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ORIGINAL PAPER

ZEB1 Induces *Ddr1* Promoter Hypermethylation and Contributes to the Chronic Pain in Spinal Cord in Rats Following Oxaliplatin Treatment

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Abstract

Application of chemotherapeutic oxaliplatin represses gene transcription through induction of DNA methylation, which may contribute to oxaliplatin-induced chronic pain. Here, *Ddr1*, which showed an increased methylation in the promoter, was screened from the SRA methylation database (PRJNA587622) after oxaliplatin treatment. qPCR and MeDIP assays verified that oxaliplatin treatment increased the methylation in *Ddr1* promoter region and decreased the expression of DDR1 in the neurons of spinal dorsal horn. In addition, overexpression of DDR1 by intraspinal injection of AAV-hSyn-Ddr1 significantly alleviated the mechanical allodynia induced by oxaliplatin. Furthermore, we found that oxaliplatin treatment increased the expression of DNMT3b and ZEB1 in dorsal horn neurons, and promoted the interaction between DNMT3b and ZEB1. Intrathecal injection of ZEB1 siRNA inhibited the enhanced recruitment of DNMT3b and the hypermethylation in *Ddr1* promoter induced by oxaliplatin. Finally, ZEB1 siRNA rescued the DDR1 downregulation and mechanical allodynia induced by oxaliplatin. In conclusion, these results suggested that the ZEB1 recruited DNMT3b to the *Ddr1* promoter, which induced the DDR1 downregulation and contributed to the oxaliplatin-induced chronic pain.

Keywords ZEB1 · DDR1 · Hypermethylation · Oxaliplatin · Chronic pain

Introduction

The use of chemotherapeutic drug oxaliplatin often leads to chronic painful neuropathy [1, 2], which is the major reason for dose reduction or discontinuation of treatment, and adversely impacts the anti-neoplastic outcomes and the quality of life in cancer patients [3]. Hence, understanding the underlying mechanism for oxaliplatin-induced chronic painful neuropathy is of substantial importance for the chemotherapy in the patients with cancer.

Discoidin domain receptors (DDRs) are members of a unique subfamily of tyrosine kinases receptor, including DDR1 and DDR2 [4]. DDR1 was initially isolated as a novel member of the receptor tyrosine kinase (RTK) family [5], and was ever identified as a gene biomarker in breast cancer cells. Further studies showed that DDR1 was widely expressed in a variety of tissues, with high levels in brain, lung and kidney, et al. [6], and participated in many pivotal cellular processes including the migration, proliferation, differentiation and survival of cell. However, whether DDR1 is involved in the chronic pain remains unclear. Although

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study showed that chemotherapeutic mitomycin C increased DDR1 through NF-κB pathway in breast cancer cell [7], the adaption of DDR1 in nociceptive pathway, as well as its potential involvement in the development of neuropathic pain, has not been reported in the setting of chemotherapeutic oxaliplatin-induced neuropathy. Studies have shown that epigenetic mechanisms such as DNA methylation play an important role in the development of chronic pain through regulating the expression of target proteins [8, 9]. Exploration of the regulation of DDR1 gene transcription in the spinal cord following oxaliplatin application may provide a new potential avenue in chronic pain management.

Zinc-finger E-box-binding homebox 1 (ZEB1) has been identified as a kind of transcription repressor/activator, which can regulate tumor invasion and metastasis [10]. Moreover, a growing body of evidence has implied a potential role of ZEB1 in epigenetic regulation during tumorigenesis [11]. For example, ZEB1 interacts with histone deacetylase 1 (HDAC1) and histone deacetylase 2 (HDAC2) to induce the transcriptional silencing of E-cadherin [12]. Furthermore, some studies have reported the correlation between ZEB1 and inflammation. For example, IL-1β is reported to promote stemness and invasion of colon cancer via activating ZEB1 [13]. In addition, it is implicated that ZEB1 may serve as a vital factor to modulate neuroinflammation [14], which is closely involved in the chronic pain [15]. Importantly, some studies showed that ZEB1 also participated in the development of neuropathic pain induced by nerve injury. For instance, various miRNAs may regulate the expression of ZEB1, which contributed to the chronic constriction injury (CCI)-induced neuropathic pain [16–18]. However, whether ZEB1 is involved in the chronic pain induced by chemotherapeutic oxaliplatin remains unclear. In addition, whether and how the ZEB1 serves as a transcriptional repressor to modulate the expression of DDR1 remains unexplored.

Methods

Animals and Behavioral Test

Male Sprague Dawley rats weighting 200–220 g were obtained from the Institute of Experimental Animals of Sun Yat-Sen University. Four rats were fed in a cage, and all animals were housed at 24 ± 1 °C and 50–60% humidity on a 12 h/12 h light–dark cycle. All experimental protocols were approved by the Local Animal Care Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animals were randomly assigned to different experimental or control groups in this study. All efforts were made to minimize the suffering and the number of rats used.

Animals were placed in a plastic box for three consecutive days (15 min per day) to allow them to adapt to the environment before testing. Von-Frey filaments that produce different forces were applied alternately to the plantar surface of hind paw. In the absence of a paw withdrawal response, a stronger stimulus was presented, while the next weaker stimulus was chosen when paw withdrawal occurred. Optimal threshold calculation by this method required five responses in the immediate vicinity of the 50% threshold.

The rats were randomly assigned to each group. The experimenter who conducted the behavioral tests was blinded to all treatments. Around 10% of rats that did not show mechanical allodynia after oxaliplatin injection were excluded from further analysis.

Drug Administration

Oxaliplatin (Sigma) was dissolved in 5% glucose to a concentration of 1 mg/ml and intraperitoneally (i.p.) injected at 4 mg/kg once per day for five consecutive days. Control animals were intraperitoneally injected with an equivalent volume of 5% glucose.

