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DnaJA1 antagonizes constitutive Hsp70-mediated stabilization of tau

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Abstract

Tau aggregation and amyloidogenesis are common hallmarks for neurodegenerative disorders called tauopathies. The molecular chaperone network constitutes the cellular defense against insults such as tau aggregation. However, chaperone effects on tau are dichotomous. Loss of tau's microtubule-binding activity facilitates an inappropriate chaperone interaction that promotes an amyloidogenic tau conformation. Conversely, other chaperones are capable of promoting tau clearance. Here, we demonstrate that a critical contributor to tau triage is the DnaJ-binding domain of Hsp70 proteins. In particular, over-expression of the constitutive DnaJ, DnaJA1, mediated tau clearance, while knockdown facilitated tau accumulation. This clearance was not specific to distinct pathogenic tau species. The activity of DnaJA1 was attenuated by concomitant increases in Hsp70. Tau reductions facilitated by DnaJA1 were dependent on the integrity of lysines known to be poly-ubiquitinated in human Alzheimer's brain. *In vivo*, DnaJA1 and tau levels were inversely correlated. The effects of DnaJA1 were partially specific: DnaJA1 reduced the levels of a polyQ protein but had no significant effect on α -synuclein levels.

These data suggest that DnaJA1 triages all tau species for ubiquitin-dependent clearance mechanisms. Moreover, the levels of DnaJA1 and Hsp70 seem to play against each other with regard to tau: as DnaJA1 levels increase, tau levels are reduced, but this can be prevented if Hsp70 levels are simultaneously induced. Thus, the DnaJ repertoire possibly represents a powerful set of genetic modifiers for tau pathogenesis. Further investigations, could provide new insights about triage decisions that facilitate or prevent amyloidogenesis of tau and other proteins associated with neurodegenerative disease.

Introduction

Molecular chaperones direct the folding, unfolding, and degradation of proteins termed clients. Chaperones are essential for proteostasis, and recent evidence suggests that they may be critical therapeutic targets in neurodegenerative diseases arising from accumulation of

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abnormal proteins like the microtubule associated protein tau¹. Excessive accumulation of tau that is abnormally hyper-phosphorylated and proteolytically-cleaved in neurons is a pathological hallmark of tauopathies, a group of more than 15 diseases, including Parkinson's and Alzheimer's diseases². Thus, developing strategies to remove abnormal tau in symptomatic patients may be clinically relevant. The chaperone super-family likely harbors several unidentified members that may be targeted to facilitate abnormal tau removal. Identifying these components could be essential to determine how tau fate decisions are made in the cell.

A major component of the chaperone network is the DnaJ/Hsp40 family of proteins. These DnaJs can deliver nascent and misfolded clients to Hsp70 proteins, while simultaneously regulating Hsp70s' ATPase turnover. These properties suggest that DnaJs have tremendous power in controlling the fate of distinct types of clients including tau. There are 41 known DnaJ proteins coded in the mammalian genome, and the importance of this diversity is just beginning to be explored. The J-domain present in all DnaJ proteins is essential for binding to Hsp70s. However, there is a vast amount of variability in sequence, structure, and function between all DnaJs that gives each one a novel functional profile to investigate¹¹.

Evidence implicating DnaJ proteins in regulating tau processing already exists. DnaJB1 (the primary Hsp40) associates with tau in a *Drosophila* model of tauopathy¹², and knockdown of this DnaJ protein stabilized tau levels in cells¹³. Moreover, targeting the DnaJ-binding region on Hsp70 with small molecules has been a powerful means to abrogate tau levels in cells and restore learning and memory functions *in vivo*.

DnaJA1 (hdj2) is the primary co-chaperone of the constitutively expressed Hsp70 variant, Hsc70¹⁸. Hsc70 is highly expressed in neurons and was recently shown to be a predominant Hsp70 family member involved in tau biology¹⁹. Moreover, oxidation, which can occur in Alzheimer's disease and with aging, can inactivate the co-chaperoning activity of DnaJA1²⁰. Thus, DnaJA1 is likely to have an important role for tau in the brain at the interface with both the constitutive and inducible Hsp70.

