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ORIGINAL RESEARCH

p63α and γ Induce TAU Phosphorylation in Cultured Mammalian Cells

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Abstract: Here we show by western blotting that transcriptionally active isoforms of p63 (p63α and p63γ) induce the phosphorylation of human 2N4R tau at the tau-1/AT8 epitope in HEK293a cells; a phospho-epitope increased in Alzheimer's disease. Confocal microscopy shows that tau and p63α are spatially separated intracellularly. Tau was found in the cytoskeletal compartment, whilst p63α was located in the nucleus, indicating that the effects of p63 on tau phosphorylation are indirectly mediated. Tau phosphorylation occurred independently of the known tau kinases, protein kinase C delta (PKCδ), c-Jun N-terminal kinase (JNK), extracellular-signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38), glycogen synthase kinase 3 (GSK3), v-akt murine thymoma viral oncogene homolog (AKT) and cyclin-dependent kinase 5 (Cdk5) and the tau protein phosphatases (PP), PP1 and PP2A-Aα/β. Considering that p63 and tau are both associated with developmental processes, these findings have ramifications for neuronal development and synaptic plasticity and also neurodegenerative diseases such as Alzheimer's disease and other tauopathies.

Keywords: p63, tau, microtubules, Alzheimer's disease, cell cycle, phosphorylation

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Introduction

Tau is the major microtubule-associated protein (MAP) in neurons and functions in the formation and maintenance of axons by influencing microtubule organization. In adult human brain, there are six isoforms of tau generated by alternative mRNA-splicing. Tau has zero, one or two amino-terminal inserts and either three or four repeats of a microtubule-binding domain situated towards the carboxy-terminus.[1] Tau splicing and phosphorylation are developmentally regulated. Only the shortest tau isoform is expressed in foetal brain[2] and foetal tau is more extensively phosphorylated than tau from adult brain.[3,4] Phosphorylated tau is less efficient at promoting microtubule assembly[5] and elevated levels of phosphorylated tau correlate with increased microtubule dynamics associated with plasticity during development.[6] Increased tau phosphorylation is also a characteristic of Alzheimer's disease (AD) and tauopathies such as frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17).[7] In these disorders, normally soluble tau is present as paired-helical filaments (PHFs), which in turn aggregate to form neurofibrillary tangles (NFTs).

p63 is a recently discovered member of the p53 family.[8] There are multiple C-terminal splice variants of p63, which include p63α, β, and γ as well as N-terminally truncated, ΔN, forms.[9] p63 isoforms induce transactivation of certain p53 target genes, whilst truncated (ΔN) forms lack the transactivation domain (TA) and function as dominant negatives.[9,10] Despite their homology the p53 family members play different biological roles. p53 is predominantly involved in the maintenance of genomic integrity through the regulation of cell cycle arrest and apoptosis, whilst p63 is involved in development. p63 is essential for epithelial development and limb formation[11,12] and is also involved in the development of the sympathetic nervous system through the elimination of superfluous neurons.[13] However, unlike p53, p63 is rarely mutated in cancer.[14]

We have previously reported that p53 immunoreactivity is increased in AD.[15] Furthermore, both p53 and p73α induce tau phosphorylation in mammalian cells[15,16] and $p53^{-/-}$ mice show a reduction in tau phosphorylation.[17] These findings prompted us to investigate the effects of p63, a related p53 family member, on tau phosphorylation in cultured mammalian cells.

