

CHIP and Hsp70 regulate tau ubiquitination, degradation and aggregation

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Molecular chaperones, ubiquitin ligases and proteasome impairment have been implicated in several neurodegenerative diseases, including Alzheimer's and Parkinson's disease, which are characterized by accumulation of abnormal protein aggregates (e.g. tau and α -synuclein respectively). Here we report that CHIP, an ubiquitin ligase that interacts directly with Hsp70/90, induces ubiquitination of the microtubule associated protein, tau. CHIP also increases tau aggregation. Consistent with this observation, diverse of tau lesions in human postmortem tissue were found to be immunopositive for CHIP. Conversely, induction of Hsp70 through treatment with either geldanamycin or heat shock factor 1 leads to a decrease in tau steady-state levels and a selective reduction in detergent insoluble tau. Furthermore, 30-month-old mice overexpressing inducible Hsp70 show a significant reduction in tau levels. Together these data demonstrate that the Hsp70/CHIP chaperone system plays an important role in the regulation of tau turnover and the selective elimination of abnormal tau species. Hsp70/CHIP may therefore play an important role in the pathogenesis of tauopathies and also represents a potential therapeutic target.

INTRODUCTION

Neurodegenerative diseases as diverse as Alzheimer's disease (AD) and Parkinson's disease (PD) share an obvious common feature—aggregation and accumulation of abnormal proteins. A large group of these diseases, known as the tauopathies, are characterized by filamentous lesions in neurons and sometimes in glia that are composed of aggregates of hyperphosphorylated microtubule-associated protein tau (tau).

Tau promotes microtubule (MT) assembly, reduces MT instability and plays a role in maintaining neuronal integrity and axonal transport (1,2). Human tau protein is encoded by a single gene on chromosome 17q21 that consists of 16 exons, and central nervous system isoforms are generated by alternative splicing involving 11 of these exons (3–7). Tau is a

phosphoprotein, predominantly expressed in neurons, where it is largely localized in axons (8). During the development of tau pathology, tau becomes hyperphosphorylated, detaches from the axonal microtubules and aggregates. The abnormal tau eventually accumulates in filamentous inclusions within neuronal cell bodies and processes. The precise sequence of events and the mechanisms involved in this process are not fully understood, but it is clear that abnormal tau accumulation and aggregation are sufficient to cause neurodegeneration. This in turn leads progressively to the onset of clinical symptoms. The primary tauopathies include Pick's disease (PiD), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP) and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). Tau also accumulates in AD, where where the tau neurofibrillary pathology (e.g. tangles and

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neuropil threads) occurs with a second protein aggregate, the amyloid plaque. The identification of exonic and intronic tau gene mutations associated with FTDP-17 established that tau dysfunction can cause neurodegeneration (9–11).

Unfolded or misfolded protein generated under diverse conditions must be either refolded by molecular chaperones, for instance Hsc/Hsp70 and Hsp40, or eliminated by the ubiquitin proteasomal system (UPS) through an energy-dependent process and concerted action of a number of molecules, including specific ubiquitin ligases. CHIP (carboxyl terminus of the Hsc70-interacting protein) is a molecule with dual function: (i) a co-chaperone of Hsp70 linked through the tetratricopeptide repeat (TPR) domain of CHIP; and (ii) possessing intrinsic E3 ubiquitin ligase activity (U-box domain) which promotes ligation/chain elongation for substrates (12–16). It is structurally similar to RING finger motifs typical of E3 ligases, like parkin. CHIP interacts functionally and physically with the stress-responsive ubiquitin-conjugating (E2 conjugase) enzyme family UBCH5. Thus CHIP is a bona fide ubiquitin ligase which provides a direct link between the chaperone and UPS and has been suggested to contribute in regulating the cellular balance between folding and degradation (17).

Recently, Imai *et al.* (18,19) showed that CHIP, Hsp70, parkin and PAELR formed a complex *in vitro* and *in vivo*. Unfolded PAELR is a substrate of the E3 ubiquitin ligase parkin and accumulation of non-ubiquitinated PAELR in the endoplasmic reticulum (ER) of dopaminergic neurons induces ER stress, leading to neurodegeneration (19). CHIP promotes the dissociation of Hsp70 from parkin and PAELR, thus facilitating parkin-mediated PAELR ubiquitination. Moreover, CHIP enhances parkin-mediated *in vitro* ubiquitination of PAELR in the absence of Hsp70. CHIP also enhances the ability of parkin to inhibit cell death induced by PAELR (18).

The role of the chaperones Hsp70/90 in tau biology has previously been examined by Dou *et al.* (20), who found an inverse relationship between tau aggregation and chaperone levels. Specifically, transgenic mice harboring the V337M tau mutation, which develop hippocampal tau aggregates, had lower levels of Hsp90 than control mice, suggesting that Hsp90 might be degraded along with aggregated tau. In addition, a small number of neurons in the hippocampus that were devoid of aggregated tau were observed to have significantly higher levels of Hsp90. The same relationship between Hsp90 and Hsp70 and tau aggregates in post-mortem samples from a single human AD brain were also reported (20).

In cell cultures transfected with tau constructs increased levels of both Hsp70 and 90, induced by treatment with geldanamycin, led to an ~80% reduction in levels of aggregated, detergent-insoluble tau. This reduction, however, was not accompanied by a decrease in total tau levels, but rather by a redistribution of tau from the insoluble fraction to the soluble fraction. The increased levels of soluble tau were accompanied by an increase in microtubule-bound tau. Reduction of Hsp70 or Hsp90 by RNAi caused the levels of the microtubule-bound tau to decrease (20).

Overall, these studies suggest that abnormal tau accumulation might be associated with perturbation of the major components of the cellular protein quality control machinery—molecular chaperones and the UPS. Hsp70/90 and other chaperones identify proteins that require proper folding,

whereas aberrant unfolded proteins are directed to the UPS. CHIP is a ubiquitin E3 ligase that is involved in ubiquitination of Hsp70-bound proteins; this generally results in their targeting to the proteasome. This is a tandem event (chaperone and UPS activity) such that perturbation in either of these systems might play a role in tau accumulation. Moreover, evidence suggests that the Hsp70/90 chaperones and ubiquitin ligases are neuroprotective and can suppress the toxicity associated with abnormal protein accumulation in *Drosophila* and mouse models of disease (21–24).

In the present study we examined the relationship between CHIP/Hsp70 and tau and the role of this chaperone system in tau degradation, ubiquitination and aggregation. We show that CHIP associates with tau through the microtubule-binding domain, is able to ubiquitinate tau and increases the level of insoluble aggregated tau. In addition, a diversity of neuronal and glial tau-related lesions in several neurodegenerative disorders have CHIP immunoreactivity. This suggests that CHIP may play a role in the formation of, or cellular response to, fibrillary tau lesions. Hsp70 also binds to tau, but has opposing effects. Hsp70 decreases tau steady-state levels and selectively reduces insoluble and hyperphosphorylated tau species. Together, these data suggest that the Hsp70–CHIP chaperone system plays an important role in tau biology and in the pathogenesis of tauopathies.

RESULTS

CHIP interacts with tau

Because Hsp70 is known to interact with tau, the major protein species in neurofibrillary pathology, we investigated whether the Hsp70 co-chaperone CHIP, an E3 ubiquitin ligase, was able to interact with and ubiquitinate tau. Although CHIP has several known substrates, none of these have been associated with neurodegenerative disease.

