

# Serine-409 phosphorylation and oxidative damage define aggregation of human protein tau in yeast

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## Keywords

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## Introduction

Protein tau is a microtubule-associated protein that is widely present in neurons and is important for the assembly and stabilization of microtubules. Tau is expressed as six isoforms derived from a single gene by alternative mRNA splicing. These isoforms differ by 29 or 58 amino acid N-terminal insertions encoded by exons 2 and 3 (0N, 1N and 2N isoforms, respectively) and by an extra C-terminal domain encoded by exon 10 (3R and 4R, respectively). The N-terminal inserts have no defined function, while the C-terminal domains are denoted as microtubule-binding domains, whereby isoform tau-4R binds with a greater affinity to microtubules than tau-3R. Besides, by alternative mRNA

## Abstract

Unraveling the biochemical and genetic alterations that control the aggregation of protein tau is crucial to understand the etiology of tau-related neurodegenerative disorders. We expressed wild type and six clinical frontotemporal dementia with parkinsonism (FTDP) mutants of human protein tau in wild-type yeast cells and cells lacking Mds1 or Pho85, the respective orthologues of the tau kinases GSK3 $\beta$  and cdk5. We compared tau phosphorylation with the levels of sarkosyl-insoluble tau (SinT), as a measure for tau aggregation. The deficiency of Pho85 enhanced significantly the phosphorylation of serine-409 (S409) in all tau mutants, which coincided with marked increases in SinT levels. FTDP mutants tau-P301L and tau-R406W were least phosphorylated at S409 and produced the lowest levels of SinT, indicating that S409 phosphorylation is a direct determinant for tau aggregation. This finding was substantiated by the synthetic tau-S409A mutant that failed to produce significant amounts of SinT, while its pseudophosphorylated counterpart tau-S409E yielded SinT levels higher than or comparable to wild-type tau. Furthermore, S409 phosphorylation reduced the binding of protein tau to preformed microtubules. The highest SinT levels were found in yeast cells subjected to oxidative stress and with mitochondrial dysfunction. Under these conditions, the aggregation of tau was enhanced although the protein is less phosphorylated, suggesting that additional mechanisms are involved. Our results validate yeast as a prime model to identify the genetic and biochemical factors that contribute to the pathophysiology of human tau.

splicing, the physiological role of protein tau is further regulated by dynamic phosphorylation through the interplay of a variety of protein kinases and phosphatases (Sergeant *et al.*, 2008; Gendron & Petrucelli, 2009; Iqbal *et al.*, 2009). The longest isoform, i.e. tau-2N/4R, has 85 putative phosphorylation sites, of which 71 have been reported to become phosphorylated, although many are only demonstrated in isolated systems. The majority of these sites are present in the proline-rich and C-terminal regions flanking the microtubule-binding domains (Sergeant *et al.*, 2008; Tremblay *et al.*, 2009). Phosphorylation of tau, especially by MARK kinases within the microtubule-binding repeats, triggers disengagement of tau to regulate microtubule assembly dynamics. This is thought not only to

decrease microtubule stability, important for the establishment of cell polarity, but also to be required for proper intracellular trafficking along the axons, because tau attached to microtubules may form a physical obstacle for vesicles moving along the microtubule tracks (Mandelkow & Mandelkow, 1998; Drewes, 2004; Gendron & Petrucelli, 2009). In addition, tau phosphorylation is developmentally regulated, as it is substantially higher in fetal brain and decreases with age (Avila *et al.*, 1994).

The importance of phosphorylation of tau gained momentum following the finding that hyperphosphorylated forms of tau are the major constituents of intraneuronal tau deposits that define neurodegenerative diseases such as Alzheimer's disease (AD), Pick's disease, progressive supranuclear palsy and frontotemporal dementia, collectively termed tauopathies (Sergeant *et al.*, 2008; Gendron & Petrucelli, 2009; Iqbal *et al.*, 2009). In these tauopathies, hyperphosphorylation of tau is believed to abrogate the physiological function of tau and to introduce conformational changes that direct tau to form paired helical filaments (PHF), which in turn further aggregate into neurofibrillary tangles (NFT) (Mandelkow *et al.*, 2003; Drewes, 2004). Interestingly, different states of tau phosphorylation appear to be associated with different stages of disease progression, as demonstrated for AD (Kimura *et al.*, 1996; Augustinack *et al.*, 2002). Both increased phosphorylation and decreased dephosphorylation may thus influence the aggregation process (Sergeant *et al.*, 2008; Gendron & Petrucelli, 2009; Iqbal *et al.*, 2009), but the exact kinases and phosphatases responsible *in vivo* and the phosphorylation sites targeted to induce aggregation of tau remain largely elusive.

Direct genetic evidence that implicated protein tau in neuronal loss and dementia came with the discovery of a diverse range of dominant mutations associated with frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17). To date, > 40 pathogenic mutations have been identified, some of which are intronic and affect mRNA splicing to alter the ratio of expression of tau-4R over tau-3R isoforms, while others are exonic and cause single amino acid substitutions or deletions that affect the ability of tau to regulate microtubule dynamics (Rademakers *et al.*, 2004; Gendron & Petrucelli, 2009). Analysis of postmortem brain samples of FTDP-17 patients confirmed the accumulation of hyperphosphorylated tau fibrils and suggested that mutations promote tau phosphorylation site-specifically (Spillantini *et al.*, 1998; van Swieten *et al.*, 1999). This is further supported by the analysis of various transgenic models (Götz *et al.*, 2009). However, studies performed to comparatively analyze the effect of tau mutations on phosphorylation and its causal relation to aggregation failed to produce consistent data. For example, when expressed in human neuroglioma H4 cells, both the tau-V337M and the tau-R406W mutants displayed a drastically reduced phosphorylation at several epitopes as compared

with wild-type tau-2N/4R (DeTure *et al.*, 2002), while in CHO cells, reduced phosphorylation was seen with tau-R406W and not with tau-V337M (Matsumura *et al.*, 1999; Vogelsberg-Ragaglia *et al.*, 2000). On the other hand, phosphorylation of recombinant tau *in vitro* with rat brain extract revealed that the R406W and V337M mutations enhanced phosphorylation at most epitopes (Alonso Adel *et al.*, 2006). This study also demonstrated that distinct tau mutants display a similar enhanced propensity to aggregate, but a more recent study presented a different picture and, for instance, failed to confirm enhanced aggregation for tau-R406W (Chang *et al.*, 2008).

The aggregation of protein tau is also influenced by oxidative stress. Indeed, oxidative stress and mitochondrial dysfunction attracted increasing interest in the field of tau-related pathologies. Oxidative damage is abundantly evident in the postmortem brain of patients, where metabolic signs of oxidative stress and markers of oxidized proteins and lipids coincide with brain regions that are affected by neurodegeneration (Perry *et al.*, 2002; Mancuso *et al.*, 2007; Moreira *et al.*, 2008; Martinez *et al.*, 2009). In addition, oxidative stress is known to increase the activity of several kinases, i.e. ERK, p38 and JNK, that are activated in the brain of patients with AD or other tauopathies, and found to phosphorylate tau (Reynolds *et al.*, 2000; Ferrer *et al.*, 2001; Sergeant *et al.*, 2008). However, several studies reported downregulation of other tau kinases and dephosphorylation of tau under oxidative stress conditions, leaving the effect of oxidative stress on tau phosphorylation to be puzzling (LoPresti & Konat, 2001; Zambrano *et al.*, 2004; Galas *et al.*, 2006). Of particular interest is that tau was also shown to be modified by products of oxidative stress *in vitro* and that this enhanced the formation of tau dimers and tau oligomers (Schweers *et al.*, 1995; Barghorn & Mandelkow, 2002; Landino *et al.*, 2004; Reynolds *et al.*, 2007).

