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## The Cell Cycle Regulator Phosphorylated Retinoblastoma Protein Is Associated with Tau Pathology in Several Tauopathies

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### Abstract

Retinoblastoma protein (pRb) is a ubiquitous 928 amino acid cell cycle regulatory molecule with diverse biological activities. One critical function of pRb is control of the G<sub>1</sub>-to-S phase checkpoint of the cell cycle. In the hypophosphorylated state, pRb suppresses the activity of E2F transcription factors thereby inhibiting transcription of cell cycle promoting genes. Upon phosphorylation, primarily by cyclin dependent kinases, phosphorylated pRb dissociates from E2F and permits cell cycle progression. We previously found phosphorylated pRb to be intimately associated with hyperphosphorylated tau-containing neurofibrillary tangles of Alzheimer disease (AD), the pathogenesis of which is believed to involve dysregulation of the cell cycle and marked neuronal death. Here, we used immunohistochemistry to investigate the presence of phosphorylated pRb in other distinct neurodegenerative diseases that share the common characteristic of hyperphosphorylated tau pathology and neuronal loss with AD. We found colocalized labeling of tau pathology and phosphorylated pRb in Pick disease and progressive supranuclear palsy (3 cases each), neurodegeneration with brain iron accumulation type 1 (2 cases) and Parkinson-amyotrophic lateral sclerosis of Guam, subacute sclerosing panencephalitis, frontotemporal dementia and Parkinsonism linked to chromosome 17 and dementia pugilistica (1 case each). These observations further implicate aberrant neuronal cell cycle progression in neurodegenerative diseases, particularly tauopathies, and suggest a novel target for therapeutic intervention.

### Keywords

Dementia pugilistica; Neurodegeneration with brain iron accumulation type 1; Neurofibrillary tangles; Pick disease; Progressive supranuclear palsy; Retinoblastoma protein; Subacute sclerosing panencephalitis; Tau pathology

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## INTRODUCTION

Retinoblastoma protein (pRb) is implicated in diverse biological functions including cellular differentiation, senescence, apoptosis, and notably, progression of the cell cycle (1). pRb is considered to be a potent tumor suppressor that exerts control of the cell cycle at the G<sub>1</sub>-to-S phase checkpoint by inhibiting the E2F family transcription factors thereby impeding the transcription of genes necessary for transition to S phase (2, 3). Importantly, the fluctuating phosphorylation status of pRb at each of its 16 potential phosphorylation sites determines its function (4); hypophosphorylated pRb intimately associates with E2F thus inhibiting cell cycle progression. The sequential phosphorylation of pRb by cyclin D-cyclin dependent kinase (CDK) 4/6 and cyclin E-CDK2 causes pRb to dissociate from E2F and advances quiescent cells to S phase (5–7). Additionally, pRb likely mediates more than G<sub>1</sub>-to-S phase checkpoint progression because sequential phosphorylation of pRb continues throughout the remainder of the cell cycle; this occurs by cyclin A-CDK2 during S phase and cyclin B-cdc2 during G<sub>2</sub>/M phases (8), before dephosphorylation occurs in late M phase by protein phosphatase 1 (9).

Inasmuch as fully differentiated mature neurons are non-proliferative, suppression of the cell cycle by hypophosphorylated pRb appears to be critical in the normal function of adult neurons. Increasing evidence suggests, however, that deteriorating neurons in neurodegenerative diseases such as Alzheimer disease (AD), abnormally initiate cell division processes (10). Specifically, aberrant expression of several cyclins, CDKs, and cyclin-dependent kinase inhibitors that may encourage expression of cell cycle promoting genes has been observed in AD (11–21). In addition, hyperphosphorylated pRb and phosphorylated minichromosome maintenance 2 (pMcm2), which is directly linked to pRb activity in cell cycle regulation, were shown to be elevated and colocalized with neurofibrillary tangles (NFTs) in AD (22, 23).

NFTs are flame-shaped intracytoplasmic inclusions composed of aggregations of hyperphosphorylated tau protein that affect proximal neurites and neuron perikarya and are hallmark pathological features of AD (24). However, tau pathology is also observed in several additional distinct neurodegenerative diseases including parkinson-amyotrophic lateral sclerosis of Guam (GP-ALS) (25), Pick disease (26, 27), progressive supranuclear palsy (PSP) (28, 29), subacute sclerosing panencephalitis (SSPE) (30, 31), frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) (32, 33), dementia pugilistica (34), and neurodegeneration with brain iron accumulation type 1 (NBIA) (35), suggesting the possibility of a common underlying pathogenic pathway for these diseases collectively referred to as tauopathies (36).

pRb hyperphosphorylated at several sites was found to be elevated and associated with NFTs in AD (22). Because AD shares several pathological characteristics such as the accumulation of hyperphosphorylated tau in NFTs, neuronal dysfunction, and eventual neuronal death with numerous other tauopathies, we investigated the presence of pRb phosphorylated at several sites in brain samples from patients with these neurodegenerative disorders.