Methods

Materials

Human embryonic kidney 293a cells (HEK293a) were from Quantum Biotechnologies (Montreal, Canada). Mouse anti-β actin was from Sigma (Poole, UK). Rabbit anti-p63, rabbit anti-total JNK, rabbit anti-total ERK, rabbit anti-total p38 and rabbit anti-total AKT were from Cell signalling (UK). Mouse anti-total GSK3α/β antibody was from Bioquote (York, UK). Rabbit anti-total tau was from Dakocytomation (Ely, UK). Tau-1 monoclonal antibody was a kind gift from Professor L. Binder (Cognitive Neurology and Alzheimer's Disease Center, Northwestern University, USA). Mouse anti-PHF-1 antibody was a gift from Dr. P. Davies (Albert Einstein College of Medicine, NY, USA). Mouse anti-AT270, mouse anti-AT8, rabbit anti-total PKCδ, mouse anti-Cdk5, mouse anti-PP1 catalytic domain, goat anti-PP2A-Aα/β catalytic domain, goat anti-mouse IgG, goat anti-rabbit IgG, donkey anti-goat IgG and foetal calf serum (FCS) were from Autogen bioclear (Wiltshire, UK). OptiMEM, low glucose Dulbecco's modified Eagle's medium (DMEM), L-glutamine, penicillin and streptomycin, Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG were from Invitrogen (Paisley, UK). p63α, p63γ, ΔNp63α, ΔNp63γ and transcriptionally inactive isoforms of p63α and γ containing point mutations in the DNA binding domain (R304H) were a generous gift from Dr K. Engeland (Universitat Leipzig, Germany). 2N4R tau containing 2 amino terminal inserts and 4 microtubule binding domain repeats was a kind gift from Professor J Woodgett (Ontario Cancer Institute, Toronto, Canada) BAX-Luc was from Dr. T. Soussi (Universite P.M. Curie, Paris). p53 and transcriptionally inactive p53R175H with a point mutation in the DNA binding domain were gifts from Dr. B. Vogelstein (John Hopkins, USA). FuGene 6 was from Roche (Burgess Hill, UK). phTK-Renilla luciferase and Dual-Glo reagents were from Promega (Southampton, UK). Enhanced chemi-luminescence reagents (ECL) were from GE Healthcare Life Sciences (Buckinghamshire, UK).

Cell Culture

HEK293a were cultured in low glucose DMEM containing FCS (10%), L-glutamine (2 mM), penicillin

(100 IU), and streptomycin (100 mg/ml). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air and were used after 1 day *in vitro* following plating.

For tau phosphorylation experiments, HEK293a cells plated in a 6 well plate (300 000 cells/well) were transfected with OptiMEM (100 µl) containing FuGene 6 (5 µl) and cDNA constructs (500 ng of each) encoding human 2N4R tau independently or in combination with the appropriate p63 isoform or empty vector to maintain constant DNA concentrations. In experiments using bisindolylmaleimide I (2 µM: a broad spectrum PKC inhibitor), cells were treated with the inhibitor 3 hours post-transfection for the duration of the experiment. Cell lysates were harvested 24 hours after transfection.

For kinase and phosphatase expression experiments, HEK293a cells plated in a 6 well plate (300 000 cells/well) were transfected with OptiMEM (100 µl) containing FuGene 6 (5 µl) and cDNA constructs (500 ng of each) encoding p63α or empty vector as a control. Cell lysates were harvested 24 hours post-transfection.

Western Blotting

Western blotting was performed according to standard protocols using the following primary antibodies; rabbit anti-total tau (1:10000), mouse anti-phospho-Ser199/Ser202/Thr205 (tau-1, 1:1000), mouse-anti-phospho-Ser202/Thr205 (AT8, 1:500), mouse anti-phospho-Ser396/Ser404 (PHF-1) (1:1000), mouse anti-phospho-Thr181 (AT270, 1:1000), rabbit anti-total PKCδ (1:2000), rabbit anti-total JNK (1:1000), rabbit anti-total ERK (1:1000), rabbit anti-total p38 (1:1000), rabbit anti-total AKT (1:1000), mouse anti-total GSK3α/β (1:1000), mouse anti-Cdk5 (1:1000), mouse anti-PP1 catalytic domain (1:1000) or goat anti-PP2A-Aα/β catalytic domain (1:2000). Primary antibodies were detected using HRP-conjugated secondary antibodies and ECL. To ensure equal loading membranes were reprobed with mouse anti-β actin. All experiments were performed in triplicate; therefore presented images are representative.

Microscopy

For sub-cellular localisation experiments HEK293a cells were transfected with human 2N4R tau (500 ng) independently or in combination with p63α (500 ng). DNA concentrations were maintained using empty control vector where appropriate. The following day the cells were fixed in ice-cold methanol and stained according to standard protocols. Cells were incubated with rabbit anti-p63 (1:500) or with anti-total tau (1:1000) before being incubated with the appropriate fluorescent secondary antibody (1:200). Nuclei were counter-stained with Hoescht 33342. Immunofluorescence was captured using a Zeiss LSM510 meta-confocal microscope. All experiments were performed in triplicate; therefore figures shown are representative of a single experiment.