The many advantages of a less complex cellular system to study in detail the molecular mechanisms leading to hyperphosphorylation and aggregation of protein tau led us to express and study human tau in yeast. Previously, we reported that the humanized yeast cells recapitulated robustly the most important aspects of a tauopathy, i.e. hyperphosphorylation, conformational change and self-aggregation of wild-type tau-2N/3R or tau-2N/4R isoforms. The ease and rapidity of genetic modification of yeast cells was then capitalized upon the finding that major pathogenic phosphoepitopes on human tau are produced by Mds1 and Pho85, the yeast orthologues of the two major mammalian tau kinases, i.e. GSK3 $\beta$  and cdk5, respectively (Vandebroek *et al.*, 2005, 2006).

In the present study, we analyzed the expression of a series of clinical FTDP-17 mutants of tau in yeast, to extend the findings of the wild-type tau isoforms and to define the molecular and phenotypic similarities as well as differences among the mutants. This uncovered the importance of serine-409 (S409)-phosphorylation to induce tau self-assembly. In

addition, we studied the influence of oxidative stress and mitochondrial dysfunction on tau aggregation and found both conditions to drastically induce the formation of tau filaments in yeast by mechanisms that appear to act additional to tau phosphorylation.

## Materials and methods

### Yeast strains and media

The *Saccharomyces cerevisiae* strains used in this study were W303-1A (Mat a *leu2-3 112 ura3-1 trp1-1 his3-11 15 ade2-1 can1-100 GAL SUC*), BY4742 (S288C Mat  $\alpha$  *his3 $\Delta$ 1 leu2 $\Delta$ 0 lys2 $\Delta$ 0 ura3 $\Delta$ 0*) and the isogenic single- or double-deletion mutants *mds1 $\Delta$* , *pho85 $\Delta$* , *mds1 $\Delta$ pho85 $\Delta$* , *sod2 $\Delta$*  and *rim1 $\Delta$* . The single *pho85 $\Delta$*  and double *mds1 $\Delta$ pho85 $\Delta$*  mutants were obtained by disruptions of *PHO85* using PCR-derived cassettes containing *KanMX*, *HIS3* or *TRP1* markers as described previously (Wach *et al.*, 1994). The *sod2 $\Delta$*  and *rim1 $\Delta$*  strains were obtained from the genome-wide yeast deletion collection (Winzeler *et al.*, 1999).

The plasmids expressing tau-2N/4R and tau-P301L were described previously (Vandebroek *et al.*, 2005, 2006). Other FTDP-17 mutations, i.e. G272V, N279K,  $\Delta$ K280, V337M and R406W, as well as the synthetic mutants S409E and S409A were introduced into the tau-2N/4R isoform using site-directed mutagenesis. All tau constructs were fully sequenced to ensure that no additional mutations were introduced during amplification. Human Gsk3 $\beta$  was inserted as an EcoRV–PvuII fragment and cdk5 as a BamHI fragment into the yeast expression vector pKT10 (Tanaka *et al.*, 1990).

Standard yeast transformation techniques were applied (Gietz *et al.*, 1992). Cells were grown at 30 °C in a selective minimal medium containing 2% glucose. To induce ROS production and oxidative stress, FeSO<sub>4</sub> was added to a final concentration of 20 mM to early exponential cells (OD = 0.5) and the cultures were allowed to grow till the mid-exponential phase (OD = 2) before harvesting and determination of the sarkosyl-insoluble tau (SinT) fraction.

### Immunological techniques

Yeast cells were grown until OD<sub>600 nm</sub> = 2.0 and then harvested by centrifugation. Samples were prepared as described previously, separated with standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), both under reducing and nonreducing conditions, or native PAGE and further analyzed using standard Western blotting techniques (Towbin *et al.*, 1979; Vandebroek *et al.*, 2005). The tau antibodies used in this study are listed in Table 1. Relative immunoreactivity was determined by densitometric comparison (software: IMAGEMASTERID, Amersham; TINA 2.08, Raytest) and normalized for total tau amounts as measured by pan-tau antibody Tau-5.

### Sarkosyl insolubility assay

Sarkosyl insolubility levels were determined as described before (Vandebroek *et al.*, 2005). Cells were homogenized with glass beads and extracts were centrifuged (30 min, 20 000 g). After *N*-lauroyl-sarkosine was added to the supernatant (final concentration 1%) and samples were incubated for 1 h, sarkosyl-soluble and -insoluble fractions were separated by centrifugation (150 000 g, 40 min). Relative quantities were determined by Western blotting using antibody Tau-5.

### Microtubule interaction studies

The method to purify phosphorylated tau from yeast has been described previously (Vandebroek *et al.*, 2005). Also, the methods to prepare taxol-stabilized microtubules from pig tubulin and to perform tau–microtubule-binding filter assays have been reported earlier (Vandebroek *et al.*, 2006).

### Statistical analysis

Statistical analysis was performed using one-way or two-way ANOVA, followed by multiple comparison tests of Tukey and Fisher.

**Table 1.** List of tau antibodies and their specificities

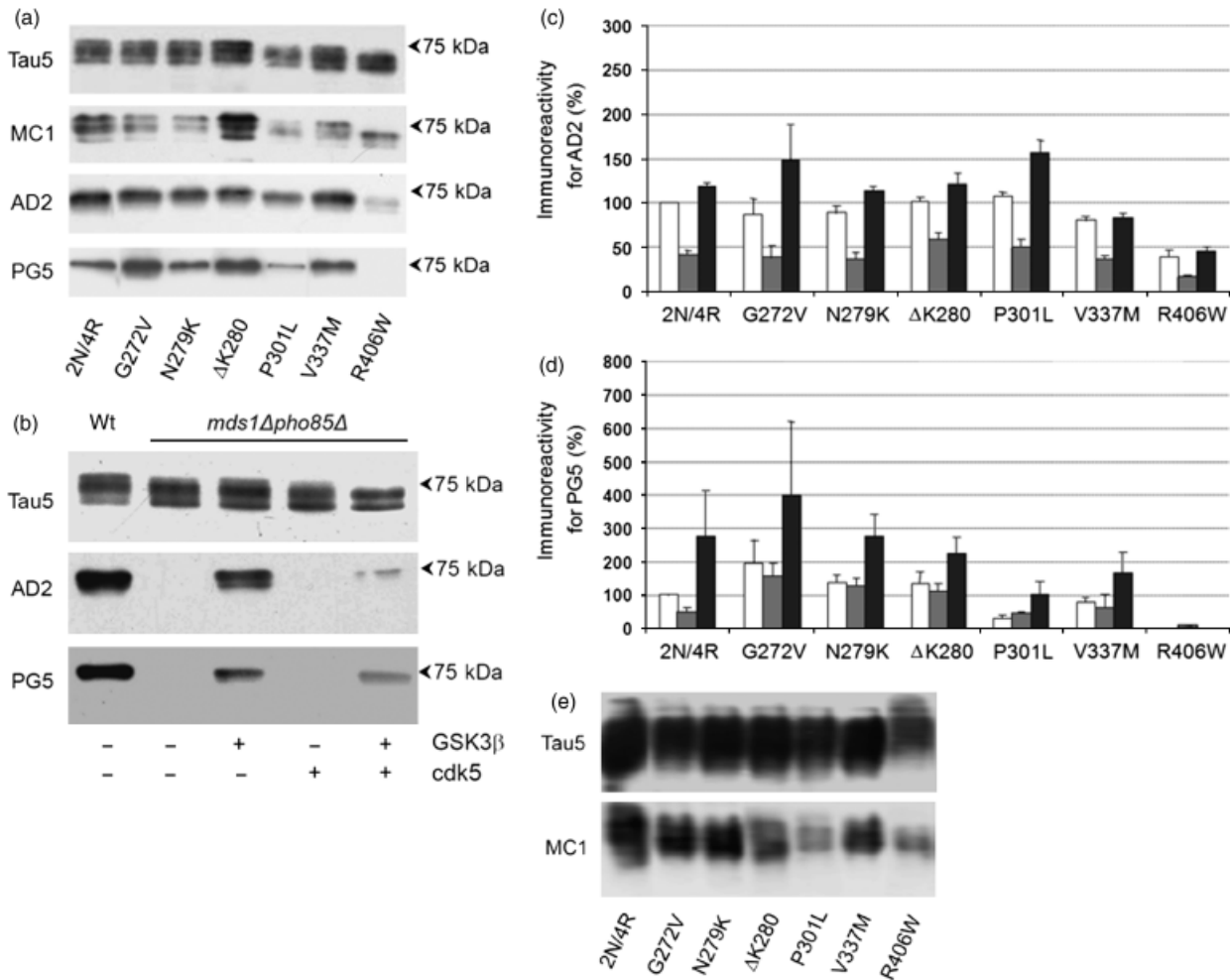
Antibody	Specificity	Source
Tau5	All isoforms	BD Pharmingen, San Diego, CA
Sheep anti-hTau (polyclonal)	All isoforms	Innogenetics, Gent, Belgium
Tau-1	Unphosphorylated Ser198/Ser199/Ser202	Chemicon, Temecula, CA
AD-2	Phosphorylated Ser396/Ser404	BIO-RAD, Hercules, CA
AT8	Phosphorylated Ser202/Ser205	Innogenetics, Gent, Belgium
AT100	Phosphorylated Thr212/Ser214	Innogenetics, Gent, Belgium
At180	Phosphorylated Thr231/Ser235	Innogenetics, Gent, Belgium
AT270	Phosphorylated Thr181	Innogenetics, Gent, Belgium
PG-5	Phosphorylated Ser-409	Gift from Peter Davies (Jicha <i>et al.</i> , 1999)
MC 1	aa 5-15/312-322 conformation dependent	Gift from Peter Davies (Jicha <i>et al.</i> , 1997)