We speculated that DnaJA1 could participate in the processes that contribute to tau triage, particularly since chemical manipulation of Hsp70's DnaJ-binding domain potentially altered tau stability. In cells and brain tissue, DnaJA1 expression inversely correlated with tau levels. DnaJA1 was inversely co-localized with peri-nuclear tau aggregates in neurons from tau transgenic mice. The anti-tau efficacy of DnaJA1 was dependent on the integrity of residues linked to ubiquitination in human disease and was abrogated when Hsp70 levels were concomitantly increased. Moreover DnaJA1 abrogated a poly-glutamine protein but had a minimal effect on α -synuclein, suggesting that some degree of specificity of DnaJA1 for client subsets exists. We conclude that DnaJA1 is a major regulator of tau stability that coordinates with Hsp70 to facilitate tau triage decisions.

Results

Pharmacological targeting of the DnaJ-binding region on Hsp70s can significantly reduce tau levels. Corroborating these results, genetic over-expression of only a flag-tagged DnaJ polypeptide in HeLa cells stably over-expressing tau caused a similar tau-reducing effects (Fig. 1A). Based on this and previous data implicating Hsp70 as a primary regulator of tau stability, we speculated that DnaJA1 may be a primary DnaJ regulator of tau turnover. To test this, we manipulated DnaJA1 levels using both knockdown and overexpression paradigms. Indeed, DnaJA1 and tau levels were inversely correlated (Fig. 1B and 1C), such that as DnaJA1 levels were reduced, tau levels increased and vice versa. DnaJA1 over-expression reduced tau by 47% (* p <0.05; Fig. 1D). The levels of all tau species investigated

were similarly affected, regardless of phosphorylation status. In addition, levels of caspase-cleaved tau were also inversely correlated with DnaJA1 levels, suggesting that even pathogenic tau species were responsive to fluctuations in DnaJA1 levels (Fig. 1E). A caspase inhibitor was used to confirm specificity of the TauC3 antibody, which only recognized tau cleaved by caspases at D421. The effects of DnaJA1 over-expression on tau were unaffected by the presence of a flag tag (Fig. 1F).

Immuno-precipitation experiments were then performed to determine if DnaJA1 could bind to tau (Fig. 2A). Flag-tagged DnaJA1 was transiently transfected in HeLa cells that stably overexpressed tau, and tau protein was immuno-precipitated. Indeed, DnaJA1 associated with tau (Fig. 2A). This interaction influenced the association of tau with Hsp70, as evidenced by increased binding of Hsp70 to tau in the presence of DnaJA1 over-expression. Co-localization experiments were performed to confirm the interaction of A1 and tau. HeLa cells stably overexpressing tau were plated on slides and transiently transfected with control plasmid or flag-tagged A1. Cells were then immuno-fluorescently stained using anti-Flag (green) and anti-tau (red) antibodies as well as Hoechst (to reveal cell nuclei in blue; Fig. 2B and 2C). The pronounced Flag-positive signal in the A1-expressing cells was absent in the control cells, as expected. Moreover, both conditions had strong tau signal (red). Merged images show a wide range of cells where the A1 or tau signal predominates, and another set where both signals co-localize (Fig. 2B). Higher magnification images of cells positive for A1 and tau showed yellow signal in the merged image (Fig. 2C); co-localization was complete in these instances (Fig. 2D Pearson coefficient >0.99). These data suggested an important role for DnaJA1 in mediating tau triage decisions.

With a relationship between DnaJA1 and tau levels established, the mechanisms employed by DnaJA1 to clear tau were further explored. A purely genetic approach was utilized to avoid off-target issues associated with chemicals that regulate clearance pathways. Based on previous work, three tau variants were designed to alter the clearance kinetics of tau and further explore the tau triage pathways being used by DnaJA1 (Fig. 2E). I308 and V309 in tau are known to be important for its binding to Hsp70 members. Correspondingly, deleting these two amino acids (Δ I308- Δ V309 tau) reduces the binding affinity of tau for Hsp70²¹. This double deletion variant was generated and termed m τ -Hsp70 (Fig. 2E and 2F). K254, K311, and K353 on tau were found to be poly-ubiquitinated when extracted from human tau tangles²². Substitutions for alanine at these lysine residues prevent ubiquitination. This 3 $\times$ mutant was generated and termed m τ -Ubq (Fig. 2E and 3B). Tau also contains two motifs within the microtubule binding domains (³³⁶QVEVK³⁴⁰ and ³⁴⁷KDRVQ³⁵¹) that are required for its targeting to lysosomes for chaperone mediated autophagy (CMA). Tau disengaging from microtubules exposes these motifs leading to its clearance by CMA. Indeed, substitutions of the first two amino acids of each CMA-motif to alanine residues prevent tau CMA²³. This 4 $\times$ mutant variant was generated and termed m τ -CMA (Fig. 2E and 3C).

