Chapter 2

Functional diversity between Hsp70 paralogs due to preferential interaction with co-chaperones.

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Abstract

Hsp70 chaperones play a central role in maintaining protein homeostasis by actively participating in a wide variety of processes including folding, anti- and dis-aggregation and degradation. Human Hsp70s comprise a family of thirteen members and act with the aid of even larger families of co-chaperones. A delicate interplay between Hsp70s and co-chaperone recruitment is thought to determine substrate fate. Yet, it has been generally assumed that all paralogs of Hsp70 have similar activity and are largely functionally interchangeable. However, we found that when expressed in cells, two highly homologous Hsp70s, HSPA1A and HSPA1L, are not functionally similar and can even have opposing effects on cellular handling of various substrates. Intriguingly, neither variations in substrate binding nor in substrate delivery by DNAJ co-chaperones play a role in this remarkable difference between these Hsp70s. Instead, the different functionality and hence substrate fate is determined by the preferential interaction of HSPA1A (and not HSPA1L), via its nucleotide binding domain, with HSPH2, an Hsp110 Nucleotide Exchange Factor, that regulates ADP/ATP exchange of Hsp70 and substrate release.
Introduction

The Hsp70 machinery is a central system of the protein quality control and it is involved in many different processes including protein folding, degradation, aggregation prevention and disaggregation (Mayer and Bukau, 2005; Kim et al., 2013; Mogk et al., 2018). This system consists of at least one member of each of three different families of chaperones: the Hsp70 (HSPA) family with ATPase activity and their co-chaperone families of DNAJs (Hsp40s) and Nucleotide Exchange Factors (NEFs) (Kampinga and Craig, 2010). Hsp70 chaperones, which are amongst the most highly conserved proteins in evolution, in humans comprise a family of 13 members (Radons, 2016). They are involved in a variety of molecular functions (Clerico et al., 2015; Mayer and Bukau, 2005) and have been reported to interact with a wide range of substrates, from non-native to native substrates, by recognizing exposed hydrophobic motifs found in most proteins (Rüdiger et al., 1997).

Hsp70 proteins consist of an N-terminal nucleotide binding domain (NBD) and a substrate interacting region, which includes a substrate binding domain (SBD) and a C-terminal domain (CTD) forming a lid that stabilizes bound substrates (Mayer and Bukau, 2005). The Hsp70 activity is based on an ATP-dependent cycle, alternating between the low-substrate-affinity ATP-bound state and the high-substrate-affinity ADP-bound state. However, intrinsic ATPase activity of Hsp70 proteins is too low for them to function independently; that is why the cycle turnover is aided by the co-chaperones DNAJs and NEFs, which stimulate ATP hydrolysis and catalyse ADP/ATP exchange respectively (Mayer, 2013). In addition, the DNAJ co-chaperones (53 members in humans) are thought to act as recruiters of substrates via interaction with their versatile substrate binding domains (Kampinga and Craig, 2010). Upon interaction with the Hsp70s, via their conserved J-domain, DNAJs together with the substrates stimulate Hsp70-ATPase activity and substrates are transferred to Hsp70s (Kityk et al., 2018). To promote substrate release, four different types of co-chaperones can stimulate nucleotide exchange in human Hsp70s: BAG (6 members), Hsp110/Grp170 (HSPH - 4 members), HspBP1/Sil1 (2 members) and GrpE (2 members) (Bracher and Verghe, 2015). Despite being structurally different and using different mechanisms, all four types of NEFs interact with the Hsp70-NBD, at different but partially overlapping sites, and stimulate nucleotide exchange of the Hsp70 ATPase.

Due to their high conservation both in evolution and within the Hsp70 family, (human) Hsp70 proteins have been used in literature as interchangeable (Warrick et al., 1999; Fernandez-Funez et al., 2016; Shukla et al., 2014; Auluck et al., 2002, 2005; Chan et al., 2000; Wong et al., 2008; McLear et al., 2008). However, specificity between Hsp70-machines
exists and different effects of the various Hsp70s have been reported (Clerico et al., 2015; Kampinga and Craig, 2010; Kakkar et al., 2014; Kampinga and Bergink, 2016). Since the recognition of substrates by Hsp70 is quite generic and since there is a lot more variability in DNAJs and NEFs, the last two families have been suggested as the ones that confer specificity to the Hsp70 system (Kampinga and Craig, 2010). However, it has not been experimentally explored to what extent (human) Hsp70 are interchangeable. Moreover, it is unclear whether different Hsp70s interact with specific co-chaperone partners, and, if so, what determines the functional outcome of these different Hsp70 complexes.

Various members of the Hsp70 machinery have been identified as suppressors of protein aggregation (Kampinga and Bergink, 2016; Kakkar et al., 2014). From the Hsp70 family, only few members (mainly HSPA1A and HSPA8) have been tested but mostly not in a comparative way. In particular, HSPA1A upregulation has been reported as highly effective in withstanding global protein aggregation induced by unfolding events such as heat shock (Stege et al., 1994) or aggregation of specific thermosensitive proteins such as luciferase (Nollen et al., 1999). However, neither HSPA1A nor HSPA8 is very effective in preventing aggregation of disease-associated amyloidogenic proteins, although results may vary depending on the system or the type of the substrate (Kampinga and Bergink, 2016; Kakkar et al., 2014). At least one exception to this is the aggregation of superoxide dismutase 1 (SOD1) mutants that cause amyotrophic lateral sclerosis (ALS) (Chattopadhyay and Valentine, 2009; Prudencio et al., 2009; Ray et al., 2004). Elevated expression of Hsp70s (HSPA1A) has been shown to suppress mutant SOD1 aggregation and even alleviated disease in murine models (Bruening et al., 1999; Koyama et al., 2006; Takeuchi et al., 2002).

Previously, we noticed that different human Hsp70 family members can have variable effects on protein aggregation of various substrates, including heat-denatured luciferase, polyglutamine proteins and mutant Parkin (Hageman et al., 2011; Kakkar et al., 2016a; Hageman et al., 2010; Rujano et al., 2007). Here, using two reported Hsp70 clients, mutant SOD1 and heat-denatured luciferase, we dissect these different effects of Hsp70s on protein aggregation. We found that two highly homologous Hsp70s, HSPA1A and HSPA1L, have opposing effects on mutant SOD1 aggregation. Strikingly, this differential activity is explicitly attributed to differences in the NBDs of the two Hsp70s, which subsequently affect their ability to functionally interact with HSPH2, an Hsp110 type of NEF, and determine substrate fate. These data suggest another layer of functional diversification within the Hsp70 machines in human cells, which is directed by Hsp70-NEF interactions. Thus, we clearly demonstrate that Hsp70s are not interchangeable but show specificity, which is not related to the substrate interaction but to their functional ATPase cycle–related complex formation with different co-chaperones.
Results and Discussion

