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G_1/S cell cycle checkpoint dysfunction in lymphoblasts from sporadic **Parkinson's disease patients.**

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Running header: Cell cycle dysfunction in non-neuronal cells from PD patients.

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ABSTRACT

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease among aging individuals, affecting greatly the quality of their life. However, the pathogenesis of Parkinson's disease is still incompletely understood to date. Increasing experimental evidence suggests that cell cycle reentry of postmitotic neurons precedes many instances of neuronal death. Since cell cycle dysfunction is not restricted to neurons, we investigated this issue in peripheral cells from patients suffering from sporadic PD and age-matched control individuals. Here, we describe increased cell cycle activity in immortalized lymphocytes from PD patients, that is associated to enhanced activity of the cyclin D3/CDK6 complex, resulting in higher phosphorylation of the pRb family protein and thus, in a G_1/S regulatory failure. Decreased degradation of cyclin D3, together with increased p21 degradation, as well as elevated levels of CDK6 mRNA and protein were found in PD lymphoblasts. Inhibitors of cyclin D3/CDK6 activity like sodium butyrate, PD-332991, and rapamycin were able to restore the response of PD cells to serum stimulation. We conclude that lymphoblasts from PD patients are a suitable model to investigate cell biochemical aspects of this disease. It is suggested that cyclin D3/CDK6-associated kinase activity could be potentially a novel therapeutic target for the treatment of PD.

1. INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting to 1.8% of population over 65 years old [1]. PD is associated with selective loss of dopaminergic neurons in the nigrostriatal pathway of the brain, and pathologically it is characterized by the presence of Lewy bodies, whose primary structural component is alpha-synuclein. Nevertheless, it has become increasingly evident that the distribution of Lewy bodies is much greater than formerly appreciated, extending to peripheral nervous systems, most notably, the enteric nervous system [2], where it occurs very early in the disease process [3]. Clinical manifestations include motor abnormalities (tremor, rigidity, slowness, balance problems), autonomic disturbances, and nonmotor symptoms (depression and cognitive impairment). The cause of PD is still unknown. Only in a minority of cases is PD determined by major gene mutations, while approximately 90% of cases are considered sporadic. Nongenetic factors, probably interacting with susceptibility genes are thought to play the most important role in sporadic PD.

The pathogenic mechanisms underlying the selective dopaminergic cell loss in PD are not well understood. Mitochondrial dysfunction, oxidative stress, and protein mishandling seem to play a central role in PD pathogenesis [4]. Moreover, a wealth of data accumulated over the past years, indicate that in PD, as well as in other neurodegenerative diseases, there is a loss of cell cycle control, leading eventually to neuronal death [5-7]. Gene and protein signature in sporadic PD have revealed significant alterations in the expression of genes linked to cell cycle regulation [8].

On the other hand, increasing evidence indicates that peripheral tissue shares significant protein/gene expression similarities to inaccessible central nervous system

(CNS) tissues [9-11] and thus may offer valuable surrogate markers for neuropsychiatric disorders. Peripheral blood mononuclear cells (PBMCs) from PD patients appear to reflect molecular events related to PD pathogenesis, i.e changes in dopamine expression, as well as in the levels of proteins involved in dopamine metabolism, have been described in peripheral cells [12]. In addition, mitochondrial dysfunction [13], and impaired ubiquitin-proteasome system [14] have been reported.

The present work was undertaken to gain insight into the possible role of cell cycle deregulation in PD. For this purpose, we have used lymphoblastoid cell lines obtained by infecting PBMCs with the Epstein Barr virus (EBV). Although the viral infection and transformation could affect the cell-cycle machinery, we have previously demonstrated that the EBV-immortalized lymphocytes retain the cellular response of either PBMCs or CD-19 B-lymphocytes upon stimulation with mitogens, finding similar changes in cell proliferation and survival, as well as in the content of certain cell-cycle regulatory and signaling proteins [15,16]. Therefore, easily accessible blood cells from sporadic PD patients appears be a useful model to study cell cycle-related events in PD pathology, obviating the difficulty of obtaining repeated blood samples from such a vulnerable population as patients suffering from PD.

We report here a serum-mediated enhancement of proliferation of lymphoblasts from sporadic PD patients associated with a cyclin D3/CDK6-dependent hyperphosphorylation of pRb. Upregulation of CDK6 expression, together with alterations in the rate of degradation of cyclin D3 and p21, resulting in higher or lower cellular content respectively, appeared to have a causative role in this phenomenon since cell treatment with specific cyclin D3/CDK6-associated kinase activity inhibitors prevented the increased rates of phosphorylation of pRb and cell proliferation observed in these cell lines.

2. MATERIALS AND METHODS

2.1. Materials

All components for cell culture were obtained from Invitrogen (Carlsbad, CA). The kinase inhibitor PD-332991 was kindly provided by Pfizer. The inhibitor of histone deacetylases sodium butyrate (NaB), cycloheximide (CHX), and rapamycin were obtained from Sigma-Aldrich. PVDF (polyvinylidene difluoride) Immobilon-P membranes for Western blots were purchased from Millipore. Rabbit anti-human antibodies, such as pRb (sc-50), p107 (sc-318), p130 (sc-317), Cyc E (sc-198), CDK6 (sc-177), CDK4 (sc-260), CDK2 (sc-748) and p16 (sc-468), and mouse anti-human antibodies α-tubulin (sc-23948) and p18 (sc-9965) were from Santa Cruz Biotechnologies. Mouse monoclonal antibody anti-human p27 was from BD Transduction Laboratories. Mouse anti-human Cyc D3 and rabbit anti-human antibodies such as Cyc D2 and p21 were obtained from Cell Signaling. Rabbit anti-human TDP-43 antibody was from Proteintech Group. Rabbit anti-human β-actin antibody was from Sigma. Mouse anti-human Lamin B was from Calbiochem. The enhanced chemiluminiscence (ECL) system was from Amersham. All other reagents were of molecular grade.

2.2. Cell lines

A total of 20 patients were diagnosed in the department of Neurology of the University Hospital 12 de Octubre (Madrid, Spain) of sporadic Parkinson disease following Diagnostic Criteria published elsewhere [17]. All patients were undergoing treatment with levodopa. The average age of the patients group was 69±10 and the age range 49-83 years old. A group of 21 age-matched individuals was used as control (average age 68±10, age range 48-83). The control group was formed of family members or caregivers of the PD patients, and showed a completely normal cognitive

and functional level. In all cases peripheral blood samples were obtained after written informed consent of the donors. PBMCs were isolated on Lymphoprep™ density-gradient centrifugation according to the instructions of the manufacturer (Axix-Shield). Cells were washed twice with phosphate buffered saline (PBS), counted, and resuspended at the desired concentration.

Establishment of lymphoblastoid cell lines was performed in our laboratory as previously described [18] by infecting PBMCs with the Epstein Barr Virus (EBV). Cells were grown in suspension in T flasks in an upright position, in approximately 10 mL RPMI 1640 (Gibco, BRL) medium that contained 2 mM L-glutamine, 100 μg/mL penicillin/streptomycin and, unless otherwise stated, 10% (v/v) fetal bovine serum (FBS) and maintained in a humidified 5% CO_2 incubator at 37 ºC. Fluid was routinely changed every 3 days by removing the medium above the settled cells and replacing it with an equal volume of fresh medium.

2.3. Determination of cell proliferation

EBV-immortalized lymphocytes were seeded at an initial cell concentration of 1 x 10^6 x mL^{-1}, and cells were enumerated everyday thereafter. The cell suspension was mixed with a 0.4% (w/v) Trypan Blue solution (Sigma), and the number of live cells was determined using a Neubauer chamber, and, for some experiments, a TC10™ Automated Cell Counter from Bio-Rad Laboratories, S.A. Cells failing to exclude the dye were considered nonviable.

In addition, cell proliferation was assessed in some experiments by the 5-bromo-2'-deoxyuridine (BrdU) incorporation method, using an enzyme-linked immunoassay kit procured from Roche. Cells (100,000 cells/well) were seeded in 96-well microtiter plates. Four hours prior to the end of the interval of measurement, BrdU (10 μM) was

added. The cells were fixed with precooled 70% ethanol for 30 min at -20 ºC and incubated with nucleases following manufacturer's recommendations. Cells were then treated for 30 min at 37 ºC with peroxidase-conjugated anti-BrdU antibody. The excess of antibody was removed by washing the cells three times, followed by the addition of substrate solution. Absorbance was measured at 405 nm with a reference wavelength of 490 nm.