To determine if CHIP and tau interact we first conducted co-immunoprecipitation experiments. Myc-tagged CHIP, parkin and Hsp70 were separately co-transfected with V5-tagged tau into HEK293 cells and then immunoprecipitation was performed with the V5 antibody. Detection of co-immunoprecipitating species was performed by western blotting with the Myc-tag antibody. Tau was found to co-immunoprecipitate with CHIP and Hsp70 (Fig. 1A). Tau also co-immunoprecipitated with parkin, an E3 ligase associated with autosomal recessive juvenile parkinsonism (25). This was not surprising given that parkin has previously been shown to interact with CHIP and that there is considerable structural homology between these two E3 ligases. To determine whether CHIP and tau interact *in vivo*, we performed co-immunoprecipitation using an antibody against CHIP in brain homogenates from transgenic mice (JNPL3 line) expressing mutant (P301L) tau (26). Western blot analysis was then performed with an antibody against tau (Fig. 1B). Tau co-immunoprecipitated with CHIP (Fig. 1B). These data clearly support the physiological and potential pathological relevance of the observed CHIP–tau interaction.

Using co-immunoprecipitation and *in vitro* binding assays, we next examined which regions of CHIP are necessary for the

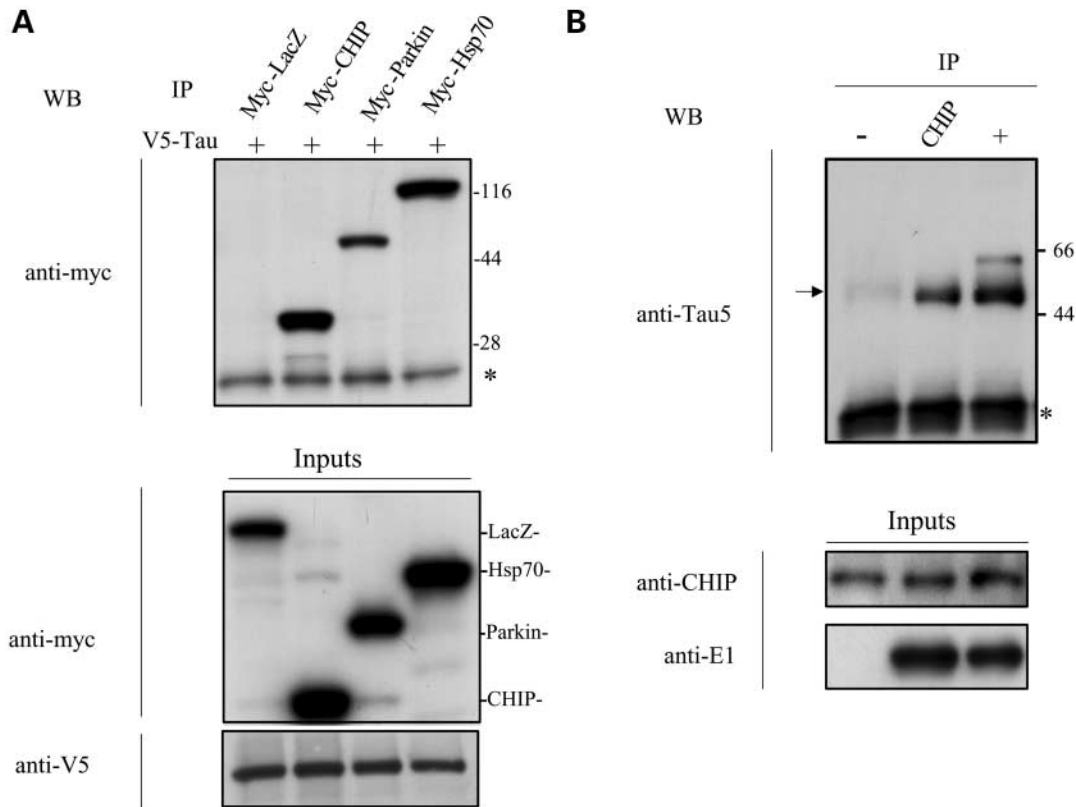


Figure 1. Tau associates with CHIP. (A) Interaction of Tau with CHIP, Hsp70 and parkin. Lysates from HEK293 cells transfected with an LacZ vector (Control), myc-tagged CHIP, myc-tagged Hsp70 or myc-tagged parkin and V5-tagged tau were immunoprecipitated with anti-V5 antibody. Immunoprecipitates (IP) and total soluble lysates (total lysate) were analyzed by western blotting (WB). (B) *In vivo* interaction of tau with CHIP in brain tissue. Mouse brain tissue from P301L transgenic mice (JNPL3) was homogenized as described in Experimental Procedures. The supernatant fractions were immunoprecipitated with anti-CHIP (CHIP), anti-E1 (+) or an irrelevant (-) polyclonal antibody. The co-precipitated tau was detected by western blotting using Tau5. An asterisk indicates the IgG light chain. The arrow indicates native tau species.

interaction with tau. CHIP contains two major structural motifs—a TPR motif and a U-box domain. The TPR motif is required for interaction with Hsc70 and Hsp90, while the U-box domain has ubiquitin ligase activity (Fig. 2A). To determine the site of interaction of CHIP with tau, we monitored the interaction of tau with these two domains of CHIP. The TPR mutant (C1; 1–189 amino acids) and U-box mutant (C2; 145–303 amino acids) both failed to bind to tau; in contrast, full-length CHIP bound strongly to tau (Fig. 2C). Although these results did not reveal a specific binding domain of CHIP with tau, it is conceivable that the interaction with tau requires both domains, as might be expected if complex formation with Hsp70 is required for the tau–CHIP interaction, or one of these domains and a third undefined region of CHIP. A series of truncated tau constructs were also generated to determine the domain of tau that interacted with CHIP (Fig. 2B). These experiments demonstrated that residues 187–311 contain the region of the tau protein necessary for interaction with CHIP. This includes the microtubule binding domains and the region immediately N-terminal (Fig. 2C).

CHIP ubiquitinates tau

To ascertain whether CHIP or parkin ubiquitinates tau, HEK293 cells were transfected with myc-tagged parkin or myc-tagged

CHIP, V5-tagged tau and HA-tagged ubiquitin (Fig. 3A). Two days later, immunoprecipitation was performed with an antibody against V5 and probed with an antibody against HA to assess the degree of tau ubiquitination. Immunoprecipitated tau showed prominent anti-HA (ubiquitin) immunoreactivity in CHIP-transfected cells, with ubiquitin positive species appearing as multiple higher molecular weight species, possibly representing oligomeric and multimeric ligations (Fig. 3A). To characterize the effect of Hsp70 on CHIP-mediated tau ubiquitination, cells were transfected as described above; however, they were also transfected with myc-tagged Hsp70 in the presence of CHIP. As shown in Figure 3B, Hsp70 attenuates CHIP activity, suggesting that Hsp70 antagonizes CHIP ubiquitination of tau. We further explored which ubiquitin lysine linkage (K48 or K68) was primarily responsible for ubiquitination of tau. Ubiquitin linkage through K48 is associated with proteasome targeting, while K68 ubiquitin linkage appears to be involved in cellular signaling/DNA repair (27). HEK293 cells were transfected with myc-tagged CHIP, V5-tagged tau and HA-tagged wild-type ubiquitin, K48 or K63 constructs. K48 and K63 refer to the particular lysine amino acid used to link the ubiquitins to each other. CHIP-mediated ubiquitination of tau did not discriminate between K48 or K63 type ubiquitin linkage suggesting that both types of linkage occur in tau (Fig. 3C). This has potential functional implications for the role of ubiquitination in tau biology. A further study showed that