## MATERIALS AND METHODS

### Brain tissue

Using a protocol approved by the Case Western Reserve University IRB, human brain tissue samples were obtained postmortem and fixed in either routine formalin or methacarn (methanol: chloroform: acetic acid; 6:3:1 v/v/v), dehydrated through ascending ethanol, embedded in paraffin, cut into 5- $\mu$ m sections, and mounted on silane-coated slides. Cases

included Pick disease hippocampus (n = 3), PSP brainstem (n = 3), GP-ALS cortex (n = 1), SSPE hippocampus (n = 1), FTDP-17 hippocampus (n = 1), dementia pugilistica cortex (n = 1, gift of Raul Rudelli, MD, Bayonne, NJ), NBIA cortex (n = 2, gift of William Halliday, MD, Toronto, Canada), and non-pathological control hippocampus (n = 10) and brainstem (n = 5). All brainstem sections were cut at the level of the pons. Due to the rarity of some diseases, very limited tissue sections were available for some cases.

### Immunohistochemistry

Tissue sections were deparaffinized with xylene and hydrated through descending concentrations of ethanol. Endogenous peroxidase activity was ablated by 30-minute incubation in 3% H<sub>2</sub>O<sub>2</sub> in methanol and non-specific antibody binding was blocked with 30-minute incubation in 10% normal goat serum (NGS) in Tris-buffered saline (TBS). The sections were incubated at 4°C overnight in primary antibody diluted in 1% NGS in TBS. Primary antibodies were rabbit polyclonal antibodies against pRb (Biosource International, Camarillo, CA) phosphorylated at serine 249 and threonine 252 (ppRb<sub>ser249/T252</sub>), serine 807 (ppRb<sub>ser807</sub>) and serine 612 (ppRb<sub>ser612</sub>), an antibody specific for pMcm2 (40/41, gift of Corrado Santocanale, PhD, Galway, Ireland) (23), mouse monoclonal antibodies to phosphorylated tau (AT8, Pierce Endogen, Rockford, IL) and glial fibrillary acidic protein (GFAP, clone GA5, Millipore, Billerica, MA). Antibody specificity was fully characterized in a previous study (22). Species-specific secondary antibodies (Millipore) and PAP complexes (Covance, Princeton, NJ) were then applied in succession. 3, 3'-diaminobenzidine (Dako, Carpinteria, CA) was used as the chromogen and imaged with a Zeiss Axiophot and Zeiss Axiocam (Carl Zeiss, Thornwood, NY).

For some experiments, tissue sections were pretreated with 70% formic acid for 5 minutes with acetate buffer and pressure cooking (Biocare Medical, Concord, CA), or treated with trypsin (400 µg/ml for 10 minutes at room temperature) to investigate effects of epitope unmasking for staining of ppRb. Further experiments were performed using double label fluorescent microscopy using AlexaFluor 488 and AlexaFluor 568 (Invitrogen, Carlsbad, CA) for detection of ppRb and AT8 or GFAP in pathological and neuritic structures and imaged using a Zeiss Axiovert microscope. Quantification of the stained structures was performed. The same fields were imaged and the structures immunostained for either ppRb alone, AT8 alone, or both were counted and expressed as the percentage of stained neurons within each group (37).

## RESULTS

Phosphorylated pRb (ppRb) was frequently associated with the characteristic pathological inclusions in several neurodegenerative diseases. Labeling of NFTs and other inclusions was verified in all cases by either colocalization with hyperphosphorylated tau and/or morphological criteria.