2.4. Preparation of whole-cell extracts and subcellular fractionation

To prepare whole-cell extracts, cells were harvested, washed in PBS and then lysed in ice-cold buffer (50 mM Tris pH 7.4, 150 mM NaCl, 50 mM NaF, 1% Nonidet P-40), containing 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium pyrophosphate and protease inhibitor Complete Mini Mixture (Roche).

To separate the cytosolic and nuclear fractions, cells were harvested, washed in PBS and then lysed in ice-cold hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM ethylendiaminetetraacetic acid (EDTA), 0.1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM PMSF and protease inhibitor mixture). After extraction on ice for 15 minutes, 0.5% Nonidet P-40 was added and the lysed cells were centrifuged at 4000 rpm for 10 minutes. Supernatants containing cytosolic proteins were separated, and nuclei were washed twice with the hypotonic buffer, and then lysed in hypertonic buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM PMSF and protease inhibitor mixture). After extraction on ice for 30 minutes, the samples were centrifuged at 15000 rpm for 15 minutes at 4 ºC. Antibodies to α-tubulin and to lamin B were used to assess the purity of the fractions.

The protein content of the extracts was determined by the Pierce BCA Protein Assay kit (Thermo Scientific).

2.5. Western Blot Analyses

50-100 μg of protein from cell extracts were fractionated on a SDS polyacrylamide gel, and transferred to a PVDF membrane which was then blocked with 1-5% bovine serum albumin (BSA) or 5% nonfat milk, and incubated overnight at 4 ºC, with primary antibodies at the following dilutions: 1:500 anti-pRb, 1:500 anti-p130, 1:500 anti-p107, 1:500 anti-CycE, 1:200 anti-CycD2, 1:500 anti-CycD3, 1:500 anti-CDK2, 1:500 anti-CDK4, 1:1000 anti-CDK6, 1:1000 anti-p21, 1:500 anti-p27, 1:500 anti-p16, 1:200 anti-p18, 1:500 anti-TDP43, 1:5000 anti-β-actin, 1:1000 anti-α-tubulin, 1:1000 anti-Lamin B. Signals from the primary antibodies were amplified using species-specific antisera conjugated with horseradish peroxidase (Bio-Rad) and detected with a chemiluminiscent substrate detection system ECL (Amersham). The specificity of the antibodies used in this work was checked by omitting the primary antibodies in the incubation medium. Protein band densities were quantified using Image J software (NIH, Bethesda, Maryland, USA) after scanning the images with a GS-800 densitometer from Bio-Rad.

2.6. Quantitative Real-Time PCR

Total RNA was extracted from cell cultures using Trizol™ reagent (Invitrogen). RNA yields were quantified spectrophotometrically and RNA quality was checked by the A260/A280 ratio and on a 1.2% agarose gel to observe the integrity of 18S and 28S rRNA. RNA was then treated with DNase I Amplification Grade (Invitrogen). One microgram was reverse transcribed with the Superscript III Reverse Transcriptase kit (Invitrogen). Quantitative real-time PCR was performed in triplicates using TaqMan™ Universal PCR MasterMix No Amperase UNG (Applied Biosystems)

reagent according to the manufacturer's protocol. Primers were designed using the Universal Probe Library for Human (Roche Applied Science) and used at a final concentration of 20 μM. The sequences of the forward and reverse primers used are the following: for CDK6 5'-tgatcaactaggaaaaatcttggac-3' and 5'-ggcaacatctctaggccagt-3'; for cyc D3 5'-ctttcctaaggctgcaaggtt-3' and 5'-caagaagccaaagccagttt-3' (these primers recognized the four transcript variants of *CCND3* gene); for p21 5'-cgaagtcagttccttgtggag-3' and 5'-catgggttctgacggacat-3'; for β-actin 5'-ccaaccgcgagaagatga-3' and 5'-ccagaggcgtacagggatag-3'.

Real time quantitative PCR was performed in the Bio-Rad iQ5 system using a thermal profile of an initial 5-min melting step at 95ºC followed by 40 cycles at 95ºC for 10s and 60ºC for 60s.

Relative mRNA levels of the genes of interest were normalized to β-actin expression using the simplified comparative threshold cycle delta *CT* method [$2^{-(\Delta CT\ gene\ of\ interest - \Delta CT\ \beta\text{-}actin}$].

2.7. Confocal laser scanning microscopy

Immortalized lymphocytes from control and PD individuals were seeded at an initial density of 1 x 10^6 cells x mL^{-1}, synchronized in serum-free RPMI medium for 24 hours, and incubated in the presence of serum for 24 hours. Cells were fixed for 15 minutes in 4% paraformaldehyde, washed in PBS/BSA and cytospinned at 700 rpm for 7 minutes onto poly-L-lysine-coated slides. Then, they were blocked and permeabilized with 0.5% Triton X-100 in PBS-0.5% BSA for 20 minutes at room temperature and incubated with rabbit anti-TDP-43 antibody (Proteintech Group). After washing with PBS, cells were incubated with Alexa Fluor 488-conjugated anti-rabbit antibody. The preparations were mounted on ProLong® Gold Antifade Reagent

with DAPI (Thermo Fisher) and visualized with the LEICA TCS-SP5-AOBS confocal microscope system (Heidelberg, Germany).

2.8. Statistics

Statistical analyses were performed on GraphPad Prism 5 for Macintosh (La Jolla, CA, USA). All the statistical data are presented as mean ± standard error of the mean (SEM). Statistical significance was estimated using an unpaired, two-tailed Student's *t*-test or, when appropriated, by one or two-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. A value of $p<0.05$ was considered significant.

3. RESULTS

3.1 Proliferative activity of lymphoblasts from control and PD patients.

We generated lymphoblastoid cell lines by transforming lymphocytes, derived from control and sporadic PD patients, with the Epstein Barr virus, and compared the proliferative activity of these cell lines by direct cell counting over time. Fig. 1A shows a time-course analysis of the rate of proliferation of lymphoblasts from control and PD patients. For these experiments, cells were seeded at an initial density of 1 x 10^6 cells x mL^{-1}, synchronized by serum-starvation, grown in the presence of 10% FBS and counted for three days thereafter. PD cells exhibited increased proliferation rates compared with those of control cells (Fig. 1A). This observation was confirmed by assessing the rate of BrdU incorporation into DNA (Fig. 1B).

3.2 Expression of key cell cycle regulatory proteins in control and PD lymphoblasts.

Cell cycle disturbances in neurodegenerative disorders appear to involve a G_1/S checkpoint failure [19]. The passage through this restriction point is regulated by several proteins, i.e, the pRb family of proteins, cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors. Therefore, we performed time-course analyses to assess possible differences in their expression levels in PD and control cells.

First, we analyzed the levels and phosphorylation status of pRb and related proteins p130 and p107, that control the passage through the restriction point in late G_1 and commitment to DNA synthesis [20]. When hypophosphorylated, these proteins associate with E2F inhibiting cell cycle progression. The phosphorylation of these proteins causes their dissociation from E2F allowing the cells to enter S phase. Hypo- and hyperphosphorylated forms of these proteins are easily distinguished in SDS-

polyacrylamide gels. Both forms were present at zero time (Fig. 2). Serum addition induced the phosphorylation of pRb, p130, and p107, reaching maximum levels at 24-48 hours as shown in the representative time-course experiments presented in Fig. 2 (upper panel). Further analyses were therefore carried out after 24 hours of serum stimulation. Levels and phosphorylation of these pocket proteins were significantly increased in lymphoblasts from PD patients relative to those found in control cells after 24 hours of serum stimulation (Fig. 2, lower panel). Levels and phosphorylation of these pocket proteins were increased in lymphoblasts from PD patients relative to those found in control cells (Fig. 2). Since pRb and related proteins are sequentially phosphorylated by different cyclin/CDK complexes: cyclin D–CDK4/6 and cyclin E-CDK2 [21,22], we analyzed the levels of those cyclins and CDKs after serum addition. As shown in Fig. 3, we didn't observe any differences in the levels of cyclin D2 or cyclin E between control and PD patients. However, we found substantial higher levels of cyclin D3, 24 h after serum stimulation, in PD lymphoblasts when compared to control cells (Fig. 3, lower panel). Cyclin D1 couldn't be detected in these cells. Moreover, we also observed increased CDK6 levels in PD cells, whereas CDK2 or CDK4 levels remained similar in control and PD patients (Fig. 4A). Fig. 4A (left panel) shows representative immunoblots of time course experiments, while Fig. 4A (right panel) shows the quantified densitometric analyses of the corresponding proteins in cell extracts prepared from cells harvested 24 hours after serum stimulation. As the regulation of these cyclin-CDK complexes may be controlled by CDK inhibitors, we also analyzed the cellular content of different members of the Cip/Kip (p21, p27) and the INK4 (p16, p18) families of CDK inhibitors. Fig. 4B depicts no differences in p27, p16 and p18 content between control and PD patients, whereas p21 levels appear to be significantly lower in PD lymphoblasts than in

control cells (Fig. 4B, right panel).