Diverse effects of various Hsp70s on mutant SOD1 aggregation

The different cytosolic/nuclear Hsp70 members HSPA1A, HSPA1L, HSPA2, HSPA6 and HSPA8 show high sequence conservation (Fig S1) and bind similar peptide motifs (Rüdiger et al., 1997; Fourie et al., 1994; Clerico et al., 2015) and hence have often been considered as functionally interchangeable. However, despite the relatively small effects of elevated cellular Hsp70 levels on various aggregation-prone substrates (Kampinga and Bergink, 2016; Kakkar et al., 2014), we noticed that the outcome in terms of client handling could differ significantly (Hageman et al., 2011; Kakkar et al., 2016a; Hageman et al., 2010; Rujano et al., 2007). To study these differences in more detail, we used mutant SOD1, a reported Hsp70 client (Bruening et al., 1999; Koyama et al., 2006; Takeuchi et al., 2002) as a model substrate. First, we developed a quantifiable fractionation method (Fig 1A) to monitor aggregation of a mCherry-tagged mutant SOD1. We used a well-known ALS-associated, aggregating mutant with the A to V substitution at position 4 (hereafter referred as SOD1\textsuperscript{A4V}) (Chattopadhyay and Valentine, 2009; Prudencio et al., 2009; Ray et al., 2004). mCherry-SOD1\textsuperscript{A4V} formed visible inclusions in cells and was partially detergent insoluble after fractionation in contrast to mCherry-SOD1\textsuperscript{WT} that showed diffuse expression and remained in the soluble fraction (Fig 1B and C). Interestingly, expression of most Hsp70s in HEK293 cells enhanced rather than reduced SOD1\textsuperscript{A4V} aggregation and only HSPA1A, showed a significant aggregation suppressing effect (Fig 1D). Largely similar results were obtained in U2OS cells, with most Hsp70 members having either no effect or enhancing SOD1\textsuperscript{A4V} aggregation and only HSPA1A leading to a significant reduction in SOD1\textsuperscript{A4V} aggregation (Fig 1E).

Differential effect on aggregation is associated with the nucleotide binding domain of Hsp70

The most striking observation was the consistent opposing effects of two of the closest paralogs HSPA1A and HSPA1L, with the former significantly reducing and the latter greatly enhancing SOD1\textsuperscript{A4V} aggregation in both HEK293 and U2OS cells (Fig 1D and E). This differential behaviour is not limited to SOD1\textsuperscript{A4V} aggregation as we previously reported that HSPA1L enhanced the aggregation of Parkin\textsuperscript{C289G} (substitution of C289 to G, a mutant associated with familial Parkinson’s disease) while HSPA1A had little effect on it (Kakkar et al., 2016a). Moreover, elevated expression of HSPA1A greatly suppressed aggregation and stimulated the refolding of heat-denatured luciferase while HSPA1L was much less efficient (Hageman et al., 2011).

HSPA1L and HSPA1A are 89% identical in their amino acid sequence and most differences
Chapter 2

A

Cell lysates in 1% NP40 (T) 20,000g

Soluble supernatant (S)  Insoluble pellet (P)

B

SOD1

α tubulin

C

mCherry-SOD1

Hoechst

Merge

mCherry-SOD1

WT

AAV

D

mCherry-SOD1

SOD1 ratio to mRFP

E

mCherry-SOD1

SOD1 ratio to mRFP

α tubulin
lie in the substrate-locking C-terminal lid domain (Fig S1). Although HSPA1A is one of the most studied human Hsp70s, not much is known about HSPA1L and its cellular functions (Radons, 2016; Daugaard et al., 2007). In contrast to HSPA1A, HSPA1L is not stress inducible, as it lacks a heat shock element in its promoter (Milner and Duncan Campbell, 1990), with very low expression in most tissues (Hageman and Kampinga, 2009; Daugaard et al., 2007). To further investigate why two very similar Hsp70s show such an opposing effect on SOD1<sup>A4V</sup> aggregation, we first generated chimeras to identify which part of the protein was responsible for this difference. Both HSPA1A and HSPA1L have three structurally distinct domains (Figs 2A and S1): the NBD (or N), the SBD (or S) and the CTD (or C) (Mayer and Bukau, 2005; Daugaard et al., 2007). Exchanging the NBD of HSPA1A with that of HSPA1L (N<sub>L</sub>S<sub>A</sub>C<sub>A</sub>) generated a protein with HSPA1L-like activity that enhanced SOD1<sup>A4V</sup> aggregation (Fig 2B). Inversely, the chimera with the NBD of HSPA1A and the SBD and CTD of HSPA1L (N<sub>A</sub>S<sub>L</sub>C<sub>L</sub>) gained an HSPA1A-like activity in suppressing SOD1<sup>A4V</sup> aggregation (Fig 2B). This pointed towards the NBD as being responsible for the opposing effect of HSPA1A and HSPA1L on SOD1<sup>A4V</sup> aggregation. Exchanging the individual SBDs or CTDs generated chimeric proteins whose activity fully depended on their NBDs further confirmed this (Fig S2A). To test if this behaviour extended to other substrates as well, we tested the chimeras in their capacity to reactivate denatured luciferase after heat shock. Similar to their behaviour against SOD1<sup>A4V</sup>, the reactivation activity segregated with the two NBDs (Fig S2B). Together these results indicate that neither the SBD nor the CTD play a role in the differential effect of these two Hsp70s on protein aggregation. Interestingly, the SBD and especially the CTD, are the most disparate domains based on the amino acid sequence (Fig S1). Since the SBD confers substrate binding, this suggests that the difference in substrate fate cannot be attributed to differential SOD1<sup>A4V</sup> binding. Consistently, both HSPA1A and HSPA1L co-immunoprecipitated efficiently with mCherry-SOD1<sup>A4V</sup> (Fig 2C).
Functional diversity between Hsp70 paralogs

In agreement with our findings, the importance of the nucleotide binding domain as a driver for functional specificity between Hsp70s has been previously noted for yeast (James et al., 1997; Sharma and Masison, 2011) and human Hsp70s (Hageman et al., 2011). However, the reason for this importance of the NBD is unclear. The NBDs of HSPA1A and HSPA1L share 91% sequence identity (Fig S1). Structural alignment utilizing previously published data (Wisniewska et al., 2010) revealed that the NBDs of HSPA1A and HSPA1L are almost identical (Fig 2D), making such a different impact on a chaperone function really remarkable. Mapping the non-conserved residues between HSPA1A and HSPA1L on HSPA1A-NBD and HSPA1L-NBD antibody was used for IP and V5 for detection of the Hsp70s. (D) Structural alignment between the NBDs of HSPA1A and HSPA1L. Structural superposition of the ribbon models after sequence alignment of HSPA1A-NBD (PDB ID: 3JXU, (Wisniewska et al., 2010); yellow-green) and HSPA1L-NBD (PDB ID: 3GDQ, (Wisniewska et al., 2010); blue) with root mean square deviation (RMSD) = 0.438 Å. Subdomains IA/B and IIA/B are marked for orientation. (E) Effect of N1 (aa1-111) and N2 (aa112-389) regions of HSPA1A or N1 (aa1-113) and N2 (aa114-391) HSPA1L NBD on SOD1\textsuperscript{A4V} aggregation. Western blots of NP40 fractions from HEK293 cells co-expressing mCherry-SOD1\textsuperscript{A4V} and V5-tagged chimeric proteins with combinations of N1 and N2 regions of NBDs between HSPA1A (yellow-green) and HSPA1L (blue). Quantification graph represents total (T) or NP40-insoluble (P) fraction western blot intensities normalized to α-tubulin and relative to mRFP control (n=2-17). In (B) & (E), error bars with s.e.m., * p=0.01-0.05, ** p=0.001-0.01, *** p<0.001.
2E). Since the N2 region contains the interaction sites of DNAJs and most interaction sites for NEFs (Fig S1), we next aimed to test whether DNAJ or NEF interactions were involved in these differential activities of HSPA1A and HSPA1L.

