The long noncoding RNA Arrl1 inhibits neurite outgrowth by functioning as a competing endogenous RNA during neuronal regeneration in rats

Dong Wang1, Yanping Chen1, Mingwen Liu1, Qianqian Cao1, Qihui Wang1, Shuoshuo Zhou1, Yaxian Wang1, Susu Mao1, Xiaosong Gu1,2, Zhenge Luo3, Bin Yu1,2*

1 Key Laboratory of Neuroregeneration of Jiangsu and Ministry of Education, Co-innovation Center of Neuroregeneration, Nantong University, Nantong 226001, China
2 Jiangsu Clinical Medicine Center of Tissue Engineering and Nerve Injury Repair, Affiliated Hospital of Nantong University, Nantong University, Nantong 226001, China
3 School of Life Science and Technology, ShanghaiTech University, Shanghai 201210, China

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* To whom correspondence should be addressed: Bin Yu: Key Laboratory of Neuroregeneration of Jiangsu and Ministry of Education, Co-innovation Center of Neuroregeneration, Nantong University, Nantong 226001, China; yubin@ntu.edu.cn; Tel: (+86)0513-85051887.

These authors contributed equally: Dong Wang, Yanping Chen, Mingwen Liu

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ABSTRACT

The intrinsic regeneration ability of neurons is a pivotal factor in the repair of peripheral nerve injury. Therefore, identifying the key modulators of nerve regeneration may help improve axon regeneration and functional recovery after injury. Unlike for classical transcription factors and regeneration-associated genes (RAGs), the function of long noncoding RNAs (lncRNAs) in the regulation of neuronal regeneration remains mostly unknown. In this study, we used RNA-Seq-based transcriptome profiling to analyze the expression patterns of lncRNAs and mRNAs in rat dorsal root ganglion (DRG) following sciatic nerve injury. Analyses using the lncRNA-mRNA co-expression network, gene ontology (GO) enrichment, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway databases indicated that the lncRNA Arrl1 decreases neurite outgrowth after neuronal injury. shRNA-mEDIATE Arrl1 silencing increased axon regeneration both in vitro and in vivo, and improved functional recovery of the sciatic nerve. Moreover, inhibiting an identified target gene of Arrl1, cyclin-dependent kinase inhibitor 2B (Cdkn2b), markedly promoted neurite outgrowth of DRG neurons. We also found that Arrl1 acts as a competing endogenous RNA (ceRNA) that sponges a Cdkn2b repressor, microRNA-761 (miR-761), and thereby up-regulates Cdkn2b expression during neuron regeneration. We conclude that the lncRNA Arrl1 affects the intrinsic regeneration of DRG neurons by de-repressing Cdkn2b expression. Our finding indicate a role for an lncRNA-microRNA-kinase pathway in the regulation of axon regeneration and functional recovery following peripheral nerve injury in rats.
Peripheral nerve injury (PNI) is a common clinical issue due to the resulting dysfunction of sensory and motor nerves. Contrary to the central nervous system (CNS), which has poor regeneration ability, the peripheral nervous system (PNS) has a restricted regeneration power following the injury (1). The differential neuronal intrinsic regeneration ability and regenerative microenvironment are two major pivotal factors in axon regeneration and functional recovery (2,3). The signal transduction and transcription response in the initiation of regeneration are well understood, and many regeneration associated genes (RAGs) are identified during the PNI (4). However, the molecular mechanisms of axon regeneration has focused mainly on transcriptional factors or RAGs, whether long non-coding RNAs (lncRNAs) have vital roles in this process remains largely unknown.

Genomic research has revealed that only 2% of genes encode coding RNA, while the others transcriptionally generates non-coding RNAs, especially lncRNAs, most of which were still largely unknown (5). With the development of RNA sequencing (RNA-seq) technology, an increasing number of lncRNAs with a relatively low expression level have been identified to have crucial functions in many physiological and pathological processes (6-8). Previous studies have demonstrated that lncRNAs are involved in the development of nervous system (9), such as Alzheimer’s disease (10) and neuropathic pain (11). However, the role of lncRNAs in peripheral nerve injury repair remains to be investigated.

The transcriptome analyses based on the RNA-seq and microarray have revealed the altered level of lncRNAs in the mouse and rat PNI models (12,13). Our previous study indicated that the level of lncRNAs was changed following sciatic nerve injury (SNI) in rat (14,15), and recent research reported that lncRNAs impacted the neuronal outgrowth by regulating the RAGs expression (16). Nonetheless, the function of lncRNAs in PNI repair and the mechanism in axon regeneration are still little understood. In this study, we identified the co-expression regulation network between lncRNAs and mRNAs following PNI. Moreover, we determined the critical role of a new lncRNA, Arrl1, in axon regeneration and functional recovery of injured sciatic nerve. Finally, we explored the mechanism underlying the regulation of Arrl1 in neuronal intrinsic regeneration.