**Results**

**Phosphopeptide mapping reveals altered phosphorylation profiles in two FTDP-17 tau mutants**

The human tau-2N/4R wild-type isoform and the clinical mutants tau-G272V, tau-N279K, tau-ΔK280, tau-P301L, tau-V337M and tau-R406W, all in the tau-2N/4R isoform, were expressed constitutively from high-copy-number plasmids in the W303-1A wild-type yeast strain. Their expression was evaluated using Western blot analysis with a polyclonal tau

antiserum (data not shown) and the pan-tau monoclonal antibody (Mab) Tau5. This revealed that the proteins are expressed as a mixture of isoforms varying in molecular weights from 64 to 72 kDa (Fig. 1). These isoforms originate from differences in phosphorylation as demonstrated previously by dephosphorylation studies (Vandebroek *et al.*, 2005). When quantified over all subforms and normalized vs. the levels of endogenous yeast alcohol dehydrogenase II, the expression levels of wild-type and mutant tau proteins appeared to be similar (data not shown). Interestingly, a detailed comparison of the electrophoretic mobility patterns detected with Tau5 for the wild-type tau protein and the



**Fig. 1.** Phosphopeptide mapping of wild-type tau-2N/4R and FTDP-17 mutant tau expressed in yeast. (a) Western blot analysis with the Mabs indicated of the total protein extracts obtained from transformed W303-1A wild-type cells expressing wild-type tau-2N/4R or FTDP-17 mutant tau. (b) Immunodetection of tau-2N/4R with the Mabs indicated in the total protein extracts obtained from W303-1A wild-type cells and isogenic *mds1Δpho85Δ* cells expressing GSK3β and cdk5 as indicated. (c, d) Relative immunoreactivity levels of protein tau for the phosphopeptide specific Mabs AD2 (c) or PG5 (d) in the total protein extracts obtained from transformed W303-1A wild-type cells (open bars) or the isogenic mutants lacking Mds1 (gray bars) or Pho85 (black bars). The analyses included wild-type tau-2N/4R and the FTDP-17 mutants tau-G272V, tau-N279K, tau-ΔK280, tau-P301L, tau-V337M and tau-R406W. All data are normalized for the total amount of tau determined with the pan-tau Mab Tau5 and are the mean with SEM of at least three independent experiments. Statistical relevance is indicated in the main text. (e) Western blot analysis and immunodetection with Tau5 and MC1 of the total protein extracts separated by native-PAGE obtained from *pho85Δ* cells that express wild-type tau-2N/4R.

clinical mutants revealed that the most phosphorylated isoform, with the lowest electrophoretic mobility, was only very weakly present in the case of tau-P301L, while being completely absent in the case of tau-R406W (Fig. 1a). This was even more prominent when immunodetection was performed with the antibody MC1, which detects a discontinuous epitope characteristic for tauopathy (Jicha *et al.*, 1997) (Fig. 1a). Combined, our data suggested that the tau-P301L and tau-R406W mutants displayed phosphorylation patterns deviating from the other FTD mutants. Importantly, neither the wild-type tau-2N/4R nor the clinical FTDP-17 mutants markedly influenced the growth properties of the transformed yeast cells (data not shown).

To assess the phosphorylation status of protein tau in detail, phosphoepitope scanning was performed with Mabs Tau-1, AT8, AT100, AT180, AT270, AD2 and PG5 (Fig. 1a; Table 1). Normalized quantification against the total tau levels, measured with Tau5, demonstrated that the epitopes defined by Tau-1, AT8, AT180 and AT270 were similarly present on wild-type and mutant tau (data not shown). In contrast, the AD2 epitope (P-S396, P-S404) was markedly lower in tau-R406W, while the PG5 epitope (P-S409) was almost absent in tau-R406W ( $P < 0.001$ ) and drastically reduced in tau-P301L ( $P < 0.05$ ) (Fig. 1a, c, d).

We previously identified the protein kinase Mds1 as the valid orthologue of GSK3 $\beta$  by its capacity to generate the AD2 and PG5 epitopes on tau-2N/4R. We then also reported that phosphorylation of the AD2 and PG5 epitope on tau-2N/4R was negatively influenced by Pho85, the orthologue of mammalian cdk5 (Vandebroek *et al.*, 2005, 2006). This then led us to the hypothesis that Pho85/cdk5 influences the phosphorylation of tau indirectly by acting as a negative regulator of Mds1/Gsk3 $\beta$ , in line with data obtained in transgenic mice (Hallows *et al.*, 2003; Plattner *et al.*, 2006). This hypothesis is also based on our current observation that the capacity of human GSK3 $\beta$  to restore the AD2 and PG5 epitopes on tau2N/4R in the yeast *mds1 $\Delta$ pho85 $\Delta$*  double-deletion strain is strongly reduced upon coexpression with human cdk5 (Fig. 1b).

