

# Pallidin protein in neurodevelopment and its relation to the pathogenesis of schizophrenia

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Received May 30, 2016; Accepted November 18, 2016

DOI: 10.3892/mmr.2016.6064

**Abstract.** Pallidin is a protein found throughout the nervous system and it has been linked to the development of schizophrenia. At the same time, it has been suggested that schizophrenia is a neurodevelopmental disease. The p38 protein participates in neuronal differentiation and apoptosis. We hypothesized pallidin and p38 play a role in neural system development and the pathogenesis of schizophrenia, and designed several experiments to test this possibility. During pull-down experiments GST-pallidin was able to bind His-Ndn (an HDAC3 binding protein) *in vitro*. In cells co-transfected with HDAC3 and p38, the transcriptional activity of p38 was significantly inhibited by HDAC3. When pallidin was overexpressed, the transcriptional activity of the endogenous HDAC3 improved significantly. Overexpression of pallidin-EGFP in HCT116 p38 wild-type cells increased the endogenous p21 protein and the mRNA levels. The decrease in the expression of endogenous p38 affected the differentiation of N2a cells. The lengths of the neurites generated in the experimental group were significantly shorter than those in the control group. We conclude that pallidin indirectly regulates the transcriptional activity of p38 during neurodevelopment by binding HDAC3 and changing its cellular localization, which leaves p38 uninhibited. Moreover, since pallidin can also affect neuronal differentiation and its variants seem to be related to an increased risk of schizophrenia, it is possible that both pallidin and p38 play a role in the pathogenesis of the disease.

## Introduction

Schizophrenia is a mental disease of unknown etiology. The patients are characterized by experiencing distortions of reality and disturbances of thought and language, and tend to withdraw from social contact (1). Neurotransmitter

disturbances are considered an important factor leading to the development of schizophrenia (2). The fact that the functions of multiple known susceptibility genes seem to relate them to the neurotransmitter system provides molecular evidence for the validity of the neurotransmitter hypothesis.

In addition to the neurotransmitter system abnormality hypothesis, a large amount of research focuses on schizophrenia as a neurodevelopmental disease. Since the early 1980s, researchers have been documenting structural abnormalities on the brains of schizophrenia patients shown on CAT images, and it has been found that the weight of brains of patients is lighter on average than those of non-affected individuals. Specialists attribute this specific finding to abnormalities occurring during early development (3).

With the progress in imaging science, studies now further show that the abnormal cerebral development of schizophrenic patients results in a decrease in volume of the lateral cerebral ventricle, of the grey and white matter, and of some special functional areas such as the hippocampus, and the prefrontal cortex. Based on imaging findings and postmortem examinations of brains, the number of neuron dendritic spines is decreased significantly in schizophrenic patients compared to non-affected individuals (4).

Pallidin is a protein widely expressed in various mammalian tissues. The earliest research showed it to be highly expressed in the brain and skeletal muscle (5). However, further studies have confirmed it is also widely expressed in the central nervous system (CNS) (6). Immunohistochemistry shows pallidin is highly expressed in the glutamatergic neurons of the hippocampus, including some large cone neurons in the neocortex, stellate cells in the entorhinal cortex, and large neurons in the cerebellar nuclei (7). The ACh neurons in the basal forebrain and cranial motor nuclear cluster, the dopaminergic neurons and GABA neurons in the substantia nigra, and the tryptamine neurons in the brainstem also exhibit a certain expression level of the pallidin protein. This widespread presence suggests its participation in diverse neural networks (8). The studies focusing on the cellular localization of pallidin have aided researchers trying to elucidate the functions of this protein. Pallidin is a cytoplasmic protein, and immunoelectron microscopy results, in the granule cell layers of the mouse hippocampal area, have shown the presence of pallidin on neural presynaptic and postsynaptic membranes, indicating that the protein may participate in the regulation of synaptic activity. Furthermore, cell biology research has found pallidin

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**Key words:** pallidin, schizophrenia, p38, HDAC3, neurite growth

in the cell nucleus, indicating that it may also exert nuclear functions (9).

p38 is a protein with important roles in cell apoptosis (10). However, a study shows that it also plays other roles in cells, including as an important transcription factor in the cell nucleus (11). p38 promotes the expression of multiple genes and regulates various functions of the cells after binding to a specific sequence on the genome. In recent years, a large amount of research points to the role of p38 in neuronal development, for instance as a result of its role in apoptosis, an essential factor in neuronal development (12). Furthermore, studies on the PCI2 cell line have shown that the transcriptional activity of p38 has a significant effect on the NGF-induced cell differentiation (13). PCI2 cells expressing exogenous p38 produce much shorter neurites than those that are produced in cells expressing p38 under control by the NGF, highlighting the importance of the interaction between these factors. Finally, two effector molecules related to neurite growth, namely small GTP enzyme Rab13 and F-actin binding protein coronin 1b, have also been found to be transcribed and are regulated by p38 during regenerating neurons (14).

The existing research shows that different enzymes can post-translationally modify newly synthesized p38 protein. Such modifications determine the downstream functionality of the resulting protein. This probably explains the dual functions attributed to p38 as an apoptosis-promoting and a development-promoting mediator. Our experiments are based on the hypothesis that both pallidin and p38 are somehow related to the pathogenesis of schizophrenia at a neurodevelopmental level.

## Materials and methods

**Cell lines and experimental animals.** All of the mammalian cell lines used (HEK293A, HCT116 and N2a) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). For culture, cells were immersed into MEM + 10% fetal bovine serum (FBS) + 1X antibiotic culture medium, and placed in an incubator containing 5% CO<sub>2</sub> at 37°C. The Sandy mice (sdy/sdy) and wild mice (WT; +/+) were purchased from the Jackson Laboratory (Sacramento, CA, USA). All animal experiments were completed under the supervision of the Animal Ethics Committee of Bingzhou People's Hospital (Shandong, China). The mice were sacrificed by decollation after the experiment and cerebral tissues were extracted for later use.

