HSP40 proteins use class-specific regulation to drive HSP70 functional diversity

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Ofrah Faust1,*, Meital Abayev-Avraham1,*, Anne S. Wentink2, Michael Mauer1,*, Nadinath B. Nillegoda3,*, Nir London3, Bernd Bukau1,*, & Rina Rosenzweig1,*. The ubiquitous heat shock protein 70 (HSP70) family consists of ATP-dependent molecular chaperones, which perform numerous cellular functions that affect almost all aspects of the protein life cycle from synthesis to degradation9,10. Achieving this broad spectrum of functions requires precise regulation of HSP70 activity. Proteins of the HSP40 family, also known as J-domain proteins (JDPs), have a key role in this process by preselecting substrates for transfer to their HSP70 partners and by stimulating the ATP hydrolysis of HSP70, leading to stable substrate binding11,12. In humans, JDPs constitute a large and diverse family with more than 40 different members2, which vary in their substrate selectivity and in the nature and number of their client-binding domains5. Here we show that JDPs can also differ fundamentally in their interactions with HSP70 chaperones. Using nuclear magnetic resonance spectroscopy4,13 we find that the major class BJDPs are regulated by an autoinhibitory mechanism that is not present in other classes. Although in all JDPs the interaction of the characteristic J-domain is responsible for the activation of HSP70, in DNAJB1 the HSP70-binding sites in this domain are intrinsically blocked by an adjacent glycine-phenylalanine rich region—an inhibition that can be released upon the interaction of a second site on DNAJB1 with the HSP70 C-terminal tail. This regulation, which controls substrate targeting to HSP70, is essential for the disaggregation of amyloid fibres by HSP70–DNAJB1, illustrating why no other class of JDPs can substitute for class B in this function. Moreover, this regulatory layer, which governs the functional specificities of JDP co-chaperones and their interactions with HSP70s, could be key to the wide range of cellular functions of HSP70.

JDPs are multidomain proteins that are characterized by the conserved signature J-domain (JD)2, which binds to the interface between the nucleotide-binding and the substrate-binding domains of HSP70 and is required for stimulation of its ATPase activity9. Canonical class A and B JDPs also comprise a glycine-phenylalanine (GF)-rich region adjacent to the N-terminal J-domain, two structurally similar C-terminal β-barrel domains (CTD1 and CTDII) that contain the substrate-binding region, and a dimerization domain5. Class A JDPs further contain a zinc-finger-like region that protrudes from CTDI (Fig. 1a).

JDPs are an intriguing example of proteins from the same family that, although structurally very similar, confer distinct activities in the cell. This functional diversity is generally thought to arise from differences in the targeting of HSP70 chaperones to substrates3,14,15. It is therefore quite puzzling that, although both class A and class B JDPs display similar affinity for amyloid fibres—such as α-synuclein and Tau—only class B JDPs have the ability to harness HSP70 to perform efficient fibre disaggregation16–18. The causes of this diverging behaviour, and hence the molecular basis behind activating HSP70 to act as a chaperone for selective substrates, remain unknown.

Interaction of class A and BJDPs with HSP70

To investigate the functional divergence between class A and class B JDPs, we first used solution nuclear magnetic resonance (NMR) to compare the interaction of HSP70 with the isolated J-domains of human DNAJA2 and DNAJB1. As these domains are known to both bind and activate the HSP70 chaperone4. In both cases, we observed selective peak broadening for J-domain residues localized at the end of helix II and the conserved HPD motif (residues 31–41 in DNAJA2 and 28–37 in DNAJB1), and in helix III (residues 47–53 in DNAJA2 and 46–51 in DNAJB1) (Fig. 1b–d, Extended Data Fig. 1a, b); this broadening indicates the proximity (that is, binding) of these residues to the HSP70 chaperone. Both interactions also closely resembled those between DnaJ (a bacterial JDP) and DnaK (the bacterial homologue of HSP70)9, demonstrating the highly conserved nature of J-D–HSP70 binding.

Given the minor differences in the J-domain-driven binding of the two co-chaperones to HSP70, we next investigated the importance of the disordered GF-rich linker, as this domain has previously been

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1Department of Structural Biology, Weizmann Institute of Science, Rehovot, Israel. 2Center for Molecular Biology of Heidelberg University (ZMBH) and German Cancer Research Center (DKFZ), DKFZ-ZMBH Alliance, Heidelberg, Germany. 3Australian Regenerative Medicine Institute (ARMI), Monash University, Clayton, Victoria, Australia. 4Department of Organic Chemistry, Weizmann Institute of Science, Rehovot, Israel. *These authors contributed equally: Ofrah Faust, Meital Abayev-Avraham. 5E-mail: bukau@zmbh.uni-heidelberg.de; rina.rosenzweig@weizmann.ac.il.
reported to be essential in bacteria for maximal stimulation of the ATPase activity of Dnak\textsuperscript{NDR}.

The NMR spectrum of DNAJ\textsubscript{2\textsuperscript{DQR}} (residues 1–111), containing both the J-domain and GF-rich regions, was very similar to that of the isolated J-domain, with additional peaks appearing in the random coil regions that corresponded to the GF linker (Fig. 1e). On addition of HSP70, the J-domain residues of DNAJ\textsubscript{2\textsuperscript{DQR}} showed the same selective peak broadening as observed upon HSP70 binding to the isolated J-domain (Fig. 1b). The GF linker residues, however, were largely unaffected (Fig. 1e), suggesting that this linker does not participate directly in the interaction with HSP70. Similar results were obtained for DNAJAL, a more abundant class A JDP (Extended Data Fig. 1c, d).

DNAJBL, a class B JDP, displayed markedly different behaviour, with no binding detected between HSP70 and DNAJBL\textsubscript{1\textsuperscript{DQR}} (residues 1–111) even after the addition of excess protonated HSP70 (Fig. 1f). This was surprising, because addition of the same concentration of protonated HSP70 to the isolated J-domain (without the GF region) caused substantial peak broadening (Extended Data Fig. 1e).

The GF region of DNAJBL inhibits JD–HSP70 binding

In addition to the lack of interaction with HSP70, the DNAJBL\textsubscript{1\textsuperscript{DQR}} construct itself also displayed a different chemical shift pattern compared with that of the J-domain alone. The greatest changes were observed in helices II and III of the J-domain, corresponding to the HSP70-binding region, and in helix IV (Extended Data Fig. 1f). Further analysis of the chemical shifts of DNAJBL\textsubscript{1\textsuperscript{DQR}} using TALOS\textsuperscript{NMR} (Extended Data Fig. 2a) and 'D\textsubscript{iso} residual dipolar coupling measurements\textsuperscript{2}\textsuperscript{2} (Extended Data Fig. 2b) revealed a stable α-helix between residues 98–106, a region that was previously suggested to be disordered. Measurement of the local backbone flexibility on the nano- to picosecond timescale further indicated that residues 93–107 in the GF-rich region are highly structured, with calculated order parameters of 0.9–1.0 (see Methods, Extended Data Fig. 2c).