To document the contribution of the two yeast kinases on phosphorylation of mutant tau, we expressed the different FTDP-17 tau mutants in strains lacking either Mds1 or Pho85 and again quantified the immunoreactivity for AD2 and PG5. This confirmed that the level of the AD2 epitope was in general twofold lower for the wild-type and mutant tau in the *mds1 $\Delta$*  strain as compared with the wild-type strain ( $P < 0.05$ ), while being slightly higher in the *pho85 $\Delta$*  strain (Fig. 1c). The level of the PG5 epitope in the different tau proteins was moderately affected in the *mds1 $\Delta$*  strain, but significantly enhanced in the *pho85 $\Delta$*  strains ( $P < 0.05$  to  $< 0.01$ ), except for tau-R406W (Fig. 1d). Intriguingly, the PG5 immunoreactivity of the mutants in the *pho85 $\Delta$*  strain tended to correlate with the distance of their mutation to the C-terminus, the exceptions being tau-P301L, which

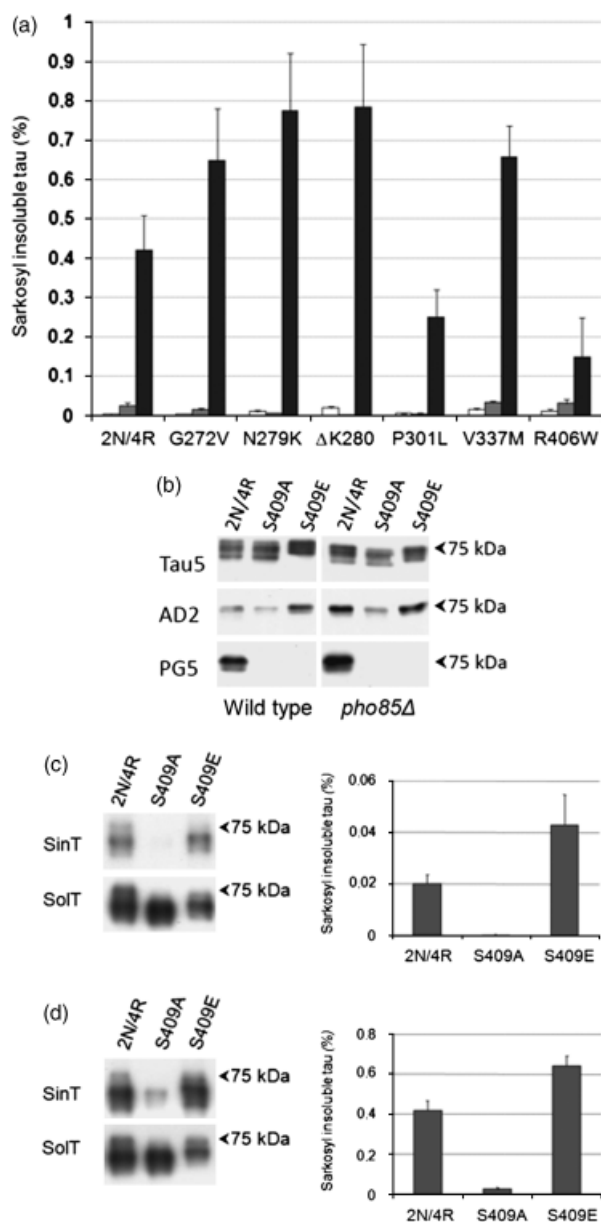
still displayed limited PG5 immunoreactivity, and tau-R406W, which still lacked this phosphoepitope. Consistent with their hampered C-terminal phosphorylation, both tau mutants also failed to display high levels of the pathological and aggregation-prone MC1 conformation upon native-PAGE analysis (Fig. 1e).

### Phosphorylation of S409 is crucial for aggregation of tau in yeast

We demonstrated previously that a small, but consistent fraction of tau-2N/4R is aggregated in yeast as determined by its sarkosyl insolubility (Vandebroek *et al.*, 2005). Quantitative analysis of this sarkosyl-insoluble fraction (SinT) in the W303-1A wild-type strain suggested that all FTDP-17 mutants aggregated more than wild-type tau-2N/4R. However, the levels remained low, which makes assessment of the biological or pathological significance difficult (Fig. 2a). In line with our previous report, the deletion of *MDS1* did not have a major impact on tau insolubility. In contrast, the deletion of *PHO85* led to a significant increase in SinT ( $P < 0.001$ ) and this for the wild-type protein as well as all clinical tau mutants (Fig. 2a). Nonetheless, the SinT levels in the *pho85 $\Delta$*  strain remained rather low for tau-P301L and tau-R406W, i.e. the two mutants with the lowest PG5 and MC1 immunoreactivity. This suggested that the phosphorylation of S409 could be a determining factor for tau aggregation.

To confirm this hypothesis, we mutagenized the epitope recognized by PG5 and expressed the synthetic tau-S409A mutant and its pseudophosphorylated counterpart tau-S409E in wild-type and *pho85 $\Delta$*  strains. Western blotting and subsequent immunodetection with PG5 demonstrated that both synthetic mutants had lost all PG5 immunoreactivity (Fig. 2b). While this result was expected for the tau-S409A mutant, it proves that in the tau-S409E mutant, glutamic acid does not substitute perfectly for a phosphorylated serine residue. Interestingly, immunodetection with AD2 was also affected in the synthetic mutants, even though the amino acid composition was not altered at this epitope (Fig. 2b). Indeed, we observed increased AD2 immunoreactivity for tau-S409E, particularly in the wild-type strain, and a decreased immunoreactivity for tau-S409A. These results indicate that the phosphorylation of both AD2 and PG5 is closely linked and that priming, i.e. the facilitation of phosphorylation of a residue through previous phosphorylation at other residues, also occurs in yeast.

We then monitored SinT levels in both strains. As compared with tau-2N/4R, these levels were drastically reduced for tau-S409A ( $P < 0.001$ ), while it was comparable or even increased for tau-S409E expressed (Fig. 2c, d). These data underscore the importance of S409 phosphorylation for the aggregation of tau. Furthermore, Western blot analysis with Tau5 of the soluble and insoluble tau fractions clearly demonstrated that mutant tau-S409A failed to



**Fig. 2.** Sarkosyl insolubility of wild-type tau-2N/4R and the FTDP-17 mutant tau in yeast. (a) Bar diagram depicting the aggregation of wild-type tau-2N/4R and FTDP-17 mutant tau as the percentage SinT obtained from transformed W303-1A wild-type cells (open bars) or the isogenic mutants lacking Mds1 (gray bars) or Pho85 (black bars). Data are given as percentages of SinT on total tau as determined with the Mab Tau-5. The analysis included wild-type tau-2N/4R and the FTDP-17 mutants tau-G272V, tau-N279K, tau-ΔK280, tau-P301L, tau-V337M and tau-R406W as indicated. (b) Western blot analysis with the Mabs indicated of protein extracts of W303-1A wild-type cells or isogenic *pho85Δ* cells expressing either tau-2N/4R or the synthetic mutants tau-S409A and tau-S409E. (c, d) Western blot analysis with Tau5 of soluble (SolT) and sarkosyl-insoluble (SinT) fractions from wild-type tau-2N/4R or the synthetic mutants tau-S409A and tau-S409E (right) and the corresponding quantifications (left) as obtained in wild-type cells (c) or *pho85Δ* cells (d). Note that more sarkosyl-insoluble extract was loaded to ensure immunodetection. Data are the mean with SEM of at least three independent experiments. Statistical relevance is described in the main text.

