Auto-regulatory J-domain interactions control Hsp70 recruitment in the oligomeric DNAJB8 co-chaperone

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Abstract

The Hsp40/Hsp70 chaperone families combine a versatile folding capacity with high specificity, which is mainly facilitated by Hsp40s. The structure and function of many Hsp40s remain poorly understood, with the oligomeric Hsp40s particularly refractory to characterization. Yet, structural insight on the architecture and dynamics of these oligomeric Hsp40s will help explain their potent ability to suppress polyglutamine and other amyloid protein aggregation. Here, our combined in vitro, in vivo, and in silico studies shed new light on the supramolecular structure of the homooligomeric Hsp40 DnaJB8 and how it regulates recruitment of partner Hsp70s. We identify in the oligomers an interaction between the J-Domain (JD) and the beta-sheet-rich C-terminal domain (CTD), causing the sequestration of the JD, and occlusion of its surface necessary for Hsp70 recruitment. We propose a new model for DnaJB8 activity in which a built-in autoinhibitory switch reversibly controls the recruitment of Hsp70. These results provide evidence that the evolutionarily conserved CTD of DnaJB8, and related Hsp40s, serves as an essential regulatory element of their pivotal role in cellular proteostasis.
Molecular chaperones play central roles in cellular homeostasis by facilitating protein folding, directing protein folding quality control, and refolding stress-denatured substrates. Structural descriptions of chaperone architecture and conformational cycles have been key to understanding their biological function. Thus, functional insight into chaperones relies on structural biology tools such as NMR, cryo-EM and X-ray crystallography. This structural information has been used to identify substrate binding sites, key conformational changes that drive substrate release, and the role of ATP hydrolysis. A molecular view of chaperones is essential to explain chaperones' biological function in protein folding and suggest new avenues for treating protein misfolding diseases.

The cellular chaperone network needs to handle a wide variety of protein substrates in numerous different (mis)folded states. This demands a combination of broad versatility and specificity in terms of substrate recognition, even though the central players Hsp70 and Hsp90 are highly conserved. This apparent contradiction is resolved by the Hsp40 (DnaJ) family of proteins, which are co-chaperones that recruit and regulate the activity of Hsp70 chaperones in refolding misfolded proteins. While the human Hsp70 family is highly conserved, the Hsp40 co-chaperone family encodes 47 diverse members, each with specialized functions in substrate recognition and presumed coordination with Hsp70. DnaJ proteins feature a J-domain (JD), which binds to Hsp70s through a conserved electrostatic interaction to trigger ATP hydrolysis by the Hsp70. This in turn initiates a conformational rearrangement in the substrate binding domain of the Hsp70 that helps capture an unfolded substrate for folding, refolding, or disaggregation.

In cases when misfolded proteins cannot be refolded, some Hsp40s help direct proteins for degradation. However, the mechanistic details of how different Hsp40s recruit Hsp70 or their substrates remain unknown. Furthermore, while it is known that the Hsp70 can interact with JDs through electrostatic surfaces it remains unclear how JD binding to Hsp70 is controlled to specific and/or activated Hsp40 proteins.
In humans, Hsp40s are classified into A, B, and C sub-families, in which members typically function as monomers, dimers, or polydisperse oligomers. For example, the A family Hsp40s (DnaJA1-A4) and a subset of B family Hsp40s (DnaJB1/B4/B5), all encode a globular domain architecture similar to the bacterial Hsp40 DnaJ. These classical Hsp40 members assemble into homo-dimers through conserved C-terminal motifs and bind unfolded substrates through conserved β-barrel C-terminal domains (CTD). Recent evidence suggests that this subset of Hsp40s can form mixed hetero-dimers driven by electrostatic contacts to expand the substrate recognition repertoire.

A different set of non-classical Hsp40 B family members (DnaJB2, DnaJB6b, DnaJB7 and DnaJB8) encode a domain architecture that is distinct from the classical dimeric DnaJ orthologs. Rather than encoding a classical β-barrel CTD, their C-terminus encodes a different architecture. Of these 4 members, the DnaJB8 and DnaJB6b proteins have been shown to assemble into oligomers in vitro and in vivo. The role of the CTD in these chaperones remains unclear, and the literature reveals conflicting results, suggesting either that it drives oligomerization or that it mediates intramolecular contacts. The functional role of the oligomeric state of DnaJB6b and DnaJB8 remains unclear, although it has been proposed to serve as a type of phase-separated storage form capable of capturing substrates. The characteristic structural and dynamic heterogeneity of the oligomers has greatly hindered efforts to study them, with recent structural studies resorting to deletion mutants to gain structural insight. A combination of variables, including large size and polydispersity, has made these chaperones recalcitrant to both classical and modern structural biology methods, including cryoEM.

The oligomeric chaperones DnaJB6b and DnaJB8 have been shown to be potent suppressors of aggregation of amyloid proteins in cells and in vitro. Despite several studies demonstrating a role for DnaJB6b in suppressing assembly of amyloid-prone substrates it is unclear exactly how it interacts with substrates, and how oligomerization...
plays a role in this process. The serine/threonine domain of DnaJB6b is important for suppression of mutant huntingtin protein aggregation in cells,\(^{27}\) and its deletion limits oligomerization yielding a stable monomer at the expense of activity\(^{23}\). In absence of a comprehensive understanding of the full-length structural behavior for this class of DnaJ chaperones, it remains unclear how they fulfill their critical function to protect the proteome from aggregation.

Here we examine in unprecedented detail the DnaJB8 variant. DnaJB8 was shown to be particularly effective at preventing formation of polyglutamine deposits, even more so than DnaJB6b, despite 63 % sequence identity\(^{22, 27}\). Notably, unlike other chaperones that inhibit mutant huntingtin aggregation\(^{28}\), DnaJB8 and DnaJB6b are thought to target and bind the expanded polyglutamine domain itself and thus are active across the whole family of polyglutamine diseases\(^{22, 27}\). The assembly of DnaJB8 into dynamic and heterogeneous high molecular weight complexes (polydisperse oligomers) has thus far prevented their detailed characterization by traditional structural biology approaches. To address this challenge, we applied a hybrid structural biology approach to understand the architecture and dynamics of the DnaJB8 oligomer. We observe that DnaJB8 assembles into large oligomers \textit{in vitro} and \textit{in vivo}. We used crosslinking mass spectrometry (XL-MS) to identify local intra-domain contacts and long-range contacts mediated by complementary electrostatic surfaces. We show that changes in ionic strength modulate the assembly and topology of oligomer, which points to the important role of charge-charge interactions. Using multidimensional solid-state NMR (ssNMR) we probe the structural and dynamic disorder of the solvated oligomers. Dramatic domain-specific differences in line widths, resolution, and peak intensity indicate that the oligomers feature a range of (dis)order, but lack highly flexible regions. The JD transitions between a strikingly ordered immobilized state and a more mobilized state in response to electrostatic disruption. We show that the JD and CTD terminal domains are monomeric in isolation, but form heterodimers when mixed, reconstituting interactions observed in oligomers. Finally, we demonstrate that the DnaJB8 JD-CTD contacts regulate the
recruitment of Hsp70. Thus, we identify and validate the first example of a DnaJ protein encoding a built-in regulatory domain that controls its recruitment (and activation) of Hsp70.

RESULTS

DnaJB8 JD binds to HspA1A using a conserved electrostatic surface

Recent crystallographic analysis of the bacterial DnaK:DnaJ complex revealed the binding mode of the complex, which validated previous insight from NMR and biochemical data. The model proposed an electrostatically driven interaction between the basic surfaces on helices 2 and 3 on the DnaJ and an acidic surface on the DnaK that spans the Nucleotide Binding Domain (NBD) and the Substrate Binding Domain (SBD). The JD includes a conserved Histidine-Proline-Aspartic acid (HPD) motif between helices 2 and 3 that recruits Hsp70 and stimulates ATP hydrolysis for substrate capture. Using the DnaK model (PDB id: 5NRO) as a template we employed Rosetta to build a homology model of the human HspA1A homolog. The HspA1A model shows good structural agreement with the DnaK homolog (Figure 1A) and also presents a conserved acidic surface that spans the NBD and SBD (Supplementary Figure 1A-C). Using the DnaK:DnaJ complex structure, we modeled the binding interface between the HspA1A homology model and the experimentally determined structure of the JD of DnaJB8 (Figure 1B-C). The basic surface on the DnaJB8 JD (Figure 1B) contacts the conserved acidic surface on the HspA1A (Figure 1C, S1D-E). Thus, conserved electrostatic contacts are likely to play a key role in the interaction between Hsp70 and Hsp40.

The DnaJB8 co-chaperone is predicted to encode three domains C-terminal to the N-terminal JD (Figure 1D): a phenylalanine and glycine (F/G) rich domain (Figure 1D, blue), a serine/threonine rich (S/T) domain (Figure 1D, cyan) and a C-terminal CTD (Figure 1D, green). Secondary structure analysis on the DnaJB8 sequence reveals the N-terminal JD fold but also predicts the CTD to adopt a five-stranded β-pleated sheet, consistent with the conformation observed in the homologous DnaJB6b (Figure 1E).
The basic surface on JD helix 2 in DnaJB8 mediates binding to Hsp70, but we see that there is surface charge complementarity to the CTD, which is similar to the same complementarity expected from the JD-Hsp70 interaction (Figure 1F). The middle F/G and S/T domains are unstructured (Figure 1G) and contain sequences with lower complexity, particularly in the S/T domain. In studies of DnaK/DnaJ, it was seen that the DnaK F/G domain does not bind directly to its Hsp70 counterpart, but it is involved in regulation of the substrate binding activity of DnaK\(^{10}\). In patients, mutations in the F/G domain of the homologous DnaJB6b (Supplementary Figure 1F) (including F89I, F93L, P96R and F100V) cause autosomal limb-girdle muscular dystrophy diseases that exhibit similar, but not identical clinical outcomes. Recent studies have shown that the JD-proximal region of the F/G domain in DnaJB6b can adopt a short helix that weakly interacts with the JD\(^{23}\), which may be relevant to the disease-causing mutations since they are found within this helix\(^{24,27}\). The low complexity S/T domain has been proposed to play a key role in the activity of DnaJB6b to inhibit polyglutamine aggregation\(^{27}\). Altogether, it is clear that charge complementarity is important for the Hsp70:Hsp40 interaction, but that there is also a role for DnaJB8 interactions and domains beyond the JD.

