Molecular and Cellular Neuroscience 68 (2015) 194–202

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

# ZDHHC17 promotes axon outgrowth by regulating TrkA–tubulin complex formation

Wei Shi [a], Fen Wang [a], Ming Gao [c], Yang Yang [d], Zhaoxia Du [a], Chen Wang [a], Yao Yao [a], Kun He [a], Xueran Chen [b,*], Aijun Hao [a,**]

[a] Key Laboratory of the Ministry of Education for Experimental Teratology, Shandong Provincial Key Laboratory of Mental Disorders, Department of Histology and Embryology, Shandong University School of Medicine, No. 44, Wenhua Xi Road, Jinan, Shandong 250012, PR China
[b] Center of Medical Physics and Technology, Hefei Institutes of Physical Science, Chinese Academy of Sciences, Hefei 230031, PR China
[c] Reproductive Medical Center of Shandong University, Shandong University School of Medicine, No. 44, Wenhua Xi Road, Jinan, Shandong 250012, PR China
[d] Infertility Center, Qilu Hospital, Shandong University School of Medicine, No. 44, Wenhua Xi Road, Jinan, Shandong 250012, PR China

ARTICLE INFO

*Article history:*
Received 21 August 2014
Revised 7 July 2015
Accepted 23 July 2015
Available online 29 July 2015

*Keywords:*
ZDHHC17
Axon outgrowth
ERK1/2
TrkA
Tubulin

ABSTRACT

Correct axonal growth during nervous system development is critical for synaptic transduction and nervous system function. Proper axon outgrowth relies on a suitable growing environment and the expression of a series of endogenous neuronal factors. However, the mechanisms of these neuronal proteins involved in neuronal development remain unknown. ZDHHC17 is a member of the DHHC (Asp-His-His-Cys)-containing family, a family of highly homologous proteins. Here, we show that loss of function of ZDHHC17 in zebrafish leads to motor dysfunction in 3-day post-fertilization (dpf) larvae. We performed immunolabeling analysis to reveal that mobility dysfunction was due to a significant defect in the axonal outgrowth of spinal motor neurons (SMNs) without affecting neuron generation. In addition, we found a similar phenotype in *zdhhc17* siRNA-treated neural stem cells (NSCs) and PC12 cells. Inhibition of *zdhhc17* limited neurite outgrowth and branching in both NSCs and PC12. Furthermore, we discovered that the level of phosphorylation of extracellular-regulated kinase (ERK) 1/2, a major downstream effector of tyrosine kinase (TrkA), was largely upregulated in ZDHHC17 overexpressing PC12 cells by a mechanism independent on its palmitoyltransferase (PAT) activity. Specifically, ZDHHC17 is necessary for proper TrkA–tubulin module formation in PC12 cells. These results strongly indicate that ZDHHC17 is essential for correct axon outgrowth in vivo and in vitro. Our findings identify ZDHHC17 as an important upstream factor of ERK1/2 to regulate the interaction between TrkA and tubulin during neuronal development.



## 1. Introduction

Successful axon outgrowth and formation primarily depend on two factors: a permissive environment and the expression of growth-associated proteins (GAPs) in neurons including transcription factors, GAP-43, and several cell adhesion and cytoskeletal proteins (Akiyama et al., 2012; Cheng and Poo, 2012; Euteneuer et al., 2013; Franze, 2013; Auer et al., 2012; Frey et al., 2000; Langhorst et al., 2008; Li et al., 2013; Ye et al., 2012). In response to appropriate signals, neurons exhibit remarkable properties that they express growth-associated proteins to promote neuron differentiation and axon outgrowth. However, the neuron-intrinsic factors involved in this precise temporal and spatial assembly are not well defined. In particular, the molecular mechanisms by which these signals regulate the assembly of complexes for cytoskeletal remodeling are largely unknown.