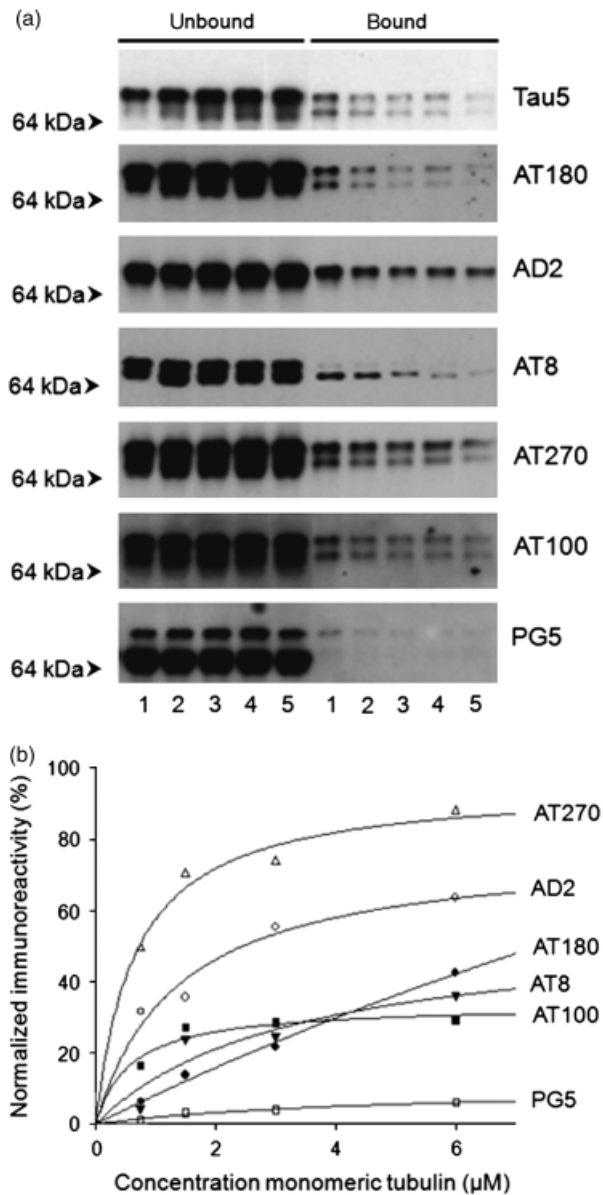
display the slow-migrating hyperphosphorylated isoform, which we identified previously as being crucial for tau to adopt the MC1 conformation and to aggregate (Vandebroek *et al.*, 2005).

### Phosphorylation of S409 is detrimental for tau–microtubule interaction

The commonly accepted hypothesis, although yet to be proven *in vivo*, posits that hyperphosphorylation of tau disrupts its binding to, and assembly of, microtubules. To elaborate on this assumption, we performed *in vitro* binding assays with taxol-stabilized porcine microtubules and recombinant tau-2N/4R isolated from the wild-type yeast strain. The qualitative analysis aimed to identify phospho-epitopes that differentiate tau bound to microtubules from soluble tau present in the unbound fraction. Our analysis clearly revealed the presence of epitopes recognized by AT180, AD2, AT8, AT270 and AT100 on protein tau bound to microtubules. In contrast, the epitope defined by PG5 was almost completely absent in the microtubule-bound tau fraction, while being abundantly present in the unbound tau fraction (Fig. 3a, b). This signifies that mainly the tau species that are not phosphorylated on S409 interact with microtubules. Combined with the observations described above, the data led us to conclude that the presence of the PG5 epitope is a decisive factor for tau aggregation and for the disengagement of tau to bind microtubules.

### Oxidative stress and mitochondrial dysfunction enhance tau aggregation independent of phosphorylation

Recent studies highlighted the involvement of oxidative stress and mitochondrial dysfunction in the etiology of different tauopathies (Melov *et al.*, 2007; Moreira *et al.*, 2008; Martinez *et al.*, 2009), while others revealed the interplay of tau phosphorylation and oxidative stress for the formation of NFT (Schweers *et al.*, 1995; Takeda *et al.*, 2000; Tremblay *et al.*, 2009). To assess the importance of oxidative stress for SinT formation in our yeast system, cells expressing the wild-type or mutant tau were challenged with the addition of ferrous sulfate in the growth medium to increase free radical production (Stadler *et al.*, 2001). This treatment increased SinT levels drastically in wild-type cells, particularly for the mutants tau-G272V, tau-N279K, tau-ΔK280 and tau-V337M (Fig. 4a). As these SinT increments were considerably higher than those observed with tau-2N/4R, the data demonstrate that FTDP-17 mutations render protein tau more vulnerable to oxidative stress-induced aggregation. Interestingly, the SinT levels of tau-P301L and tau-R406W were also significantly enhanced ( $P < 0.001$ ) and were comparable to, or even higher than, those of tau-2N/4R (Fig. 4a). In cells lacking Pho85, the addition of