3.3 Regulation of the cellular content of CDK6, cyclin D3 and p21 in PD lymphoblasts.

To evaluate the molecular mechanisms involved in the changes in the cellular content of CDK6, cyclin D3 and p21 of PD lymphoblasts, we considered whether the differences were due to an altered expression of the genes encoding those proteins or rather to a post-translational mechanism. Fig. 5 depicts the relative changes in mRNA and protein levels of CDK6, cyclin D3 and p21 following 24 hours of serum stimulation. It is shown that CDK6 mRNA levels were higher in lymphoblasts from PD patients than in control (Fig. 5A), in consonance with the protein levels observed (Fig. 5B), while mRNA levels of cyclin D3 and p21 were similar in control and PD (Fig. 5A), despite the differences found in the levels of these two proteins between control and PD cells (Fig. 5B). We then analyzed the half-life of those proteins to evaluate their stability. After 24 hours of serum addition, cells were treated with cycloheximide (CHX) to inhibit the *de novo* protein synthesis, and the cellular levels of the proteins were studied at different times thereafter. As shown in Fig. 6 (upper panel), CDK6 had a long half-life, similar in both control and PD lymphoblasts (26.1 hours and 26.5 hours respectively). Conversely, cyclin D3 and p21 had a much shorter half-life. Inhibition of protein synthesis by cycloheximide led to a rapid degradation of cyclin D3, being the rate of degradation slower in PD patients (where the half-life was 65 minutes) than in control individuals (where the half-life was 40 minutes) (Fig. 6, middle panel). On the other hand, degradation of p21 also appears to be altered in PD lymphoblasts, but in an opposite direction than cyclin D3. Half-life of the protein was 66 minutes in control cells, and 47 minutes in PD lymphoblasts,

indicating a more rapid degradation of the protein in the cells of the patients suffering from PD (Fig. 6, lower panel). Taken together, the data suggest that the increase in CDK6 protein levels may be explained by the higher expression of the corresponding gene, while differences in cyclin D3 and p21 levels seem to depend on changes in the rate of protein degradation.

3.4 Subcellular distribution of TDP-43 and CDK6 expression.

The following experiments were performed based on two pieces of information: first, it was reported that the expression of CDK6, but not of the others CDKs, is negatively regulated by the transactivation response DNA-binding protein 43 (TDP-43) [23], and second, it has been recently reported that TDP-43-positive inclusions occur in different neurodegenerative disorders including PD [24,25]. It is believed that TDP-43 inclusions result from altered trafficking of TDP-43 from the nucleus to the cytoplasm. For these reasons, we considered the possibility that in PD cells, the accumulation of this protein in the cytosol could induce the loss of the nuclear function of TDP-43 repressing the expression of CDK6. To test this idea, we carried out fractionation of cell extracts from control and PD lymphoblasts to enrich for nuclear or cytoplasmic proteins and processed them for western-blot with an anti-TDP-43 antibody. In addition, cells were analyzed by confocal laser imaging after immunostaining. Fig. 7A shows that cytosolic TDP-43 levels were significantly higher in PD cells compared to control lymphoblasts. Unexpectedly, we found that the nuclear levels of TDP-43 were not statistically different between control and PD cells (Fig. 7A). Similar results were observed with confocal laser imaging (Fig. 7C). An analogous finding was reported in a 3xTg mouse model of AD [26], suggesting that the increase of the levels in the cytosolic fraction is not simply due to a redistribution of TDP-43 from the nucleus into the cytoplasm. We studied the levels

of TDP-43 in the whole cell and observed that, in fact, total levels of the protein were increased in PD patients compared to control cells (Fig. 7B), suggesting that the increase in the cytosolic levels of the protein may be due to the general increase in the total content of the protein rather than to a redistribution of the protein from the nucleus.

3.5 Effects of sodium butyrate, PD-332991, and rapamycin on the proliferative activity of control and PD lymphoblasts.

The above-presented results point at the increased expression and activity of the complex cyclin D3/CDK6 as the key factors involved in the overstimulation of PD cells proliferation. For this reason, we were interested in analyzing the role of this complex in PD lymphoblasts and used drugs specifically targeting CDK6: sodium butyrate (NaB), to inhibit the expression of the mRNA CDK6 [27], and a specific inhibitor of CDK6-associated kinase activity developed by Pfizer, PD-332991. On the other hand, we evaluated the effects of rapamycin on proliferation of control and PD lymphoblasts. This drug has been shown to down-regulate cyclin D3 levels by activating its degradation in the proteasome [28]. As shown in Fig. 8, treatment of PD lymphoblasts with NaB (10 μM) or PD-332991 (1 μM) resulted in a significant inhibition of cell proliferation, assessed by direct cell counting (Fig. 8A) or by determining the incorporation of BrdU into DNA (not shown). These doses were selected based in previous work from our laboratory [29]. NaB and PD-332991 changed little the proliferation of lymphoblasts from control individuals. The effects of these drugs on the cellular levels of CDK6, and phosphorylation status of pRb are shown in Fig. 8B. Treatment of PD cells with NaB decreased the content of the CDK6 protein to levels similar to those of control cells. As expected, NaB was able to decrease the phosphorylation status of pRb in lymphoblasts from PD cells (Fig. 8B).

On the other hand, PD-332991, induced dephosphorylation of pRb protein without changes in the CDK6 protein levels in control and PD cells.

The effect of decreasing the cellular cyclin D3 levels by rapamycin on cell proliferation is presented in Fig. 8C. This drug partially inhibited the enhanced proliferation of PD lymphoblasts, affecting very little the proliferative activity of control cells. Fig. 8D shows the effectiveness of rapamycin in decreasing cyclin D3 levels and phosphorylation of pRb protein.

4. DISCUSSION

In this work we provide evidence that immortalized lymphocytes from sporadic PD patients show alterations in cell cycle progression and significant changes in key regulatory molecules of the G_1/S transition checkpoint. These observations are in line with previous reports from this and other laboratories indicating cell cycle deregulation in peripheral cells from patients suffering from neurodegenerative diseases as Alzheimer's disease or frontotemporal dementia [30-33]. Moreover, these cell cycle disturbances were considered systemic manifestations of the disease, as aberrant activation of cell cycle is thought to be underlying neuronal apoptosis [34,35]. Neurons are postmitotic cells that have exited the cell cycle irreversibly and if they are forced to enter the cell cycle, they die.

The increased proliferation of lymphoblasts from PD patients is accompanied by enhanced phosphorylation of pRb protein, thereby allowing the transcription of genes that promote the mechanics of the cell cycle [36]. The higher phosphorylation of pRb observed in PD cells is, most likely, due to the increased levels of the two components of the cyclin D3/CDK6 complex. Despite the fact that pRb is sequentially phosphorylated by 2 sets of protein kinases, the cyclin D/CDK4/CDK6 and cyclin E/CDK2, we did not observe differences in expression levels of other cyclins or CDKs between control and PD lymphoblasts. In addition, we found a decrease in the levels of the CDK inhibitor p21, which can also contribute to the increased phosphorylation of pRb.