**DNAJs deliver mutant SOD1 to both HSPA1A and HSPA1L**

DNAJ proteins, interact with Hsp70s through their conserved J-domain and stimulate Hsp70 ATPase activity, a step crucial for substrate transfer to the Hsp70s (Kampinga and Craig, 2010). The J-domain interacts mainly with the NBD of the Hsp70s, along with a part of the SBD and the flexible linker between the two domains (Kityk et al., 2018). The predicted contact sites on the NBD are conserved between HSPA1A and HSPA1L (Fig S1). According to the HSPA1A/HSPA1L nucleotide binding domain structure, there are a few residues in the vicinity of the J-domain contact region that are not conserved between HSPA1A and HSPA1L and that could possibly affect the Hsp70-DNAJ interaction. Substitution of various combinations of these residues of HSPA1A with the HSPA1L equivalent (mutants N3-6), led to a slight decrease in HSPA1A anti-aggregation activity, albeit not significant (Fig S3C). Those data suggested that there is probably no interference in HSPA1L-DNAJ interaction, at least for those DNAJs that are involved in SOD1\(^{A4V}\) handling. To fully exclude this possibility, we wanted to examine directly whether interaction of DNAJs was perturbed in the case of HSPA1L. We first sought to identify which DNAJs were involved in SOD1\(^{A4V}\) recruitment to the Hsp70s and would therefore be relevant to this activity. Overexpression in HEK293 cells of DNAJA (Fig 3A) or DNAJB (Fig 3B) subfamily members, which are promiscuous DNAJs for a variety of clients (Kampinga and Craig, 2010), had variable effects on SOD1\(^{A4V}\) aggregation and revealed DNAJB1, DNAJB2b and DNAJB7 as the strongest suppressors. Amongst the DNAJs that exhibited a suppressive effect on SOD1\(^{A4V}\), DNAJB1 is one of the best-characterized members for substrate delivery to the Hsp70s and stimulation of their ATPase activity (Kityk et al., 2018; Minami et al., 1996). Therefore, we focused on DNAJB1 for the subsequent studies.

To first confirm whether DNAJB1 suppresses SOD1\(^{A4V}\) aggregation via interaction with Hsp70s, we introduced a mutation in the HPD-motif of its J-domain (DNAJB1\(^{H32Q}\)), which is known to eliminate Hsp70 interaction and hence substrate delivery (Tsai and Douglas, 1996; Michels et al., 1999). Expressing DNAJB1\(^{H32Q}\) together with SOD1\(^{A4V}\) led to a massive increase in SOD1\(^{A4V}\) aggregation (Fig 3C), confirming that DNAJB1 requires Hsp70 interaction for this function.

To next examine whether inadequate delivery could play a role in the failure of HSPA1L in processing SOD1\(^{A4V}\), we immunoprecipitated GFP-HSPA1A or GFP-HSPA1L (which behave similar as their V5-tagged versions (Fig S3D)) and examined their co-precipitation with V5-
DNAJB1. Both Hsp70s showed a similar ability to interact with DNAJB1 in the presence of SOD1<sup>A4V</sup> (Fig 3D), arguing against the possibility that aggregation-enhancing effects of HSPA1L are a result of inefficient DNAJ interaction. The equal ability of HSPA1A and HSPA1L to interact with SOD1<sup>A4V</sup> (Fig 2C) is in line with the similar affinity of these Hsp70s for DNAJs (Fig 3D). Together, these results point to a step downstream of substrate delivery being fate-determining for SOD1<sup>A4V</sup> processing by either HSPA1A or HSPA1L.

**Differential binding of HSPA1A and HSPA1L to Nucleotide Exchange Factors**

Pulling-down HSPA1L, in the context of DNAJB1 over-expression, resulted in increased levels of bound SOD1<sup>A4V</sup> compared to HSPA1A (Fig 3D). These data hinted towards a reduced substrate dissociation in the case of HSPA1L. This could be either due to an intrinsic defect in ADP dissociation in the case of HSPA1L or due to the activity of NEFs. Since the nucleotide binding sites are fully conserved between HSPA1A and HSPA1L (Fig S1), a very different intrinsic ATP/ADP binding that can affect the activity seemed quite unlikely. However, we could not exclude that amino acid changes in distal sites may impede the ATPase activity in an allosteric manner. To investigate the possibility of an intrinsically defective ATPase, we generated E to Q mutations in the conserved ATP interaction sites E175 and E177 for HSPA1A and HSPA1L respectively. This Hsp70 mutation has been shown to lead to loss of ATPase activity by keeping the Hsp70 in a high substrate-affinity state, imitating a substrate-release defect (Buchberger et al., 1994; Fontaine et al., 2015; Wilbanks et al., 1994). As expected, the HSPA1A<sup>E175Q</sup> mutant dramatically increased SOD1<sup>A4V</sup> aggregation (Fig S3E), confirming that a functional HSPA1A ATPase cycle is crucial for SOD1<sup>A4V</sup> processing. HSPA1L<sup>E177Q</sup> also enhanced SOD1<sup>A4V</sup> aggregation compared to HSPA1L (Fig S3E), showing that SOD1<sup>A4V</sup> has to and can pass through HSPA1L’s ATPase cycle. Moreover, the functionality of the ATPase activity of HSPA1L has been reported before (Hageman et al., 2011; Takahashi et al., 2017). In fact, HSPA1L’s ATPase activity is required for translocation of Parkin to damaged mitochondria during mitophagy (Hasson et al., 2013). Interestingly, HSPA1L<sup>E177Q</sup> increased aggregation even more than HSPA1A<sup>E175Q</sup> (Fig S3E), suggesting that the different effects of HSPA1A and HSPA1L are likely not the result of an intrinsic ATPase activity difference.

NEFs play a crucial role in dictating the efficiency of the ATPase cycle of the Hsp70s and the subsequent substrate release (Bracher and Verghese, 2015). Therefore, we next questioned whether HSPA1L shows any distinct interaction pattern with NEFs compared to HSPA1A. Out of the various human NEFs for Hsp70s, those that are expressed in the same cellular compartment as HSPA1A and HSPA1L (cytosol/nucleus) and could therefore be interaction partners are the members of the BAG family, the three Hsp110 family members (HSPH1-3), and HSPBP1 (Vos et al., 2008; Kampinga and Bergink, 2016). To identify potential
Fig. 3

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mCherry-SOD1<sup>AAV</sup>

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α tubulin

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**C**

mCherry-SOD1<sup>AAV</sup>

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**D**

V5-DNAJB1 + mCherry-SOD1<sup>AAV</sup>

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SOD1

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Functional diversity between Hsp70 paralogs

NEFs relevant to mutant SOD1 processing, we first tested BAG1, BAG3 and BAG4 of the BAG family, and HSPH1 (Hsp105), HSPH2 (Apg-2/HSPA4) and HSPH3 (Apg-1/HSPA4L) of the Hsp110 family for their potential against SOD1\textsuperscript{A4V} aggregation. However, upon their sole overexpression, none of the NEFs that we tested inhibited SOD1\textsuperscript{A4V} aggregation; quite to the contrary, they all increased SOD1\textsuperscript{A4V} aggregation in either HEK293 or U2OS cells (Fig S4A and B). These results are consistent with earlier findings showing that high stoichiometric NEF to Hsp70 ratios have inhibitory effects on Hsp70 cycling and activity (Rauch and Gestwicki, 2014; Nollen et al., 2000; Dragovic et al., 2006; Tzankov et al., 2008; Yamagishi et al., 2000).