**Separation and culture of primary neurons.** Mouse cortical neurons were separated from the cortical tissue of neonatal mice aged 0-1 days. The cortical tissue was separated into fragments with tweezers in a 1X phosphate-buffered saline (PBS) glass dish. The tissues were incubated with 0.5% trypsin (Gibco-BRL Life Technologies Inc., Grand Island, NY, USA) for 20 min at 37°C, in an EP tube. The dislodged cells were gently centrifuged at 3,900 x g, seeded in a 24-well microplate, and cultured with Dulbecco's modified Eagle's medium (DMEM) + 10% FBS (Gibco-BRL Life Technologies Inc.) containing glutamine (3 pg/ml). After 24 h, the solution was changed to 1X NB/B27 containing glutamine (3 pg/ml) (15).

Table I. The primers used in the experiments.

Primers	Sequence
Human p21-F	5'-GCGACTGTGATGCGCTAAC-3'
Human p21-R	5'-GGGCTTCCTC□?GGAGAAG-3'
Mouse p21-F	5'-AGGAGCAAAGTGTGCCGTG-3'
Mouse p21-R	5'-GGAGTGA rAGAAATCTGTTCG-3'
Human β-actin-F	5'-GACCTGACTGACTACCT-3'
Human β-actin-R	5'-GACAGCGAGGCCAGGATGT-3'
Mouse/rat β-actin-F	5'-GACCTGACTGACTACCTC-3'
Mouse/rat β-actin-R	5'-GACAGCGAGGCCAGGATGC-3'
Human coronin 1b-F	5'-CAGAGCAAAGCCGGCATG-3'
Human coronin 1b-R	5'-GTCAGGGTGCAGGCTGTCC-3'
Mouse coronin 1b-F	5'-GTTGTGCGGCAGAGCAAATT-3'
Mouse coronin 1b-R	5'-CAAGCTGTCCAGACGGTAC-3'
Rat coronin 1b-F	5'-GTTGTGCGGCAGAGCAAAT-3'
Rat coronin 1b-R	5'-GTCAGGGTGCAGGCTGTCT-3'
Human/mouse/rat Rab13-F	5'-ACGACCACCTCTTCAAGCC-3'
Human/mouse/rat Rab 13-R	5'-AAAGCCTCATCCACTCA-3'
Human/mouse p38-F	5'-TCTGGGACAGCCAAGTCTG-3'
Human/mouse p38-R	5'-CTTCCAGTGTGATGATGGA-3'
Rat p38-F	5'-TCAGGGACAGCCAAGTCTG-3'
Rat p38-R	5'-CTTCCAGCGTGA rGATGGT-3'
Human pallidin-F	5'-CTGGTGGACAGCGAGGTG-3'
Human pallidin-R	5'-CAGAGTTCAGGAAGACGT-3'
Mouse pallidin-F	5'-CTGGTGGACAGCGAGGT-3'
Mouse pallidin-R	5'-CTCGCCTCTCTGCGATCT-3'
Mouse HDAC3-F	5'-CTGGTGGACAGCGAGGT-3'
Mouse HDAC3-R	5'-CTCGCCTCTCTGCAATCT-3'
Rat HDAC3-F	5'-ATGAAGGACCAGAAGAGATG-3'
Rat HDAC3-R	5'-GTCCTCAGAGACTGCG-3'

F, forward; R, reverse.

**Polymerase chain reaction (PCR).** Mouse cerebral tissue (1 cm<sup>3</sup>) or 10<sup>6</sup> of the mammalian cells HEK293A, HCT116 and N2a were harvested. RNA was extracted using the TRIzol method and transcribed into cDNA. Each 20 μl PCR reaction solution including DNA polymerase (1 μl), reverse transcriptase (0.35 μl), and template RNA (5 μl) was well mixed and centrifuged at 3,900 x g for 10 sec. The amplification protocol program included a reverse transcription step for 25 min at 50°C; a pre-denaturation step for 3 min at 95°C; and 5 cycles of denaturation at 95°C for 15 sec, annealing for 30 sec at 50°C, and extension for 30 min at 72°C; and finally 40 cycles of a denaturation step for 10 sec at 95°C, and an annealing step for 40 sec at 55°C. The primers used in the experiment are listed in Table I.

**Agarose gel electrophoresis.** Once the agarose gel solution cooled to about 65°C it was mixed well, carefully poured into a glass plate, and allowed to stand at room temperature until gel coagulation. The gel and the glass plate were placed in the electrophoresis tank. A 1X TAE electrophoretic buffer solution was added until the gel plate was completely immersed. The DNA sample and loading buffer were mixed. The gel plate was energized for electrophoresis at a voltage of 60-100 V immediately after loading. The electrophoresis was terminated when

the bromophenol blue reached a point ~1 cm from the lower edge of the gel plate. The gels were stained for about 20 min with ethidium bromide and rinsed for 10 min with clear water. Observations were performed under an ultraviolet lamp (Gemini Bio-Products, Woodland, CA, USA).

**GST pull-down.** The crude extract of the supernatant of the GST and GST-Tag fusion proteins (Invitrogen, Carlsbad, CA, USA) were bound to G4B. A crude extract of the supernatant of the second protein bound to the GST-Tag fusion protein was added. The samples were placed on a shaker platform for 1 h at 4°C, and then centrifuged for 5 min at 500 x g at 4°C. The supernatants were discarded. A 2X SDS gel loading buffer of appropriate amount was added to each sample. They were boiled for 5 min at 100°C, subjected to sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE), and Coomassie Brilliant Blue stained, or detected by western blot analysis (16).