A polyethylene-10 catheter was implanted into the L5/L6 intervertebral subarachnoid space after the injection of sodium pentobarbital (50 mg/kg, i.p.), and the tip of catheter was located between the levels of the L4–L6 spinal segments. The rats were allowed to recover for 5 days. Animals that exhibited hind limb paresis or paralysis were excluded from the study. The siRNA targeting DDR1 (RiboBio, China), ZEB1 (RiboBio, China), DNMT3a (RiboBio, China) or DNMT3b (RiboBio, China) were delivered intrathecally into the spinal cord of rats *via* an indwelling cannula attached to an osmotic minipump once a day for 10 consecutive days. The siRNA sequences were shown in Table 1.

For intraspinal injection of the recombinant AAV, the L4–L6 vertebrae were exposed, and the vertebral column was mounted in a stereotaxic frame. A slight laminotomy was performed, and the dura was incised to expose the spinal cord. AAV was injected into both sides of the spinal dorsal horn. The micropipette was withdrawn 10 min after viral injection, and the incision was closed with stitches.

Table 1 The target nucleotide sequences of siRNA

Gene	Sequence
Ddr1	AATTCTCCTTGAAACGGAGCC
Zeb1	GATGACGAATGCGACTCAGAT
Dnmt3a	CATCCACTGTGAATGATAA
Dnmt3b	GATCAAGCTCACGGCTGTCTA

RNA Extraction and Real-Time Quantitative PCR (qPCR)

Total RNA was extracted from the L4–L6 spinal dorsal horn tissues with TRIzol reagent (Invitrogen, USA). The reverse transcription was performed using Evo M-MLV reverse transcriptase Premix (AG, China) according to the manufacturer's protocol. The cDNA was amplified using specific primers. Real-time quantitative PCR was performed using SYBR Green Premix qPCR Kit (AG, China). The reactions were setup based on the manufacture's protocol. The relative expression ratio of mRNA was quantified by the $2^{-\Delta\Delta CT}$. The primers are listed in Table 2.

Western Blotting

Western blotting was performed as described previously. Animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) at different time points. L4–L6 spinal dorsal horn tissues were immediately removed and homogenized on ice in 15 mmol/l Tris containing a cocktail of proteinase inhibitors and phosphatase inhibitors (Beyotime, China). Protein samples were separated by gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. The blots were placed in the blocking buffer for 1 h at room temperature and incubated with primary antibody against DDR1 (1:100, sc-390,268, Santa Cruz), ZEB1 (1:1000, 21544-1-AP, Proteintech), or β-actin (1:1000, 46,967, CST) overnight at 4 °C. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody. Chemiluminescent HRP substrate (Millipore) was used to detect the immune complex. The band was quantified with computer-assisted imaging analysis system (NIH ImageJ).

Table 2 The specific primer sequences

Gene		Primers
Ddr1	Forward	GGACACCATCCTCATCAACAACCG
	Reverse	AGCAGCAACGCAGAGCCATTG
Ush1g	Forward	ACTGCCTGTCCTTCCTCGTGTC
	Reverse	GCTCTGCTTGGCTGCGATGG
Dll3	Forward	TTGTGTGTTGGCGGTGAAGATCC
	Reverse	ATGTCCCAGGTCAAGGCAGAGG
Rgs4	Forward	ACACAGAGGCAGAGAACCGAAATG
	Reverse	CACGGCAGGCAGCATGGATAC
Dmpk	Forward	AGACACCCTTCTACGCCGACTC
	Reverse	GGACACAGCAGCCCACGAATG
Mab21l2	Forward	CAAGTGCCTCTCCGTGCTGAAG
	Reverse	AGCCTCGTCCCAGTCCGTTTC
MeDIP-Ddr1	Forward	TGACTGGGATTTCCCTCTCTGGC
	Reverse	GTAACCTCACCTGCGCTGGTGAC

Immunofluorescence

Immunofluorescence was performed as previously described. Briefly, animals were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg) and immediately perfused through the ascending aorta with 4% paraformaldehyde. The L4–L6 spinal cord were removed and post fixed in the same fixative overnight. Cryostat sections (25 μm) were cut and processed for immunofluorescence with primary antibody against DDR1 (1:50, sc-390,268, Santa Cruz), ZEB1 (1:200, NBP1-05987, NOVUS), Dnmt3b (1:50, sc-376,043, Santa Cruz), NeuN (1:400, ABN78, Millipore; 1:400, MAB377, Millipore), GFAP (1:400, 3670, CST; 1:400, ab7260, Abcam), or Iba1 (1:400, ab5076, Abcam; 1:400, ab15690, Abcam). After incubation overnight at 4 °C, the sections were then incubated with cy3-conjugated and Alexa Fluor® 488-conjugated secondary antibodies for 1 h at room temperature. The stained sections were then examined with a Nikon microscope, and images were captured.

Chromatin Immunoprecipitation (ChIP) Assays

ChIP assays were performed using the ChIP Assay Kit (CST). The animals' L4–L6 spinal dorsal horn were removed quickly and placed in 1% formaldehyde for 5 min. The DNA was fragmented by micrococcal nuclease. 10 μg of digested, cross-linked chromatin was used in each immunoprecipitation assay. The chromatin solution was incubated with antibody against Dnmt3b, and the mixed complex was incubated overnight at 4 °C. Next day, the DNA was purified from the complexes after the antibody/DNA complexes were captured, washed, eluted, and reverse cross-linked. The precipitated DNA was resuspended in the nuclease-free water (50 μl), and quantitative real-time PCR was performed on the sample (2 μl) as described above. The primers are listed in Table 2.

Methylated DNA Immunoprecipitation (MeDIP)

MeDIP assays were performed as described [19]. The genomic DNA was extracted with a DNA extraction kit (TIANGEN, DP304), and ultrasound was used to break it into fragments. The 5mC DNA fragments were enriched using MeDIP kit (ActiveMotif, 55,009) following the manufacturer's instructions. After washing, elution and purification, qPCR was performed by using specific primers (Table 2).

