

BASIC SCIENCE ARTICLE



DRR1 promotes neuroblastoma cell differentiation by regulating CREB expression

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BACKGROUND: Neuroblastoma is the most common cancer in infants and the most common extracranial solid tumor in childhood. DRR1 was identified to be downregulated in poorly differentiated ganglion cells from neuroblastoma model mice. However, the roles of DRR1 in neuroblastoma remain largely unclear.

METHODS: The neuroblastoma cells were induced to differentiate, and the expression of DRR1 was detected. The expression of the neuroblastoma cell differentiation markers was analyzed in DRR1 shRNA- or DRR1-expressing vector-treated neuroblastoma cells. The downstream genes of DRR1 were screened with ChIP-seq assay. Finally, TNB1 cells were infected with DRR1 shRNA and CREB expressing vector containing lentivirus, and the expression of the cell differentiation markers, cell cycle distribution and tumor growth were analyzed.

RESULTS: The expression of DRR1 was increased in differentiated neuroblastoma cells, and downregulation of DRR1 expression inhibited the differentiation of neuroblastoma cells. Further experiments indicated that CREB is a candidate downstream gene of DRR1, and it mediates neuroblastoma cell differentiation. Moreover, overexpression of CREB rescued the effect of DRR1 shRNA on cell differentiation, cell cycle distribution and tumor growth in neuroblastoma.

CONCLUSIONS: DRR1–CREB axis modulates the differentiation of neuroblastoma cells and is associated with the outcome of neuroblastoma patients.

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IMPACT:

- DRR1 is involved in regulation of the differentiation of neuroblastoma.
- Binding with actin is essential for DRR1 to regulate neuroblastoma cell differentiation.
- CREB is a candidate downstream gene of DRR1 in regulating of the differentiation of neuroblastoma.

INTRODUCTION

Neuroblastoma is an embryonal cancer of the sympathetic nervous system, which arises from neural crest progenitor cells.¹ Gene mutations are rarely found in neuroblastoma, while gene copy number alterations are commonly detected. MYCN amplification was found in nearly 30% of neuroblastoma patients, and it is one of the most accepted mechanisms in neuroblastoma pathogenesis.^{2,3} Targeted expression of MYCN in sympathetic lineage cells caused neuroblastoma in sympathetic ganglions in a TH-MYCN transgenic mouse model.⁴ Using this TH-MYCN transgenic mouse model, we found that downregulated in renal carcinoma1 (DRR1) expression was downregulated in the hyperplastic tumor cells, when compared to that of the normal ganglion cells in the celiac sympathetic ganglions.^{5,6} Clinical data also demonstrated that decreased DRR1 expression is positively associated with high risk in neuroblastoma patients.⁶

DRR1 is a tumor suppressor gene, and its decreased expression has been found in various types of tumors.^{7–11} The roles of DRR1 in tumorigenesis have been studied, whereas the mechanism

underlying its effect is still largely unclear. Recently, DRR1 was confirmed as an actin binding protein.^{6,12–14} In glioma cells, plasma DRR1 binds to actin and promotes glioma cell invasion by activating the AKT pathway.¹³ In neurons, DRR1 binds to actin in synapses and regulates synaptosis and cognitive processes.^{12,14} Our previous study demonstrated that DRR1 binds to actin in nucleus and regulates cell cycle and cell proliferation in neuroblastoma.⁶

Actin is one of the most abundant proteins in eukaryotes. Cytoplasmic actin is important for cell shape maintenance and cell movement, and the multiple roles of nuclear actin have been attracting increasing concern.¹⁵ Increasing evidences indicate that nuclear actin plays important roles in complicated nuclear biological processes, including transcription regulation,^{16,17} chromatin remodeling,^{18,19} and DNA repair.²⁰

In the present study, our data indicated that DRR1 expression is related to the differentiation status of neuroblastoma cells. The role of DRR1 in neuroblastoma cell differentiation is associated to its binding ability with actin. Moreover, cAMP-response element-

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binding protein (CREB) was screened out as a target gene of DRR1 using chromatin immunoprecipitation sequence (ChIP-seq). Further experiments showed that CREB is involved in the regulation of DRR1-related cell differentiation, cell cycle arrest and tumor growth in neuroblastoma.

