

PH domain leucine-rich repeat protein phosphatase 1 contributes to maintain the activation of the PI3K/Akt pro-survival pathway in Huntington's disease striatum

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Dysregulation of gene expression is one of the mechanisms involved in the pathophysiology of Huntington's disease (HD). Here, we examined whether mutant huntingtin regulates the levels of PH domain leucine-rich repeat protein phosphatase 1 (PHLPP1), a phosphatase that specifically dephosphorylates Akt at Ser473. Our results show decreased PHLPP1 protein levels in knock-in models (Hdh^{Q111/Q111} mouse striatum and STHdh^{Q111/Q111} cells), in the striatum of N-terminal exon-1 mutant huntingtin transgenic mouse models (R6/1; R6/1 : BDNF +/–, R6/2 and Tet/HD94) and in the putamen of HD patients. Quantitative PCR analysis revealed a reduction in PHLPP1 mRNA levels in the striatum of R6/1 compared with wild-type mice. Coincident with reduced PHLPP1 protein levels, we observed increased phosphorylated Akt (Ser473) levels specifically in the striatum. The analysis of the conditional mouse model Tet/HD94 disclosed that after mutant huntingtin shutdown PHLPP1 levels returned to wild-type levels whereas phospho-Akt levels were partially reduced. In conclusion, our results show that mutant huntingtin downregulates PHLPP1 expression. In the striatum, these reduced levels of PHLPP1 can contribute to maintain high levels of activated Akt that may delay cell death and allow the recovery of neuronal viability after mutant huntingtin silencing.

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Huntington's disease (HD) is a neurodegenerative disorder characterized by motor dysfunction, cognitive impairment and emotional disturbances.¹ The causative mutation of HD is a dominantly heritable expansion of a trinucleotide CAG repeat in the coding region of the *huntingtin* gene, which results in long stretches of polyglutamine in the N-terminal portion of the huntingtin (htt) protein.² Although htt is ubiquitously expressed in the central nervous system,³ its mutation induces a selective regional and temporal pattern of neurodegeneration. The more severe and early degeneration affects striatal projection neurons whereas loss of cortical neurons is observed at late stages of the disease.⁴ The molecular basis that account for this specific neurodegeneration is still unknown, but different mechanisms have been proposed to contribute to the neurodegenerative process.⁵

Mature neurons have the ability to activate survival mechanisms in response to harmful stimuli. Therefore, whether a neuron lives or dies in pathological conditions is the result of a complex balance between anti- and pro-apoptotic signals,

respectively. One of the central components in signaling pathways promoting neuronal survival is the serine/threonine kinase Akt, also known as protein kinase B.⁶ This kinase is present at low levels in the adult brain,⁷ but its expression and activation increase dramatically in neurons during cellular stress or injury.⁸ For instance, activated Akt has been proposed as an important neuroprotective pathway in distinct acute^{7,9,10,11} and chronic^{12,13} models of neurodegeneration. In HD, Akt has been proposed as a crucial neuroprotective pathway because it is one of the serine/threonine kinases that phosphorylate the Ser421 of mutant htt (mhtt) attenuating its toxicity.¹⁴ In accordance, enhanced levels of phosphorylated Akt (pAkt) appear as an early striatal pro-survival response in the HD knock-in mouse model Hdh^{Q111} and in the cell model STHdh^{Q111/Q111}.¹²

Cells maintain a dynamic steady state of pAkt, balanced by the activity of kinases and phosphatases.¹⁵ Akt is activated through phosphorylation at Thr308 or Ser473¹⁶ in response to the activation of the phosphatidylinositol 3-kinase (PI3K), and its signaling is acutely terminated by dephosphorylation.

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Abbreviations: BDNF, brain-derived neurotrophic factor; FoxO1, Forkhead box 1 transcription factor; GSK3 β , glycogen synthase kinase 3 beta; HD, Huntington's disease; htt, huntingtin; mhtt, mutant huntingtin; NMDAR, *N*-methyl-D-aspartate glutamate receptor; pAkt, phosphorylated Akt; PCR, (Q)-polymerase chain reaction; PHLPP1, PH domain leucine-rich repeat protein phosphatase 1; PI3K, phosphatidylinositol 3-kinase; QUIN, quinolinic acid

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Protein phosphatase 2A (PP2A)-like phosphatases act on the Thr308 site,¹⁵ whereas the hydrophobic motif of Akt (Ser473) is directly dephosphorylated by the PH domain leucine-rich repeat protein phosphatase (PHLPP)¹⁷ resulting in effective signal termination.^{18,19} PHLPP1, a member of the PHLPP family of phosphatases, was first described as suprachiasmatic nucleus circadian oscillatory protein and shown to be highly expressed in brain neurons.²⁰ Recently, it has been shown that PHLPP1 participates in the regulation of MAPK signaling required for some forms of hippocampus-dependent memory,²¹ and that changes in total PHLPP1 protein levels are important in regulating the degree of Akt phosphorylation at Ser473 in the hippocampus.²² Because the presence of mhtt can result in alteration of gene expression,²³ in the present work we sought to determine whether mhtt could induce changes in PHLPP1 protein levels contributing to maintain high levels of pAkt (Ser473) in transgenic mouse models and in the brain of HD patients.

Results

PHLPP1 protein levels are decreased in knock-in models of HD. To investigate whether altered PHLPP1 levels could be responsible for the previously reported accumulation of pAkt (Ser473) in knock-in HD models,¹² we looked at PHLPP1 protein levels in wild-type (STHdh^{Q7/Q7}) and mutant (STHdh^{Q111/Q111}) striatal cells, and in the striatum of 5-month-old wild-type (Hdh^{Q7/Q7}) and mutant (Hdh^{Q111/Q111}) knock-in mice. As shown in Figure 1, PHLPP1 protein levels were decreased in both STHdh^{Q111/Q111} cells and Hdh^{Q111/Q111} mouse striatum compared with their respective controls. This decrease was more accentuated in the cell model (56 ± 5%, Figure 1a) than in the mouse model (36 ± 15%, Figure 1b).

We next examined whether PHLPP1 could dephosphorylate pAkt (Ser473) in neural cells because this mechanism has been described only in human non-neural cells.¹⁷ STHdh^{Q7/Q7} cells were transfected with PHLPP1, and pAkt (Ser473 and Thr308) levels were analyzed by western blot 24 h after transfection. As shown in Figure 2, pAkt (Ser473; Figure 2a), but not pAkt (Thr308; Figure 2b), levels were diminished in cells overexpressing PHLPP1, indicating that transient overexpression of this phosphatase can modulate pAkt (Ser374), but not pAkt (Thr308), levels also in neural cells.

Activation of Akt in STHdh^{Q7/Q7} and STHdh^{Q111/Q111} cells is dependent on PI3K.¹² Thus, we next assessed whether reduced PHLPP1 protein levels in STHdh^{Q111/Q111} cells could affect the rate of Akt dephosphorylation when the activation signal is blocked. To this end, we treated STHdh^{Q7/Q7} and STHdh^{Q111/Q111} cells with wortmannin, a PI3K inhibitor, and pAkt (Ser473) levels were assessed at different time points. In wild-type cells, Ser473 was dephosphorylated with a half-time of 10 min whereas in mhtt expressing cells it took about 30–40 min (Figure 2c). These data thus suggest that reduced levels of PHLPP1 participate in maintaining high levels of pAkt (Ser473) in STHdh^{Q111/Q111} cells.

N-terminal exon-1 mhtt transgenic mouse models also display reduced PHLPP1 protein levels. To find out if

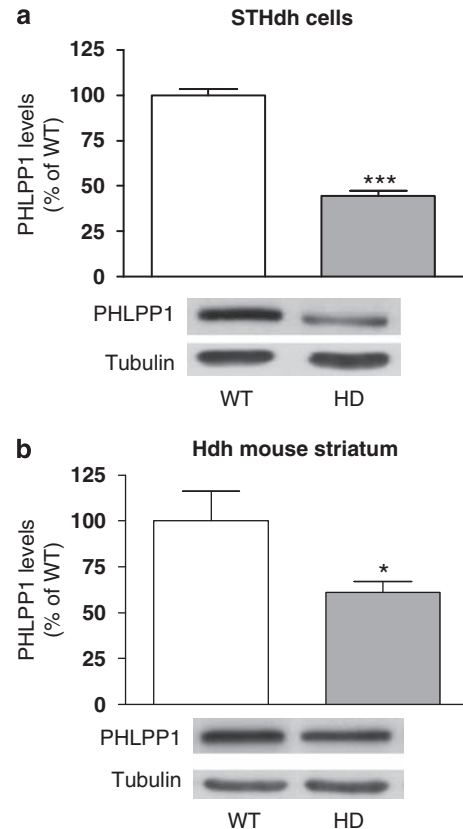


Figure 1 Knock-in models of HD display decreased levels of PHLPP1. (a) Protein extracts from wild-type (STHdh^{Q7/Q7}; WT) and mhtt (STHdh^{Q111/Q111}; HD) knock-in striatal cells, and (b) from the striatum of 5-month-old wild-type (WT) and knock-in (HD) over mice were subjected to western blot to analyze PHLPP1 protein levels. Bar graphs, obtained by densitometric analysis of western blot data, show the decrease of PHLPP1 protein levels in both STHdh^{Q111/Q111} striatal cells and knock-in mice striatum. Results (mean ± S.E.M.; n = 4–6) represent the ratio between PHLPP1 and α -tubulin levels, and are expressed as percentage of WT values. Data were analyzed by Student's *t*-test. **P* < 0.05 as compared with WT mice, and ****P* < 0.001 as compared with STHdh^{Q7/Q7} cells. A representative immunoblot is shown for each condition