Co-immunoprecipitation (Co-IP)

Co-IP was conducted using a Co-Immunoprecipitation Kit (Pierce). Spinal dorsal horn tissues were excised quickly and placed in lysis buffer. A Pierce Spin Column was placed

in a microcentrifuge tube. After addition of Amino Link Plus Coupling Resin and affinity-purified Dnmt3b antibody (10 µg, sc-376,043, Santa Cruz), the complex was incubated on a rotator at room temperature for 90–120 min to ensure antibody immobilization. Tissue lysates were added to the appropriate resin columns and incubated with gentle rocking overnight at 4 °C. The spin columns were then centrifuged and placed in new collection tubes, elution buffer was added, and the flow-through was collected by centrifugation. The immune complexes in the flow-through were analyzed by western blotting using ZEB1 antibody (1:1000, 21544-1-AP, Proteintech). All co-IP steps were performed at 4 °C unless otherwise indicated.

Statistical Analysis

All data were shown as mean ± SEM, and analyzed with SPSS 25.0. The data were analyzed using the two independent samples t test or one-way ANOVA followed by Dunnett's T3 or Tukey's post hoc test. When tests of normality were not satisfied, the permutation test was substituted. The criterion for statistical significance was $P < 0.05$. While no power analysis was performed, the sample size was determined according to our and peers' previous publications in painful behavior and pertinent molecular studies.

Results

Oxaliplatin Treatment Decreased the DDR1 Expression via Enhancing the Gene Methylation

DNA methylation plays an important role to regulate gene expression in the pathophysiological process of numerous diseases [20]. To explore the potential methylation mechanism of oxaliplatin-induced chronic pain, one methylation profile (PRJNA587622) from the SRA database was analyzed to identify the differentially methylated genes (DMGs). A total of 132 DMGs were obtained (Fig. 1a; Table S1). Next, we performed the Gene Ontology (GO) analysis and screened the top 10 terms closely related with the biological processes in the development of nervous system diseases (Fig. 1b). Among all target genes from the top 10 terms, we selected the 6 potential target genes including *Ush1g*, *Ddr1*, *Dll3*, *Rgs4*, *Dmpk* and *Mab2112* with a criterion of methylation difference > 0.1 (Fig. 1c). Next, we examined the expression of the six potential target genes in the dorsal horn at different time points following oxaliplatin treatment. PCR results showed that the level of DDR1 mRNA was significantly decreased on days 4 and 10 (Fig. 1d), and the time course of DDR1 downregulation was consistent with that of mechanical allodynia following oxaliplatin treatment (Fig. 1e). Furthermore, the MeDIP assay confirmed that the methylation level of *Ddr1* (chr20: 3,554,827–3,555,044) was significantly increased on days 4 and 10 after oxaliplatin treatment (Fig. 1f). To verify that the methylation at promoter contributed to the oxaliplatin-induced *Ddr1* downregulation, DNA methyltransferase inhibitor 5-azacytidine (5-AzaC) was intrathecally injected following oxaliplatin treatment. The results showed that treatment with 5-AzaC at dose of 10 µmol/l for consecutive 10 days abolished the oxaliplatin-induced *Ddr1* downregulation at the mRNA and protein level (Fig. 1g, h). These results suggested that the enhanced methylation in *Ddr1* promoter may contribute to the downregulation of *Ddr1* after application of oxaliplatin.

Downregulation of DDR1 in Spinal Dorsal Horn Contributed to Oxaliplatin-Induced Mechanical Allodynia

Next, we detected the expression of DDR1 protein in the spinal dorsal horn, and found that the level of DDR1 protein was significantly decreased on days 4 and 10 following application of oxaliplatin (Fig. 2a). Double immunofluorescence staining results showed that DDR1 was primarily expressed in the NeuN (neuron marker)-positive cells, but not in the Iba-1 (microglia marker)-positive cells or the GFAP (astrocyte marker)-positive cells (Fig. 2b). To further define the role of spinal DDR1 in oxaliplatin-induced chronic pain, we intraspinally injected AAV-hSyn-Ddr1-FLAG to overexpress DDR1. The results that AAV injection rescued the oxaliplatin-induced DDR1 downregulation at the mRNA and protein level on days 21, indicating the high transfection efficiency (Fig. 2c, d). The behavioral test showed that overexpression of DDR1 significantly alleviated the mechanical allodynia induced by oxaliplatin (Fig. 2e). Furthermore, the continuous intrathecal administration of siRNA targeting DDR1 induced the mechanical allodynia in naïve rats (Fig. 2f). Collectively, these results suggested that the downregulation of DDR1 was involved in the development of chronic pain induced by oxaliplatin.

DNMT3b Contributed to *Ddr1* Hypermethylation Following Oxaliplatin Treatment

Mechanistically, regulation of gene transcription by promoter methylation is accompanied by an increase of DNA methyltransferases (DNMT), including DNMT1, DNMT3a and DNMT3b [21]. So, we examined the expression of DNMTs and found that the mRNA levels of DNMT3a and DNMT3b, but not DNMT1, were significantly increased on days 4 and 10 following oxaliplatin treatment (Fig. 3a, c). Furthermore, we designed the DNMT3a siRNA and DNMT3b siRNA to observe the effect of suppression of DNMTs on DDR1 expression following oxaliplatin treatment. Intrathecal injection of DNMT3a siRNA decreased

