

AT-rich interaction domain 5A facilitates axon regeneration through docking protein 6 in the peripheral nervous system

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Abstract

Background: Peripheral nerves are easily damaged in accidental trauma due to their shallow location. Compared to the limited regeneration of the central nerve, the peripheral nerve has a certain regenerative ability after injury. However, this ability is not sufficient to achieve functional recovery. To increase the rate of regeneration after nerve injury, increasing regeneration-associated gene expression by transcription factors in neurons is necessary.

Methods: Sciatic nerve crush animal models were generated in Sprague–Dawley rats. Bioinformatics analysis and quantitative real-time polymerase chain reaction were applied to detect gene expression; immunofluorescence staining and western blotting were applied to detect protein expression. The neurite outgrowth of cultured dorsal root ganglion (DRG) neurons was performed to evaluate axon regeneration *in vitro*. Intrathecal injection of adeno-associated virus was applied to suppress or overexpress the target *in vivo*. Following transfection, immunofluorescence staining of newborn axons' marker (SCG10) in sciatic nerve after crush was used to evaluate the function of AT-rich interaction domain 5A (Arid5a) or docking protein 6 (Dok6) on axon regeneration. The binding between transcription factor (TF) and the promoter of target genes was verified by chromatin immunoprecipitation.

Result: Arid5a has high activity in specific regenerating clusters and it accumulates specifically in the nucleus of DRG neurons after sciatic nerve injury. Upon Arid5a inhibition by small interfering RNA, the outgrowth of neurites *in vitro* and the regeneration of axons *in vivo* were inhibited. In contrast, after Arid5a overexpression in rats, axon regeneration was significantly accelerated. In addition, Arid5a promotes the expression of Dok6 by binding to its promoter in DRG neurons. Suppression of Dok6 represses the neurites outgrowth of cultured DRG neurons, while its overexpression enhances axon regeneration *in vivo*. Furthermore, overexpression of Dok6 restored the impaired effect of Arid5a suppression on axon regeneration.

Conclusions: These findings indicate that axonal injury induced nucleus accumulation of Arid5a in neurons. Through Dok6, Arid5a accelerates axon regeneration of DRG neurons both *in vitro* and *in vivo*. This study enriched our understanding of the function of Arid5a in the peripheral nervous system and the transcriptional regulatory network involved in neural regeneration.

Keywords: Peripheral nerve injury; Axon regeneration; DRG neurons; Transcription factor; Arid5a; Dok6

Introduction

The neurons located in the peripheral nervous system (PNS) have the ability to sprout axons following injury, and this regenerative capacity is lost in central nervous system (CNS) neurons [1]. In addition to the microenvironment, the intrinsic regenerative capacity of neurons may be the most important factor [2]. Dorsal root ganglion (DRG) neurons belong to the PNS, and damage signals are transported into the cell nucleus following injury and subsequently evoke the transcription of regeneration-associated genes (RAGs) to achieve successful axon regrowth [3].

In our previous study, we employed laser-capture microdissection coupled with RNA sequencing (LCM-seq) to explore the gene expression profile during axon regeneration of DRG neurons [4]. Multiple RAGs, such as Atf3, Fos11, Klf6, Crem, and Arid5a, and their related transcriptional networks were identified. As reported previously, Atf3 and Jun are key

regulators of axon regeneration, several studies have emphasized their importance in the CNS and the PNS [5,6]. Although the roles of Klf6, Crem, and Fos11 in the PNS have been extensively in basic research studies, the expression of these genes in the CNS following injury has been reported to differ [7–9]. Prior to our previous study, Arid5a was not reported to be involved in nerve injury or axon regeneration.

Arid5a is a member of the Arid family, a type of nucleic acid-binding protein that is expressed in a variety of tissues and is involved in biological processes such as development, cell proliferation and differentiation, and the regulation of gene expression [10]. At present, Arid5a appears to have two different functions. Specifically, Arid5a can act as an messenger RNA (mRNA)-stabilizing protein or transcription factor in different biological processes. As an mRNA-stabilizing protein, Arid5a is known to bind the 3' untranslated region (3'UTR) of interleukin-6 (IL-6) mRNA,

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participating in immune and inflammatory responses. Lipopolysaccharide (LPS) induces the upregulation of Arid5a and IL-6 in macrophages, whereas IL-6 is suppressed in serum and Th17 cells in the context of Arid5a deficiency [11]. In addition, NF- κ B activates Arid5a gene expression and increases IL-6 expression via the stabilization of IL-6 mRNA during the early phase of LPS stimulation. Then, p38 MAPK phosphorylation of Arid5a inhibits its stabilization functions, which leads to a rapid decrease in IL-6 mRNA levels in the late phase of LPS stimulation [12]. In addition to IL-6, the mRNA of CD134 and signal transducer and activator of transcription 3 (STAT3) were also been reported to be stabilized by Arid5a in immune-related cells [13]. Arid5a could both recognized stem-loop structures in the 3'UTR of STAT3 and counteracting the RNase function of Regnase-1 to maintain the mRNA level [14].

Recently, Arid5a was found to have both DNA- and RNA-binding preferences through its extended AT-rich interaction domain (ARID) and intrinsically disordered regions (IDRs) [15]. As a transcription factor, Arid5a binds the Col2a1 promoter coupled with sox9, stimulating chondrocyte differentiation through Col2a1 [16]. In addition, Arid5a binds to the promoter of peroxisome proliferator-activated receptor gamma (Pparg) and dynamically counterregulates it to regulate energy metabolism and adipose tissue homeostasis [17]. Although our previous studies revealed the importance of Arid5a both in the PNS and CNS [4,18], the function and underlying mechanism of Arid5a in axon regeneration remain largely unknown.

Similar to our previous study, we reported that Arid5a is significantly upregulated in DRG neurons following nerve injury, and this increase mainly occurs in the nucleus. These findings indicate that Arid5a may act as a transcription factor in DRG neurons during axon regeneration. We explored the role of Arid5a both *in vitro* and *in vivo*, as well as its mechanisms in the regeneration of the axons of the PNS, in this study.

Methods

Animals

Adult Sprague–Dawley (SD) rats (12 weeks, 250–300 g, specific pathogen-free) for the sciatic nerve crush (SNC) model were provided by Nantong University Experimental Animal Center. The experimental and animal handling procedures were approved by the Administration Committee of Experimental Animals, Jiangsu Province, China (Approval No. 20190226-001).

Sciatic nerve crush injury

The anesthetized and SNC to SD rats were performed as our previous studies [19]. Firstly, rats were intraperitoneal injected with complex narcotics, which contained trichloroacetaldehyde monohydrate (85 mg/kg), magnesium sulfate (42 mg/kg), and sodium pentobarbital (17 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA). Then, the skin of the hind limbs of the rats was cut and the sciatic nerve was uncovered. The proximal side of the exposed nerve was placed on the bottom jaw of a toothless hemostat (RWD, Shenzhen, China) at 3 mm wide. The crush lasts 30 s at three clicks of the forceps, being careful not to pull the nerve. Immediately afterward, the wound was sutured, and the duration of injury was calculated. Intact rats were used as control (0 h).

Intrathecal injection of adeno-associated virus

The adeno-associated virus (AAV) serotype 2/8 constructs for suppressing rat Arid5a (AAV2/8-hsyn-EGFP-miR-Arid5a-shRNA1 and AAV2/8-hsyn-EGFP-miR-Arid5a-shRNA2) were packaged by QureGene (Hangzhou, China). The constructs for overexpressing rat Arid5a (AAV2/8-hsyn-EGFP-Arid5a) were purchased from GENECHM (Shanghai, China), and AAV serotype PHP.S used in the rescue experiments came from BrainVTA (Wuhan, China), including DOK6-overexpressing AAV (rAAV-hsyn-Dok6-P2A-mCherry-WPREs-hGH) and Arid5a-knockdown AAV (rAAV-hSyn-EGFP-miR-30a-Arid5a-shRNA3-WPREs). The short hairpin (shRNA) sequences and CDS of rat Arid5a and Dok6 are listed in [Supplemental Table 1](#). For AAV infection, rats were intrathecally injected with 10 μ l (titers of 1×10^8 + E13 for 2/8 AAV and 5×10^8 + E12 for PHP.S AAV) virus into the cerebrospinal fluid at the position between vertebrae L5 and L6. Two weeks were allowed to ensure the efficiency of the virus infection before SNC, and the process diagram can be found in [Figure 2e](#).