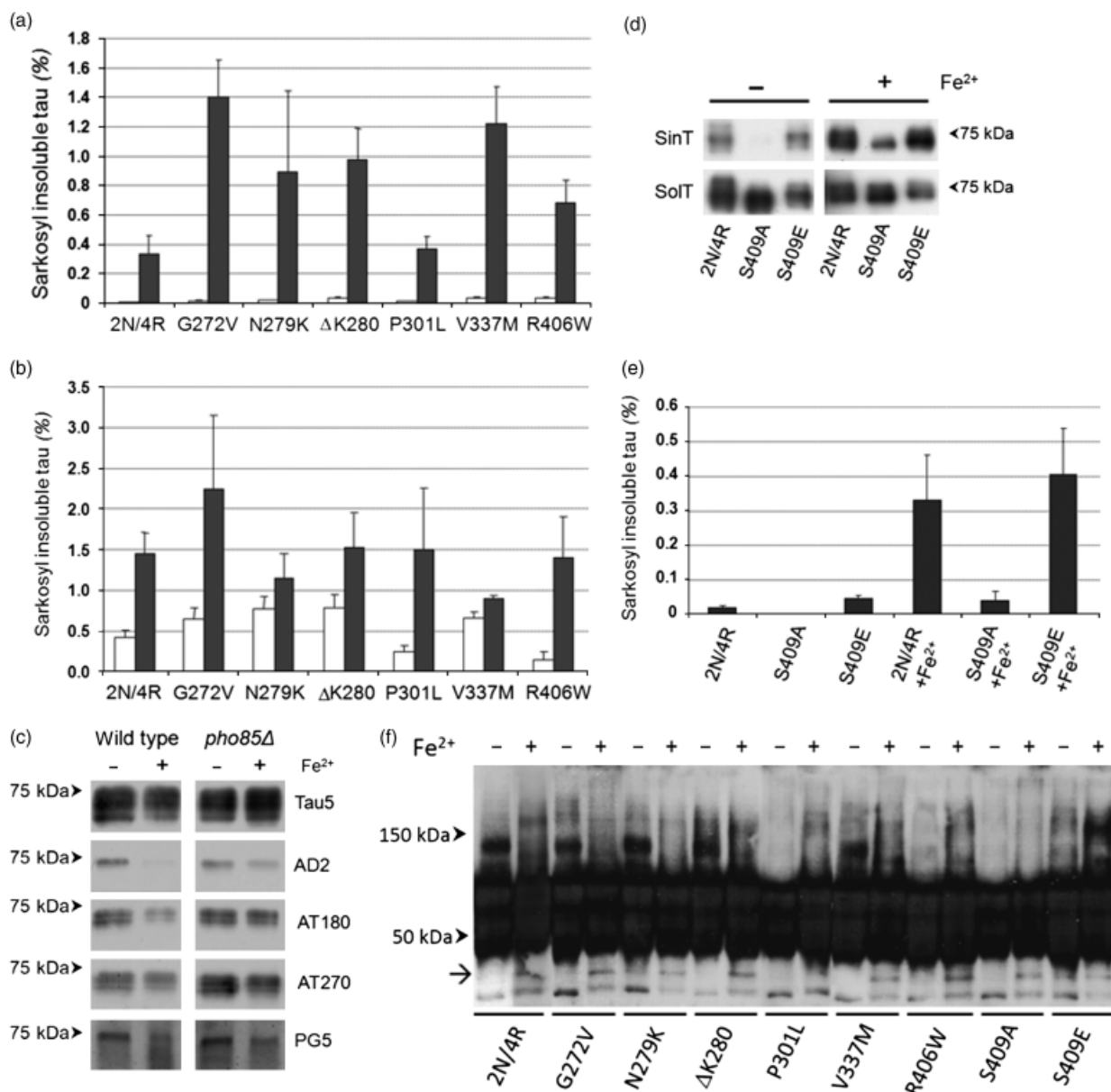


**Fig. 3.** Phosphoepitope analysis of purified tau-2N/4R upon binding to taxol-stabilized MT. (a) Western blot analysis with the Mabs indicated of MT-bound or unbound fractions of tau-2N/4R obtained from wild-type yeast cells. The concentrations of taxol-stabilized porcine MT used in the *in vitro* binding assays were 6 µM (lane 1), 3 µM (lane 2), 1.5 µM (lane 3), 0.8 µM (lane 4) and 0 µM (lane 5). (b) Relative immunoreactivity levels of protein tau present in the different microtubule-bound fractions for the indicated phospho-epitope-specific Mabs. All data are normalized for the total amount of tau determined with the pan-tau Mab Tau5.

ferrous ions to the culture medium also induced SinT for the wild-type and mutant tau (Fig. 4b), but this effect was less pronounced than in wild-type cells and, surprisingly, most obvious for tau-P301L ( $P < 0.01$ ) and tau-R406W ( $P < 0.001$ ). Combined, the data suggested that oxidative stress increases

the aggregation of tau by mechanisms acting mainly in parallel to tau phosphorylation. In fact, Western blot analysis of extracts prepared from wild-type and *pho85Δ* cells expressing tau-2N/4R indicated that tau becomes dephosphorylated upon ferrous ion treatment, especially at the AD2 and PG5 epitopes (Fig. 4c). Moreover, the treatment of wild-type cells with ferrous ions significantly induced the formation of SinT by tau-S409A ( $P < 0.001$ ) to a level that is comparable to that found for tau-2N/4R and tau-S409E in untreated cells (Fig. 4d, e). This confirms that the presence of the PG5 epitope is not strictly essential, although it facilitates tau aggregation under oxidative stress conditions.

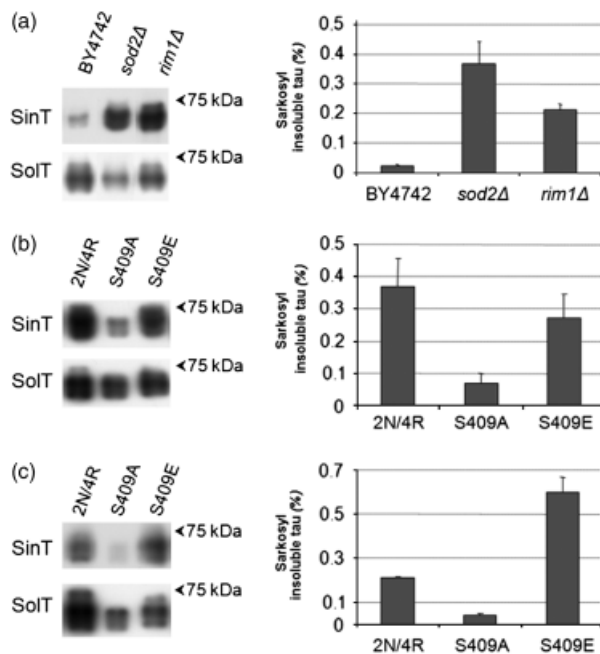
It has been shown previously that site-specific nitration and oxidation of tau influences its oligomerization and polymerization *in vitro* (Schweers *et al.*, 1995; Barghorn & Mandelkow, 2002; Landino *et al.*, 2004; Reynolds *et al.*, 2007). Especially, the oxidation of cysteine residues and the formation of intramolecular and intermolecular disulfide bridges were proposed as determining factors for PHF assembly because intramolecular bonds would lead to compacted monomers that do not assemble further, while intermolecular bonds would give rise to stabilized dimers and thereby accelerate further oligomerization (Schweers *et al.*, 1995; Barghorn & Mandelkow, 2002). To assess whether tau dimerization would explain the observed enhanced formation of SinT in yeast cells upon oxidative stress, we performed SDS-PAGE under nonreducing conditions of extracts prepared from wild-type cells. Immunodetection with the pan-antibody Tau5 revealed that in untreated cells, a significant fraction of the wild-type and mutant tau was indeed present as dimers with an approximate molecular weight of 130 kDa (Fig. 4f). In line with our data reported previously for the purification of recombinant tau from yeast strains (Vandebroek *et al.*, 2006), tau-2N/4R, tau-G272V, tau-N279K, tau-ΔK280 and tau-V337M produced markedly more dimers than tau-P301L and tau-R406. This was also the case for the pseudophosphorylated tau-S409E mutant when compared with its nonphosphorylated tau-S409A counterpart. Hence, it appears that dimer formation by protein tau is, at least in part, dependent on conformations determined by phosphorylation in the C-terminal domain. To our surprise, the treatment of the cells with ferrous ions reduced the level of tau dimers significantly and led to an oligomerization pattern characterized by a protein of tau species of approximately 120 kDa and the presence of higher-order tau oligomers. This pattern was also obvious with tau-P301L and tau-R406W and thus appeared to be independent of the capacity of tau to form dimers. Similar to that for SinT, the presence of higher-order tau oligomers was most pronounced with the synthetic tau-S409E mutant and least with the tau-S409A mutant, again indicating that phosphorylation at the PG5 epitope facilitates tau polymerization. Interestingly, we noticed a distinct band at around



**Fig. 4.** Sarkosyl insolubility of wild-type tau-2N/4R and mutant tau in yeast cells subjected to oxidative stress. (a, b) Bar diagrams showing the percentage of SinT obtained from transformed W303-1A wild-type cells (a) or *pho85Δ* cells (b) when grown in a medium without (open bars) or with (black bars) supplementation of 20 mM FeSO<sub>4</sub>. The analysis included wild-type tau-2N/4R and the FTDP-17 mutants tau-G272V, tau-N279K, tau-ΔK280, tau-P301L, tau-V337M and tau-R406W as indicated. The data are mean with SEM of three independent experiments. (c) Western blot analysis with the Mabs indicated of the total protein extracts isolated from wild-type and *pho85Δ* cells that express tau-2N/4R when grown in a medium without or with supplementation of 20 mM FeSO<sub>4</sub>. (d) Western blot analysis with Tau5 of the soluble (SolT) and sarkosyl-insoluble (SinT) fractions isolated from wild-type W303-1A cells that express wild-type tau-2N/4R, mutant tau-S409A or mutant tau-S409E when grown in a medium without or with 20 mM FeSO<sub>4</sub> (+Fe<sup>2+</sup>) supplementation as indicated. Note that more sarkosyl-insoluble extract was loaded to ensure immunodetection. (e) Quantified data of (d) represented as the mean with SEM of at least three independent experiments. Statistical relevance is described in the main text. (f) Western blot analysis with Tau5 of the total protein extracts separated by SDS-PAGE under nonreducing conditions. The analysis included extracts isolated from wild-type yeast cells that express tau-2N/4R, the FTDP-17 mutants tau-G272V, tau-N279K, tau-ΔK280, tau-P301L, tau-V337M and tau-R406W and the synthetic mutants tau-S409A and tau-S409E when grown in a medium without or with supplementation of 20 mM FeSO<sub>4</sub>. The arrow indicates the proteolytic tau fragment of 35 kDa.

