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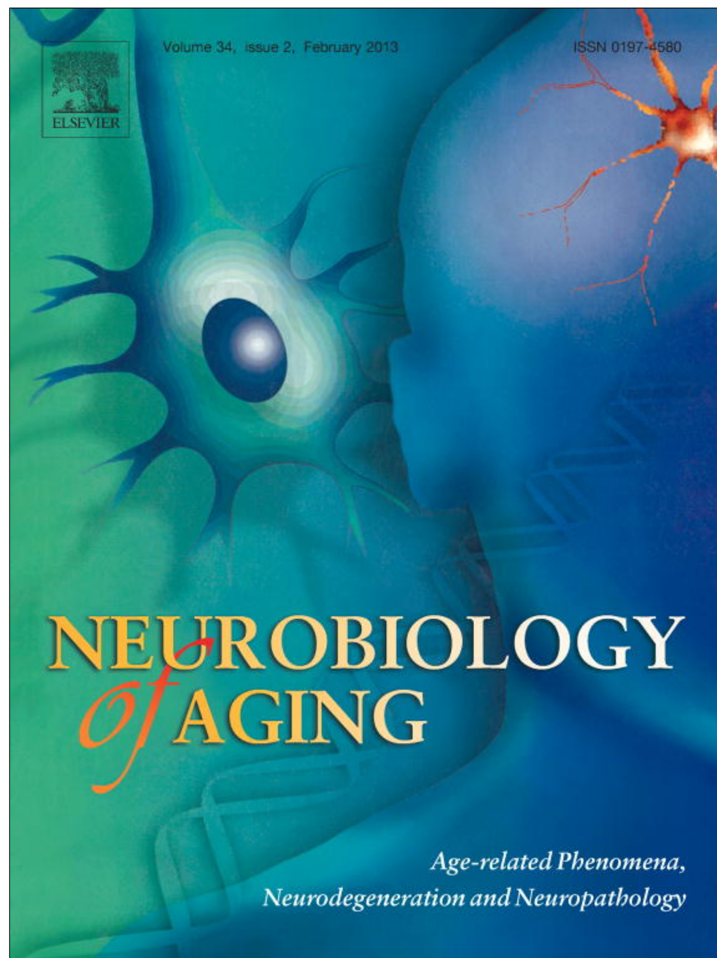

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p73 haploinsufficiency causes tau hyperphosphorylation and tau kinase dysregulation in mouse models of aging and Alzheimer's disease

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Abstract

Haploinsufficiency for the p53 family member p73 causes behavioral and neuroanatomical correlates of neurodegeneration in aging mice, including the appearance of aberrant phospho-tau-positive aggregates. Here, we show that these aggregates and tau hyperphosphorylation, as well as a generalized dysregulation of the tau kinases GSK3β, c-Abl, and Cdk5, occur in the brains of aged p73+/− mice. To investigate whether p73 haploinsufficiency therefore represents a general risk factor for tau hyperphosphorylation during neurodegeneration, we crossed the p73+/− mice with 2 mouse models of neurodegeneration, TgCRND8+/Ø mice that express human mutant amyloid precursor protein, and Pin1−/− mice. We show that haploinsufficiency for p73 leads to the early appearance of phospho-tau-positive aggregates, tau hyperphosphorylation, and activation of GSK3β, c-Abl, and Cdk5 in the brains of both of these mouse models. Moreover, p73+/−; TgCRND8+/Ø mice display a shortened lifespan relative to TgCRND8+/Ø mice that are wild type for p73. Thus, p73 is required to protect the murine brain from tau hyperphosphorylation during aging and degeneration.
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Keywords: p73; p53 family; Tau phosphorylation; Pin1; Alzheimer's disease; Aging; Neurodegeneration; GSK-3β; c-Abl; Cdk5; Tauopathy; Paired helical filaments; TgCRND8

1. Introduction

The p53 family members are transcription factors with important cellular functions such as apoptosis, survival, cell cycle, cell arrest, differentiation, and senescence. The family consists of 3 members, p53, p63, and p73, which encode multiple isoforms including or lacking the major transcriptional transactivation domain (reviewed in McKeon and Melino, 2007). In the developing nervous system, p53 regulates naturally occurring cell death and stem cell maintenance, and protection from genotoxic stress (reviewed in Jacobs et al., 2006). We recently explored the function of this family in nervous system aging, degeneration, and postnatal stem cell function and found that p73 in particular plays salient roles. In stem cells, the transactivating (TA) form of p73 is required for neural stem cell renewal (Agostini et al., 2010; Fujitani et al., 2010; Talos et al., 2010; Tomasini et al., 2008) while the truncated (ΔN) form lacking the major transactivating domain is necessary for the survival and maintenance of many peripheral nervous system (PNS) and central nervous system (CNS) neurons (Lee et al., 2004; Pozniak et al., 2000, 2002; Tissir et al., 2009; Walsh et al., 2004; Wilhelm et al., 2010; Yang et al., 2000). Intriguingly, haploinsufficiency for p73 in aged but not young mice resulted in neuronal loss, increased microglial activation, aberrant cell cycle re-entry, lipofuscin accumulation, and motor and hippocampal-associated behavioral impairments (Wetzel et al., 2008). Surprisingly, in aged p73 mice, we also observed increases in phospho-tau-positive

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aggregates (Wetzel et al., 2008), a common feature in several neurodegenerative diseases and aging (Ballatore et al., 2007). Moreover, when p73+/− mice were crossed with TgCRND8+/Ø mice, a model of Alzheimer's disease that results in plaques but not neuronal loss (Chishti et al., 2001), the brains of these animals showed neuronal death and tau phosphorylation coincident with plaque formation (Wetzel et al., 2008). Together these findings implicated p73 as a major determinant of neuronal survival, aging, and degeneration.

Several questions were raised by these findings that we have addressed here. First, were the phospho-tau-positive aggregates observed in aged p73+/− mice and in p73+/−;TgCRND8+/Ø mice by immunohistochemistry an indication of a general increase in tau phosphorylation in the CNS? Second, how does haploinsufficiency for p73 cause these abnormal phospho-tau aggregates? Our previous work showed that in aged p73+/− and p73+/− mice crossed with the TgCRND8 mice, Jun N-terminal protein kinase (JNK) activity was enhanced (Wetzel et al., 2008). While JNK is one tau kinase, GSK3β and Cdk5 are thought to be the major kinases associated with the pathogenesis of Alzheimer's disease, playing roles in amyloid precursor protein (APP) processing, synaptic dysfunctions, behavioral impairments, neuronal death, and tau phosphorylation (Ballatore et al., 2007; Hooper et al., 2008; Lee et al., 2001; Su and Tsai, 2011). In this regard, c-Abl is another kinase that is known to regulate Cdk5 and that has been recently associated with tau phosphorylation (Cancino et al., 2008, 2011; Derkinderen et al., 2005; Lee et al., 2008; Lin et al., 2007; Zukerberg et al., 2000). We therefore asked whether these 3 kinases were dysregulated in p73+/− mice. Third, is p73 haploinsufficiency a general predisposition factor for neurodegenerative cues? To answer this question, we used a second mouse model of neurodegeneration, Pin1−/− mice (Liou et al., 2003). Pin1 is a peptidyl-prolyl cis/trans isomerase that shows reduced expression levels in human Alzheimer's disease (AD) brains (Sultana et al., 2006). Because Pin1−/− mice exhibit abnormal APP processing (Pastorino et al., 2006), motor and behavioral deficits, tau hyperphosphorylation, tau filament formation, and neuronal degeneration when they are over 9 months of age (Liou et al., 2003; Lu and Zhou, 2007; Pastorino et al., 2006), it has been proposed as an Alzheimer's disease model. We therefore asked whether p73 haploinsufficiency results in the early appearance of hyperphosphorylated tau in Pin1−/− mice, as it does in the TgCRND8 AD mouse model.