**Interactions of oligomeric DnaJB8 in a cellular context**

Prior studies have highlighted the ability of DnaJB8 to assemble into oligomers but have failed to provide insights into the interactions that drive oligomer formation\(^{22,26}\). We first asked whether DnaJB8 can form oligomers in cells by expressing DnaJB8 fused to a green fluorescent protein (GFP) derivative: Clover (herein, DnaJB8-Clover) in HEK293 cells (Figure 2A). We observed the formation of fluorescent juxtanuclear puncta that localize close to the nucleus (Figure 2B). Puncta were not observed in control cells expressing Clover alone (Figure 2B). When quantified, 39.2±3.1% of the cells expressing DnaJB8-Clover contain puncta while only 0.44±0.50% of the control cells contained puncta (Figure 2C). To test the concentration dependence on puncta formation, we assayed the frequency of puncta with different levels of expression and observed 4-fold
lower protein levels reduced the DnaJB8-Clover puncta frequency to 17.5±10.3% (Figure 2C). The puncta frequency for Clover alone remained below 1% in the two experiments (Figure 2C). From microscopy images we estimate the DnaJB8-Clover puncta to have a maximum diameter of 1.0μm (Figure 2B).

We next sought to characterize biochemical properties of DnaJB8-Clover expressed in mammalian cells. To isolate the Clover protein, we used α-GFP nano-bodies allowing immunoprecipitation (IP) of the DnaJB8-Clover fusion and Clover from HEK293 cells. Western blot analysis of the purified IP fractions confirms the presence of DnaJB8 and Clover using α-GFP and α-DnaJB8 antibodies (Supplementary Figure 2A) in the DnaJB8-Clover and control cell lines. The IP purified samples were first analyzed by a proteomic mass spectrometry approach to identify any candidate proteins associated with DnaJB8-Clover. Using the CPFP software we identified 60 proteins that were enriched in the DnaJB8-Clover samples relative to the control Clover sample (Supplementary Figure 2B and Supplementary Data 1). Among the enriched proteins we found other chaperones including the eukaryotic chaperonin TRiC/CCT, Hsp90A/B, HspD1, and two Hsp70 family members: HspA8 and HspA1A (Supplementary Figure 2C). A priori, one might expect to see an enrichment in Hsp70, but the levels of Hsp70 enrichment in the IP were minor relative to the Clover controls, suggesting that the DnaJB8:Hsp70 interaction is weak under the conditions used, despite the known Hsp40:Hsp70 relationship.

Combining crosslinking with mass spectrometry can help define contact points in proteins and protein complexes, with particular importance for systems not tractable for classical structural biology methods. After isolation of DnaJB8-Clover using an α-GFP nanobody IP, we reacted the samples with a combination of adipic acid dihydrazide (ADH) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM), respectively. ADH covalently links carboxylate-carboxylate contacts via a 6-carbon bridge, while DMTMM acts as a chemical intermediate to form a direct covalent bond between lysine-carboxylate groups through dehydration. The latter reaction is referred to as a “zero-length” crosslink since no additional atoms are added in order to link the two
target groups. Crosslinking treatment yielded a high molecular weight species, consistent with an oligomeric form (Figure 2D). To identify whether inter-domain contacts were present in these oligomers, we employed XL-MS on the DnaJB8-Clover purified by IP from mammalian cells. We identified 1 carboxylate-carboxylate and 20 lysine-carboxylate crosslinks that parsed into three coarse regions: JD-JD, CTD-CTD and JD-CTD (Figure 2C, Supplementary Data 2). The three local JD contacts (Figure 2C; JD contacts in red box) linking K25-D37, K34-D37 and E41-K34 are consistent with the known structural model of the domain (Supplementary Figure 2D). Local CTD contacts were also accompanied by inter-domain JD-CTD contacts that localize to helices 2 and 3 (Figure 2C, JD-CTD contacts in grey box). Additionally, we identified a contact between K34 in the JD and D97 that is consistent with a weak interaction between the JD and a putative helix 5 of the F/G domain identified in the homologous DnaJB6b. DnaJB8-Clover oligomers isolated from mammalian cells reveal an array of inter-domain interactions, including contacts between the charge complementary JD and CTD.

DnaJB8 JD-CTD contacts are preserved in vitro

To enable a more detailed understanding of DnaJB8 oligomerization, we sought to reconstitute DnaJB8 oligomers in vitro. Full length DnaJB8 was produced recombinantly and purified as previously described. We used Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) to detect DnaJB8 oligomers forming in solution over time. We initially observe DnaJB8 species with an average radius of hydration (R_h) of 13.7±2.5nm which after 8 hours steadily increase in size, eventually reaching a maximum average R_h of 91.9±12.6nm after 15 hours (Figure 3A, Supplementary Data 3). While the average R_h is informative for interpretation of the kinetics of assembly, the derived values do not reflect real species. Partitioning the scattering data into bins with different radii reveals bona fide sizes: DnaJB8 primarily begins as a small species (5.54±2.36nm) with a small (<%1 by mass) contribution of larger species (>100nm) but as aggregation progresses the small species are converted into larger assemblies (30.6% by mass) (Supplementary Figure 3A). We next used negative-stain TEM to visualize the
DnaJB8 oligomers at 15 hours where we first observed the formation of larger species by DLS. Upon imaging the grids, we found elongated polydisperse oligomeric structures with varying lengths around 90nm, and widths of approximately 20nm along with amorphous oligomeric species (Figure 3B). These findings are consistent with prior studies on DnaJB6b and DnaJB8 showing that it can form oligomers in vitro.\textsuperscript{22, 24-25, 27, 39}

Next, we aimed to better understand the topology of these in vitro oligomers by XL-MS, using two parallel chemistries; disuccinimidyl suberate (DSS) and ADH/DMTMM (Figure 2D). Consistent with the formation of oligomers, we observe by SDS-PAGE a ladder of bands indicating the covalent trapping of intermolecular contacts (Supplementary Figure 3B). Notably, the ADH/DMTMM chemistry yielded a higher efficiency of trapping oligomeric species than DSS, which has been previously used to probe structure of the related DnaJB6b.\textsuperscript{25} XL-MS analysis of these samples showed only three crosslinks in the DSS condition (Figure 3C, Supplementary Data 2). In contrast, the ADH/DMTMM analysis yielded 18 lysine-carboxylate crosslinks and 6 carboxylate-carboxylate crosslinks that localize to three main regions (Figure 3C, Supplementary Data 2): within the JD (Figure 3C, red dashed box), within the CTD (Figure 3C, green dashed box), and contacts between JD and CTD (Figure 3C, grey dashed box). This pattern closely matches those observed in the assemblies recovered from the mammalian cells above. Within the JD, there are five crosslinks; one carboxylate-carboxylate (D33-E43) and four lysine-carboxylate crosslinks (K34-E40, K34-E41, D37-K45 and E54-K60) (Figure 3C). Out of these five crosslinks, four of them are localized around the loop between helix 2 and helix 3, where the HPD motif responsible for Hsp70 binding is located. Mapping the crosslinks onto the structure of the JD showed that the Cα-Cα distances varied across zero-length crosslink pairs from 11.6Å to 14.7Å and the single ADH pair spanned 8.9Å (Supplementary Figure 3C). These Cα-Cα distances are consistent for each chemistry, with zero-length crosslink pair distances falling under 16Å and ADH-derived distances spanning less than 20Å.\textsuperscript{37} In addition, the side chains in each amino acid pair are solvent accessible in the DnaJB8 JD structure (Supplementary Figure 3C). These data suggest that the local JD crosslinks are consistent with intramolecular contacts within a JD monomer.
The CTD region of DnaJB8 yielded two ADH-based crosslinks (E208-E224 and E210-E224) and six lysine-carboxylate zero-length crosslinks (E211-K227, E208-K227, E210-K227, E211-K223, K193-E211, and E211-K232) (Figure 3C). Amongst these, the local regions spanning E208-E211 and K223-K227 are central to the CTD and repeatedly react to peripheral sites. The third cluster of contacts linked the distal JD and CTD through four lysine-carboxylate crosslinks (K34-D212, K47-E208, K47-E211, and K60-E211) (Figure 3C, inset). These long-range crosslinks span an average sequence separation of 163.5 amino acids separated by the central unstructured F/G and S/T domains. The CTD crosslink sites are exclusively mediated through acidic amino acids; D212, E209 and E211. Interestingly, the crosslinked amino acids on the JD are all lysines (K34, K47 and K60) that localize to basic surfaces along each helix (Supplementary Figure 3D) and overlap the basic Hsp70 binding surface. Consistent with the ex vivo analysis we again identified an isolated crosslink between the JD and the proposed helix 5 in the F/G domain. Clearly, these XL-MS data point to an intricate network of inter-domain interactions in the oligomeric DnaJB8.

To further test the apparent role of electrostatically driven inter-domain interactions, we used a higher ionic strength buffer in an analogous series of experiments. Using DLS we found the average $R_h$ to remain stable at 13.7 ± 1.2 nm over time (Figure 3D, Supplementary Data 3). A closer inspection of the scattering data reveals a predominance of smaller species (7.75±0.7nm) alongside a small and unchanging contribution of larger species (<1% by mass) (Supplementary Figure 3E). TEM showed that the latter species are spherical with 10-20nm in diameter (Figure 3E). Thus, increased ionic strength inhibits the formation of the more heterogenous larger (>100 nm) structures. DLS also shows that the predominant species are more collapsed (5.54±2.36nm) in the low ionic strength condition compared to that found in the higher salt (7.75±0.7nm) (Supplementary Figure 3F). XL-MS demonstrates that while some short-range crosslinks within either the JD or the CTD are preserved, the long-range inter-domain contacts have now been lost (Figure
To control for reactivity in each buffer condition we compared the frequency of ADH driven monolinks. Monolinks occur when a chemical crosslinker reacts with a solvent exposed residue on the target protein, but no other corresponding residues are in close physical proximity to attack the other end of the crosslinker. These peptide fragments are useful for identifying isolated surfaces, as well as validating the efficiency of the crosslinker chemistry. The data show that the higher ionic strength has little effect on the chemistry of DMTMM on acidic residues or the ability of ADH to target those same residues (Supplementary Figure 3G). Thus, we observe the disruption of electrostatically driven JD-CTD interactions being accompanied by changes in the domain architecture as well as higher order assembly of DnaJB8 oligomers.