PHLPP1 protein levels are also deregulated in the striatum of N-terminal exon-1 models, we first analyzed two transgenic mouse models that express different levels of endogenous brain-derived neurotrophic factor (BDNF; R6/1 and R6/1:BDNF +/–), and show a distinct temporal pattern of disease progression.²⁴ To this end, we performed western blot in striatal protein extracts obtained from 4-, 8-, 12-, 16- and 30-week-old mice. We found that PHLPP1 levels were unchanged at 4 or 8 weeks of age when compared to wild-type levels (Figure 3a). In contrast, PHLPP1 levels were similarly decreased in R6/1 and R6/1:BDNF +/– mice at 12 (40 ± 2 and 46 ± 3%, respectively), 16 (38 ± 4 and 33 ± 3%, respectively) and 30 (52 ± 4 and 52 ± 2%, respectively) weeks of age (Figure 3a), indicating that BDNF levels have no effect on PHLPP1 expression. To determine whether the reduction of PHLPP1 levels was a general mechanism triggered by neurons in response to mhtt expression, we also determined its protein levels in the cortex and

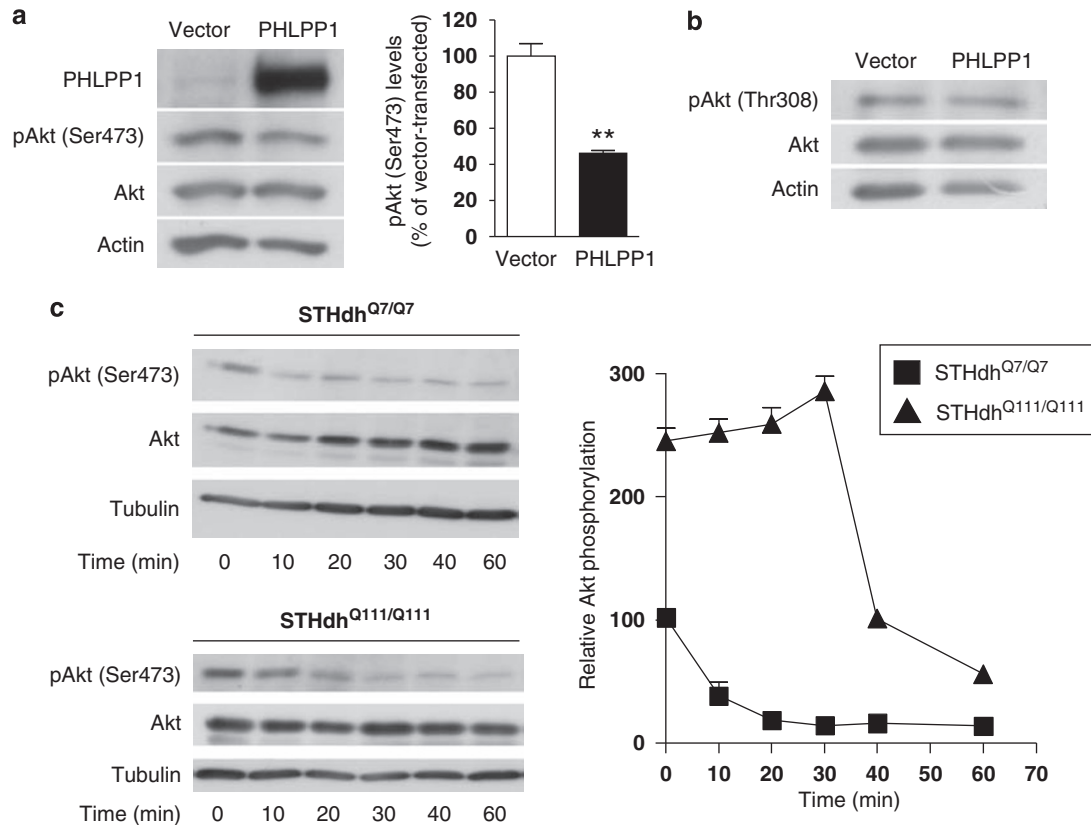


Figure 2 PHLPP1 regulates pAkt (Ser473) levels in striatal cells. **(a)** PHLPP1 overexpression reduces pAkt (Ser473) levels in STHdh^{Q7/Q7} cells. Protein levels of pAkt (Ser473) in STHdh^{Q7/Q7} cells were analyzed by western blot 24 h after transfection with a control or a PHLPP1 vector. Data are expressed as percentage of pAkt (Ser473) levels (pAkt/Akt ratio, after normalization to actin) respect to vector-transfected STHdh^{Q7/Q7} cells, and shown as mean \pm S.E.M. ($n=3$). Data were analyzed by the Student's *t*-test. $**P < 0.01$ as compared with cells transfected with a control vector. **(b)** PHLPP1 overexpression does not modify pAkt (Thr308) levels in STHdh^{Q7/Q7} cells. Protein levels of pAkt (Thr308) in STHdh^{Q7/Q7} cells were analyzed by western blot 24 h after transfection with a control or a PHLPP1 vector. **(c)** Striatal cells expressing mhtt show reduced rate of Akt dephosphorylation in the presence of wortmannin. Graph shows the time course of Akt dephosphorylation in wild-type (STHdh^{Q7/Q7}) and mhtt (STHdh^{Q111/Q111}) knock-in striatal cells treated with wortmannin (25 μ M) for 0, 10, 20, 30, 40 and 60 min. Values (obtained by densitometric analysis of western blot data) are expressed as percentage of pAkt (Ser473) levels in STHdh^{Q7/Q7} at time 0, and shown as mean \pm S.E.M. ($n=4$). Immunoblots show representative experiments

hippocampus at 12 and 30 weeks of age. Because changes observed in striatal PHLPP1 levels were similar in R6/1 and R6/1:BDNF $+/-$ mice, we analyzed cortical and hippocampal PHLPP1 levels only in R6/1 mice. Similar to that observed in the striatum, PHLPP1 levels were decreased in the cortex (12-week-old, $44 \pm 2\%$ of decrease $P < 0.01$ Student's *t*-test; 30-week-old, $30 \pm 8\%$ of decrease $P < 0.01$ Student's *t*-test) and in the hippocampus (12-week-old, $47 \pm 7\%$ of decrease $P < 0.05$ Student's *t*-test; 30-week-old, $39 \pm 6\%$ of decrease $P < 0.01$ Student's *t*-test) of R6/1 animals when compared with age-matched wild-type mice.

To know if the downregulation of this phosphatase is a common feature in N-terminal exon-1 mhtt mouse models, we next analyzed striatal PHLPP1 protein levels in R6/2 mice at 12 weeks of age. As observed in R6/1 and R6/1:BDNF $+/-$ mice, PHLPP1 protein levels were decreased ($61 \pm 10\%$ reduction) in the striatum of R6/2 mice (Figure 3b).