Reporter Assays

The transcriptional effects of the p63 isoforms were examined by reporter gene assay. Four wells of HEK293a cells plated in a 48 well plate (40 000 cells/well) were transfected by adding master-transfection mix (25 µl) to the culture medium. The master-mix contained OptiMEM (100 µl), FuGene 6 (4 µl), firefly BAX-Luc (400 ng: luciferase-based reporter DNA), phTK-Renilla luciferase (50 ng) to control for transfection efficiency and the appropriate p63/p53 isoform (800 ng). Empty vector DNA was included where necessary to maintain constant DNA concentrations. Twenty-four hours post-transfection the firefly and Renilla luciferase activities were sequentially measured using Dual-Glo reagents in a Wallac Trilux 1450 Luminometer (Perkin Elmer, UK). Firefly values were divided by the Renilla value from the same well to control for non-specific effects. Data for each set of four replica transfections was averaged, the control in each set normalized to 1 and data presented as fold increases over control. Each assay was repeated three times. Statistical analysis was performed using a one way ANOVA followed by a Tukey Post hoc. **, $P < 0.01$.

Results

The 2N4R isoform of human tau was exogenously expressed in HEK293a cells (which do not contain endogenous tau) alone or in combination with p63α, p63γ, ΔNp63α, ΔNp63γ, p63αR304H or p63γR304H. Co-expression of tau with p63α resulted in a pronounced reduction of tau-1 immunoreactivity in HEK293a cells, in comparison to cells expressing tau alone (Fig. 1A). The tau-1 antibody recognises a number of amino acids including Ser199, Ser202

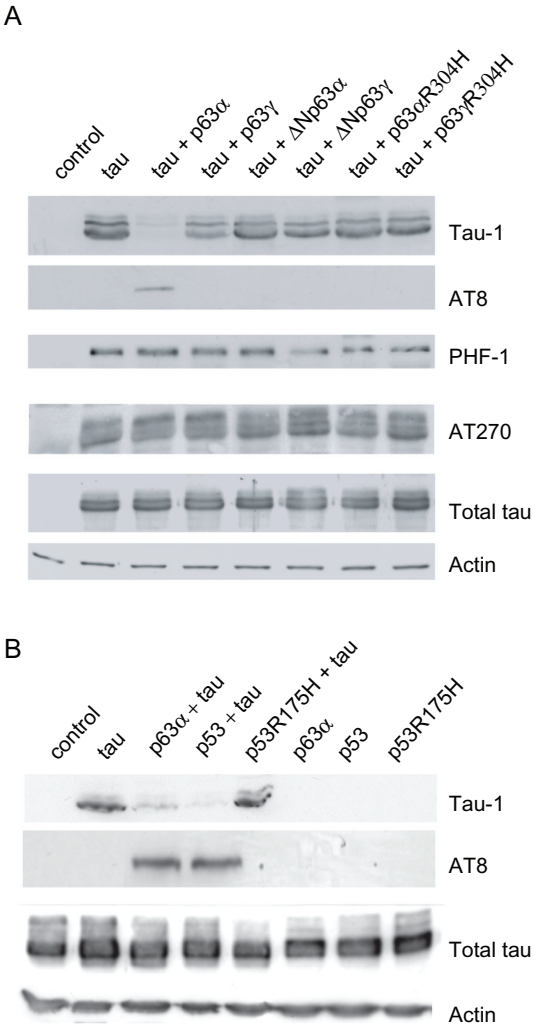

Figure 1. p63 induces tau phosphorylation in HEK293a cells. **A**) Exogenous 2N4R tau was expressed in HEK293a cells with p63α, p63γ, ΔNp63α, ΔNp63γ, p63αR304H, p63γR304H or with empty vector. Lysates were collected after 24 hours and subjected to western blotting and probed with a panel of tau antibodies as indicated. Blots were reprobed with anti-β actin to ensure equal loading. **B**) Exogenous 2N4R tau, p63α, p53 or p53R175H were expressed in HEK293a cells alone or in combination as indicated. Lysates were collected after 24 hours and subjected to western blotting and probed with either the tau-1 or AT8 monoclonal antibody or an antibody raised against total tau as indicated. Blots were reprobed with anti-β actin to ensure equal loading.