**Results**

**Identify the different expression profiles of lncRNAs and mRNAs following SNI**

SNI is a common model for studying peripheral nerve regeneration. To characterize the transcriptome changes in peripheral nerve regeneration, we implemented RNA-seq of DRG at different time point (0h, 3h, 9h, 12h, 1d, 4d and 7d) following SNI in rats. Then unsupervised cluster analysis was conducted to uncover the mRNA and lncRNA expression patterns. We identified 17 differentially expressed lncRNAs (Figure 1A). Besides, we also identified 895 differentially expressed mRNAs (Figure 1B), including regeneration associated genes (RAGs): Atf3 (17), GAP43 (18), Sox11 (19) and Gadd45a (20). The expression changes of the differentially expressed lncRNAs (Figure S1) and RAGs (Figure S2) were validated by qRT-PCR. Next, we performed co-expression analysis of lncRNAs and mRNAs to search for the key regulators, and found that 9 of the differentially expressed lncRNAs were co-expressed or potentially interact with their target genes (Figure 1C). Furthermore, Gene Ontology and KEGG pathway analysis revealed that these differential co-expressed mRNAs were enriched in neuron development or axon regeneration-related processes (Figure 1D),
such as Jak-STAT (21), cAMP (22), or MAPK signaling pathway (23,24). The expression of candidate genes involved in those pathways were also validated by qRT-PCR (Figure S2). According to the validated lncRNAs expression patterns and lncRNA feature analysis with the coding potential and ORF predictions analysis (Figure S3), we chose lncRNA NONRATT032301.1, which was named as axon regeneration related lncRNA 1 (Arrl1), for further study.

**The expression and distribution of Arrl1 in DRG neuron post SNI**

QRT-PCR results indicated that Arrl1 expression was dramatically decreased in rat DRG post-SNI (Figure 2A). FISH experiments further validated that the level of Arrl1 was reduced in day 1 and 4 following the SNI, and mainly distributed in the cytoplasm (Figure 2B). The analysis of the Arrl1 sequence in the UCSC Genome Browser (http://genome.ucsc.edu/) showed that no overlapping annotated genes were identified, and Arrl1 was located on chromosome 17p12 (Figure S3). To examine the Arrl1 distribution in DRG neuron, cytoplasmic and nuclear RNA was isolated and then analyzed by qRT-PCR. The result indicated that Arrl1 was mainly distributed in the cytoplasm (Figure 2C). The predominantly cytoplasmic distribution of Arrl1 was further validated by FISH experiment. Arrl1 was co-localized with Tuj1, a neuronal marker, in the cytoplasm (Figure 2D).

**Inhibiting Arrl1 promotes neurite outgrowth in DRG neurons in vitro**

To investigate the role of Arrl1 in axon regeneration in vitro, two Arrl1-specific shRNA were used to disturb Arrl1 expression. DRG neurons infected with adeno-associated virus (AAV) containing Arrl1-specific shRNA (KD1 or KD2) exhibited reduced level of Arrl1, compared to that infected with AAV containing negative control shRNA (NC) (Figure 3A). The immunostaining of axons with Tuj1 indicated that knockdown of Arrl1 significantly promoted axon regeneration of DRG neurons in vitro (Figure 3B to 3D). As shown in Figure 3C, two batches of Arrl1 knockdown increased the total length of axons by 107.1 % ± 12.2 % and 90.7 % ± 25.1 %, compared with the control, respectively. Moreover, the length of the longest neurite was also increased in DRG neurons infected with AAV containing Arrl1-specific shRNA (Figure 3D). Collectively, these data suggested that Arrl1 has a negative role in the regrowth of DRG neuron in vitro.

**Arrl1 regulates sciatic nerve regeneration and behavioral recovery**

As Arrl1 promoted neurite outgrowth in DRG neurons in vitro, we further explored its role in sciatic nerve regeneration and behavioral recovery in vivo. First, we intrathecally injected rats with AAV containing Arrl1-specific or negative control shRNA. Arrl1-specific shRNA can knock down Arrl1 in DRG in vivo (Figure S4). Then, the rats infected with the virus were subjected to SNI surgery. Finally, we collected the injured sciatic nerve at 3 days post-surgery, and used SCG10 to label regenerated axons (Figure 4A). The regeneration ability of sciatic nerve was significant enhanced by Arrl1 knockdown (Figure 4A to 4C). In order to assess the function of Arrl1 on the behavioral recovery, behavioral assays in rats was performed to quantify the latency of heat- or mechanical force-induced response. The rats in control and Arrl1-knockdown group exhibited similar response latency in mechanical force-evoked test at 7 days post-SNI, while the rats in Arrl1-knockdown group presented a significantly better functional recovery at 10 and 18 day post-SNI, compare to these in control group (Figure 4D). However, in the assay of heat-
induced response, the rats in Arrl1 knockdown group exhibited a moderate thermal sensory recovery at 7 day post-SNI, when compared with control group (Figure 4E). Taken together, these results demonstrated that Arrl1 knockdown promotes the sciatic nerves regeneration and improves behavioral recovery.