The results presented herein indicate that altered levels of cyclin D3 and p21 observed in PD cells are due to altered degradation of these proteins. A longer half-life of cyclin D3 protein was detected in PD lymphoblasts as compared with controls cells, while p21 seems to be degraded more rapidly in PD cells. Changes in the rate of

degradation of cyclin D3 and p21 are not due to nonspecific impairment of general protein degradation in lymphoblasts from PD cells, as half-life of CDK6 is not altered. These results are in agreement with previous work indicating that both p21 and cyclin D3 content are translationally regulated in a number of cell types [37,38]. The PI3K/Akt/mTOR pathway seems to be involved in cyclin D3 degradation and enhanced cell proliferation [28,39]. Thus, the possibility should be considered that this survival pathway is overactivated in PD cells.

Upregulation of CDK6 in PD cells appears to occur at the transcriptional level. Quantitative RT-PCR analyses demonstrated higher levels of CDK6 mRNA in lymphoblasts derived from PD patients when compared with those in control cells. The transcriptional regulation of CDK6 is achieved through distinct mechanisms. We investigated a possible relation between CDK6 expression and nuclear levels of TDP-43 protein. TDP-43 represses CDK6 expression by binding to GT repeats that are present, once or twice, in the long exons of CDK6, but not in other human CDK6 genes [23]. On these grounds, we considered the possibility of a loss of function of TDP-43 secondary to changes in the nuclear content of this protein in PD cells as it has been described to occur in frontotemporal lobar degeneration [40,41]. Significant changes in both total and cytosolic levels of TDP-43 were found in PD lymphoblasts, however, we couldn't detect diminished levels of nuclear TDP-43 to account for the higher CDK6 mRNA levels of PD cells. Nonetheless it is worth to mention that other authors reported increased expression of CDK6 by TDP-43 in CHO-K1 cells, apparently stabilizing its mRNA rather than inhibiting pre-mRNA splicing [42]. It remains to be resolved whether or not the TDP-43-mediated control of CDK6 expression is cell specific, and the role of TDP-43, if any, in the upregulation of CDK6 and enhanced proliferation found in PD cells.

On the other hand, CDK6 content appears to be negatively regulated by several miRNAs including miR-29 [43], miR-16 [44], miR-22 [45] and miR-34b [46], the levels of which have been shown to be downregulated in PD patients [47-49]. Thus, it is possible that CDK6 abundance in peripheral cells from PD patients may depend on both transcriptional and post-transcriptional events. Further work will be needed to clarify this point.

In summary, our results established a molecular link between increased CDK6 and cyclin D3 levels, pRb phosphorylation, and proliferation of lymphoblasts from sporadic PD patients. Activation of the pRb pathway has been reported in PD brain and in mouse models of PD [7], although in neurons this leads to cell death rather than to cell division. Thus, our results support the hypothesis that unscheduled cell cycle activation underlies neurodegeneration, and highlight the usefulness of peripheral cells from PD patients as potential surrogate for diagnosis and therapeutic monitoring of PD. It is suggested that therapeutic interventions aimed at ameliorating cell cycle progression activity may have a positive impact in PD. On these grounds, it is worth mentioning that rapamycin, NaB, and PD-332991 are able to restore the normal cell response by decreasing the levels of cyclin D3, CDK6 expression, or the associated kinase activity.

NaB and rapamycin have been shown to have neuroprotective properties in various PD-related experimental models in vitro and in vivo [50-53]. Rapamycin and CDK6 inhibitors are already being used as immunosuppressant and for treatment of a number of human tumors, respectively [54,55], with a good tolerance. Therefore, we believe that the cyclin D3/CDK6 complex could be considered as a novel therapeutic target for PD, and that the repositioning of these drugs towards PD treatment, may offer a significant advantage over traditional drug development.

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FIGURE LEGENDS

Figure 1

Proliferation of lymphoblasts from control and PD patients

A. Control and PD lymphoblasts were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and synchronized by serum starvation for 24 hours. On that point, 10% FBS was added and cells were incubated for 72 hours. Samples were taken every 24 hours after serum addition and counted in a Neubauer chamber. Values shown represent mean ± SEM of different experiments carried out with 20 control and 20 PD patients. Every individual was counted at least twice. B. After synchronization by serum starvation for 24 hours, 100,000 cells/well were seeded in 96-well plates in the presence of 10% FBS. After 24 hours, 10 μM BrdU was added to the medium for 4 hours. BrdU incorporation into DNA was detected according to manufacturer instructions. The magnitude of the absorbance is proportional to the quantity of BrdU incorporated into cells, which is a direct indicator of cell proliferation. Data represent the mean ± SEM of different experiments carried out with 4 control and 4 PD patients. *$p<0.05$, significantly different from control cells.

Figure 2

Retinoblastoma protein (pRb) family levels and phosphorylation status

Lymphoblasts from control and PD patients were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and synchronized by serum starvation for 24 hours. 10% FBS was added and cells were incubated for 72 hours. Four million cells were taken every 24 hours after serum addition and whole-cell lysates were prepared to perform Western Blot analyses. pRb-family protein levels were detected using phospho-specific anti-pRb, anti-p130 and anti-p107 antibodies. Representative immunoblots for time-

course experiments are shown. In the lower panel it is shown the mean±SEM of the quantification of the band densities 24 hours after serum addition, when the proteins reach the maximum levels. A total of 5 control and 5-7 PD patients were analyzed. *$p<0.05$, significantly different from control. A representative experiment is shown. pp = the hyperphosphorylated form of the corresponding protein.

Figure 3

Time course analysis of the cyclins involved in G_1-S in control and PD patients

Immortalized lymphocytes from control and PD patients were seeded at an initial density of 1 x 10^6 cells x mL^{-1}, synchronized by serum-starvation and incubated for 72 hours in the presence of 10% FBS. Whole-cell lysates where prepared every 24 hours after serum addition to analyze cyclin D2, cyclin D3 and cyclin E levels by Western Blot. Representative immunoblots for time-course experiments are shown. Values shown in the lower panel represent the mean±SEM of the quantification of the band densities 24 hours after serum addition. A total of 8-14 control and 8-16 PD patients were used in the experiments. *$p<0.05$, significantly different from control.

Figure 4

Time course analysis of the CDKs and CDK inhibitors involved in G_1-S in control and PD patients

Lymphoblasts from control and PD patients were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and synchronized by serum starvation for 24 hours. 10% FBS was added and cells were incubated for 72 hours. Whole-cell lysates were prepared every 24 hours after serum addition to analyze CDK family (CDK2, CDK4 and CDK6) levels, as well as Cip/Kip (p21, p27) and INK4 (p16, p18) families of CDK inhibitors levels by Western Blot. A, B (left panels). Representative immunoblots for time-course experiments are shown. A, B (right panels). Values shown represent the

mean±SEM of the quantification of the band densities 24 hours after serum addition. 8-14 control and 8-16 PD patients were analyzed. **p<0.01, significantly different from control.

Figure 5

Protein and mRNA levels of CDK6, cyclin D3 and p21 in control and PD lymphoblasts

Lymphoblasts from control and PD patients were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and synchronized by serum starvation for 24 hours. 10% FBS was added and cells were incubated for 24 hours to perform the following experiments: A. RNA was extracted, reverse-transcribed and subjected to qRT-PCR to analyze the differences in mRNA content. β-actin was used as housekeeping gene. Values shown represent the mean ± SEM of different experiments carried out with 8-9 control and 8-10 PD patients. *p<0.05, significantly different from control cells. B. Whole-cell extracts were prepared to analyze by Western-Blot the levels of CDK6, cyclin D3 and p21. A representative experiment is shown. Data represent mean ± SEM of different experiments carried out with 14 control and 16 PD patients. β-actin was used as loading control. *p<0.05, significantly different from control, **p<0.01, significantly different from control.