Immunofluorescence staining

After anesthetization, both L4–L5 DRGs and SNs were dissected from the rats and fixed with 4% paraformaldehyde. Twelve hours later, the DRGs and SNs were transferred to 10%, 20%, and 30% sucrose (Sigma) for gradient dehydration and then embedded in the optimum cutting temperature compound (OCT, Tissue-Tek, Tokyo, Japan). Then, the samples were sliced, and the thickness used for immunostaining was 12 μ m. Immunofluorescence Blocking Buffer (Beyotime, Shanghai, China) was used for tissue block for 1 h at 22°C, followed by primary antibody incubation at 4°C for 12–16 h. The primary antibodies used in this study included rabbit anti-Arid5A (1:200; Sigma), mouse anti-Arid5a (1:100; Novus, Colorado, USA), mouse anti-Tuj1 (1:500, R&D Systems, Emeryville, CA, USA), rabbit anti-tuj1 (1:500, Abcam, Cambridge, UK), and rabbit anti-SCG10 (1:1000, Proteintech, Chicago, USA). After being washed with phosphate buffered saline (PBS), the sections were incubated with secondary antibody for 90–120 min at 22°C. The secondary antibodies used here include: donkey anti-mouse IgG (H + L) (Alexa Fluor® 488) (1:500; Proteintech), donkey anti-rabbit IgG (H + L) (Alexa Fluor® 488) (1:500; Thermo Fisher Scientific), goat anti-rabbit IgG-Cy3 (1:500; Proteintech), and goat anti-mouse IgG (H + L) (Alexa Fluor® 647) (1:1000, Abcam). The nuclei were labeled with Hoechst 33342 (1:2000; Dojindo, Kumamoto, Japan). Images were captured by Zeiss microscopy (Axio Imager M2m), and the intensity of fluorescence was quantified by Photoshop software (version 21.2.6).

Fluorescence *in situ* hybridization

L4/5 DRGs were dissociated from the rats at 0, 3, 6, and 12 h and at 1, 3, and 7 days after SNC, and 12 μ m frozen sections were used for FISH without RNase. Ten-minute treatment of DEPC-PFA (4%) was performed followed by 30-min treatment of DEPC-protein kinase (5%) at 22°C. Compared with pre- and postwash with PBS, a 10-min treatment of triethanolamine with acetic anhydride (0.1 M) at 22°C was performed before incubation with prehybridizing solution (RiboBio, Guangzhou, China) at 42°C for 2 h. Finally, a hybridizing solution (RiboBio), containing a cy5

labeling probe of Arid5a (Sangon Biotech, Shanghai, China), was added to the slices and reacted at 42°C for 14 h. The sequence of the Arid5a probe is listed in [Supplemental Table 1](#). After being washed with 4 × standard saline citrate (SSC), 2 × SSC, and 1 × SSC for 10 min each, the tissue sections were incubated with Hoechst 33342 to label the nucleus.

Dorsal root ganglion neuron culture

The culture of DRG neurons was executed as outlined in previous descriptions [4]. In brief, the DRGs were separated from neonatal rats first and digested cells with trypsin (0.25%; Thermo Fisher Scientific) at 37°C for 0.5 h. After three blows with the tip of 1 ml in 15% bovine serum albumin, 5-min centrifugation at 900 r/min was performed to remove tissue debris. At last, the cells resuspend with neurobasal medium (2% B27, 1% L-glutamine, Thermo Fisher Scientific) and 1% penicillin–streptomycin (Beyotime), and then, they were plated on the plates that were precoated with poly-L-lysine (Sigma).

Small interfering RNAs (siRNAs) were synthesized by RiboBio (Guangzhou, China). Neuron transfection was performed with the Lipofectamine RNAiMAX reagent (Invitrogen) for 12 h according to the manufacturer's specification. The sequences of the Arid5a siRNAs and the negative control are listed in [Supplementary Table 1](#).

Real-time polymerase chain reaction

Total RNA from DRG tissues after injury or from cultured DRG neurons 48 h post-transfection was extracted with TRIzol reagent (Invitrogen). HiScript III RT SuperMix (Vazyme, Nanjing, China) was applied for complementary DNA synthesis, and ChamQ SYBR qPCR Master Mix (Vazyme) was applied for quantitative real-time polymerase chain reaction (qPCR) according to the manufacturer's instructions. qPCR was conducted via the ABI Step-One system. Relative mRNA expression levels were calculated using the comparative 2^{−ΔΔCt} method and normalized to Gapdh expression. All of the sequences of the primers that were used to amplify the target genes are listed in [Supplemental Table 1](#).

Neurite outgrowth assay

For the neurite outgrowth assay, the neurons were plated into 12-well cell culture plates or microfluidic chips (SND150, Xona Microfluidics LLC, USA) [20]. After dissociation, DRG neurons and siRNA were added to the left chamber simultaneously for 12 h. The cells were fixed with 4% paraformaldehyde 24 h later, and Tuj1 was used to label the cell bodies and axons of neurons. Images were captured by Zeiss microscopy (Axio Imager M2m). The longest length and fluorescence intensity of the neurites on each chip were measured by ImageJ (version 1.4.3.67; National Institutes of Health, Bethesda, MD, USA) and Photoshop software (version 21.2.6), respectively.

Western blot

After being infected with AAV, the DRGs were dissociated to obtain total protein using a MinuteTM Total Protein Extraction Kit (Invent Biotechnologies, Beijing, China). The Bicinchoninic Acid Protein Assay Kit (Thermo Fisher Scientific) was used for the measurement of protein concentration. Thirty micrograms of protein from each sample were separated by 10% SDS-PAGE and then electrotransferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA).

Following, the membrane was blocked with blocking buffer (Beyotime) for 1 h at room temperature and then incubated with the primary antibody for 12–16 h at 4°C. The primary antibodies used in this study include Arid5a (1:1000, Novus), Dok6 (1:400, Thermo Fisher Scientific), p-Ret (1:500, Abcam, Cat# ab51103, RRID: AB_870738), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10 000, Abcam). After being washed three times with Tris-HCl containing Tween-20, incubation of the secondary antibody lasted 2 h at 22°C. Tanon-S200 Chemiluminescent Imaging System (Tanon) and ECL Western Blotting Detection Kit (Thermo Fisher Scientific) were used to image the protein bands on the membrane. Protein expression levels were quantified by ImageJ (version 1.4.3.67).

Chromatin immunoprecipitation

To verify the binding of Arid5a to the promoters of its targets, the B35 cell line was chosen for chromatin immunoprecipitation (ChIP) assays through the Simple CHIP[®] PlusEnzymatic Chromatin IP Kit (Cell Signaling Technology, Boston, MA, USA). Briefly, the cells were first treated with 1% formaldehyde for 10 min at 20°C to crosslink the protein–DNA complexes, and a 5-min treatment with 0.125 M glycine was performed to stop the crosslinking reaction. To prevent protein degradation, a protease inhibitor cocktail was added in the following steps. After being washed with cold PBS, the cells were harvested by gentle scraping and pelleted by 5-min centrifugation at 1500 rpm/min at 4°C. Then, 10-min incubation of CHIP buffer on ice was used to release the nuclei, and chromatin breakage was achieved using micrococcal nuclease treatment for 20 min at 37°C followed by ultrasonication. Five-gram chromatin samples were used for each chromatin immunoprecipitation with IgG (negative control), anti-Arid5a, and anti-Histone 3 (positive control) at 4°C overnight. Then, the reactions were treated with ChIP-Grade Protein G Magnetic Beads and followed by low- and high-salt washing to eliminate unbound complexes. Chromatin can be isolated by CHIP elution buffer treatment for 30 min at 65°C and proteinase K treatment for 2 h at 65°C. Before qPCR, the DNA sample was purified with DNA purification spin columns for recovery of DNA and removal of protein contaminants. The primers for ChIP analysis were designed at 0–2 kb intervals upstream of a structural sequence of putative targets ([Supplemental Table 1](#)).

Bioinformatics analysis

The RNA sequencing data of rat DRGs following SNC can be acquired in the database of Genome Sequence Archive (CRA006945). Undisturbed cell clustering was performed using the Seurat R package (v3.1.1) and displayed with *t*-distributed stochastic neighbor embedding (*t*-SNE) with the top 50 principal components; the resolution used in this study was 0.7 [21].

The analysis of regulons was conducted using the Single-Cell Regulatory Network Inference and Clustering (SCENIC) R package (1.1.2-2) with default parameters [22]. Briefly, TFs and targets were identified using GENIE3, and networks were constructed by enrichment of TF-binding motifs among their targets. The activity of the regulon (area under the curve [AUC]) was calculated by the AUCCell R package. Hierarchical clustering with Euclidean distance was applied for the construction of regulon modules corroding previous studies [23].

DEGs in this study were analyzed by the Z test [24]. The DEG was recognized with a fold change (FC) > 2.0 and P -value ≤ 0.05 . Part of heatmaps and volcano plots were drawn by the omicsmart system of Gene Denovo Corporation (<https://www.omicsmart.com>).