**MiR-761 is the target molecule of Arrl1**

LncRNAs were reported to participate in the development of diseases with different manners (25,26), including the competing endogenous RNAs (ceRNA) mode (27). The target genes of Arrl1 were predicted by MRE enrichment analysis (28), and 61 candidate genes and related miRNAs were achieved. Meanwhile, RNA-seq analysis was performed following the knockdown of Arrl1 in DRG neurons. Compared with the control group, 229 genes with 1.5-fold down-regulation in Arrl1-knockdown group were selected to conduct GO analysis, which showed that most of those genes were involved in neuronal development or maturation. Then, we obtained the overlap between the target genes predicted by MRE enrichment analysis and the down-regulated gene analyzed by RNA-seq, and constructed an Arrl1-regulating ceRNA network (Figure 5A). Based on this, we selected 3 candidate target genes of Arrl1 (Cda, Cdkn2b and Kcnh4) potentially involved in neurite outgrowth and 4 related miRNA (miR-1956-5p, miR-761, miR-25-3p and miR-185-5p) (Figure 5B). Cda and Cdkn2b, but not Kcnh4 were dramatically decreased following Arrl1 knockdown (Figure 5C), and interfering Arrl1 significantly increased the miR-761 expression and moderately enhanced miR-185-5p and miR-25-3p expression, while had no effect on miR-1956-5p expression (Figure 5D). To further explore the direct relationship between Arrl1, related miRNAs and target genes, we constructed wild-type and miRNA binding sites-mutated Arrl1 (MUT1 and MUT2) luciferase plasmid, and performed luciferase assays. The result demonstrated that miR-761 and miR-25-3p significantly reduced the luciferase activity of Arrl1 in comparison with control miRNA mimic (NC), but miR-185-5p or miR-1956-5p had no notable effect. However, the mutated form (MUT1, MUT2) of miR-761 or miR-25-3p with the miRNA binding sites-mutated lost their suppressive effect on the luciferase activity, suggesting that the ability of miR-761 and miR-25-3p to regulate Arrl1 expression is dependent on them binding with Arrl1 (Figure 5E). Together, these data suggested that miR-761 and miR-25-3p are the target microRNA of Arrl1.

As shown in figure 5B, Cdkn2b has a high align score with both Arrl1 and miR-761/miR-25-3p, and Arrl1 knockdown significantly increased the Cdkn2b expression, so we proposed Cdkn2b as the target gene of Arrl1 for next study. We constructed the wild type and 3’UTR-mutated Cdkn2b (MUT) plasmid for luciferase assays. Compared with control miRNA mimic (NC), miR-761 significantly reduced the luciferase activity of Cdkn2b while miR-25-3p had no significant effect (Figure 5F). However, the miR-761 had no effect on 3’UTR-mutated Cdkn2b (MUT), suggesting that miR-761 regulates Cdkn2b expression through binding with its 3’ UTR domain (Figure 5F). Moreover, miR-761 also suppressed the Cdkn2b expression (Figure 5G). Taken together, these data suggested that Cdkn2b is the direct downstream target gene of miR-761, and miR-761 is the target molecule of Arrl1. In brief, Arrl1 works as a sponge for miR-761 targeting Cdkn2b.

**Arrl1 impacts axon regeneration through Arrl1/miR-761/Cdkn2b axis in ceRNA mode**

Because miR-761 is a key target molecule of Arrl1, we investigated the role of miR-761 in axon regeneration. We found that DRG neurons transfected with miR-761
exhibited marked increased axon outgrowth (Figure 6A), with the total and maximum length of neurite increased by 136.5% ± 27.8% and 119.2% ± 8.4%, respectively (Figure 6B and 6C). This result suggests that miR-761 promotes axonal regeneration.

We next explored the function of Cdkn2b, a downstream target gene of miR-761, in axonal growth. The Cdkn2b-specific siRNA (Si-1 or Si-2) was transfected into DRG neurons to knock down the expression of Cdkn2b (Figure 6D). DRG neurons with Cdkn2b down-regulation exhibited significant increase in the total and maximum length of neurite compared with the negative control (Figure 6E to 6G), indicating that Cdkn2b has a negative effect on axonal growth.

As the miR-761 is the target microRNA of Arrl1, we assumed that the suppressive activity of Arrl1 on axonal growth is dependent on miR-761. To test the hypothesis, we transfected DRG neurons with vehicle control AAV or that expresses Arrl1-specific shRNA, Arrl1-specific shRNA, or miR-761 inhibitor (miR-I), and performed the neurite outgrowth assay in vitro (Figure 7A). As expected, the total and maximum length of neurites were all increased in Arrl1 knockdown cells (Figure 7A to 7C). However, inhibiting miR-761 in Arrl1-knockdown cells abrogated the promoting effect of Arrl1 knockdown on axon growth (Figure 7B and 7C), suggesting that Arrl1 regulates axonal growth through miR-761. To further test the hypothesis in vivo, the miR-761 inhibitor, miR-761 antagonist (miR-A), was injected into DRG following Arrl1 knockdown in vivo. The sciatic nerve regeneration analysis was performed at day 3 post sciatic nerves crush. Consistent with the in vitro study, Arrl1 knockdown in DRG improved the sciatic nerve regeneration, while miR-761 antagonist eliminated the promoting effect of Arrl1 knockdown on sciatic nerve regeneration (Figure 7D to 7E). This result further demonstrates that Arrl1-regulated axon regeneration is dependent on miR-761.

Collectively, these data demonstrate that Arrl1 works as a sponge for miR-761 targeting Cdkn2b, and regulates axonal regeneration through Arrl1/miR-761/Cdkn2b axis in a ceRNA mode following SNI in rats (Figure 8).

Discussion

Despite the PNS having the intrinsic regeneration ability, severe PNI also induces axonal regeneration delay and a functional recovery barrier (29). Intrinsic regeneration ability is the main mechanism of nerve repair after injury (30), so it is necessary to deeply explore the mechanism of axon regeneration. Identifying novel regulators in axon regeneration could provide new potential targets for treating PNI. Here, we demonstrated that a novel long noncoding RNA, Arrl1, is decreased upon sciatic nerve injury, and functions as a sponge molecule to regulate DRG axonal growth and sensory function recovery.