35 kDa in each sample isolated from the iron-treated yeast cells, indicating that the stress treatment affected the breakdown or the clearance of tau in yeast.

Because oxidative stress eventually leads to mitochondrial dysfunction, we analyzed two mutants from the yeast genome-wide deletion collection, i.e. the *sod2Δ* strain that



**Fig. 5.** Aggregation of tau-2N/4R, tau-S409A and tau-S409E in mitochondrial yeast mutants. (a) Western blot analysis with Tau 5 (right) and the corresponding quantification (left) of soluble (SolT) and sarkosyl-insoluble (SinT) fractions isolated for wild-type tau-2N/4R expressed in BY4742 wild-type cells or the isogenic *sod2Δ* and *rim1Δ* mutants. (b, c) Western blot analysis with Tau 5 (right) and the corresponding quantification (left) of soluble (SolT) and sarkosyl-insoluble (SinT) fractions isolated from tau-2N/4R, tau-S409A or tau-S409E expressed in the *sod2Δ* (b) and *rim1Δ* mutants (c). Note that more sarkosyl-insoluble extract was loaded to ensure detection. The left panels show the corresponding quantified data. Quantified data are mean with SEM of at least three independent experiments. Statistical relevance is described in the main text.

lacks mitochondrial manganese-dependent superoxide dismutase activity (van Loon *et al.*, 1986) and the *rim1Δ* strain that lacks a single-stranded DNA-binding protein essential for mitochondrial genome maintenance (Van Dyck *et al.*, 1992). Compared with the isogenic wild-type BY4742 strain (Fig. 5a), SinT levels for tau-2N/4R were on average 16-fold higher in the *sod2Δ* strain ( $P < 0.001$ ) and 10-fold higher in the *rim1Δ* strain ( $P < 0.01$ ), which is similar to the increments observed upon ferrous sulfate treatment. Also, the increase in SinT for tau-S409A and for tau-S409E was within this range (compare Fig. 5b, c with Fig. 4d, e), again suggesting that the effects of tau-S409 phosphorylation and mitochondrial dysfunction on tau aggregation are synergistic. Note that in none of the experiments described above did we observe strong tau-related growth phenotypes.

## Discussion

An increasing number of mutations in the gene encoding protein tau are linked to the autosomal dominant-inherited

form of FTDP-17. Although these mutations have been described to affect tau-microtubuli interaction and tau aggregation, neither their mode of action *in vivo* nor how exactly the mutations lead to tau filament formation is known (Mandelkow *et al.*, 2003; Sergeant *et al.*, 2008; Gendron & Petrucelli, 2009; Iqbal *et al.*, 2009). Because all pathological aggregates of tau in the inheritable as well as in sporadic cases of tauopathy contain hyperphosphorylated tau isoforms, aberrant or increased phosphorylation is thought to be the necessary step for aggregation. We systematically and comparatively analyzed the biochemistry of six different clinical FTDP-17 tau mutants when expressed in W301-A wild-type yeast or in the isogenic mutant strains lacking Mds1 or Pho85, i.e. the orthologues of the tau kinases Gsk3 $\beta$  or cdk5, respectively. We focused on these kinases because they produce major pathogenic phospho-epitopes on tau as reported previously (Vandebroek *et al.*, 2005). However, note that other yeast kinases phosphorylate tau as well. One such example is the DYRK orthologue, Yak1, which phosphorylates tau at Y18 (data not shown).

In the strains examined, the mutant proteins tau-G272V, tau-N279K, tau- $\Delta$ K280 and tau-V337M displayed phosphorylation patterns similar to those of wild-type tau-2N/4R, while tau-P301L and tau-R406W deviated from this pattern. Particularly, tau-R406W was less phosphorylated at the AD2 epitope and both tau-P301L and tau-R406W displayed hampered phosphorylation at the PG5 epitope. The observed specific reduction in immunoreactivity of tau-R406W for AD2 is consistent with reports demonstrating decreased phosphorylation at S396/S404 of this mutant when phosphorylated *in vitro* with GSK3 $\beta$  and cdk5 (Connell *et al.*, 2001; Sakaue *et al.*, 2005), when expressed in different cell lines (Matsumura *et al.*, 1999; Perez *et al.*, 2000; DeTure *et al.*, 2002) and transgenic mice (Zhang *et al.*, 2004), or when injected in *Xenopus* oocytes (Delobel *et al.*, 2002). To our knowledge, the phosphorylation of S409 was previously not analyzed systematically in FTDP-17 mutants.

Mechanistically, the reduced AD2 reactivity of tau-R406W can be explained by the close proximity of the R406W mutation to the S396/S404 residues in the AD2 epitopes, i.e. the mutation can interfere directly with the recognition of these residues by GSK3 $\beta$  (Perez *et al.*, 2000; Li & Paudel, 2006; Tatebayashi *et al.*, 2006). Whether the R406W mutation directly interferes with the recognition of S409 to establish the PG5 epitope has not been studied, but our current data make this very likely because the tau-R406W failed to generate this epitope under conditions where other mutants displayed enhanced S409 phosphorylation. Furthermore, the formation of the AD2 and PG5 epitopes is interdependent, as demonstrated with the synthetic S409A and S409E mutants. This suggests that the phosphorylation of S409 (PG5 epitope) primes tau for or at least facilitates the subsequent phosphorylation of S396/

S404 (AD2 epitope). These observations are in line with data from the brain of AD patients, demonstrating that the formation of the PG5 epitope on tau is an early event in the pretangle stage and precedes the phosphorylation at S396, which is characteristic for NFT (Kimura *et al.*, 1996).

Besides by direct interference, mutations in tau apparently also affect phosphorylation of more distant residues, most likely by altering the conformation of tau. Evidence for this comes from our observation that the P301L mutation reduces phosphorylation of the PG5 epitope, although the mutation and phosphorylation site are separated by 107 amino acids. Recent studies show that even soluble tau can adopt the so-called 'paperclip' conformation, whereby the C-terminus folds over the microtubule-binding domain, with the N-terminus approaching the C-terminal domain (Jeganathan *et al.*, 2006). In that conformation, the C-terminus of tau is in close proximity of the P301 residue and the mutation can be envisaged to affect the paperclip conformation and affect the exposure and phosphorylation of S409. Similar long-distance effects may apply to various extents to other FTDP-17 mutations, explaining our observed inverse relation of PG5 immunoreactivity with the proximity of the mutation to the C-terminal end of protein tau.

Studies that combined FRET analysis with pseudophosphorylation of tau demonstrated that the paperclip conformation of tau became more compact when pseudophosphorylation sites were introduced both N- and C-terminally of the microtubule-binding region and that this generated the aggregation-prone MC1 conformation. Unfolding or opening of the paperclip conformation was shown to occur upon the imbalanced introduction of pseudophosphorylation sites, whereby pseudophosphorylation alone in the N-terminal moves this region away from the C-terminal region and, likewise, pseudophosphorylation alone in the C-terminal region moves this region away from the microtubule-binding repeats (Jeganathan *et al.*, 2008). Based on these data, we predicted that FTDP-17 mutants that solely affect phosphorylation in the C-terminal region would be less aggregated under normal physiological conditions. This was particularly the case for the tau-P301L and tau-R406W mutants because they combined hampered S409 phosphorylation with reduced immunoreactivity for MC1 and reduced SinT production. The clinical counterpart of this finding is reflected by the fact that despite the heterogeneity of clinical symptoms manifested by FTDP-17 mutations, most have an age of onset between 40 and 60 years. The exceptions are patients carrying the P301L and R406W mutations, in which the disease develops later, i.e. after 60 years of age (Spillantini *et al.*, 2000). In this respect, these genetic, primary tauopathies can be mechanistically compared, or even related, with the secondary tauopathy in sporadic AD patients.

