of the label was removed with a desalting column (PD-10, GE Healthcare). The BLI experiment was performed as with unlabelled proteins and the fluorescence of the protein dissociated into the buffer A with 5 mM ATP 1 mM DTT was measured using Beckman Coulter DTX 880.

BLI of direct protein-protein interactions

After initial hydration of the BLI sensor with the buffer A, the biosensor was immersed into buffer A containing 0.4 μM His-Sis1 until saturation. Subsequently, it was washed with buffer A containing 5 mM ATP and 1 mM DTT for 5 min and then immersed into the same buffer with the mix of chaperones at the indicated concentrations.

Fluorescent transmitted light microscopy

*Fluc-EGFP* (parent vector pCIneo -Fluc-EGFP) was cloned into pET22b plasmid using restriction enzymes *EcoRI* and *NdeI.* Protein was purified using Ni-NTA agarose (Protino), followed by anion exchange chromatography using Q-Sepharose (Q Sepharose Fast Flow, GE Healthcare). Luciferase-GFP (1,3mg/mL) was incubated in the buffer A with 6-M Urea at 25 °C for 15 min. Then, it was transferred to 48 °C for 10 min and 10x diluted with the buffer A containing 5 mM ATP and 1 mM DTT. After 15 min of incubation at 25°C, the reaction was initiated upon addition of the mix of chaperones at 1 μM concentration, except for Sse1 used at 0.1 μM. The final concentration of luciferase-GFP aggregates in the reaction was 0.3 μM. After 1 h of incubation with the chaperones, the reaction was arrested upon addition of 200 mM NaCl and transferred on ice. Specimens were imaged using a confocal laser scanning microscope Leica SP8X with a 100× oil immersion lens (Leica, Germany). Presented data show results from three independent experiments. Each sample within the repeat was photographed ten times. Data analysis was performed with Leica LAS X software.

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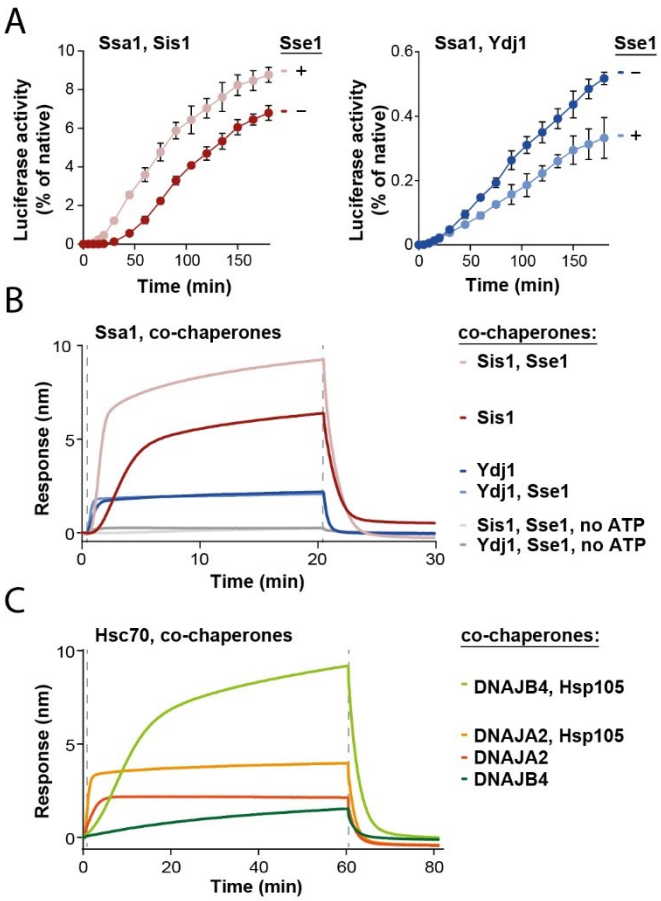


**Fig. 1. Impact of Hsp110 on protein disaggregation by the Hsp70 system depends on class of JDP.** (A) Refolding of aggregated luciferase by Ssa1-Sis1 +/- 0.1 μM Sse1 (left) or Ssa1-Ydj1 +/- 0.1 μM Sse1 (right). Error bars show SD from three independent repeats. Luciferase activity was measured at indicated time points and normalised to the native activity. (B) Sensor-bound luciferase aggregates incubated with Ssa1-Sis1 +/- 0.1 μM Sse1 or Ssa1-Ydj1 +/- 0.1 μM Sse1, with or without ATP. (C) Binding of Hsc70-DNAJB4 or Hsc70-DNAJA2 with or without Hsp105 to the heat-aggregated luciferase immobilised on the BLI biosensor. (B)-(C) dashed lines indicate the start of chaperones binding and dissociation steps.