The Gene Ontology (GO) database was used for GO biological function enrichment of DEGs. Terms were accepted with more than one gene and P -value ≤ 0.05 . The JASPAR (<https://jaspar.elixir.no/>) database was utilized to predict TF binding sites on target promoters. The reported Arid5a-related genes were identified by Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA).

Statistical analysis

The data are presented as the means $\pm$ standard deviations (SDs). The normality of the data was first tested through the Shapiro–Wilk test. If the data passed the normality test ($P > 0.05$), Student's t -test (for two groups) or ordinary one-way analysis of variance (ANOVA) (for three or four groups) was used to analyze statistical differences. If the data did not pass the normality test ($P \leq 0.05$), the Mann–Whitney test (for two groups) or the Kruskal–Wallis test (for three or four groups) was used. Multiple comparisons in ordinary one-way ANOVA and the Kruskal–Wallis test were performed through Dunnett's t -test. These analyses were performed with GraphPad Prism software (version 8.0.2; San Diego, CA, USA). * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ were considered statistically significant.

Results

Arid5a was upregulated in neurons during axonal regeneration following sciatic nerve crush

In a previous study, we explored the gene expression profile during axon regeneration. After sciatic nerve injury, we separated DRG neurons using laser-capture microdissection at 3, 6, and 12 hours (h) and 1, 3, and 7 days (d). Coupled with normal DRG neurons (0 h), we obtained 618 samples in total [4]. These cells were then sorted by cell clustering and separated into seven clusters (Figure 1a). When we analyzed the origin of the cells in each cluster, we found that the cells in Cluster 2 came from only 3 and 7 d, which coincided with the regeneration period of DRG neurons [25] (Figure 1b). In addition, we found high expression of axon regeneration-associated genes in this cluster, indicating that the neurons within this cluster may be specific axonal regeneration neurons.

To identify the transcription regulators of axon regeneration, SCENIC analysis was performed. Among the 272 regulons, 13 regulons had specific high activities in Cluster 2 (Figure 1c). Most of these molecules, such as Atf3 [26], Jun [7], Myc [27], and Smad1 [28], have been reported to participate in axon regeneration. Although the expression of Arid5a in DRG neurons was explored after SNC in our previous report, the construction and activity of the Arid5a regulon have not been investigated. Here, we analyzed the AUC of the Arid5a regulon, which represents the transcriptional activity of Arid5a. The results in Figure 1d and e revealed that Arid5a exhibits increased transcriptional activity in neurons within Cluster 2, as well as at 1, 3, and 7 d after SNC (Figure 1d and e). Consistent with the RNA-seq data, Arid5a was significantly upregulated in DRG neurons, especially at 1 and 3 d after SNC (Figure 1f).

In addition to qPCR, we analyzed Arid5a mRNA expression and localization using FISH (Figure 1g and h). Neurons but not other cells predominantly express Arid5a in the DRG. At 1, 3, and 7 d after SNC, Arid5a mRNA levels were obviously greater than that at earlier stages (3, 6, and 12 h), especially in the nuclei of neurons (Figure 1g and h). In addition, Arid5a proteins accumulated similarly in the nuclei of neurons from 1 to 7 d after SNC, whereas little ARID5A expression was noted at 0 h, as well as 3–6 h (Figure 1i and j). This upregulated expression and transcriptional activity of Arid5a following injury suggested that Arid5a may participate in the regulation of axon regeneration in DRG neurons.

Arid5a knockdown suppressed the regeneration of dorsal root ganglion neuronal axons

We found that Arid5a knockdown using siRNA impaired neurite outgrowth in adult DRG neurons. Whether it can impair the axon growth of young DRG neurons remains unknown. Here, we transfected DRG neurons from neonatal rats with two (siRNA-1 and siRNA-2) of three siRNA fragments (Figure 2a), and axon growth was detected using a microfluidic chip system (Figure 2b). As shown in Figure 2c, the neurons transfected with siRNA-1 and siRNA-2 had less neurite outgrowth. Both the length and density of newborn neurites were suppressed when Arid5a was knocked down in cultured DRG neurons (Figure 2d).

Next, we evaluated the *in vivo* impact of Arid5a knockdown on axonal regeneration by AAV-mediated Arid5a suppression. To ensure infection efficiency, AAV2/8-hSyn-EGFP-miR-Arid5a-shRNA1 (Arid5a-sh1) and AAV2/8-hSyn-EGFP-miR-Arid5a-shRNA2 (Arid5a-sh2) were intrathecally injected into adult rats 2 weeks before SNC (Figure 2e). EGFP indicated successful infection of AAV2/8 in DRG neurons, and ~30% of the neurons were labeled, indicating that myelinated larger DRG neurons exhibited greater infection efficiency (Figure 2f). Both Arid5a mRNA and protein levels were downregulated by Arid5a-sh1 and Arid5a-sh2 compared with those in the control (NC-sh) (Figure 2g and h).

After 3 days of SNC, we identified regenerated axons of DRG neurons using sciatic nerve immunofluorescence staining with SCG10, a marker of regenerating sensory axons [29]. As shown in Figure 2i, the elongation of newborn axons (SCG10+) was obviously inhibited in Arid5a-sh1 and Arid5a-sh2 rats compared with that in control animals (Figure 2i). The fluorescence intensity and maximum length of SCG10+ axons were measured to evaluate the regeneration of axons. When Arid5a was knocked down, SCG10+ axon length and density were notably reduced compared with control animals (Figure 2j). Collectively, these data indicated that Arid5a is required for the axonal regeneration following SNC.

Arid5a promotes axon regeneration following sciatic nerve crush

Given that Arid5a repression reduced axon regeneration of DRG neurons both *in vitro* and *in vivo*, we wondered whether Arid5a overexpression in neurons could increase their potential for axonal regeneration. AAV2/8-hSyn-EGFP-Arid5a (Arid5a-OE) was constructed by inserting the CDS of Arid5a into the plasmid. AAV was then delivered to the rats by intrathecal injection, followed by SNC 2 weeks later (Figure 2e). First, this AAV can infect neurons specifically and is located mainly in the nucleus. The labeling ratio was