Previous reports mainly focused on illustrating the effects of transcriptional factors or cytoskeleton-associated genes (31), such as Atf3 (32) and Klf4 (33). Only a few studies demonstrated the action of LncRNAs in nerve injury and repair. LncRNA, a multifunctional factor, participates in transcriptional modulation and post-transcriptional modification (34). In the present study, the RNA-seq analysis revealed the differential expression pattern of lncRNA and mRNA in DRG neurons following SNI. The lncRNAs-mRNAs co-expression analysis suggested an important role of lncRNAs in nerve injury repair. The functional analysis of mRNAs in the co-expression network indicates that the expression of mRNAs involved in axonal regeneration process might be regulated by some co-expressed lncRNAs. The differentially expressed lncRNAs following

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SNI might be involved in earlier repair of nerve injury. Exploring the function of differentially expressed lncRNAs might help to identify new therapeutic targets for neuroregeneration.

In this study, we identified Arrl1, which was decreased during SNI, as a pivotal negative regulator on axon regeneration. Suppressing Arrl1 expression in DRG neurons promoted the robust neurite outgrowth both in vitro and in vivo, suggesting that Arrl1 might be an intrinsic regeneration inhibitor in SNI. Recent study uncovered a conserved lncRNA, Silc1, which played a positive role in regulating mice sciatic injury repair. Knocking out Silc1 impaired axon regeneration, but the gain function of Silc1 in axon regeneration was not uncovered (16). Our previous study identified that another conserved lncRNA, uc.217, as a negative regulator in DRG neuron neurite outgrowth in vitro (14). Although we found a comparable increase in axon regeneration following Arrl1 knockdown, the effect of Arrl1 overexpression in neurite outgrowth remains to be determined.

The distribution of lncRNAs may be related to their functions. LncRNAs localized in nuclear can act as a cis-regulatory element or trans-acting element to regulate gene expression, chromatin state, or protein/RNA molecules (35,36), while lncRNAs expressed in cytoplasm can perform as a molecular sponge of miRNA and function in ceRNA mode (37). In this study, Arrl1 was mainly expressed in DRG’s cytoplasm, we proposed that ceRNA-modulated mode may underlie the mechanism of Arrl1 regulating axon regeneration. Moreover, the following bioinformatic analysis and experimental verification identified Cdkn2b as the potential downstream gene of Arrl1, which was involved in axon regeneration moderation. Previous reports indicated that the dysregulated expression of Cdkn2b impacts neurite extension in retinal ganglion cell (RGC) degeneration diseases (38). In present article, we found that Cdkn2b knockdown dramatically increased the axon outgrowth in DRG neurons. Our result demonstrated that Cdkn2b is a new potential target gene in nerve repair post-PNL.

The function of lncRNAs has been widely reported, lncRNAs directly interact with DNA, RNA and proteins, or perform as a miRNA sponge. In our work, we found a significantly increased expression of miR-761 after knocking down Arrl1. MiR-761 was firstly reported in the regulation of mitochondrial network via affecting the mitochondrial fission factor (39). Previous researches have illustrated that miR-761 is involved in tumorigenesis (40) and synaptic plasticity in hippocampal neurons(41). However, the role of miR-761 in axon regeneration is still unknown. In our study, the transfection of miR-761 significantly increased the axonal outgrowth. Meanwhile, overexpression of miR-761 reduced the Cdkn2b level, and the inhibitor of miR-761 eliminated the promoting effect of Arrl1 knockdown on axon outgrowth. Thus, we proposed that Arrl1 regulates peripheral nerve regeneration in a ceRNA mode. Collectively, we explored the downstream targets of Arrl1 and found that miR-761 is the target miRNA of Arrl1, while Cdkn2b is the downstream target of miR-761. Both miR-761 and Cdkn2b play pivotal roles in neurite outgrowth. Arrl1 regulates peripheral nerve damage repair through Arrl1/miR-761/Cdkn2b axis, but the molecular mechanism of Arrl1 differential expression post-PNI remains to be further studied.

In conclusion, our study constructed the lncRNA-mRNA co-expression network following the peripheral nerve injury, and identified a novel lncRNA, Arrl1, which can regulate the intrinsic regeneration ability of DRG neurons through sponging miR-761 to modulate Cdkn2b expression. Our finding
provides a new cognitive of lncRNA in axon regeneration and functional recovery following peripheral nerve injury.

Experimental procedures

Animals

SPF degree male Sprague Dawley (SD) rats (180-220g) were provided by Experiment Animal Center of Nantong University. All the experimental procedures involving animals were conducted in accordance with institutional animal care guidelines and approved ethically by the Administration Committee of Experimental Animals, Jiangsu Province, China.

Sciatic nerve crush and sample preparation

Twenty-four SD rats were randomly divided into six groups, and a 2 cm incision was made in the skin at the left thigh perpendicular to the femur following the intraperitoneal injection of Compound anesthetic (10 mg/kg body weight). The muscle tissue was bluntly dissected to expose sciatic nerves. Then the sciatic nerves were crushed at the 1 cm proximal to the bifurcation of tibial and fibular nerves using the fine forceps 3 times at 54 N of force. The incision was sutured after surgery. The L4-L5 DRGs were collected at 0, 3h, 9h, 12h, 1d, 4d and 7d following sciatic nerve injury.