Additional support for the importance of the PG5 epitope in determining the switch in the function of tau comes from our observation that tau isoforms lacking this epitope

preferably interact with prestabilized porcine microtubules. This extends our previous data on differential microtubule binding of phosphorylated tau isoforms produced in yeast (Vandebroek *et al.*, 2006) Consistent with the notion that FTDP-17 mutations compromise the ability of tau to regulate microtubule dynamics *in vitro* and *in cellulo* (Bunker *et al.*, 2006; Han *et al.*, 2009), we demonstrated previously that the binding of the tau-P301L mutant to stabilized porcine microtubules is less sensitive to phosphorylation than the wild-type protein. We then also showed that the mutant readily formed aggregates on the surface of these microtubules (Vandebroek *et al.*, 2006), a property similar to the inherent aggregation propensity of different tau mutants as seen in the diseased brain of tauopathy patients (Gendron & Petrucelli, 2009; Iqbal *et al.*, 2009) as well as in the brain of transgenic mice (Götz *et al.*, 2009). At first glance, the enhanced propensity to aggregate the tau-P301L mutant on prestabilized microtubules *in vitro* appears to be contradictory to our data, indicating that this mutant formed less SinT than wild-type tau-2N/4R in yeast cells under normal growth conditions. However, the conditions used for *in vitro* tau-microtubule binding do not compare with those *in cellulo*, where the reducing environment has important consequences on aggregation as discussed below. In addition, the binding of tau to microtubules was reported to facilitate its oligomerization (Makrides *et al.*, 2003), and this is known to be a driving force for subsequent PHF formation and aggregation (Sahara *et al.*, 2008). Despite considerable efforts, we were unable to demonstrate binding of tau to yeast microtubules. The reason for this might be simply because yeast microtubules differ from their mammalian counterparts by the lack of a typical taxol-binding site (Gupta *et al.*, 2003), which is known to partially overlap with the tau-binding site (Kar *et al.*, 2003). Therefore, SinT produced in our yeast cells is concluded to reflect the aggregation capacity of unbound soluble tau proteins in mammalian systems.

Increasing evidence indicates that oxidative damage and mitochondrial dysfunction significantly impact the development of neurodegenerative disorders, including AD and primary tauopathies (Melov *et al.*, 2007; Moreira *et al.*, 2008; Martinez *et al.*, 2009). We monitored the effect of oxidative stress and mitochondrial dysfunction on SinT formation of wild-type and mutant tau. Our data indicated that both conditions markedly increased tau insolubility through mechanisms that are not strictly dependent on hyperphosphorylation of tau, but rather act in parallel. In wild-type yeast cells, the increase in SinT upon oxidative stress was most pronounced for the FTDP-17 mutants, suggesting that the FTDP mutations render tau more vulnerable to oxidative stress. In the *pho85Δ* cells, the increment in SinT was less pronounced than that in wild-type cells; however, non-stressed *pho85Δ* cells already contained elevated levels of

insoluble tau, leaving less substrate with the proper conformation for oxidative stress to further induce aggregation. We addressed the question of whether the increased insolubility of tau upon oxidative stress coincided with the enhanced formation of tau dimers as suggested previously by *in vitro* experiments (Schweers *et al.*, 1995; Barghorn & Mandelkow, 2002; Landino *et al.*, 2004). The answer was apparently negative, because we observed a reduction in the level of tau dimers under conditions of oxidative stress. This was not due to a shift of preexisting tau dimers to higher-order oligomers because those FTDP mutants with low dimer formation, i.e. tau-P301L and tau-R406W, also readily formed higher oligomeric structures when challenged with ferrous ions. Although the underlying mechanisms remain to be identified, we noticed that oxidative stress led to the appearance of a distinct degradation product of protein tau of 35 kDa, indicative of altered processing or diminished clearance under this condition. Recent studies highlighted the importance of both ubiquitin-proteasome and autophagy-lysosome pathways for tau clearance and the generation of aggregation-prone tau fragments (Poppek *et al.*, 2006; Dickey *et al.*, 2007; Wang *et al.*, 2009). Consequently, we are interested in examining whether these systems cause oxidative stress-induced aggregation of tau in our model.

We further noticed that oxidative stress led to decreased phosphorylation of specific epitopes in tau-2N/4R in wild-type yeast cells and to a lesser extent in *pho85Δ* mutant cells. This is consistent with studies showing iron or peroxide ions to induce dephosphorylation of tau in primary neuronal cultures from embryonic rat and mouse brain, as well as in neuroblastoma cells (LoPresti & Konat, 2001; Zambrano *et al.*, 2004; Galas *et al.*, 2006). Those studies implicated the prolyl peptidyl isomerase, Pin1, to facilitate dephosphorylation of P-T231 in tau via PP2A (Galas *et al.*, 2006), and pointed to a role of the cdk5/p35 complex to inactivate inhibitor-2, which acts as a negative regulator of the phosphatase PP1 (Zambrano *et al.*, 2004). Both modulatory pathways are conserved in yeast. The Pin1 function is represented by the yeast orthologue Ess1 (Lu *et al.*, 1996), and our preliminary results, indeed, confirmed that disruption of the Ess1 activity leads to increased hyperphosphorylation of tau (data not shown). The cdk5/p35 complex is functionally equivalent to the yeast Pho85/Plc6,7 complex, which phosphorylates Glc8, the orthologue of mammalian inhibitor-2, thereby controlling the activity of the Glc7 phosphatase, the orthologue of mammalian PP1 (Tan *et al.*, 2003). Hence, it appears that similar mechanisms may govern oxidative stress-induced tau dephosphorylation in yeast and mammalian cells. Notably, the requirement of Pho85 for the activation of PP1 may explain why the oxidative stress-induced dephosphorylation of tau is less extensive in *pho85Δ* cells as compared with wild-type cells. Whether dephosphorylation of tau is an essential step for

tau aggregation upon oxidative stress remains to be clarified. Tau apparently regains its hyperphosphorylation status upon prolonged stress exposure (LoPresti & Konat, 2001) and this seems to involve specific stress-responsive kinases (Tremblay *et al.*, 2009; Su *et al.*, 2010). Interestingly, hyperphosphorylated tau was shown to be a poor substrate for proteasomal degradation (Poppek *et al.*, 2006; Dickey *et al.*, 2007). Therefore, it is feasible that the observed dephosphorylation of tau during oxidative stress reflects an attempt of the cells to improve the clearance of aggregation-prone tau proteins.

Oxidative stress and mitochondrial integrity are closely linked to the life span of yeast cells (Laun *et al.*, 2001; Kaeberlein *et al.*, 2007), and our data thereby relate the aggregation of tau to the problem of aging, which remains the major risk factor in AD and other sporadic tauopathies. However, we did not observe strong tau-related growth phenotypes in fermenting yeast cells treated with ferrous ions or suffering from mitochondrial dysfunctions despite higher SinT levels. Obviously, aggregation of tau is not *per se* correlated with toxicity, a conclusion that corroborates directly findings in mammalian systems and the human brain (Santacruz *et al.*, 2005; Castellani *et al.*, 2008; Terwel *et al.*, 2008). The assembly of tau into PHF and NFT must then be considered as a mechanism that converts hyperphosphorylated tau into rather inert polymers to protect or even act as a buffer against oxidative damage (Castellani *et al.*, 2008; Moreira *et al.*, 2008; Jaworski *et al.*, 2010).

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## Authors' contribution

T.Vh. and T.Vdb. contributed equally to this work.

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