Autophagy-related gene Atg5 is essential for astrocyte differentiation in the developing mouse cortex

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Abstract

Astrocyte differentiation is essential for late embryonic brain development, and autophagy is active during this process. However, it is unknown whether and how autophagy regulates astrocyte differentiation. Here, we show that Atg5, which is necessary for autophagosome formation, regulates astrocyte differentiation. Atg5 deficiency represses the generation of astrocytes *in vitro* and *in vivo*. Conversely, Atg5 overexpression increases the number of astrocytes substantially. We show that Atg5 activates the JAK2-STAT3 pathway by degrading the inhibitory protein SOCS2. The astrocyte differentiation defect caused by Atg5 loss can be rescued by human Atg5 overexpression, STAT3 overexpression, and SOCS2 knockdown. Together, these data demonstrate that Atg5 regulates astrocyte differentiation, with potential implications for brain disorders with autophagy deficiency.

Keywords astrocyte differentiation; autophagy; cortical development

Subject Categories Neuroscience; Autophagy & Cell Death; Development & Differentiation

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Introduction

In the development of the mammalian central nervous system (CNS), committed neural progenitor cells (NPCs) generate neurons and glial cells [1,2]. Recent studies have demonstrated that glial cells have additional functions, including enhanced neural plasticity and learning and facilitated garbage clearance in the brain [3,4]. It is conceivable that impaired glial cell production might cause abnormal structure formation and severe dysfunction of the CNS.

Astrocyte differentiation, as a key event in brain development, plays a vital role in the integration of brain structure construction and function. It is regulated by intrinsic epigenetic status,

transcription factors, and milieu cues in brain development [5,6]. One canonical explanation is that cortical progenitor cells can generate astrocytes responding to some extrinsic gliogenic signal dependent on cytokine-induced activation [7,8]. Previous studies have demonstrated that the interleukin (IL)-6 cytokine family, including ciliary neurotrophic factor (CNTF) and leukemia inhibitor factor (LIF), could accelerate the generation of astrocytes via the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway [9–12]. Sequential experiments have demonstrated that STATs are dimerized and translocated to the nucleus, where they directly induce the transcriptional activation of the astrocyte-specific genes, *GFAP* and *S100β*, through STAT3-binding with their promoters [9,13]. The demethylation status of the STAT3-binding site within the *GFAP* promoter is a prerequisite [12,14], and STAT-mediated transcription plays a crucial role in astrocyte differentiation [15,16].

Autophagy is an evolutionarily conserved process for the turnover and recycling of cytoplasm and organelles [17,18]. During autophagy, double-membraned autophagosomes engulf nonessential portions of the cytoplasm and organelles and fuse with the lysosome, in which damaged cell organelles or unused proteins are degraded via acidic lysosomal hydrolases. It has been reported that autophagy occurs in many physiological and pathological situations and is a key component of development and differentiation [19,20]. Autophagy can be accurately regulated by several autophagy-related (Atg) genes.

Previous studies suggest that autophagy plays a vital role in embryogenesis and some neurodegenerative diseases [21,22]. Furthermore, the deletion of *Atg5* and *Atg7* in CNS, which are essential for the initiation of autophagosomes, resulted in behavioral defects in mice caused by neurodegeneration [23–25]. Autophagy is extensively involved as a potential treatment in models of Alzheimer's disease (AD) and Huntington's disease [26,27]. However, the precise cellular processes of autophagy function in the embryonic brain development remain unknown. The previous observations raise fundamental questions: What is the relationship between autophagic machinery and astrocyte differentiation? If a

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relationship exists, what is the functional role of this interaction? The questions indicate a new research field for further investigation of these processes.

In the present study, we used several gain and loss experimental strategies to investigate the role of autophagy in the regulation of astrocyte differentiation. Here, we designed a screen to identify the function of autophagy in gliogenesis through Atg5 knockdown or overexpression and discovered that Atg5 and Atg5-related autophagy are required for the regulation of astrocyte differentiation in the developing mouse cortex. We further observed that increased levels of Atg5 could promote astrocyte differentiation by degrading SOCS2 through direct interaction between LC3 and SOCS2. We concluded that Atg5 and related autophagy can regulate the timed gliogenesis in the developing mouse cortex.

