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Proline Isomer-Specific Antibodies Reveal the Early Pathogenic Tau Conformation in Alzheimer's Disease

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Abstract

Cis-trans isomerization of proteins phosphorylated by proline-directed kinases is proposed to control numerous signaling molecules, and is implicated in the pathogenesis of Alzheimer's and other diseases. However, there is no direct evidence for the existence of *cis-trans* protein isomers in *vivo*, or for their conformation-specific function or regulation. Here we develop peptide chemistries that allow the generation of *cis* and trans-specific antibodies, and use them to raise antibodies specific for isomers of phosphorylated tau. *Cis*, but not *trans*, p-tau appears early in the brains of humans with mild cognitive impairment, and accumulates exclusively in degenerated neurons and localizes to dystrophic neurites during Alzheimer's progression. Unlike *trans* p-tau, the *cis* isomer cannot promote microtubule assembly, is more resistant to dephosphorylation and degradation, and is more prone to aggregation. Pin1 converts *cis* to *trans* p-tau to prevent Alzheimer's tau pathology. Isomer-specific antibodies and vaccines may therefore have value for the early diagnosis and treatment of Alzheimer's disease

INTRODUCTION

The reversible protein phosphorylation on certain serine or threonine residues preceding a proline (pSer/Thr-Pro) is a central signaling mechanism in diverse cellular processes in physiology and disease (Blume-Jensen and Hunter, 2001; Lu et al., 2002; Nigg, 2001). Notably, certain pSer/Thr-Pro motifs in phosphopeptides exist in two distinct *cis* and *trans* conformations (Yaffe et al., 1997) and their isomerization is especially important because Pro-directed kinases and phosphatases are *cis* or *trans* conformation-specific (Brown et al.,

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SUPPLEMENTAL INFORMATION

Supplemental Information includes six supplemental figures, extended experimental procedures, and supplemental references and can be found with this article online at

1999; Zhou et al., 2000). Moreover, phosphorylation further slows down their isomerization rate, and also renders the peptide bond resistant to conventional peptidyl-prolyl *cis-trans* isomerase (PPIases) (Yaffe et al., 1997; Zhou et al., 2000). As a unique PPIase (Lu et al., 1996), Pin1 binds to and isomerizes specific pSer/Thr-Pro motifs derived from a subset of proteins, leading us to hypothesize a novel signaling mechanism, whereby Pin1 catalytically regulates its substrate conformation after phosphorylation to control protein function (Lu et al., 1999b; Ranganathan et al., 1997; Shen et al., 1998; Yaffe et al., 1997; Zhou et al., 2000; Zhou et al., 1999).

Subsequent studies have provided supporting evidence for this new concept of post-phosphorylation conformational regulation (Liou et al., 2011; Lu and Zhou, 2007). For example, Pin1 greatly accelerates isomerization of the APP intracellular domain between the two distinct conformations, as visualized by NMR (Pastorino et al., 2006) and has profound effects on a spectrum of activities in numerous signaling molecules (Girardini et al., 2011; Liou et al., 2011; Lu and Zhou, 2007; Theuerkorn et al., 2011; Tun-Kyi et al., 2011; Yuan et al., 2011). Functionally, Pin1 regulates many cellular processes involving Pro-directed phosphorylation, with an emerging theme that Pin1 often acts on multiple targets to synergistically drive certain cellular processes to one direction (Liou et al., 2011; Lu et al., 2007; Lu and Zhou, 2007). Importantly, Pin1 deregulation contributes to an increasing number of diseases, notably cancer and Alzheimer's disease (AD) (Butterfield et al., 2006; Lee et al., 2011b; Lu and Zhou, 2007). These Pin1 functions are abolished by catalytically inactivating mutations (Lu and Zhou, 2007) or DAPK1-mediated inhibitory phosphorylation (Lee et al., 2011a), suggesting the importance of Pin1 catalytic activity. However, without a tool to directly detect *cis* or *trans*-specific protein conformation in the cell, there is no *in vivo* evidence for such two conformations for any protein, their conformation-specific function or regulation (Liou et al., 2011; Lu and Zhou, 2007).

The neuropathological hallmarks of AD are tangles made of hyperphosphorylated tau (p-tau) and plaques composed of amyloid beta-peptides (Aβ) derived from amyloid precursor protein (APP) (Ballatore et al., 2007; Goedert and Spillantini, 2006; Mattson, 2004; Spires-Jones et al., 2009). It is increasingly evident that tau pathology in AD may result from the combination of the detrimental effects from losses of tau normal function to promote microtubule (MT) assembly and toxic gains-of-function acquired by p-tau aggregates (Ballatore et al., 2007). A defining early event that disrupts tau MT function and precedes tangle formation and neurodegeneration in AD is increased tau phosphorylation, especially on Ser/Thr-Pro motifs (Ballatore et al., 2007; Goedert and Spillantini, 2006; Mattson, 2004; Spires-Jones et al., 2009). Many kinases or phosphatases are deregulated in AD brains, and modulating these enzymes can affect AD-related phenotypes (Ballatore et al., 2007; Cruz and Tsai, 2004; Dolan and Johnson, 2010). However, it is not clear how such phosphorylation becomes pathogenic and how to control it.

Recently, we have found a pivotal role for Pin1 in protecting against age-dependent neurodegeneration in AD (Lee et al., 2011b). Pin1 binds to and isomerizes the pThr231-Pro motif in tau and the pThr668-Pro motif in APP in vitro (Lu et al., 1999a; Pastorino et al., 2006; Zhou et al., 2000). Furthermore, Pin1 restores p-tau MT function and also promotes p-tau dephosphorylation and degradation (Lim et al., 2008; Liou et al., 2003; Lu et al., 1999a; Zhou et al., 2000). Pin1 also reduces amyloidogenic APP processing and toxic Aβ secretion (Pastorino et al., 2006) as well as promotes pThr668-APP degradation (Ma et al., 2011). Consequently, Pin1 knockout mice develop age-dependent tau- and Aβ pathologies, and neurodegeneration, resembling many aspects of human AD (Liou et al., 2003; Pastorino et al., 2006). By contrast, Pin1 overexpression in postnatal neurons effectively inhibits tau pathology and neurodegeneration in AD mouse models overexpressing human wild-type tau (Lim et al., 2008). Significantly in human brains, Pin1 is highly expressed in most neurons,

but is inhibited in MCI (mild cognitive impairment) and AD neurons by multiple mechanisms (Butterfield et al., 2006; Liou et al., 2003; Lu et al., 1999a), whereas the Pin1 SNP that prevents its down-regulation is associated with delayed onset of AD (Ma et al., 2010). Notably, Thr231 phosphorylation is on top of the sequential phosphoepitopes (pThr231→TG3→AT8→AT100→Alz50) during pretangle formation in AD (Luna-Munoz et al., 2007). Thus, Pin1 might accelerate *cis* to *trans* isomerization to protect against tau and Aβ pathology in AD (Lu et al., 2007; Lu and Zhou, 2007). However, there is no evidence that Pin1 actually regulates protein conformations in vivo in AD or other processes.

To detect Pin1-catalyzed conformational changes, we have developed novel peptide chemistry to generate the first antibodies (Abs) that can distinguish *cis* from *trans* pThr231-Pro conformation in p-tau and provided the first evidence that Pin1 accelerates *cis* to *trans* conversion to prevent the accumulation of the pathogenic *cis* p-tau in AD. Our findings develop the first tool to directly detect *cis*-*trans* prolyl isomerization in vivo, and suggest novel conformation-specific vaccines and Abs for treating or even preventing AD at early stages.

RESULTS

Novel Peptide Chemistry Enables to Generate *Cis* and *Trans* Conformation-Specific Abs

Proline-directed phosphorylation is a central signalling mechanism in the cell, but it is unknown whether such a phosphoprotein exists in *cis* and *trans* conformations in vivo. To address this question, we developed a novel strategy to generate the Abs that can distinguish *cis* from *trans* pThr231-Pro conformation in p-tau. NMR analysis showed that the pThr231-Pro motif in a synthetic tau peptide contained only 9% *cis* (Figure 1A and Figure S1A), making it difficult to produce *cis* specific Ab. Therefore, our strategy is to immunize rabbits with a modified pThr231-Pro tau peptide that contains a minimal structural change, but has both *cis* and *trans* contents high enough to produce *cis*- and *trans*-specific Abs, followed by separating them using affinity purification and counter-purification procedures to remove potential contamination with *cis* and *trans* locked peptides, respectively (Figure 1B).

Since the unique five-membered carbonyl ring of Pro in all natural amino acids renders Pro to adopt *cis* and *trans* conformations, we reasoned that replacing the five-membered carbonyl ring of Pro with a six-membered ring having one additional methylene group, as seen in homoproline (Pip), might increase the *cis* content and also produce Abs that would recognize endogenous tau proteins. To test this hypothesis, we used NMR analysis to determine the *cis* and *trans* contents of modified tau peptides. Indeed, a pThr231-Pip tau peptide had ~74% *cis* (Figure 1A and Figures S1A and S1B). When immunizing rabbits, the pThr231-Pip tau peptide produced Abs that recognized endogenous human and mouse tau (Figures 1E-1H). To separate *cis*- and *trans*-specific Abs, we synthesized a pThr231-Dmp (5,5-dimethylproline) tau peptide as a *cis* locked peptide because Dmp locks a peptidyl-prolyl bond in *cis* (An et al., 1999), and a pThr231-Ala tau peptide as a pure *trans* peptide because Ala adopts only *trans*, as confirmed by NMR (Figure 1A and Figures S1C and S1D).