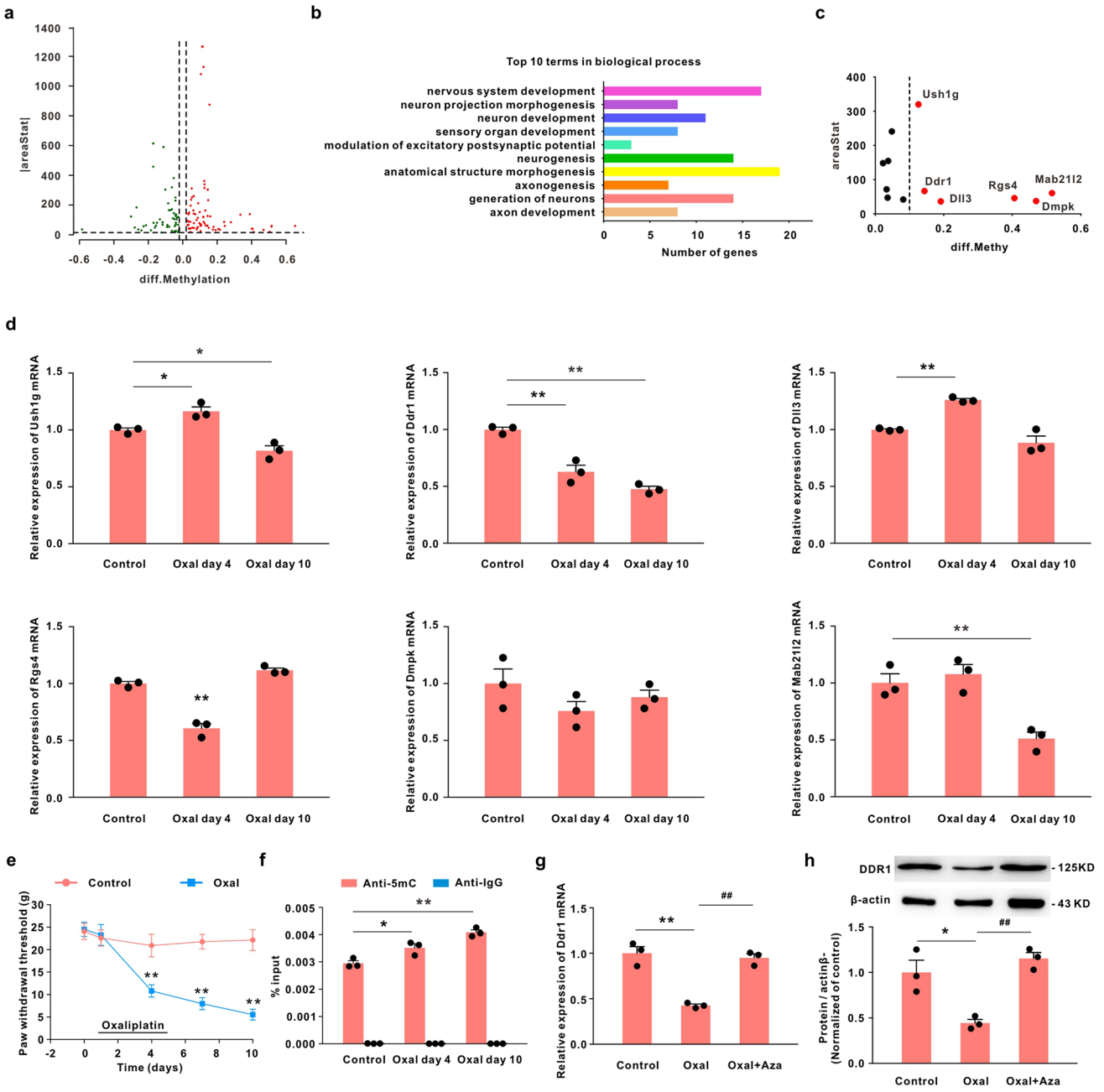


Fig. 1 The enhanced methylation of *Ddr1* gene contributed to the downregulation of the DDR1 expression following oxaliplatin treatment. **a** Volcano plots of the differentially methylated genes for methylation datasets (PRJNA587622). **b** The GO analysis showed the enrichment degree of biological process. The x-axis represents gene counts, and the y-axis represents gene function. **c** Volcano map showed six significant methylated genes. **d** The mRNA levels of *Ush1g*, *Ddr1*, *Dll3*, *Rgs4*, *Dmpk* and *Mab2112* were explored in spinal dorsal horn on days 4 and 10 following oxaliplatin treatment (n = 3 in each group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control group). **e** The hind paw withdrawal threshold of rats was examined on days 0, 1, 4, 7 and 10 following oxaliplatin treatment (n = 8 in each group; $^{**}P < 0.01$ vs. the control group). **f** MeDIP assay was performed to detect the change of DNA methylation level in specific regions of *Ddr1* promoter in spinal dorsal horn on days 4 and 10 after oxaliplatin treatment (n = 3 in each group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control group). **g** Intrathecal injection of 5-AzaC for consecutive 10 days rescued the DDR1 mRNA reduction induced by oxaliplatin (n = 3 in each group; $^{**}P < 0.01$ vs. the control group, $^{\#\#}P < 0.01$ vs. the correspondence oxaliplatin group). **h** The expression of DDR1 protein was assessed by western blot following application of 5-AzaC (n = 3 in each group; $^{*}P < 0.05$ vs. the control group, $^{\#\#}P < 0.01$ vs. the correspondence oxaliplatin group)

the DNMT3a expression (Fig. 3d), but did not affect the level of DDR1 mRNA following oxaliplatin application (Fig. 3e). However, application of DNMT3b siRNA (i.t.), which decreased the DNMT3b upregulation (Fig. 3f),

