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RESEARCH ARTICLE

GIT1 enhances neurite outgrowth by stimulating microtubule assembly

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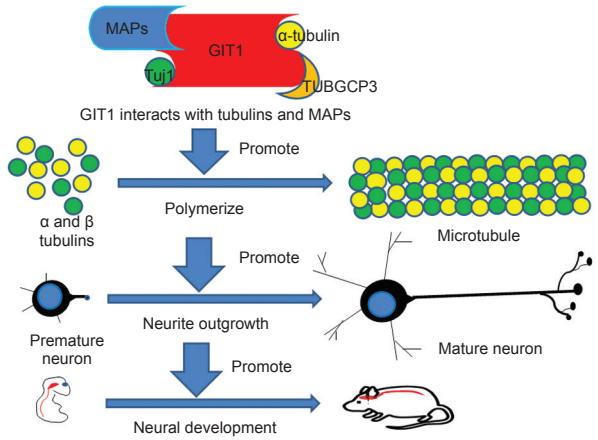
Graphical Abstract

GIT1 promotes neurite outgrowth by binding to tubulins and MAPs to control microtubule remodeling



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Abstract

GIT1, a G-protein-coupled receptor kinase interacting protein, has been reported to be involved in neurite outgrowth. However, the neurobiological functions of the protein remain unclear. In this study, we found that GIT1 was highly expressed in the nervous system, and its expression was maintained throughout all stages of neuritogenesis in the brain. In primary cultured mouse hippocampal neurons from GIT1 knockout mice, there was a significant reduction in total neurite length per neuron, as well as in the average length of axon-like structures, which could not be prevented by nerve growth factor treatment. Overexpression of GIT1 significantly promoted axon growth and fully rescued the axon outgrowth defect in the primary hippocampal neuron cultures from GIT1 knockout mice. The GIT1 N terminal region, including the ADP ribosylation factor-GTPase activating protein domain, the ankyrin domains and the Spa2 homology domain, were sufficient to enhance axonal extension. Importantly, GIT1 bound to many tubulin proteins and microtubule-associated proteins, and it accelerated microtubule assembly *in vitro*. Collectively, our findings suggest that GIT1 promotes neurite outgrowth, at least partially by stimulating microtubule assembly. This study provides new insight into the cellular and molecular pathogenesis of GIT1-associated neurological diseases.

Key Words: *nerve regeneration; GIT1; hippocampal neurons; neurite outgrowth; tubulin; microtubule-associated proteins; neural regeneration*

Introduction

GIT1 is a member of the GIT subfamily of ADP ribosylation factor (ARF)-GTPase activating protein (GAP) family of proteins (Turner et al., 2001; Kahn et al., 2008; Myers and Casanova, 2008), which are characterized by an ARFGAP domain that stimulates the GTPase activity of proteins. GIT1 also contains additional domains, including three ankyrin domains, a Spa2 homology domain (SHD), a synaptic localization domain (SLD), and a paxillin binding domain (PBD) (Zhang et al., 2003). These diverse domains enable GIT1 to interact with various proteins, and as a result, GIT1 has the capacity to play a role in multiple biological processes (Hoefen and Berk, 2006). For example, GIT1 has been reported to play a role in endocytosis (Bhanot et al., 2010; Nakaya et al., 2013; Podufall et al., 2014), adhesion (Wilson et al., 2014; Hammer et al., 2015), cell migration (Hsu et al., 2014; Penela et al., 2014; Podufall et al., 2014), proliferation (Wu et al., 2014; Xiao et al., 2014) and apoptosis (Zhang et al., 2009, 2015).

Histological studies have demonstrated that GIT1 is mainly localized to brain tissues (Schmalzigaug et al., 2007), and accordingly, the biological function of GIT1 in the nervous system has been extensively studied. Several GIT1 knockout mouse models have been developed to investigate the association between GIT1 and brain development (Schmalzigaug et al., 2009; Menon et al., 2010; Won et al., 2011). Although brain developmental defects have been observed in these mouse models, the phenotypes are not consistent. A recent report found that GIT1 deletion leads to a microcephaly-like small brain phenotype (Hong and Mah, 2015). Therefore, the functions of GIT1 in brain development are unclear and require further study.

The function of GIT1 in the development of spines and synapses has been relatively well studied, and interactions between GIT1 and specific proteins, particularly cytoskeleton-related proteins, are the foundation of neural plasticity (Zhang et al., 2005, 2013; Segura et al., 2007; Richier et al., 2010; Koles et al., 2012; Rocca et al., 2013; Lim et al., 2014; Smith et al., 2014). Neurite outgrowth plays a pivotal role in neuronal development and regeneration (Beller and Snow, 2014; Stiles et al., 2014; Villarroel-Campos et al., 2014; Baldwin et al., 2015; Dayer et al., 2015; Madl and Heilshorn, 2015). Studies on the association between GIT1 and neurite outgrowth are emerging, but inconclusive. For example, GIT1 knockout leads to reduced neurite length in mice (Menon et al., 2010), while overexpression of full-length GIT1 *in vitro* does not affect neuritic morphology (Albertinazzi et al., 2003; Za et al., 2006; Totaro et al., 2012).

