Mapping interactions with the chaperone network reveals factors that protect against tau aggregation

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A network of molecular chaperones is known to bind proteins (‘clients’) and balance their folding, function and turnover. However, it is often unclear which chaperones are critical for selective recognition of individual clients. It is also not clear why these key chaperones might fail in protein-aggregation diseases. Here, we utilized human microtubule-associated protein tau (MAPT or tau) as a model client to survey interactions between ~30 purified chaperones and ~20 disease-associated tau variants (~600 combinations). From this large-scale analysis, we identified human DnaJA2 as an unexpected, but potent, inhibitor of tau aggregation. DnaJA2 levels were correlated with tau pathology in human brains, supporting the idea that it is an important regulator of tau homeostasis. Of note, we found that some disease-associated tau variants were relatively immune to interactions with chaperones, suggesting a model in which avoiding physical recognition by chaperone networks may contribute to disease.
**Fig. 1** In vitro tau aggregation screen identifies chaperones that inhibit or promote tau aggregation. **a**, Top, domain schematic of the longest splice isoform of tau (2N4R). N1 and N2, N-terminal inserts; PP region, polyproline region; R1–R4, MBR; aggregation motifs VQIINK and VQIVYK are indicated. Bottom, schematics of tau variants assayed in screen (ON4R, ON3R and K18). Variants referenced on the ON4R tau construct are labeled: disease-associated mutations (red); PTMs including caspase cleavage site (orange), phosphorylation site (green), acetylation site (blue) and proline isomerization sites (purple). **b**, Sample result from the ThT-based tau aggregation assay. Aggregation of recombinant 0N4R tau WT (10 μM) induced by heparin in the presence of Hsc70. Data points are mean ± s.e.m. of three technical replicates; data are representative of three independent experiments. rfu, relative fluorescence units. **c**, Lag-time values for tau variants in the ThT assay. Aggregation of tau variants (10 μM) was induced with heparin, and the calculated lag time extracted from ThT curves was plotted. Data shown are mean ± s.d. of three technical replicates, representative of five independent experiments. **d**, Heatmap depicting the effect of individual chaperones on the lag-time parameter of aggregation curves. For each chaperone–tau combination, we plotted the log2 fold change in lag time when aggregated in the absence or presence of an equimolar concentration of chaperone (10 μM). Color key indicates the effect size and direction. Gray, not tested. **e**, Heatmap depicting that a subset of disease-associated mutations are resistant to chaperone protection from aggregation. Data plotted as described in **d**. **f**, Differences between Hsc70 and Hsp72 anti-aggregation effects attributed to the SBD. Aggregation of ON4R tau WT (10 μM) was induced in the presence of constructs of the Hsc70 or Hsp72 substrate binding region (aa 391–540). The effect of chaperones on the aggregation lag time (log2 fold change compared to tau-alone control) is plotted for multiple chaperone concentrations tested. Data shown are mean ± s.e.m. of three independent experiments.
with ~20 disease-associated variants of tau to understand why or how mutations and post-translational modifications (PTMs) might influence chaperone activities. From these screens, we found that the chaperone DnaJA2 is a strong inhibitor of tau aggregation and that it is active against a relatively broad number of tau variants. Furthermore, in patients with MCI and AD, we found a striking upregulation of DNAJA2 that was specific to neurons positive for aggregates. These splice isoforms include a variable number of N-terminal repeats (0N–2N), a polyproline region and either three or four microtubule-binding repeats (MBRs; R1–R4) (Fig. 1a). An important feature of the MBRs is the presence of two aggregation motifs termed PHF6* (275-VQIINK-280) and PHF6 (306-VQIVYK-311), which are required for aggregation21–23. Tau aggregation is typically measured using Thioflavin T (ThT), a dye that binds \( \beta \)-sheets. For our studies, we miniaturized the ThT assay for use in 384-well plate format to: (i) facilitate a greater number of replicates and (ii) test multiple concentrations of each chaperone. To validate this platform, we first titrated a major constitutively expressed member of the Hsp70 family, heat shock cognate 70 (Hsc70 or HSPA8), into microtubule-binding repeat (MBR) solutions of purified 0N4R wild-type tau (tauWT) and measured the aggregation rate of tau aggregation reactions (Fig. 1a and Supplementary Fig. 1). We set out to expand this dataset by comparative analysis of >30 chaperones for their effects on tau aggregation kinetics. We are subject to splicing to produce multiple isoforms16.}

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**Results**

**Select chaperones modulate tau aggregation kinetics.** To date, only a limited number of chaperones (Hsc70, Hsp27 and FKBP51) have been examined in isolation for their abilities to modulate the aggregation of tau12,13,20. We set out to expand this dataset by comparative analysis of >30 chaperones for their effects on tau aggregation kinetics. Tau is subject to splicing to produce multiple isoforms16.