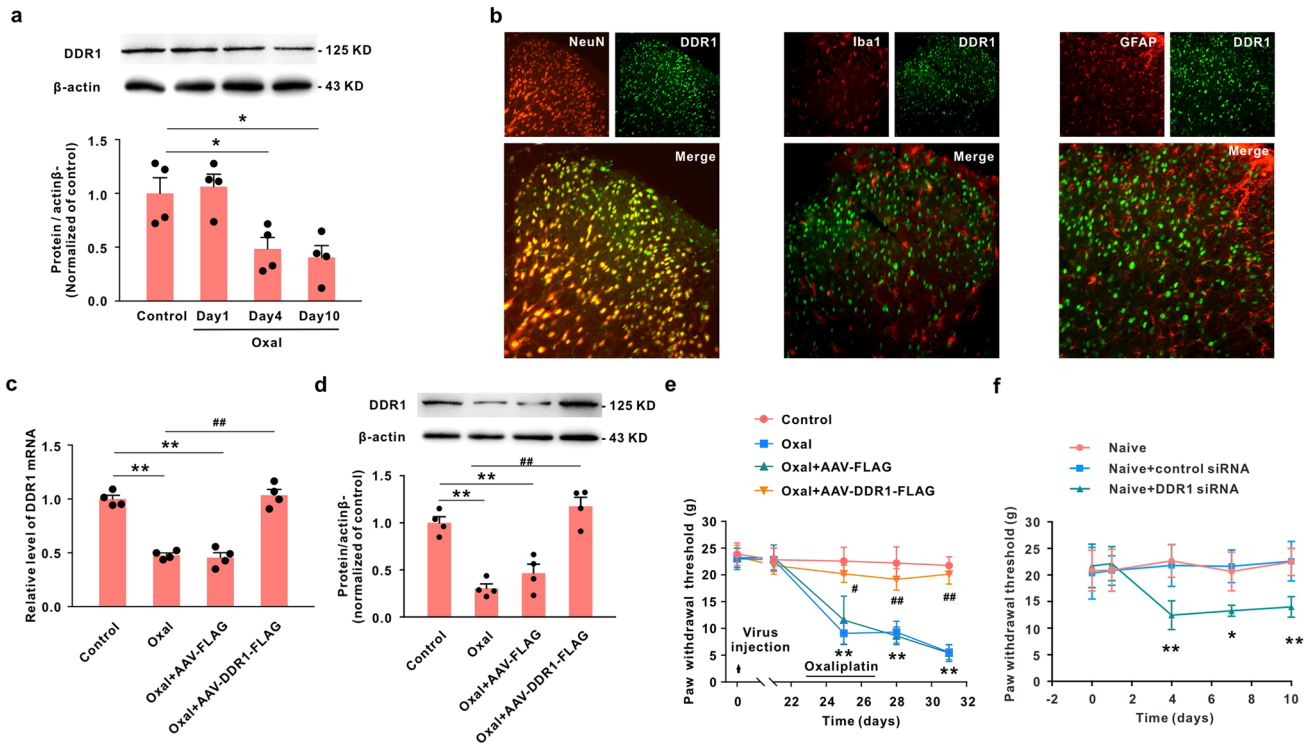


Fig. 2 The DDR1 downregulation participated in the oxaliplatin-induced neuropathic pain. **a** Representative blots and histograms showed the levels of DDR1 protein on different time points following oxaliplatin treatment (n = 4 in each group; $^{*}P < 0.01$ vs. the control group). **b** The immunofluorescence staining showed the colocalization of DDR1 (red) with NeuN (a marker for neurons), but not Iba1 (a marker for microglia) or GFAP (a marker for astrocyte) in spinal dorsal horn (scale bar = 100 μm). **c** and **d** Intraspinal injection of AAV-DDR1-Flag rescued the oxaliplatin-induced *Ddr1* downregulation of DDR1 mRNA (**c**) and protein (**d**). (n = 3 in each group; $^{**}P < 0.01$ vs. the Control group, $^{\#\#}P < 0.01$ vs. the oxal group). **e** The hind paw withdrawal threshold was markedly increased in AAV-DDR1-Flag-injected rats relative to AAV-Flag-injected rats following oxaliplatin treatment (n = 8 in each group; $^{**}P < 0.01$ vs. the control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the corresponding AAV-Flag group). **f** Application of DDR1 siRNA (i.t.) induced mechanical allodynia in naïve rats (n = 6 in each group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control siRNA group)

significantly prevented the downregulation of DDR1 mRNA and protein following oxaliplatin treatment (Fig. 3g, h). Importantly, ChIP-PCR assay showed that oxaliplatin treatment significantly increased the recruitment of DNMT3b in *Ddr1* promoter (Fig. 3i). In addition, double immunostaining studies showed that the DNMT3b was expressed in DDR1-positive cells in spinal dorsal horn (Fig. 3j). These results suggested that the enhanced recruitment of DNMT3b in the *Ddr1* promoter contributed to oxaliplatin-induced *Ddr1* hypermethylation in spinal dorsal horn.

ZEB1 Regulated the Recruitment of DNMT3b in the *Ddr1* Promoter and Contributed to the DDR1 Downregulation Following Oxaliplatin Treatment

Evidence shows that transcriptional repressor is involved in the DNMT-mediated DNA methylation [22, 23]. ZEB1, as a transcriptional repressor, is involved in many biological processes, including CNS inflammatory diseases [24]. Here, we found that oxaliplatin treatment significantly enhanced the ZEB1 expression on days 4 and 10 (Fig. 4a). Immunofluorescence staining further confirmed the upregulation of ZEB1 on day 10 following oxaliplatin (Fig. 4b). Double immunofluorescence staining showed that the ZEB1 expression was colocalizated with NeuN-positive cells, but not Iba1-positive cells or GFAP-positive cells (Fig. 4c). Moreover, co-immunoprecipitation assays revealed that oxaliplatin significantly increased the interaction between ZEB1 and DNMT3b in the spinal dorsal horn (Fig. 4d). Continuous intrathecal administration of siRNA targeting ZEB1 (10 μg/10 μl) decreased the ZEB1 upregulation (Fig. 4e) and mitigated the enhanced recruitment of DNMT3b in *Ddr1* promoter induced by oxaliplatin (Fig. 4f). MeDIP study further showed that ZEB1 siRNA application significantly inhibited the increased methylation in *Ddr1* promoter following oxaliplatin treatment (Fig. 4g). Importantly, ZEB1 siRNA significantly alleviated the oxaliplatin-induced DDR1 downregulation (Fig. 4h) and mechanical allodynia (Fig. 4i). Collectively, these results suggested that the enhanced interaction