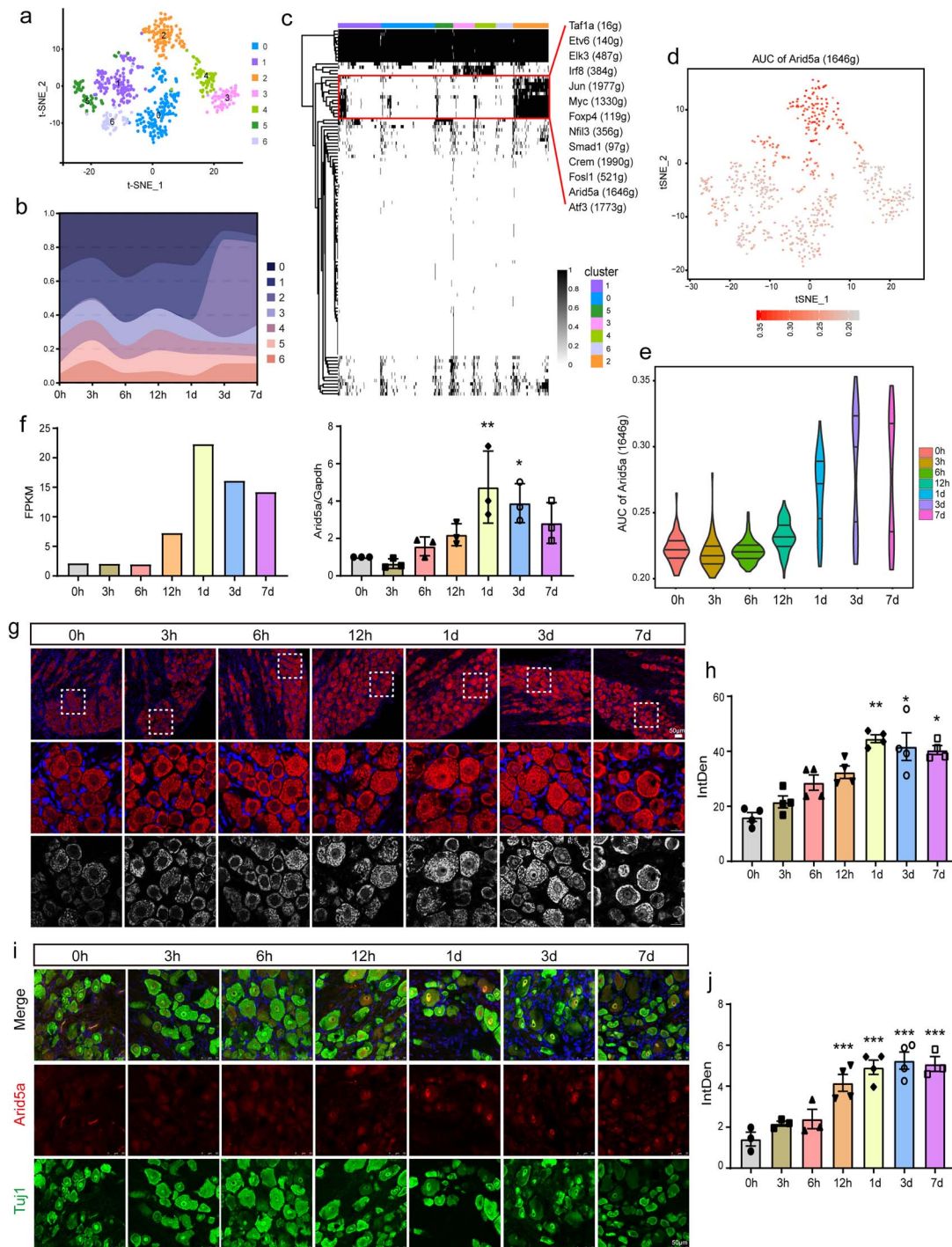


Figure 1. Arid5a was up-regulated in regeneration-associated DRG neurons following SNC. **(a)** Seven cell clusters of 627 DRG neurons after SNC are shown in a t-SNE plot. Dots represent individual cells, and colors represent cell types. **(b)** River chart showing the composition of cell clusters from each group (0 h, 3 h, 6 h, 12 h, 1 d, 3 d, and 7 d) after SNC. Colors represent the different cell clusters. **(c)** Binary activity of regulons (rows) in different groups (0 h, 3 h, 6 h, 12 h, 1 d, 3 d, and 7 d). Red box indicates those that are specifically active in Cluster 2. **(d)** Activity of the Arid5a regulon in each neuron. The dots represent individual cells, and the color reflects the activity. **(e)** Violin plot present the enrichment of Arid5a regulon activity (AUC score). **(f)** Mean Arid5a mRNA levels in each group from LCM-seq data and DRG tissue at different time points after SNC ($n=3$). **(g, h)** FISH of sections from DRGs after SNC for Arid5a (red)/ Hoechst 33343 (blue). The red signal is the cy5 labeling on Arid5a probe, and blue represents the cell nucleus. The white dotted box represents the enlarged area below (g). Fluorescence intensity statistics of Arid5a expression (red) in each group (h) ($n=4$). **(i, j)** Immunofluorescent staining of Arid5a (red), Tuj1 (green), and Hoechst 33342 (blue) in L4–L5 DRGs (i). Fluorescence intensity statistics of Arid5a expression (red) in each group (j) ($n=3$ or 4), $*P \leq 0.05$, $**P \leq 0.01$. Arid5a, AT-rich interaction domain 5A; DRG, dorsal root ganglion; SNC, sciatic nerve crush; t-SNE, t-distributed stochastic neighbor embedding; Taf1a, TATA-Box binding protein associated factor, RNA polymerase I subunit A; Elk3, ETS transcription factor ELK3; Ets6, ETS variant transcription factor 6; Irf8, interferon regulatory factor 8; Foxp4, forkhead box P4; Jun, Jun proto-oncogene; AP-1, transcription factor subunit; AUC, area under the curve; Myc, MYC proto-oncogene; Nfil3, nuclear factor, interleukin 3 regulated; Smad1, SMAD family member 1; Crem, cAMP responsive element modulator; Atf3, activating transcription factor 3; Fos1, FOS like 1; AP-1, transcription factor subunit; LCM-seq, laser-capture microdissection coupled with RNA sequencing; FISH, fluorescence *in situ* hybridization; FPKM, fragments per kilobase of exon model per million mapped fragments; IntDen, intensity density; Tuj1, tubulin, beta 3, class III; L4/5, lumbar 4/5; Gapdh, glyceraldehyde-3-phosphate dehydrogenase

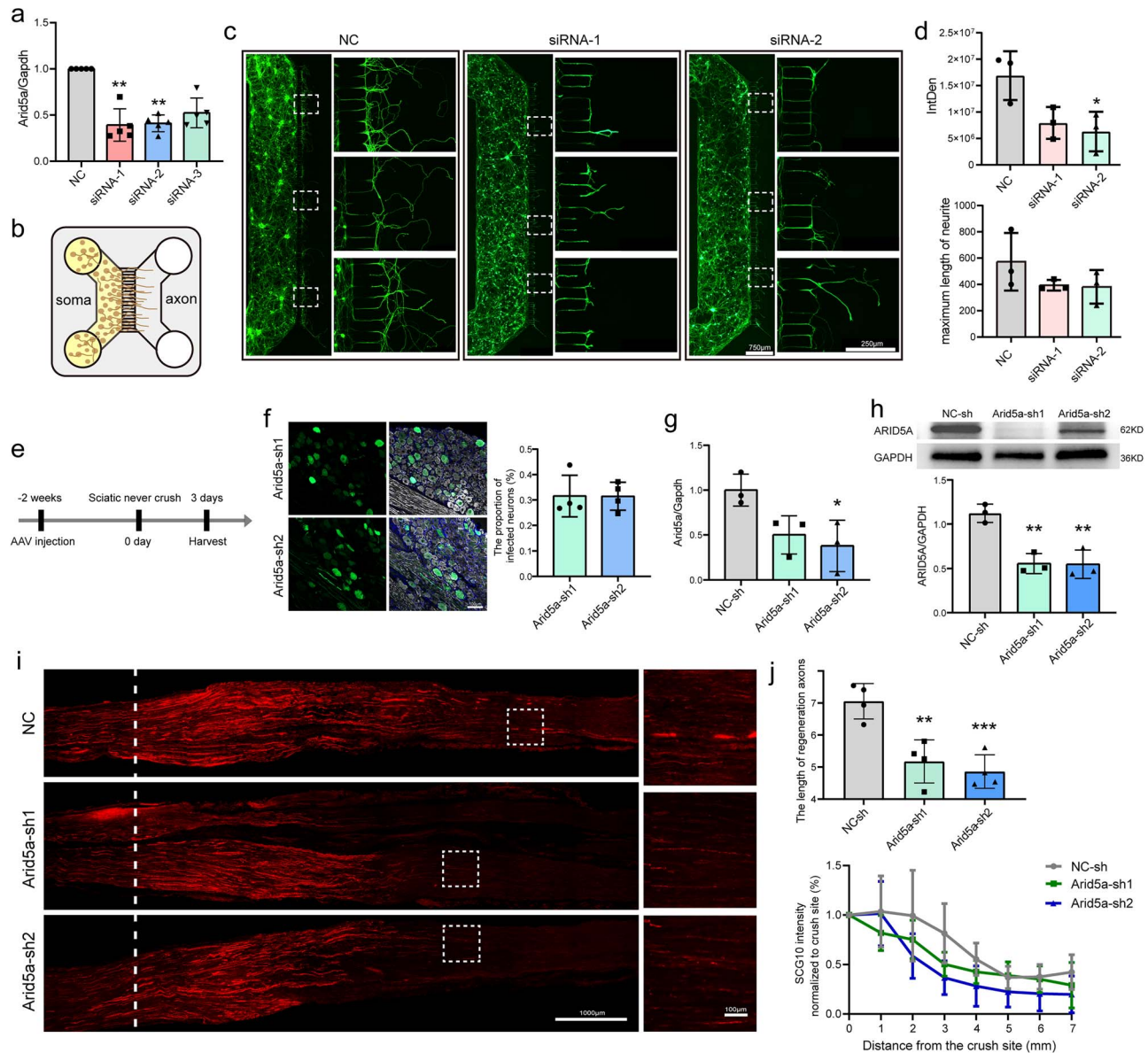


Figure 2. Arid5a is required for axon regeneration of DRG neurons. **(a)** The efficiency of Arid5a knockdown by three small interfering RNAs (siRNA-1, siRNA-2, and siRNA-3) ($n=5$). **(b)** Working diagram of microfluidic chips, in which the bodies of neurons were plated to the left and the axons were stretched to the right. **(c)** Immunostaining of TuJ1 (green) to label neurons, including the cell body and axons. The white dotted box represents the enlarged area on the right. **(d)** The histogram shows the density of new axons and the maximum length of neurites on the right of the microfluidic chips after neurons were infected with siRNA-1 or siRNA-2 targeting Arid5a ($n=3$). **(e)** Timeline for Arid5a knockdown by intrathecal injection of AAV *in vivo* and SNC. **(f)** The successful infection of AAV2/8-hSyn-EGFP-miR-Arid5a-shRNA1 (Arid5a-sh1) and AAV2/8-hSyn-EGFP-miR-Arid5a-shRNA2 (Arid5a-sh2) using intrathecal injection. The virus infection efficiency was determined based on the ratio of the number of AAV-labeled neurons (green) to the total number of TuJ1-labeled neurons (white) ($n=4$). **(g)** Expression of Arid5a mRNA after the intrathecal injection of Arid5a-sh1 or Arid5a-sh2. An intrathecal injection of normal saline (NC-sh) was used as a control ($n=3$). **(h)** Western blot showing ARID5A expression after Arid5a was knocked down by Arid5a-sh1 and Arid5a-sh2 ($n=3$). **(i)** SCG10 (red) immunostaining of sciatic nerves from NC-sh-, Arid5a-sh1-, and Arid5a-sh2-infected rats 3 days after SNC. The white dotted line on the left indicates the crush beginning site, and the white dotted box represents the enlarged area on the right. **(j)** Histogram showing the longest length of SCG10-labeled axons in the NC-sh, Arid5a-sh1, and Arid5a-sh2 groups and the intensity normalization of SCG10 to the injury initiation site ($n=4$), $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. Arid5a, AT-rich interaction domain 5A; DRG, dorsal root ganglion; SNC, sciatic nerve crush; IntDen, intensity density; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; NC, normal control; TuJ1, tubulin, beta 3, class III; AAV, adeno-associated virus; hSyn, human synapsin I; EGFP, enhanced green fluorescent protein; SCG10, stathmin 2