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Fig. 3 | Anti-aggregation chaperones bind to regions spanning the tau aggregation motifs. a, Binding sites for chaperones were identified using peptide arrays and NMR (details in Methods). A schematic of 2N4R mapping the domain and aggregation-motif positions is provided for reference (details in Fig. 1a). Location of chaperone-binding peptides from the peptide array experiments are indicated by diamonds and aligned with position in the 2N4R sequence. The binding profiles on tau for selected chaperones as probed by NMR (indicated by an asterisk (*)) were measured by titration of chaperones into 1\textsuperscript{5}N 2N4R tau. Changes in the NMR intensity ratios (1 - I/I\textsubscript{c}) for individual peaks derived from H\textsuperscript{15}N spectra (I, chaperone present; I\textsubscript{c}, tau alone) are plotted, aligned with their position in the 2N4R tau sequence. Chaperones/tau molar ratios assayed are indicated in parentheses. b, Tau binding profiles of a subset of anti-aggregation chaperones identified in the screen as probed by NMR of 15N-K18 tau (2:1 molar ratio of chaperone/tau). Changes in NMR intensity ratios I/I\textsubscript{c} plotted as described in a. Two binding regions (1 and 2, boxed by dashed lines with corresponding amino acid sequences) were identified and regions overlap with the PHF6\textsuperscript{+} and PHF6 motifs (underlined).

Fig. 1a). In contrast, a negative-control construct of Hsc70 encompassing only the nucleotide-binding domain (Hsc70\textsubscript{nBD}) had relatively little effect on aggregation kinetic parameters.

Next, we purified 18 variants of 0N4R tau to test in this format. These variants included disease-associated mutations (P301L, A152T and ΔK280) and mimics of disease-associated phosphorylation (T153E, T231E, S356E, S396E and S404E), acetylation (K174Q, K280) and mimics of disease-associated phosphorylation (T153E, T231E, S356E, S396E and S404E), acetylation (K174Q, K280 and P301L, Δ308/309, which lack the PHF6 motif, had 5- to 10-fold longer lag phases and ~2-fold slower elongation rates. These baseline studies allowed us to normalize subsequent results to the intrinsic aggregation propensity of each variant.

Each tau variant was then tested against a purified human chaperone collection. The collection contained major members of the Hsp70 and small heat shock protein (sHsp) chaperone systems. The throughput of the ThT platform also allowed us to test additional chaperones previously linked to tauopathy, including clusterin, αB-crystallin, FKBP12 and Pin1 (refs 24–27), as well as chaperones known to have roles in other protein-misfolding diseases, such as Hsp60 and HIP28,29. In addition, we assayed a select number of anti-aggregation chaperones from other organisms, including yeast Hsp104 (ref. 30) and several newly discovered chaperones from Escherichia coli and Saccharomyces cerevisae31,32. For each experiment, the purified chaperone was incubated with tau (10μM) prior to the addition of heparin. Additionally, each chaperone was tested as a negative control (Hsc70 NBD) had relatively little effect on aggregation kinetic parameters.
DnaJA2, which had not previously been linked to aggregation or function as protein disaggregases. The exceptions were Hsp60 and known to have anti-aggregation activity against other clients or lies and their structures. Despite these differences, some of them are chaperones share little similarity in terms of their chaperone family and day-to-day reproducibility, giving us confidence in the results. In addition, the negative control (Hsc70) was inactive (Fig. 1d), except for some modest, non-specific activity against the slowly aggregating 0N3R and C domain in this family of co-chaperones. We tested several hit chaperones against additional disease-associated mutations (Fig. 1f). Consistent with the original observations, the aggregation assay showed that DnaJA1 was also able to inhibit ON4R tauWT aggregation (Supplementary Fig. 2c). A construct of the conserved J domain, taken from E. coli DnaJ, had minimal effects on tau aggregation for most of the tau variants in the screen (Fig. 1d), consistent with the idea that substrate binding is often independent of the J domain in this family of co-chaperones.

Another striking observation from this analysis was that certain tau variants, such as P301L, A152T, ΔK280, D25 and D421, were relatively resistant to even the most potent chaperones (Fig. 1d and Supplementary Fig. 2d). To extend this observation, we tested several hit chaperones against additional disease-associated mutations (Fig. 1f). Consistent with the original observations, the aggregation of N279K and P301S mutants was also relatively resistant to chaperone protection when compared to that of ON4R tauWT. This result suggests that some tau variants might partially, but broadly, circumvent the anti-aggregation activities of chaperones. It is also worth noting that the tau variants lacking one aggregation motif (0N3R and Δ308/309) were protected by a wider range of chaperones.

In incubation of ~30 chaperones with ~20 tau variants revealed a fascinating pattern of effects on tau lag time (Fig. 1d) and elongation rate (Supplementary Fig. 2a). For clarity, the effects at 1:1 stoichiometry (tau/chaperone) are shown, but similar results, albeit of lower magnitude, were seen at reduced concentrations (Supplementary Fig. 2b). The first striking observation was that a subset of chaperones, including Hsc70, DnaJA2, Hsp60, clusterin and Hsp104, were the most potent suppressors of tau aggregation. Interestingly, these chaperones share little similarity in terms of their chaperone families and their structures. Despite these differences, some of them are known to have anti-aggregation activity against other clients or function as protein disaggregases. The exceptions were Hsp60 and DnaJA2, which had not previously been linked to aggregation or tau biology. DnaJA2 is closely related to another J-protein member, DnaJA1. DnaJA1 could not be tested using the screen conditions, owing to issues with solubility; however, a modified tau aggregation assay showed that DnaJA1 was also able to inhibit ON4R tauWT aggregation (Supplementary Fig. 2c).