To determine whether the interaction of DnaJA1 with Hsp70 was important for tau triage, M17 neuroblastoma cells were co-transfected with DnaJA1 and either wild-type tau (wt- τ) or m τ -Hsp70. Levels of pS396/S404 tau (pTau) and total tau within the lysates were evaluated by immunoblot. DnaJA1 potently reduced tau and pTau levels of wildtype tau (wt- τ ; Fig. 2F). Interestingly, the anti-tau efficacy of DnaJA1 was even stronger for m τ -Hsp70, suggesting that in the absence of Hsp70 binding to tau, DnaJA1-mediated tau clearance was enhanced (Fig. 2F). To confirm this, Hsp70 was overexpressed along with DnaJA1 (Fig. 2G): indeed, increased Hsp70 prevented tau clearance by DnaJA1.

The effects of DnaJA1 on the m τ -Ubq and m τ -CMA mutants were then assessed to evaluate whether DnaJA1 was utilizing these two major routes of tau clearance. M17 cells were co-

transfected with DnaJA1 and either m τ -Ubq or m τ -CMA. DnaJA1 over-expression reduced levels of wt- τ and m τ -CMA (Fig. 3A and 3B); however, m τ -Ubq was resistant to DnaJA1-mediated clearance (Fig. 3C). In fact, DnaJA1 caused m τ -Ubq to accumulate compared to control transfected cells, suggesting that the three lysine residues known to be poly-ubiquitinated in human brain were essential for tau clearance by DnaJA1.

The levels of DnaJA1 were then assessed in human brain extracts obtained from individuals who suffered from Alzheimer's disease (AD) and age-matched, non-demented controls to confirm the *in vivo* relevance of the findings in cells. Western blot analysis showed that AD brains had a 47% (* p < 0.05) reduction in the levels of DnaJA1 compared to age-matched controls (Fig. 4A and 4B), further supporting a role for DnaJA1 in tau turnover *in vivo*. Moreover, immuno-fluorescent staining was then used to assess the distribution of DnaJA1 in the brains of rTg4510 tau transgenic mice²⁴, which develop significant perinuclear pre-tangle pathology as early as 1.5 months. Co-staining for tau and DnaJA1 in brain sections from 9-month-old rTg4510 transgenic (Tg) and non-transgenic (NTG) mice revealed that neurons with robust perikaryal tau staining reminiscent of pre-tangle formation had little to no DnaJA1. DnaJA1 was observed throughout the CA3/CA2 granular layer (dashed white lines) in Tg and NTG mice (Fig. 4C and 4D), yet it was not found in neurons with pathologic tau accumulation. These findings further support the hypothesis that levels of DnaJA1 inversely correlate with tau levels.

Since DnaJA1 is known to affect a number of Hsc70 clients, cells were co-transfected with DnaJA1 and either α -synuclein or poly-glutamine of 84 repeats (Poly-Q84). These proteins were chosen because of their relevance to neurodegenerative disease { }. Poly-Q84 and α -synuclein were assessed by Western blot. DnaJA1 overexpression did not significantly reduce α -synuclein levels (22%, p > 0.05; Fig. 5A and 5C), but did reduce polyQ-84 (76%, p > 0.05; Fig. 5B and 5C). These results suggest that DnaJA1 displays specificity towards some, but not all clients involved in neurodegenerative diseases where protein aggregation is implicated.