To better understand the role of GIT1 in neurite outgrowth, we investigated GIT1 expression at different developmental stages in the mouse brain. We examined the relation between GIT1 expression levels and neurite length in primary cultures of mouse hippocampal neurons. In addition, we assessed whether nerve growth factor (NGF), a well-known neurotrophin that promotes neurite outgrowth (O'Keeffe et al., 2008; Yammine et al., 2014; Sarma et al., 2015), could upregulate GIT1 expression and rescue the axonal growth defect caused by GIT1 knockout. Furthermore, using immunoprecipitation (IP) coupled with mass spectrometry, we identified a number of novel binding partners for GIT1. Potential interactions were subsequently confirmed by co-IP and colocalization experiments. Subsequently, *in vitro* polymerization assay was performed to examine whether GIT1 directly promotes microtubule assembly.

Materials and Methods

Experimental animals

Breeding pairs of GIT1-lacZ$^{+/-}$ mice, C.129S4(B6)-*Git-1*$^{Gt(FHCRC-GT-S10-12C1)Sor}$/WeisJ were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Neither GIT1 heterozygote (HET) nor GIT1 knockout (KO) animals statistically differed in body weight or organ size from wild type (WT) mice. All experimental procedures were performed in accordance with the Guideline for the Care and Use of Laboratory Animals of the Animal Research Ethics Committee of Renji Hospital, Shanghai Jiao Tong University School of Medicine, China.

RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from various tissues, including heart, brain, spine, lung, muscle, stomach, intestine, liver, spleen, thymus, kidney, prostate, and testis of adult mice using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) and reverse transcribed using the First Strand cDNA Synthesis Kit (Takara, Dalian, China). The *Git1* cDNA was amplified with 5′-TGTGGACGAAAGCCTGATC-3′ and 5′-ATCCACCTCATCATACACATCC-3′ primers, *Git2* with 5′-CAACAATGGTGCTAACTC-3′ and 5′-CGAACGCTAACATCTGATAC-3′ primers, and *Gapdh* with 5′-ATTCAACGGCACAGTCAA-3′ and 5′-CTTCTGGGTGGCAGTGAT-3′ primers. PCR was performed using rTaq (Takara), with 25 cycles of amplification with 30 seconds denaturation at 95°C, 30 seconds annealing at 58°C and 60 seconds extension at 72°C.

Cell culture and transfection

Primary hippocampal neuron cultures were prepared as described previously (Beaudoin et al., 2012). Briefly, the hippocampus was dissected out from postnatal day 0 (P0) mouse brain and cut into small pieces, followed by 20 minutes digestion in 0.25% trypsin (Worthington, Lakewood, NJ, USA) at 37°C. After terminating the reaction with 0.1% trypsin inhibitor (Sigma-Aldrich), the digested tissue was triturated and allowed to sit undisturbed for 5 minutes, which allowed non-dissociated tissue to settle at the bottom. The upper fraction containing dissociated cells was removed to another tube, and the non-dissociated tissue was incubated with 0.1% DNase (BD Bioscience, Shanghai, China) for 5 minutes at room temperature and triturated again. The upper fraction was removed again, and this procedure was repeated three times. All the upper fractions containing dissociated cells were combined and centrifuged at 500 × *g* for 5 minutes at room temperature. The collected cells were resuspended in Neurobasal medium containing B27 supplement (GIBCO,

New York, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were then plated and cultured in a 37°C /5% CO_2 incubator. After attaching, cells were transfected using Lipofectamine® 3000 (Life Technologies, Carlsbad, CA, USA) and cultured for an additional 5 days. NGF-β (50 ng/mL; Peprotech, Rocky Hill, NJ, USA) was used as an inducer of neurite growth. To investigate the effect of NGF-β on GIT1 expression, three different concentrations of the neurotrophin were used, including 0, 25 and 50 ng/mL.

Plasmid construction

Full-length human GIT1 cDNA was obtained from the pEGFP-GIT1 plasmid (Addgene, Cambridge, MA, USA). GIT1, cGIT1, CDΔAS/SLD, nGIT1 and GIT1ΔSLD were amplified as described previously (Zhang et al., 2003), and were inserted into modified pIRESneo vectors (Takara-Clontech, Mountain View, CA, USA) with a Flag/HA tag added at the N terminal using Infusion-HD (Takara).