After Ab purification outlined in Figure 1B, the specificity of resulting *cis*- and *trans*-specific Abs was first verified using ELISA. Both *cis* (Figure 1C) and *trans* (Figure 1D) Abs specifically recognized their respective *cis* and *trans* peptides, with essentially no cross-reactivity. Their calculated Kd values for the respective *cis* and *trans* pT231-tau peptides were ~2.1 and 1.6 nM. Importantly, they did not recognize non-phoshorylated Thr231-Pro tau peptide, but both were strongly reactive to a wild-type pThr231-Pro tau peptide, as expected from the fact that *cis* and *trans* isomers in phosphopeptides can be inter-changeable

relatively easily (Pastorino et al., 2006; Zhou et al., 2000). Thus, *cis* and *trans* pThr231-Pro tau Abs are highly conformation-specific in vitro.

Next, to examine whether the *cis* and *trans* Abs recognize pThr231 in tau in vivo, we performed immunoblotting and immunostaining analyses. When tau and its T231A mutant were expressed in neuronal SY5Y cells, both Abs recognized wild-type tau, but not its T231A mutant (Figure 1E), similar to ELISA results (Figures 1C and 1D). Furthermore, robust *cis* and *trans* immunostaining signals were detected in Tau-Tg mouse brains overexpressing wild-type human tau, but not after dephosphorylation (Lu et al., 1999a) or in tau knockout mouse brains (Figure 1F). When frontal cortical sections from advanced AD patients were double immunostained with the *cis* or *trans* polyclonal Ab and monoclonal Ab recognizing total tau, all *cis* or *trans*-positive neurons were also positive for total tau, but not all total tau-positive neurons were also positive for either *cis* or *trans* p-tau (Figure 1G), indicating that some, but not all, of tau is phosphorylated on Thr231, as expected. In addition, immunostaining signals of both *cis* and *trans* pThr231-Pro Abs in AD brains were partially co-localized with those of AT180 (Figure S1E) and TG3 (Figure S1F), two Abs recognizing pT231-containing tau (Jicha et al., 1997). Thus, *cis* and *trans* Abs recognize pT231-containing tau (pT231-tau) in vivo.

Finally to determine whether these immunostaining signals are specific to *cis* or *trans* pT231-tau, we pre-absorbed the Ab with *cis*, *trans* or *cis*+*trans* pT231-tau peptide prior to immunostaining on the AD frontal cortex. Staining signals of the *cis* Ab were abolished by pre-absorption with the *cis*, but not *trans*, peptide and vice versa (Figure 1H). Of note, a monoclonal Ab that recognized only the *cis* pThr231-Dmp tau peptide, but not pThr231-Pro tau peptide in ELISA (Figure S1G) could not detect any immunostaining signal in Tau-Tg mouse brains (Figure S1H). Thus, *cis* and *trans* pT231-tau Abs are conformation- and phosphorylation-specific in vitro and in vivo.

Cis, but not *Trans*, pT231-tau Appears Early at MCI Brains, and further Accumulates and Localizes to Dystrophic Neurites as AD Progresses

In human AD, Thr231 phosphorylation is a very early tau phosphorylation event (Luna-Munoz et al., 2007) and its levels track disease progress (Ewers et al., 2007; Hampel et al., 2001). However, it is not known whether *cis*, *trans* or both pT231-tau conformations are elevated. Moreover, there is no information available on whether and which pT231-tau conformation might be detected in human brains with mild cognitive impairment (MCI). Generation of the *cis*-and *trans*-specific Abs provides the first opportunity to address these questions.

Immunostaining of human frontal cortical sections revealed that tau protein was readily detected in normal neurons and total amounts were increased with progression of neurodegeneration, especially in AD brains (Figure 2A). Although very little, if any, *cis* or *trans* pT231-tau signals were detected in 9 normal brains, they, especially *cis*, were dramatically increased in 11 AD brains (Figure 2A). Strong *cis*, but not *trans*, pT231-tau was detected in 4 out of 6 MCI cases (Figure 2A), with weaker, but clearly detectable *cis* signals in remaining 2 MCI cases (data not shown).

To confirm the above results, we performed immunoblotting analysis of the frontal cortical lysates obtained from 9 normal controls with Braak stages I and II, 6 MCI patients with Braak stages III and IV, and 11 AD patients with Braak stage V and VI to semi-quantify their *cis* and *trans* pT231-tau contents. We used *cis* and *trans* p-tau in tau-overexpressing SY5Y neuronal cells as relative standards for comparison. Again, although neither *cis* nor *trans* was detected in normal brains (Figure 2B), *cis* pT231-tau was significantly elevated in MCI brains and further accumulated as the Braak stage progresses, with much smaller

increases in *trans* p-tau (Figures 2C and 2D). These results suggest that *cis* pT231-tau is specifically elevated in MCI and AD brains. Of note, the ability of immunostaining and immunoblotting to detect *cis* and *trans* pT231-tau conformations is not unusual because many Abs raised against human AD paired helical filaments recognize AD-specific abnormal p-tau conformations in immunostaining and immunoblotting (Davies, 2000). Furthermore, certain Ser/Thr phosphorylation causes a dramatic mobility shift in SDS gels, as shown in Cdc25C (Shen et al., 1998). Moreover, both *cis* and *trans* signals were detected by immunoblotting under denatured and native conditions (Figure S2A).

The above results suggest that *cis* pT231-tau might be more pathologically relevant, which is further supported by the strikingly different subcellular localization between *cis* and *trans* p-tau in MCI and AD brains. Although both p-tau conformations were found in neuron bodies in the frontal cortex, only *cis* pT231-tau was detected in neurites at MCI and AD brains, with more and more being accumulated in dystrophic neurites as the Braak stage increased (Figures 1G and 2A and Figure S1F, yellow arrows). Notably, p-tau in dystrophic neurites was also recognized by the pT231 Ab AT180 (Figure S2B) and total tau Ab DC25 (Figure S2C), with *cis* and AT180 signals being co-localized in the neurites (Figure S1E and S2C). In sharp contrast, there was almost no or very little *trans* pT231-tau in dystrophic neurites even in advanced AD brains (Figures 1G and 2A and Figure S1F, blue arrows). Similar results were also confirmed using Abs raised in a different rabbit (Figure S1I). This different localization pattern might be pathologically significant given that missorting of p-tau to neurites and synaptic loss in the frontal cortex are early hallmarks of AD and are highly correlated with cognitive loss in AD patients (Davies et al., 1987; DeKosky and Scheff, 1990; Scheff et al., 1990). These results not only confirm the specificity of the *cis* and *trans* Abs, but also indicate that only *cis*, but not *trans*, pT231-tau is localized to pathological relevant dystrophic neurites.

Trans, but not *Cis*, pT231-tau Promotes Microtubule Assembly and Pin1 Converts *Cis* to *Trans* to Restore the Ability of *Cis* pT231-tau to Promote Microtubule Assembly

The above results suggest that *cis*, but not *trans*, pT231-tau might be pathologically more relevant to MCI and AD. We wondered whether *cis* and *trans* p-tau conformations might have any differences in biological functions or biochemical properties relevant to tau pathology in AD, and whether they might be regulated. Pin1 is the only enzyme known to isomerize the pThr231-Pro motif in tau peptide (Zhou et al., 2000), to restore the ability of pT231-tau to promote MT assembly (Lu et al., 1999a) and to promote pT231-tau dephosphorylation and degradation (Lim et al., 2008; Liou et al., 2003; Lu et al., 1999a; Zhou et al., 2000). Finally, Pin1 is inhibited by multiple mechanisms in human MCI and AD neurons (Lee et al., 2011b). Indeed, Pin1 levels were much lower in AD brains (Figures S3A and S3B), even in MAP2-positve AD neurons (Figure S3C), suggesting that lower Pin1 is unlikely due to cell death. Thus, Pin1 might protect against tau pathology in AD by preventing the accumulation of the pathogenic *cis* p-tau.

To examine this possibility, we first examined whether Pin1 would increase *cis* to *trans* isomerization of pT231-tau in vitro. Both *cis* and *trans* pThr231-Pro signals were detected in tau, but not its T231A mutant (Figures 3A-3C). Importantly, adding Pin1 significantly increased *trans*, but reduced *cis* pT231-tau content. In this assay, there may be two distinct functions embodied in Pin1: acceleration of *cis* to *trans* isomerization of the pThr231-Pro motif by the PPIase domain (Pastorino et al., 2006; Yaffe et al., 1997; Zhou et al., 2000) and *trans*-specific binding of the pThr231-Pro motif to the WW domain (Wintjens et al., 2001). When Pin1 was added, free *trans* would bind to the WW domain, depleting free *trans*. The PPIase would greatly accelerate *cis* to *trans* isomerization to maintain their equilibrium. The overall effect of Pin1 is to increase the total amount of *trans* relative to *cis*. Consistent with this notion, WW domain mutant W34A Pin1 and PPIase domain mutant K63A Pin1 did not

change the content of *cis* or *trans* (Figures S3D). Thus, Pin1 catalyzes *cis* to *trans* isomerization of pT231-tau in vitro.