In the PSP cases, ppRb<sub>ser807</sub> was found in the majority of PSP tangles that also had accumulated phosphorylated tau; it was seen in many neuropil threads in the brainstem as well (Fig. 1). The neuritic pathology was more prominent with AT8 than with the antibody to ppRb<sub>ser807</sub> in all cases. Semiquantitative analysis demonstrated that over 50% of the stained neurons contained both ppRb<sub>ser807</sub> and phosphorylated tau; an average of approximately 20% of neurons was only ppRb<sub>ser807</sub>-positive and 23% were only AT8-positive. In addition, some structures stained with AT8 displayed glial morphology (Fig. 1D, arrowheads); these were not stained for ppRb<sub>ser807</sub> (Fig. 1C). A smaller subset of PSP tangles was immunolabeled for ppRb<sub>ser612</sub> than for ppRb<sub>ser807</sub>, (Fig. 1E). In addition, the antibody to pMcm2, a key cell cycle regulator that is directly modulated by pRb activity also labeled neurons and neurites in the PSP cases (Fig. 1F), supporting the activation of cell

cycle by hyperphosphorylation of pRb. In serial sections of PSP brainstem using the rabbit polyclonal antibodies against ppRb<sub>ser807</sub> (Fig. 1G) and pMcm2 (Fig. 1H), some cells showed colocalization of both cell cycle markers. Comparisons of various pretreatments for antigen retrieval (e.g. formic acid, trypsin, and heating by pressure cooking) indicated that PSP tangles were well stained whether the tissue was left untreated or was treated using the various antigen retrieval methods (data not shown). Because the pathological structures were strongly stained with little background or non-specific staining on untreated tissue sections, most experiments were performed without pretreatments.

By double label fluorescence microscopy, many PSP tangles and a population of neurites were immunostained with both the antibody to ppRb<sub>ser807</sub> (Fig. 2A) and AT8 (Fig. 2B). Many more cell processes (which could be either neurites or glial processes containing tau) only stained for phosphorylated tau. Interestingly, some neurons in PSP cases showed high levels of ppRb<sub>ser807</sub> staining but no AT8-positive phosphorylated tau (Fig. 2D-F, white arrows). ppRb<sub>S249/T252</sub> was also found in numerous PSP tangles and neurons, some of which contained nearly undetectable levels of phosphorylated tau labeled by AT8 (Fig. 2G-I, white arrows). In an area of white matter in a PSP case with many AT8-positive processes, very few cellular processes contained both ppRb<sub>ser807</sub> and AT8 (Fig. 3A-C). Double staining with anti-GFAP indicated that most glial structures did not accumulate ppRb<sub>ser807</sub> (Fig. 3D-F).

The hippocampus and adjacent temporal cortex in the GP-ALS case contained many neurons with NFT when stained for ppRb<sub>ser807</sub> (Fig. 4A and inset). In the FTDP-17 case there was also prominent labeling of the neuritic processes with the anti-ppRb<sub>ser807</sub> antibody (Fig. 4B).

The hippocampus and adjacent temporal cortex in the SSPE case contained many NFTs with both ppRb<sub>ser807</sub> and phosphorylated tau. Many of the same neurons contained both ppRb<sub>ser807</sub> and AT8 but the tau pathology was more extensive and also included neuritic pathology and neuropil threads that were not ppRb<sub>ser807</sub>- positive (Fig. 5). Serial sections of the NBIA cases revealed many cells that showed both ppRb<sub>ser807</sub> and phosphorylated tau (Fig. 6).

Semiquantitative analysis indicated that while nearly 30% of stained neurons showed colocalization, 30% displayed ppRb<sub>ser807</sub> only and over 40% were only AT8-positive. Neuropil threads, which are AT8-positive, consistently remain unlabeled with the antibody against ppRb<sub>ser807</sub> in this and most other conditions.

NFTs in the dementia pugilistica case also showed ppRb<sub>ser807</sub> immunoreactivity. The NFTs were present in small numbers, had variable morphologic appearances and were scattered throughout the cortical section examined (Fig. 7).

Immunostaining for ppRb was seen in many Pick bodies in neurons of the hippocampus and adjacent cortex in all cases of Pick disease examined. ppRb<sub>ser807</sub> (Fig. 8A, C) and ppRb<sub>S249/T252</sub> (Fig. 8B, D) were observed in many of the same Pick bodies identified in adjacent serial sections. Pick bodies were also stained using the antibody against pMcm2 (Fig. 8E).

Non-pathological control brainstem immunostained with antibodies to pRb<sub>ser807</sub> and pRb<sub>S249/T252</sub> showed abundant healthy pigmented neurons and only light background staining (data not shown). Consistent with our previous finding (22), control hippocampal tissue from cases between the ages of 57 and 92 years demonstrated few age-associated NFTs. Younger cases between the ages of 17 and 48 years showed no specific neuronal staining.