We next determined the structure of DNAJBL\textsubscript{1\textsuperscript{DQR}} using solution NMR (Extended Data Fig. 2c–h, Supplementary Table 1). This showed that, whereas residues 1–71 of DNAJBL\textsubscript{1\textsuperscript{DQR}} adopt a similar fold to that of the isolated J-domain\textsuperscript{11}, helix V docks onto helices II and III of the J-domain, covering the HSP70-binding sites (Fig. 1g).

To determine whether this newly identified helix V is responsible for blocking the interaction with HSP70, we generated an additional truncation mutant in which the entire helix was deleted (DNAJBL\textsuperscript{1\textsuperscript{DQR}-D}). DNAJBL\textsuperscript{1\textsuperscript{DQR}-D} displayed a strong restored interaction with HSP70 and showed similar chemical shift patterns to that of the isolated J-domain, with differences observed only in helix IV (Extended Data Fig. 3a, b).

We next tested if the inhibiting helix also exists in full-length DNAJBL. Careful comparison of the chemical shifts from final methyl-TROSY (transverse relaxation-optimized spectroscopy) spectra of full-length DNAJBL, DNAJBL\textsubscript{1\textsuperscript{DQR}} and DNAJBL\textsubscript{1\textsuperscript{DQR}-D} revealed that the full-length DNAJBL adopts the same J-domain-inhibited conformation as DNAJBL\textsubscript{1\textsuperscript{DQR}} (Extended Data Fig. 3c).

This then poses the question of how this inhibition is lifted to allow HSP70 binding, as DNAJBL has long been known to both bind and activate HSP70. We therefore aimed to determine the mechanism through which this intramolecular inhibition is released in the full-length DNAJBL co-chaperone.

DNAJBL contains a second HSP70-binding site

We began by monitoring the changes in chemical shifts in full-length DNAJBL after the addition of HSP70. In line with previous observations, the addition of HSP70 to DNAJBL resulted in chemical-shift perturbations (Fig. 2a, Extended Data Fig. 4) and selective peak broadening (Fig. 2b), indicative of binding and confirming that—in the full-length

\[ \text{Fig. 1. The GF region of class B JDPs initially blocks J-domain binding to HSP70.} \]

a. Domain organization of class A and B JDPs, with boundaries indicated below. J-domain (with enlarged secondary structure arrangement below), red; GF, grey; CTDL, cyan; CTDII, blue; dimerization domain (DD), yellow; zinc-finger-like region (ZFLR) insertion into CTDL (only present in class AJDPs), pink. b. H\textsuperscript{3}N-heteronuclear single quantum correlation (HSQC) spectra of 0.2 mM DNAJ\textsubscript{2\textsuperscript{DQR}} (b) and DNAJ\textsubscript{B1\textsuperscript{DQR}} (c) alone (black), and in complex with 0.2 mM H\textsuperscript{1}H HSP70 (blue). Whereas J-domain peaks of DNAJ\textsubscript{2\textsuperscript{DQR}} are similarly affected by HSP70 interaction as in b, peaks from the GF-rich region are unchanged, indicating that the GF-rich region of DNAJ\textsubscript{2\textsuperscript{DQR}} does not bind HSP70. f. H\textsuperscript{3}N HSQC spectra of 0.2 mM DNAJ\textsubscript{B1\textsuperscript{DQR}} alone (black), and in the presence of 0.4 mM H\textsuperscript{1}H HSP70 (red). No changes were observed in the spectra of DNAJ\textsubscript{B1\textsuperscript{DQR}} upon addition of HSP70, indicating a lack of interaction. g. Cartoon representation of the ten lowest-energy solution NMR structures of DNAJ\textsubscript{B1\textsuperscript{DQR}}, with helix V coloured orange. Helix V docking onto the interface between helix II and helix III blocks the HSP70-binding sites on the J-domain of DNAJBL.
protein-inhibition by the GF region of the HSP70-binding site on the J-domain is lifted. Assignment of the chemical shifts of DNAJB1 methyl residues revealed that HSP70 binds to two distinct sites on the co-chaperone: one located at the J-domain and another in CTDI (Fig. 2b, c). Subsequent NMR experiments revealed that the HSP70–CTDI interaction can occur independently of binding to the J-domain (Extended Data Fig. 4b, c). Because the isolated CTDI from both DNAJB1 and its yeast homolog Sis1 was previously shown to interact with a conserved EVD tetrapeptide from the C termini of eukaryotic cytosolic HSP70 proteins\textsuperscript{a,23–25}, we asked if this EVD tail acts as the additional DNAJB1-binding site on HSP70 chaperones.

To test this hypothesis, we repeated the NMR binding experiment using methyl-labelled HSP70 and titrating in deuterated DNAJB1 (Fig. 2d, Extended Data Fig. 5a). The same NMR experiments were also performed with the isolated J-domain and CTDI regions of DNAJB1, because both can interact independently with HSP70 (Fig. 2d, Extended Data Fig. 5b, c). As expected, the J-domain of DNAJB1 bound to the interface between the HSP70 substrate-binding and nucleotide-binding domains, similar to the contacts observed between DnaK and the J-domain of DnaJ\textsuperscript{a}. The CTDI of DNAJB1 was indeed found to interact with the C-terminal tail of HSP70: this interaction occurred in both full-length DNAJB1 (Fig. 2d) and in a construct containing only the CTDs.

In S. aureus, the absence of the C-terminal EEVD tail is essential for proper protein folding by the combined activity of HSP70 and the class B JDP Sis1. Although, notably, not for the same activity when performed by HSP70 and class AJDPs such as Ydj1\textsuperscript{21,22}. We were therefore interested as to whether and how the interaction between CTDI and the EVD tail of HSP70 differs between class A and class B JDPs.

To investigate this, we repeated the HSP70-binding experiment using DNAJ2, a class B JDP. Like DNAJB1, DNAJ2 showed strong interaction with HSP70 through its J-domain; however, no binding was detected between the CTDs of DNAJ2 and the HSP70 C-terminal EEVD region (Fig. 2d, Extended Data Fig. 5d, f). This reveals a marked difference between the highly homologous class A and class B JDPs in their mode of binding to HSP70s. Class B co-chaperones show both intrinsic inhibition of their J-domain and an additional HSP70-binding site, whereas class A JDPs have neither.

**EEVD binding to DNAJB1 releases JD–GF inhibition**

Next, we aimed to determine whether the interaction of the CTDI of DNAJB1 with the C-terminal EEVD tail of HSP70 correlates with the release of the GF inhibition of the J-domain. To test this, we generated a peptide that corresponds to the last 20 amino acids of the C-terminal tail of HSP70. Titration of this peptide into methyl-labelled DNAJB1 resulted in chemical shift perturbations at the same CTDI residues that were found in our NMR experiments to interact with full-length HSP70 (compare Extended Data Fig. 4a, d); this indicates that HSP70 interacts with the CTDs of DNAJB1 solely through its C-terminal disordered region. In addition, the binding affinity of the peptide was rather weak (dissociation constant (K\textsubscript{D}) = 50 ± 7\,\mu\text{M}, which suggests that this interaction is transient in nature. Notably, the addition of the peptide also resulted in changes to the resonances in the DNAJB1 J-domain (Fig. 3a, indicated by an asterisk). In this region, residues in helices II and III exhibited changes on a slow timescale, indicated by a reduction in the intensity of the GF-inhibited J-domain population and a build-up of the state in which the J-domain is released (Fig. 3b).