Discussion

A role for the Hsp/c70 machinery in tau processing has been established; however, the mechanisms facilitating tau clearance or stabilization remain unknown. Here we demonstrate that DnaJA1, a major regulator of Hsc and Hsp70, mediates tau stability. Interestingly, the binding of Hsp70 to the DnaJA1-tau complex prevents tau degradation. It is therefore not surprising that blocking the DnaJ-binding region on Hsp/c70 could also enhance tau clearance. These data suggest that prevention of Hsp/c70's ATPase activity shuttles tau into a clearance pathway. In turn, our data suggest that this clearance mechanism requires ubiquitination. In addition, DnaJA1 could selectively modulate the levels of other proteins that participate in the pathogenesis of neurodegenerative diseases like polyQ. Thus, there seems to be a degree of client specificity exerted by DnaJA1, but this is more general to distinct structures rather than specific amino acid sequences. These studies also demonstrate that AD patients have lower levels of DnaJA1 in their brain. Moreover, tau transgenic neurons exhibiting tau pre-tangle structures lacked DnaJA1, while neurons that had detectable levels of DnaJA1 did not have tau aggregates. This suggests that a plentiful amount of DnaJA1 may effectively reduce the levels of aggregated tau species, thereby preventing neurotoxicity.

Our data focuses on the role of DnaJA1 as a regulator of tau fate; however, the effectiveness of DnaJA1 in reducing tau and polyQ suggests that other regulators of Hsp/c70 ATPase activity, like the 41 DnaJ proteins and their alternatively spliced variants, could hold even greater specificity for distinct pathogenic structures. The diversity of the DnaJ protein family

could be likened to proteases or E3 ubiquitin ligases, except that DnaJs lack enzymatic activity. They recognize unique clients and, rather than act directly on them, they work with Hsp70s and other cellular machinery to determine their fate. In some ways, DnaJ proteins are the quintessential chaperones in that they select a client and sort it in the cell. In diseases of proteotoxicity, where too much of an aberrant protein is being produced, DnaJs may have the capacity to be both judge and jury for a client, recognizing the abnormal accumulation and acting to remove it without requesting assistance from the rest of the chaperone system. The structural basis of client selection could also be an important tool to manipulate in future. With intrinsically disordered proteins like tau that assume many diverse conformations, any number of DnaJs may be acting on distinct tau species at any given moment in the cell. Harnessing this power to selectively dictate triage decisions for specific tau variants could provide new evidence about which species are most toxic. Another possibility is that tau accumulation itself can impact the DnaJ repertoire. In disease, where tau structure becomes constrained into filaments or oligomers, the repertoire of expressed DnaJs may be different. Thus, while the DnaJs may regulate triage decisions for tau, it is possible that tau can turn the tables and begin to regulate the DnaJs as well, altering cellular proteostasis.

Based on the properties of DnaJ proteins, we speculate that distinct DnaJ proteins may be capable of recognizing discrete post-translationally modified species. While our results implicate DnaJA1 as a major tau regulator, it is possible that other DnaJs may be even more specific for disease-associated tau species. DnaJA1 may have some client specificity, but it is also dependent on Hsp/c70 levels to serve as a gauge. Perhaps other DnaJs may not rely on this Hsp/c70 “barometer”. Here, if sufficient levels of Hsc70 were not available for DnaJA1-bound tau, DnaJA1 could triage tau for ubiquitin-dependent degradation (Fig. 3D, 3E and 4A). Thus, any imbalance in Hsp/c70 and DnaJA1 levels could dramatically impact tau accumulation. Indeed, in AD and tau transgenic mice, the absence of DnaJA1 could be a critical cause or effect of tau accumulation. Indeed, it remains unclear if tau accumulation facilitates DnaJA1 reductions or if DnaJA1 reduction cause tau to accumulate. Regardless, it would be anticipated that increasing the levels of DnaJA1 is beneficial to neurons with aberrant tau accretion. But recent work suggests that aggregation of misfolded proteins could actually be a survival mechanism employed by long-lived neurons. If this is true, then it is possible that DnaJA1-mediated abrogation of perinuclear tau aggregation may be detrimental to neuronal function.