**ssNMR on DnaJB8 oligomers reveals regions of disorder and order**

To gain additional insight into their molecular structure and dynamics, multidimensional magic-angle-spinning (MAS) ssNMR was performed on the hydrated oligomers of U-$^{13}$C, $^{15}$N-labeled DnaJB8. The MAS ssNMR signals of hydrated protein assemblies allow for the site- and domain-specific detection of mobility and (secondary) structure, even in presence of considerable disorder and heterogeneity. In 2D ssNMR spectra we observe many broad peaks, with linewidths up to 0.38 kHz, consistent with an oligomeric assembly displaying structural disorder (Figure 4A). However, strikingly, distinct subsets of narrow peaks are also detected, with 1D projections showing linewidths of 0.1 to 0.2 kHz (Figure 4C-D left). These experiments employ the cross-polarization (CP) technique, which means that the observable residues must be rigid or immobilized, lacking high flexibility. This conclusion is reinforced by a lack of signals in INEPT-based ssNMR spectra that are selective for highly dynamic peptide segments 41-43 (more below). Then, the observed narrow signals must originate from an immobilized, well-ordered subset of DnaJB8 residues. Based on the characteristic NMR shifts of amino acid types (Supplementary Figure 4A)44, we can attribute these narrow signals to residues in the JD (Table 1) while the broad peaks are dominated by signals from residues common in other domains (Table 1). The narrow signals reflect mostly $\alpha$-helical structure, unlike the broader signals that
are mostly random coil and β-sheet. Finally, with known chemical shifts of the DnaJB8 JD in solution (see also below), we prepared a synthetic 2D spectrum (Supplementary Figure 4B) that reveals a striking correspondence to the narrow ssNMR peaks (Figure 4C-D). The correspondence is such that we tentatively assign the strikingly narrow signals (Supplementary Figure 4A,B) to residues in helix 2 and helix 3 (10V, 13S, 14A, 15S, 19I, 22A, 27A, 42A, 52A). In absence of experimental solution NMR data for the other domains, we predicted estimated spectra based on their model structures. Figure 4C-D (green) shows Ala and Ser regions of their combined simulated NMR spectrum, resembling qualitatively the broader signals in our 2D ssNMR data. A particular strength of MAS ssNMR analyses of such hydrated proteins is the ability to gauge local and global dynamics. Single pulse excitation (SPE) and refocused INEPT ssNMR spectra, which enhance the more dynamic parts of samples, show surprisingly little evidence of flexible residues (Figure 4E red). The main INEPT signal at ~42ppm arises from Lys side chains, which are expected to be solvent exposed and dynamic. Since the 1D CP and SPE spectra look similar, with higher signal intensities in the former, the different domains of the protein must have a similar degree of mobility (Fig. 4E). Combined, the ssNMR data reveal oligomers that are heterogeneous in structure but lack extended flexible domains. In other words, the central F/G and ST domains are heterogenous, but also substantially immobilized within the oligomers. Uniquely ordered are parts of the JD (residues in helices 2 and 3), which appear to be well-folded and immobilized.

**Interaction sites from ssNMR**

In the JD structure (Figure 4F-I) these “ordered” residues form a surface region rich in positively charged amino acids, involving helix 2 and 3, as well as residues connecting these helices. What is responsible for stabilizing and immobilizing this part of the JD? XL-MS showed long-range contacts from the JD to the CTD, involving K34, K47, and K60 in helices 2, 3 and 4, which disappeared at elevated ionic strengths. Analogous ssNMR studies of the oligomers in PBS buffer with 285mM NaCl are shown in Fig. 4B and S4. The 2D CP-based ssNMR spectrum reproduces the broad signals of the immobilized
oligomers, but the narrow JD peaks are strikingly absent. Comparing the CP and SPE ssNMR spectra (Figure 4E), there is a clear increase in mobility, but again there is a lack of INEPT signals from highly flexible regions. Notably, although the well-ordered JD signals are no longer seen as immobilized (i.e., visible in CP ssNMR), no new “flexible” ssNMR signals were identified under high salt conditions. We interpret this as indicating that the JD gains increased mobility due to disruption of its long-range electrostatic interactions but is still folded and partly immobilized by its covalent attachment to the overall assembly. As a consequence, the JD is invisible due to intermediate timescale dynamics. Since the broad signals from the other domains are preserved, it appears that the core architecture of the oligomers persists even at high ionic strength, such that it is primarily the JD that is dislodged as electrostatic long-range interactions are weakened.

**Isolated JD and CTD are folded and monomeric**

To evaluate the potential inter- and intra-domain interactions of the JD and CTD, we produced isolated JD (residues 1-77; JD1-77) and CTD (residues 170-232; CTD170-232) (Figure 5A). By size exclusion chromatography (SEC) both constructs yielded monodisperse peaks (Figure 5B), with an estimated molecular weight of 10kDa and 8kDa for JD1-77 and CTD170-232, respectively. The CTD has been proposed to drive dimerization and multimerization in the homologous DnaJB6b, but by Size Exclusion Chromatography - Multi Angle Light Scattering (SEC-MALS) the molecular weight for the DnaJB8 CTD170-232 is unambiguously a monomer with a molecular weight of 8,376±14 g/mol (Supplementary Figure 5A). Also, by DLS in solution we estimated the JD1-77 to be 2.30±0.12 nm and the CTD170-232 to be 1.71±0.02 nm, with both stable over 15 hours (Supplementary Figure 5B, Supplementary Data 3). The purified constructs were used for XL-MS to probe the structural similarities to the corresponding domains within the full-length protein. On an SDS-PAGE gel, the crosslinked JD1-77 and CTD170-232 remained monomeric following crosslinking, while the bands broadened as a result of the chemical modification (Figure 5C). XL-MS analysis yielded 4 crosslinks for JD1-77 and 6 crosslinks.
for CTD\textsubscript{170-232} (Figure 5D, Supplementary Data 2). The identified crosslinks revealed good agreement between the local domain crosslinks observed in the full-length DnaJB8 and the isolated JD\textsubscript{1-77} and to a lesser extent for CTD\textsubscript{170-232} (Figure 5D, S5C-D).

We built 5000 predictive models of the CTD using \textit{ab initio} ROSETTA which employed fragment libraries derived from experimental structures\textsuperscript{40}. We calculated the $R_h$ for the ensemble using HYDROPPRO and the resulting values were consistent with the DLS measurement of 1.7nm (Figure 5E). The models reproducibly formed a low contact order 5-stranded $\beta$-sheet topology (Figure 5E) and the $R_h$ variation can be attributed to the more flexible termini (Figure 5E, inset). The CTD topology is defined by four $\beta$-turns stabilized by conserved asparagine/aspartate-glycine sequences (N/DG). These structures are consistent with the recently published conformation of the DnaJB6b CTD\textsuperscript{23}. Circular dichroism (CD) on the CTD sample yields spectra consistent with a predominantly $\beta$-sheet content, as predicted by our model (Supplementary Figure 7E-F).

We mapped the 14 CTD-derived crosslinks from across experiments onto the monomeric ensemble, finding that a majority of structures explain 10-11 crosslinks but only a single model explains 13 of 14 (Figure 5F). These crosslink pairs map onto each face of the $\beta$-sheet and the distances are compatible with the geometry of the crosslinking chemistry. The crosslinks that fall outside of the distance cutoff localize to the more dynamic C-terminus of CTD (Figure 5E, inset) at positions K227 and K223 (Figure 5F). Thus, our data support that both the JD\textsubscript{1-77} and CTD\textsubscript{170-232} domains are folded, monomeric and do not have intrinsic assembly properties.

**Basic surface on JD drives interaction with CTD**

In our experiments on the full-length DnaJB8 oligomers we saw that the JD and CTD interact through complementary electrostatic surfaces. We further probed this interaction by mixing the individual JD\textsubscript{1-77} and CTD\textsubscript{170-232} domains \textit{in vitro} (Figure 6A). We incubated Flourescein(FITC)-labelled JD\textsubscript{1-77} with a series of CTD\textsubscript{170-232} concentrations and measured binding affinity using a fluorescence polarization assay. The resulting binding curve revealed that the JD\textsubscript{1-77} binds to the CTD\textsubscript{170-232} with 0.575$\pm$0.115 $\mu$M affinity (Figure
Preincubated JD1-77 and CTD170-232 domains were crosslinked with ADH/DMTMM and analyzed using XL-MS. We identified local crosslinked pairs consistent with pairs observed in full length DnaJB8 and the isolated JD1-77 and CTD170-232 samples (Figure 6C). Importantly, we also reconstitute intermolecular contacts between the JD1-77 and CTD170-232 observed in full length DnaJB8 experiments. However, an increased variance in the crosslink profile may indicate that the missing proximal sequences help define the proper architecture of the full-length DnaJB8 oligomers. Solution NMR-based chemical shift mapping was used to identify the JD1-77 surface that interacts with the CTD170-232 (Figure 6D). Titration of increasing amounts of unlabeled CTD170-232 into 15N-labeled JD1-77 produced concentration-dependent shifts in a specific subset of peaks (Figure 6D-E); 33 peaks were strongly perturbed (>0.2 p.p.m.) and another 40 peaks were perturbed weakly (>0.1 p.p.m; Fig 6F). Among the strongly perturbed peaks, 27 residues have solvent accessible side chains of which fifteen are charged, with a notable overlap with the basic face in helix 2 that is implicated in Hsp70 binding (Figure 6H-I). Other residues that show strong perturbations are basic and acidic residues that wrap around the exterior face of the JD loop and helix 3 (D33, K39, E40, E41, K44, K47, E51), and residues on the charged face of helix 4 (K60, K61, R62, L64, Y65). While a few other hydrophobic residues also show strong perturbations, all are in close proximity to charged residues along each helix. Given the small size of the JD1-77, it is likely that residues in the core behind the basic surface involved in the interaction experience chemical shifts.

**Sequence properties driving interactions in DnaJB8**

Using Rosetta, we generated a full-length model of DnaJB8 combining the solution NMR structure of the JD (PDBID: 2DMX) with the ab initio model for CTD while keeping the F/G and S/T region expanded. Using the relax module in Rosetta we applied our JD-CTD XL-MS crosslink pairs as constraints to collapse the expanded full-length DnaJB8 monomer (Figure 7A and Supplementary Figure 7A). The constraints drive the collapse of the molecule to decrease the distance between the crosslink pairs (Figure 7A and Supplementary Figure 7A) from an $R_g$ of 6.65 nm in the expanded state to a much smaller...
2.45 nm (Figure 7C and Supplementary Figure 7B). Using the Hydropro software, we obtain \( R_h \) values of 9.27 nm and 4.02 nm, respectively, for each structural model. Comparing the DLS \( R_h \) size ranges for the small species in the low and high ionic strength conditions to the structure-derived \( R_h \) values suggests congruency in observed shapes dependent on presence or absence of JD:CTD contacts. The calculated \( R_h \) values for the collapsed (JD:CTD engaged) Rosetta monomer conformation is consistent with the lower end DLS size distribution obtained under low ionic strength. This is further supported by comparing the \( R_h \) of our Rosetta model to a theoretical disordered protein containing 232 amino acids to be 3.98 nm as modeled by Marsh and Forman-Kay. We calculated the spherical volume of our collapsed model to be 271.78 nm\(^3\). By using this smaller sphere and fitting multiple copies of it into the predicted volumes for larger oligomeric species, we estimate that the small species range from a monomer to 8-mer under low ionic strength conditions, which over time assemble into larger polydisperse species.

Guided by the constraints, the final model “docks” the JD onto the CTD placing the putative acidic surface on the CTD (E208-D212) in contact with the NMR-identified basic surface on the JD (K20-R29) (Figure 7A and Supplementary Figure 7B). The final model also docks Helix 5 onto the JD:CTD interface (Figure 7A). The CTD has proximal basic surfaces that flank the acidic surface, generating a characteristic alternating charge pattern that is inverted on the JD (Figure 7B). Furthermore, in the collapsed monomer, the 17 aromatic residues distributed in the F/G and S/T regions are exposed to solvent highlighting their possible role in oligomerization (Figure 7A). Although the NMR results were not employed as constraints, it is striking that the predicted interaction sites line up well with the solution and solid-state NMR data. These simulations, in conjunction with our ionic strength perturbation experiments support a model in which the JD-CTD interaction is coupled to the conformation of the F/G and S/T region with the potential to sense substrate binding and modulating the oligomer assembly.