We next used paramagnetic relaxation enhancement experiments\textsuperscript{27,28} to determine the proportion of the HSP70 EEVD-binding site on the CTDI of DNAJB1 to the JD–GF complex, where the release occurs. This was performed using nitroxide spin-probes attached either to residue 40 in helix III of the J-domain or to residue 186 in the CTDI. Substantial decreases in peak intensities were observed in the CTDI region upon spin labelling of the J-domain, and in the JD–GF region after labelling of CTDI (Fig. 3c, d; dark purple bars), indicating that in DNAJB1, the J-domain is positioned within 12–17\,\AA of CTDI. Addition of the EEVD-containing peptide, however, completely abolished the decrease in intensity in these regions (Fig. 3c, d; light purple bars). This reveals that upon peptide binding, the J-domain detaches from the CTDI and moves beyond the detection range (30\,\AA) of the paramagnetic relaxation enhancement experiments.

We similarly attached spin-labels to residues at the two ends of GF inhibitory-helix V (residues 93 and 108) and recorded the paramagnetic relaxation enhancements. These measurements show that, upon interaction with HSP70, helix V also detaches from the CTDI as well as from the J-domain (Fig. 3e, f). The direct interaction of the CTDI of DNAJB1 with the EEVD tail of HSP70 therefore releases the GF inhibition of the DNAJB1-J-domain, freeing it for subsequent HSP70-binding and activation.

**DNAJB1 binds HSP70 and substrates independently**

The CTDI region of DNAJB1 that interacts with the EEVD tail of HSP70 has previously been proposed to serve as a binding site for peptides and unfolded client proteins\textsuperscript{29–31}. This suggests the possibility that the EEVD of HSP70 mimics substrates in its binding. We therefore tested...
Fig. 1 | DNAJB1 binding to the C-terminal EEVD tail of HSP70 releases the JD–GF inhibition. a. Titration of 20-amino-acid-long HSP70 C-terminal peptide into $^{23}$CH$_3$-LYM-labelled DNAJB1, showing perturbations on the fast-exchange timescale to CTDI residues (marked by black arrows), and changes on the slow-exchange timescale to the J-domain residues (marked by asterisks). b. Addition of HSP70 EEVD-peptide (EEVDp) released the GF inhibition of the DNAJB1 J-domain, with residues L55, 120 and V54 exhibiting different chemical shifts in the GF-inhibited, docked (grey) and the free J-domain (black) conformations. In DNAJB1, the J-domain is in a fully inhibited conformation (blue) which is partially released to the free J-domain state (purple) upon addition of HSP70 C-terminal peptide. c–f. Methyl group peak intensity ratios of paramagnetic (oxidized) and diamagnetic (reduced) DNAJB1, spin-labelled at positions 40 (c), 186 (d), 93 (e) and 108 (f) in the absence (dark purple) and presence (light purple) of the HSP70 C-terminal peptide. The location of the MTSSL spin-label is indicated by the red ellipse. In the absence of HSP70, the J-domain of DNAJB1 is in close proximity to CTDI, as indicated by marked decreases in peak intensities in CTDI after spin-labeling of the J-domain (e), and in the JD–GF after labelling of CTDI (d). The addition of HSP70 C-terminal EEVD-containing peptide abolished the intensity decrease in these regions, indicating detachment of the J-domain from CTDI. Similarly, upon DNAJB1 binding to the EEVD peptide, the DNAJB1 GF region detaches both from the J-domain and CTDI (e, f). g. Residue-resolved plot of NMR signal attenuation ($I_i/I_0$) of DNAJB1 alone ($I_0$) and in the presence of HSP70 (EEVD) ($I_i$). High intensity ratios (that is, low attenuations) indicate lack of interaction. h. Residue-resolved plot of NMR signal attenuation ($I_i/I_0$), where $I_i$ and $I_0$ are signal intensities of HSP70 (EEVD)-bound and free DNAJB1 ($I_0$), respectively. Large intensity changes are observed in the J-domain residues of DNAJB1 ($I_0$), pointing to a strong interaction between this region and HSP70 (EEVD).

whether the HSP70 C-terminal tail competes with client proteins for DNAJB1 binding, and whether substrates are also capable of releasing the GF inhibition of the DNAJB1 J-domain.

Titration of a known substrate, α-synuclein$^{13}$, into DNAJB1 showed binding primarily in the CTDII—separate from the CTDI site to which the EEVD of HSP70 binds—with no changes observed for J-domain residues (Extended Data Fig. 6a, b). Thus, the release of the GF inhibition of the J-domain in DNAJB1, and the subsequent binding and activation of HSP70 by DNAJB1, is solely dependent on the interaction of the C-terminal tail of HSP70 with CTDI.

Furthermore, competition experiments—in which either the EEVD peptide is added to the DNAJB1–substrate complex or α-synuclein is added to the DNAJB1-EEVD peptide complex—showed that both substrates and the C-terminal peptide of HSP70 can simultaneously bind to DNAJB1 (Extended Data Fig. 6c, d).

**EEVD deletion abolishes HSP70–DNAJB1 binding**

We next tested the effect of the deletion of the last four residues of HSP70, which correspond to the conserved EEVD sequence, by preparing the mutant HSP70Δ(EEVD). The deletion of EEVD completely abolished the interaction between DNAJB1 and HSP70 (Fig. 3g), but had no effect on the interaction of HSP70 with DNAJA2 (Extended Data Fig. 7), reinforcing the conclusion that class A co-chaperones bind to HSP70 solely through their J-domains. The interaction of CTDI with the HSP70 C-terminal tail is therefore an essential step only for the binding and activation of HSP70 by class BJDPs. The inability of mutated HSP70 to interact with DNAJB1 also explains the previous observation that HSP70Δ(EEVD) is defective in protein refolding with class BJDPs, but not with class A co-chaperones$^{23,24}$, which possess neither the interaction site for the HSP70 EEVD nor the intrinsic J-domain inhibition that this binding is required to abate.