Fig. 4 | Tau fibrils formed in the presence of DnaJA2 have a reduced capacity to seed tau aggregation in cells. a. Representative K1B-YFP fluorescence images of Clone 1 cells transfected with Lipofectamine only (no fibrils), ON4R tauWT fibrils (WT tau, 60 ng) or ON4R tauWT fibrils (60 ng) made in the presence of DnaJA2 (WT tau + DnaJA2). b. Seeding of tau fibrils formed in the absence or presence of chaperones. The percentage of Clone 1 cells that form punctae (% cells seeded) is correlated with the amount of aggregated tau solution (micrograms of tau transfected). Unfractionated ON4R tauWT fibrils made in the presence of the indicated chaperones were transfected, and the percentage of cells seeded were plotted. Data are mean ± s.d. of three technical replicates; data are representative of three independent experiments. c. Significant reduction in seeding phenotype observed for fibrils formed in the presence of DnaJA2. Unfractionated WT, P301L and A152T ON4R tau fibrils formed in the presence or absence of the indicated chaperones were transfected into Clone 1 cells (60 ng tau), and the percentages of cells seeded were compared (mean ± s.e.m. of three independent experiments). Groups with a > 20% reduction compared to the no-chaperone control group are shaded in green. One-way ANOVA with a post-hoc Dunnett’s test, *P = 0.039, **P = 0.002, ***P = 0.0001.
It is known that an imbalance in the ratio of 4R tau versus 3R tau is linked to some tauopathies. Our results suggest that the switch toward 4R isoforms may make tau aggregation more resistant to chaperones.

One unexpected result from this analysis was the identification of chaperones that only suppressed the aggregation of a few tau variants. For example, the small HSPs (Hsp22, Hsp27 and αB-crystallin) delayed aggregation of several tau phosphomimetics, such as S356E, but not the others. Another unexpected result was the difference in activity between the two closely related Hsp70 family members Hsc70 (HSPA8) and Hsp72 (HSPA1A). Overall, Hsc70 was a much better anti-aggregation chaperone, even though it is 94% identical to Hsp72, and both Hsc70 and Hsp72 bind to tau with similar affinities. We generated analogous constructs of the isolated Hsc70 and Hsp72 substrate-binding domain (SBD) consisting of the β-sheet substrate-binding cleft and the first two α-helices of the lid (amino acids 394–540). At the highest concentrations tested (20µM), Hsc70 and Hsp72 delayed the aggregation of 0N4R tauWT to the same degree as their corresponding full-length proteins. These results suggest that the reduced anti-aggregation activity of Hsp72 can probably be attributed to the relatively few residues that differ between Hsc70 and Hsp72 in the SBD. Future studies exploring the isolated SBDs may help to shed light on the differential actions of Hsc70 and Hsp72 on tau homeostasis observed previously.

Finally, we were surprised to identify chaperones that promoted aggregation. For example, FKBP12 had no effect on the aggregation of 0N4R tauWT, but it promoted aggregation of the acetylation mimetic K280Q. These results support a model where, in some contexts, individual chaperones might facilitate tau pathology.

Some chaperones suppress lag time, others inhibit elongation rate. While we initially focused on the effects of chaperones on lag
time, our screen captured other valuable data regarding the effects on elongation rates. Studies on amyloid-β (Aβ) have been informative in elucidating the microscopic processes occurring during different phases of an amyloid aggregation reaction. From this work, stages such as primary nucleation, secondary nucleation and elongation of fibril ends all seem to contribute to the reaction. Although a parallel study on tau aggregation has not yet been reported, we hypothesized that differential effects of individual chaperones in lag time and elongation rate might be suggestive of the stage at which the chaperone was acting. When profiling chaperones for their effects on aggregation kinetics across 0N4R tau variants, we observed that the chaperones Hsc70, Hsp60 and clusterin greatly slowed both lag time and elongation rate (Fig. 2a). However, DnaJA2 and Hsp104 were less active against tau elongation (relative to their effects on lag time), whereas Hsp27 and αB-crystallin were considerably more potent on the elongation step. These unique signatures suggested that individual chaperones might suppress only a subset of microscopic processes in the aggregation reaction. Thus, we reasoned that combining chaperones with distinct activity profiles could lead to synergistic effects. To test this idea, we assayed select, pairwise combinations of chaperones (1:1 ratio) for their effects on tau lag-time kinetics. In this analysis, we asked whether the effects on lag time when two chaperones were present (5µM each) would be greater than the sum of the effect observed for each chaperone when tested alone (5µM). In general, we found that combining two chaperones led to antagonistic, not synergistic, effects on anti-aggregation activity (Fig. 2b). For example, combining Hsp72 with DnaJA2 or Hsp60 led to lower-than-predicted increases in lag time. This result might occur when chaperones compete for the same tau binding sites (as discussed below). Interestingly, a small subset of combinations showed synergistic activity, including DnaJA2 with Hsp22 or αB-crystallin, thus suggesting that these chaperones act by cooperative mechanisms.

Next, we probed whether any of the chaperones could still suppress tau aggregation once the reaction had already been initiated, and added individual chaperones at 0, 25 or 55 min post incubation. We found that delaying the addition of chaperone until 25 min into the aggregation reaction (during the lag time) still extended lag times (Fig. 2c). However, we noticed that the magnitude of the effect was smaller when compared to addition at time zero or under screen conditions (i.e., addition of chaperone at ~30 min). Delaying the addition of the chaperone until 55 min into the aggregation reaction (at the beginning of the elongation phase) largely abolished anti-aggregation activity of chaperones (Fig. 2c). Only DnaJA2 was able to significantly decrease the rate of aggregation under these conditions. Surprisingly, several chaperones, such as αB-crystallin, Hsp27 and clusterin, enhanced aggregation rates when added post-lag phase, despite having the opposite effect when added under the screening conditions. Thus, it seems likely that chaperones can affect multiple processes in the tau aggregation pathway.