Immunocytochemistry

Neurons were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100. The fixed cells were blocked with 10% donkey serum, and then incubated in primary antibody against GIT1 (goat, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Clasp2 (rabbit, 1:25; Biorbyt, San Francisco, CA, USA), microtubule-associated protein (MAP) 2 (rabbit, 1:300; Cell Signaling Technology, Danvers, MA, USA), Tuj1 (rabbit, 1:300; Sigma-Aldrich) or Flag (mouse, 1:500; Sigma-Aldrich) at 4°C overnight. After three washes in PBS, cells were incubated with the corresponding anti-mouse, anti-rabbit or anti-goat secondary antibody (Alexa Fluor 488, 594, 640; 1:600; Life Technologies) and DAPI (1:2,000; Sigma-Aldrich) away from light at room temperature for 1 hour. Images were acquired on a LSM-710 Zeiss confocal microscope (Carl Zeiss, Oberkochen, Germany). Neurite length was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) as described by Schmidt et al. (1997). Briefly, the straight-line distance from the tip of the neurite to the junction between the cell body and neurite base or to the neurite branch point was measured as an individual neurite length. The sum of all individual neurite lengths of a neuron was taken as the total neurite length per neuron, and the process with the longest individual neurite length in each neuron was considered an axon-like process.

Western blot analysis

Tissues or cells were lysed in RIPA Lysis and Extraction Buffer (Thermo, Waltham, MA, USA). The proteins were then separated on a polyacrylamide gel (Bio-Rad, Shanghai, China) and transferred onto nitrocellulose membranes (Bio-Rad). After blocking in milk, the blots were incubated in primary antibody against GIT1 (goat, 1:800; Santa Cruz Biotechnology), Clasp2 (rabbit, 1:600; Biorbyt), CRMP2 (rabbit, 1:1,000; Proteintech, Chicago IL, USA), MAP2 (rabbit, 1:1,000; Cell Signaling Technology), Tuj1 (rabbit, 1:1,000; Sigma-Aldrich), TUBGCP3 (rabbit, 1:1,000; Proteintech), MAP1B (rabbit, 1:1,000; Proteintech), Tau (rabbit, 1:1,000; Sigma-Aldrich) or beta-actin (mouse, 1:2,000; Millipore, Darmstadt, Germany) at 4°C overnight. After washing, the blots were incubated with IRDye-conjugated secondary antibody against mouse, rabbit or goat (1:10,000; Licor, Lincoln, NE, USA) away from light at room temperature for 1 hour, and visualized with an Odyssey imaging system (Licor).

Immunoprecipitation assays and mass spectrometry

Whole brains were excised from 8-week-old mice, homogenized in IP buffer (250 mM hydroxyethyl piperazine ethanesulfonic acid (pH 7.4), 100 mM NaCl, 10 mM KCl, 10 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 10% glycerine, 0.5% CHAPS and 0.5% Triton X-100) supplemented with protease inhibitor cocktail tablets (Roche, Shanghai, China), and centrifuged for 20 minutes at 12,000 × *g* at 4°C The supernatant, containing 1 mg total protein, was incubated with 10 μg anti-GIT antibody (Santa Cruz Biotechnology). The target antibody complex was immunoprecipitated with Dynabeads® Protein G (Life Technologies) and eluted with elution buffer in the immunoprecipitation kit (Life Technologies). The eluted supernatant was subjected to polyacrylamide gel electrophoresis (PAGE) for mass spectrometric detection or protein immunoblotting. For mass spectrometry, the proteins were excised from the stacking gel and digested with trypsin as previously described (Koles et al., 2012). The peptides were separated using a high performance liquid chromatography system (Agilent, Palo Alto, CA, USA) and detected with a ThermoElectron Finnigan LCQ DECA ion trap mass spectrometer (Thermo).

Tubulin polymerization assay

Purified tubulins (Cytoskeleton, Denver, CO, USA) were reconstituted in tubulin buffer (80 mM piperazine-1,4-bisethanesulfonic acid (pH 6.9), 2 mM $MgCl_2$, 0.5 mM ethylene glycol tetraacetic acid, 1 mM guanosine triphosphate and 5% glycerol), placed in a 24-well plate and pre-warmed to 37°C. Immediately, 1 or 5 μM GIT1 protein (Proteintech) diluted in tubulin buffer was added to the wells. As a positive control, 10 μM paclitaxel (Cytoskeleton), a well-known microtubule stabilizer (Yu et al., 2015) was added instead of GIT1. The blank control well contained only tubulin buffer. The tubulin polymerization curve was recorded every minute, with absorbance readings at 340 nm and 37°C. The optical density is proportional to the quantity of microtubules formed. All assays were done in triplicate.