and Thr205[18] when de-phosphorylated, therefore a decrease in immunoreactivity at this site reflects an increase in tau phosphorylation. Consistent with this, p63α induced an increase in tau phosphorylation as evidenced using the AT8 antibody, which recognizes tau phosphorylated at Ser202/Thr205. p63γ also caused a slight abatement of tau-1 immunoreactivity, although no obvious increase in tau phosphorylation was observed at the AT8 site. Baseline levels of tau immunoreactivity at the AT8 site were very low reflecting low basal phosphorylation at this site in resting cells, which is corroborated by the strong tau-1 signal. The transcriptionally inactive isoforms of p63: ΔNp63α, ΔNp63γ, p63αR304H and p63γR304H did not induce any change in tau phosphorylation at the tau-1/AT8 epitope. Furthermore, no changes in tau phosphorylation in the presence of any p63 isoform were observed using PHF-1 or AT270 monoclonal antibodies. Immunoblotting for total tau demonstrates that equal levels of tau were transfected and immunoblotting for β-actin illustrated that equal amounts of protein had been loaded across lanes. p63α caused a comparable increase in tau phosphorylation at the tau-1/AT8 epitope as p53, whilst transcriptionally inactive p53R175H had no effect on tau phosphorylation status at these sites (Fig. 1B). Furthermore, tau protein was not detectable in cells transfected with p63α, p53 or p53R175H alone.

Confocal microscopy demonstrated that p63α and tau are compartmentally segregated when co-expressed (Fig. 2). p63α (red) exhibits a diffuse nuclear localisation, whilst tau (green) is present in the cytoskeletal compartment when these cDNAs are expressed both independently (data now not shown) and in combination. This suggests that the effects of p63 on tau phosphorylation are indirect and most likely attributable to the transcription of a p63 target gene.

The transcriptional properties of the p63 isoforms were verified in HEK293a cells using BAX-Luc (Fig. 3A), a luciferase reporter derived from a known p63/p53 target gene. Both p63α and p63γ induced transcription from this promoter to a similar extent as p53, whereas ΔNp63α, ΔNp63γ, p63αR304H and p63γR304H did not exert any transcriptional effects, which is in accordance with previous reports.[10] PKCδ has recently been identified as a p63/p73 target gene,[19] although treatment with bisindolylmaleimide I (2 μM), a PKC inhibitor, did not reverse the effects of p63α, p73α or p53 on tau phosphorylation suggesting that the effects of p63 and indeed p73 and p53 are independent of this kinase (Fig. 3B). In support of this, p63α expression did not induce an increase in PKCδ levels compared with control (Fig. 3C). Furthermore, p63α did not alter the expression levels of any of the known tau kinases we examined; JNK, ERK, p38, GSK3 α/β, AKT or Cdk5 relative to β-actin (Fig. 3D). Nor were there alterations in the expression levels of the catalytic domains of the tau phosphatases, PP1 and PP2A-Aα/β, which suggests