Here, we show that haploinsufficiency for p73 leads to aberrant tau hyperphosphorylation and tau kinase activation in the aged murine brain, and causes early phospho-tau accumulation and tau kinase dysregulation in the TgCRND8 and Pin1−/− neurodegenerative models. Thus, p73 is an important neuroprotective gene in the aged or degenerating mouse brain.

2. Materials and methods

2.1. Animals

This study was approved by the Hospital for Sick Children Animal Care Committee, in accordance with Canadian Council on Animal Care guidelines. p73+/− transgenic mice (Yang et al., 2000) were maintained on a C129SvJae background as described (Pozniak et al., 2000, 2002; Wetzel et al., 2008). Alternatively, the p73+/− mice were crossed into the C57/Bl6 background for more than 10 generations, and maintained in that background. TgCRND8 hemizygous mice (Chishti et al., 2001) containing the Swedish (K670N/M671L) and Indiana (V717F) APP mutations were maintained in the C129 background as crosses with the p73+/− mice. Pin1−/− mice (Liou et al., 2003) were originally in a C57/Bl6 background, were then crossed to the p73+/− mice in the C129SvJae background and were subsequently maintained and analyzed in this mixed background. For the lifespan studies, only littermates were included.

2.2. Neuroanatomy

For histology, mice were sacrificed by sodium pentobarbital and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brains were cryoprotected and sectioned at 18 μm. In some experiments, animals were perfused, and one brain hemisphere was cryoprotected and sectioned while the other half was prepared as paraffin sections by the Toronto Centre for Phenogenomics Pathology Facility. Prior to immunocytochemistry, these paraffin sections were deparaffinized with xylene, and hydrated with ethanol. Some sections were also treated for antigen retrieval using 10 mM citrate buffer; similar results were obtained with and without retrieval. For both cryosectioned and deparaffinized sections, immunocytochemistry was performed as described (Cancino et al., 2008). Briefly, sections were washed with Tris-buffered saline (TBS) buffer, permeabilized with TBS, 0.3% Triton X-100 solution, and then incubated in TBS, 5% bovine serum albumin (BSA), 0.3% Triton X-100 for 1 hour as a blocking solution. Slides were incubated with primary antibodies in blocking solution at 4°C overnight. After TBS washes, the sections were incubated with secondary antibodies in blocking solution for 1 hour at room temperature. Finally, after TBS washes, sections were mounted in Permount solution (Thermo, Waltham, MA, USA). Digital image acquisition was performed with AxioVision (version 4.8.2) software (Zeiss, Oberkochen, Germany) on a Zeiss Axioplan 2 microscope with a Hamamatsu (Bridgewater, NJ, USA) orca-R^2 CCD video camera.

2.3. Western blot analysis and densitometry

Extracts from adult hippocampi were prepared in ice cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM ethylene glycol tet-

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raacetic acid (EGTA), 1% NP-40, 1 mM NaF, and 1 mM NaVO3). Protein was quantified by the BCA Kit (Thermo), and 20 μg of protein lysate was run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and Western blot analyses were performed as described (Cancino et al., 2011). The following antibodies were used: anti-c-Abl (K12, 1:1000), anti-Cdk5 (C-8, 1:500), anti-phospho-Cdk5 (Tyr15, 1:250), anti-GSK3β (H-76, 1:100) polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-phospho-c-Abl (Tyr412, 1:1000; Sigma, St. Louis, MO, USA); anti-phospho-GSK3β (Ser9, 1:500, Cell Signaling Technology, Danvers, MA, USA); anti-phosphorylated tau antibodies: AT8 (recognizing Ser-199- and Ser-202-phosphorylated tau, 1:500; Thermo), AT100 (recognizing Ser-212- and Thr-214-phosphorylated tau, 1:500; Thermo), AT180 (recognizing Thr-231- and Ser-235-phosphorylated tau, 1:500; Thermo), anti-pS422 (recognizing Ser-422-phosphorylated tau, 1:1000; Invitrogen, Carlsbad, CA, USA), PHF1 (recognizing Ser-396- and Ser-404-phosphorylated tau, 1:1000), and Tau5 antibody that recognizes total tau protein (total tau, 1:1000; Invitrogen). Secondary horseradish peroxidase (HRP)-conjugated antibody (1:5000) was obtained from Thermo. ImageJ (NIH, Bethesda, MA, USA) software (version 1.45) was used for scanning and quantitative analysis of Western blots.

2.4. Sarkosyl insolubility assay

Purification of sarkosyl-soluble and -insoluble tau was performed as previously described (de Calignon et al., 2012; Goedert et al., 1992; Wetzel et al., 2008) with modifications. Briefly, whole brains of 2-month-old p73+⁄+, p73+/−, p73+⁄+;TgCRND8+⁄Ø, and p73+⁄−;TgCRND8+/Ø mice were homogenized in 10 volumes of buffer H (10 mM Tris-HCl (pH 7.5), 0.8 M NaCl, 1 mM EGTA, and 1 mM dithiothreitol) and spun at 55,000*g* for 1 hour at 4 °C. Another 2 mL of buffer H was added to the pellet and samples were incubated in buffer H-1% Triton X-100 at 37 °C for 30 minutes. After the incubation, the samples were spun at 55,000*g* for 1 hour at 4 °C, the pellet was homogenized and incubated in 1% sarkosyl at 37 °C for 30 minutes and spun at 55,000*g* for 1 hour at 4 °C. The supernatant was collected as the sarkosyl-soluble fraction. Sarkosyl-insoluble pellets were extracted in buffer H. Protein concentration was determined by bicinchoninic acid (BCA) assay (Thermo) and 5 μg of sample was loaded onto 4%–20% Tris/Glicine SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with PHF1, pS422, and β-tubulin antibodies.

2.5. Statistical analysis

Statistics were performed using 2-tailed Student *t*-test for the Western blot analysis of the aging p73+⁄− versus p73+⁄+ brains. For quantitative analysis of the Western blots with the TgCRND8 hemizygous mice and Pin1−⁄− mice that were homozygous or heterozygous for p73, one-way analysis of variance (ANOVA) with Newman-Keuls post hoc test was performed. For the Kaplan-Meyer survival curves, the log-rank (Mantel-Cox) test was performed using Prism 5 (GraphPad, La Jolla, CA, USA). In all cases, error bars indicate standard error of the mean.

3. Results

3.1. Haploinsufficiency for p73 leads to aberrant tau hyperphosphorylation and tau kinase activation in the aged brain

We previously reported that in the brains of aged p73 haploinsufficient mice on a C129 genetic background, tau is hyperphosphorylated as assessed by immunocytochemistry (Wetzel et al., 2008). We initially confirmed these findings by immunostaining coronal sections through the brains of 18-month-old p73+⁄− versus p73+⁄+ mice using the tau phospho-specific antibodies AT8, AT100, and AT180, all of which recognize different phosphorylated epitopes. As we previously showed, aberrant phospho-tau-immunoreactive aggregates were present in the forebrain and hippocampus of these mice, as detected with all 3 antibodies (Fig. 1A). This analysis also revealed an increase in the levels of phospho-tau-immunoreactivity in neuronal cell bodies in the hippocampus, again as assessed with all 3 antibodies (Fig. 1B). Having confirmed our previous results, we asked whether this was specific to the C129 background; p73+⁄− mice were backcrossed into a C57/Bl6 background, and brains of 18-month-old mice analyzed. Immunostaining demonstrated that aberrant phospho-tau-immunoreactive aggregates were also seen in the C57/Bl6 background (Fig. 1C), arguing that this phospho-tau-positive aggregate formation was a robust p73-dependent aging phenotype.