RNA extraction and RNA-seq analysis

Total RNAs were extracted by TRIzol regent (Invitrogen, Carlsbad, CA, USA) following manufacturer’s instructions, and RNA-seq analysis was performed by Shanghai Biotechnology Corporation. The differential expression profiles of lncRNAs and mRNAs were determined by the bioinformatics analysis as previous reported (42). In hierarchical clustering, the Z score was calculated from the expression of lncRNAs, and the euclidean distance measure was used to compute the distance (dissimilarity) in lncRNA and the time. Gene co-expression network was built according to the normalized signal intensity of specific expression lncRNAs and mRNAs. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed to elucidate biological processes and signaling pathways associated with the correlated target genes of specific lncRNAs.

QRT-PCR

The cDNA samples were prepared by Prime-Script RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions and qRT-PCR was performed with SYBR Premix Ex Taq (TaKaRa) on an ABI system (Applied Biosystems, Foster City, CA, USA) according to standard protocols. The primers in Table 1 and Table S2 was used to validated candidate lncRNAs and genes, and the primers for target gene detection were presented in Table 2 for Arrl1 distribution analysis. The relative expression of miR-185-5p, miR-761, miR-25-3p and miR-1956-5p was quantified by a commercial qRT-PCR Primer Set designed by RiboBio (Guangzhou, China) and normalized with the U6 expression level.

Adult rat DRG neurons culture

DRG neurons were separated and maintained in vitro as previously reported (43). In detail, DRGs were dissociated steriley, and incubated with 0.25% trypsin (Invitrogen, Carlsbad, CA, USA) for 10 min with intervals trituration followed by 0.3% collagenase type I (Sigma, St Louis, MO, USA) for 90 min at 37 °C. The supernatant was purified through 15% BSA, and planted on the cell culture plate coated with poly-D-lysine and laminin (Sigma) in Neurobasal medium (Invitrogen) with B27 supplement.

AAV infection, siRNA and miRNA mimics or inhibitor transfection

DRG neurons were replanted on the
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poly-D-lysine- and laminin- pretreated coverslips and incubated for 18h after infection with AAV containing control or Arrl1-specific shRNAs for 5 days or transfection with the Cdkn2b siRNA and miR-761 mimic or inhibitor for 48 hours. The targeting site of Arrl1-specific shRNAs and Cdkn2b siRNAs was present in Table 3.

Neurite outgrowth assay

Cells were fixed and immune-stained with anti-beta-tubulin III antibody (R&D: AB_2313773) (R&D, Minneapolis, MN, USA), TuJ1 also define as a class III beta-tubulin. Neurite length was measured and quantified by Image J.

Sciatic nerve regeneration assay

Sciatic nerves were crushed as mentioned above following intrathecal injection with indicated AAV for 14 days. The sciatic nerves and L4-L5 DRGs were collected at 3 day post-SNI. The sciatic nerves were fixed and immune-stained with anti-SCG10 antibody (1:100; Abcam, Cambridge, UK, cat. number ab66155). Regenerated axon was measured and quantified by Image J.

Fluorescent in Situ Hybridization (FISH)

DRG sections or DRG neurons were fixed by 4% paraformaldehyde (PFA) (Sigma) and hybridized with 1 μg/mL DIG-labelled anti-Arr1 probe (RiboBio) at 42˚C overnight, and then incubated with anti-DIG antibody (RiboBio) and secondary antibody (RiboBio). The slides were observed and photographed using fluorescent microscopy.

LncRNA distribution assay

Cytoplasmic and nuclear RNAs from DRG neurons were isolated by the PARIS™ Kit (Life technologies, Carlsbad, USA) following the manufacturer’s protocols. The expression levels of Arrl1 in cytoplasm and nucleus were measured by qRT-PCR. To perform quantitative PCR, SYBR Green Mix was used (Takara) with validated primers which were listed in Table 2.

Luciferase reporter assay

The full-length sequence of Arrl1, the 3’ UTR sequence of Cdkn2b and the mutations of Arrl1 and Cdkn2b were constructed into the pmiR Glo vector. The indicated mutations were generated by direct DNA synthesis (GenScript, Nanjing, China). The luciferase reporter assay was performed at 48h after the reporter vectors co-transfected with indicated miRNAs into HEK-293T cells. Renilla luciferase reporter was used as an internal control, and the relative luciferase activity was normalized to Renilla luciferase activity measured by a dual luciferase reporter assay system (Promega, Madison, WI, USA). To predict the binding sites for rat miRNAs, miRanda software (http://www.microrna.org/) was used.

DRG injection

The Lumbar (L) 4/L5 intra-DRG injection was performed in ten male Sprague-Dawley (SD) rats 180-220g) at 12 day after infection with Arrl1 knockdown AAV as previous reported. In brief, the left L4 and L5 DRGs were exposed by removing the lamina of vertebra and opening the epineurium lying covered on the DRG. In the process of DRG injection, the glass needle was inserted into the ganglion, the miR-761 antagomir (2 nmol in 4 μl) was injected over a period of 5 min into the L4/L5 DRGs through the indwelling catheter attached to a 10-μl Hamilton syringe. After a delay of 2 min, the needle was removed. After 2 days, the left sciatic nerve was crushed, and obtained for analysis at 3 days later.