The zinc finger DHHC-containing (ZDHHC) proteins belong to a family of palmitoyltransferases (PATs) that catalyze protein palmitoylation, a posttranslational lipid modification affecting protein targeting, trafficking, and function (Fukata et al., 2004, 2006a). Many members of the DHHC family are expressed in neurons, but ZDHHC17 is detected far more frequently at both mRNA and protein levels in neuronal studies (Doyle et al., 2008; Heiman et al., 2008; Huang et al., 2004, 2009). This suggests that ZDHHC17 might be particularly important in neuronal regulation. Consistent with this hypothesis, ZDHHC17 is implicated in higher brain function, since mice with reduced ZDHHC17 levels displayed behavioral, biochemical, and neuropathological defects that are reminiscent of Huntington's disease (HD) (Singaraja et al., 2011; Young et al., 2012b). ZDHHC17 was recently identified as a key player in ischemic stroke and a regulator of neuronal cell death (Yang and Cynader, 2011). In addition to its well-studied function in protein palmitoylation, other roles of ZDHHC17 are just beginning

*Abbreviations:* DHHC, Asp-His-His-Cys; dpf, days post-fertilization; ERK1/2, extracellular signal-related kinase1/2; hpf, hours post-fertilization; JNK, c-Jun N terminus kinase; MAPK, mitogen-activated protein kinase; NSC, neural stem cell; PAT, palmitoyltransferase; SMN, spinal motor neuron; TrkA, tyrosine kinase.

* Correspondence to: X. Chen, Center of Medical Physics and Technology, Hefei Institutes of Physical Science, Chinese Academy of Sciences, No. 350 Shushan Hu Road, Hefei 230031, PR China.
** Corresponding author.
*E-mail addresses:* xueranchen@cmpt.ac.cn (X. Chen), aijunhao@sdu.edu.cn (A. Hao).

http://dx.doi.org/10.1016/j.mcn.2015.07.005
1044-7431/

to be appreciated, including the mediation of $Mg^{2+}$ transport (Goytain et al., 2008), regulation of $Ca^{2+}$ channel functions (Hines et al., 2010) and activation of c-Jun N-terminus kinase (JNK) pathways (Harada et al., 2003). Moreover, ZDHHC17 is responsible for palmitoylation of large conductance calcium- and voltage-activated potassium (BK) channels, which controls membrane potential and calcium influx (Tian et al., 2010). However, the roles and molecular mechanisms of ZDHHC17 in neuronal development and function are not yet fully understood.

We used *zdhhc17* morpholino (MO) in zebrafish and *zdhhc17*-specific siRNA in NSCs and PC12 cells to demonstrate that ZDHHC17 regulates spinal motor neuron (SMN) axonal formation in vivo, axonal growth in vitro, NSC differentiation, and PC12 neurite outgrowth. Collectively, our results show that ZDHHC17 is a neuron-intrinsic factor for axon growth that is crucial for the TrkA–tubulin interaction, which regulates signal transmission, including ERK1/2 phosphorylation.

## 2. Material and methods

### 2.1. Zebrafish maintenance and treatment

The zebrafish strains AB was maintained and bred according to standard procedures. We used the Esen zebrafish culture system (Esen environ science, Beijing, China) to control the temperature and the day–night cycles. All embryos were maintained at 28.5 °C without crowding (5–10 embryos/mL).

Larval movements stimulated by the touch response test were quantified using the ImageJ manual tracking plugins (National Institutes of Health, Bethesda, MD, USA).

### 2.2. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using an RNeasy kit (Qiagen, USA) according to the manufacturer's instructions. During this process, total RNA was treated with DNaseI. First-strand cDNA synthesis was carried out using Reverse Transcription System (Promega, USA), following the manufacturer's protocol. PCR was performed with Premix Taq mixture (Invitrogen, USA). β-Actin was used as an internal control. The PCR products were separated on 1.2% agarose gel by electrophoresis, stained with ethidium bromide and visualized under the ultraviolet light. The primers' sequences and the sizes of amplified products were shown in Table 1.

### 2.3. Cell culture and transfection

All reagents for cell cultures were purchased from Invitrogen.

For primary NSC culture, brains were removed from Kun Ming mouse (KM strain) embryos at E12.5. After washing and resuspension, cells were seeded at $2 \times 10^5$ cells/mL in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) medium supplemented with 2% B27 (Gibco BRL) plus 100 U/mL penicillin, 100 U/mL streptomycin, and basic fibroblast growth factor (bFGF, 20 ng/mL; R&D Systems, USA). The cells were cultured at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air. For NSC differentiation, the neurospheres were transferred into differentiation medium containing 2% fetal bovine serum (FBS) without growth factors and cultured for 7 days.

PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla. Their embryonic origin is the neural crest, which contains a mixture of neuroblastic cells and eosinophilic cells. PC12 cells were maintained in DMEM plus 10% horse serum, 5% fetal calf serum (FCS) and antibiotics at 37 °C with 5% $CO_2$ and 95% air. For differentiation, neuronal growth factor (NGF, Invitrogen) was added at 50 ng/mL under the serum conditions. Neurite outgrowth was visible 3 days later.