We therefore find that the two JDP classes, despite their high structural and sequence similarities, interact with and activate the HSP70 chaperone in different ways. Class A JDPs, such as DNAJA2 and DNAJA1, interact with HSP70 only through their N-terminal J-domains (Fig. 4a). The class B JDP DNAJB1, however, displays a more complex two-step HSP70-binding behaviour, which results from the presence of the structured helix V within the GF region. This helix, which is not found in class A JDPs, is initially docked onto the J-domain of DNAJB1, preventing it from interacting with HSP70. It is only after interaction of the DNAJB1 CTDI with the C-terminal tail of HSP70 that the J-domain is released, enabling it to bind to and activate the HSP70 chaperone (Fig. 4b).
**Fig. 4 | DNAJB1-JD–GF inhibition is essential for amyloid disaggregation.**

**a, b.** Mechanistic model of HSP70 interaction with, and activation by, class A (a) and class B (b) JDPs. c. Refolding of luciferase by HSP70 (left) and HSP70(ΔEEVD) (right), measured in the absence (grey) and presence of DNAJ2A2 (blue), DNAJ2B (red), DNAJ1B(E50A) (orange), DNAJ2B(F102A) (yellow) or DNAJ1B(ΔH5) (purple). HSP110 was included in all assays as a NEF. Reactivation after 120 min is shown as a percentage of native luciferase activity. Data are mean ± s.e.m. (n = 3).

d. Disaggregation of preformed α-synuclein fibres by HSP70 (left) and HSP70(ΔEEVD) (right) measured in the absence (black) and presence of wild-type (WT) DNAJ2B (red), DNAJ1B(E50A) (orange), DNAJ2B(F102A) (yellow) or DNAJ1B(ΔH5) (purple), with HSP110 included in all reactions as a NEF. The decrease in thioflavin T (ThT) fluorescence indicates the disaggregation of α-synuclein fibres. e. Disaggregation of α-synuclein fibres by HSP70 and DNAJ1B(ΔH5) with increasing concentrations of HSP110 (200–1,600 nM; light to dark purple). The fold excess of HSP110 relative to 100 nM is indicated. Disaggregation by wild-type DNAJ1B with the lowest concentration of HSP110 (100 nM, red) and by DNAJ2B with the highest concentration of NEF (1,600 nM, blue) are also shown for comparison.

**GF mutations restore DNAJB1–HSP70(ΔEEVD) function**

To understand the importance of the two-step binding mechanism in DNAJB1, we generated mutations designed to disrupt the JD–GF interface: a glutamic acid-to-alanine mutation at residue 50 (DNAJ1B(E50A)) and a phenylalanine-to-alanine mutation at residue 102 (DNAJ1B(F102A)) (Extended Data Fig. 8a, b). The NMR spectra of the DNAJ1B(ΔH5–GF) constructs of both mutant proteins indicated the presence of two slowly exchanging conformations, one corresponding to the GF-inhibited state and one to the free J-domain state (Extended Data Fig. 8c–f). Moreover, unlike the wild-type DNAJ1B(ΔH5–GF) construct, which displayed no binding to HSP70 (compare Extended Data Fig. 8c, d with Fig. 1f), both mutants showed strong binding to the chaperone, further demonstrating that the release of J-domain inhibition is strictly required for the interaction between HSP70 and the J-domain of DNAJB1. In addition, whereas wild-type DNAJ1B was unable to interact with HSP70(ΔEEVD), the mutants—which are no longer dependent on interaction with the EEVD tail to release the J-domain—showed tight binding to the chaperone (Extended Data Fig. 9a–c). This lack of reliance on the CTD–HSP70–EEVD interaction also explains previous observations that DNAJ1B(E50A) can partially rescue the refolding activity of HSP70(ΔEEVD)23,24. As expected, whereas the addition of wild-type HSP70 to methyl-labelled DNAJ1B mutants resulted in perturbations to both the J-domain and the CTDI regions of DNAJ1B (Extended Data Fig. 9d–f), HSP70(ΔEEVD) was found to bind only to their J-domain—again confirming that the EEVD region of HSP70 is the sole point of interaction for the CTDI of DNAJ1B.

The two DNAJ1B mutants could only partially restore the activity of HSP70(ΔEEVD) in the refolding of denatured luciferase, whereas refolding by wild-type HSP70 was as efficient with the mutants as with wild-type DNAJ1B (Fig. 4c). The reduced refolding activity of the mutants could be attributed to their lower affinity for HSP70(ΔEVEVD), resulting from the lack of CTD–HSP70 interaction (Extended Data Fig. 9), or due to the mutants having an only partially released J-domain (64% in DNAJ1B(E50A) and 36% in DNAJ1B(F102A)) (Extended Data Fig. 8e, f), or possibly a combination of both.

To discriminate between these possibilities, we designed a DNAJ1B construct that contained five mutations in helix V (H99C, M101S, F102C, F105S and F106G), thus generating an unstructured GF region bearing a strong resemblance to that of DNAJ2A. This mutant—which is denoted DNAJ1B(ΔH5) and has a perpetually released J-domain (Extended Data Fig. 10a, b)—bound HSP70(ΔEEVD) with high affinity, and fully enhanced HSP70 ATPase rates at sub-stoichiometric concentrations (Extended Data Fig. 10c, d). Like the previous two mutants, DNAJ1B(ΔH5), in conjunction with full-length HSP70, refolded denatured firefly luciferase as efficiently as did wild-type DNAJ1B (Fig. 4c), and in fact achieved higher substrate refolding rates; this explains why class AJDPs—which lack the autoinhibition that is observed in DNAJ1B—are more efficient in the refolding of misfolded substrates than are class BJ DPs. However, unlike the partially released mutants, DNAJ1B(ΔH5) also retained this same level of activity with HSP70(ΔEVEVD) (Fig. 4c), indicating that the interaction of the C-terminal tail of HSP70 with CTD of DNAJ1B does not have a role in protein refolding beyond the release of the GF inhibition of the J-domain.

**JD–GF inhibition essential for amyloid disaggregation**

It therefore seems that the mechanism of autoinhibition and release of the J-domain in DNAJ1B does not offer any functional advantage in protein folding over class A JDPs, which have an intrinsically free J-domain. We therefore investigated if this regulation might instead have an essential role in the disaggregation of amyloid fibrils, in which class AJDPs are unable to substitute for their class B paralogues22,23,25,26.

To this end, we tested the function of the DNAJ1B(E50A), DNAJ1B(F102A) and DNAJ1B(ΔH5) mutants in protein disaggregation. Wild-type DNAJ1B, together with HSP70 and the nucleotide exchange factor (NEF) HSP110, successfully disaggregated 85% of preformed α-synuclein fibrils, whereas under the same conditions the DNAJ1B(E50A) and DNAJ1B(F102A) mutants showed reduced disaggregation activity (70% and 47%, respectively). Unlike wild-type DNAJ1B, however, both mutants could still partially disaggregate α-synuclein fibres together with HSP70(ΔEEVD) (Fig. 4d). By contrast, DNAJ1B(ΔH5)—which has a fully released J-domain—was unable to promote the disaggregation of α-synuclein fibres with either wild-type HSP70 or HSP70(ΔEEVD). This mutant, however, both binds to the fibres with a similar affinity to that of wild-type DNAJ1B (Extended Data Fig. 10c) and interacts strongly with HSP70 (Extended Data Fig. 10f), recruiting it to the fibres (Extended Data Fig. 10g, h). These assays therefore strongly indicate that the autoinhibition of the DNAJ1B J-domain, and its release through the interaction of CTDI with the terminal tail of HSP70, are essential for protein disaggregation, because even a partial release of the inhibition reduces disaggregation activity.