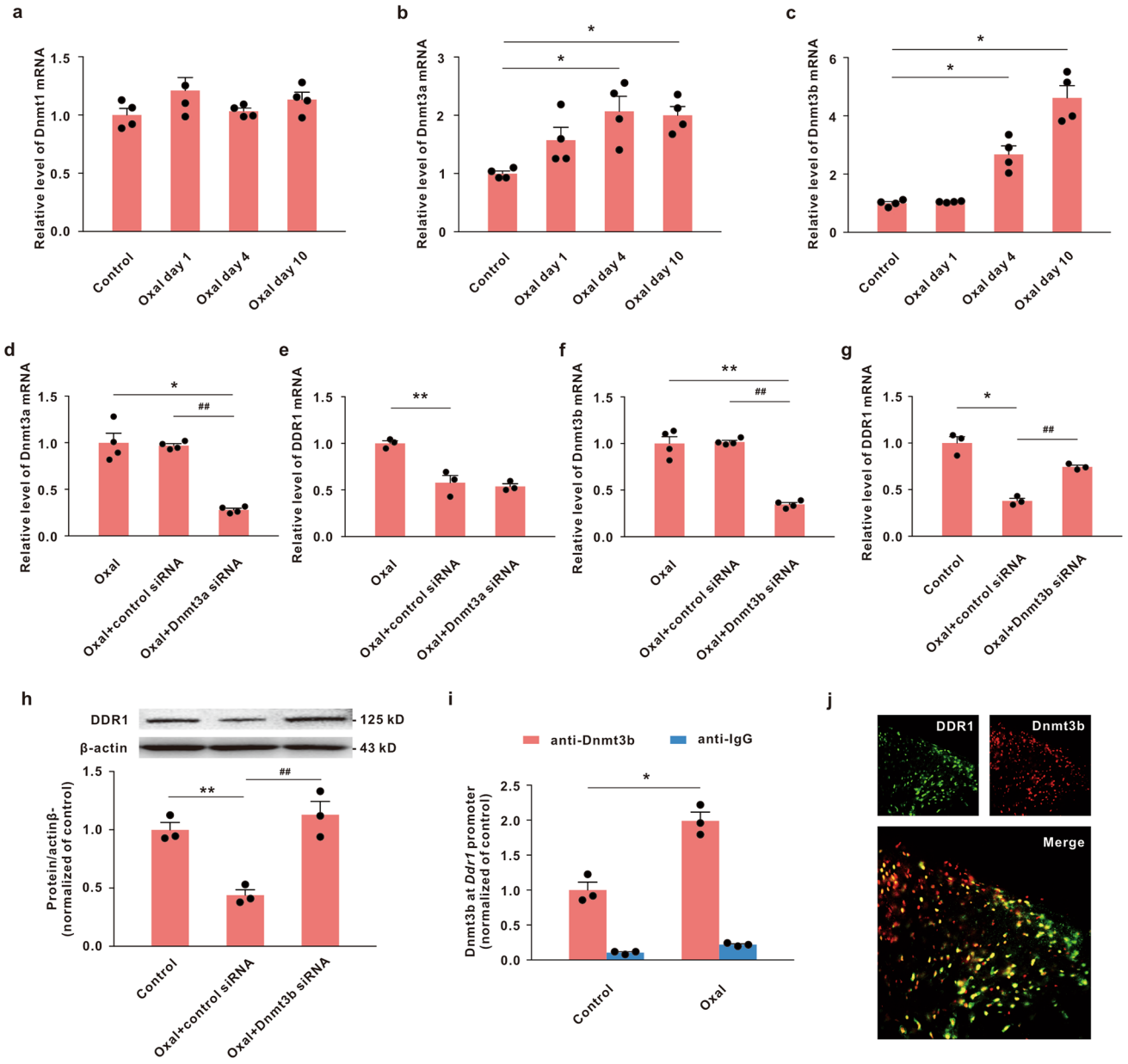
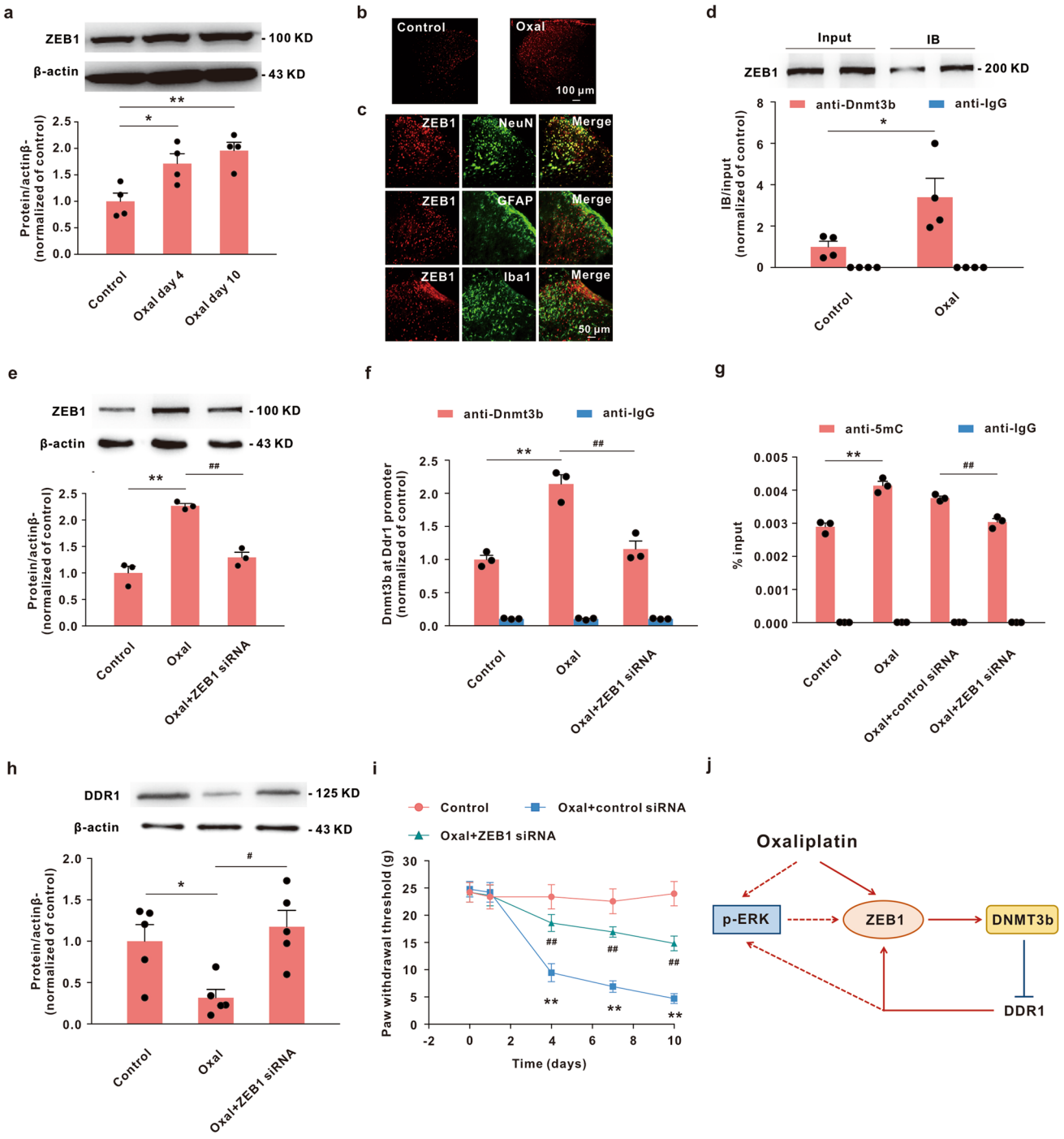