also ~30% (Figure 3a). In comparison to the control group, the expression of Arid5a was significantly upregulated in the Arid5a-OE group (Figure 3b and c). Anatomically, we found that the animals injected with Arid5a-OE had more SCG10-labeled axons than the control animals did; both the density and extension length of newborn axons were greater in the former than in the latter (Figure 3d and e).

These results indicate that Arid5a promotes axon regeneration following SNC.

Dok6 acts as a target of Arid5a in regulating axon regeneration

Given Arid5a accumulation in the nucleus of DRG neurons and its upregulated transcriptional activity after SNC,

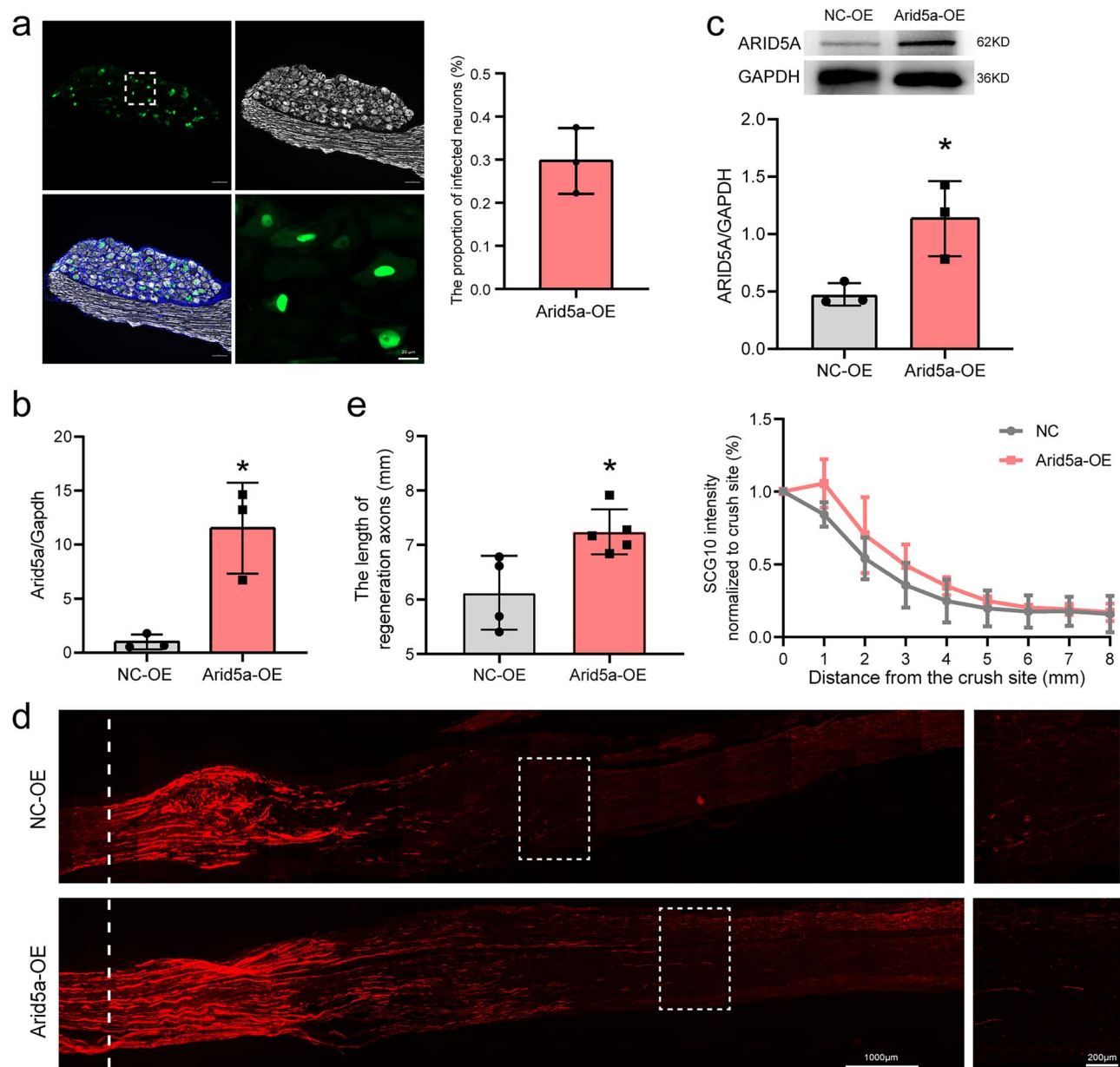


Figure 3. Arid5a overexpression promotes axon regeneration of DRG neurons *in vivo*. **(a)** Successful infection of AAV2/8-hSyn-EGFP-Arid5a (Arid5a-OE) through intrathecal injection; the white dotted box represents the enlarged area at the bottom right. The efficiency of virus infection was determined by the ratio of the number of AAV-labeled neurons (green) to the total number of Tuj1-labeled neurons (white) ($n=3$). **(b)** Arid5a expression after the intrathecal injection of Arid5a-OE. An intrathecal injection of normal saline (NC-sh) was used as a control ($n=3$). **(c)** Western blot showing ARID5A expression levels after Arid5a overexpression using Arid5a-OE, and the histogram shows the gray statistics of the strips ($n=3$). **(d)** SCG10 (red) immunostaining of sciatic nerves from NC-OE- and Arid5a-OE-infected rats 3 days after SNC. The white dotted line on the left indicates the crush beginning site, and the white dotted box represents the enlarged area on the right. **(e)** Histogram showing the maximum length of SCG10+ axons in the NC-OE and Arid5a-OE groups and the intensity normalization of SCG10 to the injury initiation site ($n=4$ or 5), $*P \leq 0.05$. Arid5a, AT-rich interaction domain 5A; DRG, dorsal root ganglion; OE, overexpression; AAV, adeno-associated virus; hSyn, human synapsin I; EGFP, enhanced green fluorescent protein; Tuj1, tubulin; beta 3, class III; NC, normal control; SCG10, stathmin 2; SNC, sciatic nerve crush; Gapdh, glyceraldehyde-3-phosphate dehydrogenase

we assumed that Arid5a may act as a transcription factor during axon regeneration. Hence, we first analyzed the hypothesized targets obtained from SCENIC. Based on the binding site analysis of Arid5a, 1645 targets were identified (Figure 4a). For the highest expression and activation of Arid5a at 3 d after SNC, we intersected these targets with differentially expressed genes (DEGs) at 3 d from LCM-seq and obtained 496 targets that were differentially expressed after injury. Most of these genes were upregulated, especially some reported axon regeneration-related genes, such as *Hamp*, *Atf3*, *Npy*, *Csrp3*, *Sprp1a*, and *Adcyap1* (Figure 4b).