**Evolutionary preservation and co-evolution**
If this interaction has biological relevance, we may expect it to be evolutionarily preserved. To probe this, we computed the extent of conservation across DnaJB8 homologs (Supplementary Figure 7C). The JD is the most conserved domain. Particularly for DnaJB6b, DnaJB2 and DnaJB7 homologs which also encode a five-stranded pleated β-sheet CTD, we found specific sites on the CTD with an elevated degree of conservation, these residues map to the N/D-G β-turns and the β-sheet surface of the CTD (Supplementary Figure 7D). In the Rosetta-generated model, the majority of these conserved CTD sites are participating in the JD-CTD interface (Supplementary Figure 7D-E). A similar sequence analysis for the homologous DnaJB6b sequence reveals similar conservation patterns and charge distributions (Supplementary Figure 7F). This supports the idea that the JD-CTD interaction has a degree of conservation across DnaJ sequences featuring homologous CTD-like domains.

Lastly, we incorporated co-evolution analysis using the Gremlin algorithm. Employing an alignment of DnaJB8 homologs we identified amino acid positions that covary, restricting analysis to only pairs showing >70% probability of being in contact. While many of the amino acid pairs covary within the JD and CTD domains, we additionally observe pairs that reflect inter-domain contacts between the JD-CTD (Figure 7C). Comparison of these long-range covariance sites reveals a striking resemblance to the XL-MS pairs identified in the full length DnaJB8 experiments. The similarity between the predicted covarying contacts (Figure 7C), conservation (Supplementary Figure 7C-D) and the XL-MS experimental contacts not only further strengthens our DnaJB8 JD-CTD model, but it also suggests that XL-MS can detect functionally important yet lower affinity interaction sites.

**JD-CTD interaction competes with Hsp70 binding**

The conserved Hsp70-JD electrostatic contacts (Figure 1) that overlap with the JD-CTD contact sites lead us to hypothesize that the observed JD-CTD interactions could regulate the recruitment of Hsp70 (Figure 8A-B). To test this hypothesis, we employed a
competition experiment leveraging a fluorescence polarization binding assay to
discriminate the JD-CTD and JD-HspA1A interactions (Figure 8C). For the JD:HspA1A
titration we determine a binding constant of $0.257 \pm 0.029 \mu M$, consistent with values
observed in the literature and similar to the JD:CTD interaction (Figure 8D, black and
green, respectively). Due to the large size difference of HspA1A (70 kDa) and the CTD
(8.7 kDa) the respective titrations saturate at different polarization values (Supplementary
Figure 8A, black and green, respectively). Leveraging this signal difference, we designed
an experiment to measure the competition of HspA1A and CTD binding to the JD. We
preincubated the FITC-labeled JD with a fixed concentration of CTD (3.125 μM) and then
subsequently titrated increasing concentrations of HspA1A. The polarization signal was
consistent with the already measured CTD-JD complex, up until HspA1A concentrations
reached 3.125μM. Past this point, the signal more closely matched that seen for the larger
HspA1A-JD complex (Supplementary Figure 8A, purple). Normalization of the
polarization data reveals a ~25-fold shift in the apparent binding constant between
JD:HspA1A when preincubated with CTD (Figure 8D). XL-MS using ADH/DMTMM was
used to identify contact points between the JD and HspA1A, revealing 3 zero-length
cross-links (K41-E513, K51-D323, and D65-K319) that are all consistent with the
structural model of the JD1-77-HspA1A interaction (Figure 8E, Supplementary Data 2).
Consistent with our findings in cells (above), incubation of HspA1A with full-length
DnaJB8 yielded intra-molecular cross-links for DnaJB8 and HspA1A, but no
intermolecular cross-links observed between the two different proteins (Supplementary
Data 2). Thus, the DnaJB8 JD uses the same basic surface to bind to its own CTD and
HspA1A. Based on our DLS data and Rosetta models of DnaJB8, the oligomer shape
changes with the engagement of the JD:CTD interaction. When the JD:CTD interaction
is preserved, DnaJB8 is capable of forming species ranging from monomer to octamer,
which proceed to larger oligomers over time (Figure 8F). The disruption of this JD:CTD
interaction liberates the HspA1A binding face of the JD and reduces the oligomer size to
a more uniform size. Considering the possible role of substrates, our model proposes that
in vivo it is the binding of Hsp40 client peptides and proteins that acts as the driving force
that disrupts the DnaJB8 oligomer and the JD:CTD interaction, thus promoting HspA1A recruitment for substrate transfer. This then represents an exciting new regulatory mechanism by which Hsp40s orchestrate the recruitment of Hsp70 (Figure 8F).

DISCUSSION

**Structure and dynamics of oligomeric DnaJB8**

Using a hybrid structural approach, we described the key molecular interactions in the oligomeric state of the Hsp40 co-chaperone DnaJB8. By DLS and TEM, we observed that DnaJB8 forms oligomers that vary in size and shape. In cells, DnaJB8 forms small puncta consistent with the size of our *in vitro* produced aggregates. We used XL-MS to capture surface contacts within and between folded domains and saw that they were similar *in vitro* and in cells. Independent of the conditions, the protein assembled into oligomeric base units with a $R_h$ range of ~4-8 nm, which is consistent with an assembly of at most eight monomers. Under normal ionic buffer conditions these assembles mature into larger polydisperse assemblies while in the presence of higher salt they stable. The collective data reveal that the core of these assemblies is formed by immobilized F/G and S/T domains, while the JD and CTD are engaged in electrostatic interactions at the surface rendering them sensitive to changes in ionic strength (Figure 8F). With ssNMR of the assembled oligomers, we showed that the F/G and S/T domains lack secondary structure, consistent with computational predictions, but should not be seen as dynamic, flexible regions in the oligomers. Dynamics-sensitive ssNMR instead showed them to be immobilized and insensitive to the destabilizing effects of elevated ionic strength. We propose a model in which self-assembly of these oligomer cores is driven by hydrophobic clustering and pi-pi stacking of aromatic side chains in the F/G and S/T domains (Figure 8F). At the periphery of this hydrophobic core are charged and hydrophilic residues, with the former engaged in electrostatic interactions that are critical for the DnaJB8 self-assembly and function.
We combined a variety of XL-MS and NMR in the solid and solution states to probe these inter-domain interactions. One of the most striking features was a newly identified interaction between the distal JD and CTD driven by electrostatics, whose perturbation had a dramatic effect on the structure, dynamics and higher order assembly of the oligomers. Analysis of the isolated JD and CTD showed a reduced mutual interaction, revealing a distinct role for the intervening domains. Indeed, our data argue that the S/T and F/G domains are central in the homo-oligomerization process, as the isolated JD and CTD are surprisingly resistant to self-assembly. These findings are distinct from published reports on the otherwise highly homologous DnaJB6b. The modeled structure of the CTD is however very similar to its DnaJB6b counterpart, featuring a pleated β-sheet topology absent of a hydrophobic core\textsuperscript{23}. Consistent with prior observations from solution NMR data we also observe weak contacts between the JD and a downstream helix in the FG domain, similarly based on electrostatics. Supporting an electrostatically driven immobilization of the JD, ssNMR analysis showed the JD mobility to be dependent on the ionic strength. Thus, we conclude that the JD is bound to charged groups on the oligomers’ surface.

**Functional role of the CTD in the oligomeric co-chaperones**

Multiple analyses reveal the isolated DnaJB8 CTD to be a monomer encoding a five-stranded pleated β-sheet topology. Our structural models for the DnaJB8 CTD are in agreement with recent cs-Rosetta simulations on the DnaJB6b CTD\textsuperscript{23}. This topology resembles the negatively charged β-sheet surface on Hsp70 that is known to interact with the JD\textsuperscript{16}. Interestingly, outside of inter-strand hydrogen bonding and polar side-chain contacts, it is not clear what forces stabilize this domain. This may explain the apparent CTD heterogeneity (unlike the JD) seen in the ssNMR spectra. In DnaJB6b the CTD itself has been implicated in driving the assembly of oligomers, even though the literature has conflicting results\textsuperscript{22-24, 26}. Söderberg et al have proposed that the minimal assembly is a dimer mediated by inter-CTD contacts\textsuperscript{25}. A recent solution NMR study showed that truncating the entire S/T region or partial truncation of the CTD blocks oligomerization\textsuperscript{23}.
Our data show that the DnaJB8 CTD is not prone to dimer formation, presumably implying a need for the other DnaJB8 domains. Considering the size of the DnaJB8 oligomeric assembly, the inability of the CTD to self-assemble indicates that this domain alone is not the driving force behind DnaJB8 oligomerization. While more experiments are required, our data suggest a possible alternate mechanism where the JD-CTD interaction is coupled strongly to the disordered F/G and S/T domains, with them together modulating oligomerization. Mimicking the effect of ionic strength modulation, it is worth noting that the lysines in the DnaJB8 and DnaJB6b CTD can be acetylated and deacetylated (via Histone Deacetylases (HDACs)) to modify these proteins’ self-assembly and function\textsuperscript{22}. The CTD architecture is conserved in a subset of B family member Hsp40s including DnaJB2, DnaJB6b, and DnaJB7, of which DnaJB6b has also been shown to oligomerize. It is tempting to speculate that the CTD in these DnaJB family members similarly serves a regulatory role in which post-translational modifications could alter the affinity for the JD, and thus indirectly alters assembly properties or Hsp70 recruitment.

**Implications for Hsp70 recruitment and substrate binding.**

DnaJB8 is responsible for the recognition and binding of particular misfolded substrates such as expanded polyglutamine domains and A\textsubscript{β}\textsuperscript{22,26-27,39}. Aside from inhibiting aggregation on its own, DnaJB8 also recruits Hsp70 for further processing of bound substrates. This necessitates a mechanism that connects Hsp70 recruitment to substrate recognition and binding. Our current findings offer a potential molecular rationale, involving auto-inhibitory interactions of the Hsp70-binding JDs within the DnaJB8 native oligomer state. The recently solved structure of the JD of DnaJ in complex with DnaK-ATP showed that the JD binds between the NBD and SBD\textsubscript{β} domains of DnaK\textsuperscript{16}. The interaction surface is characterized by charge complementarity, involving a highly negative side of DnaK and a highly positive region of the JD. The bacterial protein interface is recapitulated in our modeling of HspA1A and the DnaJB8 JD (Figure 8B-C). This involves residues involved in the DnaJB8 JD-CTD interactions, explaining the lack of Hsp70 recruitment by oligomeric DnaJB8 in cells and in vitro. This is validated by the
observed competition between Hsp70 and CTD binding to the JD (Figure 8F). In terms of the functional mechanism of DnaJB8 substrate recognition and Hsp70 recruitment, we propose the following mechanism based on these findings.