Fig. 3 DNMT3b participated in the DDR1 downregulation following oxaliplatin treatment. **a–c** The mRNA levels of DNMT1 **a**, DNMT3a **b** and DNMT3b **c** were examined in spinal dorsal horn on different time points following oxaliplatin treatment (n = 4 in each group; $^{*}P < 0.05$ vs. the control group). **d** Intrathecal injection of DNMT3a siRNA decreased oxaliplatin-induced DNMT3a upregulation (n = 3 in each group; $^{*}P < 0.05$ vs. the oxaliplatin group, $^{\#\#}P < 0.01$ vs. the control siRNA group). **e** Application of DNMT3a siRNA (i.t.) did not affect the DDR1 expression induced by oxaliplatin (n = 3 in each group). **f** Intrathecal injection of DNMT3b siRNA decreased the upregulation of DNMT3b mRNA and protein induced by oxaliplatin (n = 3 in each group; $^{**}P < 0.01$ vs. the oxal group, $^{\#\#}P < 0.01$ vs. the control siRNA group). **g** and **h** Application of DNMT3b siRNA (i.t.) prevented the mRNA and protein of DDR1 downregulation induced by oxaliplatin (n = 3 in each group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control group, $^{\#\#}P < 0.05$ vs. the correspondence oxaliplatin group). **i** The binding of DNMT3b to the *Ddr1* gene was significantly increased after oxaliplatin treatment (n = 3 in each group; $^{*}P < 0.05$ vs. the control group). **j** Double staining showed the colocalization of DDR1 and DNMT3b in the spinal dorsal horn (scale bar = 50 μm)

between ZEB1 and DNMT3b contributed to hypermethylation of *Ddr1* promoter and DDR1 downregulation induced by oxaliplatin (Fig. 4j).

Discussion

Epigenetic modification such as DNA methylation regulated the expression of pain-related genes and thus contributed to



the development of chronic pain. In the present study, we analyzed a SRA database of methylation profile, and found a remarkably increased methylation level of promoter of *Ddr1* following oxaliplatin treatment. Further MeDIP assay identified the enhancement of the hypermethylation in *Ddr1* promoter in dorsal horn, and the results from PCR and western blotting studies showed that application of DNA methyltransferase inhibitor 5-AzaC reversed the DDR1 downregulation induced by oxaliplatin. Furthermore, recovering DDR1 expression by intraspinal injection of AAV-DDR1-FLAG alleviated the mechanical allodynia induced by oxaliplatin. Importantly, we found that application of oxaliplatin significantly induced the upregulation of DNMT3b and ZEB1, and increased the interaction between DNMT3b and ZEB1 in dorsal horn neurons. Intrathecal injection of ZEB1 siRNA inhibited the increase of DNMT3b recruitment and hypermethylation at *Ddr1* promoter induced by oxaliplatin. Finally, ZEB1 siRNA prevented the DDR1 downregulation

◂**Fig. 4** ZEB1 regulated the recruitment of DNMT3b in the *Ddr1* promoter and contributed to the DDR1 downregulation following oxaliplatin treatment. **a** Representative blots and histograms showed the levels of ZEB1 protein on different time points following oxaliplatin treatment (n = 4 in each group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control group). **b** The immunofluorescence staining showed the significant upregulation of ZEB1 after oxaliplatin treatment (scale bar = 50 μm). **c** The double immunofluorescence staining indicated the colocalization of ZEB1 (red) with NeuN (a marker for neurons, green), but not GFAP (a marker for astrocytes, green) and Iba1 (a marker for microglia, green) in spinal dorsal horn (scale bar = 50 μm). **d** The co-IP showed the increased interaction between ZEB1 and DNMT3b in the dorsal horn after oxaliplatin treatment (n = 4 in each group; $^{*}P < 0.05$ vs. the control group). **e** Application of ZEB1 siRNA (i.t.) prevented the upregulation of ZEB1 protein induced by oxaliplatin (n = 3 in each group; $^{**}P < 0.01$ vs. the control group, $^{\#\#}P < 0.01$ vs. the corresponding oxaliplatin group). **f** Intrathecal injection of ZEB1 siRNA decreased the occupancy of DNMT3b in *Ddr1* promoter (n = 3 in each group; $^{**}P < 0.01$ vs. the control group, $^{\#\#}P < 0.01$ vs. the corresponding oxaliplatin group). **g** Application of ZEB1 siRNA decreased the upregulated DNA methylation level in *Ddr1* promoter in spinal dorsal horn after oxaliplatin treatment (n = 3 in each group; $^{**}P < 0.01$ vs. the control group, $^{\#\#}P < 0.01$ vs. the corresponding oxaliplatin group). **h** Application of ZEB1 siRNA significantly reversed the downregulation of DDR1 after oxaliplatin treatment (n = 5 in each group; $^{*}P < 0.05$ vs. the control group, $^{\#}P < 0.05$ vs. the corresponding oxaliplatin group). **i** Application of ZEB1 siRNA (i.t.) attenuated the oxaliplatin-induced mechanical allodynia (n = 8 in each group; $^{**}P < 0.01$ vs. the control group, $^{\#\#}P < 0.01$ vs. the corresponding oxaliplatin group). **j** A simulated diagram showed the current results and the hypothetical role of ERK

and attenuated the mechanical allodynia induced by oxaliplatin. Altogether, these results suggested that the increased recruitment of ZEB1/DNMT3b complex enhanced the methylation level of *Ddr1* promoter which suppressed the DDR1 expression in dorsal horn and mediated oxaliplatin-induced chronic pain.