Figure 6

Degradation rate of CDK6, cyclin D3 and p21 in control and PD lymphoblasts

Experiments were performed in the same conditions as Figure 5. 24 hours after serum addition, cycloheximide (CHX) (20 μg/mL) was added to the medium and cells were harvested at 0, 6, 12, 24 and 36 hours thereafter for CDK6 experiments; or 0, 0.5, 1, 1.5, 2, 3 hours thereafter for cyclin D3 and p21 experiments. Whole-cell extracts were

prepared at those times to analyze the protein content by Western-blot. A representative experiment is shown. The graph shows the decay of the protein signal, taking the levels of the protein of either control or PD lymphoblasts at time = 0 hours as 100%. Results represent the mean ± SEM of different experiments carried out with 4 control and 4 PD patients

Figure 7

Subcellular localization and content of TDP-43 in control and PD lymphoblasts

Control and PD lymphoblasts were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and serum-starved for 24 hours. After that, 10% FBS was added and cells were incubated for 24 hours. Lymphoblasts were then collected for the different experiments: A. Cells were lysed as described in Methods to separate nuclear and cytosolic fragments and then analyzed by Western Blot. Lamin B and α-tubulin antibodies were used as loading and purity control of the nuclear and cytosolic fractions, respectively. A representative experiment is shown. Data represent mean ± SEM of different experiments using 11 control and 11 PD patients. **$p<0.01$ significantly different from control cytosol. B. Whole-cell extracts were prepared to analyze the total content of TDP-43 of the cells by Western Blot. A representative experiment is shown. Data represent mean ± SEM of different experiments carried out with 12 control and 12 PD patients. β-actin was used as loading control. **$p<0.01$ significantly different from control. C. Subcellular localization of TPD-43 was also studied by confocal microscopy. Cells were stained with anti-TDP-43 antibody followed by secondary antibody labeling with Alexa Fluor 488. DAPI was included in the mounting media to stain the nucleus. Merged images show a predominant nuclear localization of TPD-43, being cytosolic localization higher in PD patients than in control cells. Magnification: 63X.

Figure 8

Effects of NaB, PD-332991, and rapamycin on the proliferative activity of control and PD lymphoblasts

For all the experiments, control and PD lymphoblasts were seeded at an initial density of 1 x 10^6 cells x mL^{-1} and synchronized by serum starvation for 24 hours. On that point, 1 μM PD-332991, 10 μM NaB, or 30 ng/mL rapamycin plus 10% FBS were added. A. Cells were incubated for 72 hours. Samples were taken every 24 hours after serum and drugs addition and counted using an Automated Cell Counter from Bio-Rad. Graph represents the number of cells in the different conditions after 72 hours of serum addition. Values shown represent mean ± SEM of different experiments carried out with 8 control and 8 PD patients. ** $p<0.01$ different from control cells, +$p<0.05$ different from untreated PD cells, ++ $p<0.01$ different from untreated PD cells. Every individual was counted at least twice. B. After 24 hours of serum and drugs addition, whole-cell extracts were prepared to analyze by Western Blot the cellular content of CDK6 and the phosphorylation status of pRb protein. A representative experiment is shown. C. Experimental conditions were identical to those described in A. Rapamycin was added at the concentration of 30 ng/mL. Data represent the mean ± SEM of different experiments carried out with 4 control and 4 PD patients. **$p<0.01$, significantly different from control cells, ++$p<0.01$ different from untreated PD cells. D. A representative experiment showing the effects of rapamycin on cyclin D3 levels and phosphorylation of pRb.

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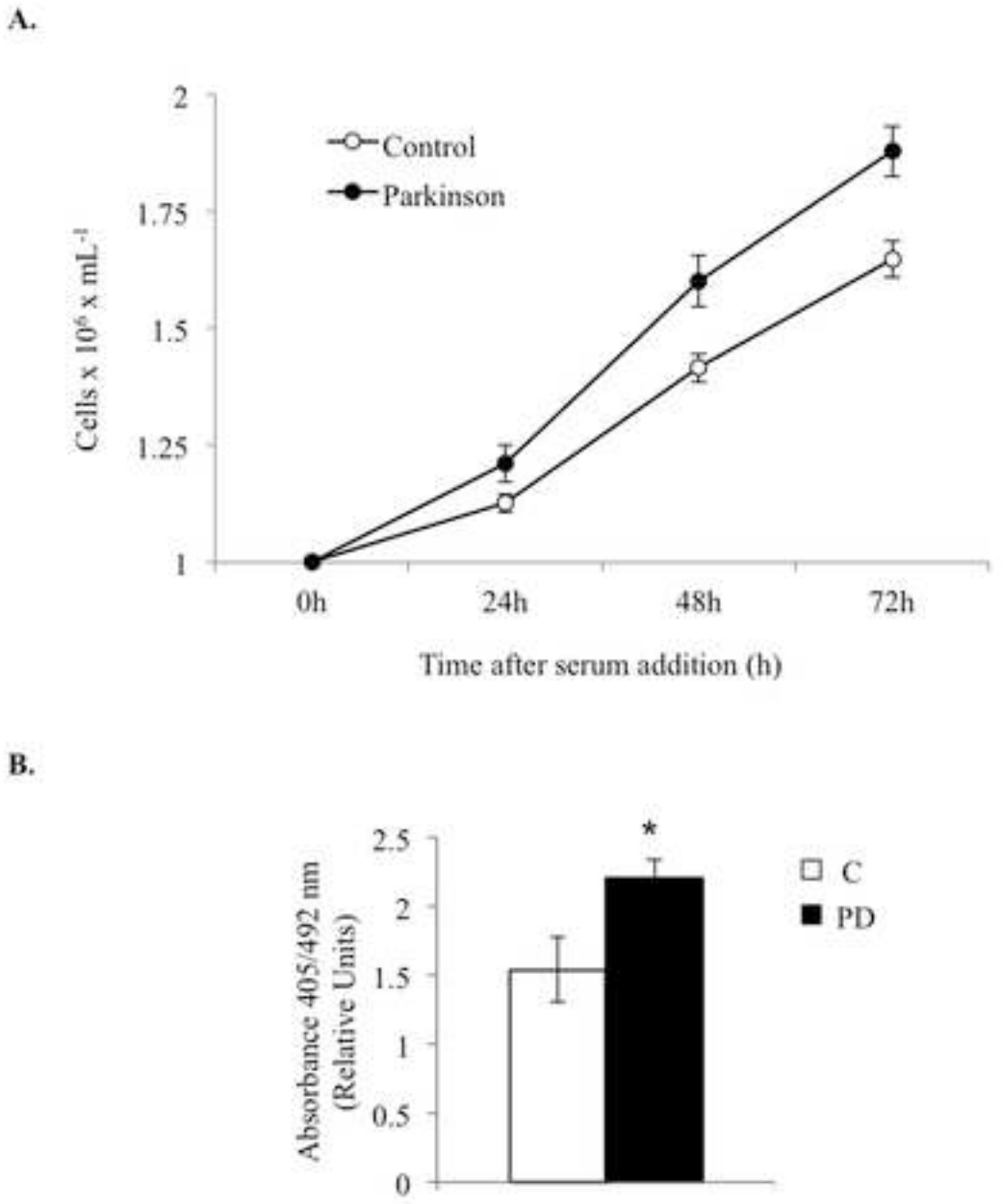