We next examined whether such Pin1-catalyzed *cis* to *trans* isomerization would affect the ability of p-tau to promote MT assembly, a major tau function that is lost in AD (Ballatore et al., 2007), using FITC-labeled tubulin, as described (Lu et al., 1999a; Nakamura et al., 2001). As expected, MT assembly was greatly increased by tau, but not Cdc2 phosphorylated tau, which was restored by PP2A (Figures 3D and 3G). Furthermore, Pin1 effectively restored the ability of p-tau to promote MT assembly (Figures 3D and 3G). In contrast, Cdc2-treated T231A tau could still promote MT assembly and its ability to affect MT function was not affected by Pin1 (Figures 3E and 3H), indicating the essential role of T231 phosphorylation for Pin1 to regulate p-tau. These results confirm the previous findings that phosphorylation of tau on Thr231 by Cdc2 disrupts its ability to promote MT assembly (Lu et al., 1999a), that Pin1 restores the MT function of pT231-tau (Lu et al., 1999a), and that the ability of Pin1 to regulate MT function depends on T231 phosphorylation (Lim et al., 2008; Zhou et al., 2000). More importantly, the ability of Pin1 to restore p-tau MT function was fully blocked by incubation of Pin1-treated p-tau with *trans* Ab, but not *cis* Ab (Figures 3F and 3I). This effect of the *trans* Ab was dependent on Pin1 action because application of *trans* or *cis* Ab to p-tau without Pin1 did not affect MT assembly (Figure S3E). Thus, *cis*, but not *trans*, p-tau loses normal function to promote MT assembly and Pin1 catalyzes *cis* to *trans* isomerization to restore p-tau MT function.

C*is* p-Tau Is more Resistant to Protein Dephosphorylation and Degradation, and also more Prone to Protein Aggregation than the *Trans*

In addition to loss of tau normal function, p-tau in AD is more resistant to dephosphorylation and degradation, and more prone to protein aggregation, resulting in toxic gains-of-function (Ballatore et al., 2007). PP2A is a major Pro-directed Ser/Thr phosphatase in brain lysates that preferentially dephosphorylates pSer/Thr-Pro motifs in *trans* in synthetic pT231-tau peptides, as shown using chymotrypsin to chemically remove the *trans* conformation (Zhou et al., 2000). To directly examine whether *cis* pT231-tau is specifically resistant to dephosphorylation by PP2A, we used the conformation-specific Abs. *Trans*, but not *cis*, pThr231-Pro tau peptide was readily dephosphorylated by PP2A (Figure 4A), but they both were rapidly dephosphorylated by CIP (Figure 4B), a calf intestinal alkaline phosphatase that has no conformation-specificity towards its substrates (Zhou et al., 2000). Moreover, *cis* locked pThr231-Dmp tau peptide was much more resistant to dephosphorylation by PP2A than *trans* pThr231-Pro tau peptide (Figure S4A), although it was robustly dephosphorylated by CIP (Figure S4B). These results provide further evidence that PP2A is a Pro-directed phosphatase preferentially dephosphorylating pSer/Thr-Pro motifs in *trans* (Zhou et al., 2000).

Since Thr231 phosphorylation increases tau protein stability and aggregation (Lim et al., 2008), we next compared protein stability of *cis* and *trans* pT231-tau in neuronal cells and mouse brains using the cycloheximide chase (Lim et al., 2008). C*is* p-tau was much more stable than the *trans*; after 12 hr, *cis* p-tau was not reduced, whereas *trans* p-tau was reduced by ~75% (Figures 4C and 4D). Given the ability of Pin1 to increase *trans* but decrease *cis* in p-tau protein (Figures 3A and 3C), we might expect that Pin1 knockdown (Pin1 KD) would increase *cis*, but decrease *trans* p-tau levels, as well as further increase *cis* p-tau stability. Indeed, at time 0, *cis* increased, but *trans* decreased in Pin1 KD cells, as compared to control cells (Figures 4C and 4D). Furthermore, *cis* was significantly more stable and had a longer half-life than *trans* in Pin1 KD SY5Y cells than that in control cells (Figures 4C and 4D). Essentially the same results were obtained when inhibitors of CDKs, JNKs and GSK-3, which are implicated to phosphorylate tau on Thr231, were added together with CHX to assess preexisting p-tau (Figure S4C). In brain slice cultures from Tau-Tg mice, *cis* p-tau

had a longer half-life than the *trans*, being ~24 vs. ~6 hr (Figures 4E and 4F). Thus, *cis* p-tau is more stable than the *trans* in vitro and in vivo, and Pin1 converts *cis* to *trans* to promote p-tau turnover.

To examine the effects of p-tau conformations on its aggregation, we compared the contents of *cis* and *trans* pT231-tau in sarcosyl-soluble and -insoluble fractions. In Tau-Tg mouse brains, *trans* p-tau was almost all in the soluble fraction, with very little, if any insoluble *trans* (Figures 4G and 4H). However, *cis* p-tau was found almost equally between the insoluble and soluble fractions (Figures 4G and 4H). Similar results were also obtained in human MCI brains. As compared with *trans* p-tau, the *cis* was found much more in the insoluble fraction than in the soluble fraction for total p-tau and cleaved p-tau (de Calignon et al., 2010) (Figures 4I-4L), indicating that *cis* p-tau is more prone to protein aggregation.

The above results suggest that *cis* pT231-tau in Tau-Tg mouse brains and human AD brains might be stable under denatured condition. Indeed, *cis* pT231-tau in the insoluble fraction of Tau-Tg mouse brains was recognized by *cis*, but not *trans*, Ab in immunoblotting not matter SDS and boiling were used or not (Figure S4D). Furthermore, when we used *cis* Ab to immunoprecipitate *cis* pT231-tau from the insoluble fraction of AD brains, where *cis* pT231-tau exhibited a range of molecular weights bigger than IgG (Figures S4E and S4F), it was detected only by *cis* and total tau Abs, but not by *trans* Ab (Figure S4F). Thus, *cis* p-tau is more resistant to dephosphorylation, degradation and more prone to aggregation than the *trans*.

Cis, but not *Trans*, pT231-tau Is not only Correlated with Reduced Pin1 Levels, but also Fully Overlapped with Neurofibrillary Degeneration in the AD Hippocampus

The above results indicate that *cis*, but not *trans*, pT231-tau not only localizes to the pathologically relevant subcellular location, but also displays both losses of tau normal function as well as gains of toxic function. A critical question is whether Pin1 regulates the content of the *cis* and *trans* pT231-tau in neurons in vivo.

To address this question, we started with investigating the relationship between Pin1 levels and *cis* or *trans* pT231-tau levels in subregions of the AD hippocampus. As shown (Liou et al., 2003), we found that Pin1 was highly expressed in the CA2 region, but was dramatically reduced in the CA1 region (Figures 5A-5C, and Figure S5A). Furthermore, neurons that were positive for PHF-1, a solid marker of neurofibrillary neurodegeneration in AD, was prevalent in the CA1, but not CA2 region (Figures 5F and 5H and Figures S5B and S5C), as documented (Liou et al., 2003). Importantly, in the CA2 region where Pin1 was highly expressed, *trans*-positive neurons were dominant and only few *cis*-positive neurons were detected (Figures 5A and 5D and Figure S5A). However, in the CA1 region where Pin1 was barely expressed, *cis*-positive neurons were greatly increased with the number similar to that of *trans*-positive neurons (Figures 5B and 5E and Figure S5A). The Pin1-catalyzed *cis*-*trans* conversion in vivo is likely because both *cis* and *trans* forms were present in the same neurons, as demonstrated by immunostaining of two mirror sections of the AD hippocampus with *cis* or *trans* Ab and total tau Ab as a common indicator (Figure S5D).

More importantly, almost all *cis*-positive cells were also positive for PHF-1 in both CA2 (Figures 5F and 5G) and CA1 regions (Figures 5H and 5I). However, 74% of *trans*-positive cells in the CA2 region were negative for PHF-1 (Figures 5F and 5G), with only 26% *trans*-positive cells being positive, which might be expected because Thr231 is unlikely to be the only phosphorylation site that contributes to neurofibrillary neurodegeneration in AD. Thus, *cis*, but not *trans*, pT231-tau is fully overlapped with neurofibrillary degeneration and also correlated with reduced Pin1 levels in the AD hippocampus.

Whereas Pin1 Overexpression Increases *Cis* to *Trans* Conversion of the pT231-Tau, Pin1 Knockout Decreases the Conversion in AD Mouse Models

The above results suggest that Pin1 might prevent the accumulation of the pathogenic *cis* pT231-tau in the brain. To directly test the possibility, we examined the effects of Pin1 overexpression in postnatal neurons on the *cis* and *trans* contents of pT231-tau by crossing Thy1-Pin1 transgenic (Pin1-Tg) mice with Tau-Tg mice (Ishihara et al., 1999; Lim et al., 2008). Indeed, Pin1 overexpression significantly increased *trans* pT231-tau, but reduced *cis* pT231-tau, when compared with littermates that only overexpressed tau, as normalized using total tau (tau5 Ab) (Figures 6A and 6B) or total protein (tubulin Ab) (Figure S6A). These results were further confirmed by immunostaining on the cerebral cortex (Figures 6C and 6D). Thus Pin1 overexpression reduces *cis*, but increases *trans* pT231-tau levels in mouse brains, consistent with its ability to increase *cis* to *trans* conversion in vitro (Figures 3A and 3C) and to suppress neurofibrillary neurodegeneration in mice (Lim et al., 2008).