## DISCUSSION

In this study, we sought to investigate the potential role of hyperphosphorylated pRb in neurodegenerative diseases with hyperphosphorylated tau pathology and neuronal loss. We found that ppRb immunoreactivity was 1) elevated in all disease cases compared to controls, 2) often colocalized with hyperphosphorylated tau-containing NFT pathology in all diseases studied, 3) was also often present in neurons that did not contain phosphorylated tau, and 4) colocalized with hyperphosphorylated tau in Pick bodies in Pick disease cases. Because the presence of pRb indicates that cells are bypassing the G<sub>1</sub>-to-S phase checkpoint, these data suggest that vulnerable neurons in GP-ALS, Pick disease, PSP, SSPE, FTDP-17, dementia pugilistica, and NBIA aberrantly re-enter the cell cycle throughout the disease course. Importantly, pRb must be phosphorylated at several of its 16 potential phosphorylation sites in a cumulative manner for disinhibition of E2F transcription factors to occur and the cell cycle to progress (38). Evidence indicates S249/T252 and S807 only become available for phosphorylation after several other sites are phosphorylated due to conformational masking (39). Specifically, initial phosphorylation on sites T356, T373, S811, T821, and T826 must occur to invoke a conformational change in the pRb molecule that makes S249/T252 and S807 available to CDKs (38). In addition, hypophosphorylated pRb is tightly localized to the nucleus, whereas hyperphosphorylated pRb releases to a cytoplasmic distribution (40). Cytoplasmic pRb labeling was predominantly observed in the cases analyzed in this study. Therefore, our results indicate that the pRb isoforms evaluated represent hyperphosphorylated pRb that is dissociated from E2F and, as such, represents a valid indicator of cell cycle progression. Furthermore, the antibodies used in this investigation were fully characterized and shown to have high specificity in a previous study (22), lending additional support to the validity of our present findings. pRb phosphorylation by CDKs probably occurs in the nucleus after which the CDK-pRb complex translocates to the cytoplasm (22). Evidence indicates pRb phosphorylation occurs independent of, or prior to, NFT formation in AD (41), however, CDKs have also been shown to phosphorylate tau in vitro (42-44) (Fig. 9). Our finding in the present study that a number of neurons in PSP and other diseases express high levels of ppRb, yet have not accumulated phosphorylated tau supports this concept. Consequently, accumulating pRb-CDK in the cytoplasm may be necessary for NFT formation and explain the colocalization of ppRb and NFTs observed.

We also found that pMcm2 is in pathological structures in both PSP and Pick disease, and that it is often present in the same cells with ppRb (Fig. 1). CDK 2 is required for the phosphorylation of Mcm2 responsible for regulating DNA replication; this is another G<sub>1</sub>/S phase checkpoint control protein along with pRb that is tightly regulated by E2F (45, 46). Thus, this finding further supports the dysregulation of pRb and subsequent activation of cell cycle in the vulnerable neurons in tauopathies. In addition, extracellular signal-regulated kinase and p38, which are activated in AD and mediate tau phosphorylation (47-51), also phosphorylate pRb. The combined effect of CDKs, extracellular signal-regulated kinase, and p38 may, therefore, represent a link between the aberrant phosphorylation increased in tau and pRb in these tauopathies (52, 53). Further investigation into the presence of kinase(s) that mediate both tau and pRb phosphorylation in tauopathies is certainly warranted. In this regard, Cdk5 might be one candidate because multiple studies demonstrate that Cdk5 is one of the major tau kinases (54, 55) and, importantly, also phosphorylates pRb and subsequent cell cycle activation (Fig. 9) (56, 57).

Irrespective of the mechanism by which hyperphosphorylated pRb accumulates in tau pathology within the tauopathies investigated, the implications of this observation are intriguing. Although neurons were historically viewed as quiescent non-proliferating cells, our results on pRb and pMcm2 add to increasing evidence that suggests neurons may re-enter the cell cycle throughout the course of various neurodegenerative diseases. Because

the various tauopathies investigated in this study and AD all involve progressive neuronal dysfunction ultimately resulting in neuronal death, re-entry into the cell cycle may be a major neurodegenerative mechanism in these diseases. In support of this notion, pRb phosphorylation occurs prior to neuronal death in animal models of ischemia (58, 59), and the forced activation of neuronal cell cycle re-entry in vivo causes severe neuronal cell loss in a transgenic mouse model (60). Although many questions remain and several future studies are justified, our finding of pRb in various tau pathologies of GP-ALS, Pick disease, PSP, SSPE, FTDP-17, dementia pugilistica, and NBIA further elucidates the role of pRb in neurodegeneration, supports the notion of a common underlying pathological pathway present in tauopathies, and suggests a novel potential target for therapeutic intervention.