Statistical analysis

Data are expressed as the mean ± SEM. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Inter-group comparisons were analyzed by one-way analysis of variance followed by Tukey's test. $P < 0.05$ was considered statistically significant.

Results

GIT1 was more abundantly expressed in the developing brain than other tissues

To investigate the relative GIT1 abundance in various tissues,

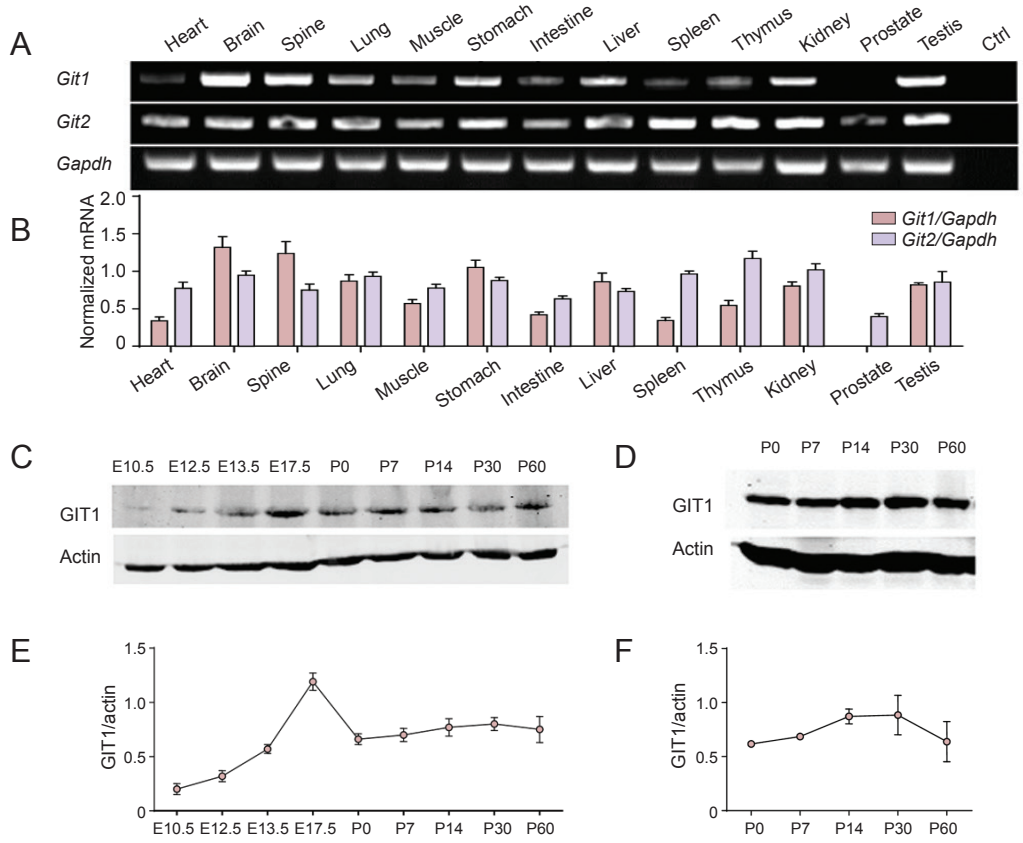

Figure 1 GIT1 was highly expressed in the nervous system.
(A) Expression profiles of *Git1* and *Git2* in 2-month-old mice, as assessed by RT-PCR. Ctrl: Negative control. (B) The relative mRNA expression levels of *Git1* and *Git2* in 2-month-old mice, normalized to *Gapdh*. The data are expressed as optical density ratio of *Git*/*Gapdh* (n = 3; data are given as mean ± SEM). (C) GIT1 protein expression in the whole brain from embryonic day (E) 10.5 to postnatal day (P) 60. (D) GIT1 expression in the mouse hippocampus at P0, 7, 14, 30 and 60. Quantification of GIT1 expression in the whole brain (E) and hippocampus (F) were normalized to β-actin. Data represent the mean ± SEM of three independent observations.