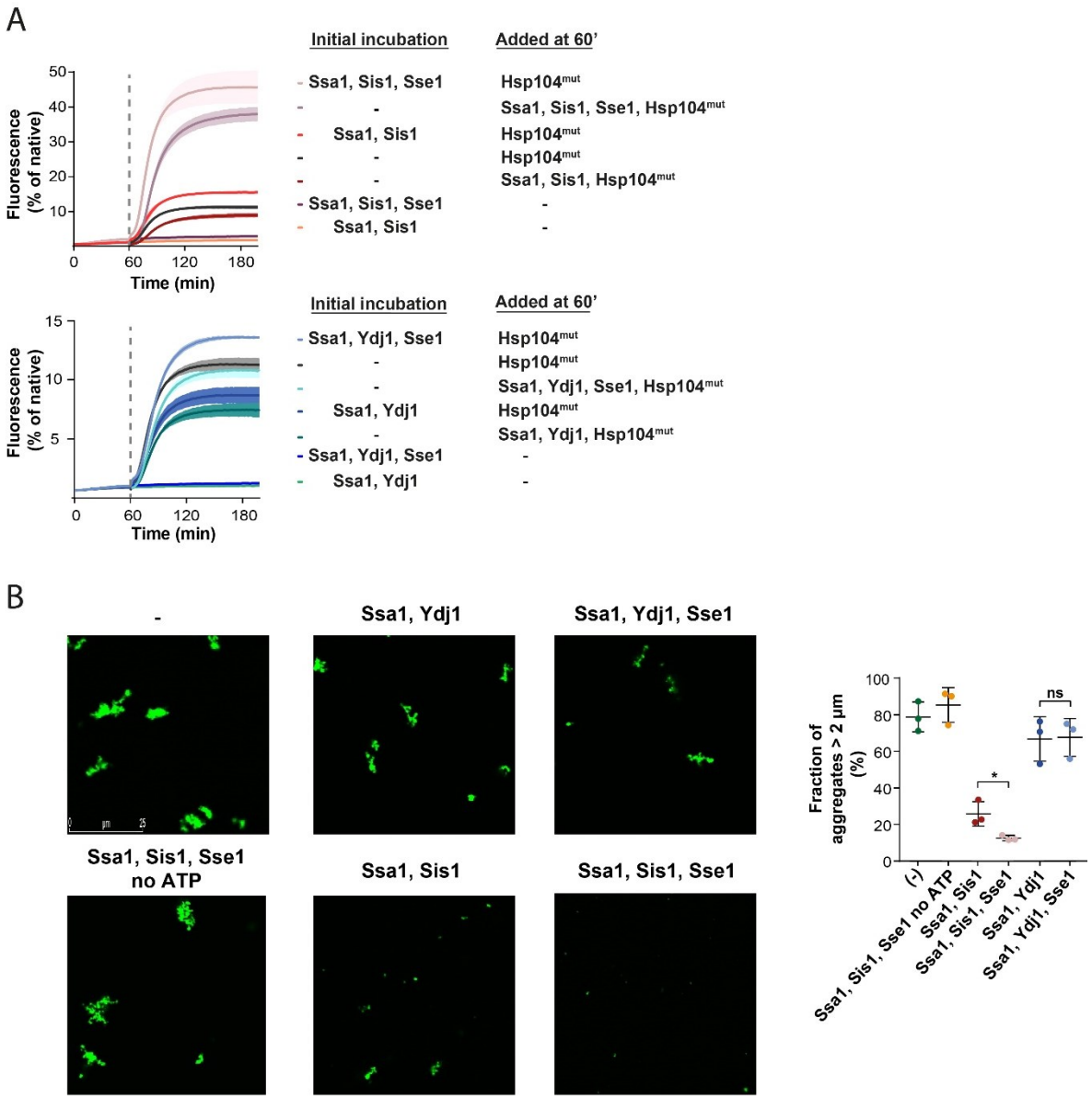


**Fig. 2. Sse1 promotes modification of aggregates by the Hsp70 system.** (A) Initial incubation of heat-aggregated GFP aggregates with yeast Hsp70 system, followed by the addition of the Hsp104 D484K F508A variant. Dashed lines indicate the beginning of the incubation with the Hsp104 variant. (B) Fluorescence microscopy images of FLUC-EGFP monitored upon addition of Ssa1-Sis1 +/- 0.1 µM Sse1 or Ssa1-Ydj1 +/- 0.1 µM Sse1. Left panels show controls of the luciferase-GFP aggregates alone and upon the addition of the Hsp70 system without ATP. Quantification of the fraction of aggregates > 2 µm is from three independent replicates. Two-tailed t test was performed: *$p < 0.05$, ns: not significant.