**Cell transfection (lipofection method).** Six-well microplates were inoculated with  $1 \times 10^5$ - $3 \times 10^5$  cells (HEK293A, HCT116 and N2a) per well. For transfection, the cells were cultured in the incubator overnight until the cell density reached 50-80% confluence. The transfection procedure was done following the manufacturer's instructions. Briefly, 1-4  $\mu$ g plasmid DNA (Sangon Biotech Co., Ltd., Shanghai, China) was used for transfections. DMEM was used to prepare solutions containing plasmid DNA-liposome compounds. The cells were washed with DMEM prior to the transfections. Next, 200  $\mu$ l of the DNA-liposome compound solution were mixed with 0.8 ml DMEM and added to the cell incubation wells. The cells were placed back in the incubator for 4-6 h. Finally, 1 ml of the culture solution at a serum concentration equivalent to 2 times the normal was added to the cells in each well. Transfections were allowed to proceed for 18-24 h prior to changing the growth media.

**Co-immunoprecipitation.** Collected lysis buffers of supernatants containing lysed cell proteins were added to appropriate protein A or G gel columns (Bio-Rad Laboratories, Inc., Hercules, CA, USA) coupled with antibody. The protein extracts were incubated with the gel while shaking the mixture for over 5 h at 4°C.

The columns were then centrifuged for 3-5 sec at the highest speed at 4°C. The supernatant was carefully pipetted and discarded. Washing buffer (1  $\mu$ l) was added to the columns. The washing proceeded for 20 min at 4°C. The washing steps were repeated once. The columns were centrifuged for 3-5 sec at the highest speed at 4°C. The supernatants were carefully pipetted out and discarded. Finally, a 2X SDS gel loading buffer solution of appropriate amount was added to the samples. The samples were boiled for 5 min at 100°C and then subjected to the SDS-PAGE analysis.

**Western blot analysis.** The pre-extracted mammalian cell proteins were subject to SDS-PAGE. Next, the gels were placed in a sandwich with transfer membrane in the electrical transfer tank (120 V 320 mA). The transfer proceeded for 60-90 min. The membranes were placed in a dish containing 25 ml blocking buffer. After 2 h on a shaker platform at

room temperature, the primary antibodies were added: Anti-pallidin, HDAC3, his-Ndn, F-actin, GAPDH, and p21 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The reactions were left incubating overnight at 4°C. The next day, each membrane was washed 3 times for 5 min with TBST on a shaker at room temperature. Appropriate secondary antibodies (Boster Inc., Wuhan, China) were added. Incubations took place on a shaker for 1 h at 37°C. The membrane was washed 3 times x 5 min with TBST. A developer was used to observe the results on a platform.

**Immunocytochemistry.** The mammalian cells (HEK293A, HCT116 and N2a) were washed 3 times x 5 min with PBS after growing on glass slides. A goat serum working solution was added carefully with a dropper. The slides were blocked for 20 min at 37°C. The goat anti-pallidin (1:500; cat. no. PA5-18160); rabbit polyclonal anti-HA-Ndn (1:500; cat. no. MA1-012X), mouse polyclonal anti-EGFP (1:500; cat. no. MA1953), and rabbit polyclonal anti-pal-EGFP (1:2,000; cat. no. PA527854) were added. The cells were allowed to stand overnight at 4°C, and then were washed 3 times for 5 min with PBS the next day. The specific secondary antibody polyclonal FITC (1:1,000; cat. no. A-11090; Invitrogen Life Technologies) was added. The reactions were incubated for 2 h at 37°C, stained with Hoechst for 5 min and washed with PBS 3 times for 5 min each. The sections were placed on clean glass slides, air dried, mounted with the anti-fluorescent quenching mounting solution, and conserved away from light at 4°C for microscopic examination.

**RNA interference.** The small RNA interference primers (Google Biotechnology Co., Ltd., Shanghai, China) were designed as follows: 5'-AAGUGACAAGUCAAGAGAAGCAA-3' (target to human pallidin mRNA); 5'-AAGUGAUAA GUCAAGAGAAGCAA-3' (target to mouse pallidin mRNA); 5'-AAGCGACAAGUCAAAAAGAAGCAA-3' (target to rat pallidin mRNA); and 5'-CCACUUGAUGGAGAGUAUU-3' (target to mouse p38 mRNA). The cell densities were allowed to reach 40-60% confluence before transfection. The following solutions were prepared: Solution A with 2-10  $\mu$ l Lipofectamine Oligofectamine (Invitrogen) transfection reagent and 80  $\mu$ l Opti-MEM, which was incubated for 5 min at room temperature; and solution B with 4-8  $\mu$ l of siRNA and 80  $\mu$ l Opti-MEM. Solution A and B were mixed well together. The mixtures were allowed to stand for 30 min at room temperature. The cells were washed with Opti-MEM. Then 0.8  $\mu$ l Opti-MEM were added to each liposome mixture and the transfection mixture was added to the cells. The cultures were incubated for 4-6 h. Then the culture medium was replaced with a fresh one at 18-24 h after transfection. The cells were harvested 4-72 h after transfection.