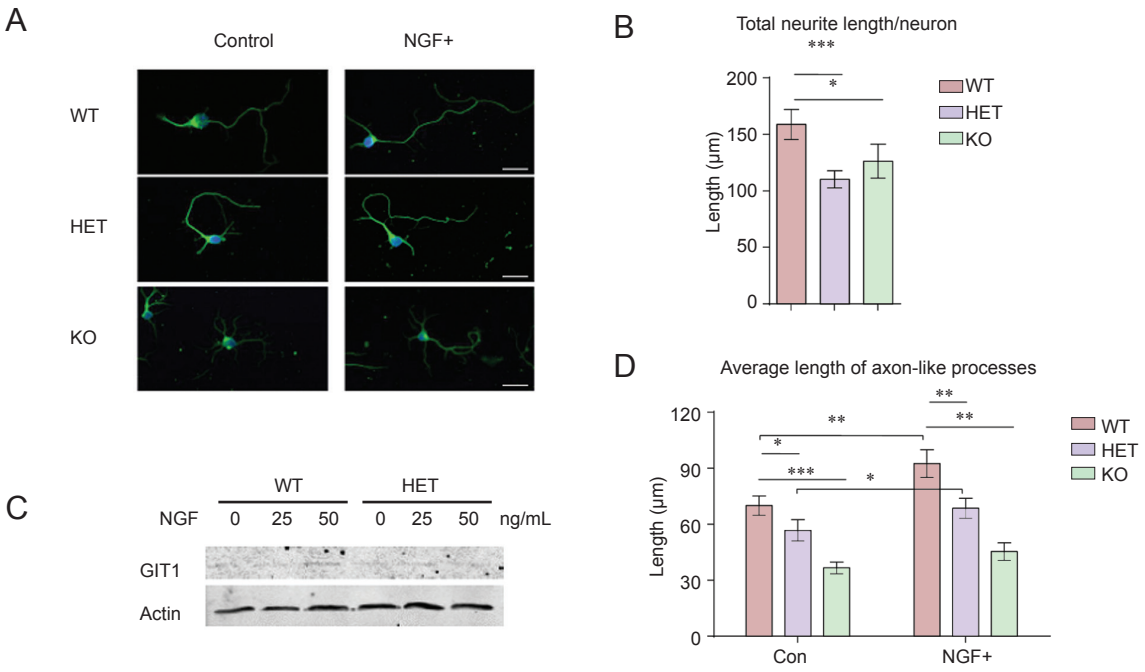

Figure 2 Neurite growth was impaired in GIT1 knockout neurons.
(A) Hippocampal neurons isolated from wild type (WT), GIT1 heterozygote (HET) and GIT1 knockout (KO) mice at postnatal day (P) 0 were cultured for 5 days in control medium (left) or medium containing 50 ng/mL nerve growth factor (NGF) (right). Neurons were immunostained with anti-Tuj1 (green) antibody and DAPI (blue). Scale bar: 20 μm. Quantification of total neurite length per neuron (B) and average length of axon-like processes (D) of WT, HET and KO hippocampal neurons *in vitro*. The effects of NGF treatment are shown in Figure D. n = 30–50 cells; data are expressed as the mean ± SEM. The experiments were repeated three times. (C) Western blot of lysates from P0 WT and HET hippocampal neurons cultured for 5 days in the presence of 0, 25 or 50 ng/mL NGF. Anti-GIT1 and anti-actin antibodies were used to detect target protein expression. *P < 0.05, **P < 0.01, ***P < 0.001. One-way analysis of variance followed by Tukey's test.