To finally determine whether endogenous Pin1 is a rate-limiting factor that controls the levels of *cis* and *trans* pT231-tau conformations in vivo, we determined the effects of Pin1 knockout on *cis* and *trans* contents of pT231-tau by crossing Pin1 knockout mice with Tau-Tg mice. Both immunoblotting (Figures 6E and 6F and Figure S6B) and immunostaining (Figures 6G and 6H) analyses showed that loss of Pin1 function significantly increased *cis* pT231-tau, but decreased *trans* pT231-tau in brains. These results are consistent with the ability of Pin1 knockout to induce tau pathology and neurodegeneration (Liou et al., 2003), directly opposite to Pin1 overexpression (Lim et al., 2008). These results were also confirmed in a condition where the intensities of the *cis* and *trans* signals are almost identical between different Tau-Tg mouse groups (Figure S6C). These results together indicate that *cis*, but not *trans*, pT231-tau is the pathogenic conformation in MCI and AD, but is converted by Pin1 to the non-pathological *trans*, providing the first structural evidence how the isomerase Pin1 protects against AD tau pathology.

DISCUSSION

Pin1-catalyzed *cis-trans* isomerization of pSer/Thr-Pro motifs is widely proposed to regulate many physiological and pathological processes, but there is no direct evidence in vivo. We have developed a novel technology to generate novel *cis* and *trans* specific Abs to provide the first evidence that *cis*, but not *trans*, pT231-tau is pathogenic in MCI and AD and that Pin1 protects against tau pathology by converting it to the non-toxic *trans*. These results provide the first tool to study *cis-trans* prolyl isomerization and their conformation-specific function and regulation, and suggest novel conformation-specific diagnoses and therapies for AD and other diseases.

Conformation-Specific Abs as a First Tool to Study *Cis-Trans* Prolyl Isomerization

Emerging evidence suggests that both phosphorylation-dependent and -independent *cis-trans* prolyl isomerization functions as a new molecular timer in many biological and pathological processes (Lu et al., 2007; Theuerkorn et al., 2011). Most of these studies are based on some structural analyses in vitro, in combination with mutational analyses in cells. Probably the most direct evidence so far to document *cis*- and *trans*-specific function is to replace the key Pro8 in the neurotransmitter 5-HT3 receptor with unnatural Pro analog having varying *cis* or *trans* preference and then assay 5-HT3 activity in Xenopus oocytes, leading the conclusion that *cis-trans* isomerization switches on or off the channel (Lummis et al., 2005). However, the functions of endogenous *cis* and *trans* proteins still remain to be determined.

To apply the above approach to study the conformational regulation after phosphorylation would be challenging, if not impossible, because such Pro analog motifs are unlikely to be

phosphorylated in vivo to become Pin1 substrates (Brown et al., 1999). To detect Pin1-catalyzed protein conformational changes, we reasoned that Abs would only recognize either *cis* or *trans*, but not both of a pSer/Thr-Pro motif in a protein based on the completely distinct structures of these two conformations in pThr688-Pro-containing APP peptides, as revealed by NMR (Pastorino et al., 2006).

A key step to develop such conformation- and phospho-specific Abs is to increase the *cis* content of pSer/Thr-Pro motifs in the antigen with the smallest change possible because ~90% of pSer/Thr-Pro motifs in a synthetic peptide are in *trans* (Lu et al., 2007). We have here discovered that simply increasing the Pro ring by one methylene group in a pT231-tau peptide is sufficient to dramatically increases the *cis* content to 74%, and importantly, to generate phospho-specific Abs that recognize endogenous phosphoproteins. Moreover, we have developed *cis* or *trans* locked peptides to purify *cis* and *trans*-specific Abs. The resulting Abs turn out to be highly specific with little cross-reactivity. We have since used this technology to successfully generate conformation-specific Abs against several other proteins (data not shown). Albeit the development of an antibody-independent identification of *cis* and *trans* protein conformations in vivo will be useful, we believe that this approach can be widely used to study *cis* and *trans* specific function and regulation of a peptidyl-prolyl bond, including a non-phosphorylated one.

Conformation-Specific Localization, Biological Function and Pathological Significance in AD

We have uncovered for the first time the striking differences in the subcellular localization, biological function and pathological significance of *cis* and *trans* p-tau conformations in AD (Figure 7). Although neither *cis* nor *trans* pT231-tau is detected in the healthy brain, *cis*, but not *trans*, p-tau appears early in MCI neurons and further accumulates as AD progresses. Furthermore, *cis*, but not *trans*, p-tau is fully associated with neurofibrillary degeneration and also localizes to the pathologically relevant dystrophic neurites in AD brains. Moreover, *cis*, but not *trans*, p-tau not only loses tau normal function, but also gains tau toxic function, two major properties that are known to contribute to tau pathology in AD (Ballatore et al., 2007). These results indicate that *cis*, but not *trans*, p-T231-tau is pathogenic in AD.

Pin1-Catalyzed *Cis* to *Trans* Isomerization Prevents the Accumulation of the Pathogenic p-Tau Conformation in AD

We have provided the first structural evidence that Pin1 protects against tau pathology by converting the pathogenic *cis* p-tau to the non-pathogenic *trans* (Figure. 7). These results provide the first molecular explanation why Pin1 overexpression promotes tau dephosphorylation and degradation, and inhibits tau aggregation and tau pathology, whereas Pin1 knockout has the opposite effects in model systems (Hamdane et al., 2006; Lim et al., 2008; Liou et al., 2003). These results are highly relevant to human AD because Pin1 is inhibited in MCI and AD neurons by multiple mechanisms (Lee et al., 2011b) and preventing Pin1 inhibition is associated with delaying AD onset (Ma et al., 2010). Furthermore, Pin1 is phosphorylated and catalytically inactivated by DAPK1 (Lee et al., 2011a), which is genetically linked to human AD (Li et al., 2006) and whose deletion improves learning and memory in mice (Yukawa et al., 2006). Thus, lack of sufficient Pin1 to convert *cis* to *trans* contributes to pathogenic p-tau accumulation and tau aggregation, eventually leading to tangle formation and neurodegeneration in AD.

Potential Novel *Cis* and *Trans* Conformation-Specific Disease Diagnoses and Therapies

Our exciting new insight into the role and regulation of p-tau conformations in AD also suggest novel approaches for early diagnosis and treatment of AD (Figure 7). For example, Thr231 phosphorylation is a very early phosphorylation event in human AD (Luna-Munoz

et al., 2007) and its levels in cerebrospinal fluids tracks AD progression, but with large individual variations, making it difficult to become a standardized test (Ewers et al., 2007; Hampel et al., 2001). Our findings that *cis*, but not *trans*, pT231-tau appears early in MCI and is pathogenic suggest that *cis* pT231-tau and its ratio with *trans* might be a better and easier standardized biomarker, especially for early diagnosis and patient comparison. Furthermore, overexpressing Pin1 or preventing Pin1 inhibition in neurons might be a new approach to reduce the *cis* to *trans* pT231-tau ratio to prevent tau pathology at early stages. Finally, active or passive immunization against some pSer/Thr-Pro motifs in tau including the pThr231-Pro motif can reduce tau aggregates and memory deficits in mouse models (Boimel et al., 2010; Boutajangout et al., 2011; Boutajangout et al., 2010). However, our findings that only *cis*, but not *trans*, pT231-tau is pathogenic and 90% of regular synthetic pT231-tau peptides is in *trans* suggest that it might be more specific and effective and safer to develop conformation-specific vaccines or Abs specifically targeting *cis* pT231-tau for treating or even preventing AD at early stages. These studies would also further validate the conformation-specific significance in AD. Given the critical role of Pin1 and other isomerases in regulating many other proteins in physiology and disease (Lee et al., 2011b; Liou et al., 2011; Theuerkorn et al., 2011), it would be interesting to determine whether prolyl isomerization also regulates these protein conformations and whether these conformational switches might be exploited for developing novel diagnostic and therapeutic procedures.

EXPERIMENTAL PROCEDURES

Synthesis of Tau Peptides

Peptides used for the experiments are wild-type phosphorylated Thr231-Pro tau (KVAVVRpTPPKSPS), non-phosphorylated Thr231 tau (KVAVVRTPPKSPS), *cis* lock-in phosphorylated Thr231-Dmp tau (KVAVVRpT(5,5-dimethyl-L-proline)PKSPS), *trans* lock-in phosphorylated Thr231-Ala tau (P232A)(KVAVVRpTAPKSPS) and phosphorylated Thr231-Homoproline (pThr231-Pip) tau (CKKVAVVRpT(Pip)PKSPSSAK), which are synthesized by a commercial source.

NMR Spectrometry

Amide region of TOCSY spectra of tau-derived peptides (sequence KVAVVR-pT231-X232-PKSPS) were used. Pro, Pip, Dmp, or Ala was incorporated into the sequence following pThr231. 2D 1H-1H TOCSY spectra (mixing time of 70 ms) were taken and the population of the *cis* isomer was determined by comparing peak volumes. Peaks used included the gamma, beta, and alpha protons of pThr231 as seen from the amide proton in both the *cis* and *trans* states. *Cis* and *trans* isomers were assigned by identifying characteristic through-space NOEs between Thr231 and Xaa232 protons in 1H-1H ROESY spectra.