To ask whether this immunostaining reflected a general increase in neural tau phosphorylation, we performed Western blot analysis with the same 3 phospho-tau antibodies as well as PHF1 and pS442 antibodies to phosphorylated tau on hippocampal lysates prepared from 18-month-old p73+/+ and p73+/− mice in the C129 background. As a control, we probed the same lysates with an antibody against total tau protein, the Tau5 antibody. This analysis demonstrated an increase in tau phosphorylation as detected by all 3 antibodies (Fig. 1D). Quantification by scanning densitometry of phosphorylated tau relative to total tau demonstrated a robust and significant increase in tau phosphorylation in hippocampi of aged p73+⁄− mice as compared with their wild type counterparts (Fig. 1E).

These data indicate that in aged mice, p73 haploinsufficiency causes tau to be aberrantly phosphorylated at multiple sites. In this regard, we previously identified JNK as one tau kinase that is dysregulated and hyperactivated in the brains of aged p73+⁄− mice (Wetzel et al., 2008). To ask if tau kinase dysregulation is a common feature of p73

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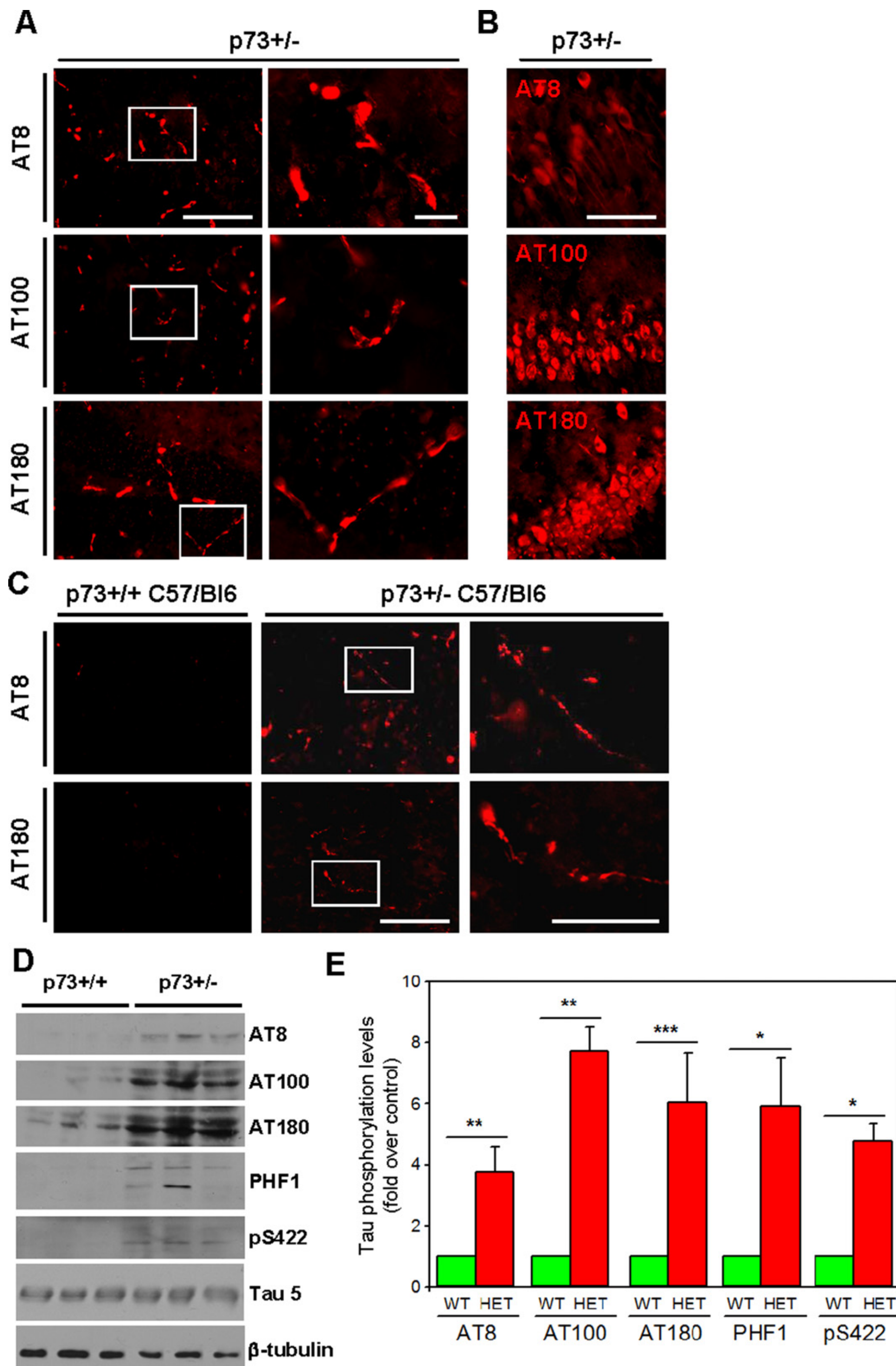

Fig. 1. p73 haploinsufficiency causes tau hyperphosphorylation in the aging mouse brain. (A) Micrographs of phospho-tau-positive aggregates in the hippocampus of 18-month-old p73+/− mice in a C129 background, as detected by immunostaining of paraformaldehyde-fixed cryostat sections with three different phospho-tau antibodies, AT8, AT100, and AT180. The boxed areas in the left panels are shown at higher magnification in the right panels. Scale bars = 50 and 10 μm in the left and right panels, respectively. (B) Micrographs showing phospho-tau immunoreactivity in the cell bodies of CA1 hippocampal neurons in sections of 18-month-old p73+/− hippocampi immunostained with AT8, AT100, and AT180 antibodies. Scale bar = 50 μm. (C) Immunostaining as in (A) of sections through the hippocampus of 18-month-old p73+/+ versus p73+/− mice in a C57/Bl6 background. The boxed areas

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heterozygosity, we focused on GSK3β, Cdk5, and c-Abl, tau kinases that are associated with the formation of paired helical filaments in Alzheimer's disease and tauopathies (Ballatore et al., 2007). We performed Western blot analyses with hippocampal lysates of 18-month-old p73+/− versus p73+/+ mice using the phosphorylation and activation-specific antibodies, anti-phospho-GSK3β (ser9; phosphorylation at this site is downregulated when GSK3β is activated), phospho-Cdk5 (Tyr15), and phospho-c-Abl (Tyr412). This analysis demonstrated that these 3 tau kinases were all activated in the p73+/− versus p73+/+ hippocampi. Specifically, the level of GSK3β ser9 phosphorylation was decreased, while the levels of tyrosine phosphorylation of c-Abl and Cdk5 were both increased in p73+/− lysates (Fig. 2A). Analysis of the same lysates with antibodies specific for total kinase protein levels, combined with quantification by scanning densitometry confirmed these changes (Fig. 2B). Thus, p73 haploinsufficiency leads to tau hyperphosphorylation and tau kinase dysregulation in the brains of aging mice.

3.2. p73 haploinsufficiency causes early phospho-tau accumulation, tau kinase dysregulation, and decreased lifespan in the TgCRND8 murine model of Alzheimer's disease

To ask if p73 haploinsufficiency causes tau kinase dysregulation during neurodegeneration as well as aging, we turned to the TgCRND8 mouse model, where human APP containing the Swedish and Indian mutations is overexpressed in neurons, leading to development of plaques but not phospho-tau-positive aggregates or neuronal loss in the first few postnatal months (Bellucci et al., 2007; Chishti et al., 2001). We previously crossed these mice with the p73+/− mice and showed that p73 heterozygosity led to neuronal loss and the appearance of phospho-tau-positive aggregates at 1.5–2 months of age, when plaques appeared in these mice (Wetzel et al., 2008). We initially repeated these results by immunostaining cryostat sections from 1.5-month-old TgCRND8+/Ø mice that were either wild type or heterozygous for p73 with the phospho-tau-specific antibodies AT8, AT100, and AT180. As previously reported (Wetzel et al., 2008), the TgCRND8+/Ø mice that were heterozygous but not wild type for p73 displayed aberrant phospho-tau-positive aggregates in the hippocampus and cortex at this age (Fig. 3A; Supplementary Fig. 1 for anti-Tau5 control). These phospho-tau-positive murine aggregates were readily detected in fixed cryostat sections, but were largely undetectable in paraffin sections from the same animals (Supplementary Fig. 2).