One trivial reason for DnaJA2's anti-aggregation properties might be that it binds to the accelerant heparin. To test this possibility, we induced tau aggregation with an unrelated accelerant, arachidonic acid, and found that DnaJA2 retained its ability to suppress aggregation (Supplementary Fig. 3a). Moreover, although DnaJA2 had some weak affinity (~2µM) for heparin in vitro (Supplementary Fig. 2b), increasing heparin levels did not abolish DnaJA2's anti-aggregation activity (Supplementary Fig. 2c).

Finally, we tested whether chaperones could disassemble mature fibrils (incubated >20h), as measured by a decrease in ThT signal. The Bukau group pioneered studies of chaperone complexes that have disaggregase activity on α-synuclein amyloids, but we found that none of the chaperones by themselves were able to reverse aggregation of 0N4R tauWT fibrils (data not shown). This result is not surprising, as the Bukau group showed that only combinations of chaperones, plus ATP, creates disaggregate activity.

We have not yet identified the combination of chaperones and ATP that might act as a putative disaggregate for tau fibrils.

### Chaperones bind to hotspots within the tau polypeptide.

Our screen results prompted us to ask where chaperones might bind on tau. Specifically, we wondered which chaperones might bind directly to the PHF6 and PHF6* regions in the MBRs that are known to be required for tau aggregation. Previous studies have mapped where one or two chaperones bind to individual clients in vitro, but little is known about client recognition in a broader context. Peptide arrays have previously been used to map the binding sites of Hsp70s and Hsp90 on tau, and these interaction sites have been confirmed and extended by NMR spectroscopy.

Inspired by these approaches, we mapped the binding sites on tau for our broader collection of chaperones. Briefly, a tau peptide array was generated with 15-mer peptides in four amino acid steps that covered the longest adult isoform of tau, 2N4R. We then prepared a set of His-tagged purified chaperones from the aggregation screen that were compatible with the solubility and tagging requirements of the platform. The binding of the His-tagged chaperones (10µM) was measured with fluorescently labeled anti-His antibodies. After removing false positives that bound anti-His antibody and/or Hsp70, we annotated true binding sites as regions composed of at least two contiguous 15-mer peptides (Fig. 3a and Methods). We found that the previously assayed chaperones Hsp72 and Hsp90 bound sites overlapping both aggregation motifs PHF6* (aa 275–280) and PHF6 (aa 3–311) and near the polyproline–R1 boundary (aa 220–242) (Supplementary Fig. 4a, b), as expected. Strikingly, we found that a number of other chaperones, including DnaJA1, DnaJA2, Hsp27 and FKBP51 also bound peptides in or around PHF6* and PHF6 (Fig. 3a). We also noted that there were additional high-frequency chaperone-binding sites in the polyproline region (aa 220–242) and the N terminus (aa 5–23). These regions were also interesting because they correspond to multiple AD-associated phosphorylation sites, a known Pro325 isomerization site and an N-terminal region previously linked to tauopathy. The peptides that bound to multiple chaperone families shared no overall similarity in characteristics such as charge, pi, aliphatic index or hydrophobicity (Supplementary Table 2), consistent with the relatively broad binding properties expected from these different structural classes.

Next, we carried out NMR studies on Hsp72, Hsp90, DnaJA2, Hsp27 and DnajB4 by titrating them into solutions of N-labeled WT 2N4R tau (Fig. 3a). This experiment was important because peptide arrays cannot mirror the broader context of surrounding regions in polypeptides. Despite this complication, we found that the location of the interactions, as measured by NMR, generally supported the peptide array results. Specifically, Hsp72, Hsp90 and DnaJA2 seemed to bind regions that spanned the polyproline and MBRs (R1–R4), in alignment with previous NMR studies when available. However, compared to the peptide array, the NMR-based study also picked up broader footprints for Hsp72, Hsp90 and DnaJA2, which often encompassed the R3 region containing the PHF6 motif. Interestingly, no binding was observed for Hsp27, suggesting that it may modulate tau aggregation through recognition of non-nomeric forms of the protein.

To further explore the interaction of anti-aggregation chaperones with the PHF6 and PHF6* regions of tau, we carried out further NMR studies. Specifically, we titrated Hsc70, DnaJA2, clusterin and Hsp60 into solutions of N-labeled WT K18 tau (Fig. 3b). Owing to the higher resolution of this construct, we observed more discrete binding footprints. Specifically, we found that Hsc70, DnaJA2, clusterin, and Hsp60 bound to the region overlapping the PHF6 motif, along with weaker additional binding to the PHF6* region. Thus, a major mechanism of anti-aggregation activity seems to be the direct binding to tau in the aggregation-prone sequences.
A subset of chaperones suppresses formation of tau ‘seeds’. Recent studies have demonstrated that tau aggregates can have prion-like activity, including the ability to induce aggregation of soluble tau when introduced to cells\(^{16,19}\). Indeed, propagation of aggregates may be a potential mechanism for disease progression in a variety of neurodegenerative diseases\(^{41}\). Because we found that several chaperones slowed tau aggregation, we wondered whether this activity would suppress prion-like character. In support of this idea, centrifugation (100,000 g) of the tau samples co-incubated with chaperones showed that some, such as Hsc70, Hsp72 and DnaJA2, increased tau solubility (Supplementary Fig. 5a,b). However, even small, soluble tau species have been determined to be capable of nucleating aggregation, so we did not consider the ability to block overall aggregation a sign of anti-prion-like activity\(^{42}\). Accordingly, we used a ‘clone’ cell line developed in the Diamond laboratory, in which K18 tau is stably expressed as a fusion to yellow fluorescent protein (K18-YFP)\(^{41}\). In this model, the K18-YFP is distributed evenly in the cytoplasm but forms fluorescent puncta upon transfection with aggregated tau from patient brain or recombinant sources (Fig. 4a). Importantly, tau seeds can be titrated into this system, and the number of cells with puncta can be quantified (Fig. 4b). As a control, we first made sure that none of the individual chaperones reduced the number of puncta when added to preformed fibrils (Supplementary Fig. 5c). This result suggested that the chaperones themselves did not reduce seeding when added exogenously.