Production and Purification of Abs

Rabbits were immunized with pThr231-Homoproline (pThr231-Pip) tau peptide that was coupled to KLH with N-terminal Cys. The resulting sera was purified with pThr231-Homoproline (pThr231-Pip) tau peptide. For *cis* conformation-specific pT231-tau Abs, the resulting bound fraction was purified twice with *cis* pThr231-Dmp tau peptide to collect the bound fraction, followed by counter-purification with pThr231-Ala tau peptide, with the unbound fraction as *cis* conformation-specific pThr231-Pro tau Ab. For *trans*-specific pThr231-Pro tau Ab, the Ab was purified twice with wild-type pThr231-Pro tau peptide to collect the bound fraction, followed by counter-purification with pThr231-Dmp tau peptide with the unbound fraction as *trans*-specific pThr231-Pro tau Ab.

Human Brain Specimen

Brains from 11 patients with AD (Braak stage V and VI) (mean age ± SE: 78.4 ± 3.1 years old), 6 MCI (Braak stage III and IV) (84.4 ± 5.6) and 9 healthy controls (Braak stage I and II) (78.0 ± 6.5) were used for the analyses. All AD subjects met the clinical, and neuropathological National Institute on Aging-Reagan Institute (NIA-RI) criteria, for AD. Our studies for human samples have been approved by our Institutional Review Board.

Transgenic Overexpression and Gene Knockout Mice

Tau-Tg mice (Ishihara et al., 1999), Tau-Tg+Pin1-Tg mice and Tau-Tg+Pin1 KO mice in C57BL/6 background were generated, as described (Lim et al., 2008; Liou et al., 2003). Tau knockout mice (Dawson et al., 2001) were purchased from Jackson Laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

Novel peptide chemistry enables to generate *cis* and *trans* conformation-specific antibodies C*is*, but not *trans*, p-tau appears early, accumulates and localizes to dystrophic neurites in AD C*is*, but not *trans*, p-tau not only loses tau normal function, but also gains toxic function in AD Pin1 accelerates *cis* to *trans* conversion to prevent pathogenic p-tau accumulation in AD

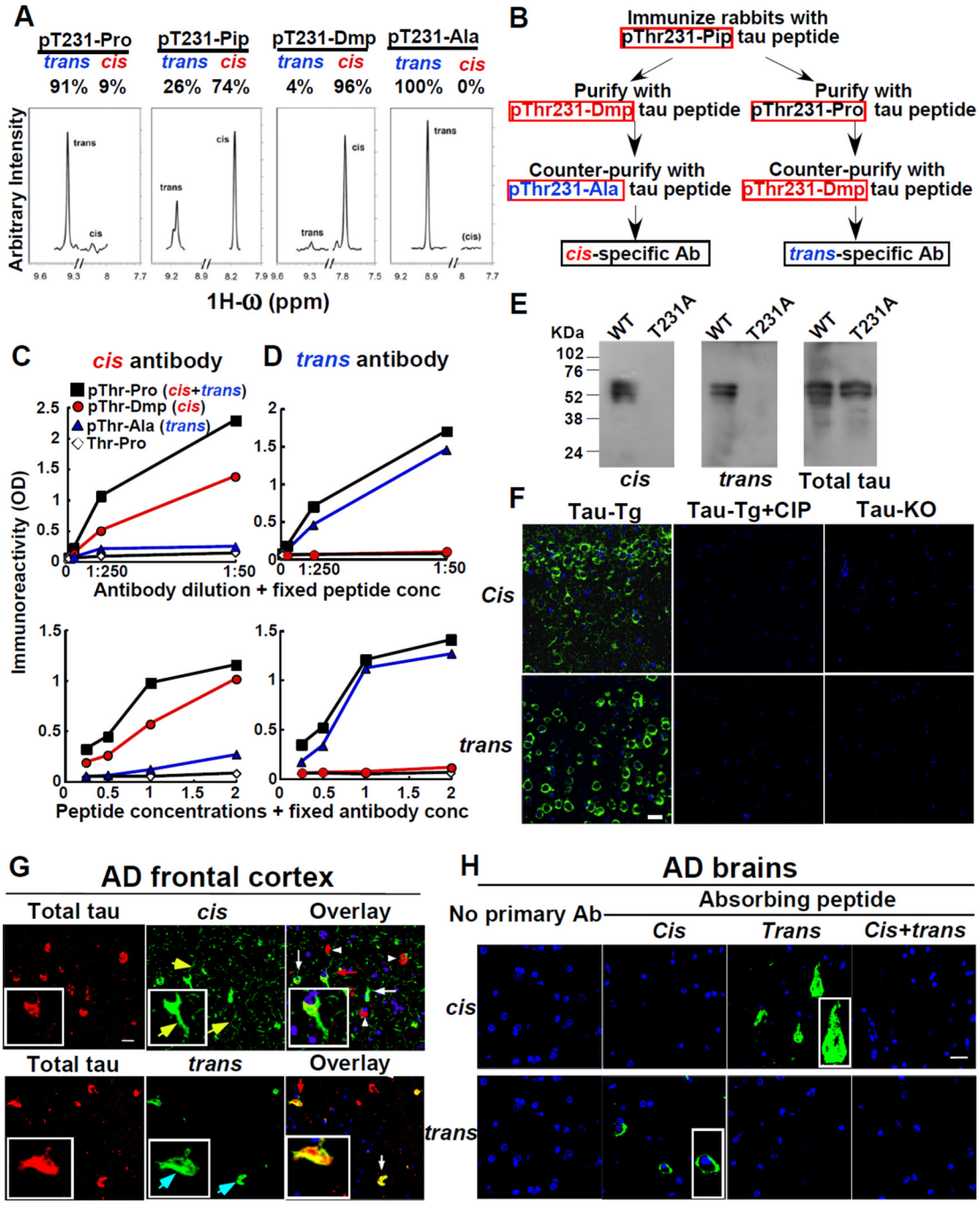

Figure 1. Novel Peptide Chemistry Enables to Generate Abs that Distinguish *Cis* from *Trans* pThr231-Pro Conformation in p-Tau

(A) Proline modifications increase the prevalence of the *cis* isomer, as determined by NMR.

(B) The scheme for Ab purification.

(C, D) Specific recognition of the *cis* and *trans* pThr231-Pro tau Abs by *cis* (pThr231-Dmp) and *trans* (pThr231-Ala) peptides, respectively, while both Abs recognize wild-type pThr231-Pro (*cis*+*trans*) tau peptide.

(E) T231A point mutation abolishes the ability of *cis* or *trans* pThr231-Pro tau Ab to recognize tau in SY5Y cells.

(F) *Cis* and *trans*-pThr231-Pro tau Abs are tau- and phosphorylation-specific, as shown in Tau-Tg brains after dephosphorylation by CIP or in Tau knockout mouse brains.
(G) *Cis* and *trans* pThr231-Pro tau Abs recognize tau in AD brains. White arrows and arrowheads indicate total tau-positive neurons with and without *cis* or *trans* pThr231-Pro tau expression, respectively. Red arrows indicate the neurons shown in insets. Yellow arrows point to dystrophic neurites labeled with the *cis*, and blue arrow to almost exclusive neuronal body localization for the *trans*.
(H) *Cis* and *trans* Abs are conformation-specific with little cross-reactivity in AD brain sections. Only the *cis* and *trans* Abs that were pre-absorbed with *trans* and *cis* peptides, respectively, show signals. Scale bar, 20 μm.
See also Figure S1.