A parallel study has identified that recruitment of HSP70 molecules, at high density, to α-synuclein fibrils is key to efficient amyloid disaggregation. We therefore tested whether the ability of JDPs to cluster HSP70 chaperones is likewise dependent on the autoinhibitory mechanism. The proximity of HSP70 chaperones on a fibril surface
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was measured using Förster resonance energy transfer experiments in the presence of DNAJB1, DNAJA2 or the mutant DNAJB1(D15S), which has a functional HSP70-binding site on CDT1 but does not contain the autoinhibitory helix V. Notably, the assay showed that only the combination of wild-type DNAJB1 and wild-type HSP70 fostered efficient clustering (Extended Data Fig. 10f), demonstrating that both the CF inhibition of the J-domain and its docking onto the CTD are vital for efficient targeting and clustering of HSP70 onto the amyloid fibres.

We next asked whether artificially improving the clustering of HSP70 around the reduced-efficiency DNAJB1 mutants could restore their disaggregation activity. We therefore repeated the disaggregation assays with an excess of Hsp110, because it has been shown that Hsp110 displays a biased NEF activity towards HSP70 molecules that are not densely packed onto the fibrils, thereby actively increasing the proportion of efficiently clustered HSP70 molecules. The addition of increasing concentrations of the NEF Hsp110 partially rescued the disaggregation activity of the DNAJB1(D15S) mutant (Fig. 4e), but had no such effect on DNAJA2.

These experiments establish why class AJDPs, which have similar affinity for α-synuclein fibres to that of DNAJB1 (Extended Data Fig. 10e) but possess neither a second HSP70-binding site nor a similar autoinhibitory mechanism, are incapable of participating in amyloid disaggregation. Overall, our results show that the DNAJB1 chaperone has evolved a regulatory mechanism for precise control of the targeting of HSP70 to client proteins—a mechanism that is both unique and vital to the functions of HSP70. Moreover, because the inhibitory helix V region is also highly conserved across cytosolic class BJDPs, this regulation probably governs the activity of all members of this family—a key example being DNAJB6, in which mutations in this region result in distal myopathy disorders. Notably, of the class B family there are only two members that do not contain the helix V region: the ER-localized DNAJB9 and DNAJB1L. However, these proteins are known to be co-chaperones for HSPA5 (also known as BIP), which does not contain the corresponding EVD tetrapeptide at its C terminus that is required to release the JD–CF inhibition.

It therefore seems that we have just begun to scratch the surface in understanding this newly identified layer of regulation, and its importance in the many functions of class B chaperones in the cell.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-020-2906-4.


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Methods

No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

Labelling and purification of J-domain and JD–GF constructs of DNAJ1, DNAJ2 and DNAJB1

DNAJ1 (residues 1–69), DNAJ1 (residues 1–109), DNAJ2 (residues 1–71), DNAJ2 (residues 1–115), DNAJB1 (residues 1–72), DNAJB1 (residues 1–111) and DNAJB1 (residues 1–96) wild-type and mutant constructs were expressed in BL21 (DE3) cells (Novagen) and grown in M9 minimal media supplemented with 3.1μM Cl or 3.1μM Cl/3Cl-glucose for 13NO or 13NO.labelled sample, respectively. Cells were grown at 37 °C to an optical density (OD) of 1.0 and induced overnight at 25 °C with 1 mM IPTG.

All proteins were expressed with an N-terminal 6×His-Smt3-tag purified on a HisTRAP column (GE Healthcare). The tag was subsequently cleaved using Ulp1 enzyme overnight at 4 °C, and the cleaved protein was further separated from the uncleaved protein and the enzyme using a HisTRAP affinity column. The untagged protein was then concentrated and loaded onto a size-exclusion Superdex 75 16/60 column (GE Healthcare) equilibrated with 50 mM HEPES, 100 mM KCl, pH 7.4, 2 mM DTT and 0.03% NaN3. Purity and composition of the proteins were confirmed by SDS–PAGE and electrospray ionization mass spectrometry (ESI-MS).

Labelling and purification of full-length HSP70, DNAJ2 and DNAJB1 proteins

HSP70 (also known as IISP8) or HSP70 variants were expressed in Escherichia coli BL21CodonPlus (DE3)-RIPL cells transformed with pET-sumo plasmids. Full-length DNAJB1 variants were expressed in BL21 (DE3) cells (Novagen). The cells were grown at 37 °C in M9 D0 media, supplemented with 13HCl glucose as the sole carbon source. Methyl labelling of the ile-δ1-15CH3, Val-leu-15CH3, 15CD3, and Met-e-15CH3 (refered to as IILY2) was achieved following previously reported procedures.

Stereospecific labelling of DNAJB1 Leu-δ1-15CH3 and Val-γ1-15CH3 was performed by addition of 20 μM L-methyl-4-15CH3-α-tocotrienol (NMRBio) at OD 0.9. After 45 min, isoleucine precursors and 15CH3 labelled methionine were added, and 15 min later protein production was induced by 1 mM IPTG for 16 h at 25 °C.

Unlabelled HSP70, DNAJ2 and DNAJB1 proteins or their variants were grown in Luria Bertani broth (LB) medium to OD of 0.8 and induced overnight with 1 mM IPTG at 25 °C.

All constructs were expressed with an N-terminal 6×His-Smt3-tag. After expression, bacteria were collected and lysed by French press, and the proteins were purified on a HisTRAP column (GE Healthcare), followed by cleavage of the purification tag using Ulp1 protease at 4 °C. The cleaved proteins were concentrated and loaded onto HiLoad 16/60 Superdex 200 pg gel filtration column (GE Healthcare) equilibrated with 25 mM HEPES pH 7.5, 150 mM KCl and 0.03% NaN3. Subsequently, proteins were concentrated, aliquoted, snap-frozen in liquid nitrogen and stored at −80 °C. The purity of all proteins was confirmed by SDS–PAGE.

NMR spectroscopy

All NMR experiments were carried out at 25 °C on a Bruker AVANCE III 600 MHz, 18.8T (800 MHz) or 11.7T (1,000 MHz) Bruker spectrometers equipped with triple resonance single (2) or triple (x, y, z) gradient cryoprobe. The experiments were processed by NMRPipe48 or Bruker TopSpin, and analysed via Sparky49 or CCPNMR. All NMR experiments involving HSP70 protein were carried out in the presence of an ATPase-deficient T204A mutant of HSP70 and in the presence of an ATP regenerating system.46

Assignments of J-domain and DNAJB1 constructs

Backbone 1H, 15N and 13C resonance assignments were carried out on a sample of DNAJB1, DNAJB1 or DNAJB1 in 300 mM HEPES pH 7.5, 50 mM KCl, 0.03% NaN3, and 10% D2O buffer. DNAJB1 and DNAJB1 were assigned using a 3D HNCA, CBCA(CO)NH and HN(CA)CO experiments, recorded on a 600 MHz magnet, resulting in the unambiguous assignment of 68 out of 68 non-proline residues for DNAJB1 at 94 out of 104 for DNAJB1. DNAJB1 assignments were obtained using 3D HNCA, CBCA(CO)NH, HN(CA)CO and HNCO experiments acquired at 800 MHz, resulting in the assignment of 71 out of the 74 non-proline residues. The assignments of DNAJB1 were available through the Biological Magnetic Resonance Data Bank (ID 19163).