To examine whether decreased PHLPP1 protein levels in the N-terminal exon-1 mhtt transgenic mice were directly associated with a downregulation of mRNA expression, we performed quantitative (Q)-polymerase chain reaction (PCR) assay in samples obtained from the striatum of wild-type and

R6/1 animals at 12 weeks of age. Consistent with reduced levels of striatal PHLPP1 protein at this age, Q-PCR analysis revealed a reduction in the expression of PHLPP1 in the striatum of R6/1 compared with wild-type mice ($28 \pm 9\%$ reduction; $P < 0.05$ Student's *t*-test).

pAkt levels are increased in the striatum of N-terminal exon-1 mhtt transgenic mouse models during the neuropathological process. PHLPP1 downregulation could result in high levels of activated Akt.¹⁷ Thus, we looked at pAkt (Ser473) protein levels in the same mouse models and ages where PHLPP1 protein levels were determined. In agreement with changes in PHLPP1 levels, we observed increased levels of pAkt (Ser473)/Akt ratio in the striatum of R6/1 and R6/1:BDNF $+/-$ mice at 12 (61 ± 12 and $70 \pm 15\%$, respectively), 16 (63 ± 9 and $81 \pm 16\%$, respectively) and 30 (155 ± 26 and $183 \pm 35\%$, respectively) weeks of age without changes in total Akt protein levels (Figure 4a). No differences were observed in pAkt (Ser473) levels between R6/1 and R6/1:BDNF $+/-$ mice, indicating that BDNF levels are not regulating the activation of Akt in these HD models. Interestingly, at the

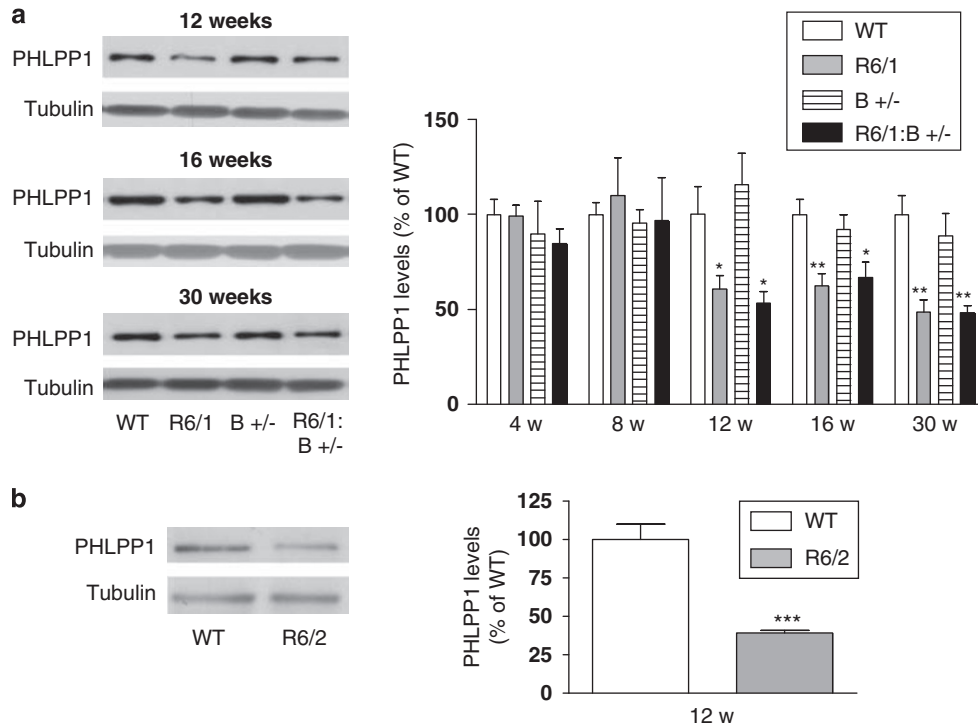


Figure 3 PHLPP1 levels are decreased in the striatum of N-terminal exon-1 mhtt transgenic mouse models. **(a)** PHLPP1 levels were analyzed by western blot of protein extracts obtained from the striatum of wild-type (WT), R6/1, BDNF +/– (B +/-) and R6/1 : BDNF +/- (R6/1 : B +/-) mice at different stages of the disease progression (from 4 to 30 weeks (w) of age), and **(b)** from the striatum of R6/2 mice at 12 weeks of age. Representative immunoblots show protein levels of PHLPP1 and α -tubulin in 12-, 16- and 30-week-old WT, R6/1, BDNF +/- and R6/1 : BDNF +/- mice and in 12-week-old R6/2 mice. Figures show the decrease in striatal PHLPP1 levels in R6/1 and R6/1 : BDNF +/- mice with respect to their littermate controls at different stages of the disease progression, and in R6/2 mice at 12 weeks of age. Values (obtained by densitometric analysis of western blot data) are expressed as percentage of WT mice (PHLPP1/ α -tubulin ratio), and shown as mean \pm S.E.M. ($n=4-6$). In **a** data were analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test. * $P<0.05$ and ** $P<0.01$ as compared with WT and BDNF +/- mice. In **b** data were analyzed by Student's *t*-test. *** $P<0.001$ as compared with WT mice

latest stage of the disease progression (30 weeks) both R6/1 and R6/1 : BDNF +/- animals showed an increase in the pAkt (Ser473)/Akt ratio that was significantly higher than that observed at earlier stages (Figure 4a). To know whether these changes were specific to the phosphorylation of Akt at Ser473, we also analyzed pAkt (Thr308) levels. Because changes in pAkt (Ser473) were similar in R6/1 and R6/1 : BDNF +/- mouse striatum, levels of pAkt (Thr308) were analyzed only in wild-type and R6/1 mice striatum at different stages of the disease progression. No changes in pAkt (Thr308) were detected at 4 and 8 weeks of age whereas the striatum of 12- and 30-week-old R6/1 mice displayed a similar increase of pAkt (Thr308) levels (12 weeks, $244 \pm 48\%$ of wild-type levels; 30 weeks, $230 \pm 36\%$ of wild-type levels; $P<0.01$ Student's *t*-test). Next, we examined whether pAkt (Ser473) was localized in neurons or glial cells by immunohistochemical analysis. As shown in Figure 4b, pAkt (Ser473) was only detected in striatal neurons in both wild-type (Figure 4b2) and R6/1 mice (Figure 4b3). The morphology of striatal neurons labeled with anti-pAkt (Ser473) antibody was similar to that of striatal DARPP-32-positive neurons (compare Figures 4b2 and b3 with Figure 4b1), indicating that the enhancement of pAkt (Ser473) levels in R6/1 mice occurs in medium-sized striatal projection neurons.

An enhancement of the pAkt (Ser473)/Akt ratio was also observed in the striatum of 12-week-old R6/2 mice compared to their littermate controls ($60 \pm 10\%$; Figure 4c). This increase was significant although total Akt levels were also significantly increased ($35 \pm 6\%$; $P<0.05$ Student's *t*-test). In contrast, despite PHLPP1 downregulation, no changes in pAkt (Ser473) protein levels were observed in the cortex ($119 \pm 12\%$ of wild-type levels) or in the hippocampus ($104 \pm 4\%$ of wild-type levels) of 30-week-old R6/1 mice. These data show that in these HD mouse models, the PI3K/Akt pathway is specifically regulated in the striatum.

R6/1 mouse striatum displays increased pAkt levels both in the cytosol and in the nucleus. Activated Akt can translocate from the plasma membrane to the cytosol and nucleus,²⁵ regulating different target proteins within each of these cellular compartments. Thus, we analyzed pAkt (Ser473) levels in striatal cytosolic and nuclear fractions from 12- and 20-week-old mice. As we found no differences in pAkt (Ser473) levels between R6/1 and R6/1 : BDNF +/- during disease progression (Figure 4a), we looked at the intracellular localization of pAkt (Ser473) only in wild-type and R6/1 animals. As shown in Figure 5, 12- and 20-week-old R6/1 animals exhibited higher pAkt (Ser473) levels than wild-type mice, both in the cytosol (increases of 81 ± 20 and