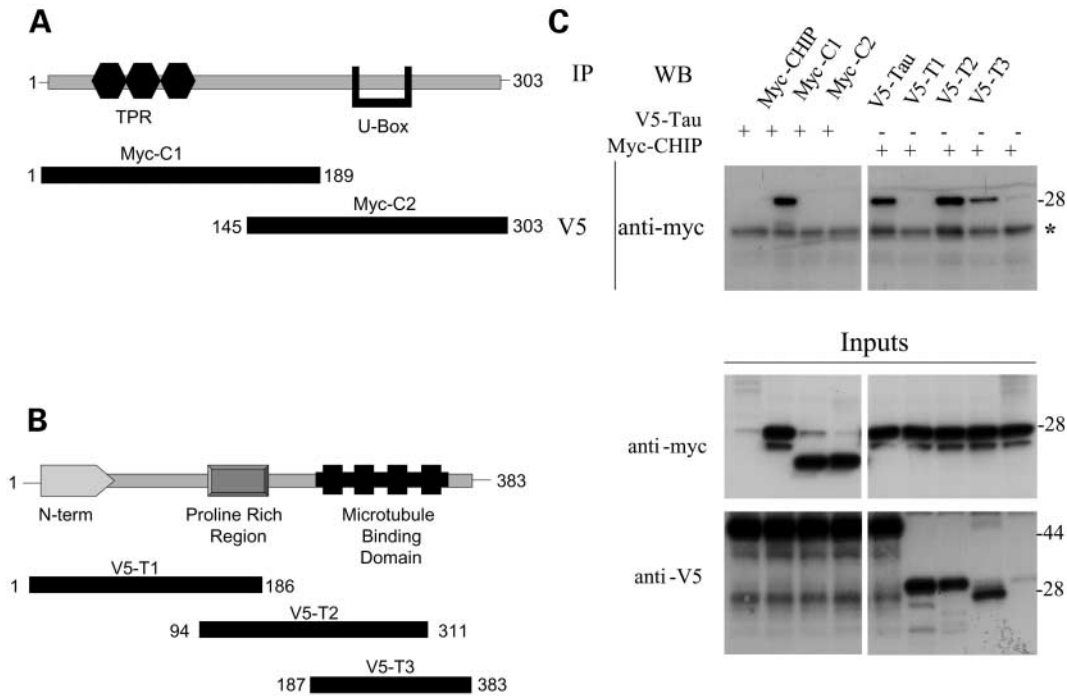


Figure 2. CHIP preferentially interacts with the microtubule-binding domain of tau and tau binding requires full-length CHIP. (A) Diagrammatically representation of full-length CHIP and the two structural domains (C1 and C2) used to determine the Tau binding. (B) Diagrammatically representation of tau and the three domains (T1, T2 and T3) used to determine the CHIP binding domain. (C) Lysates prepared from HEK293 cells transfected with V5-tagged tau and various myc-tagged CHIP domain constructs and various V5-tagged tau domains constructs and myc-CHIP and subjected to IP with anti-V5 followed by anti-myc immunoblotting. Lysates (inputs) were immunoblotted with either anti-V5 or anti-myc antibodies. The asterisk indicates IgG light chain. A representative result from three experiments is shown.

the amount of multimeric ubiquitinated tau (>200 kDa) increased dramatically after the cells were treated with the proteasome inhibitor MG-132, suggesting that a proportion of tau is degraded through the proteasome (Fig. 3D). In particular, it would appear that tau carrying long ubiquitin chains in the soluble fraction is degraded by the proteasome.

To verify the functional interaction between CHIP and tau, we reconstituted the ubiquitination reaction *in vitro*. In this experiment, immunoprecipitated CHIP or parkin and recombinant His-tagged tau were combined with other essential components for *in vitro* ubiquitination, including ATP and E2 conjugases. Again, immunoprecipitated CHIP, but not parkin, ubiquitinated tau (Fig. 4), demonstrating that tau is a substrate of CHIP. Further, when the western blot was re-probed with an antibody against the His-tag on tau, a ladder of species was observed, confirming that tau was directly ubiquitinated by CHIP. Finally, we observed that, in the absence of additional cellular components, Hsp70 had no impact on tau ubiquitination by CHIP; this is in contrast to the attenuation associated with increased Hsp70 activity observed in the *in vivo* ubiquitination studies. This suggests that *in vivo* an additional component of the complex or a particular Hsp70 conformation is required to down regulate tau ubiquitination by CHIP.

CHIP immunoreactivity in human neurodegenerative tauopathies

To evaluate the potential pathological significance of the interaction between tau and CHIP, we examined the

immunolocalization of CHIP in postmortem brain sections of different human tauopathies, including AD, PSP, CBD, FTDP-17 and PiD, as well as JNPL3 transgenic mice that express mutant (P301L) tau. CHIP immunoreactivity was detected in a wide range of tau-positive lesions in both neurons and glia, including neurofibrillary tangles (NFTs; Fig. 5A) and dystrophic neurites in neuritic plaques (Fig. 5B) of AD, Pick bodies in PiD (Fig. 5C), globose NFTs (Fig. 5D) and tufted astrocytes (Fig. 5E) in PSP, and oligodendroglial coiled bodies and thread-like processes in CBD (Fig. 5F).

To confirm CHIP co-localization with tau lesions, serial sections from PiD, were immunostained with anti-phospho-tau (CP13; Fig. 5G) and with antibodies anti-CHIP (Fig. 5H), and to ubiquitin 3–39 (Fig. 5I).

The degree of CHIP immunoreactivity correlated to the predominant isoform of tau in the lesions, with 3R tauopathies showing more immunoreactivity than 3R+4R tauopathies or 4R tauopathies. Specifically, there was more robust CHIP immunoreactivity in Pick bodies (3R) than in NFTs in AD (3R+4R). Almost all Pick bodies were also ubiquitin-immunoreactive on adjacent sections stained for ubiquitin. In adjacent sections of AD stained for ubiquitin, most of the CHIP-positive NFTs were also ubiquitin-immunoreactive. The major exception was extracellular NFT, which had no CHIP-immunoreactivity yet variable ubiquitin immunoreactivity. Pre-tangles, neurons with non-fibrillar abnormal phospho-tau immunoreactivity, were negative for CHIP (data not shown). There were only a few NFTs stained in PSP, CBD and FTDP-17. These were all 4R tauopathies and neurofibrillary lesions in these disorders had