Because the phospho-tau-positive aggregates could only be visualized in fixed cryostat sections, we asked whether this immunostaining accurately reflected an increase in the levels of phosphorylated tau by Western blot analysis of lysates from hippocampi of 1.5-month-old TgCRND8+/Ø mice in either the p73+/+ or p73+/− backgrounds. As controls, we used p73+/+ mice of the same age. This analysis demonstrated a robust increase in phosphorylated tau as detected with the AT8, AT100, PHF1, and pS422 antibodies, and a smaller increase with the AT180 antibody (Fig. 3B). Quantification by scanning densitometry revealed that these increases were significant relative to levels of total tau or β-tubulin proteins in the same samples (Fig. 3C). The increase in phosphorylated tau in 2-month-old p73+/−;TgCRND8+/Ø mice as compared with p73+/+;TgCRND8+/Ø mice was also evident in sarkosyl-insoluble fractions prepared from whole brains and probed in Western blot analyses with anti-PHF1 or pS422 (Supplementary Fig. 3).

We next asked whether tau kinases were dysregulated in the brains of these crosses, as they were in the aged p73+/− mice. Lysates from hippocampi of 1.5-month-old TgCRND8+/Ø mice in either the p73+/+ or p73+/− backgrounds were probed on Western blot analyses with antibodies for GSK3β phosphorylated on serine 9, c-Abl phosphorylated on tyrosine 412, and Cdk5 phosphorylated on tyrosine 15. The same lysates were also probed for total levels of these 3 kinases, and the levels of protein phosphorylation were normalized to total kinase levels by scanning densitometry (Fig. 4A and B). This analysis demonstrated that regulation of these 3 tau kinases differed with regard to genotype. For GSK3β, a change in ser9 phosphorylation was observed in TgCRND8+/Ø hippocampi, irrespective of the p73 genotype. In contrast, for both c-Abl and Cdk5, relative levels of activation were similar between the control and p73+/+;TgCRND8+/Ø mice, but were significantly increased in hippocampi of p73+/−;TgCRND8+/Ø mice. To define the cell type responsible for these increases, we immunostained coronal cryostat sections of the hippocampus. This analysis showed increased phospho-c-Abl and phospho-Cdk5 immunoreactivity in hippocampal neurons of p73+/−;TgCRND8+/Ø hippocampi relative to p73+/+;TgCRND8+/Ø mice (Fig. 4C).

While we were performing these studies, we noted that the p73+/−;TgCRND8+/Ø mice apparently lived shorter lives than did the p73+/+;TgCRND8+/Ø mice.

in the middle panels are shown at higher magnification in the right panels. Note that the phospho-tau-positive aggregates are only present in the brains of aged p73 heterozygous mice. Scale bars = 50 and 25 μm in the middle and right panels, respectively. (D) Western blot analysis of equal amounts of protein from hippocampal lysates of 18-month-old p73+/+ and p73+/− mice in the C129 background, probed with AT8, AT100, AT180, PHF1, or pS422 antibodies to detect phosphorylated tau, or with an antibody against total tau (Tau5) or β-tubulin. Each lane derives from an independent animal. (E) Quantification by scanning densitometry of the relative level of tau phosphorylation/total tau in Western blot analysis similar to that seen in (D) ($n = 3$ for each sample; $^{**}p < 0.01$, $^{***}p < 0.001$).

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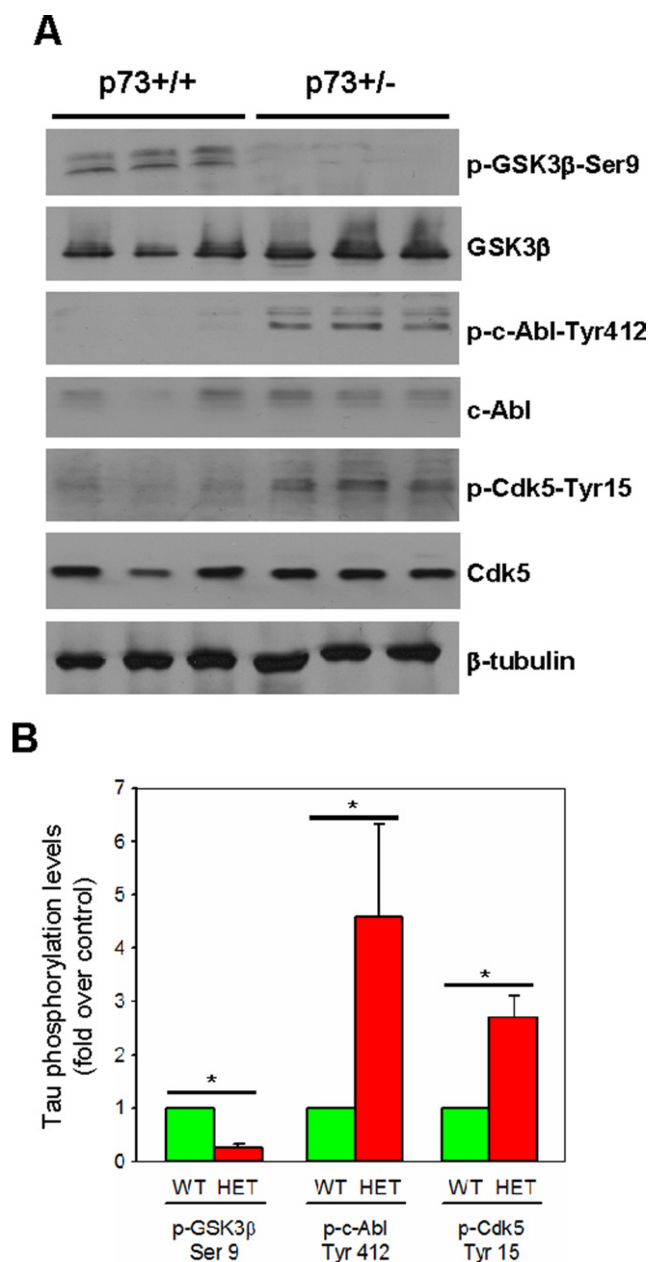

Fig. 2. p73 haploinsufficiency causes tau kinase dysregulation in the aging mouse brain. (A) Western blot analysis of equal amounts of protein from hippocampal lysates of 18-month-old p73+/+ and p73+/− mice in the C129 background, probed with antibodies for GSK3β phosphorylated at serine 9, c-Abl phosphorylated at tyrosine 412, or Cdk5 phosphorylated at tyrosine 15. To obtain an indication of the relative levels of kinase activation, the same lysates were analyzed on Western blot using antibodies that recognized total GSK3β, c-Abl, or Cdk5. The same lysates were also analyzed for levels of β-tubulin as a control for equal loading. Note that for GSK3β, ser9 phosphorylation is decreased when the kinase is activated, while for c-Abl and Cdk5, tyrosine phosphorylation is increased when the kinases are activated. Each lane derives from an independent animal. (B) Quantification of the relative levels of GSK3β, c-Abl, and Cdk5 phosphorylation as assessed by scanning densitometry of Western blots similar to those seen in (A) (n = 3 for each sample; $*p < 0.05$).

We therefore performed Kaplan-Meyer survival curves comparing littermates of these 2 genotypes (Fig. 4D). This analysis demonstrated that the p73+/+;TgCRND8+/Ø mice lived, at most, 11 months, with a median lifespan of 7.5 months. In contrast, the p73+/−;TgCRND8+/Ø mice lived to only 6 months of age, with a median lifespan of 4 months. Because both p73+/+ and p73+/− mice lived past the 14 month time frame of the experiment (Fig. 4D, data not shown), then these findings indicate that p73 haploinsufficiency predisposes the TgCRND8 mice to a decreased lifespan as well as to premature development of phospho-tau-positive aggregates in their brains.