Next, to see whether chaperones could suppress seeding activity, we transfected clone 1 cells with unfractionated 0N4R tau\(^{17,19}\) fibrils formed in the presence of individual chaperones (1:1 ratio), mirroring the initial ThT assay conditions. Strikingly, we found that Hsc70 and DnaJA2 were the only chaperones effective at reducing tau prions (Fig. 4c). DnaJA2 was particularly effective in this platform, and it even suppressed tau prions formed from P301L and A152T mutants. Thus, these results suggest that DnaJA2 probably acts on tau during the aggregation process to lower both its aggregation and its seeding potential.

DnaJA2 is upregulated in affected neurons of MCI and AD patients. We asked whether DnaJA2 was linked to tau aggregate pathology in vivo. To test this idea, brain slices from patients with MCI or AD were labeled with antibodies for phospho-tau (AT8, green) and DnaJA2 (red). In addition, we used propidium iodide (PI) to indicate nuclei (blue) and FSB to stain amyloid (white). First, we confirmed that MCI samples had sparse tau pathology as marked by AT8 positivity (Supplementary Fig. 6a), whereas samples from patients with AD had greater pathology (Supplementary Fig. 6b). In MCI samples, neurons positive for AT8 and/or FSB staining (Fig. 5a,b) had strikingly increased levels of DnaJA2 compared to surrounding neurons in the same sample or non-demented controls (Fig. 5c). Interestingly, the DnaJA2 staining does not colocalize with the AT8 or FSB signals in the soma or neurites (Fig. 5a,b) of affected MCI neurons; rather, it was interspersed or surrounding the marked regions of pathology (Supplementary Fig. 6c). Quantification of DnaJA2 levels showed increased staining for DnaJA2 in 80% of AT8-positive neurons in MCI samples (Fig. 5d) and 31% of AT8-positive neurons in AD (Fig. 5e). These results suggested that DnaJA2 might be a protective factor that diminishes in AD neurons. We also noted that, in AD samples, there was prominent DnaJA2 staining at sites just adjacent to neurons, which are potentially surrounding glial cells (Supplementary Fig. 6d).

Next, we compared the DnaJA2 results to staining with a control marker of AD57. Thus, our findings raise the interesting possibility that chaperones associate with tau and which ones tune its levels. However, we were interested in learning which chaperones physically bind to tau and how tau variants (mutation, PTMs) impact this molecular recognition. Biochemical and biophysical approaches allowed us to broadly examine tau variants and their direct binding to candidate chaperones. On the other hand, this approach is reductionist in some important ways; for example, chaperones rarely work by themselves, and many of them operate in an ATP-rich environment with a full complement of stress responses associated signaling factors and crowding agents. None of those features are replicated in our studies. Despite this limitation, we uncovered some previously unknown relationships that could have implications for chaperone biology and tauopathies.

Forging new connections between chaperones, tau, and disease-related pathology. Although DnaJA2 is known to be a co-chaperone of the Hsp70s, it had not previously been linked to tau homeostasis. However, our results suggest that it could be an early marker of tau pathology. Previous transcriptome analyses of brain tissues found a moderate decrease in DnaJA2 mRNA transcripts coincident with age and AD\(^{2}\); however, our results suggest that DnaJA2 protein levels can be highly variable even between individual neurons within the same sample. For example, we found that specific neurons with tau pathology had dramatically higher DnaJA2 levels compared to those of neighboring cells, which would probably not be clear in whole-brain transcriptome studies. Follow-up studies will be aimed at testing the hypothesis that DnaJA2 is an early protective factor that limits tau aggregation. Specifically, it seems possible that deficiencies in either its levels or its function may coincide with nucleation sites for pathology.

Other chaperones also warrant further investigation. For example, multiple studies have identified the CLU gene encoding clusterin as a significant risk factor for AD\(^{44}\). Clusterin, a secreted protein, has reported anti-aggregation activities towards Ap, another amyloido-genic protein implicated in AD. Tau is thought to be secreted from neurons\(^{46}\), and elevated tau in the cerebrospinal fluid is a diagnostic marker of AD\(^{42}\). Thus, our findings raise the interesting possibility that clusterin has another protective function to bind and sequester extracellular tau. Another potentially interesting chaperone is the prolyl isomerase FKBP12. In AD samples, the overall expression of FKBP12 is decreased, but a strong colocalization pattern of NFTs with FKBP12 was observed\(^{2}\). There is a report that FKBP12 delays
aggregation of a short tau peptide containing the PHF6 aggregation motif; however, we found no evidence of anti-aggregation activity in any of the longer tau constructs used in our study. In contrast, our findings suggest that FKBP12 might promote tau aggregation, at least for some acetylated variants.