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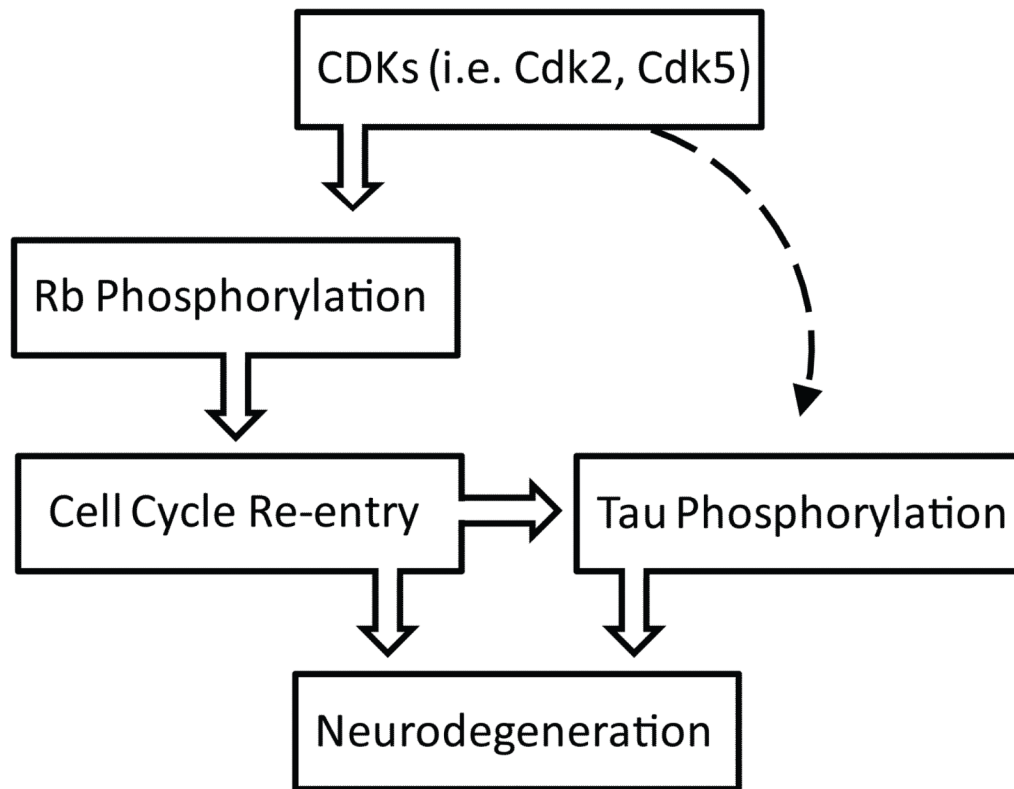
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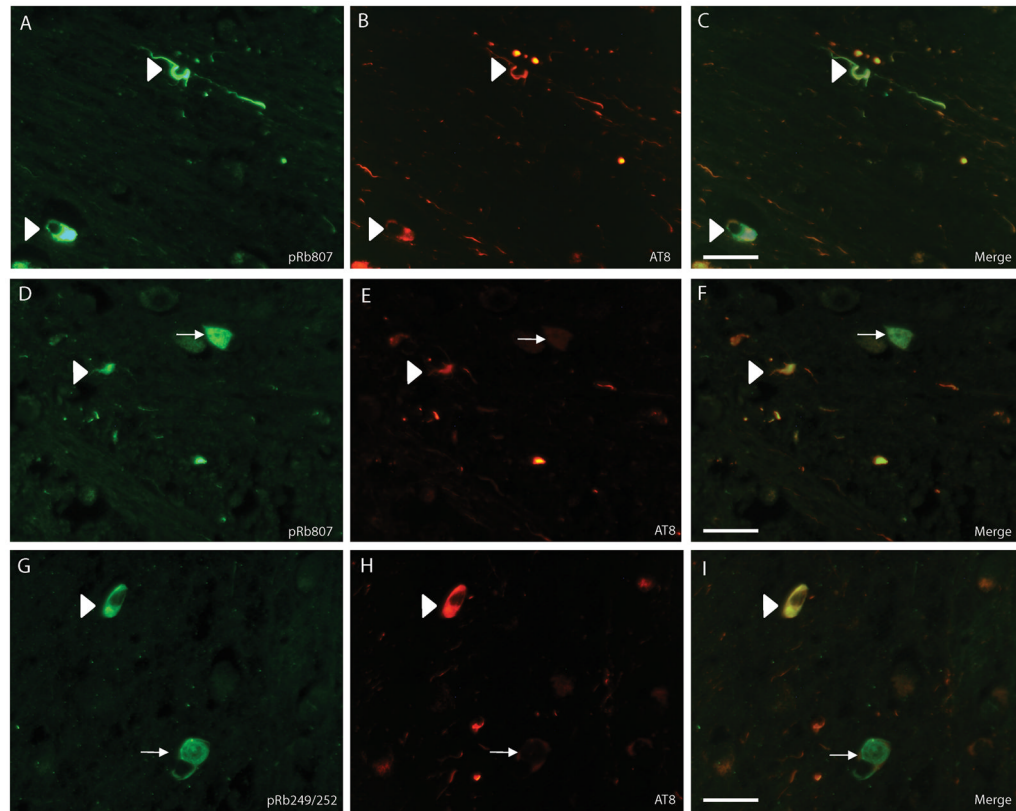
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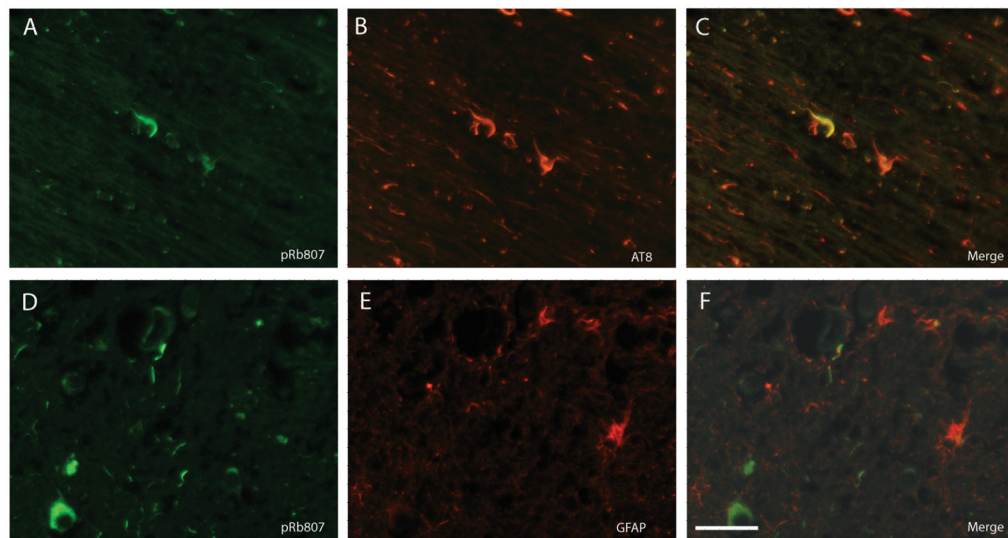
**Figure 1.**