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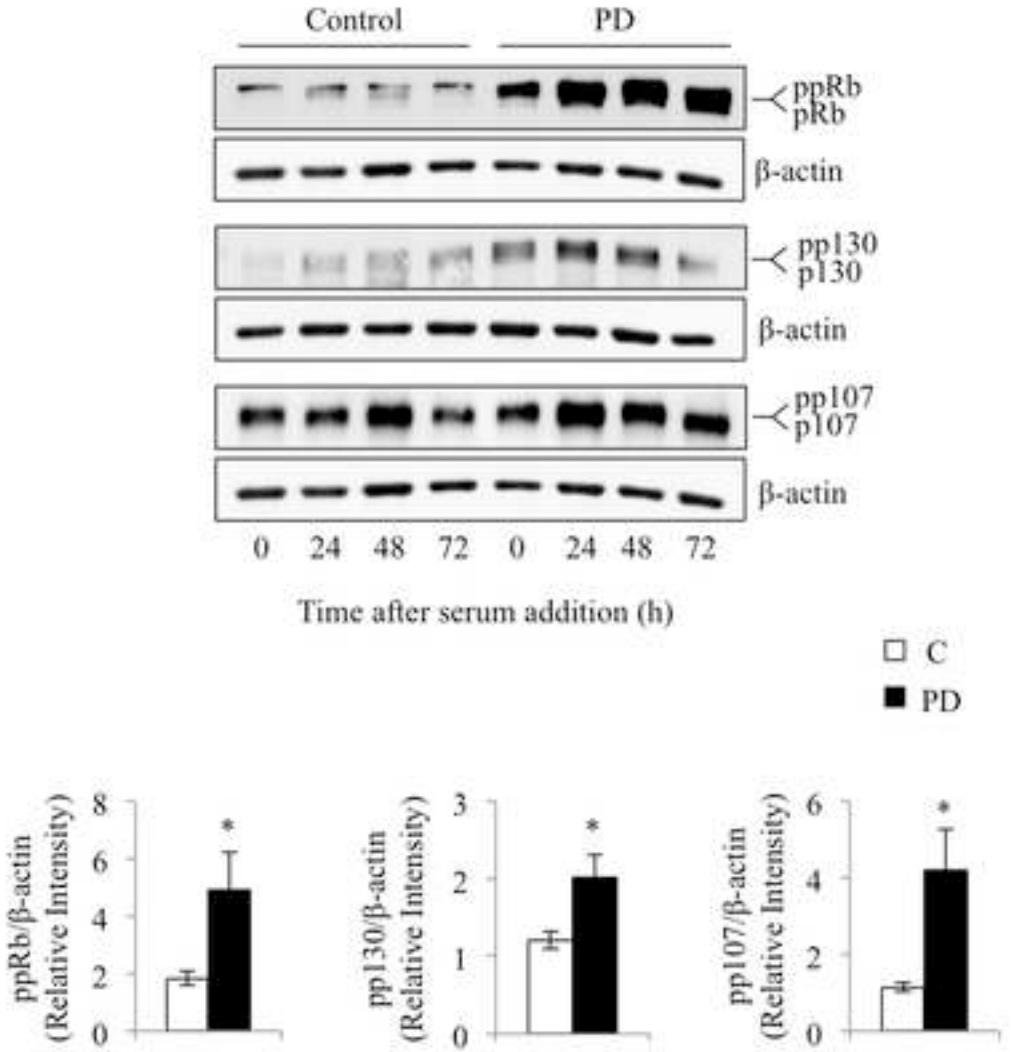

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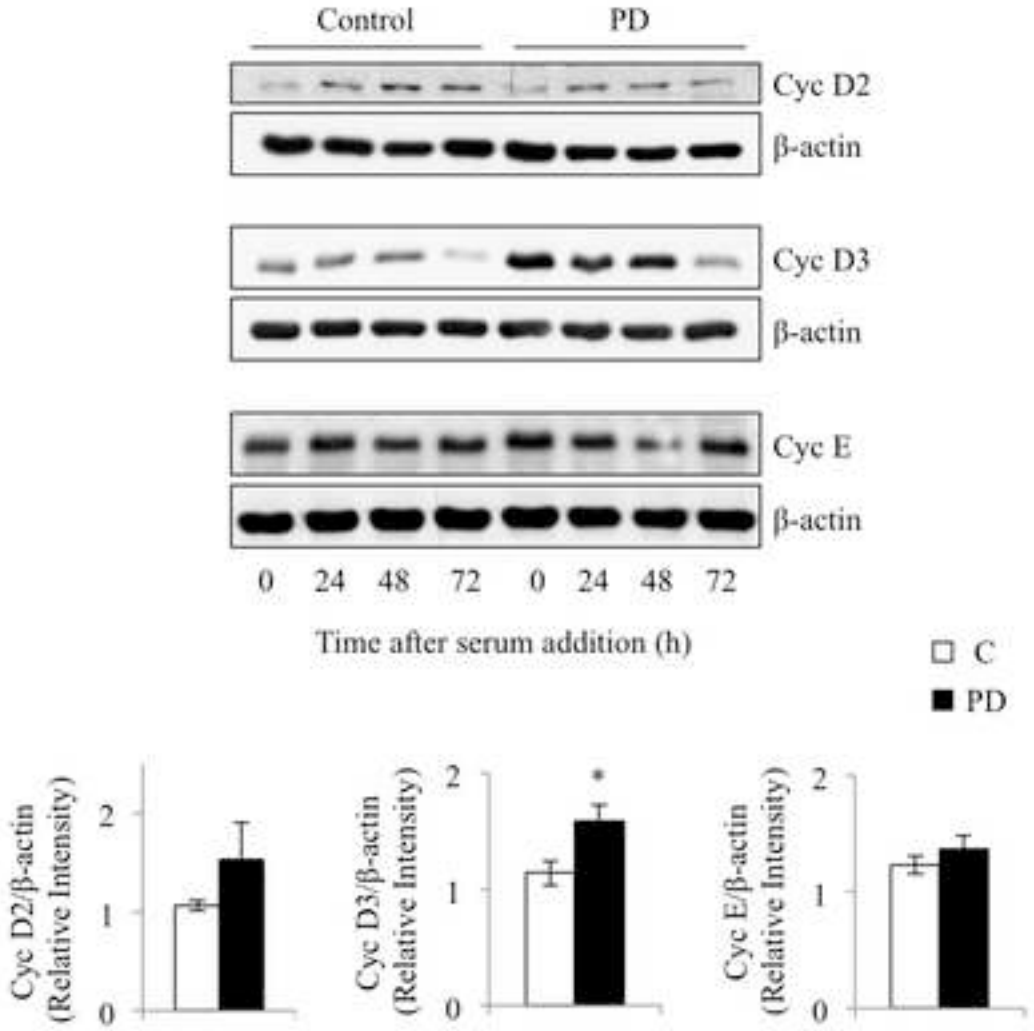

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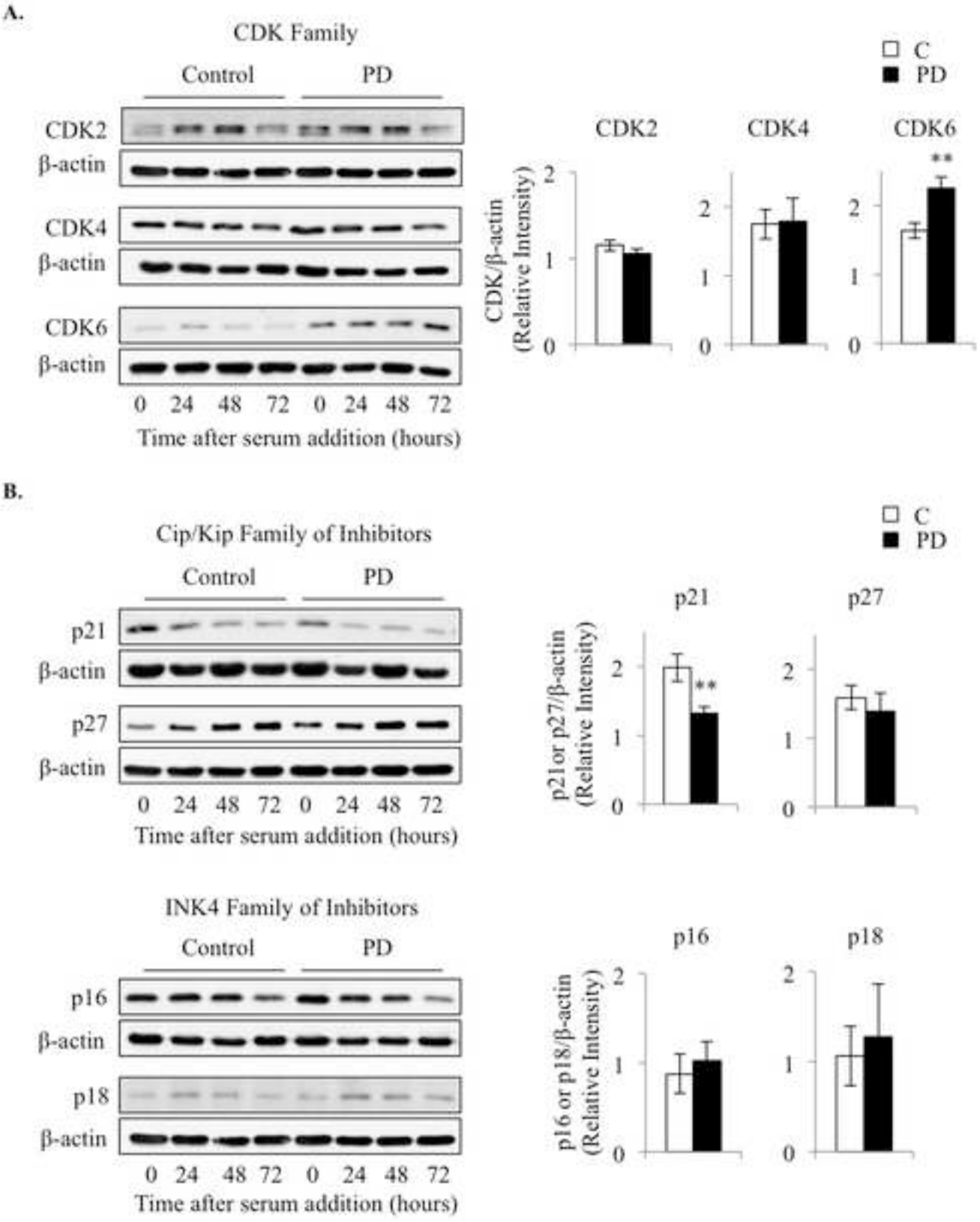