Do disease-related mutations and PTMs in tau evade chaperone function? Tau pathology has been linked to point mutations, truncations and aberrant PTMs. Why do some variants, such as P301L, cause disease? We wondered whether some of these variants might ‘avoid’ the chaperone system, possibly contributing to an imbalance in their own homeostasis. Indeed, we observed a striking reduction in anti-aggregation activity for particular tau mutations, such as P301L and D421. Of the 50 + tau mutations linked to tauopathy, P301L is the most common, whereas accumulation of the D421 fragment is prevalent in patients with AD and is correlated with cognitive decline. Thus, we hypothesize that some tau variants, but likely not all, lose affinity for key surveillance chaperones, such as DnaJ2A. Over time, this failed recognition might contribute to increased tau levels and its deposition. It is likely that many other factors also contribute, but it is compelling to envision that a failure of surveillance chaperones is one issue.

Methods
Methods, including statements of data availability and any associated accession codes and references, are available at [https://doi.org/10.1038/s41594-018-0057-1](https://doi.org/10.1038/s41594-018-0057-1).

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Author contributions
S.-A.M., C.A.D. and J.E.G. conceived and designed the study. S.-A.M., R.F., C.C., J.O., H.K., A.G. and T.A. acquired data. S.-A.M., R.F., C.C., J.O., H.K. and O.J. analyzed and interpreted data. S.-A.M., F.T.F., M.R.W., M.Z., J.E.G. drafted and/or revised the manuscript. N.J., V.A.A., B.M.D., J.N.R., J.L., F.T.F., M.R.W. and M.Z. contributed reagents.

Competing interests
The authors declare no competing interests.

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Methods

Recombinant protein purification. Methods for purifying individual chaperones are referenced in Supplementary Table 3. Protein purity was confirmed by SDS–PAGE. Modifications to published methods are detailed as follows. For tau purification, sodium chloride (500 mM) and the small-molecule arachidonic acid (10 mM) were included in the growth media prior to induction to improve expression and minimize degradation. Expression was induced with 200 mM IPTG for 3.5 h at 30 °C. To purify tau, cells were lysed via a microfluidizer (Microfluidics), and then the lysate was boiled for 20 min. The clarified supernatant containing tau was dialyzed over night in 20 mM MES, pH 6.8, 50 mM NaCl, 1 mM EGTA, 1 mM MgCl₂, 2 mM DTT, 0.1 mM PMSF) then purified by cation exchange with a pre-elution step of four column volumes of 15% elution buffer (Buffer A with 1 M NaCl) to remove degradation products. Following a 15–60% gradient elution, pure tau fractions were pooled and concentrated prior to snap freezing aliquots for storage at −80 °C. For K18 tau NMR experiments, tau was purified as described above, except protein was expressed in M9 minimal media containing sodium chloride (500 mM) and 1% glycerol. Purified ‘N’-labeled K18 tau was dialed overnight into 20 mM ammonium bicarbonate solution, then lyophilized and stored at 4 °C. Naive Hsp60 containing its mitochondrial import signal was overexpressed in E. coli BL21(DE3) cells induced with 0.25 M IPTG for 4 h at 37 °C. Cells were resuspended in 50 mM Tris- HCl, pH 8, 10 mM imidazole and 500 mM NaCl and lysed with a microfluidizer. The cleared lysate was applied to a Ni-NTA resin (Qiagen) and eluted with buffer containing 300 mM imidazole. The His-tag was cleaved during overnight dialysis in 50 mM Tris, pH 7.5, 1 mM DTT at 4 °C. Hsp60 was purified to homogeneity in a Superdex 200 16/600 column (GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl and eluted using a linear gradient of 50 mM to 1,000 mM of NaCl. Hsp60 was snap frozen in liquid nitrogen and stored at −80 °C.

WT Pln1 was cloned into the pMSCG10 vector and transfected into BL21 (DE3) cells. Cultures were grown at 37 °C to an approximate OD of 2, and then expressed with 1 mM IPTG overnight at room temperature. Cell pellets were collected and lysed in 100 mM Tris, 200 mM NaCl, 10% glycerol and 1 mM reduced GSH, pH 8.4, then loaded onto a GSTrap 4B column. After purification, TEV protease was added overnight. Cleaved Pln1 was concentrated and purified by size-exclusion chromatography on a Superdex 75 column (10 mM HEPES, 100 mM NaCl, 10% glycerol, 1 mM DTT, pH 7.5). S. cerevisiae Hsp104/pProEX-HT-B was overexpressed with a TEV protease-cleavable N-terminal His-tag in E. coli BL21 (DE3) RIL (Agilent) by inducing cells with 0.5 mM IPTG for 6 h at 37 °C. Cells were resuspended in 25 mM Tris- HCl, pH 7.5, 300 mM NaCl, 5% glycerol, 30 mM imidazole and 5 mM β-mercaptoethanol and lysed using a microfluidizer. The cleared lysate was applied to a Ni-Sepharose FF column (GE Healthcare), and proteins were eluted using a linear gradient of 30 mM to 500 mM imidazole. The His-tag was cleaved with His-TEV protease while dialyzing the protein sample in 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% glycerol and 1 mM DTT overnight at 4 °C. After removing the liberated His-tag and His-TEV protease by passing over a Ni-Sepharose FF column, Hsp104 was purified by anion exchange chromatography and eluted using a linear gradient of 50 mM to 1,000 mM of NaCl. Hsp104 was further purified by size-exclusion chromatography using a Superdex 200 10/300 column (GE Healthcare) pre-equilibrated in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% glycerol and 1 mM DTT. Hsp104 hexamers were concentrated, snap frozen in liquid nitrogen and stored at −80 °C.