**Cell differentiation experiment and detection of the process of growth.** N2a was cultured in complete medium (10% FBS + DMEM) for 24 h. The plasmid or siRNA (as above) was transfected with Lipofectamine 2000 or Oligo Lipofectamine for 6 h. Next, cells were cultured with DMEM containing 2% FBS to induce cell differentiation. The new processes on the cell membranes were observed under a microscope. Approximately 200-250 cells were counted in each experiment. The primary

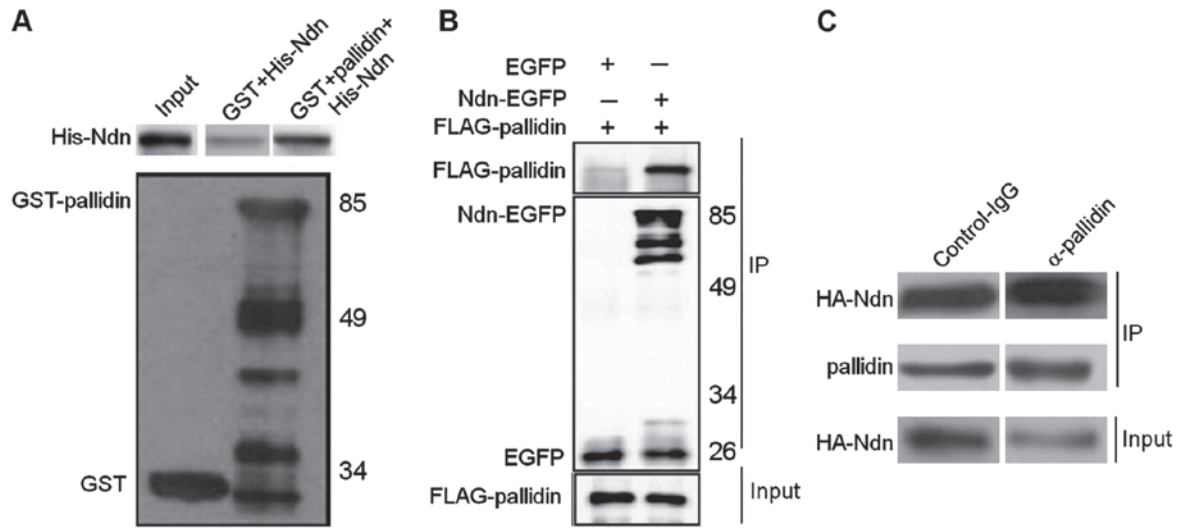


Figure 1. Identification of binding between HDAC3 and pallidin. (A) GST-pallidin co-immunoprecipitates with His-Ndn *in vitro* whereas the control GST does so only to a smaller extent. (B) FLAG-pallidin also co-precipitates with Ndn-EGFP when Ndn-EGFP was precipitated by the GFP antibody. (C) HA-Ndn and the endogenous pallidin co-precipitated, whereas the control serum was not able to precipitate as much HA-Ndn. It seems possible that HDAC3 and pallidin could be directly interacting.

cortical neurons, separated from the neonatal mice, were inoculated into a 24-well microplate. The solution was replaced at 24 h. The cells were stained with Tuj (Biosynthan GmbH, Berlin, Germany) to show the neuronal processes 5 days after being cultured with NB/B27.

**Detection of living cells with the MTT method.** The plasmid at a determined concentration was used for transfections at a cell confluence of 50–60%. The original culture medium was pipetted out after 36–48 h. Cells were washed 2 times with 1X PBS. Five hundred microliters of MTT diluted with phenol red-free MEM was added to obtain a final concentration of 0.5 mg/ml. The reactions proceeded for 2–3 h at 37°C. Then, the excess MTT was absorbed, and 500  $\mu$ l of 0.04 M acidulated isopropanol was added. The cell culture dish was gently shaken back and forth until the bluish violet crystal formazan was completely dissolved in the acidulated isopropanol. The supernatant was transferred to a 96-well ELISA plate (Thermo Fisher Scientific, Inc., Beijing, China). The optical density was determined at 570 nm (Thermo Fisher Scientific, Waltham, MA, USA).

**Statistical analysis.** The measurement data were expressed with mean  $\pm$  standard deviation. The significance of the average differences between two groups of the data were tested with the t-test of two independent samples. The measurement data were expressed as a percentage. The Chi-square test was used for comparisons among groups. The differences were considered statistically significant when  $P < 0.05$ . The SPSS 16.0 software was used to process the statistical data.

## Results

**Identification of binding between HDAC3 and pallidin.** The experimental results showed that GST-pallidin co-immunoprecipitates with His-Ndn *in vitro*, whereas, not with the control GST alone. To further verify the binding between pallidin

and HDAC3 in the mammalian cell lines, FLAG-pallidin and EGFP or Ndn-EGFP proteins were co-expressed in a human embryonic renal cell line (HEK293A). Subsequently, a co-immunoprecipitation experiment was conducted. The results indicated that FLAG-pallidin also co-precipitates with Ndn-EGFP when Ndn-EGFP was bound to the GFP antibody; as a negative control, EGFP alone was not able to co-precipitate FLAG-pallidin (Fig. 1B). Next, a dysbindin antibody (which can also bind pallidin) was used to precipitate the endogenous pallidin in a mouse neuroma mother cell line (N2a) transfected with HA-Ndn. The results showed that HA-Ndn and the endogenous pallidin co-precipitated, whereas the control IgG antibody was not able to precipitate as much HA-Ndn (Fig. 1C).