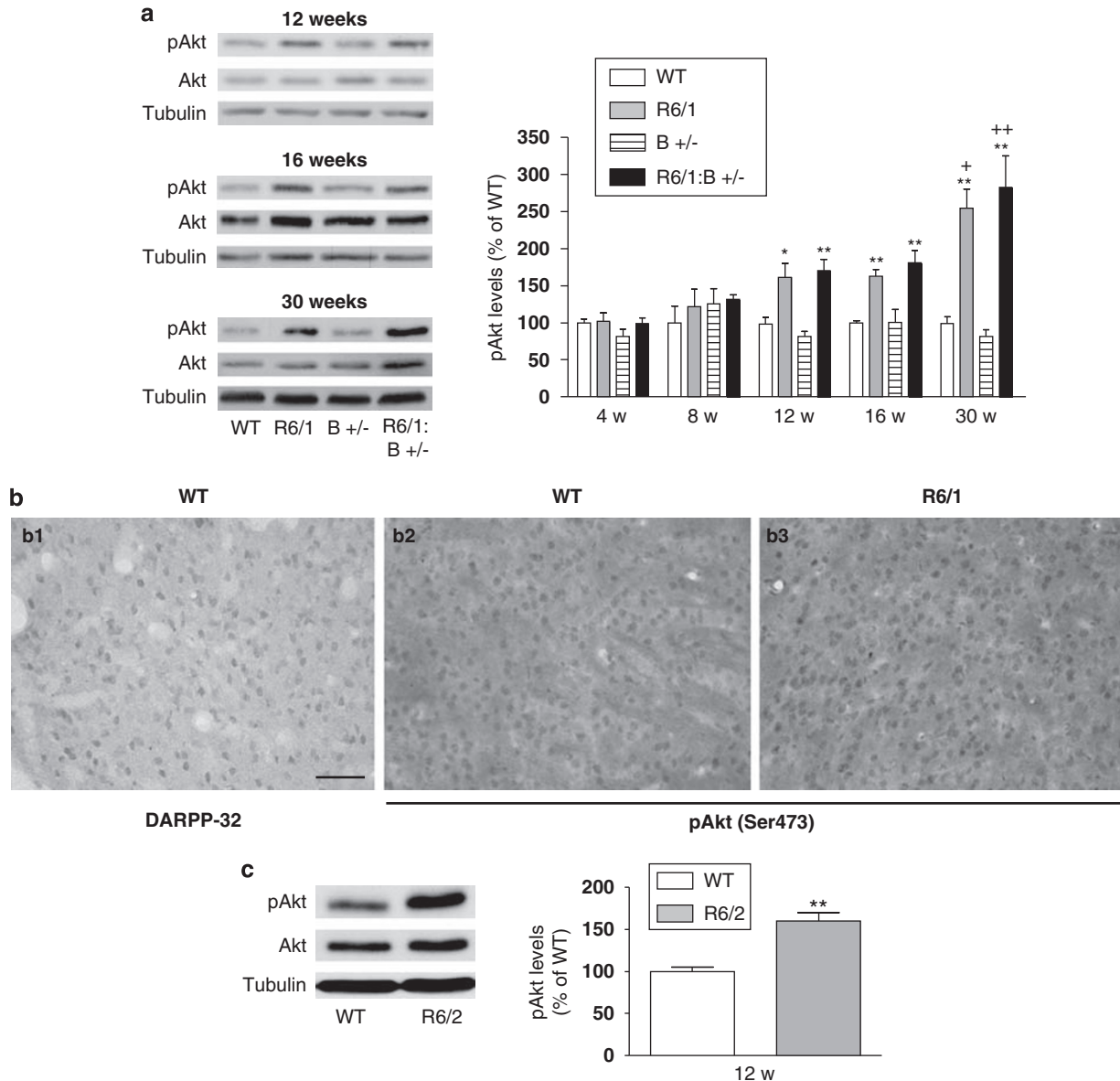


Figure 4 Akt phosphorylation status is increased in the striatum of R6 mice at ages where PHLPP1 levels are decreased. **(a)** Striatal samples from wild-type (WT), R6/1, BDNF +/– (B +/-) and R6/1 : BDNF +/- (R6/1 : B +/-) at different stages of the disease progression (from 4 to 30 weeks (w) of age) were blotted with the pAkt (Ser473) antibody, and then re-probed for total Akt and α -tubulin to obtain the ratio of pAkt (Ser473). Representative immunoblots show protein levels of pAkt (Ser473), Akt and α -tubulin in 12-, 16- and 30-week-old WT, R6/1, BDNF +/- and R6/1 : BDNF +/- mice. Bar graphs were obtained by densitometric quantification of the western blot data and show the increase of pAkt (Ser473) levels in R6/1 and R6/1 : BDNF +/- mice at 12, 16 and 30 weeks of age when compared with their corresponding controls. The results are expressed as percentage of WT mice (pAkt (Ser473)/Akt ratio, after normalization to α -tubulin), and data shown are the mean \pm S.E.M. ($n = 4-7$). Data were analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test. * $P < 0.05$ and ** $P < 0.01$ as compared with WT and BDNF +/- mice; + $P < 0.05$ and ++ $P < 0.01$ as compared with 12- and 16-week-old R6/1 and R6/1 : BDNF +/- mice. **(b)** DARPP-32 and pAkt (Ser473) were examined by immunohistochemistry in the striatum of 20-week-old mice. Photomicrographs show DARPP-32-positive neurons in WT mice striatum (b1), and pAkt (Ser473)-positive neurons in WT (b2) and R6/1 (b3) mice striatum. Scale bar, 75 μ m. **(c)** Striatal samples from 12-week-old wild-type (WT) and R6/2 mice were blotted with the pAkt (Ser473) antibody, and then re-probed for total Akt and α -tubulin to obtain the ratio of pAkt (Ser473). Representative immunoblots show protein levels of pAkt (Ser473), Akt and α -tubulin in wild-type and R6/2 mouse striatum. Bar graphs were obtained by densitometric quantification of the western blot data and show the increase of pAkt (Ser473) levels in 12-week-old R6/2 mice when compared with their controls (WT). The results are expressed as percentage of WT mice (pAkt (Ser473)/Akt ratio, after normalization to α -tubulin), and data shown are the mean \pm S.E.M. ($n = 4-7$). Data were analyzed by Student's *t*-test. ** $P < 0.01$ as compared with WT mice

$91 \pm 11\%$, respectively; Figure 5a) and in the nucleus (increases of 102 ± 20 and $164 \pm 35\%$, respectively; Figure 5b).

Increased Akt activation is associated with inactivation of pro-apoptotic targets. If the elevated level of Akt

phosphorylation in the striatum of N-terminal exon-1 mhtt transgenic mice is physiologically relevant, it follows that phosphorylation of proteins regulated by Akt may also be upregulated. Thus, we analyzed the phosphorylation status of glycogen synthase kinase 3 beta (GSK3 β ; Ser9) and Forkhead box 1 transcription factor (FoxO1; Ser256), two

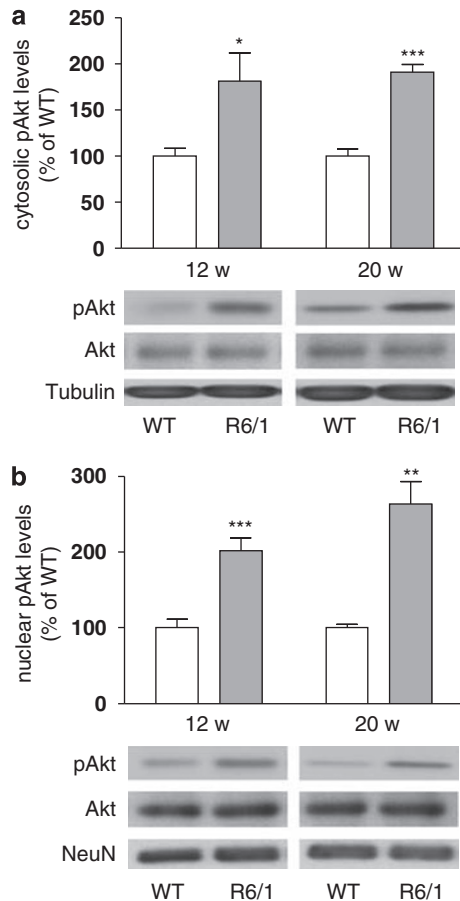


Figure 5 Levels of pAkt are increased both in the cytosolic and in the nuclear fraction in the R6/1 mouse striatum. Levels of pAkt (Ser473) protein were analyzed by western blot in cytosolic (a) and nuclear (b) fractions prepared from the striatum of wild-type (WT) and R6/1 mice at 12 and 20 weeks (w) of age. Representative immunoblots show protein levels of pAkt (Ser473), Akt and α -tubulin in the cytosolic fraction (a) and of pAkt (Ser473), Akt and NeuN in the nuclear fraction (b), in WT and R6/1 mice. The western blot data were quantified by densitometric analysis, and the results expressed as percentage of normalized WT values. Data are the mean \pm S.E.M. ($n=5-8$) and were analyzed using Student's *t*-test. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ as compared with WT mice

well-known downstream targets of the Akt pathway that are involved in neuronal death.⁶ Consistent with Akt activation, the pGSK3 β /GSK3 β ratio was significantly increased in the R6/1 striatum at 12 ($125 \pm 23\%$), 16 ($76 \pm 23\%$) and 30 ($69 \pm 27\%$) weeks of age due to a large enhancement of pGSK3 β levels (Figure 6a).

FoxO1 phosphorylation status determines its cellular localization.²⁶ Akt phosphorylates FoxO1 resulting in its nucleus–cytosol shuttling and cytoplasmic retention. Therefore, to investigate whether pAkt (Ser473) was acting on FoxO1, we determined FoxO1 levels in nuclear fractions and pFoxO1 levels in cytosolic fractions extracted from the striatum of 12- and 20-week-old wild-type and R6/1 mice. We found that the striatum of R6/1 mice exhibited increased cytosolic pFoxO1 levels both at 12 ($164 \pm 57\%$; Figure 6b) and at 20 ($265 \pm 62\%$) weeks of age (Figure 6c), consistent with a significant decrease in total FoxO1 levels in nuclear fractions (Figure 6c). Altogether, these results show that Akt activation

translates into pro-survival signaling by means of GSK3 β and FoxO1 inactivation (Figure 6).