Results and Discussion

Atg5 expression in brain development

According to previous studies, autophagy and its related genes, such as *Atg5*, *Atg7*, and *Ambra1*, are crucial to CNS, embryogenesis, and organ development [23–25], but the virtual function of autophagy on astrocyte differentiation is largely unknown. To explore whether Atg5 is a key regulator of astrocyte differentiation, we obtained cerebral tissue from five different stages to analyze the expression of Atg5 and related proteins (Fig 1A, Supplementary Fig S1A). The results showed that Atg5 expression increased from E12 to E15 and gradually decreased after E19. The level of autophagy, which is indicated by the autophagy markers LC3II/LC3I, also increased from

Fig 1. Atg5 expression in the developing mouse brain.

A Western blot analysis showing Atg5, LC3, and GFAP protein expression levels in the brain cortex during the late embryonic cortex development.
 B Immunostaining showing the Atg5 expression in the developing brain from E12 to P2.
 C Western blot analysis revealing the effect and efficiency of knockdown by Atg5 shRNA *in vitro*. A notable reduction of Atg5 and LC3 protein expression was observed in NPCs infected with lentivirus.
 D GFAP staining showing less astrocyte number after Atg5 knockdown. E12 NPCs were cultured with LIF (50 ng/ml) for 2 (2D), 4 (4D), and 6 days (6D). Quantification of cells differentiating into astrocytes over the total cells was measured and plotted.
 E Western blot showing that expression of GFAP was sharply reduced. NPCs infected with control or Atg5 shRNA virus were cultured and treated with LIF for 4 days.
 Data information: The data are represented as means $\pm$ SD, $n = 3$, $*P \leq 0.05$, Student's *t*-test. Scale bar: 50 μ m. Atg5, autophagy-related gene 5; E, embryonic day; GFAP, glial fibrillary acidic protein; LC3, microtubule-associated protein 1 light chain 3; P, postnatal day. See also Supplementary Fig S1.

E12 to E15 and then decreased from E19. GFAP expression was increased during this period (Fig 1A, Supplementary Fig S1A). It demonstrates that astrocyte differentiation occurs at the late embryonic development stage and the initiation of astrocyte differentiation corresponds to the expression of Atg5 and the autophagy level. The immunostaining results of Atg5 (Fig 1B), LC3 (Supplementary Fig S1B), and GFAP (Supplementary Fig S1C) showed that Atg5 expressed highly but not restricted in the ventricular zone (VZ)/subventricular zone (SVZ) and peaked expression was observed at E15, consistent with the results of the Western blot analysis. These data suggest that Atg5 might be involved in broad brain development including astrocyte differentiation.

Atg5 knockdown represses astrocyte differentiation *in vitro*

To determine whether Atg5 regulates astrocyte differentiation, a series of Atg5 knockdown experiments were performed. At 72 h after cells infected with lentivirus in culture, we observed that Atg5 shRNA constructed in lentiviral vector dramatically inhibited Atg5 expression and reduced LC3 levels (Fig 1C, Supplementary Fig S2A), indicating defects in autophagy. After *in utero* electroporation (IUE) combined with Atg5 shRNA knockdown plasmid (Supplementary Fig S2B), the LC3-II/LC3-I levels were decreased compared with the control *in vivo* (Supplementary Fig S2C). An examination of astrocyte differentiation *in vitro* revealed that E12 NPCs successfully differentiate into GFAP-expressing astrocytes under differentiation conditions for 6 days, and LIF (50 ng/ml) significantly promotes GFAP levels (Supplementary Fig S2D). Next, we explored the function of Atg5 on astrocyte differentiation under the same culture conditions using Atg5 knockdown. Primary NPCs infected with Atg5 shRNA lentivirus showed that astrocytic differentiation was significantly impaired. After 4 days, Atg5 knockdown resulted in 49.8% reduction of astrocyte differentiation (Fig 1D). The result of Western blot shows that the GFAP protein level was decreased in Atg5 knockdown cells (Fig 1E, Supplementary Fig S2A). Collectively, these results suggest that Atg5 and basal autophagy participate in astrocyte differentiation *in vitro*.

Atg5 knockdown induces astrocyte differentiation defects *in vivo*

According to the previous reports, astrocytes first appeared at E15–16 and achieved the highest number during the neonatal period [28]. To further investigate whether Atg5 regulates astrocyte differentiation *in vivo*, control or Atg5 shRNA constructs were electroporated together with a GFP-expressing vector into the lateral ventricle of embryos at different development stages, and the brains sections were analyzed at E18, P0, and P8. The results show that Atg5 knockdown caused a substantial reduction of GFAP-positive astrocytes in the VZ/SVZ at E18 or P0 (Fig 2A and B). Furthermore, Atg5 knockdown induced robust morphological defects. In control group, GFP-positive cells with typical astrocyte morphology were readily detected in the upper layer of the cortex. Contrastingly, most of Atg5 knockdown cells remained in the VZ/SVZ and rare morphologically mature astrocytes were observed (Fig 2E). These results indicate that Atg5 deficiency might obstruct astrocyte generation in the developing mouse cortex.