In conclusion, understanding the diversity of the DnaJ family could be critical for therapeutic development around the chaperone network. While inhibitors of Hsp90 are currently in clinical trials and have shown early success, some issues pertaining to off-target effects and toxicity have surfaced. Hsp70 inhibitors are also in development and have shown pre-clinical efficacy for tauopathies¹⁷. Nevertheless, the same concerns may arise for Hsp70 inhibitors given the large number of clients and structural redundancies of its family members. Thus, a better understanding of specificity in protein triage decisions is needed. The findings presented here suggest that DnaJ proteins could be the molecular switches of the chaperone system. These proteins not only select clients, but they also seem to facilitate triage decisions depending on the surrounding environment. If warranted, DnaJs can stabilize clients; however, they also have the capacity to remove clients via ubiquitin-dependent and possibly other clearance pathways. Ultimately, a better understanding of this important protein family could improve therapeutic development for the chaperone family and lead to specific therapies for neurodegenerative diseases.

Materials and Methods

Plasmids

Poly-glutamine-84 ataxin 3 was provided by Dr. Henry Paulson (University of Michigan). DnaJA1 and Hsp70 were made in the pCMV6 plasmid backbone. Tau mutants were generated in wildtype tau in a pcDNA3.1 vector. Wild type synuclein and tau were provided by Dr. Michael Hutton (Mayo Clinic).

Antibodies

Antibody to pTau is PHF1, which recognizes pS396/S404, and it was a generous gift from Dr. Peter Davies. Antibody to caspase-cleaved tau, Tau C3, recognizes the 421 amino acid fragment of tau, and it was a generous gift from Dr. Lester Binder. Antibody to total tau and Flag rabbit polyclonal antibody were purchased from SantaCruz Biotechnology, Inc. (sc-5587; sc-807). Flag mouse monoclonal antibody was obtained from Sigma, St. Louis, MO. (F3040). Rabbit polyclonal Anti- α -synuclein antibody was obtained from Cell Signaling Technologies, Danvers, MA (2272S;2642). Anti-actin antibody was obtained from Sigma (A2066). Anti-GAPDH antibody was obtained from BIODSIGN International, Saco, ME (H86504M). Anti-Hsp70 was procured from Stressgen, Ann Arbor, MI and Stressmarq Biosciences Inc, BC, Canada (ADI-SPA-810;SMC-100B). Anti-mouse monoclonal and anti-rabbit polyclonal secondary antibodies were obtained from Southern Biotech, Birmingham, AL (1030-05;4010-05). All antibodies were used at 1:1000 dilution, except for PHF1 and Tau C3, which were used at a 1:500 and 1:250, respectively. Secondary antibodies conjugated to HRP and anti-goat secondary antibody conjugated to a fluorophore (488 nm or 594 nm) were purchased from Southern Biotechnology. Alexa Fluor antibodies (Invitrogen) were optimized and diluted according to manufacturer's specifications.

Cell culture, transfections and protein recovery

HeLa cells stably transfected with V5-tagged 4R0N Tau, HeLa cells, and M17 cells were grown as previously described (12). Transfections were performed with Lipofectamine 2000 reagent (LFM; Invitrogen). The cells were harvested in Mammalian Protein Extraction Reagent (M-PER) buffer (Pierce) containing 1 $\times$ protease inhibitor mixture (Calbiochem), 100 mM phenylmethylsulfonyl fluoride, and 1 $\times$ phosphatase inhibitor II and III cocktails (Sigma).

Co-immunoprecipitations (IP)

IP experiments with the indicated antibodies were performed as described previously (12). Briefly, HeLa cells stably over-expressing V5-tagged wildtype human tau were transfected with 6 μ g of each plasmid and Lipofectamine 2000. After 48 hours, the cells were harvested in M-PER buffer. Lysates were pre-cleared for 1 hour at 4°C with 25 μ L of Protein G beads. The lysates were loaded onto spin columns and the filtrate was collected. The lysates were incubated with 2 μ g of SantaCruz anti-total tau antibody for one hour. Fifty μ L of Protein G were added and incubated with rotation at 4°C overnight. Protein G beads were pelleted and washed five times with Co-IP Buffer (100mM Tris HCl, 150mM NaCl). Samples were subjected to western blot analysis.

Western blots

Western blot analysis was performed as previously described (12). Protein samples were prepared using 2 $\times$ Laemmli's sample buffer. Samples were boiled for 5–10 minutes and then loaded onto a 10% or 18 % Tris-glycine gels (Invitrogen) or a 18-well, 10% criterion gels (Bio-Rad). The gels were transferred onto nitrocellulose membranes and then blocked

for 1 hour at RT with 7% milk. The membranes were incubated with primary antibodies with dilutions stated above.