**Pallidin changes localization and influences the transcriptional activity of p38.** Immunofluorescence results indicated that overexpression of pallidin-EGFP caused a change in the cellular distribution of HA-Ndn, from being present normally in both the cytoplasm and nucleus to being present only in the cytoplasm (Fig. 2A). During previous experiments we observed that when exogenous p38 was overexpressed, the activity of the reporter gene was markedly increased. By contrast, the transcriptional activity of p38 was significantly inhibited by HDAC3 in the cells co-transfected with HDAC3 and p38. The transcriptional activity of endogenous HDAC3 increased significantly when pallidin was overexpressed (data not shown). Overexpression of pallidin-EGFP in the HCT116 p38 wild-type cell line increased the endogenous p21 protein and mRNA levels (Fig. 2B and C). The small RNA interference method was used to decrease the expression level of endogenous pallidin and observe the effects on the expression level of endogenous p21 in the HCT116 p38<sup>+/+</sup> cell line. A decrease in p21 was still significant even after the p38 activator doxorubicin (DOX) was used to promote the transcriptional activity of endogenous p38 (Fig. 2D). Various different plasmids were transfected into HCT116 p38<sup>-/-</sup> cells as shown in Fig. 2E. It



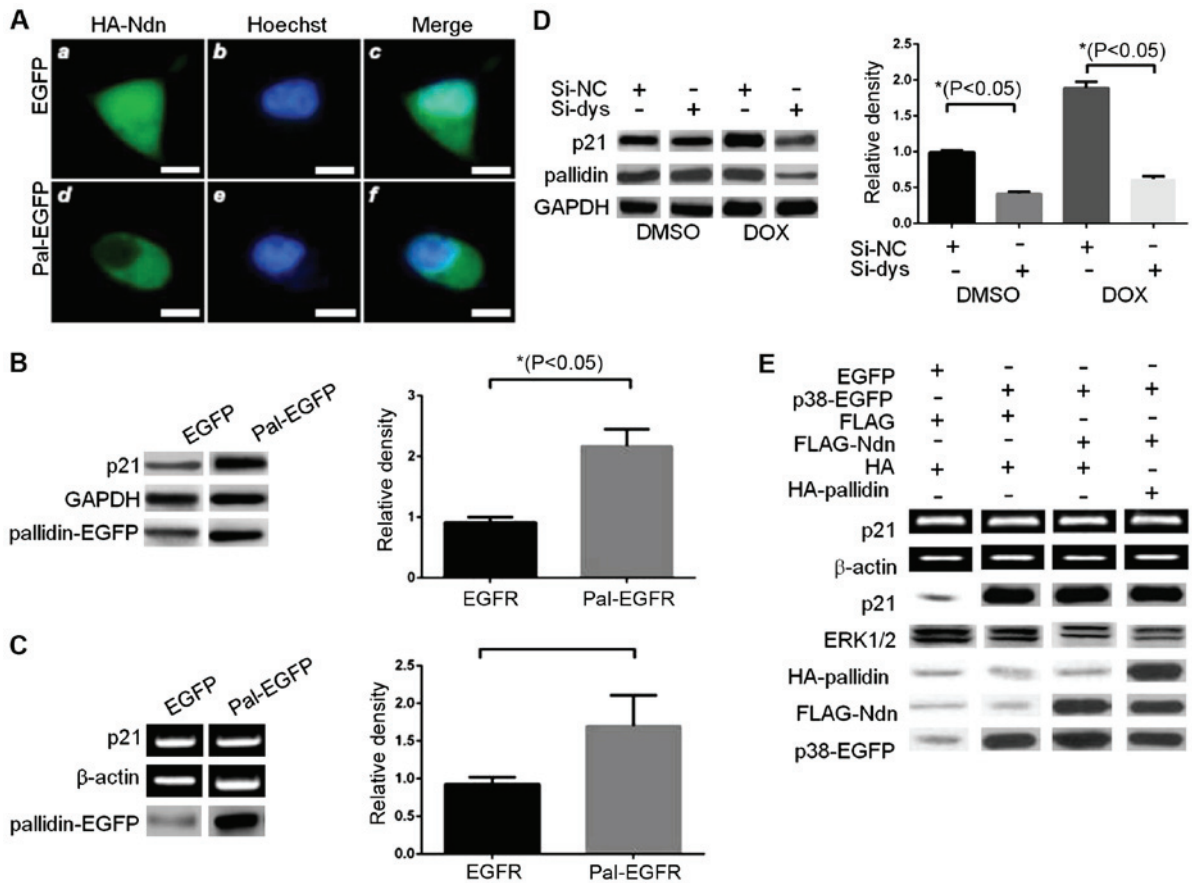


Figure 2. Pallidin changes HA-Ndn localization and influences the transcriptional activity of p38. (A) The immunofluorescence results showed that overexpression of pallidin-EGFP caused HA-Ndn to disappear from the nucleus and be present only in the cytoplasm of the transfected cells. (B and C) Overexpression of pallidin-EGFP in the HCT116 p38 wild-type cell line increased the endogenous p21 protein (western blot analysis) and mRNA (quantitative PCR) levels. (D) The small RNA interference method was used to decrease the expression level of endogenous pallidin and observe the effect on the expression level of the endogenous p21 protein in the HCT116 p38<sup>+/+</sup> cell line. The decrease in p21 protein was significant even upon activation of endogenous p38 transcription by doxorubicin (DOX). (E) Different combinations of overexpressing plasmids were transfected into HCT116 p38<sup>-/-</sup> cells. Pallidin was able to reverse the decrease of the p21 protein and RNA levels in the HCT116 p38<sup>-/-</sup> cell line as a result of its inhibitory effect on HDAC3.

also indicated that pallidin was able to reverse the decrease of the p21 protein and RNA levels as a result of inhibitory effect of HDAC3. This coincided with the previous result of the reporter gene. In conclusion, pallidin was able to influence the transcriptional activity of the downstream p38 by changing the intracellular localization of HDAC3.