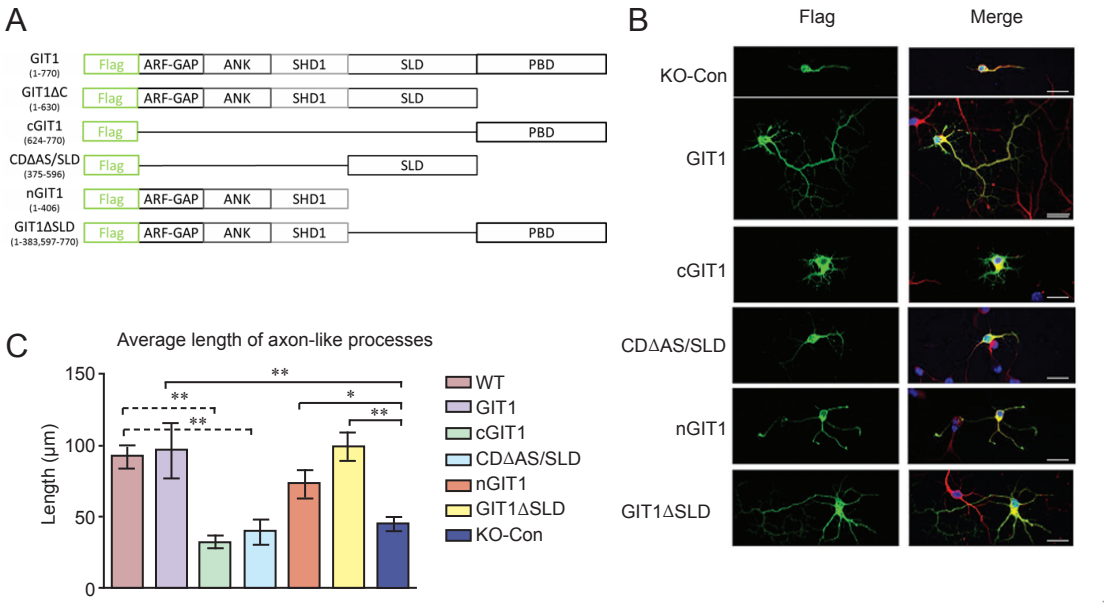

Figure 3 Axonal growth in GIT1 knockout (KO) hippocampal neurons and the effects of overexpression of GIT1 or GIT1 deletion constructs.
(A) Schematic diagram of the full-length GIT1 and different GIT1 deletion constructs: cGIT1, CDΔAS/SLD, nGIT1 and GIT1ΔSLD. The Flag-tag was attached to the N terminal of the constructs. (B) KO hippocampal neurons were cultured for 5 days after transfection with the constructs, and then stained with anti-Flag (green) and anti-Tuj1 (red) antibodies and DAPI (blue). Scale bars: 20 μm. (C) Average length of axon-like processes in KO hippocampal neurons cultured for 5 days after transfection with the GIT1 deletion constructs. Cells were analyzed using ImageJ software. $n = 3$ cells; data are expressed as the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. One-way analysis of variance followed by Tukey's test. The experiments were repeated three times. Con: Control.

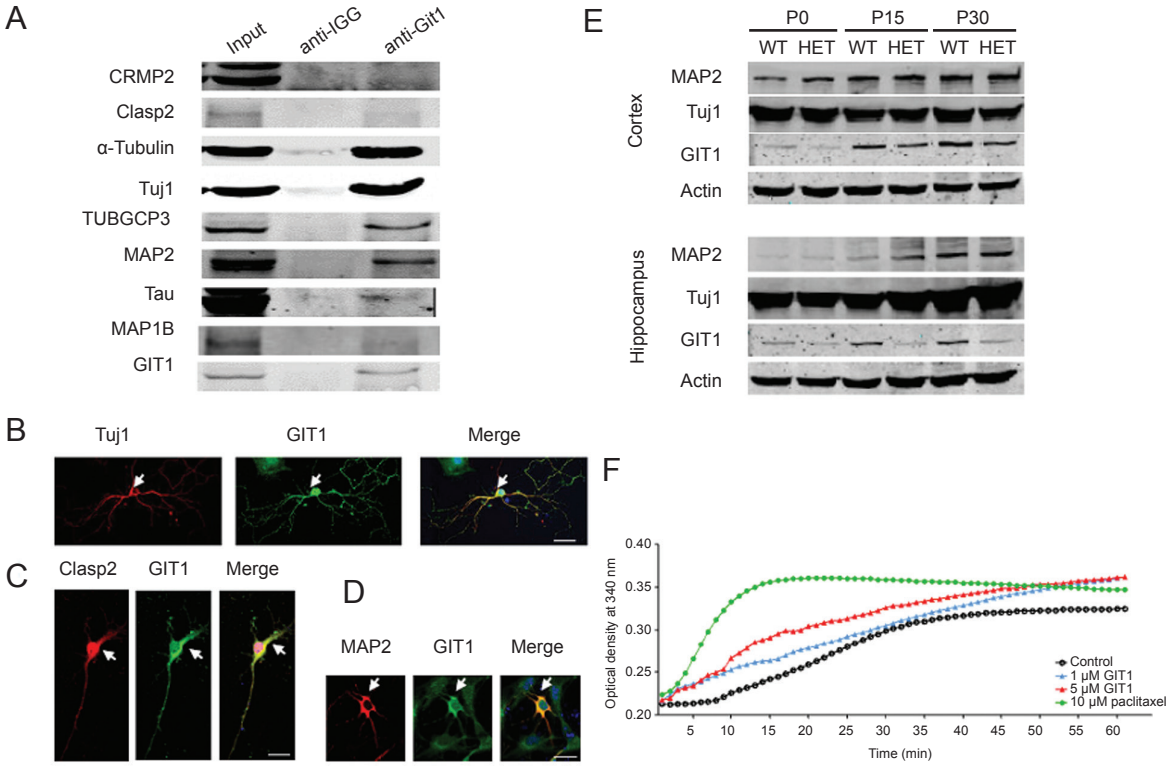

Figure 4 GIT1 interacted with tubulins and microtubule-associated proteins, and stimulated microtubule assembly.
(A) Brain extract from 8-week-old mice was immunoprecipitated with the GIT1 antibody, and western blots were probed by antibodies against CRMP2, Clasp2, α-tubulin, Tuj1, TUBGCP3, MAP2, Tau, MAP1B and GIT1. The images show the colocalization of Tuj1 (red) and GIT1 (green) (B), Clasp2 (red) and GIT1 (green) (C), and MAP2 (red) and GIT1 (green) (D) in primary cultured hippocampal neurons on postnatal day (P) 0. Scale bars: 20 μm. (E) Expression levels of Tuj1, MAP2 and GIT1 in the cortex and hippocampus of wild-type (WT) and GIT1 heterozygote (HET) mice on postnatal day (P) 0, P15 and P30. (F) GIT1 activates tubulin polymerization *in vitro*. Result represents means of three independent observations. min: Minutes.