GO enrichment analysis revealed that these targets primarily participated in developmental processes (GO:0032502, GO:0048856, GO:0044767, and GO:0048869), cell motility (GO:0048870, GO:0006928, GO:0061674, GO:0040011, and GO:0051179), and the cell response to stress and stimulus (GO:0006950 and GO:0050896), which were highly correlated with the neuron response and axon regeneration after injury (Figure 4c). To further narrow the range of candidate targets, IPA, a database of reported relationships, was applied. Among the 144 genes associated with Arid5a, 5 overlapped with previous putative targets (Figure 4d). These genes

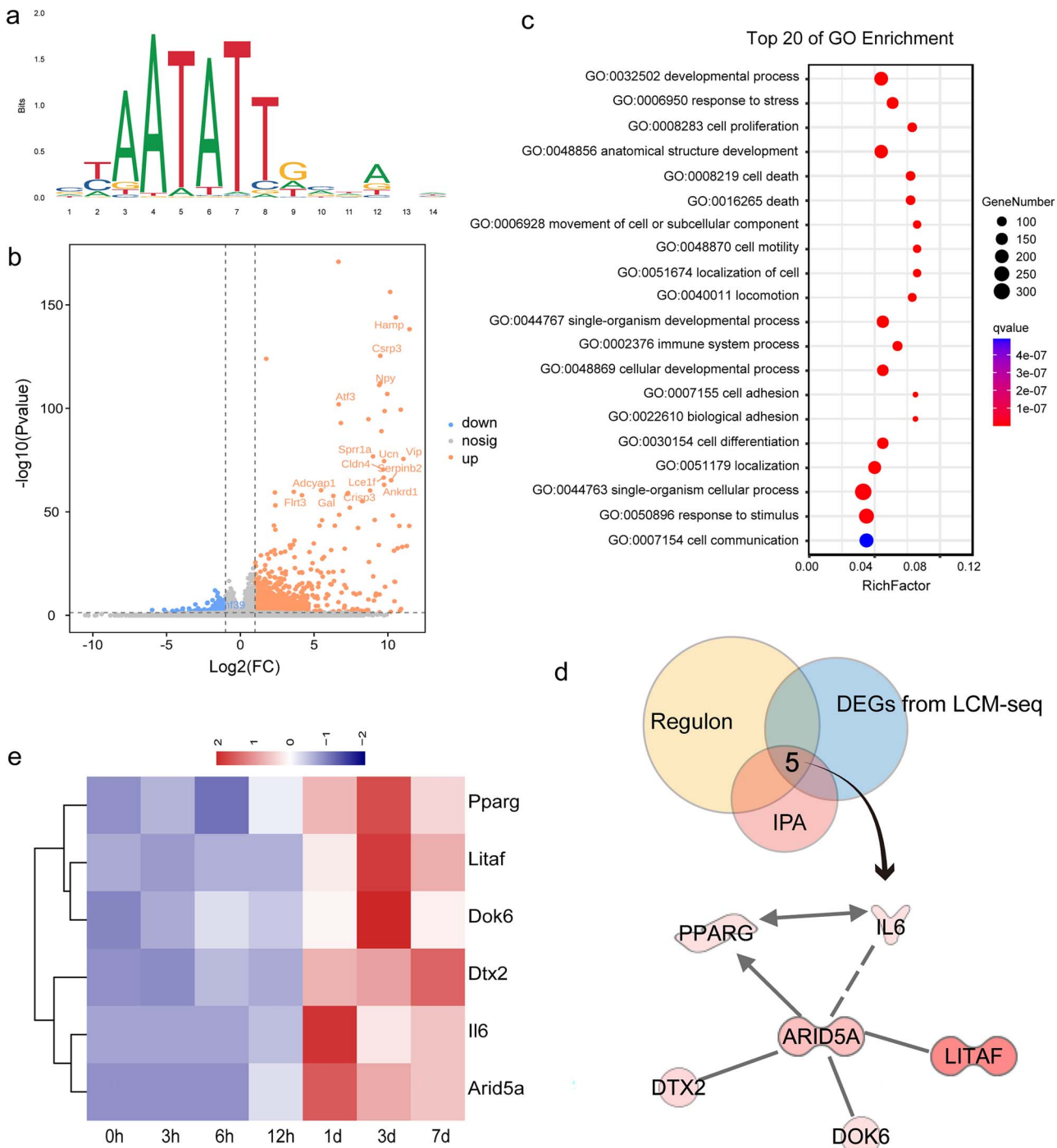


Figure 4. Predicted targets of Arid5a. **(a)** Sequence logo plots of reference (JASPAR) and identified position weight matrix for Arid5a. **(b)** Volcano plot displaying up- and down-regulated targets of Arid5a at 3 days after injury compared to the normal group; orange represents upregulated genes labeled in orange, blue represents downregulated genes, and gray represents nondifferentially expressed genes. **(c)** Heatmap showing the top 20 categories GO enrichment of the putative targets. **(d)** Five candidates were selected from the intersection of SCENIC analysis (Regulon) DEGs obtained from the LCM-seq and IPA databases (IPA). The line between molecules represents their interactions from the IPA. **(e)** Heatmap showing the expression of the candidates from LCM-seq data. Arid5a, AT-rich interaction domain 5A; JASPAR, the JASPAR database; Hamp, hepcidin antimicrobial peptide; Csrp3, cysteine and glycine-rich protein 3; Npy, neuropeptide Y; Atf3, activating transcription factor 3; Sprr1a, small proline-rich protein 1A; Ucn, urocortin; Vip, vasoactive intestinal peptide; Cldn4, claudin 4; Lce1f, late cornified envelope 1F; Serpinb2, serpin family B member 2; Adcyap1, adenylate cyclase-activating polypeptide 1; Flrt3, fibronectin leucine rich transmembrane protein 3; Gal, galanin and GMAP prepropeptide; Crisp3, cysteine-rich secretory protein 3; Ankrd1, ankyrin repeat domain 1; GO, gene ontology; FC, fold change; Pparg, peroxisome proliferator-activated receptor gamma; Litaf, lipopolysaccharide-induced TNF factor; Dok6, docking protein 6, Dtx2, deltex E3 ubiquitin ligase 2; Il6, interleukin 6; DEGs, differentially expressed genes; LCM-seq, laser-capture microdissection coupled with RNA sequencing; IPA, ingenuity pathway analysis