Western blot

Protein extracts were prepared from primary cultured DRG neurons. Equal amounts of protein were electrophoresed on 12% SDS-PAGE and then transferred onto a nitrocellulose membrane (Bio-Rad). Blots were probed with antibody against Cdkn2b (Origene, cat. number TA312926; 1:1000). Image J was used to quantify the results of Western blot.
Behavioral assessment

The behavioral assessment was performed as reported previously (44). For hotplate test, rats were placed on the glass surface for an acclimation period in 5 min. A focused heat light source linked to a timer (model 33 Analgesia Meter; IITC/Life Science Instruments, Woodland Hills, CA, USA) was used to radiate the plantar surface of hind paw, and quick hind paw raising action was considered to be a positive response. Each paw was tested for five individual times, and the mean value was calculated.

For the von Frey test, all experimental animals were adapted to environment for 30 min before the test, the plantar surface of left hind paw were vertically stimulated by a series of von Frey filaments (Stoelting, Wood Dale, IL). The filament was bent for 5s with a sufficient force on the central of plantar surface, and paw flinching or brisk withdrawal was considered as a positive response. Every test was repeated two times in each rat, and the mean value was calculated.

Statistics analysis

The experiment data statistics were analyzed by unpaired, 2-tailed Student’s t test or ANOVA with Tukey post hoc test, $p < 0.05$ was considered statistically significant. All quantitative data are expressed as mean ± standard errors (SD).

Data availability

All the data are contained within the article.
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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.
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FOOTNOTES
The abbreviations used are: TFs, transcriptional factors; RAGs, regeneration associated genes; DRG, dorsal root ganglion; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; Cdkn2b, cyclin dependent kinase inhibitor 2B; Arrl1, axon regeneration related lncRNA 1; ceRNA, competing endogenous RNA; PNI, Peripheral nerve injury; CNS, central nervous system; PNS, peripheral nervous system; lncRNA, long non-coding RNA; KD, knock down; RGC; retinal ganglion cell.
Table 1. Primers for qRT-PCR validation of candidate lncRNAs

| NO. | LncRNA               | Forward (5’-3’)          | Reverse (5’-3’)         | Size |
|-----|----------------------|--------------------------|-------------------------|------|
| 1   | NONRATT032155.1      | CTGCCAAGGTCAGAAGAGGG     | TCTGTAGCCAGTTTAATTCCTTG | 114  |
| 2   | NONRATT032941.1      | AAACAACGAGCATCGAAGAGTTG | ATTTCCTCCGTGAGCTGCTCT  | 116  |
| 3   | NONRATT033029.1      | CCACTCCGAACCTCTCGTG     | CAGCTCGAGTGACTGACAGG    | 185  |
| 4   | NONRATT031542.1      | CATAAGCACCACAGCTCGAT    | AGTCCCTTGAGCCTTCTCCA    | 95   |
| 5   | NONRATT031464.1      | GAGCGGCTGACCGAAGGTAAG   | GAGCTGAGGCCAGAAACC      | 123  |
| 6   | NONRATT032795.1      | CCCGTGGGATGGAGCTAAAG    | CTGTTTGCTGCTCCACTCT     | 147  |
| 7   | NONRATT031482.1      | GTCGACTTCCTCCTCCTGAA    | GGCATGTCAGCAGTGCTAGT    | 91   |
| 8   | NONRATT033484.1      | TAGAAACGGAGGAGTGACGGG   | CTTCCCATTGAGGCCCTTTG    | 187  |
| 9   | NONRATT032644.1      | AAAGAGCACTGGGAGGGGATTT  | AGTGTCCCGAAGAAGAGCTT    | 85   |
| 10  | NONRATT031980.1      | GTGCTTTCTTTGCCTTGTTG    | AAACTTTGCCAGCATCCCG     | 209  |
| 11  | NONRATT032735.1      | ATGGCCCAAAAAAGCCAAGT    | GGCCGATGTTAGACACTTTT    | 163  |
| 12  | NONRATT032301.1      | CGCCTTGGCAATAGCCTTACC   | ACCACGTGTCAGCAACAGT     | 97   |
| 13  | ENSRNOT00000087471   | AGGAAGGGCCATGCTCGAAG    | GGATAGCTGCAACAAGGCA     | 223  |
| 14  | ENSRNOT0000076322    | TCTCCCTGGGAAGCTTGCTTG   | GTCAGAGTGGTGGCTTCTA     | 208  |
| 15  | ENSRNOT0000077098    | CTCCAGGCGCAGTGCTGAAGA   | CTCCACCTTGATGCTGGCTCT   | 86   |
| 16  | ENSRNOT0000080401    | TGGAGCCTCCTGGTTCCTCA    | GGCAGAAAAATAACCCACCTCT  | 137  |
| 17  | ENSRNOT0000076902    | AGGCCATTCAGATCCGCTCA    | CCCACGGTGTGGTCTTTATG    | 249  |
### Table 2. Primers for qRT-PCR validation of Arrl1 distribution and target genes