In the non-stressed native state, the DnaJB8 forms oligomers in which the JD is engaged in electrostatic interactions and thus not available for very effective Hsp70 binding. Substrate binding to DnaJB8 is expected to disrupt the normal state of the oligomer complex and could cause the release of the immobilized JD (in a manner analogous to the ionic strength experiments). As a consequence, the Hsp70-binding helix-turn-helix motif of the JD becomes available. This would enable the recruitment of Hsp70 to the loaded DnaJB8 protein (Figure 8F). The substrate could do so by disrupting the oligomer structure altogether. However, our data illustrate that complete loss of the oligomers is not required for a mobilization of the JD. The oligomers must be stabilized by self-self interactions that are not disrupted by ionic strength, and do not require the involvement of the JD. We propose that the more hydrophobic F/G domain may form this salt-resistant core structure, with the CTD and JD being more surface exposed. Thus, it may be possible to recruit Hsp70 to an oligomeric state of DnaJB8, without a need for complete solubilization of the protein. Although we employed ionic strength to manipulate the sequestration of the JD helix-turn-helix motif, it is tempting to speculate that this could also be affected by changes in the S/T and/or F/G domains. Here, it is noteworthy that negative residues in helix 5 (E97/D98) in the F/G domain interact with the JD (XL-MS data). A disruption of the JD-CTD interaction may therefore be coupled to low complexity sequences located in the S/T domain that are thought to mediate substrate binding. Future mechanistic and structural studies on this complex co-chaperone with substrates will reveal the interplay between oligomer dynamics, substrate binding and recruitment of Hsp70.
CONCLUSIONS

We present a hybrid structural biology studies of DnaJB8 oligomers to reveal a new role for specific electrostatic contacts that mediate the homo-oligomeric assembly in vitro and in vivo. Despite a heterogeneous structure of the oligomers, our use of complementary XL-MS, (ss)NMR studies and in silico analyses provided a novel perspective on the fundamental structure of the protein complex and its function. We show an exciting interplay between contacts that drive self-assembly and regulate recruitment of Hsp70 to the JD, pointing to an orchestrated auto-inhibitory mechanism involving the latter. Future studies will be focused on the interplay between DnaJB8 conformation, substrate binding, and Hsp70 recruitment. This study highlights how hybrid structural methods can reveal novel insight in a dynamic and polydisperse oligomeric chaperone.

MATERIALS AND METHODS

Sequence and structural analysis

Analysis of protein sequences was performed using Local CIDER. Secondary structure prediction was calculated using PSIPRED and the extent of disorder was calculated using DISOPRED. An ensemble of 5000 HspA1A structural models was produced using ab initio Rosetta using the DnaK (PDB:5NRO) conformation as a template. Briefly, the HspA1A sequence was aligned to the DnaK sequence to identify regions with loop insertions and deletions. The HspA1A fragment library was produced using the fragment picker. The lowest scoring model was used to produce a complex between HspA1A and the DnaJB8 JD. The structural images were produced using PyMOL.

Cell biological and biochemical analysis of DnaJB8-Clover cell lines

The human DNAJB8 protein-coding sequence was cloned using Gibson assembly into a modified FM5 lentiviral expression plasmid in which the UbC promoter was replaced by a CMV promoter, the linker sequence was replaced by “GSAGSAAGSGEF” and the YFP was replaced by mClover3. The resulting gene produced a DNAJB8-mClover3 fusion protein. In parallel, we produced a construct that expresses the fluorescent protein...
(mClover3) but lacks DNAJB8. Both plasmids were separately co-transfected into HEK293T cells along with helper plasmids (pCMV-VSV-G and psPAX2) to produce lentivirus, which was harvested after 48hrs and used to produce polyclonal cell lines that expressed either DNAJB8-mClover3 or mClover3. For crosslinking experiments, cells from a confluent 10cm² cell culture dish were pelleted and lysed using an insulin syringe in 1xPBS with 1mM DTT, 1mM PMSF, 1x EDTA-free Protease Inhibitor Cocktail (Roche), and 1% Digitonin. After spinning at 1000xg for 10min., the lysate was recovered and incubated with a polyhistidine tagged GFP-nanobody (purified as described previously³⁴) for 2.5 hours at 4°C. The lysate was then incubated with 25μL NiNTA beads (Clontech) (for 1 hour at 4°C for binding. The beads were washed five times with 300μL 1xPBS. The buffer for each wash was removed after pulse spinning the beads via centrifugation. The beads were preincubated for 5 minutes at 37°C and a final concentration of 57mM ADH and 36mM DMTMM were added to each sample. Following a one-minute incubation with chemical crosslinkers, the reaction was quenched with 1mM ammonium bicarbonate. After another pulse spin to remove the buffer, the beads were resuspended in elution buffer, (8M Urea, 0.5M imidazole, pH 7.5). After a final pulse spin, the supernatant was retained and analyzed by mass spectrometry.

**Analysis of Mass Spectrometry Results**

Each Thermo .raw file was converted to .mzXML format for analysis using an in-house installation of xQuest⁵⁷. Score thresholds were set through xProphet⁵⁷, which uses a target/decoy model. The search parameters were set as follows. For grouping light and heavy scans (hydrazide crosslinks only): Precursor mass difference for isotope-labeled hydrazides = 8.05021 Da for ADH-d₀/d₈; maximum retention time difference for light/heavy pairs = 2.5 min. Maximum number of missed cleavages = 2, peptide length = 5-50 residues, fixed modifications = carbamidomethyl-Cys (mass shift = 57.02146 Da), mass shift of light crosslinker = 138.09055, mass shift of monolinks = 156.1011 Da, MS¹ tolerance = 15 ppm, and MS² tolerance = 0.2 Da for common ions and 0.3 Da for crosslink ions; search in enumeration mode. For zero-length crosslink search: maximum number of missed cleavages = 2, peptide length = 5-50 residues, fixed modifications carbamidomethyl-Cys (mass shift = 57.02146 Da), mass shift of crosslinker = -18.010595 Da, no monolink mass specified, MS¹ tolerance = 15 ppm, and MS² tolerance = 0.2 Da.
for common ions and 0.3 Da for crosslink ions; search in enumeration mode. The FDRs of all *in vitro* experiments range from 0.05 to 0.33.

**Western blot analysis**

10μL aliquots of the HEK control, Clover, and DnaJB8-Clover cell-lines were removed from the elution and loaded onto a 4-12% Bis-Tris SDS-PAGE gel for western blotting. Upon running the gel to completion, the gel transferred onto a transfer membrane soaked in Novoblot transfer buffer. Following the transfer, the membrane was soaked in milk blocking buffer for 1 hour at room temperature. For immunolabelling, we added 1:2000 dilution of polyclonal Anti-GFP (rabbit) (Rockland) in milk and incubated the membrane shaking at room temperature for 2 hours. The primary antibody solution was dumped and the membrane washed three times for 10 minutes each with 1xTBST before adding the polyclonal Anti-Rabbit Peroxidase (Rockland) at a 1:5000 dilution in milk. The membrane was incubated with secondary antibody at room temperature for 1 hour before removing the antibody solution. The membrane was washed 3 times in 5-minute intervals with 1xTBST and finally one 5-minute wash with 1xTBS. The membrane was soaked in 1mL of Luminol enhancer and peroxide solution for 1 minute before imaging.

**In cell analysis of DnaJB8-Clover and Clover cell lines**

HEK293T cells expressing either DNAJB8-mClover3 or mClover alone were plated at 300,000 cells per well in media (10%FBS, 1%Pen/Strep, 1%GlutaMax in DMEM) in a 6-well glass bottom plate (Cellvis, P06-1.5-N). After 30 hours, cells were stained with Hoescht33342 at a final concentration of 2ug/mL in cell media for 30 minutes at 37C and 5%CO2. The plate was placed on an InCell6000 with a heated stage and fifty fields of view were imaged under DAPI and FITC channels at 60X magnification (Nikon 60X/0.95, Plan Apo, Corr Collar 0.11-0.23, CFI/60 lambda). Images were exported as TIFF files for downstream analysis.
Identification of factors bound to DnaJB8

DnaJB8-Clover was purified from HEK293 cells as described previously\textsuperscript{34} using a GFP-nanobody construct with a His tag. The pulldown was analyzed via LC-MS/MS, resulting in mass spectra with mass peaks for all proteins in solution. The resulting data was submitted to the CPFP\textsuperscript{35} server for peak assignment and protein identification. The UTSW CPFP server assigns protein peptides to peaks in submitted mass spectra by searching the NCBI and EBI protein databases for mass spectra matches to known protein sequences. The output results assign each identified protein a value in sequence coverage, peptide-spectrum match (PSM), unique peptide hits, and matched isotope pattern chromatograms (MICs). Using a Clover only cell line as a control, we compared MIC scores for relative protein abundance in the DnaJB8-Clover sample to determine which proteins were more abundant in the presence of DnaJB8 during the pulldown and used sequence coverages >70% and PSMs>50 to restrict the list. Hits identified in the DnaJB8-Clover pull downs that were enriched relative to the Clover controls were input into the string database\textsuperscript{58} to build network interaction maps based on interactions described in the literature.

Recombinant expression and purification of DnaJB8

The vector used for DnaJB8 expression was a pET-29b vector containing the gene for human DnaJB8, a T7 promoter to activate DnaJB8 expression, a His tag region at the end of the gene, and a gene for kanamycin resistance. This vector construct was transformed into \textit{E. coli} BL-21 (DE3) cells and plated onto 2xLB plates containing 0.05mg/mL Kanamycin. 12mL of 2xLB, 0.05mg/mL Kanamycin were prepared and inoculated with a single colony from the plate. This small culture was incubated overnight at 37°C while shaking at 220rpm. In the morning, the 12mL culture was added to 1L of 2xLB supplemented with 0.05mg/mL Kanomycin and incubated at 37°C while shaking at 220rpm. Once OD600 reached 0.6-0.8 A.U., 1mL of 1M IPTG was added to induce DnaJB8 expression. After incubation for an additional 4 hours, the cells were harvested by spinning down the culture at 4,000g for 20 minutes.
The resulting cell pellet was resuspended in 25mL 1xPBS and 1mM PMSF in preparation for insoluble separation. The resuspended cells were sonicated at 30% power, 5X pulse for 10 minutes using an Omni Sonic Ruptor 4000 (Omni International). The lysed cells were pelleted at 10,000g for 30 minutes and the supernatant was discarded. The insoluble pellet was rinsed with 1xPBS, 0.75%Tween-20 and again pelleted at 10,000g for 30 minutes.