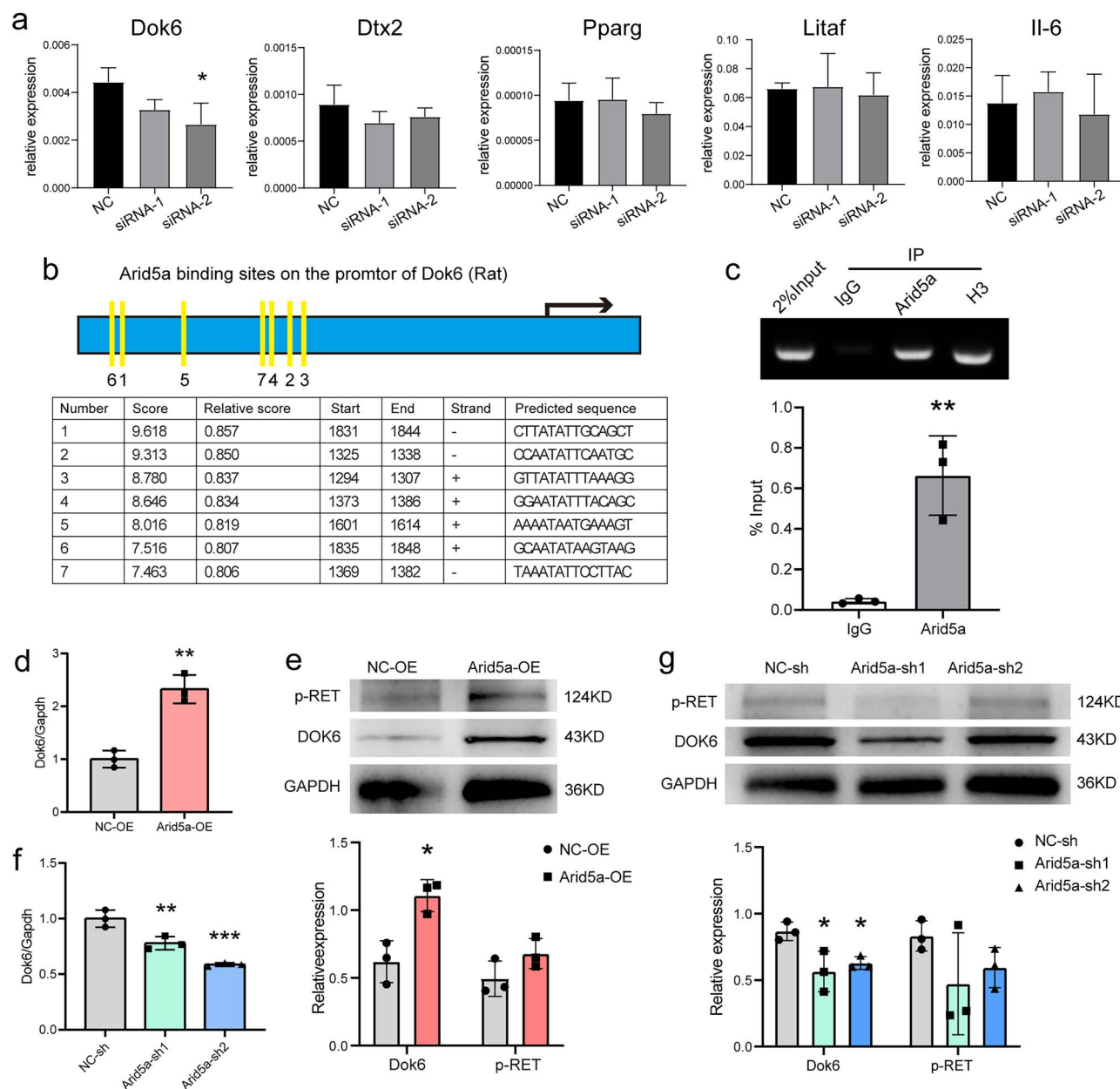


Figure 5. Dok6 is the target of Arid5a in promoting axon regeneration of DRG neurons. **(a)** qPCR was used to detect the expression of five candidate targets after cultured DRG neurons were transfected with Arid5a siRNA ($n=3$). **(b)** Predicted binding sites of Arid5a on the Dok6 promoter; the specific location and sequence of each site are listed below. **(c)** ChIP was performed using antibodies against Arid5a with qPCR across the Dok6 promoter, ($n=3$). **(d)** Dok6 expression in rats injected with Arid5a-OE AAV ($n=3$). **(e)** Western blot showing p-RET and DOK6 expression after Arid5a overexpression using Arid5a-OE, and the histogram shows the gray statistics of the strips ($n=3$). **(f)** Dok6 expression in rats injected with AAV targeting Arid5a-sh1 or Arid5a-sh2 ($n=3$). **(g)** Western blot showing ARID5A expression after Arid5a was knocked down by Arid5a-sh1 and Arid5a-sh2, and the histogram shows the gray statistics of the strips ($n=3$), $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. Dok6, docking protein 6; Arid5a, AT-rich interaction domain 5A; DRG, dorsal root ganglion; qPCR, quantitative real-time polymerase chain reaction; AAV, adeno-associated virus; Dtx2, deltex E3 ubiquitin ligase 2; Pparg, peroxisome proliferator-activated receptor gamma; Litaf, lipopolysaccharide-induced TNF factor; Il6, interleukin 6; IgG, immunoglobulin G; H3, histone H3; IP, immunoprecipitation; ChIP, chromatin immunoprecipitation; NC, normal control; RET, ret proto-oncogene; OE, overexpression; Gapdh, glyceraldehyde-3-phosphate dehydrogenase

included Pparg, Litaf, Dok6, Dtx2, and Il6 and presented similar expression patterns to Arid5a (Figure 4e).

Next, we detected the expression of these five candidates after knocking down Arid5a in cultured DRG neurons. Only Dok6 was significantly downregulated when neurons were transfected with siRNA-1 or siRNA-2 (Figure 5a). Hence, we next wanted to determine whether Arid5a binds to the Dok6 promoter. Seven binding sites for Arid5a were located on

the Dok6 promoter according to predictions from the JASPAR database (Figure 5b), and ChIP analysis verified binding between them (Figure 5c).

In addition, we detected Dok6 expression when Arid5a was knocked down or overexpressed *in vivo* and found Dok6 was upregulated in the Arid5a-OE group (Figure 5d and e) but downregulated in the Arid5a-sh1 and Arid5a-sh2 groups (Figure 5f and g). Dok6 is an intracellular adaptor that

promotes Ret-mediated neurite growth [30]. Hence, we also detected the relative expression of phosphorylated RET after Arid5a overexpression or knockdown *in vivo*. Along with Dok6, p-RET was upregulated in rats infected with the Arid5a-OE AAV (Figure 5e) but impaired when Arid5a was knocked down (Figure 5g). However, the difference of p-RET was not significant. These data indicate that Dok6 may act downstream of Arid5a to promote the regeneration of DRG neuron axons.

Arid5a promotes axon regeneration through Dok6

To determine the regulatory relationship between Arid5a and Dok6, we first investigated the function of Dok6. Similar to Arid5a, suppressing Dok6 through siRNA impaired neurite outgrowth in cultured DRG neurons *in vitro* (Figure 6a and b). In the construct, Dok6 overexpression using AAV (rAAV-hsyn-Dok6-P2A-mCherry-WPREs-hGH) had a positive effect on neurite outgrowth (Figure 6c). Furthermore, the knockdown of Arid5a with Arid5a-sh3 (rAAV-hSyn-EGFP-miR-30a-Arid5a-shRNA3-WPREs) impaired newborn neurites, which was reversed by transfecting neurons with AAV of Dok6-OE simultaneously in cultured DRG neurons (Figure 6c).

To verify these results *in vivo*, AAVs of Arid5a-sh3 and Dok6-OE were delivered into rats through intrathecal injection. Two weeks after infection, the rats were subjected to SNC. Three days later, the immunofluorescence staining results revealed that the AAV of Arid5a-sh3 had a greater infection efficiency (~40%) than did the AAV of Dok6-OE (~30%), and ~15% of the neurons were infected with both Arid5a-sh3 and Dok6-OE (Figure 6d). As demonstrated using SCG10 labeling, the length and density of regenerated axons in the Dok6-OE group were greater than those in the rats treated with NaCl (NC) (Figure 6e and f).

As previously observed, Arid5a knockdown using the intrathecal injection of Arid5a-sh3 significantly impaired axon regeneration (Figure 6g and h). However, this phenomenon can be reversed by Dok6 overexpression. No significant difference was detected between normal rats and the rats that were simultaneously injected with Arid5a-sh3 and Dok6-OE AAVs (Figure 6g and h). These results indicate that both Arid5a and Dok6 promote axon regeneration after SNC. As the target of Arid5a, Dok6 promotes the role of Arid5a in axon regeneration.

Discussion

Initially, we sought to define the subtype of neurons by classical markers of DRG neurons, such as *Nefh* (response to the NF cluster), *Ret* (response to the NP cluster), calcitonin gene-related peptide (CGRP) (response to the PEP cluster), and *TH* (response to the TH cluster) [31]; however, these clusters were not obviously distinguished using these markers with our data. We assume that the samples obtained by LCM may contain one or more cells (both neurons and glia), differential genes in glial cells may dilute changes in gene expression in neurons. Additionally, it been reported that classical marker of neurons reduced upon injury [6], which may due to the transcriptional reprogramming of DRG neurons after injury. Hence, new markers or better analytical methods should be explored to solve this problem.

On the basis of the higher expression of regeneration-related genes in Cluster 2, we termed Cluster 2 a regeneration-related state of neurons. However, not all of the neurons from

3 and 7 d were collected in Cluster 2. According to previous studies, injuries to the sciatic nerve include crush and transection. Transection can lead to ~90% DRG neuron injury, whereas crush can lead to ~50% DRG neuron injury [6]. Consistent with this finding, 57.7% and 50.5% of the neurons from 3 and 7 d, respectively, formed Cluster 2 (Figure 1a), and these damaged neurons are the same as those we studied in terms of their ability to regenerate axons.

In this study, we assumed that Arid5a acts as a TF during axon regeneration, but we cannot reclude Arid5a as an RNA-stabilizing protein. The regulatory effect of Arid5a on the transcription of IL-6 has been studied previously [32]. The latter has been reported to participate in axon regeneration [33]. Our LCM-seq results revealed that IL-6 was also significantly upregulated in DRG neurons from 1 to 7 d after SNC. The rapid increase in IL-6 at 1 d indicated the possibility of post-translational modification of IL-6 in DRG neurons. Although IL-6 mRNA levels slightly changed after Arid5a was knocked down in cultured DRG neurons, its expression was not detected *in vivo* in the context of Arid5a knockdown or overexpression at both the mRNA and protein levels. Whether Arid5a can bind to IL-6 mRNA to stabilize it or the IL-6 promoter to regulate its transcription in DRG neurons still needs to be verified and will be investigated in the future.

As a candidate target of Arid5a, Dok6 not only has binding sites for Arid5a located in its promoter but is regulated by Arid5a both *in vivo* and *in vitro*. As a substrate of tyrosine kinases, Dok6 participates in neurite outgrowth and axon guidance through RET or Ntrk3 [30,34]. Docking protein 1 (DOK1), 2, 4, 5, and 6 adaptor proteins all interact with the phosphorylated 1062 tyrosine (Y1062) [35,36]. Phosphorylated Y1062 of RET activates multiple signaling pathways, such as JNK pathways [37,38] and the PI3K/AKT pathway [39,40]. Furthermore, these pathways also strongly influence axon regeneration [41]. These studies highlight the importance of the Dok6/RET pathway in axon regeneration, but the slight alteration in p-RET in this study suggests the possibility that other targets or pathways act downstream of Arid5a. The downstream pathways and upstream regulators of Arid5a still need to be explored.