| Gene   | Forward (5’-3’)                      | Reverse (5’-3’)                      | Size |
|--------|-------------------------------------|-------------------------------------|------|
| GAPDH  | CCTCATTGACCTCAACTACATG              | CTTCCTCATGGTGGTGGAAGAC              | 215  |
| U6     | CTCGCTTCGGCAGCACA                   |AACGCTTACGAATTGCGT                  | 94   |
| β-actin| GTCACCAACTGGGACGAT                  |GAGGCATACAGGGACAACA                 | 209  |
| Arrl1  | CGCGTGCAAATAAGCTTACC                | ACCAGCCTTGAAGCAAAACGT              | 97   |
| Cda    | TGCTCTCTGTGAAGCCAAGCAG              | TCTACGTGCACCCAGAGAAG               | 109  |
| Kcnh4  | TGCAGGCTGAACCAAGAGATT               | CAAGTGGAATCAGGGTTG                 | 128  |
| Cdkn2b | GACAGGTGGAGACGGGTGC                 | GCCCATCATCATGACCTGGA               | 98   |

### Table 3. Interference sequence (IS) designed for target gene

| Target Gene | IS NO. | Target Sequence (5’-3’)                     |
|-------------|--------|---------------------------------------------|
| Arrl1       | shRNA-1| CCGTGTGCACGTAAGTTCTCTCT                    |
| Arrl1       | shRNA-2| CGTGTGCACGTAAGTTCTCTC                      |
| NC          | shRNA  | GCCTAAGGTAAAGTCGCCCTCG                     |
| Cdkn2b      | siRNA-1| CGGTAGACTTAGCTGAAGA                       |
| Cdkn2b      | siRNA-2| CGATCCAGGTCTAGATGAT                       |
| NC          | siRNA  | GGCTCTAGAAAAAGCCTATGC                     |
Figure 1. The transcriptome sequence and bioinformatic analysis of the rat DRG post sciatic nerve injury. (A and B) Identification of the significantly differentially expressed lncRNAs and mRNAs at 0h, 3h, 9h, 12h, 1d, 4d and 7d following sciatic nerve injury. The red rectangular frame in Figure 1A indicated NONRATT032301.1. (C) The co-expression network of the differentially expressed lncRNAs-mRNAs in Figure 1A and 1B. (D) Gene Ontology and KEGG pathway analysis for differential co-expression genes from Figure 1C.
Figure 2. The expression and distribution of Arrl1 in DRG neuron following sciatic nerve injury. (A) The mRNA level of Arrl1 expression in DRGs at indicated time point after SNI. Values represent means ± SD (n = 3). "***" for $p < 0.01$, "****" for $p < 0.001$. (B) The fluorescence intensity of Arrl1 in DRGs at 0d (D 0), 1d (D 1) and 4d (D 4) post-SNI. The DRG was fixed and subjected to FISH assay with indicated probe. Red for Arrl1, and blue for nucleus, bar=100 μm. (C) The distribute proportion of Arrl1 in DRG neuron nucleus and cytoplasm in the absence of sciatic nerve injury. Cytoplasmic and nuclear RNAs from DRG neurons were isolated and then analyzed the Arrl1 expression by qRT-PCR. β-actin was used as a positive control for cytoplasm-distributed gene and U6 was used as a positive control for nuclear-distributed gene. Values represent means ± SD (n = 3). (D) Arrl1 colocalizes with Tuj1 in cytoplasm of DRG neuron in the absence of sciatic nerve injury. The DRG neurons were fixed and subjected to FISH assay with indicated antibody. Red for Arrl1, green for Tuj1 (a marker for neuron), and blue for nucleus, bar=50 μm.
Figure 3. Knockdown of Arrl1 increased the neurite outgrowth *in vitro*. (A) The expression of Arrl1 in DRG neurons infected with AAV containing negative control shRNA (NC), Arrl1-specific shRNA-1 AAV (KD1) or Arrl1-specific shRNA-2 AAV (KD2). The level of Arrl1 expression was detected by qRT-PCR using RNA isolated from the DRG neurons. (B) Immunostaining for Tuj1 in DRG neurons infected with indicated AAV. Red for Tuj1 (a marker for neuron), bar=100μm. (C and D) Quantify the total and maximum neurite length by normalized with NC group in Figure 3B. All the data are shown as mean ± SD (unpaired, 2-tailed Student’s t test; n = 3). *** for p < 0.001, **** for p < 0.001.
Figure 4. Arrl1 knockdown promotes sciatic nerve regeneration and sensory recovery following PNI. (A) Immunostaining for SCG10 in sciatic nerves of rats infected with AAV containing related shRNA at 3 days post-SNI. NC (negative control shRNA), KD1 (Arrl1-specific shRNA-1), KD2 (Arrl1-specific shRNA-2). Red for SCG10 (a marker for regenerated sensory axon). The white dotted line in the left of the image represents crush site, and the white triangle represents the leading edge of regenerated axon. bar=1000μm. (B) Quantitation of the normalized SCG10 fluorescence intensity from the crush site toward the distal end. Values represent means ± SD (unpaired, 2-tailed Student’s t test; n = 3). * for p < 0.05, ** for p < 0.01. (C) Quantify the maximum regenerated axon length from the injury site by normalized with NC group in Figure 4A. Values represent means ± SD (unpaired, 2-tailed Student’s t test; n = 3 for each group). * for p < 0.05. (D and E) Assessment of mechanical and thermal sensory recovery of indicated rats at different time point after SNL. Values represent mean ± SD (n=5; *** for p < 0.05; two-way ANOVA).
Figure 5. MiR-761 is the target molecular of Arrl1. (A) Construction of the potential Arrl1-regulating ceRNA network. (B) The align score between target genes and related miRNAs in Arrl1-regulating ceRNA network. (C and D) The level of three target genes and four miRNAs expression in DRG neurons infected with AAV containing negative control shRNA (NC), Arrl1-specific shRNA-1 AAV (KD1) or Arrl1-specific shRNA-2 AAV (KD2). (E) The interaction analysis of the Arrl1 and three candidate miRNAs. The HEK293T cells were transfected with indicated Arrl1 and miRNA. Then the transfected cells were subjected to luciferase analysis. pmirGLO-Arrl1 (wild type Arrl1), MUT1 (miR-761 binding sites-mutated Arrl1), MUT2 (miR-25-3p binding sites-mutated Arrl1), NC (control miRNA mimic). (F) The interaction analysis of the Cdkn2b and miR-25-3p or miR-761. The HEK293T cells were transfected with indicated Cdkn2b and miRNA. Then the transfected cells were subjected to luciferase analysis. MUT (3’UTR-mutated Cdkn2b), NC (control miRNA mimic). (G) The mRNA levels of Cdkn2b expression...
Arrl1 regulates neurite outgrowth in DRG neurons transfected with control miRNA mimic (NC) or miR-761 mimic (miR-761). All the data are shown as mean ± SD (unpaired, 2-tailed Student’s t test; n = 3). “*” for *p* < 0.05, “**” for *p* < 0.01, N.S. for non-significant.