Moreover, given that astrocyte differentiation is initiated and continued during late embryonic development, E16 embryos were

electroporated and analyzed at postnatal day 8. Atg5 knockdown prohibited astrocyte production and maturation (Supplementary Fig S2E). These results indicate that Atg5 knockdown persistently represses astrocyte differentiation, suggesting that astrocyte differentiation is permanently affected when Atg5 is suppressed. We subsequently investigated whether Atg5 is involved in regulating astrocyte-restricted precursor cells, utilizing CD44 as an astrocyte precursor cell marker [12]. The results demonstrate that Atg5 knockdown led to a profound decrease of astrocyte precursor cells and the effectiveness was sustained postnatally (Fig 2C and D). In addition, Atg5 knockdown at E16 after the initiation of astrocyte generation also induced a reduction in the number of progenitor cells (Supplementary Fig S2F). The data suggest that Atg5 is necessary for the specification of astrocyte progenitor cells, and Atg5 knockdown cause astrocyte differentiation defects.

In addition, to rule out the possibility that the reduction in astrocytes reflects cell death, we detected the degree of cell death *in vivo* and *in vitro*. The confocal images and statistical analyses showed that Atg5 knockdown does not affect cell death (Supplementary Fig S2G and H). The results suggest that the reduced number of astrocytes and astrocyte precursor cells did not result from cell death. Taken together, these results clearly show that astrocyte differentiation is markedly affected when Atg5 is knocked down.

Atg5 overexpression enhances astrocyte differentiation

The astrocyte differentiation defects caused by Atg5 loss prompted us to perform reciprocal Atg5 gain-of-function experiments. To further analyze the role of Atg5 during astrocyte differentiation, we created an Atg5 overexpression plasmid to efficiently increase Atg5 expression levels. The results of the Western blot analysis confirmed that the level of autophagy was more than doubled compared with the control after Atg5 overexpression (Fig 3A). The results of immunocytochemistry and Western blot showed that Atg5 significantly promoted astrocyte differentiation and increased the expression of GFAP *in vitro* (Fig 3B and C). The amount of GFAP-positive cells increased by 106 and 86% after 4 and 6 days, respectively, compared with control cells in culture (Fig 3B). Additional *in vivo* experiments involved the electroporation of E14 dorsal cortices with the Atg5 construct or control plasmid and analysis at E18 through immunocytochemistry for GFAP, CD44, and BLBP (Fig 3D–F). The results demonstrated that the number of astrocytes, astrocyte precursor cells, and radial progenitor cells increased by 66.1, 64.5, and 110% after Atg5 overexpression. Collectively, astrocyte differentiation is facilitated through Atg5 overexpression both *in vitro* and *in vivo*. To further examine Atg5 as a key regulator in astrocyte differentiation, we generated a human Atg5 (hAtg5) overexpression plasmid and examined the effects of the coexpression of hAtg5 with control or mouse Atg5 (mAtg5) shRNA in astrocyte differentiation. The defects in NPCs differentiation, resulting from mAtg5 knockdown, were rescued after hAtg5 overexpression (Fig 3G, Supplementary Fig S3A–C).

Atg5 conditional knockout impairs astrocyte differentiation

To further study the function of Atg5 in astrocyte differentiation, the Atg5 gene was conditionally knocked out *in vitro* and *in vivo*. NPCs isolated from Atg5^{fllox/fllox} mice [25] were infected with lentivirus and cultured for 4 days. Little expression of GFAP and LC3 caused by

Fig 2. Atg5 knockdown represses astrocyte differentiation *in vivo*.

A, B Atg5 knockdown resulting in a decrease of GFAP-positive cells analyzed in E18 and P0 brain after control, and Atg5 shRNA plasmids were electroporated at E14. The graphs on the right show the percentages of double-labeled GFAP/GFP cells relative to the number of GFP-positive cells.

C, D Atg5 knockdown leading to reduced astrocyte-restricted precursor cells, as demonstrated by CD44 staining. The percentages of CD44⁺GFP⁺ cells are shown in the graph.