Human brain samples

Fresh-frozen human cerebellar and medial temporal gyrus specimens were obtained from the Brain Donation Program at Sun Health Research Institute in Sun City, Arizona. Subjects with a clinical history of dementia were diagnosed as Alzheimer's using neuropathologic consensus criteria, including those published by CERAD²⁸ and the NIA-Reagan Institute (1997). Control subjects did not have a clinical history of dementia and did not meet neuropathologic criteria for AD or other neurologic disorders. The average post-mortem intervals were 2.6h for AD and 2.3h for control specimens. The average age for the AD specimens was 87 years, and the average age for the controls was 88 years. Dr. Tom Beach (Sun Health, Phoenix, AZ) provided Alzheimer's disease and normal human brain tissue samples (medial temporal gyrus). The postmortem interval was between 2.5 hours and 3 hours. The samples were age and gender matched. Human brain tissues were homogenized in M-PER buffer and centrifuged at $13000 \times g$ for 10 min at 4°C. Supernatants were collected and results were subjected to Western blot analysis.

Animals

All procedures involving experimentation on animal subjects were done in accord with the guidelines set forth by the Institutional Animal Care and Use Committee of the University of South Florida. The rTg4510 and parental mice were maintained and genotyped as described previously (25). Mouse brain tissues were homogenized in Hsio buffer, and later processed for Western Blot analysis.

Immunohistochemistry (IHC) and Microscopy

Mouse brains were harvested for IHC analysis as described previously (25); cell culture immunofluorescence preparation and analysis was performed as described²⁹. Staining was analyzed with the Zeiss AxioImager. Z1 and AxioVision software using 56/0.16 or 406/0.75 dry ECPlan-NeoFluar objectives where specified. Images were captured at room temperature with an AxioCam MR3 camera. Staining was analyzed with the Zeiss AxioImager. Z1 and AxioVision software using 56/0.16 or 406/0.75 dry ECPlan-NeoFluar objectives where specified. Images were captured at room temperature with an AxioCam MR3 camera. Fluorochromes used were DsRed, FITC, and DAPI. Images were modified in Adobe Photoshop by adjusting the brightness and contrast to all channels equally and simultaneously. Co-localization scatter analysis was performed on all images taken from non-transgenic and transgenic brain slices. Images at 20× magnification corresponded to areas within the CA2, CA3, and frontal cortex areas. Moreover, 63× images included five, one um z-stacked images. These included the use of the apotome (Zeiss) camera as a means to obtain sharper, confocal-like images.

Quantification and Statistical Analyses

Quantification of all blots was performed using ImageJ software as previously described (43, 44). Graphs are plotted based on relative intensity values. Statistical analyses were performed by Student's t tests as indicated in the figure legends.

Highlights

- DnaJA1 triages all tau species for ubiquitin-dependent clearance mechanisms.
- DnaJA1 and tau levels are inversely proportional; Hsp70 prevents this.

- The DnaJ repertoire is a powerful set of genetic modifiers for tau pathogenesis.

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Figure 1. The DnaJ-binding domain on Hsp70 and its main DnaJ protein, DnaJA1, play a critical role in tau stability

HeLa cells stably over-expressing tau were transiently transfected with 2 μ g of plasmid corresponding to flag-tagged DnaJ sequence (A), siRNA to DnaJA1 (B), 2 mg flag-tagged DnaJA1 (C and E), and 2 μ g of either flag-tagged or untagged DnaJA1 (F). (A) Representative western blot of tau in cells over-expressing the DnaJ domain peptide. (B) Representative immunoblot of tau in cells transfected with siRNA for DnaJA1. (C) Representative western blot of tau in cells over-expressing DnaJA1. (D) Quantification graph of (C) indicating a 47% reduction in tau levels (* $p < 0.05$). (E) Sample western blot of

pathogenic tau species in cells over-expressing DnaJA1. (F) Representative immunoblot of tau in cells overexpressing flag-tagged versus untagged DnaJA1.