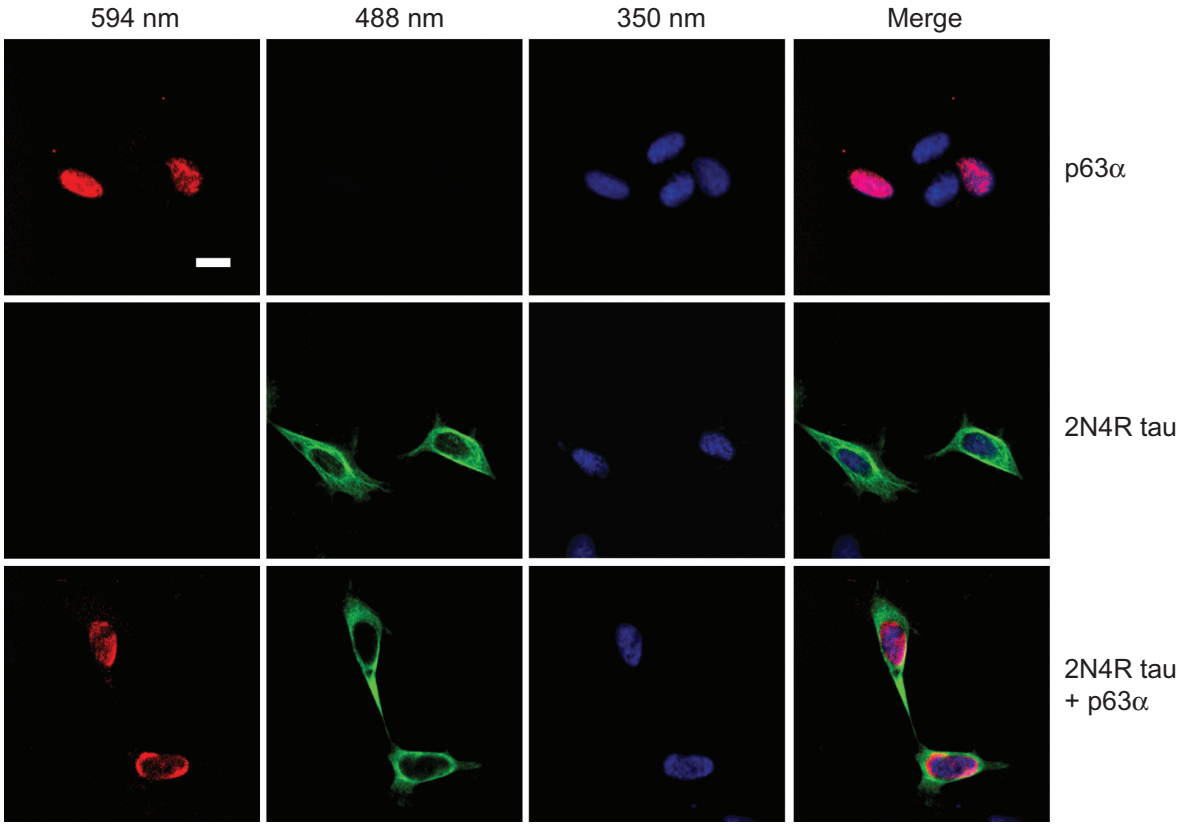

Figure 2. Sub-cellular distribution of tau and p63α in HEK293a cells. Exogenous 2N4R tau and p63α were co-expressed in HEK293a cells and detected using the appropriate antibodies. Tau (green) was found in the cytoskeletal compartment whilst p63α (red) was present in the nucleus. Nuclei were counterstained with Hoescht (blue). Scale bar represents 10 μm.

that the effects of p63 on tau phosphorylation do not involve increased transcription of these genes. There were also no changes in the phosphorylation state of JNK, ERK, p38, GSK3α/β, AKT or Cdk5 following transfection of p63α, indicating that p63 does not modulate kinase activity through the transcription of an unknown mediator (data not shown).

Discussion

Here we show that p63α and to a much lesser extent p63γ induce the phosphorylation of human 2N4R tau at the tau-1/AT8 epitope in HEK293a cells, an epitope that is highly phosphorylated in Alzheimer's disease.[20] Similarly, we have previously shown that in HEK293a cells p53 induces the phosphorylation of human 2N4R tau at the tau-1/AT8 epitope and that p73α induces tau phosphorylation at the PHF-1 and tau-1/AT8 epitopes.[15,16] We infer that the effects of p63 on tau phosphorylation are indirect and this is also proposed to be the case for p53 and p73;[15,16] as evidenced by the compartmental segregation of tau and the p53 family members. Furthermore, p63 isoforms lacking transcriptional properties (ΔNp63α, ΔNp63γ, p63αR304H and p63γR304H) did not effect tau phosphorylation and nor do transcriptionally inactive p53 or p73 mutants.[16] However, the precise mechanism through which p63 isoforms, and indeed the other family members, induce tau phosphorylation remains to be determined. Potential mechanisms include the increased expression of a tau kinase or the reduced expression of a tau phosphatase, although we have ruled out the involvement of PKCδ, JNK, ERK, p38, GSK3α/β, AKT, Cdk5 and the tau phosphatases, PP1 and PP2A-Aα/β.

Our findings are relevant to both neurodevelopment and neurodegeneration. During development p63-induced tau phosphorylation might facilitate synaptic modeling and connectivity, thereby mediating neurogenic processes. Conversely, pathological activation of p63 and/or other p53 family members in the adult brain might mediate neurofibrillary changes contributing to neuronal loss in disease. Aberrant re-entry into the cell cycle leading to neuronal cell death instead of regeneration is one mechanism thought to