Side chain chemical shift assignments for DNAJB1 were obtained using (H(CO)NH-TOCSY, H(CO)NH-TOCSY, H(CO)NH-TOCSY, H(CO)NH-TOCSY, H(CO)NH-TOCSY, H(CO)NH-TOCSY, H(CO)NH-TOCSY, H(CO)NH-TOCSY) spectra recorded on per-deuterated protein samples dissolved in 100% D2O, 25 mM HEPES, pH 7.5, 50 mM KCl and 0.03% NaN3. The assignment of DNAJB1 methyl groups was obtained via (H(CO)NH-TOCSY, H(CO)NH-TOCSY, H(CO)NH-TOCSY, H(CO)NH-TOCSY, H(CO)NH-TOCSY, H(CO)NH-TOCSY, H(CO)NH-TOCSY, H(CO)NH-TOCSY) and methyl–methyl NOE experiments.

In order to assign the methyl residues of DNAJB1 CTDs (residues 154–340), 12 lie to Lue or Lue/Val to lie point mutations were made (117L, 119L, 120S, 126L, 126V, 127E, 127V, 128H, 129L, 130L, 131L). A combined analysis of methyl–TROSY spectra of mutant and wild-type (WT) proteins and a 3D methyl–methyl NOE dataset (mixing time of 200 ms; correlations of the form 1H(CO)NOE–1H(CO)H) was recorded. The observed pattern of intra- and intermolecular NOEs was carefully compared with the network of short-range methyl–methyl distances in the crystal structure (PDB: 3AGY). Stereospecific assignment of Val and Leu methyl groups was achieved by labelling only the δ1-Leu and γ1-Val moieties (proS labelling; NMRBio). A DNAJB1 construct lacking the dimerization domain was used to distinguish between inter- and intradimer NOEs, as well as to validate the assignments of the dimerization domain methyl groups. These combined approaches enabled unambiguous assignment of 95 out of 99 methyl peaks (64 methyl residues).

Backbone dynamics

Measurements of backbone 1H R1 and R2 relaxation rates and steady-state heteronuclear 1H–15N NOEs (hNOEs) were carried out on a 3 mM uniformly 15N-enriched DNAJB1 or sample at 25 °C, pH 7.4, 18.8T using hard- and soft-pulse experiments. The 1H–15N data were acquired using relaxation delays ranging from 2–1,500 ms whereas 1H R1, R2 rates were quantified using relaxation delays from 2–100 ms. R1 values were calculated from R1, R2 rates according to the equation R1 = R1cos2θ + R1sin2θ, where θ = arctan(ωγ/ωα), ωα is the spin-lock field strength (1.0 kHz). Values of relaxation rates were obtained by nonlinear least-squares fitting of the experimental data to a mono-exponential decay function, Aexp(−RT, T) = [R1(R2)]H–15N NOE values were calculated by comparing the peak intensities with and without 1H saturation of 6 s.

Values of R1, R2, and NOE were fitted using the model-free approach with Modelfree software assuming isotropic overall motion, to extract a single correlation time (τc) of 9.5 ns and residue-specific values of order parameters (S).
Paramagnetic relaxation enhancement measurements

Labelling of DNAJB1\textsuperscript{19,35} with a spin-label was achieved by introducing a single cysteine mutation at the desired position (G40, S56 or G105). Purified mutants, stored in 5 mM DTT, were buffer-exchanged into 1 ml of degassed buffer (25 mM HEPES, pH 7.5, 50 mM KCl, 1 mM EDTA, 0.03% NaN\textsubscript{3}), before incubation overnight at 4 °C with threefold molar excess of MTSL spin-label (Toronto Research Chemicals 5-(2,2,5,5-tetramethyl-2,5-dihydro-imidazol-1-yl)methyl methanesulphonothioate). The labelling reaction was terminated by exchanging the sample into the NMR buffer. All samples were submitted for mass confirmation (ESI-MS) to ensure proper labelling, and complete labelling was achieved for all constructs.

The reduced samples were generated through the addition of 100 mM ascorbic acid, pH 7.4 at 4 °C overnight, followed by buffer exchange to the NMR buffer.

Amide 1H cross-relaxation rates were recorded in the oxidized (R\textsuperscript{ox}) and reduced (R\textsuperscript{red}) states to obtain the paramagnetic relaxation enhancement (PRE) rate, R\textsuperscript{ox} = R\textsuperscript{ox} - R\textsuperscript{red}. Relaxation rates were measured using an H\textsuperscript{1}/H\textsuperscript{15} HSQC pulse sequence at 800 MHz using a ten-point measurement, ranging from 0 to 40 ms\textsuperscript{23}. Measured peak intensities in two-dimensional correlation maps were fitted to extract R\textsubscript{nr} with errors estimated from the covariance matrix method\textsuperscript{10}.

Correlations disappeared for some residues in the oxidized state; in these cases a lower bound for R\textsuperscript{ox} was estimated from the relation

\[
\frac{I_{\text{exp}}}{I_{\text{red}}} = \exp \left( -2 R_{\text{red}} \tau \right) \times \left( \frac{R_{\text{ox}}}{R_{\text{red}}} \right)^{2}
\]

where I\textsubscript{red} is the intensity of the correlation in the 15N-H spectrum recorded of the protein with the spin-label in the reduced state while I\textsubscript{exp} is set to 3 times the noise floor. The value of \tau is the time for transfer of magnetisation between H\textsuperscript{1} and 15N spins and is set to 5.3 ms. No notable changes in R\textsubscript{nr} rates were obtained from back-to-back measurements (differences were within experimental errors) and mass-spectrometric analysis showed that samples with the MTSL spin-label remained attached to the protein after each experiment. Site-specific methyl H\textsuberscript{1}F\textsubscript{2} rates were converted to distances from the proton in question to the paramagnetic centre using the formula\textsuperscript{25}:

\[
r = \left( \frac{4 \tau_{1} + 3 \tau_{2}}{1 + \omega \tau_{2}} \right)^{1/6}
\]

where \(\beta = 1.23 \times 10^{-14} \text{ m}^3 \text{ s}^{-1}\) and \(\omega\) is set to 9.5 ns (calculated through backbone relaxation data).

Distance constraints were calculated for each construct, resulted in 88 constraints in total.