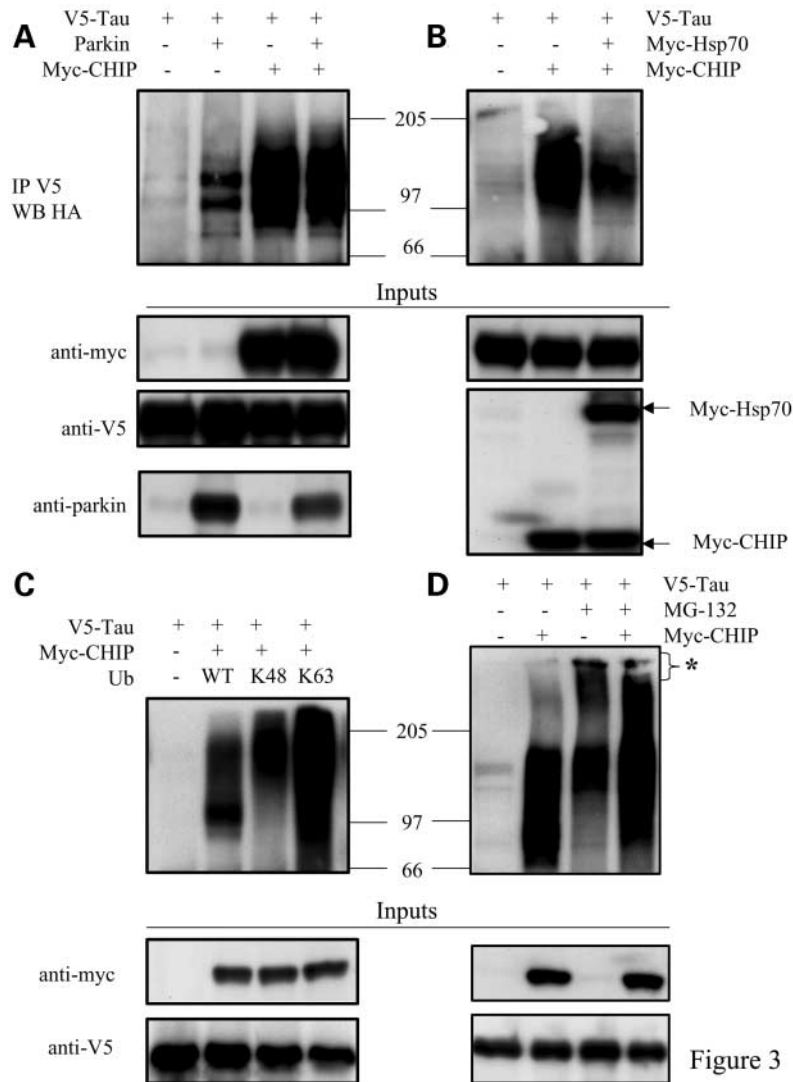


Figure 3

Figure 3. Tau tends to be ubiquitinated *in vivo*. (A) CHIP, not parkin, mediates tau ubiquitination. V5-tagged Tau cDNA combined with empty vector, untagged parkin or myc-tagged CHIP and HA-Ub construct were transfected into HEK293 cells. Immunoprecipitates with anti-V5 mAb (V5-IP) immunoblotted with HA mAb to assess the amount of ubiquitination. Total soluble lysates (inputs) were analyzed by western blotting using anti-myc, anti-V5 or anti-parkin. (B) Hsp70 attenuates CHIP activity. V5-tagged Tau cDNA combined with empty vector, myc-tagged CHIP and myc-tagged Hsp70 and HA-Ub construct were transfected into HEK293 cells. Immunoprecipitates with anti-V5 mAb immunoblotted with HA mAb to assess the amount of ubiquitination. Total soluble lysates (inputs) were analyzed by western blotting using anti-myc, anti-V5 or anti-parkin. (C) CHIP ubiquitinates tau through K48 and K63 ubiquitin linkages. V5-tagged Tau cDNA combined with myc-tagged CHIP (or vector as a control) and HA-tagged wild type or K48 or K63 ubiquitin mutants were transfected into HEK293 cells. Immunoprecipitates with anti-V5 mAb immunoblotted with HA mAb to assess the amount of ubiquitination. Total soluble lysates (inputs) were analyzed by western blotting using anti-myc or anti-V5. (D) Proteasome inhibition increases CHIP-mediated ubiquitination of tau. V5-tagged Tau cDNA combined with either empty vector or myc-tagged CHIP and HA-tagged ubiquitin were transfected into HEK293 cells. Thirty-six hours post-transfection cells were exposed to MG132 (5 μ M, 12 h). Immunoprecipitates with anti-V5 and immunoblotted with HA mAb to access the amount of ubiquitination. Total soluble lysates (inputs) were analyzed by western blotting using anti-myc or anti-V5.

almost no ubiquitin immunoreactivity. Only a few glial lesions in the latter 4R tauopathies were CHIP-immunoreactive. Overall, the number of CHIP immunoreactive lesions was 50–70% for PiD, 5–10% for AD and 1–5% for both PSP and CBD. These data suggest a role for CHIP in pathologies involving tauopathies in humans. Similar to humans, neurofibrillary lesions in spinal cord sections of JNPL3 mice, which contain mutant 4R tau, were weakly immunoreactive for CHIP and ubiquitin, yet strongly positive for phospho-tau (data not shown).

Controls for specificity of CHIP included omission of primary antibody and absorption with CHIP synthetic peptide. These sections showed no immunoreactivity in Pick bodies (Fig. 5K) or NFTs (data not shown).

Effects of CHIP and Hsp70 on accumulation of detergent-insoluble tau

To examine the impact of CHIP-mediated ubiquitination on tau aggregation, COS-7 cells were transfected with a mutant

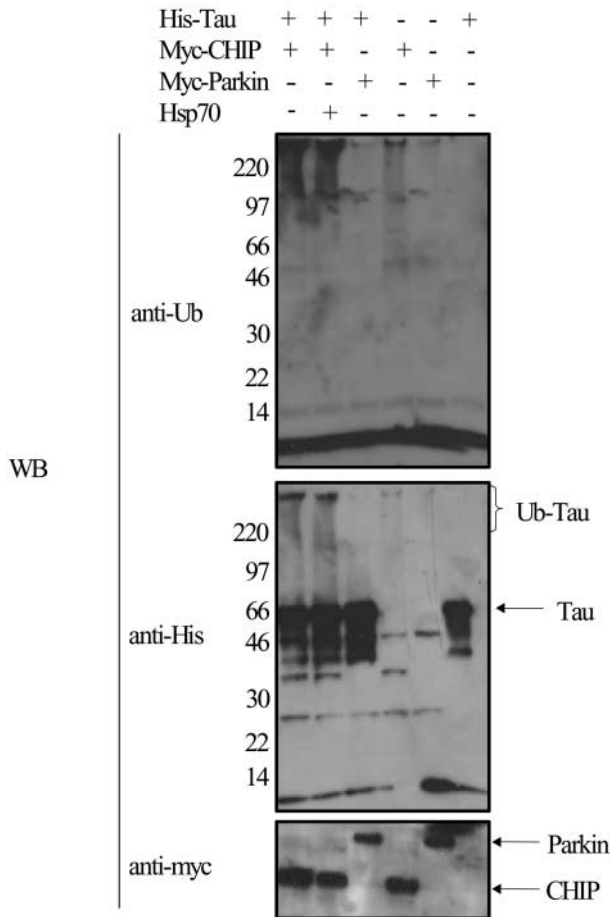


Figure 4. *In vitro* ubiquitination of tau by CHIP. The ubiquitination of Tau by CHIP (or parkin) was reconstituted by using immunoprecipitated myc-tagged parkin and myc-tagged CHIP from transfected HEK293 cells with the addition of purified UBCH7 or UBCH5b and the additional essential components for *in vitro* ubiquitination (see Materials and Methods). The reaction products were analyzed by western blot analysis with both anti-ubiquitin and anti-His antibodies. Inputs were immunoblotted with anti-myc antibodies. Brackets and molecular marker at 220 kDa indicate both Tau and ubiquitin-positive high molecular species. All experiments were replicated twice with similar results.