Different NEF types have been found to interact with different sites in the NBD of Hsp70s (Polier et al., 2008; Schuermann et al., 2008; Sondermann et al., 2001; Arakawa et al., 2010; Xu et al., 2008; Shomura et al., 2005). (Fig S1). All NEFs have several contact sites in the region aa112-389 or aa114-391 of HSPA1A or HSPA1L respectively (Fig S1), which we found to be crucial for their differential activity (Fig 2E). Combinations of amino acid substitutions that in total included every one of the non-conserved residues between HSPA1A and HSPA1L (mutants N3-10) were unable to indicate a certain group of amino acids as being responsible for the differential activity of HSPA1A and HSPA1L (Fig S3C). However, since the interaction surface with the various NEFs is quite broad, functional differences between the two Hsp70s could be the result of more global changes in the NBD, affecting interactions at multiple sites. For this reason, we examined the ability of the different NEFs to interact with HSPA1A or HSPA1L in the presence of SOD1\textsuperscript{A4V} using co-immunoprecipitation. BAG1 and BAG3, used as two representatives of the BAG family, were efficiently co-immunoprecipitated with HSPA1A and HSPA1L (Fig 4A). Similarly, HSPBP1 exhibited comparable binding to both HSP70s (Fig 4B). In sharp contrast, however, all three members of the Hsp110 family of NEFs (HSPH1, HSPH2 and HSPH3) showed a strong
Figure 4. Hsp110 NEFs show increased interaction with HSPA1A. (A-C) Interaction of HSPA1A or HSPA1L with NEFs of the (A) BAG, (B) HSPBP1 or (C) Hsp110 families, in the presence of SOD1A4V. GFP-HSPA1A or GFP-HSPA1L were transiently expressed in HEK293 cells with either (A) HA-BAG1 or FLAG-BAG3, (B) untagged-HSPBP1, (C) V5-tagged HSPH1, HSPH2 or HSPH3. GFP nanotrap was used for native immunoprecipitation of the Hsp70s. Western blots using the indicated antibodies are shown. Quantification graph of binding represents ratios of IP intensities of (A) HA/GFP for BAG1 (n=3) and FLAG/GFP for BAG3 (n=2), (B) HSPBP1/GFP for HSPBP1 (n=1) and (C) V5/GFP for HSPH1-3 (n=3 for each), all relative to HSPA1A measurements. In all graphs, error bars with s.e.m., * p=0.01-0.05, ** p=0.001-0.01, *** p<0.001.
Functional diversity between Hsp70 paralogs

binding preference to HSPA1A over HSPA1L (Fig 4C). Co-immunoprecipitation of HSPH2 with the HSPA1A/HSPA1L NBD chimeras confirmed that this differential binding is coupled to the NBDs of the two Hsp70s (Fig S4C).

Human Hsp110s have around 60% identity between them and Hsp110 contact sites are very broad on Hsp70-NBD interaction surface possibly explaining why our NBD mutations could not point us to a certain location on the NBD (Fig S3C). The differential binding of the two Hsp70s to Hsp110 NEFs raised the possibility that functionally different Hsp110-Hsp70 interactions might play a role in the differential ability of HSPA1A and HSPA1L to suppress mutant SOD1 aggregation. In fact, mass spectrometry analysis of soluble immunoprecipitated mCherry-SOD1\textsuperscript{A4V} revealed endogenous HSPH2 among SOD1\textsuperscript{A4V} interactors (Fig S4D and Table S1). Notably, HSPA1A co-expression led to association of all Hsp110 family members with SOD1\textsuperscript{A4V}, something that did not happen upon HSPA1L co-expression, suggesting that HSPA1A attracts all Hsp110 chaperones towards SOD1\textsuperscript{A4V} and this might be crucial for its aggregation suppressing activity. HSPH1 and HSPH2 are abundant while HSPH3 is expressed in low quantities in most tissues (Hageman and Kampinga, 2009). Since they all interact with HSPA1A, they might compete for HSPA1A binding. Mutant SOD1 has been found to interact with Hsp110 chaperones in an ALS mouse model (Wang et al., 2009), something we also observed and which seemed to be enhanced by HSPA1A co-expression (Fig S4D). Moreover, Hsp110s has been reported to extend survival in mutant SOD1 ALS mouse model (Nagy et al., 2016) and rescue transport defects in mutant SOD1-containing squid axoplasm (Song et al., 2013). Importantly, this preference of Hsp110s over HSPA1A did not seem to be specific for mutant SOD1 as a substrate. Similar results were obtained when we co-immunoprecipitated HSPH2 with HSPA1A or HSPA1L without SOD1\textsuperscript{A4V} co-expression (Fig S4E), suggesting that this partnership is not limited to SOD1\textsuperscript{A4V} and might be involved in handling other substrates too.

**HSPH2 is necessary for HSPA1A-mediated reduction of mutant SOD1 aggregation**

Next, we assessed whether any of the Hsp110s is required for the activity of HSPA1A towards mutant SOD1 aggregation. Surprisingly, only depletion of HSPH2 in HSPA1A-overexpressing cells strongly diminished the aggregation-suppressing effect of HSPA1A, showing that HSPH2 plays an important role in HSPA1A activity against SOD1\textsuperscript{A4V} aggregation (Fig 5). Loss of HSPH1 or HSPH3 resulted in a drop in SOD1\textsuperscript{A4V} aggregation irrespective of which of the two Hsp70s was co-expressed, suggesting that these two Hsp110s might compete with HSPH2 for Hsp70 interaction and either are less efficient or lead to a different processing pathway. Importantly, loss of HSPH2 together with HSPA1L overexpression did not result in further increase in SOD1\textsuperscript{A4V} aggregation, suggesting that upon HSPA1L interaction SOD1\textsuperscript{A4V}
follows a pathway independent of HSPH2 (Fig 5). Together, these data suggest that a crucial reason for the functional difference between HSPA1A and HSPA1L in suppressing SOD1<sup>A4V</sup> aggregation is their different functional interaction with HSPH2.

The drop in SOD1<sup>A4V</sup> aggregation upon increased expression of HSPA1A and the importance of HSPH2 can be attributed to several activities. For example, Hsp110s together with HSPA1A have been found to be crucial components of a disaggregation machine (Rampelt et al., 2012; Mattoo et al., 2013; Gao et al., 2015; Nillegoda et al., 2015; Shorter, 2011). Moreover, we noticed that increasing HSPA1A levels led to a decrease of both insoluble and total levels of SOD1<sup>A4V</sup> (Fig 1D and E), which is pointing towards a degradation pathway for ultimate substrate clearance. HSPA1A has been previously reported to suppress aggregation by promoting degradation of aggregating proteins like other SOD1 mutants (Urushitani et al., 2004), mutant Parkin (Kakkar et al., 2016a), or polyglutamine proteins (Wang et al., 2013; Bailey et al., 2002). Interestingly, a very recent study in yeast also showed that Hsp110s target Hsp70 substrates for degradation (Kandasamy and Andréasson, 2018a), further supporting efficient substrate disposal upon HSPA1A-Hsp110 interaction.