3.3. Haploinsufficiency for p73 causes early phospho-tau accumulation and tau kinase dysregulation in a second neurodegenerative model, the Pin1−/− mice

These data indicate that p73 haploinsufficiency accelerates tau kinase dysregulation and thus formation of phospho-tau-positive aggregates in a neurodegenerative mouse model where mutant human APP is expressed. We therefore asked whether p73 haploinsufficiency would confer a similar accelerated phenotype on a second model of neurodegeneration, the Pin1−/− mice. Pin1 is a peptidyl-prolyl cis/trans isomerase whose loss of function has been associated with aberrant APP processing, amyloid-β production and, importantly for this study, with pathological tau phosphorylation (Liou et al., 2003; Lu and Zhou, 2007; Pastorino et al., 2006). Thus, Pin1−/− mice present with progressive age-dependent degeneration so that by 9 months of age, they display motor and behavioral deficits, tau hyperphosphorylation, tau filament formation, and neuronal degeneration. We first confirmed that, in 9-month-old Pin1−/− mice, phospho-tau-positive aggregates were present, as reported (Liou et al., 2003). To do this, we immunostained coronal cryostat sections through the forebrain of these mice with the AT8, AT100, and AT180 phospho-tau-specific antibodies, comparing them with control Pin1+/+ mice and to 9 month old p73+/− mice, where phospho-tau-positive aggregates are not yet detectable (Wetzel et al., 2008). This analysis demonstrated the presence of phospho-tau-positive aggregates as detected by all 3 antibodies in the cortex and hippocampus of the 9-month-old Pin1−/− mice, but not the 9-month-old p73+/− mice (Fig. 5A), consistent with previous reports (Liou et al., 2003). Interestingly, these aggregates were very similar in appearance to those seen in the 18-month-old p73+/− and the 1.5-month-old p73+/−; TgCRND8+/Ø brains.

To determine whether p73 heterozygosity accelerated the appearance of these phospho-tau-positive aggregates in a Pin1−/− background, we crossed these mice, and at 3 months of age immunostained coronal cryostat sections with the 3 phospho-tau antibodies. No phospho-tau aggregates were seen in Pin1−/− mice that were wild type for p73 (Fig. 5B), consistent with the lack of aggregates in Pin1−/− mice at this age (Liou et al., 2003). In contrast,

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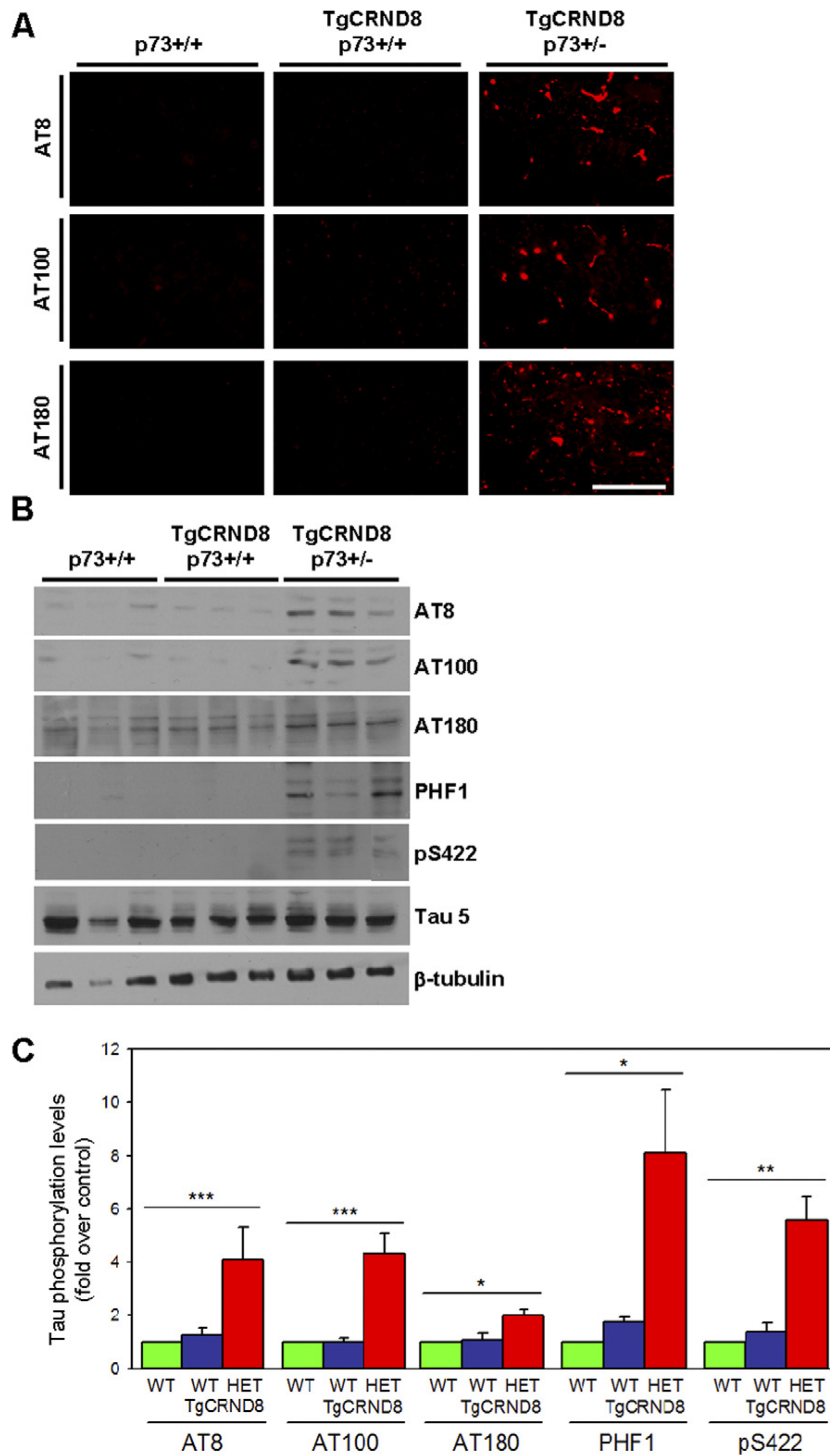

Fig. 3. p73 haploinsufficiency causes early tau hyperphosphorylation in the brains of TgCRND8 mice. (A) Micrographs of phospho-tau-positive aggregates in the cortex of 1.5-month-old p73+/+ mice, p73+/+;TgCRND8+/Ø mice, or p73+/−;TgCRND8+/Ø mice, all in a C129 background, as detected by immunostaining of paraformaldehyde-fixed cryostat sections with 3 different phospho-tau antibodies, AT8, AT100, and AT180. Note that only mice that are heterozygous for p73 and hemizygous for the TgCRND8 transgene display the phospho-tau-positive aggregates at this age. Scale bar = 50 μm. (B) Western blot analysis of equal amounts of protein from hippocampal lysates of 1.5-month-old p73+/+ mice, p73+/+;TgCRND8+/Ø mice, or p73+/−; TgCRND8+/Ø mice, all in the C129 background, probed with AT8, AT100, AT180, PHF1, or pS422 antibodies to detect phosphorylated tau, or with an antibody against total tau (Tau5) or β-tubulin. Each lane derives from an independent animal. (C) Quantification by scanning densitometry of the relative level of tau phosphorylation/total tau (n = 3 for each sample; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