(P301L) tau expression construct in the absence or presence of either CHIP or Hsp70. Total tau was extracted and then fractionated into triton-soluble and insoluble pools. Transfection of CHIP dramatically increased the accumulation of high molecular weight aggregated tau species in the detergent (triton) insoluble fraction (Fig. 6), detected by western blot analysis with the Tau5 and E1 antibodies. The apparent molecular weight of these aggregated tau species ranged from ~90 kDa to large enough to be retained in the stacking gel. In contrast, expression of Hsp70 selectively reduced the amount of detergent insoluble tau to the point where little or no tau partitioned into the detergent-insoluble fraction. Although the level of insoluble tau was selectively reduced by Hsp70 transfection, there was no corresponding increase in detergent-soluble tau levels, which were also reduced relative to non-transfected cells (Fig. 6).

Induction of Hsp70 reduces tau levels *in vitro* and *in vivo*

To further explore the relationship between Hsp70 and tau steady-state levels *in vitro* we upregulated Hsp70 through geldanamycin (GA) treatment and activated heat shock factor 1 [(HSF-1 (+))] transfection (28). GA is a naturally occurring benzoquinone ansamycin that specifically binds to and interferes with the activity of the molecular chaperone Hsp90 (29), a negative regulator of heat-shock factor 1 (HSF-1), which regulates the transcription of several molecular chaperones, including Hsp70 (28,30). M17 human neuroblastoma cells, which express significant levels of endogenous tau, were either treated with GA or transfected with a mutant (constitutive active) form of HSF-1 (mHSF-1). As shown in Figure 7, treatment with GA reduced tau levels in a dose-dependent manner. Similar results were observed in cells transfected with mHSF-1. Both GA and mHSF-1 increased Hsp70 levels, with mHSF-1 causing the greater induction of the molecular chaperones (Fig. 7). HSF-1 (+), but not GA, also caused an increase in Hsp40 levels, suggesting that Hsp40 is unlikely to be necessary for the reduction of steady-state tau levels produced by these two treatments.

Based on the results described above, Hsp70 appears likely to be involved in regulating tau metabolism, especially the turnover of triton-insoluble species. To obtain additional evidence supporting this idea, we assessed the amount of endogenous tau in the brains of old mice overexpressing the inducible form of Hsp70 (31). There was no significant difference in age between transgenic and control littermates (30.6 ± 5.1 and 28.3 ± 2.1 months of age for non-transgenic and TgHsp70i mice, respectively). Whole brain homogenates from three non-transgenic and three tgHsp70 mice were homogenized and separated into 1% Triton X-100-soluble or -insoluble fractions. The fractions were then immunoblotted using Tau46, a polyclonal antibody to a carboxyl terminal epitope in tau that detects all forms of human and mouse tau (Fig. 8A). The amount of tau was normalized to β -actin levels in the brain of each mouse. Tau levels in TgHsp70 mice in both the soluble and insoluble fractions were significantly lower (~50% lower in both fractions) compared with NT mice (Fig. 8A and B). Moreover, high molecular weight triton-insoluble tau species present in the stacking gel that were observed in the very old NT mice were absent in the age-matched TgHsp70 mice (Fig. 8B). Tau levels were normalized to β -actin from the same gel from either the soluble or insoluble fractions with all the mice in the study. Statistical significance was estimated using Student's *t*-test for difference between NT and tgHsp70i mice in both fractions ($*P < 0.01$, $**P < 0.001$).

DISCUSSION

In the current study, we identified tau as a substrate for the ubiquitin ligase-chaperone protein CHIP. We concluded that tau is an authentic substrate of CHIP from the following evidence: first, CHIP interacts with tau and is specifically ubiquitinated by CHIP *in vivo* and *in vitro* in the presence of the E2 conjugase, UbcH5b. Second, proteasome inhibition augmented CHIP-mediated tau ubiquitination and promoted the insolubility of tau in triton-X-100 detergent. Finally, CHIP