E Defects in astrocyte differentiation and astrocyte maturation after Atg5 knockdown. E16 embryonic brains electroporated with control and Atg5 shRNA plasmids and analyzed at P3. The left of each panel, confocal images of GFP⁺ cells. Arrowhead, GFP-labeled astrocytes. The right of each panel, a higher-magnified view of the boxed region, shows morphological differences (scale bar: 20 μ m).

Data information: The data are represented as means $\pm$ SD (A–D), $n = 3$, * $P \leq 0.05$, Student's t -test, scale bar (A–D): 50 μ m. Ctrl, control; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein. See also Supplementary Fig S2.

Fig 3. Overexpression Atg5 leads to excessive astrocyte differentiation.

- A** Western blot analysis revealing a notable increase of Atg5 and LC3 protein expression in cells infected with Atg5 overexpression lentiviruses.
- B** NPCs were treated in cell differentiation medium with LIF (50 ng/ml) for 2, 4, and 6 days. The cells were stained with anti-GFAP antibody to display astrocyte differentiation. The percentages of cells differentiating into astrocytes were measured.
- C** Western blot analysis showing the expression of GFAP protein was increased after Atg5 overexpression. NPCs infected with control or Atg5 overexpression lentiviruses were cultured and treated with LIF for 4 days.
- D-F** GFAP, CD44, or BLBP immunostaining demonstrating that Atg5 increases the number of astrocyte, astrocyte-restricted precursor cells, and glial progenitor cells at E18 cortices separately which were electroporated at E14 cortices. The graphs show the proportion of GFAP⁺GFP⁺ astrocytes, CD44⁺GFP⁺ cells, or BLBP⁺GFP⁺ in electroporated cortices.
- G** Human Atg5 overexpression rescued astrocyte differentiation defects caused by mouse Atg5 knockdown.

Data information: The data are presented as means ± SD, $n = 3$, * $P \leq 0.05$, Student's t -test. Scale bar: 50 μ m. See also Supplementary Fig S3.

Atg5 knockout was shown in Western blot. And the percentage of GFAP-labeled astrocytes was reduced to 2% only (Fig 4A). Moreover, the *Atg5*^{fllox/fllox} mice brains were electroporated with Cre or control plasmid at E16 or P0. GFAP immunocytochemistry confirmed that GFAP-positive cells were reduced by 42.6% after the deletion of *Atg5* (Fig 4B). Subsequently, immunocytochemistry for CD44 and FGFR3 revealed that the number of astrocyte-restricted precursor cells was reduced by 44.86 and 59.8%, respectively (Fig 4C and D). BLBP and GLAST immunostaining showed the radial progenitor cell numbers were reduced by 45.7 and 38.6% (Fig 4E and F). To further analyze whether gliogenesis in postnatal pups is regulated through Atg5, the brains of neonatal *Atg5*^{fllox/fllox} mice were electroporated and analyzed at P8. The results demon-

strated a strong reduction in GFAP-positive astrocytes due to Atg5 knockout (Fig 4G). Furthermore, mature astrocytes were not observed in cortices electroporated with the Cre construct. In contrast, cortices electroporated with control plasmid exhibited a clear astrocytic morphology (Fig 4H). Taken together, these results suggest Atg5 knockout induces defects in the number and morphology of astrocytes.

Atg5 regulates astrocyte differentiation via the SOCS2-JAK2-STAT3 signaling pathway

Cell lineage specification is regulated by both external and intrinsic cues in the developing brain [29]. Studies have shown that the

Figure 4. Atg5 knockout results in astrocyte differentiation defects.

- A** *Atg5* knockout impairs astrocyte differentiation *in vitro*. NPCs isolated from *Atg5*^{f/f} mice were infected with control and Cre virus and cultured for 4 days. Left, Western blot showing GFAP and LC3 expression declined drastically. Right, very low astrocyte number was demonstrated by GFAP staining.
- B** GFAP immunostaining showing a decrease in the number of astrocytes after *Atg5* knockout. The graph shows quantification of GFAP⁺GFP⁺ cells.
- C, D** CD44 and FGFR3 immunostaining showing a decrease of astrocyte-restricted precursor cells due to *Atg5* knockout. The graph shows the proportion of CD44⁺GFP⁺ and FGFR3⁺GFP⁺ cells, respectively.
- E, F** BLBP and GLAST immunostaining demonstrating the reduction in radial progenitor cells after *Atg5* knockout. The quantifications of BLBP⁺GFP⁺ and GLAST⁺GFP⁺ are shown in the graphs.
- G** *Atg5* knockout also results in the persistent disruption of astrocyte number. Control or Cre constructs were electroporated into the brains of *Atg5*^{f/f} newborn pups (P0) and analyzed at P8. The graph shows quantification of GFAP⁺GFP⁺ cells.
- H** *Atg5* knockout leads to astrocyte differentiation defects persistently. The brains electroporated with Cre plasmid did not show typical mature astrocytic morphologies (scale bar: 20 μ m).