Overall, our data show that Hsp70 paralogs, despite being highly conserved, can have different functionalities depending on the co-chaperone context and that specifically

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**Figure 5.** HSPA1A activity on SOD1<sup>A4V</sup> aggregation depends on HSPH2 nucleotide exchange factor. Effect of Hsp110 knock-down on SOD1<sup>A4V</sup> together with overexpressing HSPA1A or HSPA1L. NP40 fractionation of HEK293 cells co-transfected with mCherry-SOD1<sup>A4V</sup>, V5-HSPA1A or V5-HSPA1L and siRNA for HSPH1-3 or mock. Western blots using the indicated antibodies are shown. Quantification graph represents total (T) or NP40-insoluble (P) fraction western blot intensities relative to siMock control for each group (n=6-8). Error bars with s.e.m. * p=0.01-0.05, ** p=0.001-0.01, *** p<0.001.
the type of NEF plays a crucial role in regulating the fate of a substrate in the Hsp70-cycle. Importantly, our data reveal that different Hsp70s act with preferred NEFs. This implies that Hsp70s themselves co-determine substrate fate through a pre-set selection of co-chaperones and that, at least for certain substrates, binding or delivery to Hsp70 plays a less determining role. This pre-set coupling of Hsp70s with co-chaperones suggests limitations in flexibility in order to maximize efficient substrate handling. This enables an optimized system to maintain protein homeostasis. However, between different cell types or under different conditions (e.g. heat- or oxidative- stress), the types of substrates or the fates of the substrates can vary and this might require differently optimized Hsp70-NEF couples. This can be accomplished by changing the relative expression patterns of Hsp70s and NEFs, that likely influences co-chaperone context and thus the fate of substrates. There are examples of such a switch. For example, upon various types of stress, the ratio between the NEFs BAG1 and BAG3 is flipped, changing substrate fate from proteasome- to autophagosome-mediated degradation (Minoia et al., 2014). It is well known that expression of chaperones is mendable, thereby changing the capacity of the system. However, our data explain that relative changes in expression of fixed Hsp70 and NEF pairs not only change the machinery capacity but also change substrate fate. In addition, our data urge for clear specification of Hsp70 member identification in future chaperone studies and careful re-evaluation of possible conflicting existing literature data on Hsp70 functions, especially in cellular or in vivo systems.

Materials and Methods

Gene cloning, plasmids, and siRNAs
GFP- or V5-HSP70s (HSPAs), V5-DNAJs, V5-HSP110 (HSPHs) and HSPBP1 cloned into pcDNA5/FRT/TO (Invitrogen) vector plasmid were previously described (Hageman et al., 2010, 2011). HA-BAG1 and FLAG-BAG3 encoding plasmids were previously described (Minoia et al., 2014). Luciferase construct (Cyt/Nuc-superluc-eGFP) was previously described (Hageman et al., 2007). pcDNA5-FRT/TO-mCherry-SOD1WT was generated by combining mCherry from a pcDNA3.1(+)−mCherry vector (kind gift from Dr. B. Giepmans, University Medical Center Groningen, NL) and SOD1WT amplified from pEBB-FLAG-SOD1WT (kind gift from Dr. B. van de Sluis, University Medical Center Groningen, NL) previously described (Vonk et al., 2010), into a pcDNA5-FRT/TO backbone plasmid. pcDNA5-FRT/TO-mCherry-SOD1AV as well as FRT/TO-V5-HSPA1A mutants (Figure S3C) constructs were generated using QuikChange XL Site-Directed Mutagenesis Kit (Agilent), according to manufacturer’s instructions. Domain and sub-domain swaps between HSPA1A and HSPA1L were constructed by PCR amplification of the domain of interest with flanking
restriction sites and re-insertion of the replacing fragment by ligation. All primers used for cloning are listed in Table S2. All generated constructs were verified by sequencing. For gene knockdown, 50 nM of the following siRNAs were used: siGENOME SMARTpool siRNAs (Dharmacon) for HSPH1 (M-004972-00), HSPH2/HSPA4 (M-012636-02), HSPH3/HSPA4L (M-012636-02) and siGenome Non-Targeting siRNA (Pool #1, D-001206-13, Dharmacon) was used as mock siRNA negative control.

**Cell cultures and transfections**

HEK293 cells (human embryonic kidney) stably expressing the tetracycline repressor (Flp-In T-REx HEK293, Invitrogen) and U2OS cells (human osteosarcoma - a kind gift from Dr. C. Dinant) were cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (Greiner Bio-One) and penicillin/streptomycin (Gibco). Cells were transiently transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. Expression in Flp-In T-REx HEK293 was induced by 1 μg/ml tetracycline. All cell lines are frequently checked for mycoplasma contamination.

**NP40 fractionation**

48 or 72 hours after transfection, cells were washed with PBS and harvested in lysis buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 1% NP40 (Igepal-CA 630, Sigma) and complete protease inhibitor cocktail (Roche). Cell lysates were sonicated at 50% input for 5 sec, protein concentrations were measured with DC protein assay (Bio-Rad), equalized and a part of each sample was kept as the total (T) fraction representation. The remaining part of the sample was centrifuged at 20000 g for 30 min at 4°C and supernatant was kept separately as the (S) fraction. The pellet was washed once with lysis buffer and after another centrifugation at 20000 g for 30 min, supernatant was discarded and the pellet was resuspended in 1/3 of initial volume lysis buffer with sonication, representing the (P) fraction. In all three fractions, 4x Laemmli sample buffer (8% SDS, 40% glycerol, 20% 2-mercaptoethanol, 0.001% bromophenol blue) was added and samples were boiled for 5 min and kept at -20°C until use.

**Immunoprecipitations**

For GFP-Trap immunoprecipitation with crosslinking, cells were harvested in cold PBS, pelleted at 3800 g for 3 min and incubated with 1 mM DTSP (3,3-Dithiodipropionic acid di(N-hydroxysuccinimide ester)) cross-linking reagent (Sigma) in PBS for 30 min on ice. To quench cross-linking, 2 mM glycine was added and incubated for 15 min. After centrifugation for 5 min, 3800 g at 4°C, the cells were washed once with PBS, pelleted again and snap-frozen in liquid nitrogen. Pellets were resuspended in lysis buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 1.5 mM MgCl₂, 0.5% NP40 (Igepal-CA 630, Sigma), 3%
glycerol, 0.9 mM DTT (Dithiothreitol, Sigma) and complete EDTA-free protease inhibitor cocktail (Roche) and lysates were homogenized by passing through a 26G needle or by sonication (50% input, 5 sec). After spinning twice at 20000 g for 10 min at 4°C to clear lysates from cell debris, a portion of the supernatant was collected for input measurement before adding GFP-Trap® magnetic agarose beads (gtma, Chromotek) to it. Extracts were incubated with beads at 4°C for 2 hours under gentle agitation, followed by one wash with lysis buffer without DTT, 3 washes with lysis buffer and one wash with lysis buffer containing 300 mM NaCl. Laemmli sample buffer was added to the beads and input samples, which were boiled for 5 min and kept at -20°C until use.