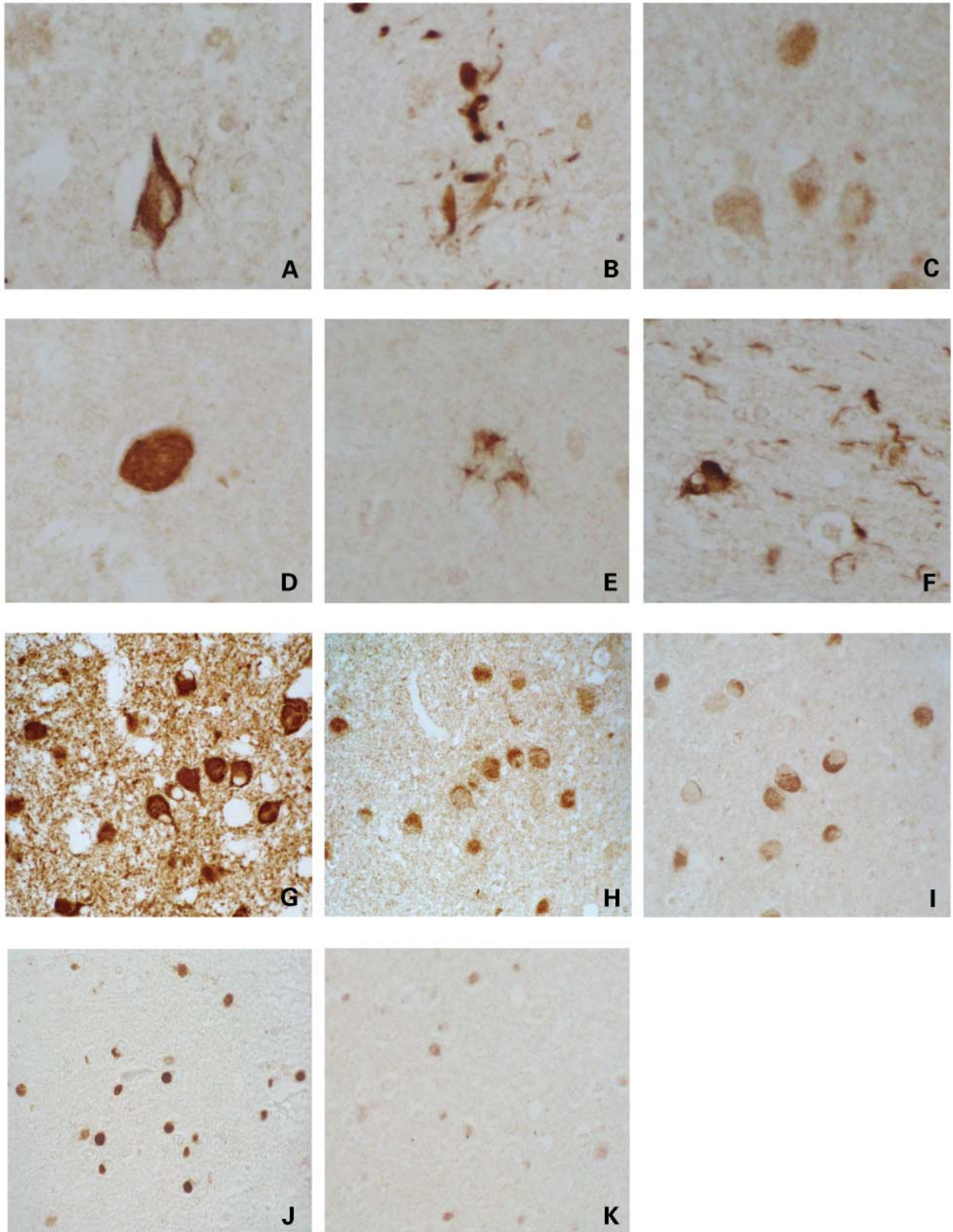


Figure 5. CHIP immunoreactivity is present in a diversity of neurodegenerative tauopathies. Immunostaining of CHIP in several tauopathies. (A) NFT in AD, (B) dystrophic neurites in senile plaques in AD, (C) Pick bodies in PiD, (D) globose NFT in PSP, (E) tufted astrocyte in PSP and (F) oligodendroglial coiled bodies and threads in CBD. CHIP co-localization with tau and ubiquitin was visualized using serial sections immunostained for phospho-tau with CP13 (G), CHIP (H) and anti-ubiquitin 3–39 (I). CHIP staining in PiD case (J) and preabsorption with CHIP synthetic peptide (K).

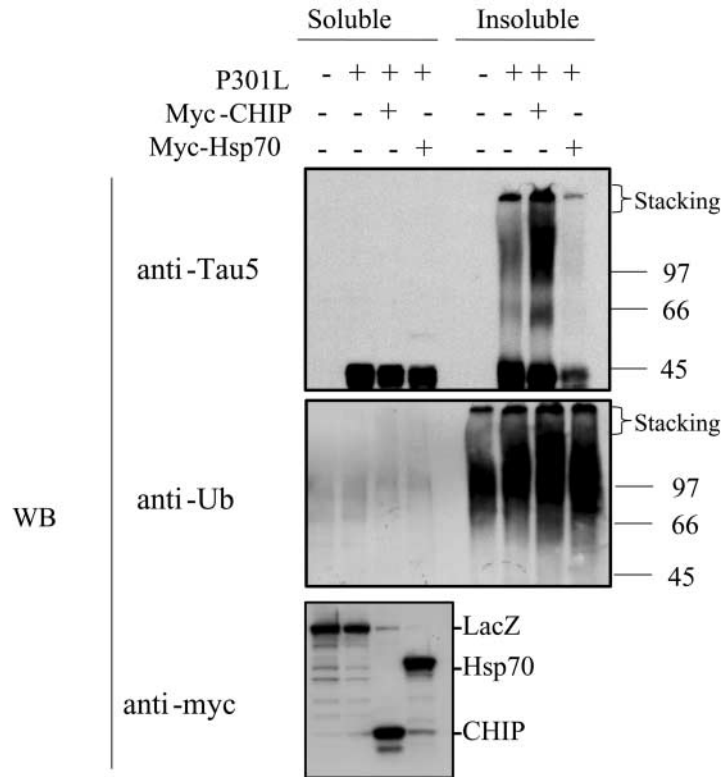


Figure 6. CHIP increases, Hsp70 suppresses tau aggregation. CHIP increases and Hsp70 decreases the accumulation of insoluble Tau in the COS7 cells. Cells transfected with vector plasmid (lacZ) or construct for mutant tau (P301L) combined with construct for mock (Control), myc-tagged CHIP or myc-Hsp70, were lysed and separated into 1% Triton X-100-soluble (S) or -insoluble (I) fractions, then immunoblotted with Tau5. Membrane was stripped and re-probed with anti-ubiquitin. Soluble fraction was used to demonstrate expression of respective constructs.

immunoreactivity was present in a range of tau lesions in several neurodegenerative tauopathies, especially those in which 3R tau is present in pathologic lesions, such as PiD and AD. The tauopathies with CHIP immunoreactivity were also those that have been shown in other studies to have ubiquitin-positive lesions (32). In contrast, disorders such as PSP or CBD in which lesions show almost no ubiquitin immunoreactivity were also negative for CHIP (35–38).

In this study, we found that CHIP mediated ubiquitination of tau. Moreover, both CHIP and Hsp70 interact with tau, suggesting that these two proteins act in concert to control tau metabolism. In fact, overexpression of Hsp70, in cells, attenuated the ubiquitination of tau induced by CHIP (Fig. 3B). These results suggest that Hsp70 and CHIP interact at the functional and/or cellular level. Interestingly the negative effect of Hsp70 on tau ubiquitination was not observed with *in vitro* assays, suggesting that additional chaperones that interact with the Hsp70/CHIP complex might play a role *in vivo*. Furthermore, it appears that a proportion of the tau ubiquitinated by CHIP via K48 ubiquitin linkage is consistent with the observed evidence of proteasome degradation of poly-ubiquitinated tau; however, tau was also ubiquitinated via K63 ubiquitin linkage, suggesting an alternative cellular fate for these species possibly including altered distribution and aggregation.

Although Dou *et al.* (20) reported that increased levels of Hsp70 reduce tau aggregation, which is in accord with results

of the present study, they did not determine if this was a result of a reduction in tau levels (Figs 7 and 8), as our data indicate, rather than a redistribution of tau. Our data would further suggest that the CHIP and Hsp70 levels are critical to tau physiology such that excess CHIP would promote tau aggregation whereas Hsp70 would suppress it. In our model systems, insoluble tau aggregates represented a small fraction of total tau protein, which is a consistent observation in several tauopathies, including AD. Hsp/Hsc70 may protect against tau aggregation, neurofibrillary degeneration and neurotoxicity. Our data argue that Hsp70 (with CHIP) may be a critical determinant of normal tau degradation and may possibly be involved in the pathogenesis of human tauopathy. In this scheme, molecular chaperones would mediate tau degradation and directly or indirectly prevent tau aggregation and the toxicity associated with this protein. The balance between CHIP and Hsp70 levels may well be critical. Dai *et al.* (33) have recently shown that CHIP regulates activation of Hsp70 through induced trimerization and transcriptional activation of HSF-1. The activation of HSF-1 by CHIP emphasizes that a single protein (i.e. CHIP) within the complex can regulate major and often diametrically opposed chaperone activities (Hsp70) to alter the metabolism of substrate, in this case tau (33).