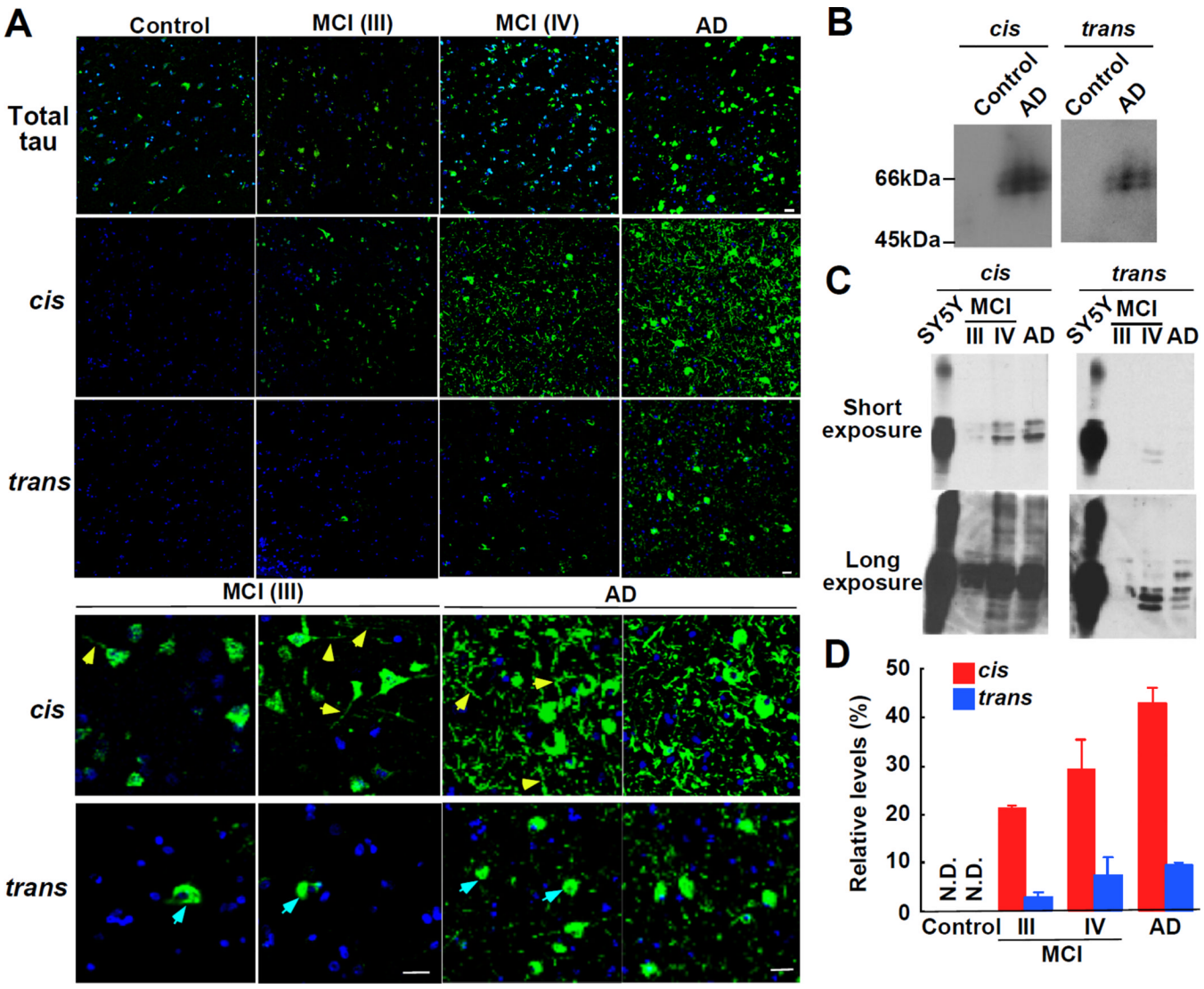

Figure 2. *Cis*, but not *Trans*, pT231-tau Appears Early at MCI Brains, and further Accumulates and Localizes to Dystrophic Neurites as AD Progresses

(A) There was little, if any, *cis* or *trans* pT231-tau signal in age-matched normal brains, but they, especially *cis* conformation, were dramatically increased in AD brains. Strong *cis*, but not *trans*, pT231-tau was detected in MCI cases. Note, many dystrophic neurites (yellow arrows) are labeled with the *cis*, but not the *trans*, which is almost exclusively located at neuron bodies (blue arrows). Scale bars, 20 μm.

(B-D) The *cis* is much more abundant than the *trans*, especially in MCI. Frontal cortex lysates from AD and age-matched normal control (B), Braak stages III and IV (MCI) and Braak stages V and VI (AD) (C) were subjected to immunoblotting analysis with *cis* and *trans* Abs, with short and long exposures being shown in top and bottom panels, respectively. Intensities of *cis* or *trans* signals of tau-overexpressed SY5Y cells and human brain tissues at each Braak stage were semi-quantified, and the percentages of the signals of human samples relative to that of SY5Y cells were expressed as means ± SE (D).

See also Figure S2.

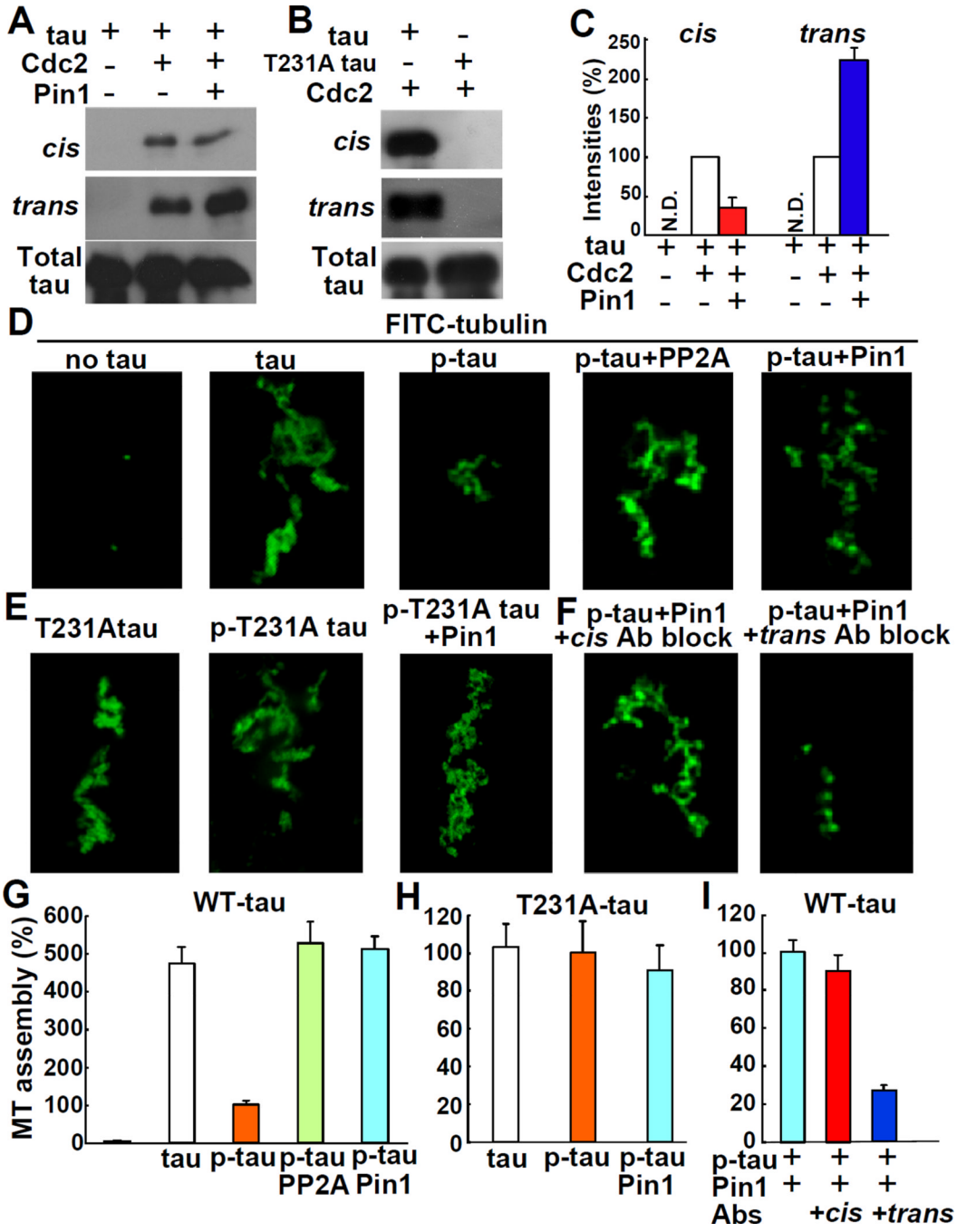

Figure 3. *Trans*, but not *Cis*, pT231-tau Promotes MT Assembly and Pin1 Converts *Cis* to *Trans* to Restore the Ability of *Cis* pT231-tau to Promote MT Assembly

(A-C) Addition of Pin1 to p-tau decreases *cis*, but increases *trans* pThr231-Pro levels. Recombinant tau (A, B) and its T231A mutant (B) were phosphorylated by Cdc2 or control buffer and then incubated with Pin1 before subjecting to immunoblotting analysis with conformation-specific Abs, with the relative signal intensities being quantified (C).

(D-I) *Trans*, but not *cis*, pT231-tau promotes MT assembly. FITC-labeled tubulin was treated with wild-type or T231A mutant tau, p-tau, p-tau+PP2A or p-tau+Pin1, followed by assaying MT assembly using confocal microscope (D, E). Addition of Pin1 or PP2A restored the ability of p-tau to promote MT assembly (D, G). The ability of T231A-p-tau to

promote MT assembly was not affected by Pin1 addition (E, H). Incubation of Pin1-treated p-tau with the *trans*, but not *cis* Ab blocked MT assembly (F, I). MT assembly of each treatment relative to that of p-tau (G, H) and p-tau+Pin1 (I) was quantified.
See also Figure S3.