Increased pAkt levels contribute to prevent excitotoxic-induced cell death in R6/1 striatum. N-terminal exon-1 mhtt transgenic mouse models are resistant to excitotoxicity induced by intrastriatal injection of quinolinic acid (QUIN), an *N*-methyl-D-aspartate glutamate receptor (NMDAR) agonist.²⁷ We therefore asked whether increased levels of pAkt (Ser473), and inactivation of GSK3 β and FoxO1 proapoptotic pathways, correlate with less neuronal loss and the resistance to excitotoxicity in R6/1 mice. To address this question, we analyzed pAkt (Ser473) levels 4 h after intrastriatal QUIN injection in 12-week-old R6/1 mice. No changes in pAkt (Ser473) levels were observed after intrastriatal QUIN injection in wild-type animals compared with the non-injected side (Figures 7a and b). In contrast, pAkt (Ser473) levels after QUIN injection in the R6/1 mouse striatum were about threefold higher than in the contralateral striatum (Figures 7a and b). Furthermore, PHLPP1 levels were not affected by the QUIN-induced striatal lesion, either in wild-type or in R6/1 mice (Figure 7a). These results suggest that the activation of the PI3K/Akt pathway in R6/1 mice striatum may participate in the reduced neuronal loss observed after QUIN injection.

Changes in striatal PHLPP1 and pAkt levels are differentially reverted by suppressing transgene expression in Tet/HD94 mice striatum. To determine whether PHLPP1 downregulation depends on the continuous expression of mhtt, we quantified PHLPP1 protein levels in Tet/HD94 mice. Striatal PHLPP1 protein levels were examined in 22-month-old mice from three different groups: wild-type, Tet/HD94 with no pharmacological intervention (gene-ON) and Tet/HD94 after 5 months of doxycycline administration that turns off the transgene expression (gene-OFF). As observed in the striatum of R6/1, R6/1:BDNF +/– and R6/2 mice, PHLPP1 protein levels were significantly decreased in the Tet/HD94 gene-ON group when compared with wild-type levels ($35 \pm 8\%$ reduction; Figure 8a). Interestingly, by suppressing transgene expression, PHLPP1 protein levels completely returned to wild-type levels (Figure 8a). We next analyzed whether pAkt (Ser473) levels were modified in the same experimental conditions. Consistent with results obtained in R6 mice, we observed an increase in pAkt (Ser473) levels in Tet/HD94 mouse striatum with respect to wild-type animals accompanied by the consequent enhancement of the pAkt/Akt ratio ($200 \pm 40\%$; Figure 8b). In contrast to that observed for PHLPP1 protein levels, the increase in pAkt/Akt ratio was only partially reverted by suppressing transgene expression, with a significant reduction of pAkt (Ser473) levels ($100 \pm 5\%$) compared with gene-ON group, but being still significantly higher than in wild-type mice (Figure 8b).

PHLPP1 protein levels are decreased in the putamen of HD patients. To know whether changes in PHLPP1 also occur in HD patients, we analyzed its protein levels in the putamen of human HD brains. As observed in the striatum of HD transgenic mouse models, PHLPP1 protein levels were also significantly decreased in human samples to

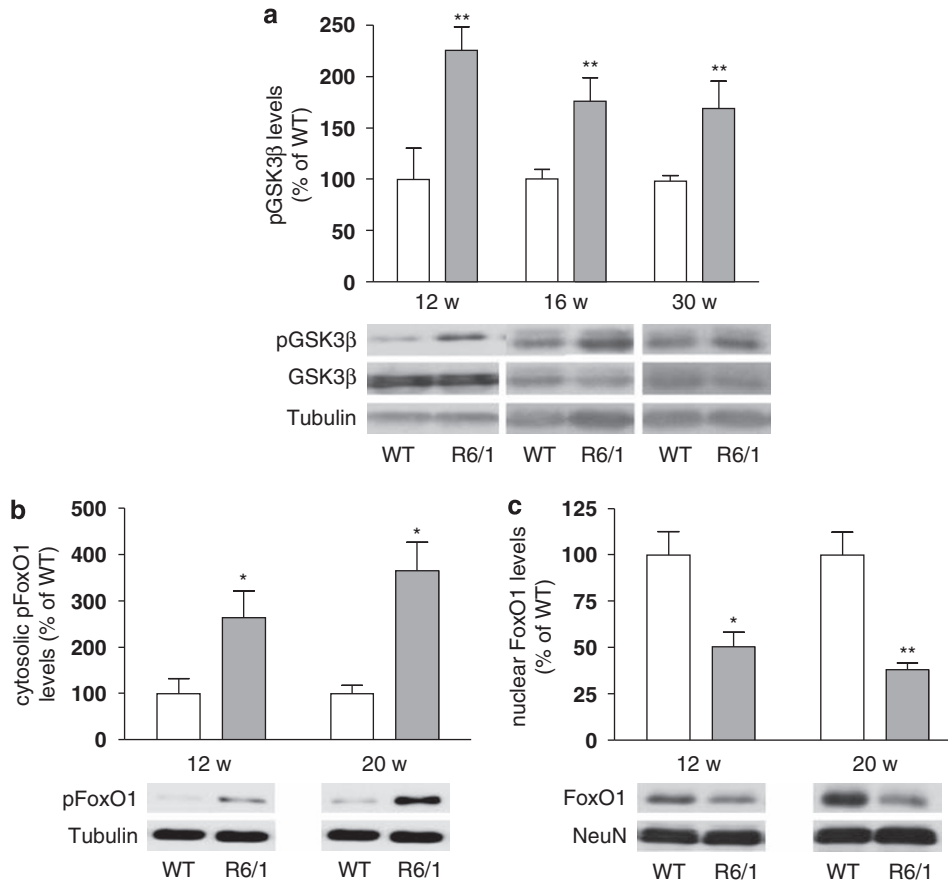


Figure 6 R6/1 striatum displays increased levels of inactivated GSK3 β and FoxO1. (a) Levels of pGSK3 β (Ser9) were analyzed by western blot in the striatum of 12-, 16- and 30-week-old (w) mice. Analysis of the band intensities (shown in the bar graph) indicates that the pGSK3 β /GSK3 β ratio is significantly increased in R6/1 striatum at different stages of the disease progression. Values are expressed as percentage of wild-type (WT) mice (pGSK3 β /GSK3 β ratio after normalization to α -tubulin), and data shown are the mean \pm S.E.M. ($n = 5-7$). Data were analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test. ** $P < 0.01$ as compared with the corresponding WT. Representative immunoblots show pGSK3 β , GSK3 β and α -tubulin protein levels in striatal samples of WT and R6/1 mice at 12, 16 and 30 weeks of age. Striatal protein levels of cytosolic pFoxO1 (Ser256) (b) and nuclear FoxO1 (c) were analyzed by western blot in 12- and 20-week-old WT and R6/1 mice. Quantification of the western blot data was performed by densitometric analysis. Values were expressed as percentages of WT mice (pFoxO1/ α -tubulin ratio or FoxO1/NeuN ratio) and shown as mean \pm S.E.M. ($n = 4-8$). Data were analyzed two-way ANOVA followed by Bonferroni's *post hoc* test. * $P < 0.05$ and ** $P < 0.01$ as compared with WT mice. Representative immunoblots show levels of pFoxO1 and α -tubulin in the cytosolic fraction (b), and of FoxO1 and NeuN in the nuclear fraction (c) obtained from the striatum of WT and R6/1 mice at 12 and 20 weeks of age

13.35 \pm 11.08% of control cases (Figures 9a and b). We next analyzed whether pAkt levels were also modified in human HD brain but we were unable to detect pAkt in these samples.

Discussion

The results of the present study show that PHLPP1 protein levels are reduced in the striatum of all transgenic HD mouse models analyzed (Hdh^{Q111/Q111}; R6/1; R6/1:BDNF^{+/-}; R6/2; Tet/HD94), in the HD cell model STHdh^{Q111/Q111}; as well as in the putamen of HD patients. Furthermore, decreased levels of PHLPP1 were also observed in the cortex and hippocampus of R6/1 mice. In addition, we show that reduced PHLPP1 protein levels depend on the presence of mhtt because in the Tet/HD94 mouse striatum they return to control levels when mhtt expression is turned off. Here, we also show that pAkt (Ser473) protein levels are increased in

the striatum of the N-terminal exon-1 mouse models according to previous results obtained in knock-in models.¹² Higher and sustained pAkt (Ser473) levels in the striatum during the progression of the disease could be attributed in part to the reduction of PHLPP1 protein levels as both events occur simultaneously. Consistent with high levels of active Akt, the phosphorylation of GSK3 β and FoxO1 was increased in the striatum of R6/1 mice. Finally, we also show that intrastriatal QUIN injection in R6/1, but not in wild-type, mice upregulates pAkt (Ser473) protein levels, which can contribute to the absence of striatal cell death observed in these animals after an excitotoxic injury.