Although CHIP has been implicated in the ubiquitination of several substrates, including unfolded CFTR, glucocorticoid receptor and androgen receptor (14–16), tau is the first CHIP substrate that has been implicated in a number of

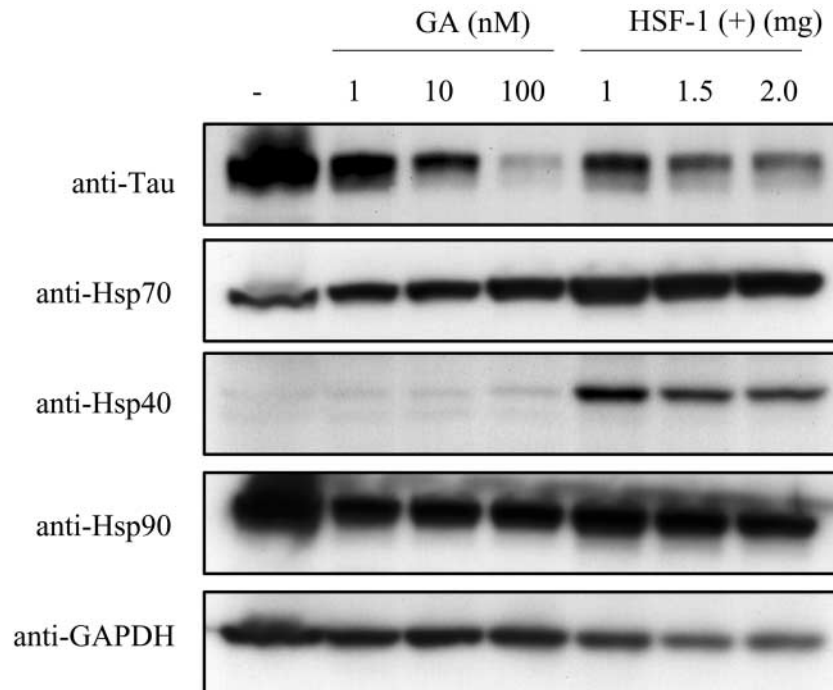


Figure 7. Induction of molecular chaperones decreases tau steady-state levels. M17 human neuroblastoma cell lines expressing endogenous tau were treated with geldanamycin (GA) at 1, 10 and 100 nM or transfected with activated heat shock factor 1 at 1, 1.5 and 2 μ g DNA for 48 h. Cells were lysed and immunoblotted with Tau5 mAb. Membranes were stripped and sequentially re-probed with monoclonal antibodies to Hsp70, Hsp40, Hsp90 and GAPDH (for protein loading).

neurodegenerative diseases. It is also clear that the another member of this chaperone complex, Hsp70, is involved in tau metabolism. Although many questions remain, the multiple effects of the Hsp70/CHIP chaperone system on tau biology make it of interest as a potential therapeutic target for the human tauopathies including AD.

MATERIALS AND METHODS

Expression vectors, cell culture and antibodies

cDNAs for parkin, tau (4R0N \pm P301L), Hsp70 and CHIP were cloned into the mammalian expression vector pcDNA3.1.1 (Myc- or V5-tagged). Deletion constructs targeted for the respective domains were cloned into similar expression vectors. Ubiquitin constructs were obtained from Dr Ted Dawson (Johns Hopkins). A mutated cDNA sequence encoding an HSF1 lacking residues 203–315 was inserted into vector pcDNA3.1 to prepare expression construct HSF1(+). The integrity of all constructs was confirmed by automated sequencing.

COS-7 and HEK-293 cells were maintained in Optimem (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies), heat inactivated. Cells were transfected using Lipofectamine 2000 (Life Technologies) or FuGene6 (Roche) incubated for 48 h and treated as previously described. Human M17 Neuroblastoma cell lines stably overexpressing vector, wild-type tau (4R0N) and P301L cell lines were maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum heat inactivated, glutamine, and 500 μ g ml⁻¹ G418.

CHIP polyclonal antibody was obtained from Abcam; HA and Myc antibodies was obtained from Roche; parkin antibody was

obtained from Cell Signaling; Tau5 was generously provided by Dr Binder (Northwestern University); Hsp70 antibody was obtained from Stressgen abs; E1, human specific tau and Tau46 antibody were obtained from Dr Shu-Hui Yen (Mayo Clinic); CP13 (phospho-tau ser202) was obtained from Peter Davies (Albert Einstein College of Medicine) and ubiquitin 3–39 from Signet. HRP-coupled anti-mouse and anti-rabbit secondary antibodies were obtained from Jackson ImmunoResearch. Ubiquitin polyclonal antibodies were obtained from Dako. His monoclonal antibody was obtained from Calbiochem. LB509 to α -synuclein (Zymed, San Francisco, CA, USA). Hsp40 and Hsp90 were obtained from BD Transduction laboratories. Hsp70 was obtained from Stressgen.

Ubiquitination assays

In vitro. Reactions were performed in 50 μ l mixture containing 50 mM Tris-HCl, pH 7.5, 2.5 mM MgCl, 10 mM DTT, 4 mM ATP, 10 μ g ubiquitin (Sigma), 500 ng of E1 (Calbiochem, San Diego, CA, USA), 200 ng of UbcH7 or UbcH5b (Affintiresearch, Exeter, UK), immunoprecipitated myc-tagged parkin or myc-tagged CHIP and 2 μ g recombinant monomeric his-tagged tau (Dr Binder). Reactions were carried out for 2 h at 37°C before terminating with an equal volume of 2 \times SDS sample buffer. The reaction products were then subjected to western blot analysis with anti-ubiquitin (Dako, Carpinteria, CA, USA), Tau5 or anti-His antibodies.

In vivo. HEK293 cells were transfected with 4 μ g of V5-tagged tau or 4 μ g Myc-tagged parkin, Myc-tagged CHIP or Myc-tagged CHIP and Myc-tagged Hsp70 and 4 μ g of