The insoluble cell pellet was resuspended in 50mL lysis buffer (8M Guanidinium HCl, 50mM HEPES, 20mM Imidazole, 1mM DTT pH 7.5) and sonicated at 30% power, 3X pulse for 1 minute to solubilize the DnaJB8 from the insoluble pellet. After a 30-minute incubation at room temperature, the cellular debris was pelleted at 15,000g for 30 minutes. The resulting supernatant was mixed with 2mL HisPur™ Ni-NTA Resin (Thermo Scientific) for 1 hour before being loaded onto a gravity column. The column was washed with an additional 50mL of lysis buffer, followed by 50mL of a second wash buffer (50mM HEPES, 20mM Imidazole, 1mM DTT pH 7.5 in H₂O). The protein was eluted with 30mL of elution buffer (50mM HEPES, 500mM Imidazole, 1mM DTT pH 7.5 in H₂O) and collected in 2mL fractions. After selecting for fractions with high purity, the DnaJB8 solution was loaded into 3.5kDa cutoff Biotech CE Dialysis Tubing (Spectrum Labs) and dialyzed overnight at 4°C in 50mM ammonium formate to minimize assembly. The protein was then lyophilized and stored at -80°C for future use.

**Dynamic Light Scattering**

All samples were prepared at 1.2mg/mL in 1xPBS, 1mM DTT, pH 7.4. All protein samples were filtered through a 0.22μm PES sterile filter and loaded in triplicate onto a 384 well clear flat-bottom plate. The plate was loaded into a Wyatt DynaPro Plate Reader III and set to run continuously at room temperature at a scanning rate of 1 scan per 15 minutes, with 1 scan composed of 10 acquisitions. The data were analyzed using the Wyatt Dynamics software version 7.8.2.18. Light scattering results were filtered by Sum of Squares (SOS) <20 to eliminate statistical outlier acquisitions within each scan. For DnaJB8 in 1xPBS(150mM) buffer, one of the triplicates contains partial data due to high
SOS values. This is a result of increasing polydispersity and heterogeneity, which is consistent with oligomers of that size. Average $R_h$ readings at each time point were reported and further averaged across triplicate wells. Data for the smallest particles was reported by filtering out any signal that was greater than 10nm for runs with JD1-77 and CTD170-232, while more broad analyses such as the full DnaJB8 protein did not consider size cutoffs.

**Transmission Electron Microscopy**

5μL of sample at 200μM was loaded onto grids. 200 mesh formvar grids are prepared through glow discharge followed by the application of each 5μL sample onto each grid. After 30 seconds, the grid is blotted by filter paper followed by washing the grid with 5μL of ddH$_2$O. After another 30 seconds, the grid is blotted again followed by the addition of 5μL of 2% uranyl acetate for negative staining. After 1 minute, the grid was dried for the final time and loaded into a FEI Tecnai G2 Spirit Biotwin TEM. All images were captured using a Gatan 2Kx2K multiport readout post column CCD. The images were acquired at the UT Southwestern EM Core Facility.

**Cross-Linking Reagents**

All crosslinking reagents used are commercially available: ADH (Sigma-Aldrich), and mixed light and deuterated ADH (ADH-h$_8$/d$_8$) (Creative Molecules). DMTMM (Sigma-Aldrich). For all crosslinking experiments, stock solutions were made of each crosslinking reagent. A 1:1 ADH-d$_0$/ADH-d$_8$ solution was made at 100mg/mL in 1xPBS pH 7.4 (Sigma-Aldrich). DMTMM (Sigma-Aldrich) was prepared at a 120mg/mL concentration in 1xPBS pH 7.

**Cross-Linking Mass Spectrometry**

The ex-vivo purified DnaJB8 was dialyzed to remove excess imidazole, and transferred into 1xPBS pH 7.4 buffer. For the full-length DnaJB8 experiments, lyophilized DnaJB8 was resuspended in either 1xPBS(150mM) or 1xPBS(285mM) to a concentration of
100μM. The JD1-77 and CTD170-232 constructs were purified into 1xPBS buffer, and were prepared for XLMS experiments at 100μM each. 2μM HspA1A in 1xPBS pH 7.4 buffer and mixed with either 40μM DnaJB8 or 40μM JD1-77 for XLMS experiments. All samples were incubated at 37°C while shaking at 350 rpm for 30 minutes. Final concentrations of 57mM ADH do/d8 (Creative Molecules) and 36mM DMTMM (Sigma-Aldrich) were added to the protein samples with final concentrations of 8.3 mg/mL and 12 mg/mL respectively, and incubated at 37°C with shaking at 350 rpm for 30 minutes. The reactions were quenched with 100mM ammonium bicarbonate and incubated at 37°C for 30 min. Samples were lyophilized and resuspended in 8M urea. Samples were reduced with 2.5mM TCEP incubated at 37°C for 30 min followed by alkylation with 5mM iodoacetimide for 30 minutes in the dark. Samples were diluted to 1M urea using a stock of 50mM ammonium bicarbonate and trypsin (Promega) was added at a 1:50 enzyme-to-substrate ratio and incubated overnight at 37°C shaking at 600 rpm. 2% (v/v) formic acid was added to acidify the samples following overnight digestion. All samples were run on reverse phase Sep-Pak tC18 cartridges (Waters) eluted in 50% acetonitrile with 0.1% formic acid. 10μL of the purified peptide fractions was injected for LC-MS/MS analysis on an Eksigent 1D-NanoLC-Ultra HPLC system coupled to a Thermo Orbitrap Fusion Tribrid system. Peptides were separated on self-packed New Objective PicoFrit columns (11cm x 0.075mm I.D.) containing Magic C18 material (Michrom, 3μm particle size, 200Å pore size) at a flow rate of 300nL/min using the following gradient. 0-5min = 5 %B, 5-95min = 5-35 %B, 95-97min = 35-95 %B and 97-107min = 95 %B, where A = (water/acetonitrile/formic acid, 97:3:0.1) and B = (acetonitrile/water/formic acid, 97:3:0.1). The mass spectrometer was operated in data dependent mode by selecting the five most abundant precursor ions (m/z 350-1600, charge state 3+ and above) from a preview scan and subjecting them to collision-induced dissociation (normalized collision energy = 35%, 30ms activation). Fragment ions were detected at low resolution in the linear ion trap. Dynamic exclusion was enabled (repeat count 1, exclusion duration 30sec).
Solid state NMR analysis

Chaperone oligomers were prepared in PBS buffer with different NaCl concentrations. Lyophilized DnaJB8 uniformly labeled with $^{13}$C and $^{15}$N ($U^{-13}$C, $^{15}$N) was re-suspended in 1ml of PBS buffer in which the final NaCl concentration was 100mM or 285mM. Each sample was packed into a 3.2mm MAS NMR rotor (Bruker Biospin) by sedimentation, using a previously described ultracentrifugal packing tool. Sedimentation was done at 175,000 x g force in an Optima L-100 XP Ultracentrifuge using a SW-32 Ti centrifuge rotor for 1 hour. Subsequently, excess of the supernatant fluid was removed. Sample tubes were washed with another 1mL buffer solution after which a second packing step using same parameters was performed. Finally, supernatant was removed, spacers were placed on the top of the hydrated sedimented protein oligomers, and rotors were closed with the cap, sealed with a small amount of epoxy to avoid sample dehydration.

Experiments were performed on Bruker 600 MHz and 750 MHz spectrometers, at 277 K temperature using triple-channel (HCN) 3.2mm MAS EFree probes. Single pulse excitation (SPE) measurements were performed using a 6µs $90^\circ$ pulse on $^{13}$C, 3s recycle delay, 4k scans and TPPM proton decoupling of 83 kHz during acquisition.

1D Cross-Polarization experiments (CP) were performed using a 3.1µs $90^\circ$ $^1$H pulse, 2ms contact time, recycle delay of 3s, 4k scans and TPPM proton decoupling of 83 kHz during acquisition. Both SPE and CP experiments ran for 3.4 hours.

The $^{13}$C-$^{13}$C 2D CP-DARR experiments were performed using 25ms mixing time, 0.4ms contact time, 3.1µs $90^\circ$ proton pulse, 6µs $90^\circ$pulse on $^{13}$C, TPPM proton decoupling of 83 kHz, recycle delay of 2.8s, 1k scans. This experiment took 4 days and 20 hours. The amino acid type and secondary structure were predicted using the PLUQ program based on the to chemical shifts in the 2D experiment. Linewidth calculation was done using the SPARKY program.

Simulations and synthetic NMR spectra

The structure of the DnaJB8 JD in solution was determined previously using solution NMR. The synthetic spectrum of this domain was generated using the corresponding...
solution NMR chemical shifts from the BMRB (entry 11417). To simulate 2D NMR spectra of the other three domains MD simulations were performed on full length DnaJB8. The starting DNAJB8 conformation was produced using rosetta with a fully expanded conformation of the F/G and S/T domains while keeping the JD and CTD in the folded conformations. MD simulations were carried out in Desmond and the simulation box was prepared in Maestro. Estimated chemical shifts of the resulting structural models were generated using the SPARTA+ package.

Recombinant Expression and Purification of J Domain and C-Terminal Domain

Both vector constructs containing DnaJB8 JD and CTD respectively were cloned into pET-29b using Gibson assembly. These vectors were transformed into E. coli BL-21 (DE3) cells and plated onto 2xLB plates with 0.05mg/mL Kanamycin. 12mL of 2xLB with 0.05mg/mL Kanamycin were prepared and inoculated with a single colony from each plate. These small cultures were incubated overnight at 37°C while shaking at 220rpm. In the morning, the 12mL culture was added to 1L of 2xLB supplemented with 0.05mg/mL Kanamycin and incubated at 37°C while shaking at 220rpm. Once OD600 reached 0.6-0.8 A.U., 1mL of 1M IPTG was added to induce protein expression. After incubation for an additional 4 hours, the cells were harvested by spinning down the culture at 4,000g for 20 minutes. For preparing 15N JD, a single colony was inoculated into 10mL 2xLB supplemented with 0.05mg/mL Kanamycin and incubated for 7-8 hours at 37°C while shaking at 220rpm. The 10mL culture was then mixed into 100mL of M9 minimal media (42mM Na2HPO4, 22mM KH2PO4, 8.5mM NaCl, 0.1mM CaCl, 2mM MgSO4, 1E-4% Thiamine, 0.4% Glucose, 187mM NH4Cl, 0.05mg/mL Kanamycin) and incubated overnight at 37°C while shaking at 220rpm. In the morning of the following day, the cells were spun down at 2,000g for 10minutes and resuspended in 20mLs of M9 minimal media containing 15N labeled NH4Cl in place of the unlabeled molecule. This was immediately added to 1L of M9 minimal media with 15N labeled NH4Cl and allowed to incubate at 37°C shaking at 220rpm. Once OD600 reached 0.6-0.8 A.U., 1mL of 1M IPTG was added to induce protein expression. After incubation for an additional 4 hours, the cells were
harvested by spinning down the culture at 4,000g for 20 minutes. The cell pellets were resuspended in 20mL of soluble wash buffer (SWB) (50mM KPO$_4$, 300mM NaCl, 10% glycerol, 1mM PMSF, 10mM BME, pH 8) and sonicated at 30% power, 5X pulse for 10 minutes using an Omni Sonic Ruptor 4000 (Omni International). After incubation at room temperature for 1 hour, the cell lysate was spun down at 15,000g for 30 minutes to separate the soluble supernatant from the insoluble pellet. The supernatant was mixed with 2mL TALON® Metal Affinity Resin (Clontech) and incubated at 4°C for 1 hour. The protein-resin slurry was loaded onto a gravity column and washed with an additional 40mL of SWB. This was followed by subsequent washes: 20mL SWB with 0.5% tritonX-100, 20mL SWB with adjusted 700mM NaCl, 20mL SWB with 0.1mM ATP and 5mM MgCl$_2$, and an additional 40mL of SWB. The protein was eluted with 16mL SWB with 200mM Imidazole into 2mL fractions. After selecting for fractions with high purity, the protein solution was loaded into 3.5kDa cutoff Biotech CE Dialysis Tubing (Spectrum Labs) and dialyzed overnight at 4°C in 1xPBS to restore native folding. Both domain constructs were further enriched by running on a GE Superdex 200 Increase 10/300 column in 1xPBS 1mM DTT pH 7. The protein was aliquoted and flash frozen in liquid nitrogen and stored at -80°C for future use.