Here we show that the expression of distinct forms of mhtt, full-length and N-terminal exon-1, downregulates PHLPP1 in striatal cells. Moreover, a reduction in the levels of this phosphatase also occurs in cortical and hippocampal cells expressing N-terminal exon-1 mhtt with 115 CAG repeats. The analysis of PHLPP1 protein levels in R6/1 mice revealed that this downregulation first occurs at presymptomatic stages

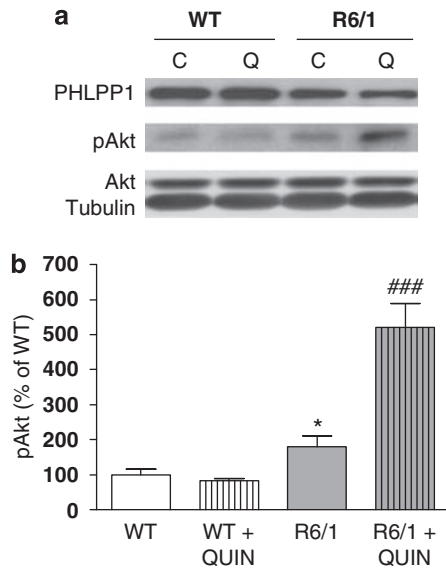


Figure 7 Intrastratial QUIN injection in R6/1, but not in wild-type, mice increases pAkt (Ser473) protein levels. (a) Representative immunoblots show protein levels of PHLPP1, pAkt (Ser473), Akt and α -tubulin in 12-week-old wild-type (WT) and R6/1 mice striatum, 4 h after QUIN injection. C, striatum contralateral to the lesion; Q, QUIN-injected striatum. (b) Analysis of the band intensities (shown in the bar graph) indicates that QUIN injection only increases pAkt (Ser473) protein levels in R6/1 mouse striatum. Values are expressed as percentage of wild-type mice (pAkt (Ser473)/Akt ratio after normalization to α -tubulin), and data shown are the mean \pm S.E.M. ($n=5$). Data were analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test. * $P < 0.05$ compared with non-injected wild-type striatum (WT); ### $P < 0.001$ compared with QUIN-injected wild-type striatum (WT + QUIN). R6/1, non-injected R6/1 striatum; R6/1 + QUIN, QUIN-injected R6/1 striatum

(12 weeks) and is maintained until late stages of the disease (30 weeks) in all regions examined, suggesting that regulation of PHLPP1 protein levels by mhtt is part of the pathogenesis of the disease. Furthermore, our results show a regulation of PHLPP1 expression by mhtt at the transcriptional level because both PHLPP1 mRNA and protein were decreased in the R6/1 mouse striatum at 12 weeks of age. Transcriptional deregulation has been proposed as an important pathogenic mechanism in HD.²³ The presence of an expanded polyglutamine domain in the protein can disrupt transcription through several mechanisms such as interaction or sequestration of transcription factors.²⁸ For instance, it has been recently shown *in vitro*, and in the striatum and cortex of R6/2 mice that NF-Y is one of the transcription factors sequestered by mhtt.²⁹ It is worth noting that when searching for promoter regions in the *PHLPP1* gene (using the Matrix Search for transcription factor binding sites, Match public version 1.0, with the cut-off selection set to minimize the sum of both false-positive and false-negative results) we identified NF-Y as a possible regulator of PHLPP1 expression. Because the interaction between mhtt and NF-Y leads to a reduction of HSP70 gene expression,²⁹ it is tempting to speculate that this mechanism could also be involved in the downregulation of PHLPP1 mRNA levels.

Reduced levels of PHLPP1 can result in increased levels of pAkt (Ser473) as has been previously shown in some cancer cell lines.¹⁷ Furthermore, it has been recently described that

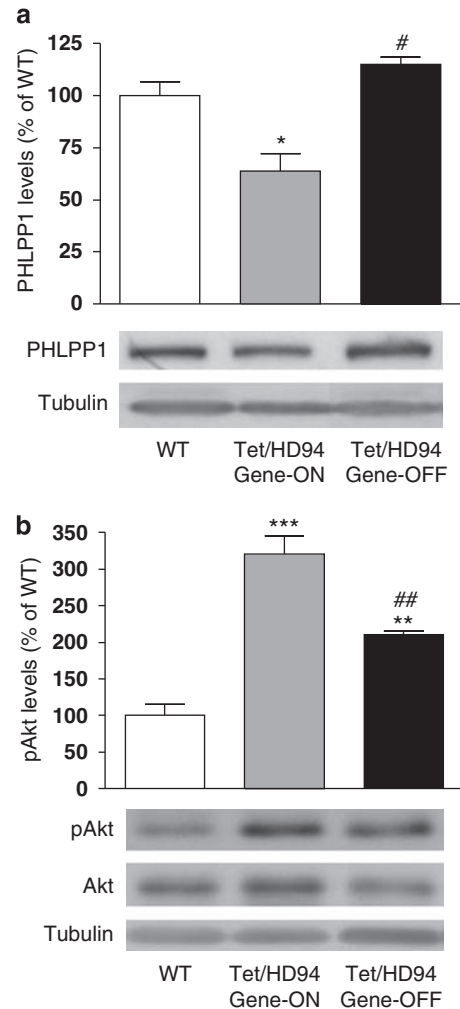


Figure 8 Suppression of mhtt expression in Tet/HD94 mice differentially affects PHLPP1 and pAkt levels. PHLPP1 and pAkt (Ser473) protein levels were analyzed by western blot in the striatum of 22-month-old wild-type (WT) and Tet/HD94 mice, either with no pharmacological intervention (gene-ON) or after 5 months of transgene shutdown by doxycycline administration (gene-OFF). Figures show the densitometric measures of PHLPP1 (a) and pAkt (Ser473) (b) normalized to α -tubulin or Akt/ α -tubulin ratio, respectively. Results are expressed as percentages of WT \pm S.E.M. for four animals per condition. Data were analyzed by one-way ANOVA followed by Bonferroni's *post hoc* test. * $P < 0.05$ and *** $P < 0.001$ as compared with WT mice; # $P < 0.05$ and ## $P < 0.01$ as compared with Tet/HD94 gene-ON mice. Immunoblots show representative experiments

regional variations in PHLPP1 protein levels in rat hippocampus could account for the differences in pAkt (Ser473) levels.²² In agreement, in the striatum of all HD models analyzed, we observed increased pAkt (Ser473) levels in the same conditions where PHLPP1 protein levels were reduced. Furthermore, we also detected increased pAkt (Thr308) levels in R6/1 mouse striatum at 12 and 30 weeks of age. Changes in pAkt (Thr308) levels suggest that phosphatases that regulate the dephosphorylation of Akt at Thr308, such as PP2A, could be also affected by mhtt expression. However, we detected similar levels of PP2A in wild-type and R6/1 mice striatum at 4, 8, 12, 16 or 30 weeks of age (data not shown). Thus, as

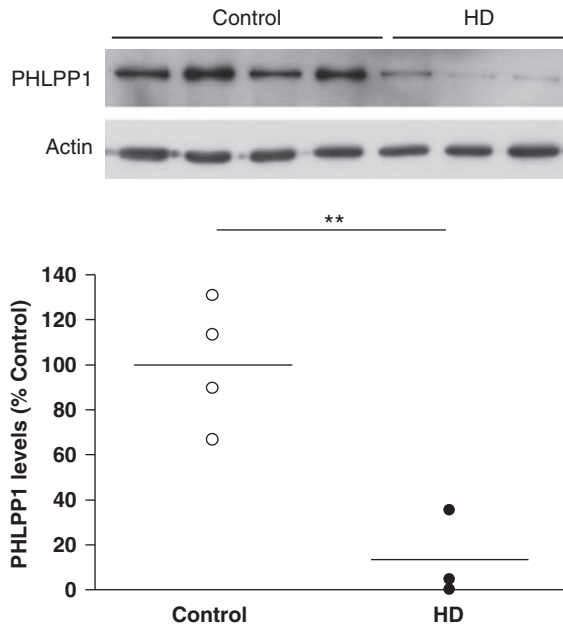


Figure 9 The putamen of HD brains shows decreased levels of PHLPP1. PHLPP1 protein levels were analyzed by western blot in samples from the putamen of control and HD post-mortem brains. Immunoblots of PHLPP1 and actin in control and HD samples are shown. Quantification of the western blot data was performed by densitometric analysis. The densitometric measures of PHLPP1 levels were normalized to actin and expressed as percentage of control. Results are shown as mean \pm S.E.M. Data were analyzed using Student's *t*-test. ** $P < 0.01$ as compared with control