Figure 2. DnaJA1 abrogates tau independently of Hsp70

(A) Tau-overexpressing HeLa cells were transfected with DnaJA1, tau was immuno-precipitated, and samples were subjected to western blot analyses. Representative western blot showing that tau immuno-precipitates with DnaJA1, and this association favors recruitment of Hsp70 by 2.76 fold. Input immunoblots confirm DnaJA1-dependent tau abrogation. HeLa cells stably overexpressing tau were transfected with either a control plasmid or Flag-tagged DnaJA1 and consequently immuno-fluorescently stained with anti-Flag and anti-tau antibodies as well as with Hoechst reagent to reveal cell nuclei (blue). (B) Low magnification (5 $\times$) images of cells; scale bar = 80 μ m. (C) Images at higher magnification of an A1-transfected cell (scale bar = 25 μ m). (D) Co-localization plot of 2C showing complete co-localization (Pearson coefficient > 0.999). (E) Diagram depicting three tau mutants used in figures 2F, 3B, and 3C. The tau mutant mT-Ubq has K/A substitutions at 254, 311, and 353, thereby preventing ubiquitination at these sites. The mutant mT-Hsp70 cannot bind to Hsp70 due to the deletion of I308 and V309. The tau mutant mT-CMA has replacements of amino acids at 336, 337 and 350, 351 for alanine residues, targeting it for chaperone-mediated autophagy (CMA). (F) Representative western blots showing tau abrogation by DnaJA1 in M17 cells co-transfected with wildtype tau (wt- τ) or mT-Hsp70; DnaJA1 cleared mT-Hsp70 more potently than wt- τ . (G) Representative immunoblot showing that DnaJA1-mediated clearance of tau and pTau is reversed by co-expression of Hsp70. Samples were obtained from tau-overexpressing HeLa cells co-transfected with DnaJA1 alone or in combination with Hsp70.

Figure 3. DnaJA1-mediated abrogation of tau requires ubiquitination

M17 cells were transiently co-transfected with DnaJA1 and the indicated tau variants. Representative western blots showing that in the presence of DnaJA1, wt- τ (A) and m τ -CMA (B) were decreased, while m τ -Ubq (C) accumulated.

Figure 4. DnaJA1 levels and distribution are inversely correlated with AD pathology and tau aggregates

(A) Representative immunoblot of DnaJA1 levels in human AD and age-matched control brain tissue. (B) Quantitative analysis of (A) shows that DnaJA1 is reduced by 47% (* $p < 0.05$). (C–E) Immunofluorescent staining of tau (red) and DnaJA1 (green), in the CA2-CA3 hippocampal region of nine-month old non-transgenic (NTG; C) and rTg4510 (Tg; D and E) mice. White dotted lines highlight the granular layer of the hippocampus. (C) DnaJA1 (green) is expressed in cells of the neuronal layer (arrowheads) in NTG mice, while tau (red) is virtually undetectable. (D) Distribution of DnaJA1 (green) remains unchanged in Tg mice. (E) Higher magnification images show no proximity of DnaJA1 with tau signal.

(F) Co-localization plot shows inverse co-localization between tau and DnaJA1. Scale bar for 20× (C and D) and 63× (E) images equals 50 μm and 10 μm , respectively. Three mouse brains were analyzed for each condition.

Figure 5. A1 displays selectivity for some, but not all, disease-relevant, aggregate-prone clients HeLa and M17 cells were co-transfected with DnaJA1 and either α-synuclein or 84-repeat poly-glutamine (polyQ84). Cells were harvested 48 h post-transfection. (A and B) Representative western blots of lysates from HeLa cells show that DnaJA1 did not significantly affect α-synuclein levels; in contrast, DnaJA1 significantly reduced polyQ84. (C) Quantification graph of (A and B) showing that DnaJA1 reduced α-synuclein and polyQ84 by 22% ($p > 0.05$) and 76% ($*p < 0.05$), respectively.

Figure 6. Schematic representation of DnaJA1-mediated abrogation of tau

Under normal conditions, tau provides stabilization to microtubules. However, in tauopathies and upon dissociation and re-conformation into an unfolded and toxic state, tau undergoes chaperone triage by DnaJA1. If Hsc/p70 protein is abundant, it will form a complex with tau and DnaJA1; this partnership will preserve tau from being degraded. On the other hand, in the absence of Hsc/p70, DnaJA1 chaperones tau for clearance via a ubiquitin-dependent pathway.