we first performed semi-quantitative RT-PCR to obtain Git1 and Git2 mRNA expression profiles. We found that Git1 was expressed most abundantly in the brain and spine, weakly in the heart and spleen, and was almost undetectable in the prostate; however, Git2 was expressed ubiquitously in all 13 examined tissues (**Figure 1A**, **B**). This suggests that GIT1 likely has very important functions in the nervous system. Therefore, we subsequently examined GIT1 protein expression levels at different stages of brain development by western blot assay (**Figure 1C**, **E**). GIT1 could be detected at embryonic day 10.5 (E10.5), and thereafter, expression levels rapidly increased, reaching a maximum at E17.5. At P0, the expression level decreased to roughly half of the peak amount, and was maintained until P60 with little fluctuation. In the hippocampus, GIT1 expression levels increased starting at P7, peaking at P30, and then tended to decrease by P60, consistent with the time window of hippocampal development (Gu et al., 2012) (**Figure 1D**, **F**).

GIT1 KO inhibited neurited extension

To evaluate GIT1's role in neurite outgrowth, primary hippocampal neurons were isolated and cultured *in vitro* for 5 days. Neurons were immunolabeled for Tuj1, a neuron-specific marker (**Figure 2A**). Compared with WT neurons, both GIT1 KO and GIT1 HET neurons showed a significant reduction in total neurite length per neuron (**Figure 2B**). The neurite outgrowth defect was more obvious when another important morphological characteristic was measured, axonal length (**Figure 2D**). Because NGF enhances the expression of GIT1 (**Figure 2C**), we examined whether NGF could rescue the defects in axonal growth in the GIT1 mutant. As shown in **Figure 2D**, NGF treatment caused a significant increase in the average length of axon-like processes in WT and HET, but not GIT1 KO, neurons. Interestingly, there was a greater increase in WT neurons than in HET neurons, indicating that the increase in the length of axon-like processes induced by NGF treatment is dependent on Git1 gene dosage.

GIT1 overexpression rescued the axonal growth defects in GIT1 KO neurons, and the N terminal was sufficient for this effect

To further examine the neurite outgrowth-promoting effect of GIT1, we performed GIT1 overexpression experiments in the hippocampal neuronal cultures. As shown in **Figure 3B** and **C**, overexpression of GIT1 in primary hippocampal neurons isolated from GIT1 KO mice resulted in a complete rescue of the axon growth defect. The length of axon-like processes in these cells was comparable to that in WT neurons after 5 days in culture. To determine which domains of GIT1 mediate this effect, we made various constructs of the different functional domains. These GIT1 constructs were designated cGIT1, CDΔAS/SLD, nGIT1 and GIT1ΔSLD, each with a Flag tag at the N terminal (**Figure 3A**). Each construct was transfected into GIT1 KO neurons, followed by immunostaining for Tuj1 (red) and Flag (green) (**Figure 3B**). Similar to GIT1 overexpression, transfection of nGIT1 or GIT1ΔSLD (GIT1 with the SLD domain deleted) effectively rescued the axon outgrowth defect in GIT1 KO neurons. In sharp contrast, all other GIT1 fragments failed to do so (**Figure 3C**). These results demonstrate that nGIT1 is sufficient for the axon growth promoting effect of GIT1.

GIT1 binded to tubulins and MAPs and promoted microtubule assembly

To screen for proteins that interact with GIT1, GIT1 was immunoprecipitated from brain extracts and mass spectrometry was performed. Many tubulins and MAPs were identified (**Supplementary Table 1 online**). We verified these results by co-IP experiments. As shown in **Figure 4A**, tubulins, including α-tubulin, Tuj1 and TUBGCP3 (a member of the γ-tubulin complex), and MAPs (Clasp2, Tau, MAP1B and MAP2) were co-precipitated by the GIT1 antibody. Some of the interactions were further confirmed by immunofluorescence colocalization experiments. In hippocampal neurons, GIT1 was clearly colocalized with Tuj1 (**Figure 4B**), Clasp2 (**Figure 4C**) and MAP2 (**Figure 4D**). Furthermore, western blot assay was performed to examine the expression of Tuj1 and MAP2 in the cortex and hippocampus in both HET and WT mice at P0, P15 and P30 (**Figure 4E**). Notably, lower GIT1 protein levels were detected in the cortex and hippocampus of HET mice compared with WT animals at the different developmental time points. In contrast, no substantial difference was seen in expression levels of Tuj1 or MAP2 between WT and HET mice, indicating that GIT1 does not affect levels of these proteins. These results suggest that GIT1 may affect neurite outgrowth by impacting tubulin polymerization. We, therefore, evaluated the capacity of purified tubulin to form microtubules in the presence of GIT1 *in vitro* (**Figure 4F**). When incubated with 1 μM GIT1, tubulin started to polymerize by as early as 2 minutes and displayed rapid polymerization during the first 15 minutes. In comparison, in the control, the start of tubulin polymerization was delayed to ~6 minutes. In the presence of 5 μM GIT1, tubulin polymerization was increased significantly. As expected, the positive control paclitaxel dramatically accelerated tubulin polymerization. These data indicate that GIT1 promotes microtubule assembly *in vitro* in a concentration-dependent manner.