Residual dipolar coupling measurements

 Backbone amide 1\textsuperscript{3}D\textsubscript{0} residual dipolar couplings (RDCs) were measured using a 300 \mu M sample of DNAJB1\textsuperscript{19,35} aligned in 4.2% v/v C\textsubscript{6}E\textsubscript{6} (PEG/hexanol (Sigma) or 16 mg ml\textsuperscript{-1} bacteriophage p11 (Asla Scientific). The sample was diluted in 50 mM HEPES PH 7.4, 150 mM NaCl buffer because a higher salt concentration was necessary to reduce the strength of electrostatic interactions between the alignment medium and the protein. 1\textsuperscript{3}D\textsubscript{0} RDCs were measured using IPAP (in-phase anti-phase) HSQC experiments\textsuperscript{20} on a 1.000 MHz Bruker spectrometer and ranged from +30 to -18 Hz.

CS-Rosetta calculations

The structure of the DNAJB1\textsuperscript{19,35} was generated using CS-Rosetta\textsuperscript{33,34}. As a first step, the pick-frags application from the CS-Rosetta toolbox 3.0 was used for fragment selection, based on the backbone chemical-shift data\textsuperscript{35}. Overall, 546 chemical shifts were used as input (95 \textsuperscript{13}C\textsubscript{0} shifts, 84 \textsuperscript{13}C\textsubscript{p} shifts, 94 \textsuperscript{15}N\textsubscript{2} shifts, 94 \textsuperscript{1H}\textsubscript{a} shifts, and 84 \textsuperscript{1H}\textsubscript{a} shifts). The fragment library containing 3- and 9-residue fragments was assembled by scoring both against a library of fragments with chemical shifts predicted from SPARTA\textsuperscript{36}, and against chemical shifts predicted from secondary structure elements.

We then used Rosetta’s Ab initio Relax protocol with the chemical shifts, 1,271 NOEs and 88 distance restraints derived from \textsuperscript{1}H\textsubscript{a} PRE measurements as inputs, to generate 250,000 starting models. Only PRE constraints were included in the preliminary centroid mode step.

The resulting lowest energy 500 models (by total score) were then subjected to local simultaneous refinement of backbone and side chain conformations (Rosetta-relax), incorporating 95 RDCs. Each such run generated 500 models, with the same constraints as the original ab initio modelling. The top 10 models of the resulting approximately 250,000 models (by total score) are shown in Fig. 1g and submitted to the PDB (ID: 6ZSN). Quality analysis of the structures was performed using PDBS 1.5 validation software suite\textsuperscript{37} and PROCHECK-NMR\textsuperscript{38}.

NMR chemical shift perturbations

The interaction of DNAJB1 with HSP70 or HSP70 C-terminal peptide (the last 20 amino acids of the HSP70; GGGAPPSGAGGAPTIEEEDV) was monitored by 2D H\textsuperscript{1}/H\textsuperscript{15}C heteronuclear multiple-quantum correlation (HMQC) methyl-TROSY experiments\textsuperscript{24}. Deuterated HSP70 protein (400 \mu M) or unlabelled HSP70 peptide (800 \mu M) was added to methyl labelled DNAJB1 samples (200 \mu M monomer concentration).

Chemical shift perturbations were calculated from the relation

\[
\Delta \delta = \left( \frac{\Delta \delta_{\text{H}}}{\alpha} \right)^{2} + \left( \frac{\Delta \delta_{\text{C}}}{\beta} \right)^{2}
\]

where \(\Delta \delta_{\text{H/C}}\) is the shift change between methyl group \textsuperscript{1}H (15C) nuclei in apo and fully saturated forms of the protein. \(\alpha (\beta)\) is one standard deviation of the methyl (15C) chemical shifts (separate values of \(\alpha (\beta)\) are used for different methyl groups), as tabulated in the Biological Magnetic Resonance Data Bank (http://www.bmr.b.wisc.edu). Chemical shift perturbations greater than one standard deviation from the mean were considered significant.

NMR titrations

To estimate dissociation constants for the interaction of the DNAJB1 with HSP70 or HSP70 C-terminal peptide (GGGAPPSGAGGAPTIEEEDV), U-[\textsuperscript{2}H\textsubscript{4},1\textsuperscript{5}C\textsubscript{3}]DNAJB1 samples (200 \mu M) were titrated with increasing amounts of deuterated HSP70 (50, 100, 150, 200, 300, 300 and 800 \mu M) or unlabelled C-terminal peptide (20, 40, 80, 120, 160, 200, 240, 280, 320, 360, 400, 480, 600, 1,000, 1,650 \mu M), and the positions of cross-peaks monitored by recording 2D H\textsuperscript{1}/H\textsuperscript{15}C HMBC spectra.

The \(K_{d}\) values for these titrations were calculated by a nonlinear least-squares analysis using the equation

\[
\Delta \delta = \Delta \delta_{\text{MAX}} \left( \frac{[L]}{[L] + [P] + K_{d}} \right)^{4} \Delta \delta_{\text{MAX}} - 4 \frac{[P] [L]}{2 [P]}
\]

where \([P]\) and \([L]\) are the total protein (NMR-labelled) and ligand (unlabelled) concentrations at each aliquot, \(\Delta \delta\) is the change in peak position after each aliquot and \(\Delta \delta_{\text{MAX}}\) is the change in shifts between apo and fully bound states of the protein. Binding isotherms were quantified separately for \textsuperscript{1}H and \textsuperscript{13}C chemical shifts.

HSP70 ATPase activity determination

HSP70 phosphate-release rates after ATP hydrolysis were measured under steady-state conditions by monitoring the change in fluorescence of the phosphate-binding protein (PBP) A197C mutant, which was labelled at Cys197 using 7-diethylamino-3-[N-(4-maleimidoethyl)carbamoyl]coumarin (MDCC, CDX-D0198 from Adipogen). Fluorescence
was measured in a Synergy H1 plate reader by exciting at 430 nm and measuring at 465 nm. All reactions contained PBP, 0.25 μM HisP70 in 50 mM HEPES pH 7.5, 25 mM KCl, 10 mM MgCl₂, and 2 mM DTT, and varying concentrations (2.5, 10, 50, 100, 125, 250 and 500 nM) of the JDPs. After the plate was incubated at 37 °C for 10 min, the reactions were started by injection of 100 μM ATP to each well. Wells were then read every 40 s for the first 20 min, and every 2 min for the next 40 min. For each plate, a series of five phosphate concentrations with PBP alone was measured to generate a calibration curve, which was used to correlate fluorescence to concentration of the released phosphate. All ATPase assays were performed in triplicate.