For SOD1 antibody immunoprecipitation, cells were washed once with PBS, pelleted at 3800 g for 3 min and snap-frozen in liquid nitrogen. Pellets were resuspended in lysis buffer containing 50 mM HEPES pH 7.5, 80 mM KCl, 0.4% NP40 (Igepal-CA 630, Sigma), 0.5 mM DTT, 10% glycerol, complete EDTA-free protease inhibitor cocktail (Roche) and lysates were homogenized by passing through a 26G needle. After spinning twice at 20000 g for 10 min at 4°C to clear lysates from cell debris, a portion of the supernatant was collected for input measurement and the rest was separated in two parts, the IP sample and the control sample and SOD1 antibody (IFL-154), sc-11407, Santa Cruz) was added to the IP sample. Both IP and control samples were incubated at 4°C for 2 hours under gentle agitation followed by incubation with protein A/G-PLUS agarose beads (sc-2003, Santa Cruz) for 1 hour at 4°C under gentle agitation. Beads were washed four times with lysis buffer and Laemmli sample buffer was added to the beads (IP and control) and input samples, which were boiled for 5 min and kept at -20°C until use.

**Western blot and antibodies**

Equal amounts of proteins were loaded into 10-12% SDS-PAGE gels. Proteins were transferred onto PVDF membranes and blotted with the primary antibodies: GFP Living colors mouse monoclonal (JL-8), 632381, Clontech); V5 tag mouse monoclonal (46-0705, Invitrogen); SOD1 rabbit polyclonal (IFL-154), sc-11407, Santa Cruz), FLAG mouse monoclonal (IM2), 035K6196, Sigma), HSPH1/HSP105 rabbit monoclonal (EPR4576), ab109624, Abcam), HSPH2/HSPA4 rabbit monoclonal (EPR14166), ab185692, Abcam), HSPH3/HSPA4L rabbit polyclonal (ab87241, Abcam), alpha-tubulin mouse monoclonal (IB-5-1-2, T5168, Sigma), beta-actin mouse monoclonal (8H10D10, Cell Signaling), HSPBP1 mouse monoclonal (1D5), NBP2-01168, Novus Biologicals) and rat monoclonal HA-Peroxidase (12013819001, Roche). After incubation with the appropriate HRP-conjugated secondary antibody (Amersham), visualization was performed with enhanced chemiluminescence (ECL) and Hyperfilm (Amersham) or ChemiDoc Imaging System (Bio-Rad). Quantification of western blots was performed with either ImageJ (https://imagej.nih.gov/ij/) or Image

Functional diversity between Hsp70 paralogs
Lab (Bio-Rad) software. In all quantifications, each band’s intensity was normalized by dividing to the appropriate loading control. For each experiment, each sample value was normalized to a control sample and ratios were plotted on graphs. For statistical analysis, one sample t-test was performed between the control and each sample for most graphs, except graphs on figures 2B, 2E and 3C where one-way ANOVA together with Dunnett’s multiple comparison test were used to determine differences between the designated samples. Statistical analysis and graphs were done with GraphPad Prism 5.0 (GraphPad Software).

Luciferase reactivation assay
Cells were transfected with luciferase reporter (Cyt/Nuc-superluc-eGFP) and control or chaperone constructs in 1:9 luciferase to chaperone ratio. 24 hours after transfection, cells from each sample were trypsinized and divided into six coated tubes, three to be used as control samples and three as heat-shock (HS) samples, and kept at 37°C in complete medium. 48 hours after transfection, equal volume of complete medium supplemented with 40ug/ml cycloheximide and 40mM MOPS (3-(N-Morpholino)propanesulfonic acid) was added to the cells and incubated for 30 min at 37°C. Thereafter, HS samples were transferred into a water bath at 45°C, incubated for 30 min and then returned at 37°C and left to recover for 2 hours. After 2 hours, all samples (control and HS), were transferred on ice and lysed with equal volume of BLUC lysis buffer (25 mM Tris/H$_3$PO$_4$ pH 7.8, 10 mM MgCl$_2$, 1% (v/v) Triton X-100, 15% glycerol, and 1 mM EDTA). Samples were then transferred to -80°C for at least 30 min to complete lysis by freezing and then thawed and kept on ice until measurement. Luciferase activity was measured for 10 sec by injecting the substrate (BLUC, 1.25 mM ATP, and 0.087 mg/ml D-luciferin) using a Sirius Luminometer (Berthold Detection Systems). Three measurements from three tubes (technical replicates) were done per condition per sample and the average was taken as final measurement. For statistical analysis, one-way ANOVA together with Dunnett’s multiple comparison test were used to determine differences between the designated samples. Statistical analysis and graphs were done with GraphPad Prism 5.0 (GraphPad Software).

Molecular structure modelling
Molecular structure figures were prepared with PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC, https://pymol.org) and structural alignment between HSPA1A and HSPA1L NBDs were performed using align command in PyMOL.

Fluorescent microscopy
48 hours after transfection, cells grown on coverslips expressing mCherry-SOD1 proteins were fixed with 2% paraformaldehyde in PBS for 15 min, incubated with Hoechst 33342
(Invitrogen) for 5 min to stain nuclei and mounted on microscopy slides. Microscopy was performed with a TissueFAXS (TissueGnostics) Zeiss AxioObserver Z1-based fluorescence microscope using a Zeiss Plan-Apochromat 63x/1.40 Oil, DIC objective and image acquisition was performed with a CMOS-color PL-B623 Pixelink 3.1 Megapixels camera. Brightness/contrast corrections were done with ImageJ (NIH, [https://imagej.nih.gov/ij/](https://imagej.nih.gov/ij/)).

**Mass spectrometry**

Cells were washed once with PBS, pelleted at 6000 rpm for 3 min and snap-frozen in liquid nitrogen. Pellets were resuspended in lysis buffer (50 mM HEPES pH 7.5, 80 mM KCl, 0.4% NP40 (Igepal-CA 630, Sigma), 0.5 mM DTT, 10% glycerol, complete EDTA-free protease inhibitor cocktail (Roche)) and lysates were homogenized by passing through a 26G needle. After spinning twice at 20,000 g for 10 min at 4°C to clear lysates from cell debris, they were incubated with RFP-Trap® magnetic agarose beads (Chromotek) at 4°C for 2 hours under gentle agitation. Beads were washed four times with lysis buffer before Laemmli sample buffer was added. Samples were boiled for 5 min and were sent to mass spectrometry facility for analysis. Mass spectrometry data were analysed using PEAKS Studio 8.5 (Bioinformatics Solutions Inc.).