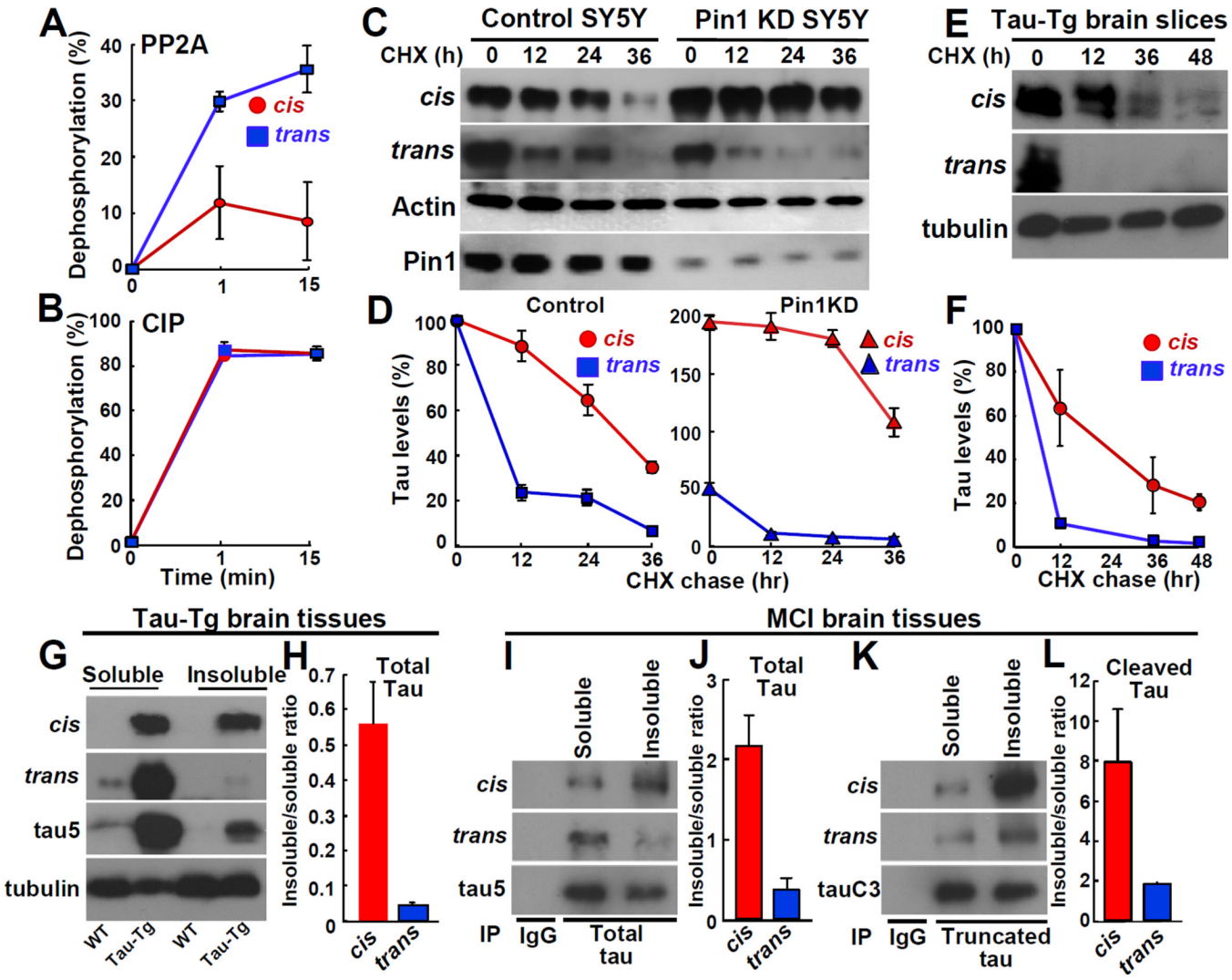

Figure 4. *Cis* pT231-tau Is More Resistant to Protein Dephosphorylation and Degradation, and More Prone to Protein Aggregation than the *Trans*

(A-B) *Cis* pT231-tau is more resistant to dephosphorylation by PP2A, but not CIP.

(C-D) *Cis* pT231-tau is more stable than the *trans* and Pin1 KD increases *cis*, but reduces *trans* in neuronal cells.

(E, F) *Cis* pT231-tau is more stable than the *trans* in brain slice cultures of Tau-Tg mice.

(G-L) *Cis* pT231-tau is more prone to protein aggregation than the *trans* in Tau-Tg mouse brains

(G, H) and human MCI brains (I-L) for total tau (tau5) (I, J), or cleaved tau (tauC3) (K, L), as determined by the sarcosyl fractionation. The amounts of *cis* and the *trans* in the insoluble fraction relative to the soluble fraction were quantified (H, J, L).

See also Figure S4.

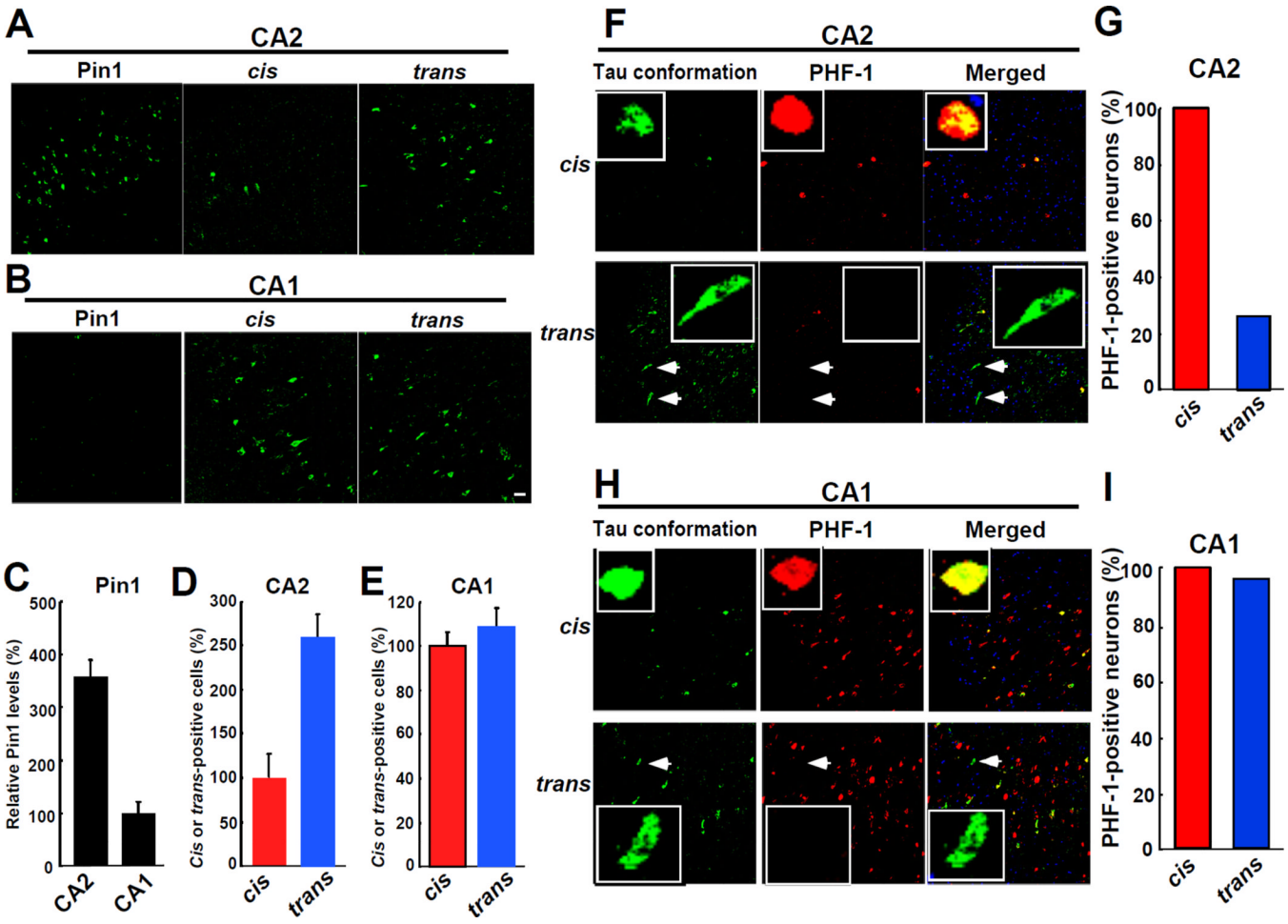

Figure 5. *Cis*, but not *Trans*, pT231-tau Is not only Correlated with Reduced Pin1 Levels but also fully Overlapped with Neurofibrillary Degeneration in the AD Hippocampus

(A-E) *Cis*, but not *trans*, pT231-tau is correlated with reduced Pin1 levels. CA2 (A) and CA1 (B) subregions of the AD hippocampus were immunostained with Pin1 and *cis* or *trans* Ab. Relative Pin1 levels (C) and the numbers of *cis* or *trans* positive neurons in the CA2 (D) or CA1 (E) region are quantified. In the CA2 subregion where Pin1 was highly expressed, *trans*-positive neurons were dominant (A, C, D), but in the CA1 subregion where Pin1 was barely expressed, both *cis*- and *trans*-positive cells were found (B, C, E).

(F-I) *Cis*, but not *trans*, pT231-tau signals are fully overlapped with PHF-1 signals. Double immunostaining images of *cis* or *trans* Ab with PHF-1 in the CA2 (F, G) and CA1 (H, I) regions showed that all *cis*-positive cells were also PHF-1 positive in both CA2 and CA1 regions, but 74% of *trans*-positive cells in the CA2 region were not positive for PHF-1. Arrows indicate *trans*-positive and PHF-1-negative neurons. Scale bars, 20 μm.

See also Figure S5.