**Solution NMR with $^{15}$N-labeled JD and CTD**

The $^{15}$N-labeled JD was exchanged into 20mM Tris 100mM NaCl 1mM DTT pH 7 buffer in preparation for solution NMR. Each HSQC run was done for 4 hours at 1 scans/min with the temperature fixed at 299K. After each run, unlabeled CTD was titrated into the sample at 1:1, 1:3, and 1:6 ratios sequentially. All scans were collected on an Agilent DD2 600MHz instrument at the UT Southwestern Biomolecular NMR Facility. Each spectrum was converted into a readable format and phase corrected using NMRPipe$^{64}$. Peak assignments were based on the deposited information from BMRB (11417). The software Sparky$^{61,65}$ was used to analyze the peak shifts across all spectra.

**Circular Dichroism**
Recombinant CTD\textsubscript{170-232} domain constructs were transferred into 10mM NaPO\textsubscript{4}, 150mM NaF, pH 7.4 buffer at a concentration of 40\textmu M. The experiment was run using a Jasco J-815 Circular Dichroism instrument with a PMT detector using a 10 mm quartz cuvette. 6 accumulations were taken at a speed of 50 nm/min along the UV spectrum from 190 nm to 300 nm. Spectra analysis was done using the BeStSel online software\textsuperscript{66-67} to determine secondary structural composition.

**SEC-MALS**

CTD\textsubscript{170-232} construct at a concentration of 6.6 mg/mL in 1xPBS was filtered through a 0.1 \textmu m filter to remove larger impurities. The sample was further filtered using a 0.22 \textmu m centrifugal filter before 100 \textmu L was applied to a Superdex 200 Increase 10/300 column equilibrated in 1xPBS. The column was inline with a Shimadzu UV detector, a Wyatt TREOS II light-scattering detector, and a Wyatt Optilab tREX differential-refractive-index detector. The flow rate was 0.5 mL/min. The data were analyzed with Wyatt’s ASTRA software version 7.1.0.29. SEDFIT was used to calculate the dn/dc of the protein.

**CTD model generation**

Fragment libraries for the CTD sequence were generated using the Robetta server. 5000 models were produced using the \textit{ab initio} protocol and clustered to identify unique conformations. Lowest scoring models from the top clusters showed high structural similarity. The identified crosslinks from the CTD in full length DnaJB8 or isolated CTD were evaluated for consistency with each model in the ensemble. For acid-acid and acid-lysine crosslinks a distance threshold of 21 and 16 Å, respectively, was considered as satisfied and consistent with the chemistry. Distances were calculated using a custom script in MATLAB ver.R2019a and the crosslink pairs were visualized using PyMOL. The HYDROPRO software was used to calculate radii of hydration from structural models.

**Fluorescence polarization**
JD$_{1-77}$ was labelled with 10x FITC-maleimide in 1xPBS 1mM TCEP for 2 hours at room temperature. Excess dye was removed and the reaction quenched using a Zeba™ spin desalting column. For all experiments, 0.2μM JD$_{1-77}$ was incubated in triplicate with a titration gradient of Hsp70(150μM-0μM) or CTD$_{170-232}$(150μM-0μM) in 1xPBS, 1mM TCEP, pH 7.4. For competition experiments, labelled JD$_{1-77}$ was mixed with 3.125μM CTD in triplicate and incubated at room temperature for 1 hour before adding a titration gradient of Hsp70(150μM-0μM). Fluorescence polarization readings were taken with excitation at 494nm and emission at 525nm.

**Modeling of full length DNAJB8 using Rosetta and XL-MS restraints**

Given the globular conformations of the JD and CTD we considered how the crosslinks identified for the full-length protein could guide the JD-CTD interaction. Using Rosetta we assembled a monomeric conformation leveraging the JD and CTD conformations while keeping the F/G and S/T regions full expanded. This starting model was then used in a relax protocol in conjunction with crosslinks as constraints to produce an ensemble of collapsed conformations. For acid-acid and acid-lysine contacts 21 and 16 Å distance thresholds were used as restraints.

**Conservation mapping**

DNAJB8 homolog sequences were identified using Blast$^{68-69}$ and the sequences were aligned using Clustal Omega$^{70}$. The protein sequence alignment and structure of DNAJB8 JD (PDBID 2DMX) were used as input in Al2Co$^{71}$ to map the conservation onto the structural models. The conservation was mapped onto the models in PyMOL.

**Coevolutionary variation analysis**

The GREMLIN software$^{50-52}$ was used to identify covarying amino acid pairs from a DNAJB8 protein sequence alignment. A probability of 0.7 was used to threshold the data to identify amino acids with strong coupling.
Recombinant expression and purification of HspA1A

HspA1A gene was cloned into the pMCSG7 plasmid and transformed into BL-21 (DE3) cells and plated onto 2xLB plates with 0.1mg/mL ampicillin. 12mL of 2xLB with 0.1mg/mL ampicillin were prepared and inoculated with a single colony from each plate. These small cultures were incubated overnight at 37°C while shaking at 220rpm. In the morning, the 12mL culture was added to 1L of 2xLB supplemented with 0.1mg/mL ampicillin and incubated at 37°C while shaking at 220rpm. Once OD600 reached 0.6-0.8 A.U., 1mL of 1M IPTG was added to induce protein expression. The cells continued to incubate overnight at 12°C shaking at 220rpm. After incubation, the cells were lysed using a PandaPlus 2000 homogenizer (GEA) by pressing the cells with 10,000p.p.m pressure. The lysate was spun at 15,000xg for 45 minutes to remove insoluble cell components, and the resulting supernatant was mixed with 2mL TALON® Metal Affinity Resin (Clontech) and incubated at 4°C for 1 hour. The slurry was spun down at 700xg for 2 min to remove the majority of the buffer and the beads were added onto a gravity column. The beads were washed with 6CV of wash buffer (50mM Tris, 500mM NaCl, 10mM imidazole, 5mM βME, pH 8) and eluted with 5mL of elution buffer (50mM Tris, 500mM NaCl, 300mM imidazole, 5mM βME, pH 8). HspA1A containing fractions were confirmed by SDS-PAGE and pooled together for desalting. Desalting/buffer exchange was performed using a PD-10 desalting column (GE Healthcare), where HspA1A fractions were transferred into anion-exchange wash buffer (50mM Tris, 20mM NaCl, 1mM DTT, pH 8.75). The protein was loaded onto a HiTrap Q HP anion exchange column (GE Healthcare) and eluted across a gradient of anion-exchange elution buffer (50mM Tris, 1M NaCl, 1mM DTT, pH 8.75). HspA1A-containing fractions were once again combined and loaded onto a Superdex™ 200 Increase 10/300 GL (GE Life Sciences) size exclusion column, where HspA1A was further purified and transferred into 1xPBS, 1mM DTT, pH 7.4 buffer for all subsequent experiments.

Code availability
All DnaJB8 simulations were carried out with ROSETTA (available at https://www.rosettacommons.org/). All DNAJB8 molecular dynamics simulations were performed using Desmond (available at https://www.deshawresearch.com/resources_desmond.html). Sequence charge distributions were calculated using localCIDER (available at https://pappulab.github.io/localCIDER/).

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Author Contributions

B.D.R., I.M., P.V.D.W., and L.A.J. conceived and designed the overall study. B.D.R. performed in vitro protein assays, cell models, crosslink mass spectrometry, and ROSETTA simulations. I.M. and P.V.D.W. performed ssNMR experiments and analyzed the data. S.B. performed DNAJB8 CTD experiments and ROSETTA simulations. J.V.A produced mammalian cell lines and collected microscopy images. B.D.R. performed electron microscopy. B.D.R., I.M., P.V.D.W., and L.A.J. wrote the manuscript, and all authors contributed to its improvement.

Competing Interests

The authors declare no competing interests.
References


Table 1. Residue distribution per domain with net charge for each domain.

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### Figure Legends

#### Figure 1. Electrostatic interactions drive chaperone and co-chaperone interactions. (A) Structural superposition of a HspA1A structural model (blue) with a crystal structure of DnaK-DnaJ (green and cyan, respectively; PDBID: 5NRO) shows good agreement. (B-C) Electrostatic surface potential of DnaJB8 JD docked into the JD binding site on HspA1A is shown in cartoon representation and is colored in black. Binding mode is based on the DnaJ-DnaK coordinates. Basic surface on helix 2 docks onto the HspA1A surface. Electrostatic surface potential of HspA1A with docked DnaJB8 JD is shown in cartoon representation and is colored in black. The HspA1A surface presents an acidic face that complements the basic DnaJB8 JD surface. Highly acidic potential is shown in red and highly basic is shown in blue. (D) Cartoon schematic of DnaJB8 colored according to domain annotation: JD (red), F/G rich (blue), S/T rich (cyan), and CTD (green). (E) PSIPRED secondary structure analysis of the DnaJB8 sequence. Probabilities for helix, sheet, and random coil assignments are colored pink, yellow and blue, respectively. (F) Charge-based analysis of the DnaJB8 sequence. Sequence charge distribution of DnaJB8 is shown using Net Protein Charge per Residue (NPCR). Basic regions are shown in blue and acidic regions are shown in red. Basic face of helix 2 is highlighted along with the corresponding acidic face of the CTD. The helices for JD are shown as grey rectangles. (G) DISOPRED analysis of the DnaJB8 sequence. Regions above the red-dashed line are predicted to be disordered.