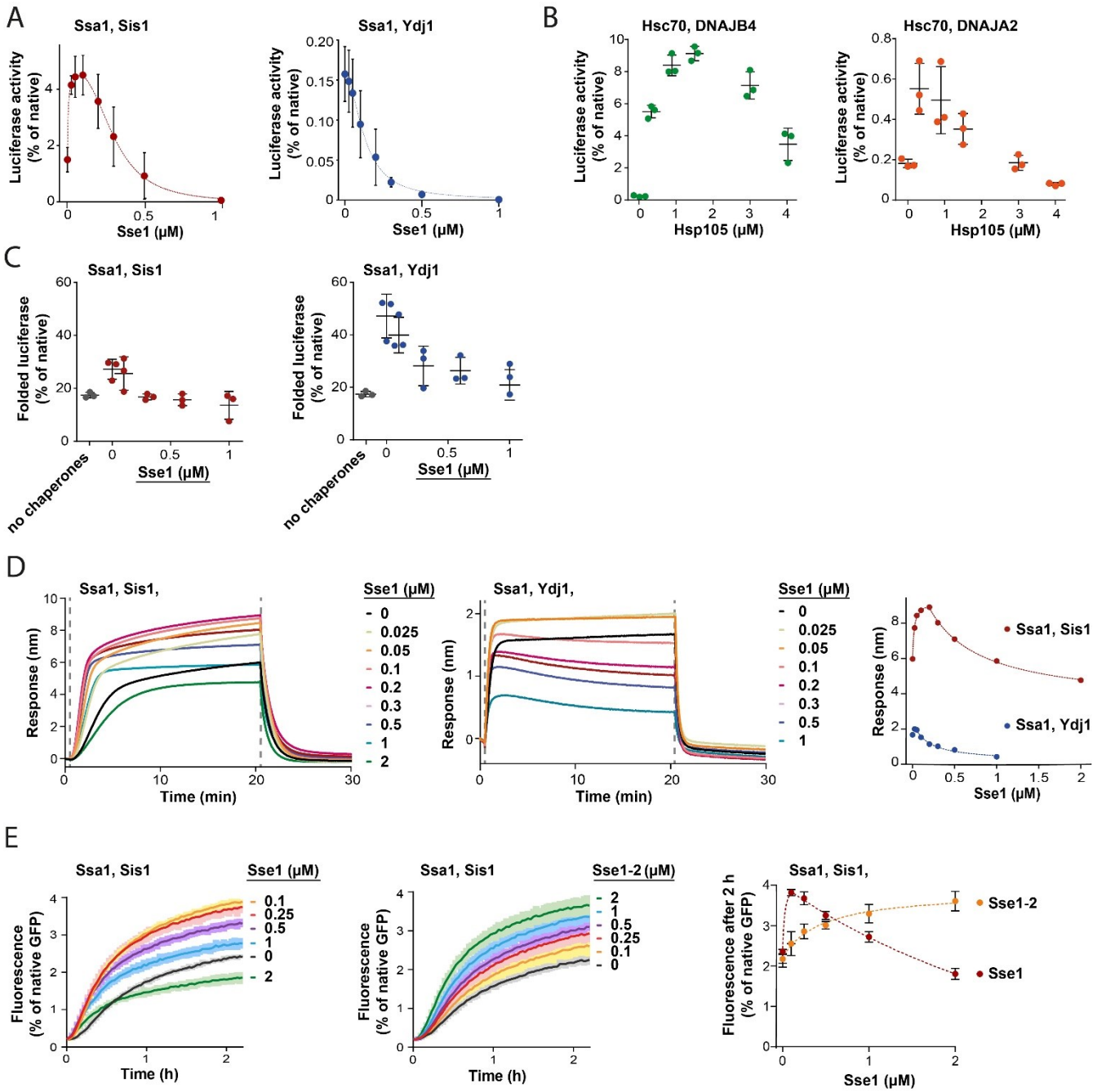


**Fig. 3. Susceptibility of Hsp70 to Hsp110 depends on JDP class and phase of disaggregation.** (A) Sse1 titration in the refolding of aggregated luciferase by Ssa1-Sis1 (red) or Ssa1-Ydj1 (blue). Activity of luciferase was measured after 1 h and normalised to the native protein. (B) Incubation of Hsc70-DNAJB4 (green) or Hsc70-DNAJA2 (orange) with increasing concentrations of Hsp105 and luciferase aggregates. Luciferase activity was measured after 4 h and normalised to the activity of native protein. (C) Spontaneous folding of non-aggregated luciferase diluted from 5 M GuHCl (grey) alone or with addition of the Hsp70 system comprising Ssa1-Sis1 (red) or Ydj1-Ssa1 (blue) at increasing concentrations of Sse1. Activity of luciferase was measured after 2 h and normalised to the native protein. (D) Binding of Ssa1-Sis1 or Ssa1-Ydj1 in the presence of Sse1 at the indicated concentrations to the sensor covered with luciferase aggregates. Right panel shows a plot of the binding signal prior to the dissociation step of Ssa1-Sis1 (red) or Ssa1-Ydj1 (blue) with concentrations of Sse1. (E) Renaturation of heat-aggregated GFP by Ssa1-Sis1 in the presence of Sse1 or Sse1-2 at the indicated concentrations. Right panel shows the plot of GFP activity after 2 h of incubation in the presence of Sse1 (orange) or Sse1-2 (red). Dashed lines show the

fitting of the *[Agonist] vs response* model to the data from the stimulation and inhibition phases separately using the *GraphPrism* Software.