**Protein recondensation**

Recombinant firefly luciferase (0.2 μM) was incubated for 40 min at 30 °C in denaturation buffer (25 mM HEPES-KOH pH 7.5, 50 mM KCl, 10 mM MgCl₂, 2 mM DTT, 6 M guanidinium chloride). To start the recondensation reaction, the denatured luciferase was diluted 150-fold into recondensation buffer (25 mM HEPES, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.1 mg ml⁻¹ BSA, 1 mM ATP, 20 mM creatine phosphate, 6 μg creatine kinase) supplemented with the indicated chaperones and incubated at 30 °C. Luminescence was measured after 0, 10, 20, 40, 80 and 120 min by addition of 50 μM of luciferase reagent (Promega) to 9 μl of the recondensation reaction on a Synergy H1 plate reader. The concentration of HSP70 and HSP10 was kept constant at 2 and 0.1 μM, respectively. The JDP concentration used in each condition is indicated below:

DNAJB1::HSP70 (0.5:1), DNAJB1(DH5)::HSP70 (0.2:1), DNAJB1 (50A)::HSP70 (0.5:1), DNAJB1(F102A)::HSP70 (0.5:1), DNAJB1::HSP70(ΔEEVD) (2:1), DNAJB1(DH5)::HSP70(ΔEEVD) (2:1), DNAJB1 (50A)::HSP70(ΔEEVD) (2:1), DNAJB1(F102A)::HSP70(ΔEEVD) (2:1), DNAJB2::HSP70 (0.5:1), DNAJB2::HSP70(ΔEEVD) (0.5:1).

**Förster resonance energy transfer experiments**

Fluorescent labelling of HSP70(S494C) and HSP70(ΔEEVD,S494C) was performed at position 494 (S494C) in an otherwise cysteine-free background (C267A, C574A, C603A) and purified as described. Before labelling, chaperones were incubated with 10 mM DTT for 30 min, followed by exchange into a reducing-agent-free buffer by passing the protein through a desalting column (PD MiniTrap G-25, GE Healthcare). Labelling was achieved by incubating the proteins with tenfold molar excess of Alexa Fluor 488 C5-maleimide (Invitrogen) or Alexa Fluor 594 C5-maleimide (Invitrogen) fluorescent dye for 2 h at 25 °C and in the presence of 0.5 mM TCEP. Excess label was removed by a desalting column (PD MiniTrap G-25, GE Healthcare). Labelling efficiencies were determined by absorbance and exceeded 90%.

Förster resonance energy transfer (FRET) experiments were performed with 200 nM of mixed donor (AF488) and acceptor (AF594) and incubated with 20 μM α-synuclein fibrils and indicated J-domain proteins in 50 mM HEPES-KOH pH 7.5, 50 mM KCl, 5 mM MgCl₂, 2 mM DTT and 2 mM ATP. Samples were allowed to equilibrate for 1 h at 25 °C. Emission spectra between 500 and 700 nm were recorded using a CLARIOstar plate reader (BMG LABTECH) at an excitation centre at 480 nm with a bandwidth of 10 nm and emission bandwidth of 10 nm. FRET efficiencies were determined from the ratios between donor and acceptor fluorescence I₅₉₀nm/I₄₈₀nm.

To quantify the effect of the different J-domain proteins on HSP70 clustering, FRET efficiencies were measured in the presence of an excess of the indicated JDP (5 μM) and expressed as a fraction of the FRET efficiency measured with a stoichiometric concentration of the same JDPs (200 nM).

**Fluorescence anisotropy measurements**

Steady-state equilibrium binding of DNAJ2, DNAJB1, and DNAJB1(DH5) chaperones to preformed α-synuclein fibres was measured by fluorescence anisotropy using 200 nM of fluorescently tagged chaperones (DNAJB1(G194C-AF594), DNAJB1(DH5,G194C-AF594), or CCGFPC- DNAJA2-FLASH40). Samples were allowed to equilibrate for 1 h at 22 °C and data was acquired by a CLARIOstar plate reader (BMG LABTECH) with excitation at 590 nm with a bandwidth of 50 nm, emission at 675 nm with a bandwidth of 50 nm and dichroic filter of 639 nm for AF594 detection and excitation at 482 nm with a bandwidth of 16 nm, emission at 530 nm with a bandwidth of 40 nm and a dichroic filter at 504 nm for FLASH detection. Data were fit to one-site binding model using GraphPad Prism 6.

Steady-state equilibrium binding of DNAJB1 and DNAJB1(DH5) to HSP70(T204A) was measured by fluorescence anisotropy using 25 nM of fluorescently tagged chaperones (DNAJB1(G194C-AF488), DNAJB1(DH5,G194C-AF488)). Samples were allowed to equilibrate for 30 min at 37 °C and measurements were performed on a Tecan SPARK 10M plate reader in black, flat-bottom 384-square well plates. The excitation filter was centred on 485 nm with a bandwidth of 20 nm, and emission filter was centred on 535 nm with a bandwidth of 25 nm. Data were fit to a one-site binding model using OriginPro v.2018.

Steady-state equilibrium binding of HSP70 to α-synuclein fibrils as a function of JDP concentration was measured by fluorescence anisotropy using 200 nM of fluorescently tagged HSP70(S494C-AF488) (C267A, C574A, C603A) preincubated for 1 h at 22 °C with 0.2 μM preformed α-synuclein fibrils and the indicated concentrations of DNAJB1, DNAJB1(DH5) or DNAJA2. Anisotropy measurements were performed in a CLARIOstar plate reader (BMG LABTECH) with excitation at 482 nm with a bandwidth of 16 nm, emission at 530 nm with a bandwidth of 40 nm and a dichroic filter at 504 nm.

**Co-sedimentation assay**

HSP70 (10 μM) and preformed α-synuclein fibrils (10 μM) were incubated with the indicated concentration of DNAJB1, DNAJB1(DH5) or DNAJA2 and 2 mM ATP for 1 h at 22 °C. Samples were centrifuged at 10,000g for 10 min, the supernatant was removed and pellets were gently washed with buffer (50 mM HEPES-KOH (pH 7.5), 50 mM KCl, 5 mM MgCl₂, and 2 mM DTT). The content of the pellet fractions was analysed by SDS–PAGE.

**Disaggregation assay**

Disaggregation of amyloid fibrils by chaperones was monitored by changes in thioflavin T (ThT) fluorescence over a 12–16 h period using a FLUOstar Omega plate reader (BMG LABTECH), excitation at 440 nm, emission at 480 nm. 1 μM preformed α-synuclein fibrils were incubated with 2 μM HSP70, 1.0 μM DNAJB1 (50A), 0.1 μM HSP110 (unless otherwise indicated), 2 mM ATP, an ATP-regeneration system (8 mM PEP and 20 μg pyruvate kinase), and 30 μM ThT at 30 °C in 50 mM HEPES-KOH (pH 7.5), 50 mM KCl, 5 mM MgCl₂, and 2 mM DTT. Background ThT fluorescence of buffer and chaperones was subtracted, and all intensities were normalized to the fluorescence intensity at t = 0 min. Data shown are representative traces of 3 independent experiments.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability**

The data that support the findings of this study are available from the corresponding authors upon reasonable request. NMR chemical shifts have been deposited in the Biological Magnetic Resonance Data Bank under the following accession codes: 50169 for DNAJB1, 50168 for DNAJA2 and 50167 for DNAJB1. The structure of DNAJB1 has been deposited to the Protein Data Bank (PDB) under accession code 6ZSN. Source data are provided with this paper.
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Correspondence and requests for materials should be addressed to R.R. or R.R.

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