Figure 6. MiR-761 and Cdkn2b regulates axon regeneration in vitro. (A) Immunostaining for Tuj1 in DRG neurons transfected with control miRNA mimic (NC) or miR-761 mimic (miR-761). Red for Tuj1, bar=100μm.

(B and C) Quantification of neurite length by normalized with NC group in Figure 6A. (D) The protein level of Cdkn2b by western blot.
Cdkn2b in DRG neurons transfected with negative control siRNA (NC), Cdkn2b-specific siRNA-1 (Si-1) or Cdkn2b-specific siRNA-2 (Si-2). Upper panel: Quantitative analysis of results of Western blot. Lower panel: Results of Western blot. (E) Immunostaining of Tuj1 in DRG neurons transfected with negative control siRNA (NC), Cdkn2b-specific siRNA-1(Si-1) or Cdkn2b-specific siRNA-2(Si-2), bar=100μm. (F and G) Quantification of neurite length by normalized with NC group in Figure 6E. All the data are shown as mean ± SD (unpaired, 2-tailed Student’s t test; n = 3). “*” for p < 0.05 and “**” for p < 0.01. 
**Figure 7.** Arrl1 regulates axon regeneration through Arrl1/miR-761/Cdkn2b axis in ceRNA mode. (A to C) Effect of inhibiting miR-761 on neurite outgrowth in Arrl1-knockdown DRG neurons in vitro. The DRG neurons were infected with AAV containing negative control shRNA (NC), Arrl1-specific shRNA-2 (KD2). Then the KD2-infected DRG neurons were further transfected with miR-761 inhibitor (miR-I). The neurite outgrowth of the DRG neurons was visualized by immunostaining of Tuj1 (A), and quantify the total and maximum neurite length by normalized with NC group in Figure 7A (B and C). Red for Tuj1, bar=100μm. (D to F) Effect of inhibiting miR-761 on sciatic nerve regeneration in Arrl1-knockdown DRGs in vivo. The miR-761 inhibitor for in vivo application, miR-761 antagomir (miR-A), was transfected into DRGs by DRG injection following Arrl1 knockdown in vivo. Immunostaining of SCG10 to label the regenerative sciatic nerve. Red for SCG10. The white dotted line in the left of the image represents crush site, and the white triangle represents the leading edge of regenerated axon, bar=1000μm (D). Image J was applied to quantify the normalized fluorescence intensity of SCG10 from the crush site toward the distal end (E), and quantify the maximum regenerated axon length from the injury site by normalized with NC group in 7D (F). All the data are shown as mean ± SD (unpaired, 2-tailed Student’s t test; n = 3). “*” for p < 0.05 and “**” for p < 0.01 between the NC and KD2 groups, “#” for p < 0.05 and “##” for p < 0.01 between the KD2 and KD2+miR-A groups or KD2+miR-I groups.

**Figure 8.** Graphical demonstration for the mechanism underlying Arrl1-regulated axon regeneration post-SNI. The expression of Arrl1 is decreased following sciatic nerve injury, attenuating the sponge effect on miR-761 and leading to increased miR-761. Increased miR-761 negatively regulates downstream target gene, Cdkn2b, which is an inhibitor in neurite outgrowth, thus promoting the initiation of axon regeneration.
The long noncoding RNA Arrl1 inhibits neurite outgrowth by functioning as a competing endogenous RNA during neuronal regeneration in rats
Dong Wang, Yanping Chen, Mingwen Liu, Qianqian Cao, Qihui Wang, Shuoshuo Zhou, Yaxian Wang, Susu Mao, Xiaosong Gu, Zhenge Luo and Bin Yu

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