Serial sections from the brainstem in a case of progressive supranuclear palsy (PSP). (A-D) Colocalization of ppRb<sub>ser807</sub> (A, C) and AT8 labeling of hyperphosphorylated tau (B, D) in PSP tangles. Higher magnification of boxes from (A) and (B) are shown in C and D, respectively. Glial tau pathology (arrowheads in D) is demonstrated with the AT8 antibody. (E) pRb<sub>612</sub> is also localized to a population of PSP tangles and neurites. (F) Phosphorylated minichromosome maintenance 2 (pMcm2), another cell cycle marker, is specifically localized to PSP tangles and neurites. (G, H) Both ppRb<sub>ser807</sub> (G) and pMcm2 (H) are present in the same neurons seen in adjacent serial sections. Asterisks denote landmark vessels and arrows identify the same neurons across serial sections. Scale bars = 50  $\mu$ m.



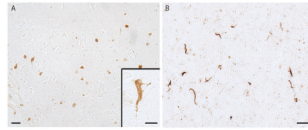
**Figure 2.**

Double label fluorescence microscopy in progressive supranuclear palsy (PSP). (A-C) A large number of PSP tangles show both ppRb<sub>ser807</sub> (A, green) and AT8 (B, red) marked with white arrowheads and there are also many AT8-positive neurites that do not contain ppRb<sub>ser807</sub> (C, merged image). (D-F) There are a few neurons that are strongly immunoreactive for ppRb<sub>ser807</sub> (D, green), but lack AT8 (E, red) (white arrow) and evident in merged image (F). White arrowheads indicate neurons with colocalization. (G-I) The same pattern is found using an antibody specific for ppRb<sub>S249/T252</sub> (G, green). Many neurites only stain with antibody AT8 (H, red); some PSP tangles display only ppRb<sub>S249/T252</sub> and not phosphorylated tau (I, merged image, white arrow); some PSP tangles show colocalization (white arrowheads). Scale bars = 50  $\mu$ m.