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**Author contributions**

DS performed all the experiments, except the experiments in Fig S4A and S4B; MK performed the experiments in Fig S4A and S4B. HHK and SB supervised the experiments. JB provided technical assistance. DS, HHK and SB designed the experiments, analysed the data and wrote the manuscript.
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Vonk, W.I.M., C. Wijmenga, R. Berger, B. Van De Sluis, and
Chapter 2


**Supplementary Information**

DnaK (E.c)  
---MKIGIDGLTSCAVINMDTTPVLENAGDRTTPSSIIAYQDGETLHGQPARK 56

SSA1 (S.c)  
---MSKAVGIDGLTSCAVINMDTTPVLENAGDRTTPSSIIAYQDGETLHGQPARK 56

HSPA1A (H.s)  
---MKAAIGIDGLTSCAVINMDTTPVLENAGDRTTPSSIIAYQDGETLHGQPARK 56

HSPA1L (H.s)  
---MSARGPAIGIDGLTSCAVINMDTTPVLENAGDRTTPSSIIAYQDGETLHGQPARK 56

HSPA2 (H.s)  
---MQAPRELAVIDGLTSCAVINDTTPVLENAGDRTTPSSIIAYQDGETLHGQPARK 56

HSPA6 (H.s)  
---MSKPVAVGIDGLTSCAVINDTTPVLENAGDRTTPSSIIAYQDGETLHGQPARK 56

HSPA8 (H.s)  
---MSKPVAVGIDGLTSCAVINDTTPVLENAGDRTTPSSIIAYQDGETLHGQPARK 56

DnaK (E.c)  
---QAVTNPQNTLFAIKRLIGRRFQDEEVQRDVSIMPFKIIAADNGD---AWVEVKG--QKMAPP 11

SSA1 (S.c)  
---QAAMNPSTTVDFALKRLIGRNFDEVQADMKHFPFKQVINDGKPQIQVEFKGETKNFTPE 11

HSPA1A (H.s)  
---QVALNPQNTLFAIKRLIGRKFDEVQADMKHFPFKQVINDGKPQIQVEFKGETKNFTPE 11

HSPA1L (H.s)  
---QVAMNPQNTLFAIKRLIGRKFDEVQADMKHFPFKQVINDGKPQIQVEFKGETKNFTPE 11

HSPA2 (H.s)  
---QVAMNPQNTLFAIKRLIGRKFDEVQADMKHFPFKQVINDGKPQIQVEFKGETKNFTPE 11

HSPA6 (H.s)  
---QVAMNPQNTLFAIKRLIGRKFDEVQADMKHFPFKQVINDGKPQIQVEFKGETKNFTPE 11

HSPA8 (H.s)  
---QVAMNPQNTLFAIKRLIGRKFDEVQADMKHFPFKQVINDGKPQIQVEFKGETKNFTPE 11

DnaK (E.c)  
---QISAEVLKKMKKTAEDYLGEPVTEAVITVPAYFNAPDQTDAEQATKDAGRIAGLEVKRIINEPT 17

SSA1 (S.c)  
---QISSMVLKMKKTAEDYLGEPVTEAVITVPAYFNAPDQTDAEQATKDAGRIAGLEVKRIINEPT 17

HSPA1A (H.s)  
---EISSMVLKMKKTAEDYLGEPVTEAVITVPAYFNAPDQTDAEQATKDAGRIAGLEVKRIINEPT 17

HSPA1L (H.s)  
---EISSMVLKMKKTAEDYLGEPVTEAVITVPAYFNAPDQTDAEQATKDAGRIAGLEVKRIINEPT 17

HSPA2 (H.s)  
---EISSMVLKMKKTAEDYLGEPVTEAVITVPAYFNAPDQTDAEQATKDAGRIAGLEVKRIINEPT 17

HSPA6 (H.s)  
---EISSMVLKMKKTAEDYLGEPVTEAVITVPAYFNAPDQTDAEQATKDAGRIAGLEVKRIINEPT 17

HSPA8 (H.s)  
---EISSMVLKMKKTAEDYLGEPVTEAVITVPAYFNAPDQTDAEQATKDAGRIAGLEVKRIINEPT 17

DnaK (E.c)  
---DFDSRLINYLVEEFKKDQGIDLRNDPLAMQRLKEAAEKAKIELSSAQQTDVNLPYITADA 29

SSA1 (S.c)  
---DFDNRLVNHFIQEFKRKHKKDSEGQKRAVRRLRTACERAKRTLSSSAQTSVEIDSLFE-- 28

HSPA1A (H.s)  
---DFDNRLVNHFIQEFKRKHKKDSEGQKRAVRRLRTACERAKRTLSSSAQTSVEIDSLFE-- 28

HSPA1L (H.s)  
---DFDNRLVNHFIQEFKRKHKKDSEGQKRAVRRLRTACERAKRTLSSSAQTSVEIDSLFE-- 28

HSPA2 (H.s)  
---DFDNRLVNHFIQEFKRKHKKDSEGQKRAVRRLRTACERAKRTLSSSAQTSVEIDSLFE-- 28

HSPA6 (H.s)  
---DFDNRLVNHFIQEFKRKHKKDSEGQKRAVRRLRTACERAKRTLSSSAQTSVEIDSLFE-- 28

HSPA8 (H.s)  
---DFDNRLVNHFIQEFKRKHKKDSEGQKRAVRRLRTACERAKRTLSSSAQTSVEIDSLFE-- 28

DnaK (E.c)  
---TGPKHMNIKVTRAKLESLVEDLVNRSIEPLKVALQDAGLSVSDIDDVILVGGQTRMPMVQ 35

SSA1 (S.c)  
-----GIDYFSTTSRAFEELCQLDLRSTLPDEKVLKRDCAKLSQDVQDLVGLGSTRIPKQV 34

HSPA1A (H.s)  
-----GIDYFSTTSRAFEELCQLDLRSTLPDEKVLKRDCAKLSQDVQDLVGLGSTRIPKQV 34

HSPA1L (H.s)  
-----GIDYFSTTSRAFEELCQLDLRSTLPDEKVLKRDCAKLSQDVQDLVGLGSTRIPKQV 34

HSPA2 (H.s)  
-----GIDYFSTTSRAFEELCQLDLRSTLPDEKVLKRDCAKLSQDVQDLVGLGSTRIPKQV 34

HSPA6 (H.s)  
-----GIDYFSTTSRAFEELCQLDLRSTLPDEKVLKRDCAKLSQDVQDLVGLGSTRIPKQV 34

HSPA8 (H.s)  
-----GIDYFSTTSRAFEELCQLDLRSTLPDEKVLKRDCAKLSQDVQDLVGLGSTRIPKQV 34

DnaK (E.c)  
---KKVAEFF-GKEPRKDVLNPSDEAVGIAAVAQGGLVTGQD----VODVLLLDVTPLSIGEITM 40

SSA1 (S.c)  
---KLTVDFNGKEPNRRNPSDEAVGIAAVAQGGLVTGQD----VODVLLLDVTPLSIGEITM 40

HSPA1A (H.s)  
---KLQDFFNGKEKDNSDEAVGIAAVAQGGLVTGQD----VODVLLLDVTPLSIGEITM 40

HSPA1L (H.s)  
---KLQDFFNGKEKDNSDEAVGIAAVAQGGLVTGQD----VODVLLLDVTPLSIGEITM 40

HSPA2 (H.s)  
---KLQDFFNGKEKDNSDEAVGIAAVAQGGLVTGQD----VODVLLLDVTPLSIGEITM 40

HSPA6 (H.s)  
---KLQDFFNGKEKDNSDEAVGIAAVAQGGLVTGQD----VODVLLLDVTPLSIGEITM 40

HSPA8 (H.s)  
---KLQDFFNGKEKDNSDEAVGIAAVAQGGLVTGQD----VODVLLLDVTPLSIGEITM 40

**Linker SBD**
Figure S1. Hsp70 sequence alignment. Clustal omega sequence alignment of the main cytosolic/nuclear Hsp70s of E.coli (DnaK), S. cerevisiae (Ssa1) and H. sapiens (HSPA1A, HSPA1L, HSPA2, HSPA6, HSPA8). Identical residues between HSPA1A and HSPA1L are shown in grey. Symbols indicate the predicted interaction sites with ATP/ADP and co-chaperones, based on previously published data from various Hsp70 and co-chaperone homologues: (Δ) ATP/ADP (Kityk et al., 2012; Flaherty et al., 1990, 1994; Wisniewska et al., 2010; Sriram et al., 1997; Arakawa et al., 2011; Qi et al., 2013), (#) DNAJ (Kityk et al., 2018), (♦) Hsp110 (Polier et al., 2008; Schuermann et al., 2008), (α●) BAG (Sondermann et al., 2001; Arakawa et al., 2010; Xu et al., 2008) and (■α) HSPBP1 (Shomura et al., 2005).