*The 90-110 amino acid sequences of pallidin are participating in HDAC3 regulation.* In co-immunoprecipitation experiments both pallidin-EGFP and pallidin-N-EGFP co-precipitated FLAG-HDAC3. However, neither pallidin-C-EGFP nor the negative control EGFP, were able to co-precipitate FLAG-HDAC3 (Fig. 3A). We established two pallidin mutants with either the CCD or LZM domains deleted. Co-immunoprecipitation assays were conducted after the two mutants and the full-length pallidin were co-transfected with FLAG-HDAC3. The result showed that both mutants lost their capacity to bind to HDAC3 (Fig. 3B). The full-length pallidin-EGFP enabled HA-HDAC3 to exhibit significant cytoplasmic localization. On the contrary, the pallidin-ALZM-EGFP without its leucine zipper was not able to influence the subcellular localization of HA-HDAC3. HA-HDAC3 exhibited uniform distribution in the cytoplasm

and nucleus in the cells co-transfected with the mutant (similar to those transfected with EGFP control) (Fig. 3C).

*Pallidin influenced growth of the differentiated cellular processes by regulating the downstream gene product of p38.* The levels of mRNA in the p38 downstream target genes was detected with RT-PCR after the expression level of endogenous p38 was knocked down. At the same time, a western blot analysis demonstrated that the expression levels of the two effect proteins coronin 1b and Rab13 decreased (Fig. 4A). The two mRNA molecules of coronin 1b and Rab13 transcriptionally induced by p38 decreased with the reduction in pallidin (Fig. 4B). Meanwhile, the expression levels of p38 and HDAC3 were not influenced by pallidin (Fig. 4C). Our results showed that the changes in pallidin can affect the transcriptional activity of p38 during cell differentiation. The decreased expression of endogenous p38 influenced the degree of N2a cellular differentiation. The length of the nervous processes (neurites) generated in the cells of the experimental group was significantly shorter than the length of the processes in the control group. Similar to the endogenous p38, the decrease of endogenous pallidin also produced the same effect in the N2a cell line (Fig. 4D).

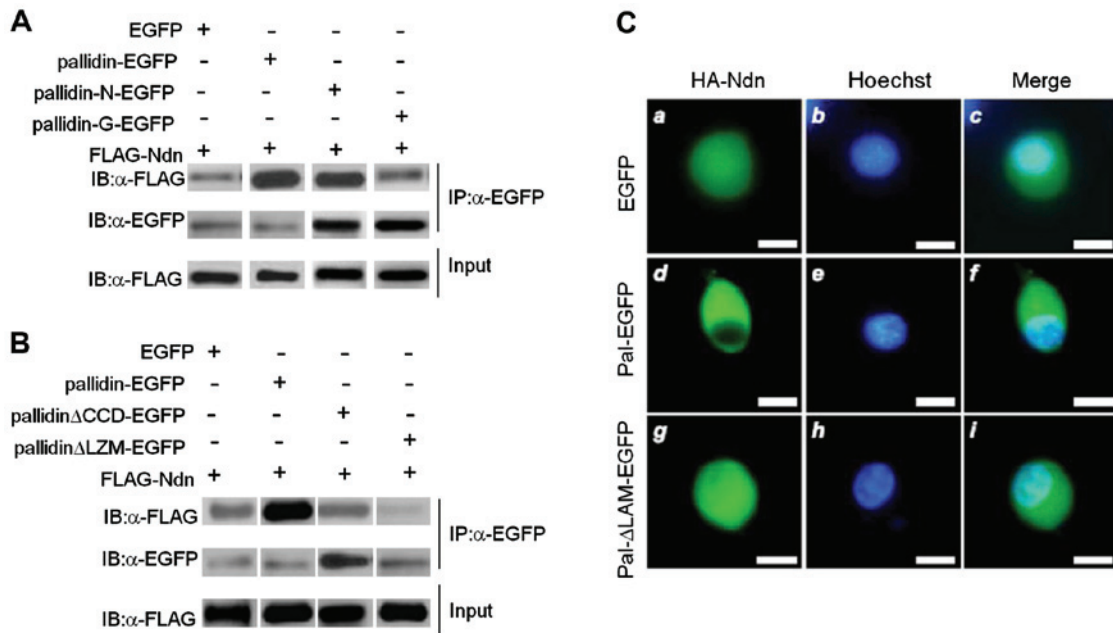


Figure 3. The 90-110 amino acid sequences of pallidin participating in HDAC3 regulation. (A) Both pallidin-EGFP and pallidin-N-EGFP co-precipitate FLAG-HDAC3. By contrast, neither pallidin-C-EGFP nor the negative Control EGFP co-precipitated with FLAG-HDAC3. (B) Co-immunoprecipitation assays were conducted after the pallidin mutants with their CCD or LZM domain deleted were co-transfected with FLAG-HDAC3. Both mutants lost their capacity to bind to HDAC3. (C) A pallidin- $\Delta$ LZM-EGFP without its leucine zipper was not able to influence the subcellular localization of HA-HDAC3. HA-HDAC3 exhibited a uniform distribution throughout the cytoplasm and nucleus in the cells co-transfected with the mutant.