previously shown in cancer cell lines,¹⁷ chronically reduced levels of PHLPP1 could affect the phosphorylation state of both Ser473 and Thr308 in striatal cells. In contrast to what happens in the striatum, we did not observe changes in pAkt (Ser473) levels in the cortex and hippocampus of R6/1 mice although PHLPP1 levels were also downregulated. Thus, these results indicate that a reduction in the levels of this phosphatase may not be enough to increase pAkt (Ser473) levels. In the striatum of Tet/HD94 mice, we observed that after shutting down the expression of mhtt PHLPP1 protein levels returned to wild-type levels but pAkt (Ser473) upregulation was only partially reduced strengthening the idea that an additional regulation, such as activation of kinases, is likely to participate in increasing pAkt (Ser473) levels in striatal cells. Akt phosphorylation depends on PI3K activation.¹⁶ The main signaling pathways controlling PI3K activation, and consequently Akt phosphorylation, are those initiated by the binding of trophic factors to their receptors.⁶ However, here changes in pAkt (Ser473) levels could not be related with endogenous levels of BDNF, although we cannot rule out the possible participation of other trophic factors. Neurons can also activate PI3K in response to NMDAR stimulation³⁰ or to oxidative stress.³¹ Interestingly, these inducers have been shown to participate in HD.^{32,33} Moreover, striatal membrane fractions from the N171-82Q HD mouse model show increased levels of PI3K,³⁴ which could result in higher levels of Akt activation. In the HD cell model STHdh^{Q111/Q111} enhanced NMDAR signaling upregulates Akt activity,¹² and here we show that this also occurs in R6/1 mice striatum as

increased pAkt (Ser473) levels were observed after QUIN injection. The activation of Akt through NMDAR stimulation³⁰ or by oxidative stress³¹ is transient, but here we show that increased pAkt (Ser473) levels are maintained through the course of the disease. The levels of pAkt (Ser473) may depend upon a dynamic balance between PHLPP1 levels and Akt-activating mechanisms. Therefore, our results suggest that the PI3K/Akt pathway is activated as a stress response to ameliorate the detrimental effects of mhtt expression, probably mediated by changes in NMDAR stimulation or by increased oxidative stress, and reduced levels of PHLPP1 contribute to maintain high levels of activated Akt over time. This is supported by the observation that after inhibition of PI3K, pAkt levels at Ser473 were maintained longer in STHdh^{Q111/Q111} cells than in wild-type cells.

One of the important findings of this study is that R6/1 mice displayed enhanced levels of pAkt (Ser473) in the striatum, but not in the cortex or hippocampus, suggesting a specific response of striatal neurons to mhtt expression. Similarly, pAkt (Ser473) levels are increased in cellular and mouse models of spinocerebellar ataxia type 1,³⁵ another CAG repeat disorder. Thus, it seems that a common consequence of expressing proteins with a polyglutamine tract expansion is the deregulation of cell signaling pathways involving Akt. This observation was extended to the striatum of other N-terminal exon-1 mouse models by showing increased levels of pAkt (Ser473) in R6/1 : BDNF +/−, R6/2 and Tet/HD94 mice. In good agreement with our results, the striatum of Hdh^{Q111} knock-in mice, and the striatal HD cell model STHdh^{Q111/Q111} show enhanced levels of pAkt (Ser473).¹² However, Akt activation status in 6-month-old YAC128 mice and in 6- and 10-week-old R6/2 mice was previously shown to be unchanged.³⁶ Our observation that the activation of the Akt pathway in the presence of mhtt is a brain region-dependent effect explains why that study failed to find changes in pAkt (Ser473) levels as active Akt was measured in whole brain lysates.³⁶ Akt activation has been largely shown as one of the main mechanisms to prevent neuronal death during injury.⁸ Accordingly, Akt prevents neuronal death induced by mhtt,¹⁴ and increasing Akt expression has beneficial effects in *Drosophila* models of HD.^{37,38} Furthermore, in an acute rat model of HD showing massive cell death a decrease of pAkt levels occurs before neuronal loss.³⁹ Here we show that Akt is still activated at late stages of the disease and that these changes are occurring in striatal medium-sized spiny projection neurons, the most affected in HD. Therefore, our results suggest that increased Akt activation could counteract mhtt toxicity, raising the threshold above which apoptotic signals can occur. In line with this hypothesis, although N-terminal exon-1 models used in the present study display an increase in pro-apoptotic proteins of the Bcl-2 family,^{40,41} they show little, if any, cell death in the striatum.^{24,41–44} Similarly, the Hdh^{Q111} knock-in mouse model of HD show enhanced levels of pAkt in the striatum¹² with no overt cell death.⁴³ Moreover, our results showing increased levels of pAkt (Ser473) in the Tet/HD94 mouse striatum further support the importance of maintaining active Akt, which can participate in delaying irreversible cell loss to allow the recovery of neuronal functionality after transgene shutdown.⁴⁴ It would be interesting to know whether regulation of active Akt levels also occurs

in HD patients during disease progression, but the difficulty in obtaining human brain samples lacking massive neuronal degeneration makes complex this type of analysis.

Htt itself is one of the targets of active Akt. Phosphorylation of mhtt at Ser421 by pAkt abrogates its toxicity.¹⁴ However, all HD mouse models expressing the N-terminal exon-1 mhtt lack the Ser421 Akt phosphorylation site. Therefore, in these conditions, activated Akt should be regulating other targets, such as GSK3 β or FoxO1 proteins, that participate in apoptosis.⁶ In fact, we detected increased levels of pGSK3 β and pFoxO1, indicating that pAkt inactivates these proteins in the striatum of R6/1 mice. Notably, GSK3 β or FoxO1 inactivation by Akt has been shown to be neuroprotective in several types of brain injury, such as ischemia^{9,10} or traumatic lesion,¹¹ thereby suggesting a neuroprotective function for Akt mediated by inactivation of GSK3 β and FoxO1 also in the R6/1 model of HD. Supporting this idea, the resistance of striatal cells of R6/1 mice to excitotoxic-induced cell death can in part be related to the presence of increased levels of pAkt (Ser473), and inactivation of GSK3 β and FoxO1. In line with this, the phosphorylation status of Akt has been shown to determine neuronal survival or death after cerebral ischemia^{9,10} and to participate in the different vulnerability of CA1 and CA3 hippocampal neurons to cellular stress.²²

In summary, in this work we show that one of the cellular responses to full-length or N-terminal exon-1 mhtt expression is the reduction of PHLPP1 protein levels, which is initiated at early stages and persists during the course of the disease. In addition, only striatal cells respond to the stress caused by mhtt activating the PI3K/Akt pathway. Signals that promote Akt phosphorylation together with decreased levels of PHLPP1 operate in conjunction to maintain high levels of Akt activation in striatal cells, which participate in the inhibition of the pro-apoptotic pathways involving GSK3 β and FoxO1 proteins. We also show that increased pAkt levels are still detected at late stages of the neurodegenerative process offering a mechanistic explanation to the modest neuronal death described in these HD mouse models. Our data thus support the relevance of the PI3K/Akt pro-survival pathway and suggest its use as a therapeutic approach to delay striatal neuronal death as reduced PHLPP1 protein levels are indeed occurring as a part of the pathophysiology of HD.

Materials and Methods

Cell cultures. Conditionally immortalized wild-type (STHdh^{Q7/Q7}) and mutant (STHdh^{Q111/Q111}) striatal neuronal progenitor cell lines expressing endogenous levels of normal and mhtt with 7 and 111 glutamines, respectively, have been described elsewhere.¹² Cells were grown at 33°C in Dulbecco's modified Eagle's medium (Gibco, Renfrewshire, Scotland, UK) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 2 mM L-glutamine and 400 μ g/ml G418 (Geneticin; Invitrogen, Carlsbad, CA, USA). To analyze pAkt levels in the presence of the PI3K inhibitor wortmannin (0.25 μ M; Calbiochem, La Jolla, CA), we incubated striatal cells in Dulbecco's modified Eagle's serum-free medium for 3 h and then exposed them to the inhibitor during 10, 20, 30, 40 and 60 min. Wild-type striatal cells were transfected with human PHLPP1 cloned into pCDNA3HA vector (generously provided by Dr. Alexandra Newton)¹⁷ using Lipofectamine 2000 (Invitrogen), as instructed by the manufacturer. At 14 h after transfection the medium was replaced by Dulbecco's modified Eagle's serum-free medium, and cells cultured for further 10 h before cells extracts were prepared.