Data information: Bar graphs (B–G), means $\pm$ SD, $n = 4$, $*P \leq 0.05$, Student's *t*-test. Scale bar (A–G): 50 μ m. FGFR3, fibroblast growth factor receptor 3; GLAST, glial high affinity glutamate transporter.

Fig 5. Atg5 regulates astrocyte differentiation via modulating SOCS2-JAK-STAT signaling pathway.

- A E12 NPCs infected with lentiviruses for Atg5 knockdown, Atg5 overexpression, and controls were cultured for 4 days. Western blot analysis of the total JAK2, pJAK2, total STAT3 and pSTAT3 protein expression in these groups with or without 30-min LIF treatment. In the right panel, the percentage of pJAK2/JAK2 and pSTAT3/STAT3 was calculated.
- B GFAP protein level was rescued through STAT3 overexpression in Atg5 knockdown cells. NPCs were infected with different lentivirus solutions and cultured for 4 days. Western blot and the quantification of GFAP protein expression are shown.
- C The expression of SOCS2 at different periods was elevated after Atg5 knockdown. Cell lysates were harvested from cells after be transfected with Atg5 shRNA 36 or 96 h.
- D Defective pSTAT3 protein expression induced by Atg5 shRNA was rescued with SOCS2 shRNA. NPCs were transfected with control and SOCS2 shRNA, or cotransfected with Atg5 shRNA and control or SOCS2 shRNA.
- E Co-immunoprecipitation of LC3 and SOCS2. 293FT cells were cultured under normal or starvation condition, treated with or without lysosomal inhibitors (E64d and pepstatin A), immunoprecipitated with a LC3 antibody, and immunoblotted with a SOCS2 antibody.
- F Association between LC3 and SOCS2 when Flag-tagged LC3 was overexpressed. 293FT cells transfected with Flag-tagged LC3 were cultured under normal or starvation condition and treated with or without lysosomal inhibitors. The lysates were subjected to immunoprecipitation with anti-Flag-LC3 and immunoblotted with anti-SOCS2 antibody.

Data information: The data are represented as means $\pm$ SD, $n = 3$, $P \leq 0.05$, $**P \leq 0.01$, Student's t -test. JAK2, Janus kinase 2; SOCS2, suppressor of cytokine signaling 2; STAT3, signal transducer and activator of transcription 3. See also Supplementary Fig S4.

activity of the JAK-STAT signaling pathway is significantly improved during astrocyte differentiation [30]. We hypothesize that the pJAK and pSTAT might be activated through Atg5 during astrocyte differentiation. To examine it, E12 NPCs infected with Atg5 shRNA, Atg5 and the corresponding controls were cultured for 4 days with or without LIF for 30 min before being harvested. Atg5 knockdown inhibited pJAK2 and pSTAT3 (Fig 5A), while Atg5 overexpression led to activation of JAK2 and STAT3 (Supplementary Fig S4A). The reduced expression of GFAP and the number of GFAP⁺ cells due to Atg5 shRNA could be partly rescued through STAT3 overexpression *in vitro* (Fig 5B, Supplementary Fig S4B). Furthermore, STAT3-C (constitutively activated STAT3, A661C/N663C) overexpression could effectively rescue the deficits of astrocytes number *in vivo* (Supplementary Fig S4C). These data suggest that Atg5 directly or indirectly affects pJAK2 and pSTAT3 and ultimately regulates astrocyte differentiation.