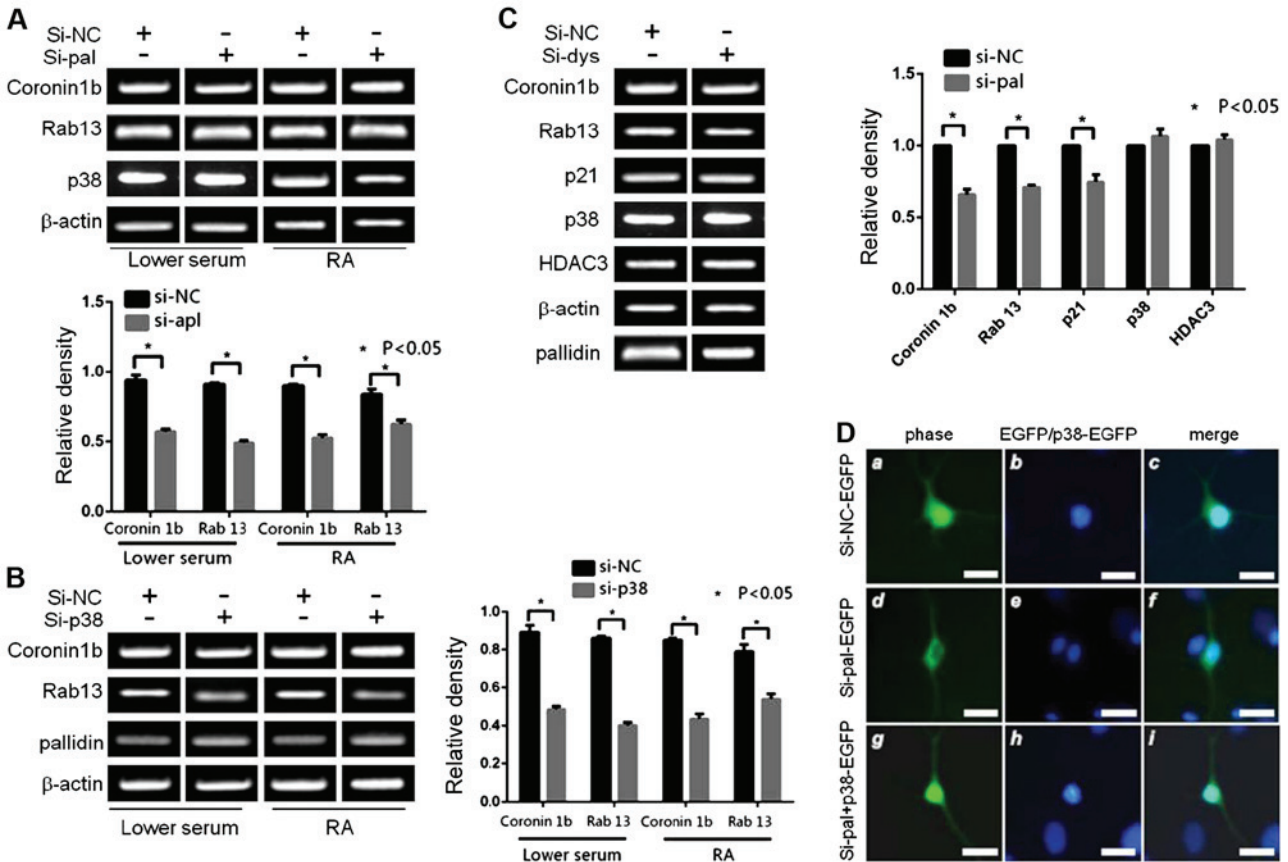


Figure 4. Pallidin influences growth of the differentiated cell processes by regulating the downstream gene product of p38. (A) The levels of mRNA in the downstream target genes were detected by RT-PCR after the expression level of endogenous p38 was knocked down. The results demonstrated that the expression levels of the two effect proteins, namely coronin 1b and Rab13, both decreased. (B) The two effector molecules coronin 1b and Rab13 transcriptionally expressed by p38 were also decreased with the reduction in pallidin. (C) The expression levels of p38 and HDAC3 were not influenced by pallidin. (D) The decreased expression of endogenous p38 influenced the degree of N2a cellular differentiation. The length of the nerve processes generated in the experimental group was significantly shorter than that in the control group. Similar to the endogenous p38, the decrease in endogenous pallidin also produced the same effect in the N2a cells.