HD mouse models. Homozygous mutant Hdh^{Q111/Q111} and wild-type Hdh^{Q7/Q7} knock-in mice were obtained from matings between male and female Hdh^{Q111/Q7}

heterozygotes as described previously.⁴⁵ R6/1 and R6/2 heterozygous transgenic mice expressing exon-1 of mhtt with 115 and 150 CAG repeats, respectively, were obtained from Jackson Laboratory (Bar Harbor, ME, USA). R6/1 mice were cross-mated with BDNF heterozygous mice (BDNF +/–)⁴⁶ to obtain R6/1:BDNF +/– mice, as previously described.²⁴ Conditional Tet/HD94 mice express a chimeric mouse/human exon-1 with 94 CAG repeats under the control of the bidirectional tetO responsive promoter.⁴⁷ To turn off mhtt expression, we treated 17-month-old wild-type and Tet/HD94 mice with doxycycline in drinking water during 5 months (2 mg/ml for 4 months followed by 0.5 mg/ml for 1 month; gene-OFF group).⁴⁴ Some animals were left without intervention (gene-ON group). All mice used in the present study were housed together in numerical birth order in groups of mixed genotypes, and data were recorded for analysis by microchip mouse number. Experiments were conducted in a blind-coded manner respect to genotype. All mice were genotyped by PCR as described previously.^{24,42,45,47} The animals were housed with access to food and water *ad libitum* in a colony room kept at 19–22°C and 40–50% humidity, under a 12:12 h light/dark cycle. All animal-related procedures were in accordance with the National Institute of Health Guide for the care and use of laboratory animals, and approved by the local animal care committee of the Universitat de Barcelona (99/01), and the Generalitat de Catalunya (99/1094).

Striatal lesions. Wild-type and R6/1 mice (12-week-old) were anesthetized with pentobarbital (50 mg/kg) and QUIN (10 nmol; Sigma Chemical Co., St Louis, MO, USA) was intrastrially injected at the following coordinates relative to bregma: AP + 0.6 mm, ML + 2 mm and 2.7 mm below the dural surface with the incisor bar at 3 mm above the interaural line. QUIN was injected over 2 min and the cannula was left in place for additional 5 min. Animals were killed 4 h after intrastriatal QUIN injection for biochemical analysis.

Post-mortem brain tissues. Brain tissues were obtained from the University of Barcelona and Institute of Neuropathology Brain Banks (Barcelona, Spain) following the guidelines of the local ethics committees. Cases, with and without clinical neurological disease, were sampled and processed using the same protocol. PHLPP1 protein levels were analyzed in three patients with HD (with death at end-stage disease at 28, 59 and 60 years; post-mortem intervals 4–13 h) and four control cases (39, 64, 68 and 71 years; post-mortem intervals 3–9 h).

Total protein extraction. Wild-type STHdh^{Q7/Q7} and mutant STHdh^{Q111/Q111} striatal neuronal progenitor cells were washed once with phosphate-buffered saline (PBS), and total cellular proteins were extracted by incubating cells in lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 10 mM EGTA, 150 mM NaCl, protease inhibitors (2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ μ l aprotinin, 1 μ g/ μ l leupeptin) and phosphatase inhibitors (2 mM Na₃VO₄, 100 mM NaF). Animals were deeply anesthetized and killed by decapitation at the age of 5 months (wild-type Hdh^{Q7/Q7} and mutant Hdh^{Q111/Q111} knock-in mice), 12 weeks (wild-type and R6/2 mice), 4, 8, 12, 16, 20 or 30 weeks (wild-type, R6/1, BDNF +/– and R6/1:BDNF +/– mice) or 22 months (wild-type, gene-ON and gene-OFF Tet/HD94 mice). The brain was quickly removed and the striatum, cortex and hippocampus were dissected out and homogenized in lysis buffer (as for cell protein extraction). Post-mortem human tissue was also homogenized using the same lysis buffer. Samples were centrifuged at 15 000 \times g for 20 min at 4°C, the supernatants were collected and protein concentration was measured using the Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis. Western blot was performed as described elsewhere.⁴¹ Proteins were denatured in 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 140 mM β -mercaptoethanol and 0.1% (w/v) bromophenol blue, heated at 100°C for 5 min and resolved in denaturing polyacrylamide gels. Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Massachusetts, MA, USA), and washed twice in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). After blocking (TBS-T solution plus 5% bovine serum albumin and 5% skimmed milk) at room temperature for 1 h, membranes were blotted overnight at 4°C with the following primary antibodies: anti-PHLPP1 (1:500; Cayman Chemical, Ann Arbor, MI, USA), anti-PP2A (1:7000; BD Transduction Laboratories, San Jose, CA, USA), anti-pAkt (Ser473, Thr308; 1:1000), anti-pGSK3 β (Ser9; 1:4000) anti-GSK3 β (1:5000), anti-FoxO1 (1:1000), anti-pFoxO (Ser256; 1:1000) and anti-panAkt (1:1000) from Cell Signaling (Beverly, MA, USA). Incubation with mouse monoclonal antibodies against α -tubulin (1:100 000; Sigma Chemical Co.), actin (1:10 000; MP Biochemicals, Aurora, OH, USA) or NeuN (1:1000; Chemicon, Temecula, CA, USA) was performed to

obtain loading controls. After primary antibody incubation, membranes were washed with TBS-T and incubated for 1 h at room temperature with the appropriated horseradish peroxidase-conjugated secondary antibody (1:2000; Promega, Madison, WI, USA), and the reaction was finally visualized with the Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Western blot replicates were scanned and quantified using a computer-assisted densitometric analysis.

Q-PCR assay. Total RNA was extracted from 12-week-old wild-type and R6/1 striatal samples and cDNA synthesized as described elsewhere.⁴¹ The cDNA was then analyzed by Q-PCR using the following TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA): 18S (Hs99999901_s1), and PHLPP1 (Mm01295848_m1). Reverse transcriptase (RT)-PCR was performed in reaction buffer containing 12.5 μ l Brilliant Q-PCR Master Mix (Stratagene, La Jolla, CA, USA), 1.25 μ l TaqMan Gene Expression Assays and 10 ng of cDNA. Reactions were as follow: 40 cycles of a two-step PCR; 95°C for 30 s and 60°C for 1 min, after initial denaturation at 95°C for 10 min. All RT-PCR assays were performed in duplicate. To provide negative controls and exclude contamination by genomic DNA, we omitted the RT in the cDNA synthesis step, and samples were subjected to the PCR reaction in the same way for each TaqMan Gene Expression Assay. The RT-PCR data were analyzed and quantified using the Comparative Quantitation Analysis program of the MxProTM Q-PCR analysis software version 3.0 (Stratagene) with the 18S gene expression as internal loading control. Results were then normalized respect to wild-type values.

Immunohistochemistry. For immunohistochemical analysis, wild-type and R6/1 mice at 20 weeks of age ($n = 3$ for each condition) were deeply anesthetized and immediately perfused transcardially with saline followed by 4% paraformaldehyde/phosphate buffer. Brains were removed and postfixed for 1 to 2 h in the same solution, cryoprotected by immersion in 30% sucrose/PBS and then frozen in dry ice-cooled isopentane. Serial coronal cryostat sections (30 μ m) through the whole striatum were collected in PBS as free-floating sections. After treatment with H₂O₂ (0.3% in PBS, 10% methanol) for 15 min and blocking with 5% normal horse serum and 0.2% BSA for 2 h, sections were incubated with anti-pAkt (Ser473; 1:50; Cell Signaling) antibody for 16 h at 4°C. After washing, sections were incubated with biotinylated secondary antibodies (ABC kit; Pierce, Tattenhall, UK) and then with avidin-biotin complex. Finally, they were developed with 0.05% diaminobenzidine, 0.01% NiCl₂ and 0.02% H₂O₂. As negative controls, some sections were processed as described above in the absence of primary antibody. DARPP-32 immunohistochemistry was performed as previously described.²⁴

Subcellular fractionation. Both cytosolic and nuclear fractions were extracted from fresh striatal samples. Tissue was homogenized in lysis buffer (4 mM HEPES, 0.32 M sucrose, 2 mM PMSF, 10 μ g/ml aprotinin, 1 μ g/ml leupeptin, 2 mM Na₃VO₄, 0.1 mg/ml benzamidine), and centrifuged at 800 \times g for 10 min. The resulting supernatant was centrifuged at 10 000 \times g for 15 min to obtain a cytosol/light membrane supernatant that was further centrifuged at 100 000 \times g for 15 min to obtain the cytosolic fraction (supernatant). The pellet resulting from the initial centrifugation was resuspended in lysis buffer and centrifuged at 800 \times g for 10 min. The pellet, containing washed nuclear fraction, was then resuspended in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 5 μ M ZnCl₂, 10 mM EGTA, 2 mM PMSF, 10 μ g/ml aprotinin, 1 μ g/ml leupeptin, 0.1 mg/ml benzamidine, 2 mM Na₃VO₄ and 100 mM NaF) and incubated for 30 min at 4°C in a tube rotator. Finally, after centrifuging for 20 min at 15 000 \times g, the supernatant was collected and stored at -80°C until use. Protein concentrations were determined as above.

Statistical analysis. Statistical analysis was performed using the one- or two-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test, or Student's *t*-test, as appropriate and indicated in the figure legends.

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