Compared with *in situ* injection, intrathecal injection of AAV has the advantages of easy operation and less damage and has been assessed in several clinical trials [42]. However, the infection efficiency of the virus remains unsatisfactory, and the maximum infection efficiency of the virus was ~60% in this study. In particular, when the two types of AAV were injected simultaneously, fewer than 20% of the neurons were infected with both. Although we extended the infection time to 4 weeks, we did not obtain better results. Compared with the high infection efficiency of the central nervous system [43], the vector construction and serotype selection of AAV still need to be improved. Furthermore, knockout or knockdown mice must be generated to investigate the function of Arid5a and the regulatory relationship between Arid5a and Dok6 in axon regeneration, potentially providing more robust support for this study.

Conclusions

Upon axonal injury, Arid5a showed higher activity in specific regenerating clusters of DRG neurons. Its upregulation and nuclei accumulation in neurons potentially promote axon regeneration following nerve injury. As a target, Dok6 could

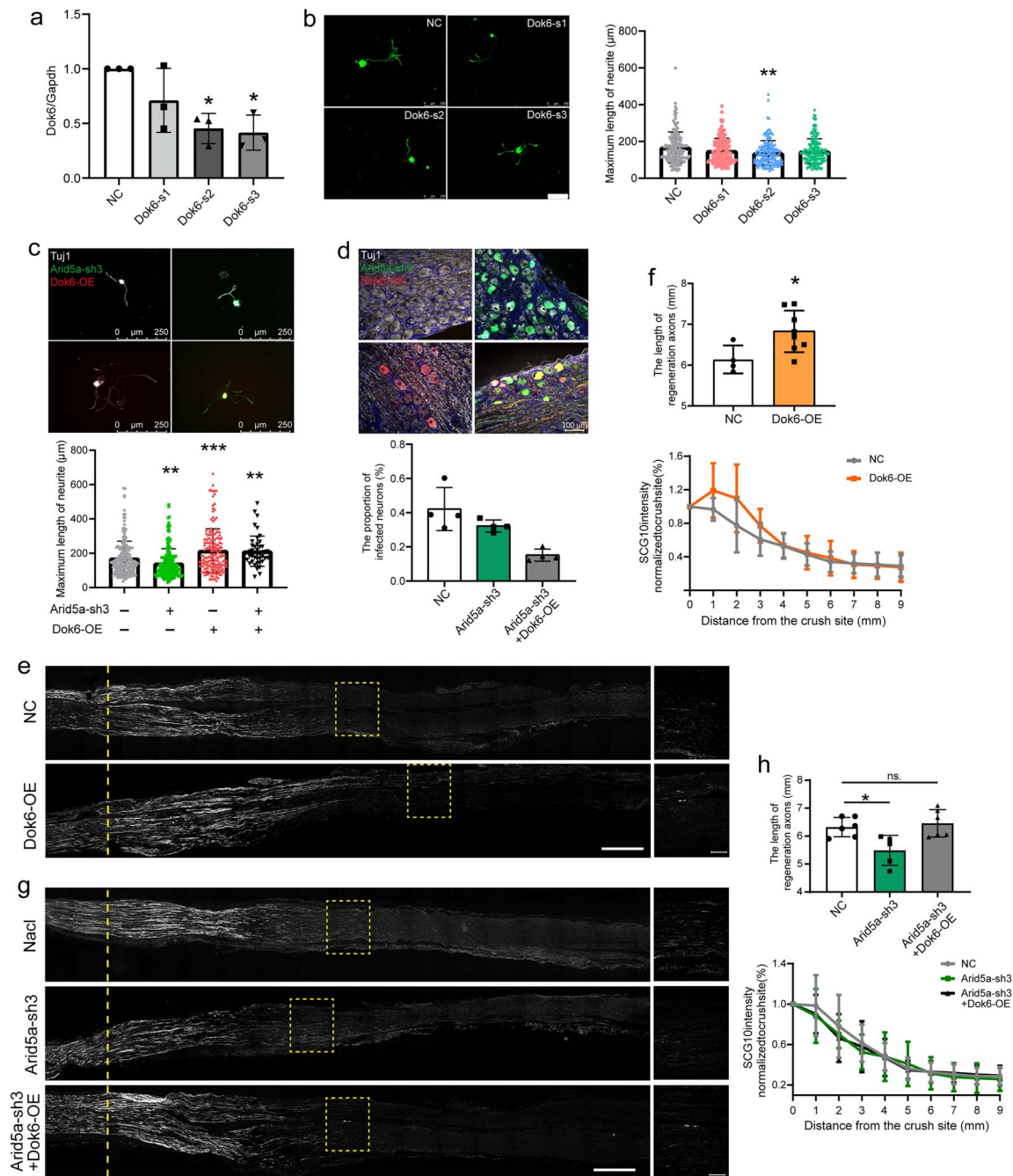


Figure 6. Dok6 overexpression reverses the suppressive effect of Arid5a knockdown on axon regeneration of DRG neurons. **(a)** qPCR was used to detect the knockdown efficiency of Dok6 by three siRNAs (Dok6-s1, Dok6-s2, Dok6-s3) in cultured DRG neurons ($n = 3$). **(b)** Effect of Dok6 siRNA on neurite outgrowth in cultured DRG neurons. Tuj1 (green) labeled neurons after transfection with Dok6-s1, Dok6-s2, or Dok6-s3; scale bar: 100 μ m. The histogram shows the quantification of the maximal length of neurite per neuron. **(c)** Cultured DRG neurons were subjected to AAV-mediated knockdown of Arid5a (rAAV-hSyn-EGFP-miR-30a-Arid5a-shRNA3-WPREs, Arid5a-sh3, green) and overexpression of Dok6 (rAAV-hSyn-Dok6-P2A-mCherry-WPREs-hGH, red) separately or simultaneously. PBS was used as a control (NC), and Tuj1 (white) was used to label the neurons. Scale bar: 250 μ m. The histogram shows the quantification of the maximal length of neurite per neuron. **(d)** Successful infection of Arid5a-sh3 (green) and Dok6-OE (red) through intrathecal injection; scale bar: 100 μ m. The efficiency of virus infection was determined based on the ratio of the number of AAV-labeled neurons (green or red) to the total number of Tuj1-labeled neurons (white) ($n = 4$). **(e)** SCG10 (white) immunostaining of sciatic nerves from rAAV-hSyn-mCherry-WPRE-hGH (NC-) and Dok6-OE-infected rats 3 days after SNC. The yellow dotted line on the left indicates the crush beginning site, and the yellow dotted box represents the enlarged area on the right. Scale bars: 1000 μ m (left) and 200 μ m (right). **(f)** Histogram showing the maximum length of SCG10+ axons in the NC and Dok6-OE groups and the intensity normalization of SCG10 to the injury initiation site ($n = 4$ or 8). **(g)** SCG10 (white) immunostaining of sciatic nerves from Arid5a-sh3 with or without Dok6-OE; NaCl was used as a control (NC). The yellow dotted line on the left indicates the crush beginning site, and the yellow dotted box represents the enlarged area on the right. Scale bars: 1000 μ m (left) and 200 μ m (right). **(h)** Histogram showing the maximum length of SCG10+ axons in the NC, Arid5a-sh3, and Arid5a-sh3 + Dok6-OE groups, and the intensity normalization of SCG10 to the injury initiation site ($n = 5$ or 6), $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. Dok6, docking protein 6; Arid5a, AT-rich interaction domain 5A; DRG, dorsal root ganglion; OE, overexpression; AAV, adeno-associated virus; hSyn, human synapsin I; EGFP, enhanced green fluorescent protein; WPREs, woodchuck hepatitis virus post-transcriptional regulatory elements; qPCR, quantitative real-time polymerase chain reaction; NC normal control, OE overexpression, Tuj1, tubulin; beta 3, class III; SCG10, stathmin 2; ns, no significance; Gapdh, glyceraldehyde-3-phosphate dehydrogenase

mediate the effect of Arid5a in promoting axon regeneration in the PNS. Our study revealed the expression and role of Arid5a in DRG neurons after injury, which involves the enrichment of the transcriptional regulatory network in neural regeneration. This study provides new insights and therapeutic targets for the treatment of peripheral nerve injury in the clinic.

Author contributions

Study conception and design, data analysis, and manuscript draft: L.L.Z.; experiment implementation: Z.X.R., L.L.Z., and W.X.H.; manuscript revision: X.S.G. All authors have read and approved the final manuscript. Zhixian Ren (Investigation, Methodology, Visualization [equal]), Weixiao Huang (Investigation, Methodology, Project administration [equal]), Xiaosong Gu (Conceptualization, Supervision [equal]), and Lili Zhao (Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Validation, Visualization [equal])

Supplementary data

Supplementary data are available at *Burns & Trauma* online.

Ethics approval

This study involving animal handling procedures was approved by the Administration Committee of Experimental Animals, Jiangsu Province, China (Approval No. 20190226-001) and performed following institutional guidelines.

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Conflict of interest

None declared.

Data availability

Data supporting the results of this study can be obtained from the corresponding author upon reasonable request.

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