In vitro tau aggregation. All proteins in the aggregation assay were dialyzed overnight in 4°C with D2O buffer (Dulbecco’s, 1 mM DTT). Tau (10 μM) was pre-incubated in the presence or absence of chaperones (2.5–20 μM) for 30 min at 37 °C. ThT (Sigma) at a final concentration of 10 μM was added, and aggregation was induced by the addition of a freshly prepared heparin sodium salt solution (Santa Cruz) at a final concentration of 1 mM DTT. For non-induced controls, assay buffer was added in place of heparin solution. In assays using arachidonic acid as an aggregation inducer, heparin was replaced with arachidonic acid (150 μM) in 5% ethanol, and non-induced controls contained 5% ethanol. Aggregation reactions were carried out at 37 °C with continuous shaking and monitored via ThT fluorescence (excitation, 444 nm; emission, 485 nm; cutoff, 532 nm). Empty spots were used as negative controls. Binding was defined as >2 consecutive peptides with fluorescence signals greater than one standard deviation above the mean in replicate experiments. Peptides that bound to the anti-His6 antibody alone or to an Hsp70 construct lacking its SBD (Hsp70noSBD) were identified as false positives and excluded from further analysis. Positive sites were restricted to those composed of two or more consecutive binding peptides. The one exception was peptide (as 5–29), which was included because the peptide preceding it was a false positive.

NMR spectroscopy. For K18 tau–chaperone binding studies, all proteins were dialyzed overnight into NMR buffer. NMR buffer used in experiments was 30 mM sodium phosphate, pH 6.8, 10 mM NaCl, 1 mM TCEP, 10% (vol/vol) D₂O or, alternatively, 25 mM HEPES, pH 7.4, 10 mM KCl, 5 mM MgCl₂, 1 mM TCEP, 10% (vol/vol) D₂O). Prepared samples contained 50 μM ‘N’-labeled tau and 50 or 100 μM unlabeled chaperone in NMR buffer. 2D 1H-15N heteronuclear single quantum coherence (HSQC) spectra were recorded at 10°C on a Bruker Avance AV800 spectrometer equipped with a cryoproxy. 16 scans were acquired per τ1 and spine flip angle of 2,100 and 100° for τ2 and they were used in the ‘H and ‘N dimensions, respectively. Spectra were processed using NMR and Sparky based on deposited tau assignments (Barre 2013, BioMagResBank accession number 19253). Signal intensity ratios were calculated using Prism. For 2N4R tau interaction studies, ‘H-15N correlation spectra were acquired on 30 μM ‘N’-labeled tau and up to ten equivalents of unlabeled chaperone at 5°C. For 2N4R tau aggregation controls, ‘H spectrum was acquired on 0.5 μM ‘N’ labeled tau. For experimental spectra, 50 mM sodium phosphate, 10 mM HEPES, 10 mM NaCl, 1 mM DTT, 10% (vol/vol) D₂O, pH 6.8, was used for Hsp27 and β-cryotin. Spectra were processed in Topspin and analyzed in Sparky.

Tau cellular seeding assay. The HEK293 cell line stably expressing the microtubule-binding repeat region of tau fused to V5-FLAG (clone 1) was kindly provided by M. Diamond. The cell line stock tested negative for mycoplasma. Cells were plated at a density of 2,000 cells/well in 384-well clear black plates. For experiments with Lipofectamine 2000 (Thermo Fisher Scientific) used to introduce fibrils, a ratio of 1 μg tau protein/0.3 μL Lipofectamine 2000 was used. Following a 48-h treatment, nuclei were stained in live cells with 0.0125 µg/well of Hoechst 33342 (Thermo Fisher Scientific) for 1 h. Images for DAPI and FITC channels from three regions per well were captured using an InCell 6000 (GE HealthCare). The images were processed using InCell Developer software (GE HealthCare) with an algorithm developed to identify live cells with intracellular aggregates larger than 0.89 m². Greater than 600 cells per well, assayed in triplicate, was analyzed in three independent experiments.

Fibril sedimentation. Tau aggregation samples were centrifuged at 100,000g for 1 h at 4°C. Supernatants were collected, and pellets were suspended in aggregation assay buffer overnight at room temperature with shaking. SDS sample buffer

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Articles

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(1×final) was added to equal fractions of each reaction and loaded on 4–15% SDS–PAGE gels. A set of standards of known tau protein amounts was present on each gel. Proteins were visualized with Coomassie blue, and images of stained gels were used to quantify the bands with Image lab software (Bio-Rad).

Human tissue samples. Seven formalin-fixed brain tissue samples were obtained from neuropathologically confirmed cases of Alzheimer's disease (Braak stage V–VI), mild cognitive impairment (Braak stage II), or non-demented aged control. Medial frontal cortex and inferior temporal cortex regions were utilized for this study. Tissue was obtained from the University of California San Francisco Neurodegenerative Disease Brain Bank. All procedures for animal use were approved by the UCSF Institutional Review Board’s Human Research Protection Program.