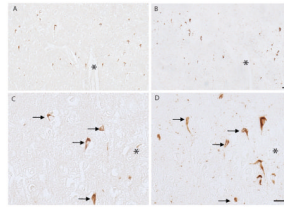


**Figure 3.**

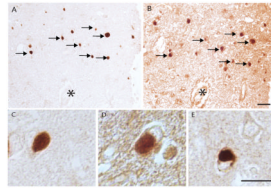
Double label immunofluorescence in another case of progressive supranuclear palsy. (A-C) Very few AT8-positive neurites (B, red) contain ppRb<sub>ser807</sub> (A, green, and merged image, C). (D-G) Even in fields with many ppRb<sub>ser807</sub>-positive neurites (D, green) the vast majority do not colocalize with astrocytic processes marked using an antibody to glial fibrillary acidic protein (E, red, and merged image F). Scale bar = 50  $\mu$ m.



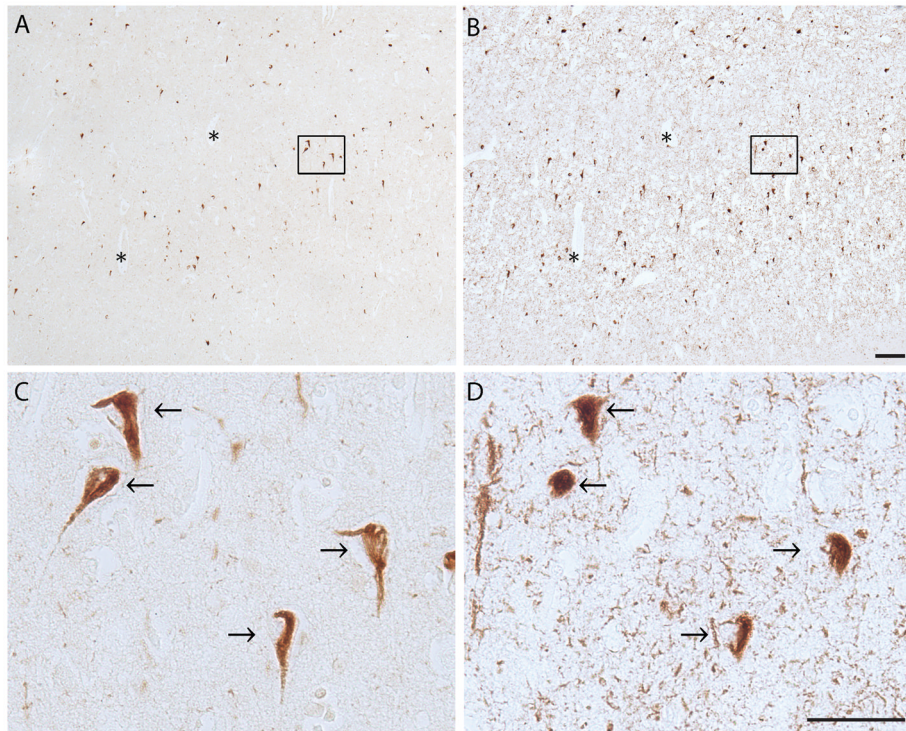
**Figure 4.** Parkinson-amyotrophic lateral sclerosis of Guam (GP-ALS) hippocampus and adjacent cortex and frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17). **(A)** In a case of GP-ALS there is substantial ppRb<sub>ser807</sub> in neurofibrillary tangles (NFTs). Higher magnification (inset) reveals the morphology of the NFTs containing ppRb<sub>ser807</sub>. **(B)** In the tau mutation disorder FTDP-17, the neuritic pathology is prominently labeled with the antibody against ppRb<sub>ser807</sub>. Scale bars: **A, B** = 50  $\mu$ m; Inset = 20  $\mu$ m.