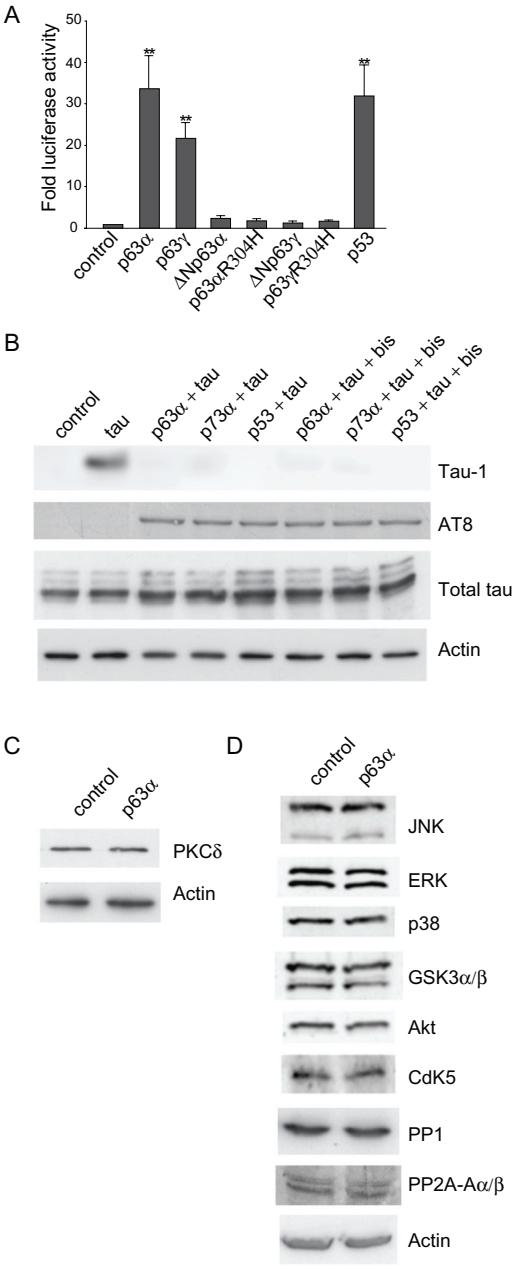

Figure 3. Exploration of the transcription-dependent mechanism of p63-induced tau phosphorylation in HEK293a cells. **A**) HEK293a cells were transfected with BAX-Luc with either empty vector or p63α, p63γ, ΔNp63α, ΔNp63γ, p63αR304H, p63γR304H or p53 as indicated. Twenty-four hours post transfection cells were lysed and luciferase activity determined. Statistical analysis was performed using ANOVA followed by the Tukey post hoc. $^{**}P < 0.01$. **B**) HEK293a cells were transfected with 2N4R tau with either p63α, p73α, p53 or empty vector in the presence and absence of bisindolylmaleimide I (bis: 2 μM), which was added as indicated three hours post-transfection. Lysates were collected after 24 hours and subjected to western blotting using either the tau-1 or AT8 monoclonal antibody or an antibody raised against total tau as indicated. **C**) HEK293a cells were transfected with empty vector or p63α and lysates were collected after 24 hours and subjected to western blotting with an antibody raised against total PKCδ. **D**) HEK293a cells were transfected with empty vector or p63α and lysates were collected after 24 hours and subjected to western blotting with antibodies raised against total JNK, ERK, p38, GSK3α/β, AKT, Cdk5, PP1 or PP2A-Aα/β. Blots were reprobed with anti-β actin to ensure equal loading.

contribute to neurodegenerative processes in AD.[21] We propose that the activation of p53 family members and subsequent tau-phosphorylation plays a pivotal role in neuronal death in AD as neurons attempt to re-enter the cell cycle, but fail. Accordingly, mitotic neuroblastomas have been shown to express highly phosphorylated tau (as indicated using tau-1, AT8, AT180 and PHF-1 antibodies) compared to neuroblastomas in interphase.[22] Phosphorylation events during mitosis are thought to mediate reorganization of the microtubule network, a necessary process for cell division.

Previously, we have demonstrated that p53 is upregulated approximately 2 fold in the superior temporal gyrus of Alzheimer's patients compared to control subjects.[15] This is consistent with several other studies that have reported an increase in p53 immunoreactivity in sporadic AD[23,24] especially in subpopulations of cortical neurons undergoing neurofibrillary degeneration.[25] We were unable to detect an upregulation of p63 or p73 isoforms in AD tissue (data not shown). However, a recent report shows that p73 is aberrantly expressed in hippocampal neurons in AD, with p73 being expressed in the nucleus in AD patients as opposed to the cytoplasm in control subjects.[26] Therefore, nuclear accumulation of p73 and/or p63 (without an overall change in expression level) might account for transcriptionally-driven changes in tau phosphorylation in some instances. Consequently, deregulation of p53 family members, consistent with their apoptotic role in degenerative disorders, might promote excessive and prolonged tau phosphorylation, which in turn might precipitate the formation of NFTs thereby contributing to cell death. Thus, modulation of p53 family-dependent cell death pathways might be of therapeutic benefit in AD and in other age related neurological disorders.

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Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors

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