#### Figure 2. DnaJB8 forms oligomers in cells. (A) Cartoon schematics for the DnaJB-Clover and Clover constructs used in the mammalian experiments. DnaJB8 is colored as in Figure 1. Clover is colored in green. (B) Representative images of cells expressing DnaJB8-Clover and Clover. Clover and DAPI fluorescence signal are shown in green and blue, respectively. 5um scale bar is shown in white. (C) Quantification of DnaJB8-Clover and Clover puncta in high and low protein level expressing cell lines. In each analysis at least 2000 cells were counted. (D) Western blot analysis of DnaJB8-Clover and Clover proteins isolated from mammalian cell lines. Total (T), elutions (E) and crosslinked elutions (XL) of each condition are shown. Western blot was probed with GFP antibodies. (E) XL-MS contact map of DnaJB8-Clover crosslinks (orange boxes) identified using DMTMM and ADH. The axes are colored in red and green for JD and CTD, respectively. Crosslink pairs between JD-CTD, JD-JD and CTD-CTD are shown in dashed boxes colored grey, red and green, respectively.

#### Figure 3. DnaJB8 forms oligomers in vitro. (A) DLS time-course of DnaJB8. Average radius of hydration (R_h) of triplicate samples starts at ~15nm and increases over time to ~90nm. An arrow indicates the time point where DnaJB8 was observed under TEM. (B) TEM image of DnaJB8 oligomers. 200nm scale bar is shown in black. Length of oligomeric assemblies is ~90nm with a ~20nm width. (C) XL-MS contact map of DnaJB8 crosslinks identified using DMTMM and ADH (black) and DSS (grey). The axes are colored in red and green for JD and CTD, respectively. Crosslink pairs between JD-CTD, JD-JD and CTD-CTD are shown in dashed boxes colored grey, red and green, respectively. (D) DLS time-course of DnaJB8 under elevated ionic strength conditions (285mM NaCl). Average R_h of triplicate samples is ~13.7±1.2 nm. (E) TEM image of DnaJB8 oligomers. 50nm scale bar is shown in black. The oligomers appear as spherical masses with an average diameter of ~15nm. (F) Contact map of DnaJB8 crosslinks identified using ADH/DMTMM in the presence of high ionic strength. The axes are colored in red and green for JD and CTD, respectively. JD-JD and CTD-CTD crosslinks are shown in dashed boxes colored in red and green, respectively.

#### Figure 4. Solid-state NMR of DnaJB8 oligomers at physiological and high ionic strength.
(A) 2D $^{13}$C-15N ssNMR spectrum of U-$^{13}$C, 15N-labeled DnaJB8 oligomers in PBS, using 25 ms DARR mixing. (B) Corresponding 2D ssNMR spectrum at elevated ionic strength (285mM NaCl).

(C-D) Boxed Ala and Ser regions from panel “A”. The experimental peak pattern (black) is well resolved and is similar to the expected pattern of the folded JD in solution (red; middle). Green spectra (right) represent simulated signals predicted for Ala and Ser in our models of the non-JD domains, shown with enhanced broadening reflecting the heterogeneity seen in the experiments.

1D spectra on far left show slices through the experimental 2D data, along with selected peak widths (in kHz). (E) 13C 1D spectra at physiological (top) and elevated ionic strength (bottom) that show rigid residues (black, CP), rigid and mobile residues (green, SPE) and only mobile residues (red, INEPT). See also text. (F) Electrostatic surface potential of the DnaJB8 JD. Strongly positive area surrounding helix 2 is highlighted by the grey ellipse. Highly positive and negative potentials are colored blue and red, respectively. White color represents neutral charge. (G) The same view showing the location of Ala with narrow peaks at physiological salt levels (panel “A”) which disappear at high ionic strength (panel “B”). (H,I) Same model after 90° y-rotation showing two additional smaller positively charged regions and the nearby location of Alanines that disappear at high ionic strength.

**Figure 5. Isolated JD and CTD proteins are monomeric.** (A) Cartoon schematic for the full length DnaJB8 and the two domain fragments: JD1-77 and CTD170-232. Domains are colored as in Figure 1. (B) SEC chromatograms of JD1-77(red), CTD170-232(green) and LMW standards (blue). JD1-77 and CTD170-232 elute at apparent molecular weights of 14kDa and 6.5kDa, respectively. (C) SDS-PAGE coomassie gel of crosslinked JD1-77 and CTD170-232 crosslinked with either DMTMM only or additionally with ADH. (D) Contact map of ADH/DMTMM crosslinks identified for JD1-77(red), CTD170-232(green) and full length DnaJB8 (black). The axes are colored in red and green for JD and CTD, respectively. Crosslink pairs between JD-JD and CTD-CTD are shown in dashed boxes colored in red and green, respectively. (E) Rosetta ab initio models of CTD. The structural models are consistent with $R_e$ values derived from DLS (dashed line). Structural overlay of low energy scoring models reveals consistent pleated $\beta$-sheet fold. Models are shown in cartoon representation and are colored in green. (F) Histogram of the number of intra-domain crosslinks that are consistent with crosslink chemistry geometry (“satisfied”) in the ensemble of 5000 models. One model satisfies 13 out of 14 possible crosslinks identified in our experiments. Crosslinks are mapped onto best matching CTD structural model. CTD is shown in cartoon representation and is colored in white. Sites of crosslink are shown as spheres and are colored red or blue for D/E and K, respectively. Dashed yellow lines connect linked amino acid pairs.

**Figure 6. JD and CTD interact through charge complementary surfaces.**

(A) Schematic of the JD1-77 and CTD170-232 constructs used in fluorescence polarization and XL-MS binding experiments. (B) Fluorescence polarization (FP) titration measuring interaction between JD1-77-FITC and a concentration range of unlabeled CTD170-232. FP experiments were performed in triplicate and shown as averages with standard deviation. Non-linear regression fit of the binding curve reveals a $K_{d,app}$ of 0.575μM for this interaction. (C) Contact map of ADH/DMTMM crosslinks identified from an incubated JD1-77 and CTD170-232 sample (grey) and full length DnaJB8 (black). The axes are colored in red and green for JD and CTD, respectively. Helix 2 and 3 are shown in grey on the x-axis. Crosslink pairs between JD-CTD are shown in a dashed box colored in grey. (D) Schematic for the chemical shift experiment with U-15N JD and titrated with unlabeled CTD. Domains are colored as in Figure 1. HSQC solution NMR spectrum of 15N-labeled JD1-77 against a titration of CTD170-232: 0x (yellow), 1x (red), 3x (purple), 6x (blue). DnaJB8 JD peak assignments were transferred from deposited data (BMRB:11417). (E) Insets of peaks with highest observed chemical shifts: H31, L28, L26, R29. Peaks were colored as in D. (F) Histogram of chemical shifts by residue from 1-77. Average chemical shift value of $\sim$0.019ppm is
denoted by the red line (excludes prolines). (G) DnaJB8 JD structure illustrating the location of helix 2 (pdbid:2DMX). Structure is shown in cartoon representation and colored in white. (H) Mapping chemical shifts onto the DnaJB8 JD structure. JD is shown in surface representation and is colored according to chemical shifts from low (0.0 ppm) colored in yellow to high colored in red (0.04 ppm). (I) Electrostatic potential mapping onto DnaJB8 JD structure. JD is shown in surface representation and colored according to electrostatic. Highly acidic potential is shown in red and highly basic is shown in blue.

**Figure 7. Sequence and structural properties of the JD-CTD interaction.** (A) XL-MS-based refinement of full length expanded DnaJB8 monomer. Cartoon representation of DnaJB is shown in fully expanded conformation and collapsed conformation, colored by domain as in Figure 1. Aromatic amino acids in the F/G and S/T domains are shown as spheres and colored according to the domain. Residues in Helix 5 (H5) are shown as spheres and colored in magenta. (B) Charge complementary surfaces on the JD and CTD in the full-length DnaJB8 model mediate the interaction. Highly acidic potential is shown in red (- sign) and highly basic is shown in blue (+ sign) and colored by conservation. (C) GREMLIN sequence-based covariance analysis identified high confidence covarying amino acids on DnaJB8 that localize within the JD (red sector), within CTD (green sector) and across JD-CTD (grey sector). XL-MS identified for full length DnaJB8 (black) overlap with the covarying regions. Covarying positions localizing to amino acids in F/G domain are shown in brown and co-localize with crosslinks to this surface (black). (D) Three amino acids: D37, E43, and S50 are shown to co-vary with the CTD (red spheres).

**Figure 8. CTD and HSPA1A compete for the same basic binding surface on DnaJB8 JD.** (A-B) Acidic surfaces on HspA1A and CTD interact with basic surface on DnaJB8 JD. In the fully extended DnaJB8 conformation, the CTD (green) is not engaged with the DnaJB8 JD (red) and is accessible to HspA1A binding. In the collapsed DnaJB8 conformation, the CTD (green) is with the engaged DnaJB8 JD and not accessible to HspA1A binding. DnaJB8 is shown in cartoon and is colored according to domains as in Figure 1. HspA1A is shown in cartoon representation and is colored in blue. (C) Experimental workflow of experiments to determine competition between Hsp70 and CTD for JD binding. (D) Normalized FP binding curves measuring affinity between JD-CTD (green) and JD-Hsp70 (grey). Preincubation of JD-CTD followed by addition of Hsp70 (purple) shows delay in binding consistent with a competitive binding model. FP experiments were performed in triplicate and shown as averages with standard deviation. Fit was performed using non-linear regression analysis in GraphPad Prism. (E) JD-HspA1A crosslinks mapped onto JD-Hsp70 model. JD and HspA1A are shown in cartoon representation and colored white and black respectively. Sites of crosslink are shown as spheres and are colored red or blue for aspartic/glutamic and lysines, respectively. Dashed yellow lines connect linked amino acid pairs. (F) Schematic of proposed DnaJB8 model. Individual domains are shown as JD (red spheres), CTD (green spheres), F/G+S/T (light blue spheres), and both Hsp70 (dark blue spheres) and substrate (purple lines) are shown. DnaJB8 domains are displayed relative to the R
values derived from DLS experiments (Hsp70 not drawn to scale). DnaJB8 forms a fundamental oligomeric species through aromatic contacts in the F/G and S/T domains ranging from monomer to octamer. In the JD:CTD engaged state, these structures grow over time to form larger polydisperse oligomers (>100nm). The JD:CTD disengaged state (bottom) demonstrates our hypothesis where substrate bound to a uniform minimal unit of DnaJB8 disrupts the JD:CTD interaction. This leads to the recruitment of Hsp70 along the JD-CTD binding face, and the handoff of substrate to Hsp70.
Figure 2

A) J-Domain, F/G, S/T, CTD, Clover

B) DnaJB8-Clover, Clover

C) Clover, DAPI

D) α-GFP

E) DnaJB8-Clover XL-MS
Figure 3

A) DnaJB8 150mM NaCl

B) TEM image of DnaJB8 150mM NaCl

C) DnaJB8 150mM NaCl

D) DnaJB8 285mM NaCl

E) TEM image of DnaJB8 285mM NaCl

F) DnaJB8 285mM NaCl