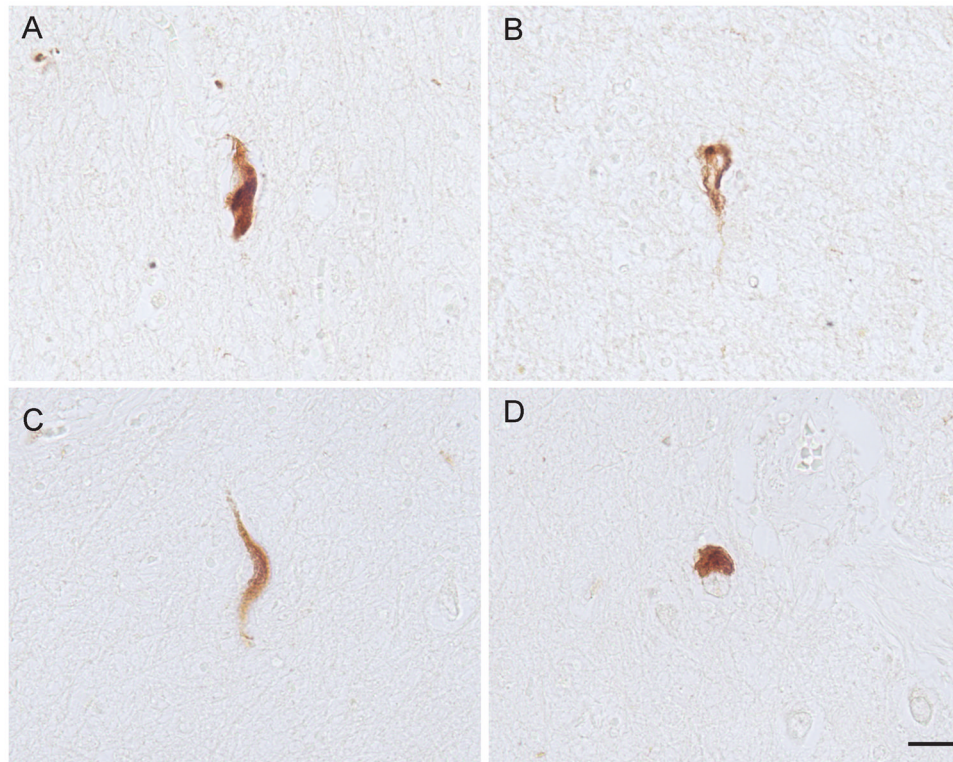
**Figure 5.** Serial sections from the hippocampus of a case of subacute sclerosing panencephalitis (**A-D**). There is colocalization of ppRb<sub>ser807</sub> (**A**) and hyperphosphorylated tau (**B**) in NFTs. Higher magnification demonstrates colocalized labeling of ppRb<sub>ser807</sub> (**C**) and hyperphosphorylated tau (**D**) in many of the same neurons across serial sections. Asterisks denote landmark vessels. Scale bars = 50  $\mu$ m.



**Figure 6.** Neurodegeneration with brain iron accumulation type 1. (**A-D**) Sections immunostained with antibody to ppRb<sub>ser807</sub> (**A, C**) and AT8 staining for hyperphosphorylated tau (**B, D**) show widespread and abundant colocalized labeling of neurofibrillary tangles. Higher magnification of boxes from **A** and **B** reveal colocalization in many of the same neurons (arrows). Asterisks denote landmark vessel. Scale bars: **A, B** = 100  $\mu$ m; **C, D** = 50  $\mu$ m.

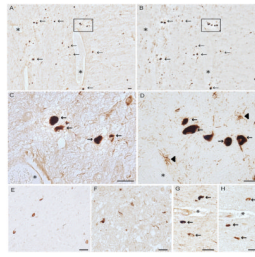


**Figure 7.** Neurofibrillary tangles (NFTs) in the cortex from a case of dementia pugilistica. (A-D) NFTs immunolabeled for ppRb<sub>ser807</sub> display the characteristic morphologies found in the disorder. Scale bar = 20  $\mu$ m.



**Figure 8.** Pick disease. (**A, B**) Pick bodies throughout the hippocampus and cortex accumulate ppRb<sub>ser807</sub> (**A**) and many of the same Pick bodies are also immunopositive for ppRb<sub>S249/T252</sub> (**B**, arrows). Asterisks denote landmark vessel. (**C-E**) Higher magnification images show well-defined Pick bodies immunostained for ppRb<sub>ser807</sub> (**C**), ppRb<sub>S249/T252</sub> (**D**), and the cell cycle marker phosphorylated minichromosome maintenance 2 (**E**). Scale bars: **A, B** = 50  $\mu$ m; **C-E** = 20  $\mu$ m.





**Figure 9.**

Hypothetical mechanism of pRb regulation in tauopathies. The activation of Cdks induces the hyperphosphorylation of pRb and subsequent degradation, which leads the progression of cell cycle to S-phase. The re-activation of cell cycle in post-mitotic neurons might induce hyperphosphorylation of tau and neuronal cell loss. In addition, Cdks, particularly Cdk5, might phosphorylate tau independent of cell cycle regulatory effects (broken line). Cdk5 can directly phosphorylate tau.