Immunohistochemistry and confocal imaging. Formalin-fixed samples were embedded in paraffin using standard procedures. Sections (8 μm) were cut, photobleached using a multiperpendicular LED lamp, deparaffinized, then processed for immunohistochemistry. Sections stained with antibodies were pretreated by incubation in heated 0.01 M sodium citrate buffer as an epitope-retrieval step. Following blocking with 10% normal goat serum, sections were incubated with primary antibody overnight at 4 °C (see Supplementary Table 4 for antibody incubation in heated 0.01 M sodium citrate buffer as an epitope-retrieval step). Sections stained with antibodies were pretreated by incubation in heated 0.01 M sodium citrate buffer as an epitope-retrieval step. Primary antibody detection was performed using goat secondary antibodies with conjugated AlexaFluor488 or AlexaFluor647 (A-21245, Life Technologies). All immunohistochemistry-stained sections were subsequently stained with 2.5 μM Fluorostyrylbenzene (FSB; Congo red derivative) (Santa Cruz) in 1×PBS and washed with propidium iodide (Sigma) before mounting. Samples were visualized using a 40× water-immersion lens (1.1 NA) or 63× oil-immersion (1.4 NA) in sequential scan mode on a Leica SP8 confocal microscope equipped with HyD detectors. 8-bit image z stacks (1-μm steps) were collected at 1,024×1,024 or 2,048×2,048 pixel resolution. Images were processed using NIH ImageJ.

Isothermal calorimetry (ITC). Chaperones were dialyzed into ITC buffer overnight (40 mM HEPES, pH7.4, 150 mM NaCl). Protein concentrations were determined via BCA assay (Pierce). The experiment was carried out at 25°C on a MicroCal VP-ITC where heparin at 200 μM or 1 mM (based on an average MW of 9,500) was titrated into a 100 μM cell solution of Hsc70 or DnaJ2. Calorimetric parameters were calculated using Origin 7.0 software and fit with a one-site binding model.

Dot blot. Purified recombinant chaperones (1.5 μg or 0.75 μg total) were spotted on nitrocellulose, stained with Ponceau S to verify transfer, and then processed by immunoblot. The membrane was blocked in 4% milk solution then incubated with DnaJ2 antibody (1:20,000) at 4 °C overnight. The membrane was incubated with goat anti-mouse HRP (1:40,000, Cell Signaling Technology) for 45 min prior to chemiluminescence signal detection with ECL Select (Amersham) by a Chemidoc Touch gel imaging system (Bio-Rad).

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

Data availability. Mutations and post-translational modifications in tau mimicked in this study are described in refs 46–48 and listed in Supplementary Table 1. Purification of individual chaperones is described in refs 49–53 and listed in Supplementary Table 3. The datasets generated and analyzed in the current study are available from the corresponding author upon reasonable request.

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- Clearly defined error bars
- State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection
For all data collection, instrument data was obtained using the manufacturer recommended software

Data analysis
The Grace plotting program and customized scripts written in Perl were used to analyze aggregation kinetic data as described in Materials and Methods. InCell Developer software (GE Healthcare) was used to process images from cellular assays. NMR spectra were processed/analyzed using Topspin and Sparky as described in Material and Methods. Images from immunohistochemistry stained human patient samples were analyzed with NIH Image. ITC data processed using Origin 7.0 software. Statistical analyses performed with Prism (GraphPad software)

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Life sciences

Study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size       | No sample size calculation was performed for aggregation screens however, reproducibility between replicates and individual experiments was verified during the optimization of the technique (see supplementary data). |
|-------------------|---------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions   | No data was excluded from analysis                                                                                                                                                        |
| Replication       | All attempts at replication were successful. Experiments were also replicated with different batches of reagents (e.g. aggregation inducers), protein preparations (e.g. Hsc70, DnaJA2), and cell line stocks (e.g. clone 1 cells) |
| Randomization     | Samples were from protein preparations (verified by SDS-PAGE). Sample groups divided according to protein identity.                                                                 |
| Blinding          | Investigators were not blinded to group allocation                                                                                                                                         |

Materials & experimental systems

Policy information about availability of materials

n/a Involved in the study

☑ Unique materials
☑ Antibodies
☑ Eukaryotic cell lines
☐ Research animals
☐ Human research participants

Unique materials

Obtaining unique materials

Protein expression constructs or purified proteins used in the study are available from the authors. The clone 1 cell line is available with permission from Marc Diamond (UT Southwestern). Human patient samples were received from William W. Seeley and the UCSF Neurodegenerative Disease Brain Bank.

Antibodies

Antibodies used

Detailed information regarding antibodies used in the study are provided in the Materials and Methods.

Validation

DnaJA2 antibody (Ongene) was tested for specificity against a panel of other Hsp40 family members by dot immunoblot in this study. The DnaJB4 antibody (Atlas Antibodies) was used in the Human Protein Atlas Project. Hsc70 and Hsp72 antibody (Enzo) checked for cross reactivity by immunoblot. According to the manufacturer, Hsp27 antibody (StressMarq) has been used for IHC/IF in Unger et al (2017), Kötter et al. 2014, Periera et al. 2018. The AT8 antibody (Pierce/ThermoFisher) has been used for IHC/IF staining results in over >250 publications as referenced on the manufacturer’s product webpage.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

The clone 1 cell line was obtained from Marc Diamond [UT Southwestern]
Authentication

The cell line was authenticated through replication of results in phenotypic assays developed for the cell line by the laboratory of Marc Diamond (UT Southwestern).

Mycoplasma contamination

Original cell line stock tested negative for mycoplasma using commercial kit (Mycoalert, Lonza)

Commonly misidentified lines
(See iCLAC register)

N/A

Method-specific reporting

n/a | Involved in the study
---|---
☑️ | ChIP-seq
☑️ | Flow cytometry
☑️ | Magnetic resonance imaging