To further reveal the relationship between Atg5 and the JAK-STAT signaling pathway, a series of experiments were performed to investigate changes of inhibitors of JAK/STAT including SOCS1, SOCS2, SOCS3, and SHP-2 [31,32]. The greatest decrease of SOCS2 (the suppressor of cytokine signaling 2, a member of STAT-induced STAT inhibitors) was observed when Atg5 was overexpressed (Supplementary Fig S4D). Next, we observed that Atg5 knockdown elevated the expression of SOCS2 (Fig 5C), and Atg5 overexpression decreased SOCS2 (Supplementary Fig S4F). We further found that SOCS2 knockdown (Supplementary Fig S4E) resulted in the increased expression of pSTAT3 and could restore decreased pSTAT3 levels to normal caused by Atg5 knockdown (Fig 5D). To further analyze the relationship between SOCS2 and Atg5 *in vivo*, we investigated whether SOCS2 downregulation could rescue astrocyte differentiation defects caused by Atg5 loss. Our data show that SOCS2 knockdown restored the number of GFAP-positive astrocytes when Atg5 was inhibited (Supplementary Fig S4G). The results show that Atg5 downregulates SOCS2 and raise the possibility that LC3, the key component of autophagy, might bridge this connection and degrade SOCS2. To test this possibility, the interaction between LC3 and SOCS2 was investigated by co-immunoprecipitation experiments. Either in normal or in starvation condition, the interaction between LC3 and SOCS2 was observed (Fig 5E). The interaction was also examined when LC3 was overexpressed (Fig 5F). Furthermore, more SOCS2 protein was pulled down by LC3 when cells were treated with lysosomal inhibitors (E64d and pepstatin A), which interferes with autolysosomal formation. In addition, the co-localization of LC3 and SOCS2 was observed in cells cotransfected with Flag-tagged LC3 and HA-tagged SOCS2 (Supplementary Fig S4H). These data suggest that the regulation of Atg5 on SOCS2 is carried out by the interaction between LC3 and SOCS2 and the role of Atg5 in astrocyte differentiation is mediated by autophagy. We propose that when Atg5 is overexpressed, LC3 is activated and SOCS2 is degraded through autophagy, resulting in the absence of the suppression of SOCS2 on STAT3. Then, JAK2-STAT3 pathway is activated, and astrocyte differentiation is regulated during embryonic brain development.

Emerging evidence supports the notion that autophagy plays an important role in the central nervous system [23–25], but the virtual function of autophagy in gliogenesis during late brain development is largely unknown. Astrocytes support nearby neurons,

and continually modify the chemical environment of neighboring cells. During development, astrocytes could influence synapse formation and function and nerve cell migration to achieve brain structure [33,34]. Here, we demonstrate that the autophagy-related gene Atg5 could strongly regulate the generation of glial cells in the developing mouse cortex. This conclusion is based on the results of *in vitro* and *in vivo* loss- and gain-of-function models. In the present study, we show that knockdown Atg5 could block the potency of the gliogenesis of radial progenitor cells via the JAK-STAT signaling pathway. At the same time, we observe that knockdown Atg5 decreases the number of Nestin-positive progenitor cells located at VZ/SVZ at E19 (Supplementary Fig S5A), which mainly generate astrocytes at late brain developmental stage. Because we do *in utero* electroporation at E16 and analyze at P2, when neurogenesis is almost finished, the Pax6-positive precursor cells and the Tuj1-positive neurons do not reveal a significant difference (Supplementary Fig S5B and C). Together, we provide multiple lines of evidence supporting a role of Atg5 in regulating astrocyte differentiation through the modulation of STAT3 activity. These findings shed light on the molecular mechanisms underlying astrocyte differentiation in the developing mouse brain.

Materials and Methods

Detailed information can be found in Supplementary Methods.

Mice

All animal experiments were approved by the Animal Care and Use Committee of Institute of Zoology, Chinese Academy of Sciences.

In utero and postnatal electroporation

The mixture that contains target plasmids, EGFP-encoding plasmid, and fast green was microinjected into the lateral ventricle. Embryos were electroporated with five 50-ms pulses at 40 V with a 950-ms interval, and neonatal pups were electroporated with four pulses at 90 V.

Statistical analysis

Images were acquired on a Zeiss LSM 780 confocal system and analyzed by Adobe Photoshop, and the 'Ipwin 32' was used for cell counting. The software Odyssey V3.0 was used for quantification of Western blot bands. *P*-values were calculated using the Student's *t*-test. All bar graphs are plotted as mean $\pm$ SD. Statistical significance: NS, not significance, $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Supplementary information for this article is available online: <http://embor.embopress.org>

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Author contributions

SW, BL, and JJ designed research; SW, BL, HQ, XL, QL, ZS, WX, and FJ performed research; SW, BL, and FJ analyzed data; SW, BL, and JJ wrote paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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