Discussion

Although GIT1 has been reported to stimulate neurite outgrowth, the observations have been inconsistent (Za et al., 2006; Totaro et al., 2012). Our present findings strongly suggest that GIT1 promotes neurite outgrowth, especially of axon-like processes.

We found relatively high expression levels of GIT1 in the nervous system compared with other tissues, which correlates well with neuritogenesis in the brain. Although GIT1 is detected in most tissues at various levels, its expression is predominantly in the brain, indicating that GIT1 has important functions in this organ. This hypothesis is further supported by the fact that GIT1 expression dramatically

increases from E12.5 and remains at a high level throughout embryogenesis, during which neuronal migration and axon and dendrite growth and sprouting occur. Hence, GIT1 is strongly correlated with neuritogenesis. Furthermore, GIT1 is highly expressed in the hippocampus, which peaks at around P30, consistent with a critical role in synaptogenesis. These findings provide additional support for an important role of GIT1 in spine and synapse development. Compared with GIT1, GIT2, a homolog, has a quite different expression pattern. GIT2 is ubiquitously expressed in all tissues, without any specificity. GIT2 expression in the hippocampus is consistently low and is not correlated with synapse formation (data not shown). Therefore, it is unlikely that GIT2 expression and synaptic plasticity are closely related.

Another major finding of the present study is that both GIT1 KO and GIT1 overexpression significantly affect neurite length. Our results show that GIT1 is required for axonal extension and that NGF enhances axonal growth in neurons from WT and HET, but not KO, mice. Furthermore, we found that the expression of GIT1 can be stimulated by NGF, a well-known neurite outgrowth promoter. This suggests that the neurite outgrowth stimulating effect of NGF may be mediated by GIT1, although the underlying mechanisms are unknown.

Albertinazzi et al. (2003) found that transfection of full-length p95-APP1 (GIT1) did not have an obvious effect on neurite outgrowth, although some deletion constructs did. p95-APP1 with an SHD domain deletion (p95-ΔSHD) and p95-APP1 with a C-terminal truncation after the SHD domain (P95-C) tended to stimulate neurite extension. However, an N-terminal construct truncated after the SHD domain (p95-N) and a C-terminal construct truncated before the SHD domain (p95-C2) completely blocked neurite outgrowth. In the current study, we investigated the function of various GIT1 deletion constructs on neurite outgrowth. We found that the SLD domain (CDΔAS/SLD) and the PBD domain (cGIT1) alone had no effect on neurite extension. In contrast, nGIT1 (corresponding to p95-N) and GIT1-ΔSLD (GIT1 with an SLD domain deletion) significantly stimulated axon-like neurite extension. Therefore, we conclude that the GIT1 N-terminal is sufficient to stimulate axonal extension, and that the effect can be enhanced by the PBD domain and weakened by the SLD domain.

The molecular mechanisms by which GIT1 stimulates neurite outgrowth remain unclear. Albertinazzi et al. (2003) proposed that activation of the GIT1-Pix-Rac1B-PAK signaling pathway triggers growth cone actin remodeling to drive neurite extension. Our current results suggest the presence of a novel mechanism, in which GIT1 promotes neurite extension by inducing microtubule remodeling. By mass spectrometry, we found that many tubulins and MAPs were co-immunoprecipitated by the GIT1 antibody, and these interactions were confirmed by co-IP and colocalization experiments. The tubulin polymerization assay revealed that GIT1 facilitates tubulin polymerization in a concentration-dependent manner, without directly affecting expression levels of Tuj1 or MAP2. Taken together, our findings support the concept that GIT1 affects neurite growth by modulating both actin and microtubule dynamics. Our working model is that GIT1 binds to tubulins and MAPs to control microtubule remodeling, thereby promoting neurite outgrowth.

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Conflicts of interest: *None declared.*

Supplementary data: *Supplementary information is available at http://www.nrronline.org.*

Plagiarism check: *This paper was screened twice using Cross-Check to verify originality before publication.*

Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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