For protein expression and *zdhhc17* RNAi, the transfection of expression plasmids and pSuper-*zdhhc17* siRNA was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

### 2.4. Immunoprecipitation and immunoblotting

Cells were washed once in PBS and lysed 30 min at 4 °C in IP buffer (0.5% Nonidet P-40, 500 mM Tris·HCl (pH 7.4), 20 mM EDTA, 10 mM NaF, 2 mM benzamidine, and a mixture of protease inhibitors) and centrifuged 15 min, 13,000 rpm at 4 °C. The supernatants were incubated with adequate antibody(1 μg) at 4 °C overnight and incubated 2–4 h at 4 °C with anti-protein A-coupled Sepharose beads. The proteins were then eluted and analyzed by Western blotting as described previously (Zhao et al., 2011).

The primary antibodies were as follows:

For Western blotting: anti-ERK1/2 (1:2000, Cell Signaling Technology), anti-p-ERK1/2 (1:2000, Cell Signaling Technology), anti-p38 (1:1000, Cell Signaling Technology), anti-p-p38 (1:1000, Cell Signaling Technology), anti-JNK (1:1000, Cell Signaling Technology), anti-p-JNK (1:1000, Cell Signaling Technology) and anti-β-actin (1:2000, Sigma).

For immunoprecipitation: anti-TrkA (Abcam), anti-tubulin (Santa Cruz Biotechnology) and anti-GFP (Santa Cruz Biotechnology).

### 2.5. Constructs and mutagenesis

*Zdhhc17* siRNA primers were annealed and inserted into the HindIII/BglII sites of the pSUPER vector (Oligo Engine). The primers used to generate *zdhhc17* siRNA were as follows. Complementary oligonucleotides: 5′-GATCCCC**GGAGATACAAGCACTTTAA**TTCAAGAGATTAAAGTGCTTGTATCTCCTTTTTGGAAA-3′, and 5′-AGCTTTTCCAAAAAGGAGATACAAGCACTTTAATCTCTTGAA**TTAAAGTGCTTGTATCTCC**GGG-3′ (corresponding to nucleotides 2039–2057 of mouse zdhhc17 mRNA). The primers used to generate scrambled siRNA (control siRNA) were as follows: 5′-GATCCCC**GATAAGAACAGCGGCTATA**TTCAAGAGATATAGCCGCTGTTCTTATCTTTTTA-3′ and 5′-AGCTTAAAAAGATAAGAACAGCGGCTATATCTCTTGAA**TATAGCCGCTGTTCTTATCGGGGAT**CGGG-3′.

Zebrafish full-length *zdhhc17* cDNA was amplified using 5′-CGCGGATCCATGGCGGACGCTCTGGTTGGATATG-3′ forward and 5′-CCGCTCGAGCACCAGCTGGTATCCTGAGCCGGAC-3′ reverse primers and cloned into pCS2-GFP expression vectors using BamHI and XhoI restriction sites included in the primers (underlined).

Mouse full-length *zdhhc17* cDNA was amplified with 5′-CCCAAGCTTATGCAGCGGGAGGAGGGATTTAACA-3′ forward and 5′-CTAGTCTAGACTACACAAGCTGGTACCCAGATCC-3′ reverse primers and cloned into a pCS2 + expression vector using the HindIII and XbaI restriction sites included in the primers (underlined). *Zdhhc17*Δ ankyrin domain was generated by the deletion of nucleotides encoding amino acids 89–257 using an Easy Mutagenesis System (Transgen Biotech).

**Table 1**
Primers for RT-PCR and RNA probe synthesis.

| Genes | Primer sequence (5′–3′) | | Product length (bp) |
|---|---|---|---|
| *Danio rerio zdhhc17* | Sense primer | CAAGACAAGGTCATCTCTCCA | 575 |
| | Anti-sense primer | CAAATCAGCAATAAATCCCAC | |
| *Mus musculus dhhc17* | Sense primer | CATGGGTGGGTAACTGTGTAGGTG | 352 |
| | Anti-sense primer | GGGCTTTCAATAGATGTTGTCGTA | |
| *Rattus norvegicus dhhc17* | Sense primer | GGATGAGTACGATACCGAAACG | 460 |
| | Anti-sense primer | TCCAAACTGAGCAGCCAAGTGG | |