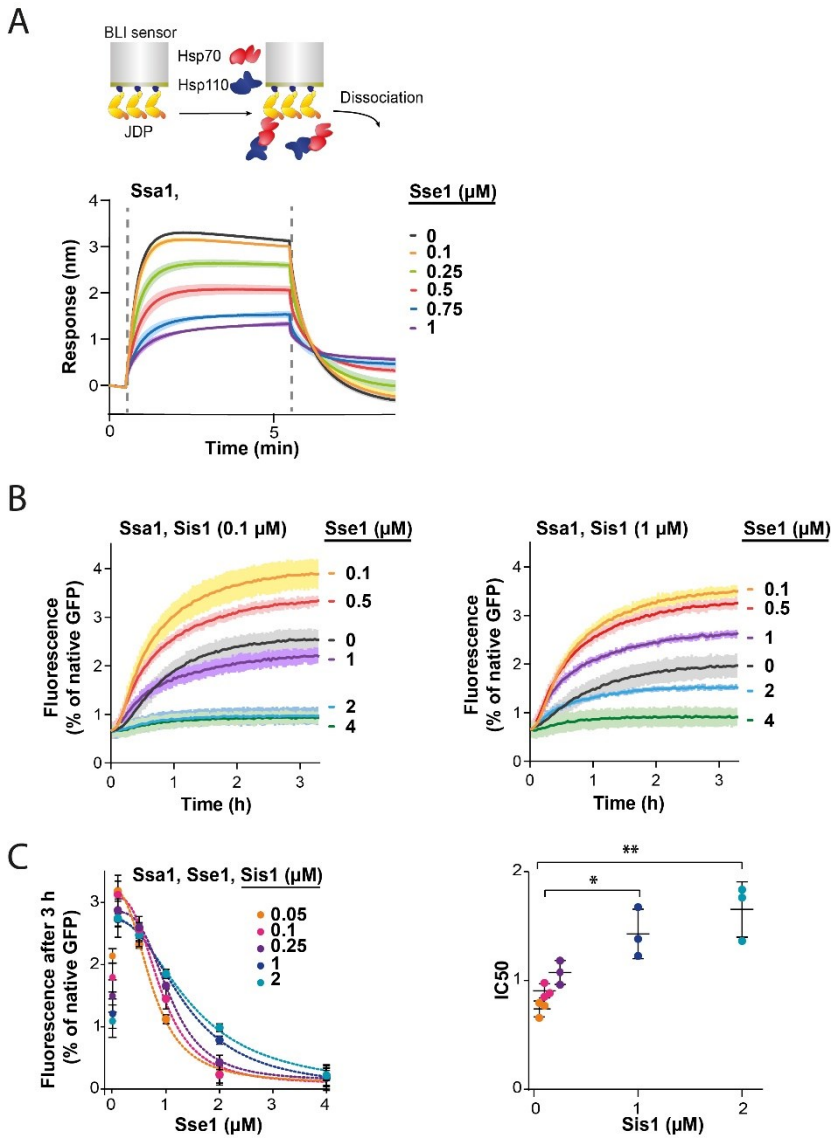


**Fig. 4. Hsp110 and class B JDP show apparent competition for Hsp70.** (A) Upper panel shows the scheme of the BLI experiment. Binding of Ssa1 in the presence of increasing concentrations of Sse1 to Sis1 immobilised on the BLI sensor through the $His_6$–SUMO tag. Lines are the average of three replicates and the shades designate standard deviation. Dashed lines indicate addition of chaperones to sensor-bound Sis1 and dissociation step. (B) Titration of Sse1 in renaturation of heat-aggregated GFP by Ssa1-Sis1, where Sis1 was used at 0.1 μM (left) or 1 μM (right) concentration. (C) Plot of GFP activity after 3h processed by Ssa1 and simultaneously increasing concentration of Sis1 and Sse1 (left). IC50 of Sse1 was determined by fitting the [*Inhibitor] versus response* model to the data from three experiments using the *GraphPrism* Software (dashed lines). Two-tailed t test: *$p < 0.05$, **$p < 0.01$.

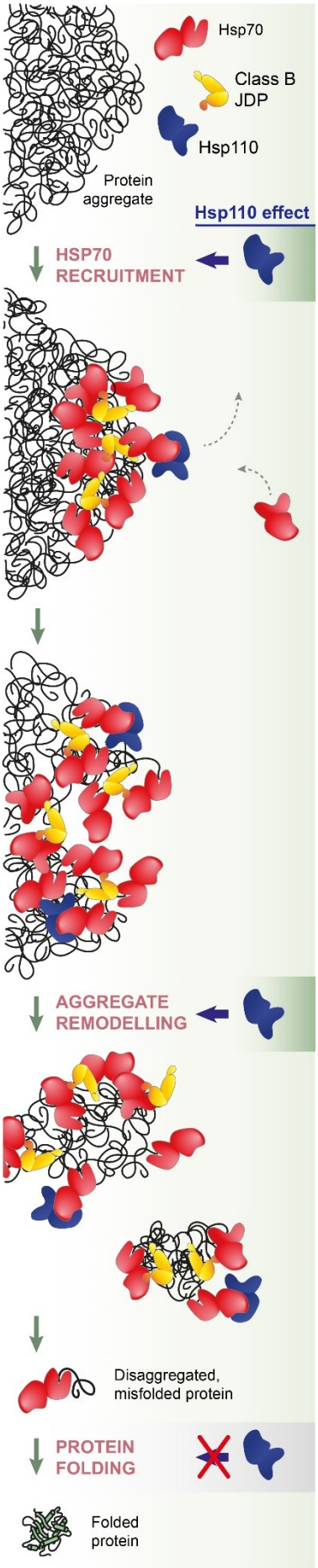
Hsp70
Class B
JDP
Hsp110
Protein
aggregate
Hsp110 effect
HSP70
RECRUITMENT
AGGREGATE
REMODELLING
Disaggregated,
misfolded protein
PROTEIN
FOLDING
Folded
protein

**Fig. 5. Hsp110 impact on Hsp70-dependent disaggregation.** Hsp110 increases Hsp70 binding to the aggregate surface specifically in the presence of class B JDP and improves aggregate remodelling into smaller assemblies but does not aid final protein folding. Hsp110 role at the two former stages significantly stimulates protein disaggregation (dark green shades). By slightly facilitating Hsp70 dissociation from the substrate and JDP, the sub-stoichiometric level of Hsp110 might gradually uncover new Hsp70-binding sites, e. g. buried under the chaperone complex, potentially leading to more abundant and effective Hsp70 recruitment (dashed grey arrows).