Studies showed that chemotherapy may induce the change of cytosine methylation in promoter regions of many genes [25, 26]. For example, oxaliplatin treatment changed the DNA methylation profile in ovarian cancer or cervical cancer [27, 28]. In the present study, we found that oxaliplatin treatment significantly enhanced the methylation level in the promoter of *Ddr1* and decreased the DDR1 expression in spinal dorsal horn. Furthermore, inhibition of methylation by using 5-AzaC (i.t.) prevented the downregulation of DDR1 protein induced by oxaliplatin. These results suggested that oxaliplatin treatment increased the methylation of *Ddr1* gene, which leading to DDR1 downregulation in spinal dorsal horn. Although DDR1 is found in high level in brain, their functions have not been established in chronic pain. We further found that DDR1 was expressed in the spinal dorsal horn neurons, and the time course of DDR1 reduction was consistent with that of mechanical allodynia following oxaliplatin treatment. Notably, the overexpression of DDR1 significantly attenuated the oxaliplatin-induced mechanical allodynia. DDR1, as one of tyrosine kinase receptors (RTKs), can inhibit ERK signaling pathways in mesangial cells [29], and accumulating evidence indicates that the activation of ERK signaling pathways plays a critical role in chronic pain [30]. In addition, studies show that various regulatory mechanisms, including receptor/ligand internalization and subsequent degradation or dephosphorylation by phosphatases, exist to negatively mediate the function of RTKs [31]. Hence, it is possible that the downregulation of DDR1 can enhance the activity of various painful signaling pathways such as ERK/MAPK to mediate the chronic pain following oxaliplatin.

DNA methylation is mediated by a family of DNMTs, including DNMT1, DNMT3a and DNMT3b, and these DNMTs are expressed widely in most tissues in tissue- and condition-specific manner [22]. The present study showed that oxaliplatin treatment significantly increased the expression of DNMT3a and DNMT3b, whereas only DNMT3b may participate in oxaliplatin-induced DDR1 downregulation in spinal dorsal horn. The results from double immunofluorescence staining showed that DNMT3b was expressed in DDR1-positive cells, and suppression of DNMT3b upregulation by DNMT3b siRNA restored the expression of DDR1 in dorsal horn in the rats with oxaliplatin treatment. Moreover, ChIP-PCR assay showed an increased DNMT3b occupancy in *Ddr1* promoter in dorsal horn after oxaliplatin treatment. Our findings suggested a pivotal role of DNMT3b-triggered DNA methylation at the *Ddr1* promoter to induce *Ddr1* gene silencing in dorsal horn after oxaliplatin treatment.

ZEB1, as a transcriptional regulator, is involved in many biological processes. In the present study, we found that oxaliplatin treatment increased the ZEB1 expression in the spinal dorsal horn neurons. Various of regulators, including NF-κB or Ras/Erk, mediated the ZEB1 expression in differential settings. For example, the activation of ERK/MAPK pathway enhanced the ZEB1 expression, by which it increased the invasive potential of prostate cancer cells [32]. In addition, the activation of ERK/MAPK pathway played an important role in chronic pain [33], and oxaliplatin treatment activated the NF-κB singling pathway in spinal dorsal horn [34]. Then, we speculated that the ZEB1 upregulation may potentially result from the activation of NF-κB or ERK/MAPK signaling pathway following oxaliplatin. In general, a homeostatic balance between physiological pain and pathological pain was maintained by properly responding to extracellular signaling molecules. This balance can be disrupted in the presence of persistent extracellular stimulation. While previous studies showed that the activation of ERK signaling upregulated the expression of ZEB1 in cancer cells, DDR1 exhibited the potency to inhibit ERK signaling pathway in mesangial cells. Considering our finding that upregulation of ZEB1 mediated the DDR1 reduction in dorsal horn after oxaliplatin treatment, a positive feedback loop involving

ZEB1 upregulation and DDR1 reduction potentially existed to induce ERK activity in dorsal horn neurons in the setting of chemotherapeutic-induced neuropathic pain, while further studies were needed. We further found that inhibition of ZEB1 significantly attenuated the mechanical allodynia in the modeled rats, suggesting the involvement of ZEB1 in oxaliplatin-induced chronic pain. This was consistent with the previous reports that the ZEB1 upregulation contributed to the nerve injury (CCI)-induced neuropathic pain [16–18]. In general, ZEB1 demonstrate the capability to downregulate or upregulate the expression of its target genes by differential epigenetic mechanisms, including DNA methylation, histone modifications, and recruitment of different co-suppressors or co-activators through SID, CID, or CBD [35]. For instance, recruitment of histone deacetylases HDAC1/2 following direct ZEB1 binding onto the *CDH1* gene promoter leads to repression of CDH1 transcription [36]. Here, we found that oxaliplatin increased the interaction between ZEB1 and DNMT3b, and application of ZEB1 siRNA inhibited the DNMT3b occupancy and the hypermethylation in *Ddr1* promoter induced by oxaliplatin. Importantly, ZEB1 siRNA also alleviated the DDR1 downregulation and mechanical allodynia in the rodents treated with oxaliplatin. These results suggested that ZEB1 induced the recruitment of DNMT3b to *Ddr1* promoter, which consequently increased the methylation of *Ddr1*, repressed of DDR1 expression in dorsal horn neurons, and contributed to chronic pain following oxaliplatin treatment.

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Data Availability The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All experimental protocols were approved by the Local Animal Care Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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