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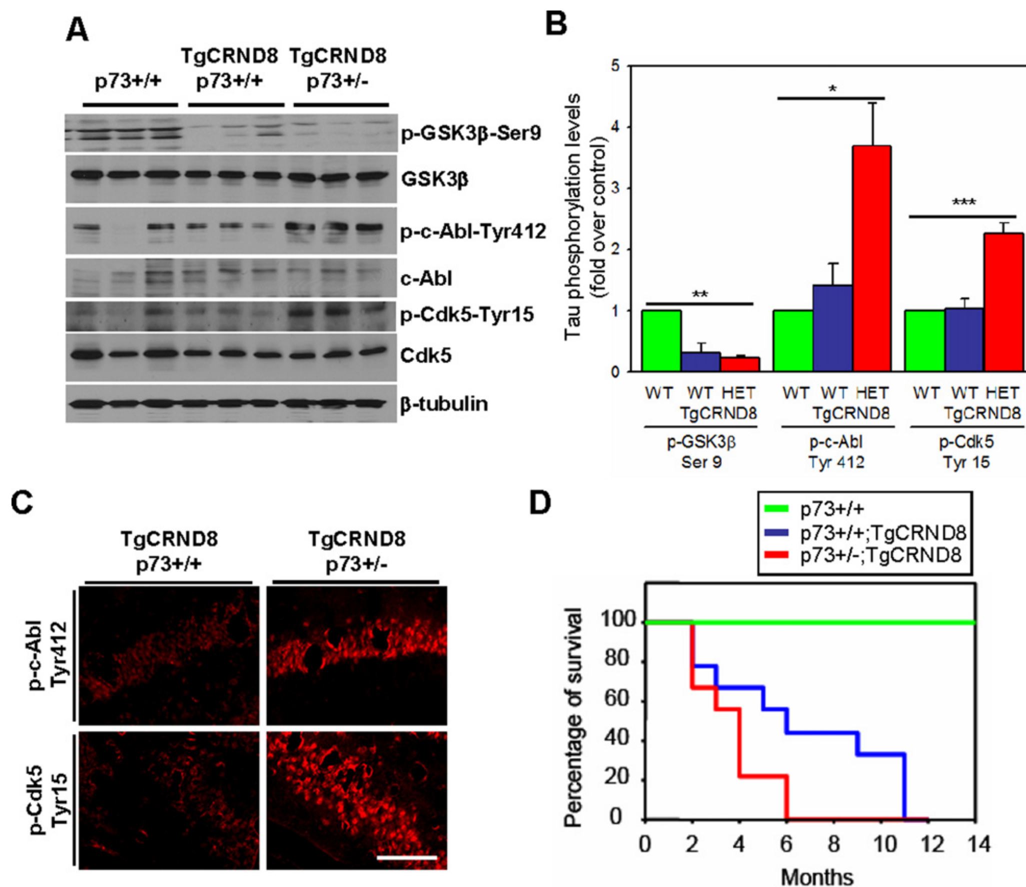

Fig. 4. p73 haploinsufficiency causes tau kinase dysregulation in the 1.5-month-old TgCRND8 mouse brain. (A) Western blot analysis of equal amounts of protein from hippocampal lysates of 1.5-month-old p73+/+ mice, p73+⁄+;TgCRND8+⁄Ø mice, or p73+/−;TgCRND8+⁄Ø mice all in the C129 background, probed with antibodies for GSK3β phosphorylated at serine 9, c-Abl phosphorylated at tyrosine 412, or Cdk5 phosphorylated at tyrosine 15. To obtain an indication of the relative levels of kinase activation, the same lysates were analyzed on Western blot using antibodies that recognized total GSK3β, c-Abl, or Cdk5. The same lysates were also analyzed for levels of β-tubulin as a control for equal loading. (B) Quantification of the relative levels of GSK3β, c-Abl, and Cdk5 phosphorylation as assessed by scanning densitometry of Western blots similar to those seen in (A) ($n = 3$ for each sample; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). (C) Micrographs showing phospho-tau immunoreactivity in the cell bodies of CA1 hippocampal neurons in sections of 1.5-month-old p73+/−;TgCRND8+/Ø hippocampi immunostained with phosphorylation-specific antibodies for c-Abl and Cdk5. Scale bar = 100 μm. (D) Kaplan-Meyer survival curves of p73+/+ mice (green line), p73+/+;TgCRND8+⁄Ø mice (blue line), and p73+/−;TgCRND8+/Ø mice (red line), all in the C129 background ($n = 16$ for each genotype; $**p = 0.0057$).

phospho-tau-positive aggregates were observed with all 3 antibodies in the brains of p73+/−;Pin1−/− mice (Fig. 5B), indicating that p73 haploinsufficiency accelerated their development. To confirm these findings, we performed Western blot analysis on hippocampal lysates from 3-month-old p73+/−;Pin1−/− versus p73+/+;Pin1−/− mice with the AT8 and AT100 antibodies. For comparison, we also analyzed lysates from 3-month-old wild type mice, and from 9-month-old Pin1−/− mice. As expected, 9-month-old Pin1−/− mice displayed higher phospho-tau levels than did 3-month-old wild type mice as detected with the AT8, AT100, PHF1, and pS422 antibodies (Fig. 5C and D). Consistent with previous data, and with our immunostaining, levels of phospho-tau in 3-month-old p73+/+;Pin1−/− mice were similar to those seen in wild type (p73+/+;Pin1+/+) mice. In contrast, levels of phospho-tau were significantly increased in hippocampi of 3-month-old p73+/−;Pin1−/− mice relative to wild types, and were similar to the 9-month-old Pin1−/− mice (Fig. 5C and D).

These results demonstrate that, as seen in the TgCRND8 model, p73 haploinsufficiency accelerated the development of aberrant phospho-tau-positive aggregates in the Pin1−⁄− mice. To ask whether this was due to dysregulation of tau kinases, we performed Western blot analyses using phosphorylation-specific antibodies for GSK3β, c-Abl, and Cdk5, comparing hippocampal lysates from the same groups that were analyzed immunocytochemically. As seen for phospho-tau, the relative levels of phospho-GSK3β, phospho-c-Abl, and phospho-Cdk5 were all altered in 9-month-old Pin1−⁄− hippocampi relative to 3-month-old wild type mice (Fig. 6A and B) while at 3 months of age, p73+/+;Pin1−⁄− tau kinase activation levels were similar to those seen in wild type mice of the same age, and were lower than in the 9-month-old