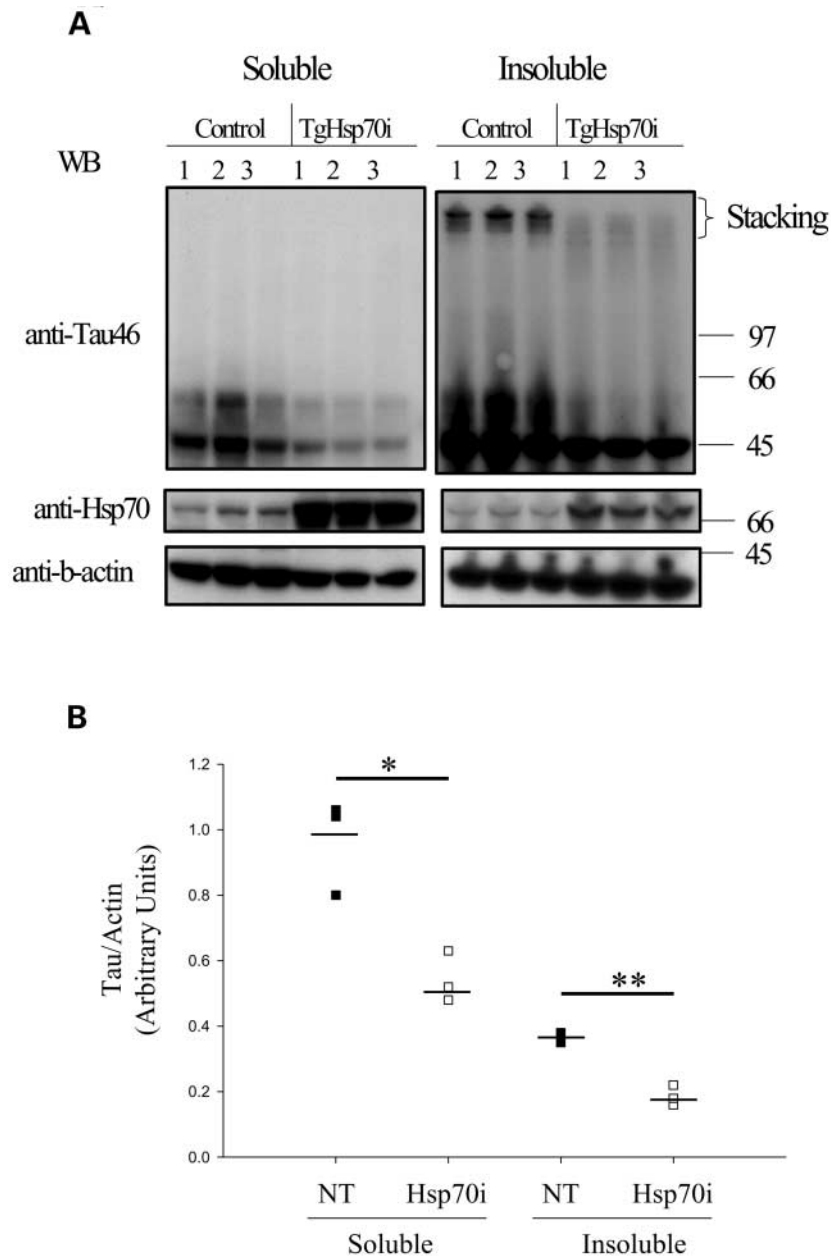


Figure 8. Tau levels in brains of mice overexpressing inducible form of Hsp70. (A, B) Whole brain tissues from 30-month-old normal (NT) or transgenic mice expressing the Hsp70 transgene were separated into 1% Triton X-100-soluble or -insoluble fractions, then immunoblotted with Tau46. Membrane was stripped and re-probed with anti-Hsp70 and anti-β-actin (control for protein loading). There was no significant difference between mice (30.6 ± 5.1 and 28.3 ± 2.1 months of age for NT and TgHsp70i, respectively). Tau normalized to β-actin from the same gel from either the soluble or insoluble fractions on all mice examined. Statistical significance was estimated using Student's *t*-test for difference between NT and tgHsp70i mice in both fractions. * $P < 0.01$; ** $P < 0.001$.

pRK5-HA-wild-type ubiquitin, pRK5-HA-K48-ubiquitin, or pRK5-HA-K63-ubiquitin plasmids. After 48 h, immunoprecipitation was performed with an antibody against V5. The precipitates were submitted to western blotting with an antibody against HA.

Co-immunoprecipitation

For co-immunoprecipitation from cell cultures, HEK293 cells were transfected with 4 μg of each plasmid. After 48 h, cells

were washed with cold PBS and harvested in immunoprecipitation buffer (0.1% Triton X-100, 2 μg/ml aprotinin, 100 μg/ml PMSF, 100 mM NaCl in 50 mM Tris-HCl, pH 7.2). The lysate was sonicated, pre-cleared for 1 h at 4°C with 25 μl of protein G (Pierce) and centrifuged at 14 000 rpm. The supernatants were incubated with 2 μg of an antibody against V5 (Life Technologies) and 60 μl of protein G and rocked at 4°C overnight. The protein G beads were pelleted and washed three times with immunoprecipitation buffer. The precipitates were resolved on SDS-PAGE gel and subjected to western blot

analysis. Bands were visualized with chemiluminescence (Pierce, Rockford, IL, USA).

For co-immunoprecipitation of proteins from mice, whole brains from adult mice expressing the P301L transgene brain (or non-transgenic mice as controls) were homogenized in 4 vols of ice-cold PBS containing 320 mM sucrose and 0.1% Triton X-100 with protease inhibitor cocktail (Sigma). The tissue homogenate was centrifuged at 37 000g at 4°C for 20 min. The supernatant was collected and 500 µg of protein was used for immunoprecipitation with one of the following antibodies: anti-CHIP, anti-E1 or anti-GFP (irrelevant antibody). The precipitates were subjected to western blot analysis and immunoblotted with Tau5.

M17 human neuroblastoma cells were either treated with GA (100 nM) or transfected with mHSF-1 (1, 1.5 and 2 µg DNA) for 48 h. Cells lysates were 1% Triton-PBS plus protease inhibitors. The precipitates were subjected to western blot analysis and immunoblotted with Tau5 and HSPs 70, 40 and 90.

Immunohistochemistry

Pre-absorption and specificity testing of polyclonal anti-CHIP antibody. CHIP peptide was re-suspended in 1% BSA in PBS to a concentration of 1 mg mL⁻¹. The peptide solution was added to diluted CHIP antibody in TBST to obtain a final dilution of 1 : 500. The mixture was rocked for 1 h at room temperature. The mixture was centrifuged at 13 500g for 2 min. The supernatant was separated from the pellet. Serial sections (5 µm thick) from a PiD case were deparaffinized and rehydrated in xylene and graded series of alcohol (100, 100, 95 and 70%). Antigen retrieval was performed in dH₂O in steam bath for 30 min. The sections were allowed to cool. The supernatant and diluted CHIP antibody (1 : 500 in TBST) were used for immunohistochemistry on the DAKO Autostainer (DakoCytomation, Carpinteria, CA, USA) using the DAKO EnVision HRP system on the serial sections. DAKO Liquid DAB Substrate-Chromogen system was the chromogen. The slides were then dehydrated and coverslipped.

Single antibody staining. Paraffin serial sections (5 µm thick) were used for immunohistochemistry from, diffuse Lewy body disease ($n = 6$), multiple system atrophy ($n = 2$) JNPL3 and littermate control mice (one each), AD ($n = 2$), PiD ($n = 4$), PSP ($n = 2$), CBD ($n = 2$), and FTDP_17 ($n = 2$). The sections were then processed the same as above. Primary antibodies used in the serial sections were: CHIP (1 : 250), CP13 (1 : 500) and 3-39 (1 : 200 000). In Lewy body disease and multiple system atrophy cases anti-synuclein (LB509, 1 : 100) replaced CP13. All antibodies were diluted in DAKO Antibody Diluent with background reducing components.

Fractionation experiments

For triton soluble/insoluble fractionation experiments, cells or tissue were lysed in a buffer containing PBS with 1% Triton X-100 and a cocktail of protease inhibitors. After sonication, cells were centrifuged at 100 000 g at 4°C for 30 min. Triton X-100 insoluble pellets were dissolved in a buffer containing 1% Triton X-100/1% SDS. The soluble and insoluble fractions

were used in western blot analysis using the antibodies described in the figure legend.

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NOTE ADDED IN PROOF

Very recently Shimura *et al.* (34) reported that tau binds to Hsc70 and phosphorylation is a requirement for ubiquitination by CHIP. In addition, CHIP was able to rescue phosphorylated tau-induced toxicity. Our data, while generally consistent with the findings of Shimura *et al.*, extends our understanding of the interaction between the Hsp70/CHIP chaperone system and tau by demonstrating the *in vivo* co-localization of CHIP with tau lesions in human patients with tauopathy and further by exploring the antagonistic action of Hsp70 and CHIP on tau ubiquitination and aggregation. In addition, while Shimura *et al.* report that CHIP ubiquitination is dramatically enhanced by GSK-3β driven phosphorylation of tau, our data show that GSK-3β phosphorylation is not absolutely required for CHIP to ubiquitinate tau.

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