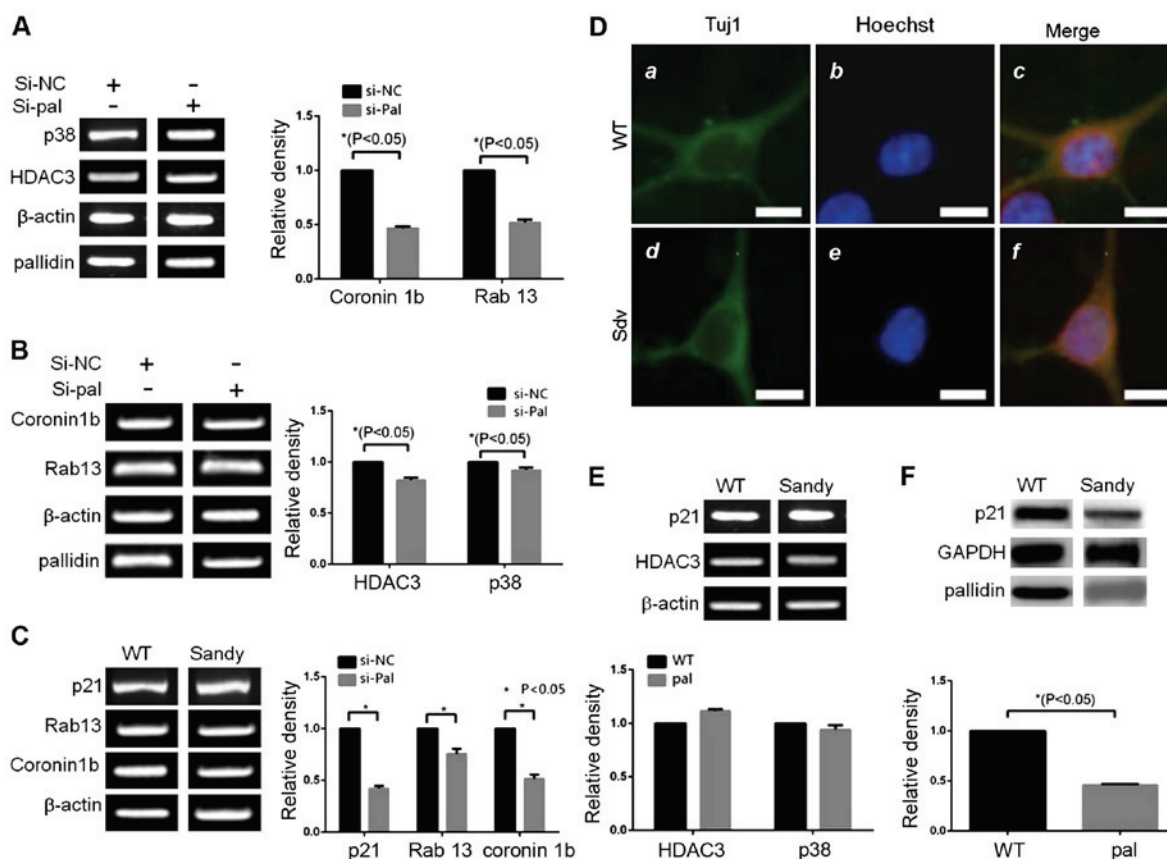


Figure 5. The role of pallidin in regulating the neurite growth. (A) In the primary culture rat cerebral cortex neurons, when the expression level of pallidin decreased as a result of small RNA interference, the expression levels of coronin 1b and Rab13, the downstream transcription target genes of p38, also decreased. (B) The expression levels of p38 and HDAC3 did not change. (C) The detection of the sandy mice without pallidin also indicated that the mRNA levels of p21, coronin 1b, and Rab13 decreased significantly when compared with those in the wild-type (WT) mice at the same age. (D) The length of the neurites of the cortical neurons of the sandy mice decreased significantly at 5 days after the neurons were cultured *in vitro* when compared to that of the wild mice (decreased by ~20%,  $P=0.0068$ ). No change in body size was observed. (E and F) The p21 protein level in the hippocampal region of the sandy mice decreased significantly when compared with that in the WT mice. There was no significant difference in expression level between p38 and HDAC3.

**The role of pallidin in regulating the process growth.** In the primary culture of rat cerebral cortex neurons, when the expression level of pallidin decreased as a result of small RNA interference, the expression levels of coronin 1b and Rab13 (the downstream transcription target genes of p38) also decreased. The expression levels of p38 and HDAC3 did not change (Fig. 5B). The detection of the sandy mice without pallidin also indicated that the mRNA levels of p21, coronin 1b, and Rab13 decreased significantly when compared with those in the wild-type (WT) mice at the same age (Fig. 5C). The p21 protein level in the hippocampal region of the sandy mice decreased significantly when compared with that in the wild mice (Fig. 5E). Similarly, there were no significant differences in expression levels between p38 and HDAC3 (Fig. 5F). The length of the processes of the cortical neurons of the sandy mice decreased significantly at 5 days after the neurons were cultured *in vitro* when compared with the processes in the WT mice (decreased by ~20%,  $P=0.0068$ ). No change in body size was observed (Fig. 5D).

## Discussion

In this study, we investigated the new functions of the schizophrenia-related protein pallidin. Regulating the transcriptional activity of p38 influences the neural system development.

We have demonstrated that pallidin promotes the transcriptional activity of the p38 inhibited by intranuclear HDAC3 by retaining HDAC3 in the cytoplasm. HDAC3 is a known transcription inhibitor (17). It inhibits the transcriptional activity by being bound to proteins such as E2F1 and p38, in the cell nucleus (5,8,18). It regulates the functions of neurons by inhibiting important intranuclear transcription factors (19,20).

This study shows that dysbindin-1 (a pallidin homologue) can be directly bound to HDAC3 *in vitro*. The results also show that overexpression of pallidin can change the distribution ratio of HDAC3 in the cytoplasm/nucleus by retaining HDAC3 within the cytoplasm. The inhibition of p38 transcription by HDAC3 is reduced with lower amounts of HDAC3. Hence, pallidin can change the transcriptional activity of p38 by regulating HDAC3. A pallidin-ALZM mutant variant lacking 90-110 amino acid sequences (21) demonstrates that pallidin can change the HDAC3 location. The mutant cannot bind HDAC3 and therefore, does not affect its location. Thus, we discuss a new function of pallidin: It influences the transcriptional activity of p38 by being bound to HDAC3 and changing the location of HDAC3.

In the knock-down experiments of the endogenous pallidin, we observed the same result as when knocking down the endogenous p38: The reduction significantly interferes with the cellular differentiation. Overexpression of p38 in the



pallidin knocked down cells can restore the cellular differentiation ability.

These results indicate that it is possible that pallidin influences the growth of the nerve processes via p38. The *in vivo* data also indicate that the genes related to neural development downstream of p38, coronin 1b and Rab13 decreased in the pallidin knocked down sandy mice. Moreover, the cortical neurons of the sandy mice also show alterations in neuronal process growth characteristics. Therefore, we believe that the endogenous pallidin plays a role in maintaining the transcriptional activity of p38 during neurodevelopment and guaranteeing the expression of necessary genes. It has been reported that Rab13 promotes neuronal development by binding and antagonizing the neuronal process growth inhibiting factors (22-24). In addition, studies have indicated that knockdown of coronin 1b can promote aggregation of F-actin in leading edge and influence the morphology of the plate-shaped filopodia on the cells (25,26). Concentrations of both effector molecules, Rab13 and coronin 1b decrease with overexpression of pallidin. Thus, the abnormal arrangement of F-actin in the sandy mice may be associated with the decrease of coronin 1b and Rab13.

Based on the results of our experiments, it seems quite possible that pallidin influences the growth of the nerve processes via p38, thereby participating in neuronal development. An abnormally developed nervous system may also increase the risk for schizophrenia. Given that pallidin has been linked to the pathogenesis of schizophrenia, and we found pallidin to function with p38 during neuronal development, our study argues for schizophrenia as a neurodevelopmental disease.

## References

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