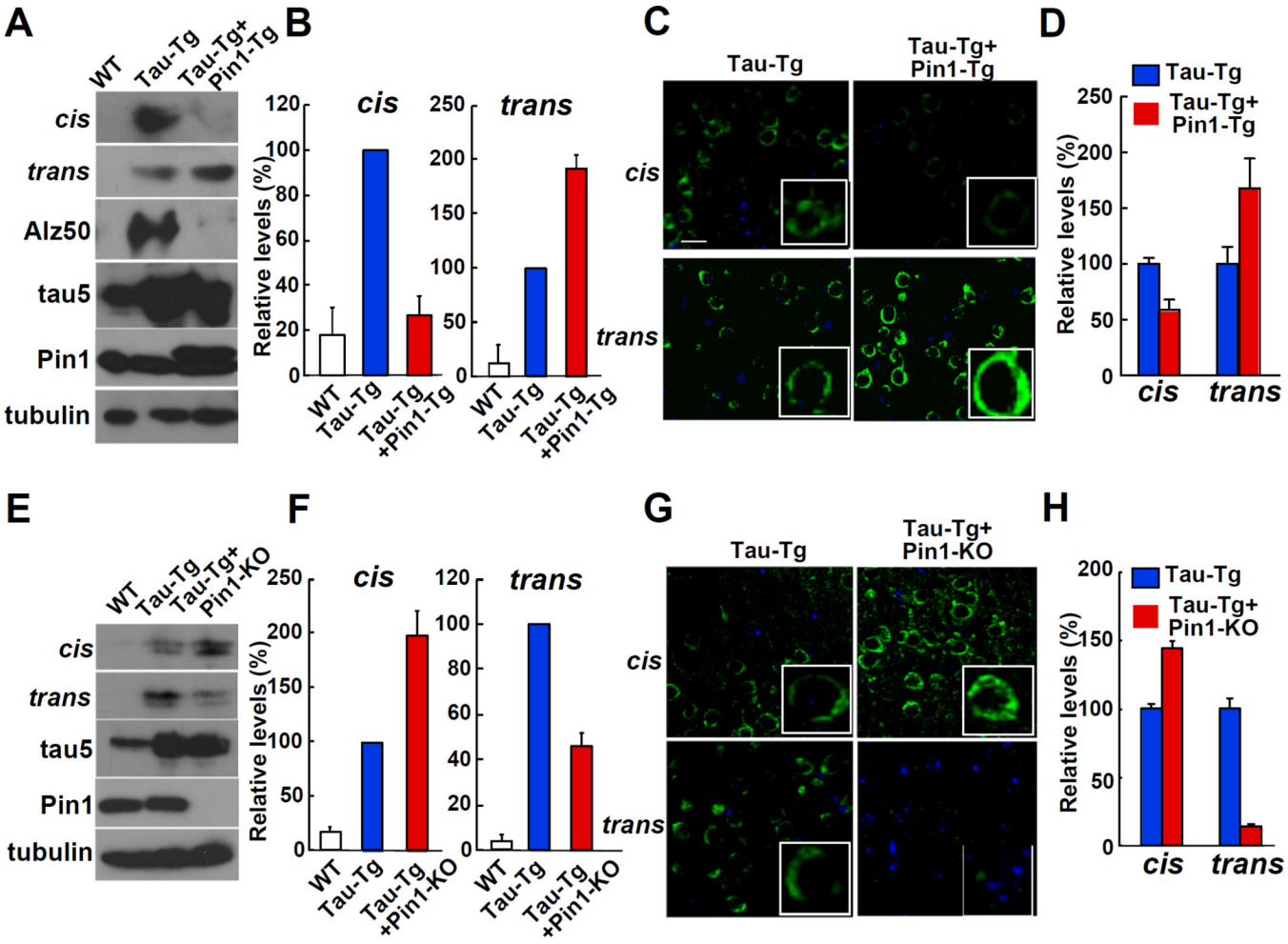

Figure 6. Whereas Pin1 Overexpression Increases the *Cis* to *Trans* Conversion of pT231-Tau, Pin1 Knockout Reduces the Conversion in AD Mouse Models

(A-D) Pin1 overexpression decreases *cis* pThr231-Pro tau, but increases *trans* pT231-tau in tau transgenic mouse brains. Both immunoblotting (A, B) and immunostaining (C, D) analyses of the cerebral cortex of wild-type littermates (WT), tau transgenic (Tau-Tg), and tau and Pin1 double transgenic (Tau-Tg+Pin1-Tg) mice showed higher *trans* pThr231-Pro tau, but lower *cis* pT231-tau signals in Tau-Tg+Pin1-Tg than those in Tau-Tg mice.

(E-H), Pin1 knockout increases *cis* pThr231-Pro tau, but decreases *trans* pT231-tau in tau transgenic mouse brains. Both immunoblotting (E, F) and immunostaining (G, H) analyses of the cerebral cortex of mice revealed higher *cis* pThr231-Pro tau, but lower *trans* pT231-tau signals in Tau-Tg and Pin1 KO mice (Tau-Tg+Pin1-KO) than those in Tau-Tg mice. Scale bars, 20 μm.

See also Figure S6.

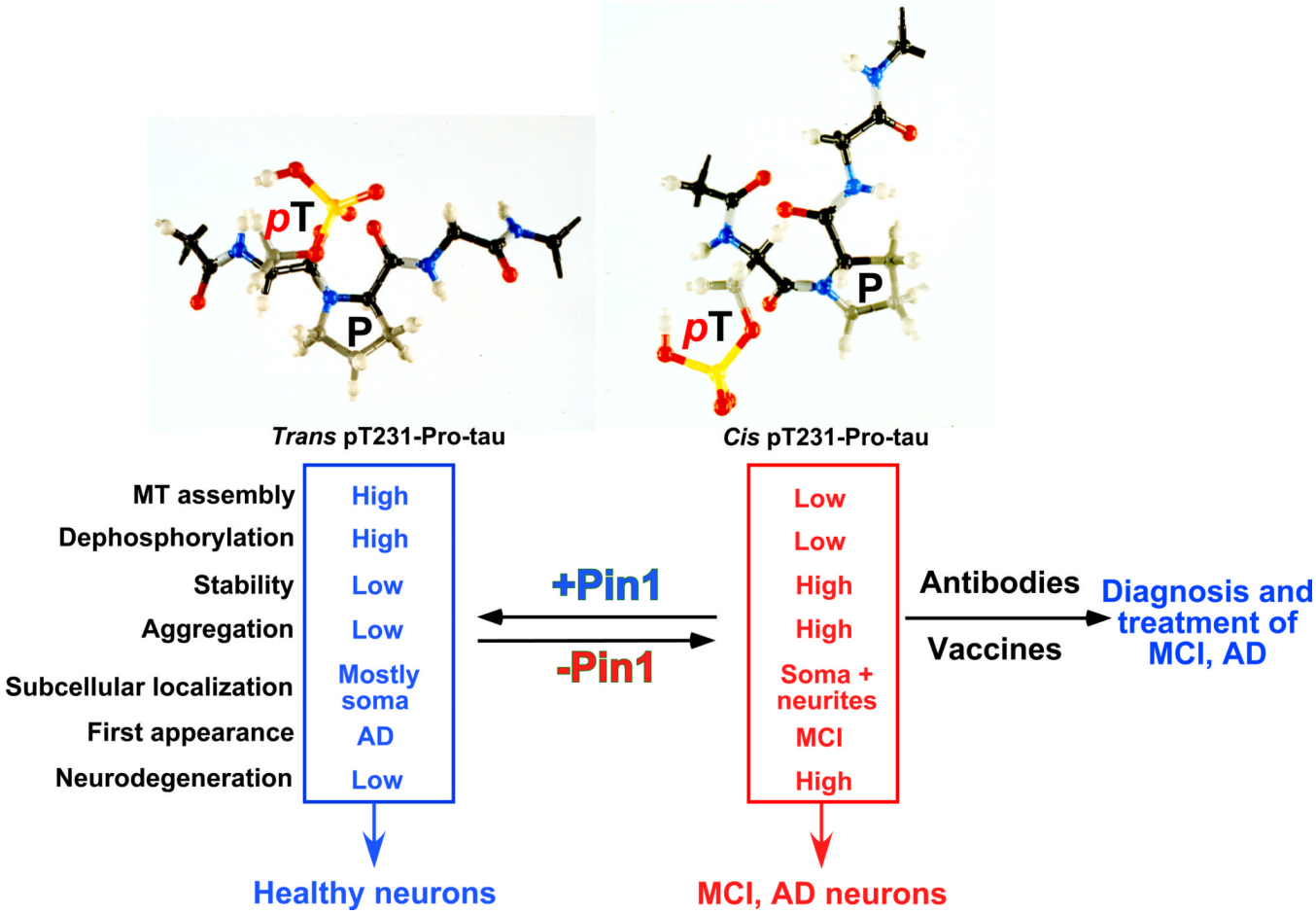

Figure 7. Pin1 Prevents the Accumulation of the Pathogenic *Cis* pT231-tau Conformation in AD by Converting It to the Non-pathogenic *Trans*, and Vaccines or Abs Specifically Targeting *Cis* pT231-tau Might Be Developed for Early Diagnosis and Treatment of AD

pT231-tau protein exists in the two completely distinct *cis* and *trans* conformations, as depicted in cartoons of the primary backbone structures. *Cis*, but not *trans*, pT231-tau loses normal function and also gains toxic function. Pin1 prevents the accumulation of the pathogenic *cis* pT231-tau conformation in AD by converts it to the non-pathogenic *trans*. Conformation-specific antibodies and/or vaccines against the pathogenic cis pT231-tau might be developed for the diagnosis and treatment of AD, especially at early stages.