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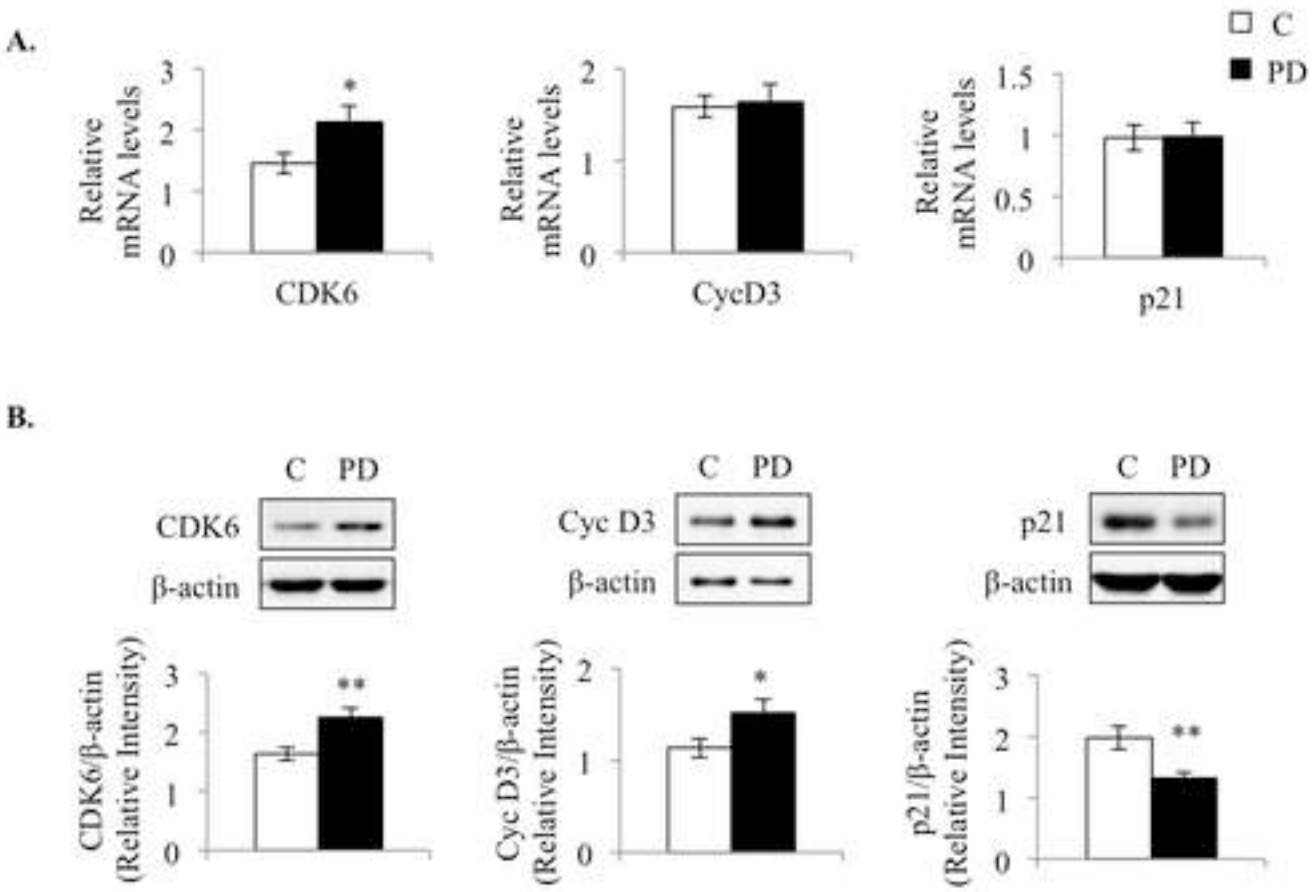

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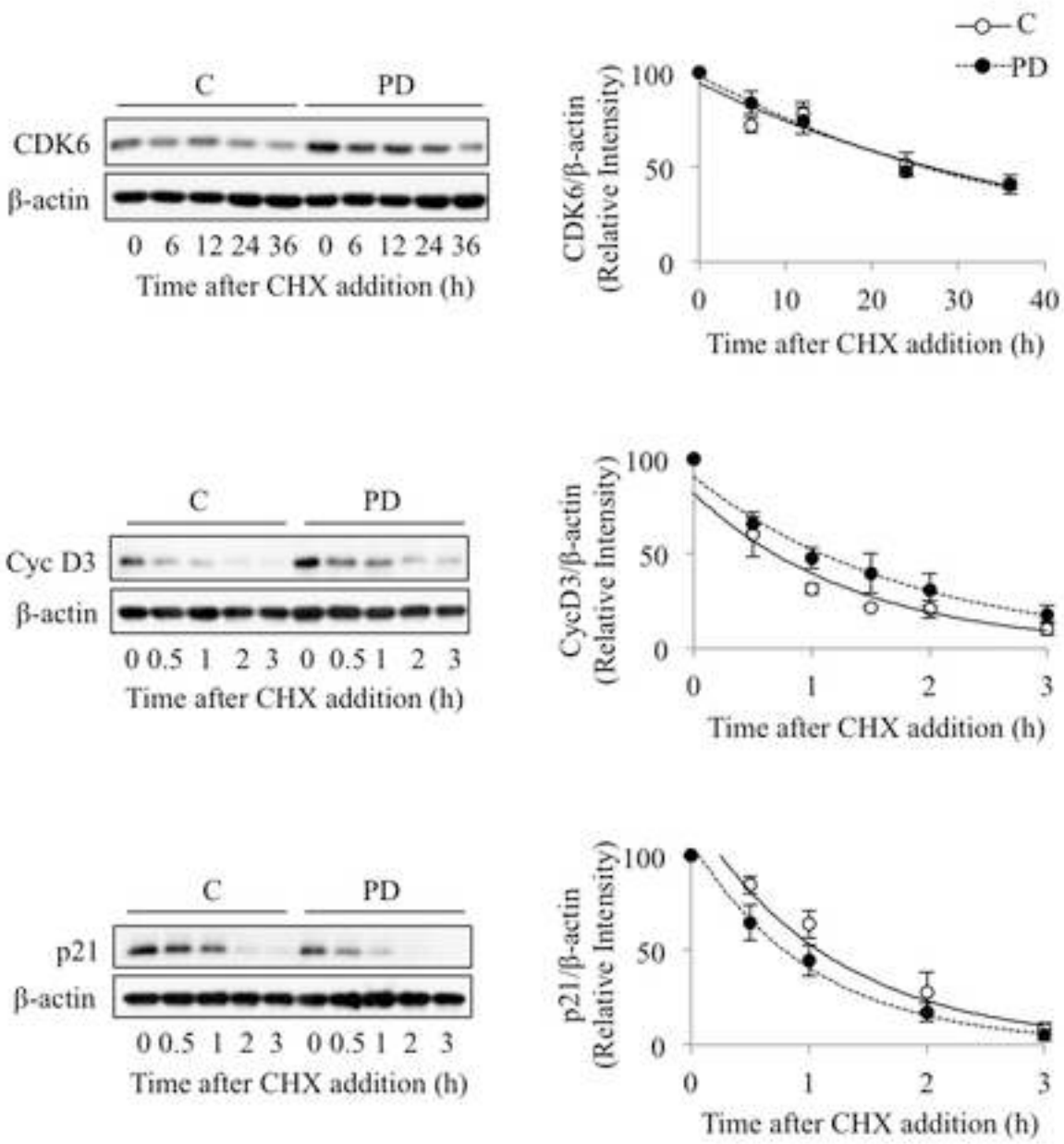