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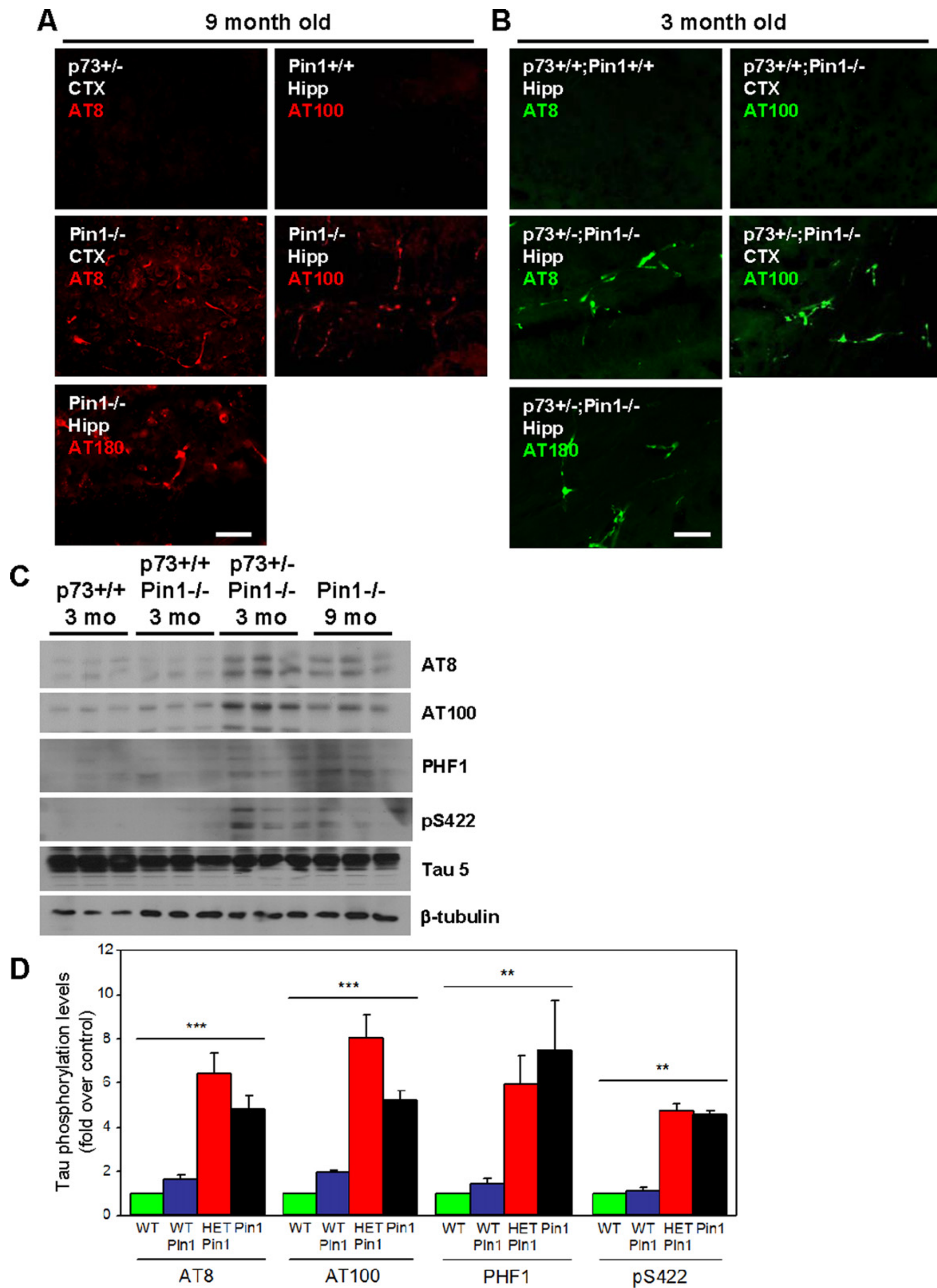

Fig. 5. p73 haploinsufficiency causes early formation of phospho-tau-positive aggregates in the brains of Pin1−/− mice. (A) Micrographs of phospho-tau-positive aggregates in the hippocampus (Hipp) and cortex (CTX) of 9-month-old p73+/− mice, Pin1+/+ mice, or Pin1−/− mice, as detected by immunostaining of paraformaldehyde-fixed cryostat sections with the AT8, AT100, and AT180 antibodies. Note that only the Pin1−/− mice display phospho-tau-positive aggregates at this age, as previously reported (Liou et al., 2003). Scale bar = 50 μm. (B) Micrographs of phospho-tau-positive aggregates in the CTX or Hipp of 3-month-old p73+/+;Pin1+/+, p73+/+;Pin1−/−, or p73+/−;Pin1−/− mice, as detected by immunostaining with the AT8, AT100, and AT180 antibodies. Note that at this age only the Pin1−/− mice that are heterozygous for p73 display these phospho-tau-positive aggregates. Scale bar = 50 μm. (C) Western blot analysis of equal amounts of protein from hippocampal lysates of 3-month-old p73+/+ mice, p73+/+;Pin1−/− mice,

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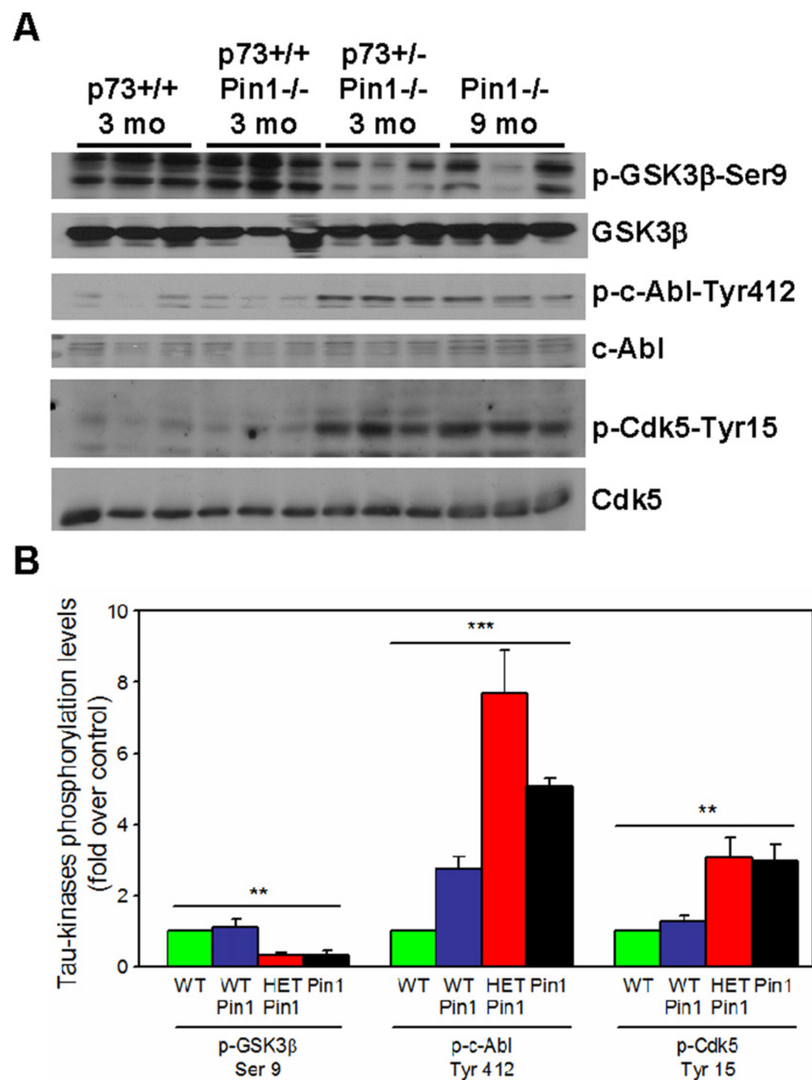

Fig. 6. p73 haploinsufficiency causes tau kinase dysregulation in the hippocampus of Pin1−/− mice. (A) Western blot analysis of equal amounts of protein from hippocampal lysates of 3-month-old p73+/+ mice, p73+/+;Pin1−/− mice, or p73+/−;Pin1−/− mice probed with antibodies for GSK3β phosphorylated at serine 9, c-Abl phosphorylated at tyrosine 412, or Cdk5 phosphorylated at tyrosine 15. As a positive control, lysates from 9-month-old Pin1−/− hippocampi were also analyzed. To obtain an indication of the relative levels of kinase activation, the same lysates were analyzed on Western blots using antibodies that recognized total GSK3β, c-Abl, or Cdk5. The same lysates were also analyzed for levels of β-tubulin as a control for equal loading (data not shown). (B) Quantification of the relative levels of GSK3β, c-Abl, and Cdk5 phosphorylation as assessed by scanning densitometry of Western blot analysis similar to those seen in (A) ($n = 3$ for each sample; ** $p < 0.01$, *** $p < 0.001$).

Pin1−/− mice (Fig. 6A and B). In contrast, the 3-month-old p73+/−;Pin1−/− mice showed levels of activation of these 3 kinases that were similar to those seen in the 9-month-old Pin1−/− mice (Fig. 6A and B), indicating that p73 haploinsufficiency accelerated activation of tau kinases in the Pin1−/− brains.

4. Discussion

The results described here provide 3 lines of evidence in support of the idea that p73 is an important neuroprotective gene for the aging and degenerating mammalian brain. First, we show that haploinsufficiency for p73 leads to aberrant

or p73+/−;Pin1−/− mice probed with AT8, AT100, PHF1, or pS422 antibodies to detect phosphorylated tau, or with an antibody against total tau or β-tubulin. As a positive control, lysates from 9-month-old Pin1−/− hippocampi were also analyzed. Each lane derives from an independent animal. (D) Quantification by scanning densitometry of the relative level of tau phosphorylation/total tau in Western blot analysis similar to that seen in (C) ($n = 3$ for each sample; ** $p < 0.01$, *** $p < 0.001$).