Figure S2. Hsp70 domain and subdomain differences affecting the functionality of HSPA1A or HSPA1L. (A) Effect of SBD or CTD swapped chimeras between HSPA1A and HSPA1L on SOD1\textsuperscript{A4V} aggregation. Western blot of NP40 fractionation of HEK293 cells co-expressing mCherry-SOD1\textsuperscript{A4V} and V5-tagged chimeric proteins with swapped SBDs or CTDs between HSPA1A (yellow-green) and HSPA1L (blue). (B) Effect of NBD chimeras on heat-denatured luciferase reactivation. HEK293 cells were transfected with luciferase reporter and mRFP (control) or V5-tagged HSPA1A, HSPA1L and their NBD-swap chimeras. Cells were heat-shocked for 30 mins at 45\textdegree C and luciferase activity was measured after heat shock and compared to non-heat shocked control. The ratio of HS to non-HS luciferase activity is plotted (n=2). Error bars with s.e.m., * p<0.05, ** p<0.01.
Figure S3. Hsp70 domain and subdomain differences affecting the functionality of HSPA1A or HSPA1L. (A) Positions of the non-conserved residues between HSPA1A and HSPA1L NBDs. Ribbon model of HSPA1A-NBD (PDB ID: 3JXU, (Wisniewska et al., 2010); yellow-green) and the positions of the residues that are not conserved in HSPA1L (red spheres). Subdomains IA/B and IIA/B are noted for orientation. Nucleotide (ADP) is colored in light blue. (B) Interaction surface of HSPA1A or HSPA1L NBDs. Surface representations of HSPA1A-NBD (PDB ID: 3JXU, (Wisniewska et al., 2010); yellow-green) and HSPA1L-NBD (PDB ID: 3GDQ, (Wisniewska et al., 2010); blue). (legend continued on next page)
(Figure S3 legend continued) For the non-conserved residues, hydrophobic surfaces are colored in yellow, negatively charged in red, positively charged in light blue and the remaining in white (PyMOL script YRB, Hagemans et al., 2015). Subdomains IA/B and IIA/B are marked for orientation. (C) Effect on SOD1\textsuperscript{A4V} aggregation of substitutions of HSPA1A-NBD residues with the HSPA1L equivalent in the vicinity of DNAJ (N3-6) or NEF (N7-10) interacting sites. Western blots of NP40 fractionation of HEK293 cells co-expressing mCherry-SOD1\textsuperscript{A4V} and V5-tagged HSPA1A mutants. Quantification graph represents total (T) or NP40-insoluble (P) fraction western blot intensities relative to mRFP control (n=1-14). Error bars with s.e.m., ‘p≤0.01-0.05, ‘‘p≤0.001-0.01, ‘‘‘p≤0.001. (D) Effect of V5-versus GFP-tagged Hsp70s. Western blot of NP40 fractionation of HEK293 cells co-expressing mCherry-SOD1\textsuperscript{A4V} and V5- or GFP-tagged HSPA1A and HSPA1L. Endogenous SOD1 is used as a loading control. (E) HSPA1A or HSPA1L ATPase-deficient mutants on SOD1\textsuperscript{A4V} aggregation. Western blots of NP40 fractionation of HEK293 cells co-expressing mCherry-SOD1\textsuperscript{A4V} and V5-tagged HSPA1A (yellow-green) or HSPA1L (blue), their NBD swaps or their ATPase-deficient mutants, HSPA1\textsuperscript{AE175Q} and HSPA1\textsuperscript{LE177Q} respectively.
Figure S4. NEF effect on SOD1<sup>A4V</sup> aggregation and binding to Hsp70s. (A) Screen of Hsp110 family members for suppressors of SOD1<sup>A4V</sup> aggregation. NP40 fractionation of HEK293 (representative western blot and graph) or U2OS (graph only) cells co-expressing mCherry-SOD1<sup>A4V</sup> and V5-HSPH1-3. Quantification graphs represent total (T) or NP40-insoluble (P) fraction western blot intensities relative to mRFP control (n=5 for U2OS and n=3-6 for HEK293). (B) Screen of BAG family members for suppressors of SOD1<sup>A4V</sup> aggregation. NP40 fractionation of HEK293 (representative western blot and graph) or U2OS (graph only) cells co-expressing mCherry-SOD1<sup>A4V</sup> and HA-BAG1, FLAG-BAG3 (legend continued on next page).
Quantification graphs represent total (T) or NP40-insoluble (P) fraction western blot intensities relative to mRFP control (n=2 for U2OS and n=2 HEK293). 

**C** Interaction of HSPH2 with HSPA1A/HSPA1L-NBD swaps in the presence of SOD1A4V. Co-expression of GFP-tagged NBD swapped chimeras with V5-HSPH2 and mCherry-SOD1A4V followed by native immunoprecipitation of Hsp70s with GFP nanotrap and western blots using the indicated antibodies.

**D** List of Hsp110 interactors of SOD1A4V by mass spectrometry. mCherry-SOD1A4V was co-expressed with either empty vector, V5-HSPA1A or V5-HSPA1L and soluble mCherry-SOD1A4V was precipitated using RFP nanotrap. Samples were subjected to mass spectrometry analysis and Hsp110 interactors were identified for each sample. Number of spectral counts and unique peptides are noted for each protein.

**E** Interaction of HSPH2 with HSPA1A or HSPA1L without the presence of SOD1A4V. Co-expression of GFP-HSPA1A or GFP-HSPA1L with V5-HSPH2 followed by native immunoprecipitation of Hsp70s with GFP nanotrap and western blots using the indicated antibodies. In **A&B**, error bars with s.e.m., * p=0.01-0.05, ** p=0.001-0.01, *** p<0.001.
### Table S1. List of Hsp110 interactors of mCherry-SOD1-A4V after immunoprecipitation with RFP-trap and mass spectroscopy analysis.

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**Analysis parameters (PEAKS studio software):**
- Peptides: -10lgP ≥ 0
- Proteins: -10lgP ≥ 0 and ≥ 0 unique peptides
- De novo only ALC% ≥ 50 and -10lgP ≤ 0
### Table S2. Primers used in this study.

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