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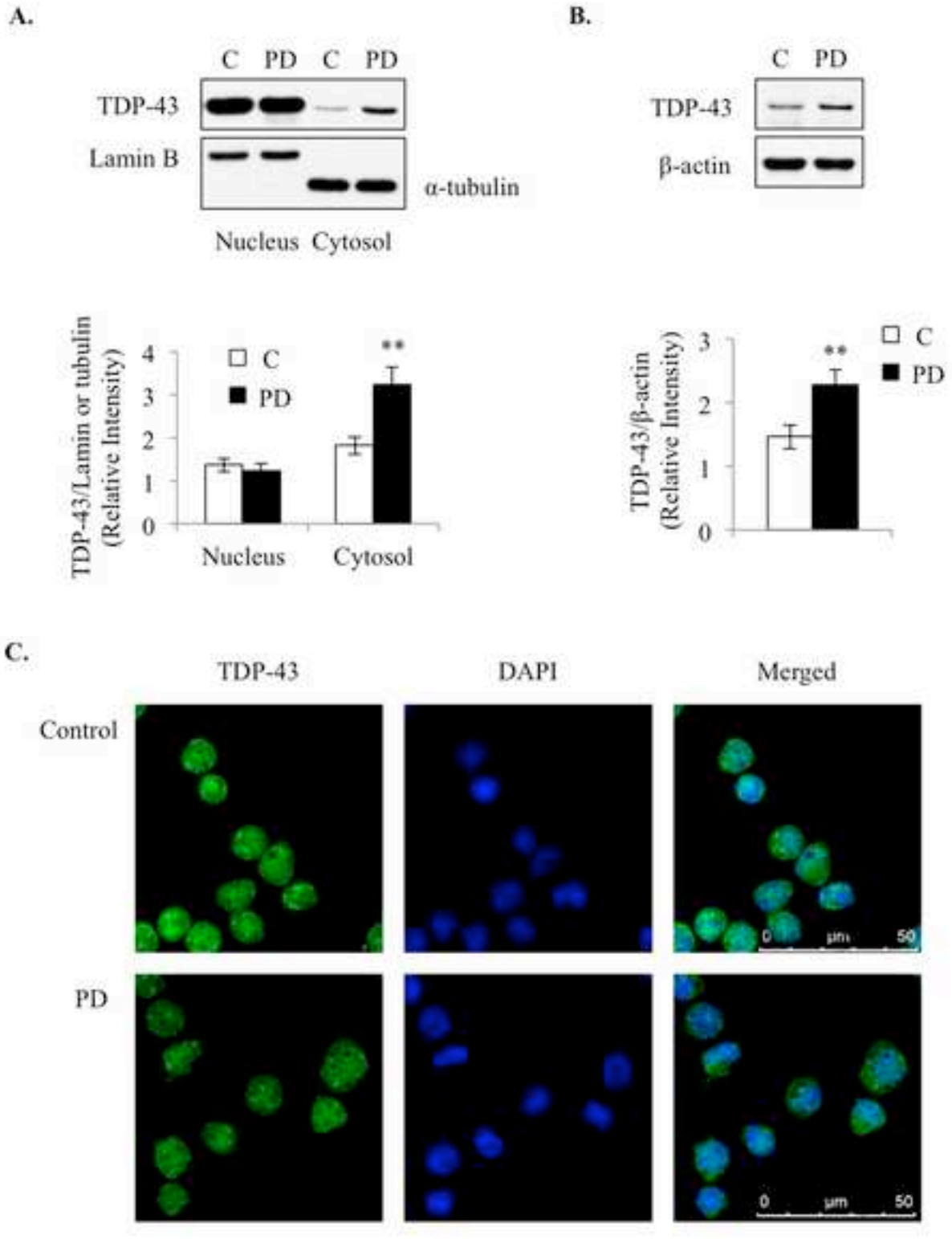

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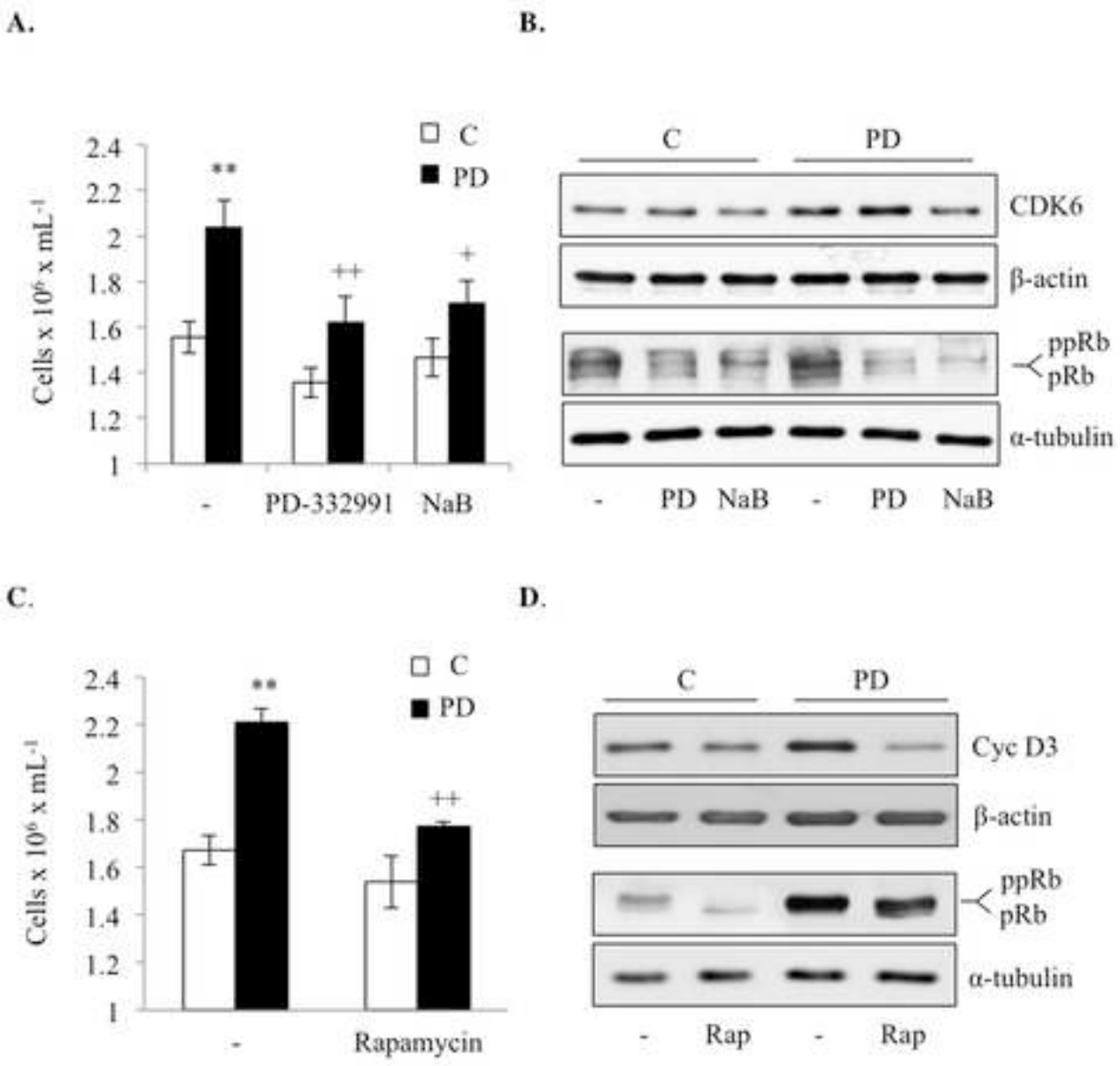

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