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tau hyperphosphorylation in the aging murine brain, potentially in response to aberrant activation of the tau kinases GSK3β, Cdk5, and/or c-Abl. Second, we show that p73 heterozygosity causes early phospho-tau accumulation, tau kinase dysregulation and decreased lifespan in the TgCRND8 mice, a well described animal model for Alzheimer's disease. Third, we have examined a second age-dependent murine neurodegeneration model that displays high tau phosphorylation, the Pin1−/− mouse, and show that p73 haploinsufficiency causes early tau hyperphosphorylation and tau kinase dysregulation in this model as it does in the TgCRND8 mouse model. Thus, as indicated by our previous work (Wetzel et al., 2008), p73 is a potent neuroprotective gene, and decreases in p73 levels predispose the nervous system to tau hyperphosphorylation and neurodegeneration.

One major conclusion of our work is that p73 haploinsufficiency represents a general predisposition factor for neurodegenerative cues, at least in mice. In particular, we now show that, as we have seen for the TgCRND8 Alzheimer's disease mouse model (Wetzel et al., 2008 and data presented here), p73 haploinsufficiency leads to premature tau hyperphosphorylation and tau kinase dysregulation in Pin1−/− mice. Pin1 is a peptidyl-prolyl cis/trans isomerase whose loss of function has been associated with aberrant APP processing, amyloid-β production and, perhaps more importantly, pathological tau phosphorylation (Liou et al., 2003; Lu and Zhou, 2007; Pastorino et al., 2006). Pin1 loss of function is thought to produce these alterations by inducing conformational changes in key downstream protein targets including tau and tau kinases (Keeney et al., 2011; Park et al., 2012; Zhou et al., 2000). Thus, the Pin1−/− mice are a progressive age-dependent neurodegenerative model that is completely distinct from models such as the TgCRND8 mice that involve overexpression of human APP. However, despite their completely different underlying pathological mechanisms, p73 haploinsufficiency leads to early tau hyperphosphorylation and formation of phospho-tau-positive aggregates in both models, suggesting that it may well represent a general predisposition factor for at least some aspects of neurodegeneration.

A second conclusion of this and our previous work is that p73 may act as a predisposition factor for neurodegeneration, at least in part by causing aberrantly increased tau phosphorylation. Dysregulation of tau phosphorylation is a major cause of abnormal tau function and aggregation, ultimately leading to tangle formation, one of the main hallmarks of Alzheimer's disease and tauopathies like Pick's disease and Frontotemporal dementia with Parkinsonism (Ballatore et al., 2007). While tau protein is highly modified posttranslationally, its ability to bind microtubules is largely controlled by phosphorylation (Johnson and Stoothoff, 2004) and, as a consequence, dysregulation of tau kinases is frequently associated with tau malfunction and neurodegeneration. In this regard, we previously demonstrated that phospho-tau-positive aggregates were present in the aging p73+/− murine brain, and that similar aggregates occurred early in TgCRND8 mice that were haploinsufficient for p73 (Wetzel et al., 2008). Here we show that p73 haploinsufficiency causes the appearance of these phospho-tau-positive aggregates and a robust overall increase in tau phosphorylation. This increase occurs not just during aging and in young TgCRND8 mice, but also in young (3-month-old) Pin1−/− mice that do not display enhanced tau phosphorylation when wild type for p73. Interestingly, these filamentous phospho-tau-positive aggregates are similar in appearance in all of these models, and are similar to the aggregates observed at 9 months of age in Pin1−/− mice. It is not yet known, however, whether the appearance of phospho-tau aggregates resulting from p73 heterozygosity is a cause or consequence of the neurodegeneration. It will be informative in this regard to know whether degeneration still occurs in p73 heterozygous mice lacking one or both copies of the mouse tau gene.

Why does p73 haploinsufficiency cause tau hyperphosphorylation? We previously associated the appearance of phospho-tau-positive aggregates with increased activation of JNK, which is known to phosphorylate tau (Atzori et al., 2001; Johnson and Nakamura, 2007; Yoshida et al., 2004) and which is directly regulated by p73 (Lee et al., 2004). However, while JNK phosphorylates tau, its contribution to human tau pathologies and Alzheimer's disease is still unclear. Instead, two other tau kinases, GSK3β and Cdk5, have been widely associated with the pathogenesis of Alzheimer's disease, because they are known to participate in aberrant APP processing, synaptic dysfunction, behavioral impairment, neuronal death, and tau phosphorylation in this neurodegenerative disorder (Hooper et al., 2008; Su and Tsai, 2011). We therefore asked whether these two kinases were dysregulated by p73 haploinsufficiency. We also analyzed the activation of the c-Abl tyrosine kinase, which has recently been associated with tau phosphorylation, can control Cdk5 activity, is localized in hippocampi and tangles of Alzheimer's disease brains, and when overexpressed as a constitutively active protein results in neurodegeneration in mice (Cancino et al., 2008, 2011; Derkinderen et al., 2005; Estrada et al., 2011; Jing et al., 2009; Lee et al., 2008; Lin et al., 2007; Schlatterer et al., 2011a, 2011b; Zukerberg et al., 2000). Data presented here indicate that all 3 of these tau kinases are dysregulated by p73 haploinsufficiency in the two mouse models of neurodegeneration studied here. Whether this is because p73 directly regulates all of these kinases, as we previously observed for JNK (Lee et al., 2004), or is an indirect consequence of the neuronal death and degeneration that occurs when p73 is haploinsufficient (Wetzel et al., 2008) is still an open question. However, direct or indirect, dysregulation of these three tau kinases and JNK is likely to account for the aberrant tau phosphorylation and phospho-tau-positive aggregates that we report here.

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One final surprising result of our study is that p73 haploinsufficiency decreased the life span of TgCRND8 mice. In this regard, the TgCRND8 mice have previously been shown to have a reduced life span (Chishti et al., 2001), but the cellular mechanisms for this are still unclear. One potential explanation for our data are that the enhanced CNS degeneration we report impinges upon basic neural functions that are necessary for life. A second explanation is that these animals die as a consequence of degeneration of their peripheral nervous systems. We previously showed that aged p73+/− mice frequently display a prolapsed penis and/or rectum, likely due to a large decrease in peripheral parasympathetic neurons and sympathetic innervation (Wetzel et al., 2008). While we have not observed a similar phenotype in the p73+/−;TgCRND8+/Ø mice, it is still possible that they display other peripheral neural phenotypes that could lead to a shortened life span (Pozniak et al., 2000; Walsh et al., 2004).

We and others have previously shown that ΔNp73 is a key survival protein for neurons in both the developing and adult nervous systems, and that even haploinsufficiency for p73 makes the nervous system vulnerable to long-term stresses such as aging and potentially degeneration (Lee et al., 2004; Pozniak et al., 2000, 2002; Tissir et al., 2009; Walsh et al., 2004; Wetzel et al., 2008; Wilhelm et al., 2010; Yang et al., 2000). Here, we show that p73 haploinsufficiency predisposes the nervous system to aberrant tau hyperphosphorylation and the formation of phospho-tau-positive aggregates. Whether this also occurs in humans that have decreased levels of neural p73 as a consequence of either genetic or environmental changes is not yet known. While there is an association between decreased ΔNp73 expression and AD (Li et al., 2004), and one study showed individuals with copy number variations (CNV) at the p73 genomic locus (Wong et al., 2007), these observations have not been observed in subsequent copy number variation studies. Confirmation of a role for p73 heterozygosity in human dementia awaits large-scale studies determining whether ΔNp73 protein levels are indeed decreased in this population.

Disclosure statement

All authors disclose no conflicts of interest.

Appropriate approval and procedures were used concerning animals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2012.04.010.

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