MATERIAL AND METHODS

Plasmids

Lentivirus-based non-targeting shRNA and specific shRNAs for DRR1 and CREB were obtained from Sigma (St. Louis, Missouri). The information regarding shRNAs is shown in Supplementary Table S1. DRR1-Venus and its mutants were generated as previously reported.⁶ CREB expression vectors was constructed as previously reported.²¹

Cell culture and transfection

The neuroblastoma cell lines, TNB-1, SH-SY5Y, and N2A were obtained from RIKEN Cell Bank (Tsukuba, Japan), and cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 media (Thermo Fisher, Waltham, MA) supplied with 10% heat-inactivated fetal bovine serum (Thermo Fisher). HEK293T cells and HeLa cells were obtained from Beyotime Biotechnology (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher) supplied with 10% heat-inactivated fetal bovine serum (FBS). Transfection experiment with plasmids in neuroblastoma cell lines was performed with lentivirus packaged by HEK293T cells as previous reported.²

Real-time PCR

Total RNA extraction from cultured cells and real-time PCR were carried out as previously described.²² The information of primers is shown in Supplementary Table S2.

Immunofluorescence staining

Immunofluorescence staining was performed as previously described.⁵ Briefly, the cells were washed with phosphate-buffered saline (PBS) and were fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were then incubated with blocking solution (0.5% normal goat serum plus 0.3% Triton X-100 in PBS) at room temperature for 1 h at room temperature. After washing the cells with PBS, the cells were incubated with the primary antibodies (TH: 1:100, sc-25269, Santa Cruz Biotechnology, Santa Cruz, CA; GAP43: 1:100, orb536647, Biorbyt, Cambridge, UK) in blocking solution overnight at 4 °C, followed by incubation with the appropriate fluorochrome-conjugated secondary antibodies (goat anti-mouse IgG Alexa Fluor 488, 1:500, R37120; goat anti-rabbit IgG Alexa Fluor 594, 1:500, R37117; Invitrogen, Carlsbad, CA) for 1 h at room temperature. After washing with PBS, the images were taken.

Immunoprecipitation

Immunoprecipitation was carried out as previously described.²² Briefly, HeLa cells were transfected with DRR1 expressing vectors of different lengths by Lipofectamine 3000 (Invitrogen). The cell lysate was prepared at 72 h after transfection and was incubated with anti-GFP antibody (MBL, Nagoya, Japan) and protein G Sepharose (GE Healthcare, Pittsburgh, PA) at 4 °C for 6 h. The mixture was centrifuged and washed with PBS for 3 times. Then the sample was mixed with sample buffer, and was subjected to Western blotting with anti-β-actin antibody (1:1000, A5441; Sigma-Aldrich).

ChIP-seq and ChIP assay

ChIP-seq and ChIP assay were performed as previously described.²³ Primers used for ChIP assay are listed in Supplementary Table S2.

Flow cytometry

Cell cycle analysis was performed as previously described.⁶ Briefly, TNB1 cells infected with indicated vectors (1×10^6) were harvested and washed with cold PBS. The cells were then fixed with 70% ethanol and were resuspended with PBS. The cells were treated with 100 μg/ml of RNase for 30 min at 37 °C, followed incubation with 50 μg/ml of propidium iodide (PI) at 4 °C for 30 min. The data were obtained from a flow cytometer (BD Biosciences, New Jersey) and were analyzed with the CellQuest software (BD Biosciences).

Xenograft

TNB1 cells (5×10^6 cells in 50% Matrigel; BD Biosciences) were subcutaneously inoculated into the left flank of 8-week-old athymic BALB/c nude mice (Liaoning Changsheng biotechnology, China). Tumor volumes were calculated with the following formula: volume (mm^3) = (width)² × length/2.¹

Statistical analysis

The results are presented as the mean ± standard deviation (SD), and the *p* value was calculated with Student's *t* test.

RESULTS

Expression of DRR1 was increased in differentiated neuroblastoma cells

In the previous study, we found that poorly differentiated cells showed decreased DRR1 expression in the ganglions from 2-week-old MYCN transgenic mice.⁶ To investigate the role of DRR1 in neuroblastoma cell differentiation, we cultured SH-SY5Y cells with 0.1% FBS, and then detected the expression of DRR1. The results showed that the neurites in 0.1% FBS cultured cells were longer than that in 10% FBS cultured cells, and the expression of tyrosine hydroxylase (TH), growth associated protein 43 (GAP43) and DRR1 was increased in the 0.1% FBS cultured cells when compared with that of 10% FBS cultured cells (Fig. 1a, b). TH and GAP43 are the differentiation markers of neuroblastoma cells. The similar result was confirmed in 0.1% FBS cultured N2A cells (Fig. 1c, d). We then treated TNB1 cells with retinoic acid (RA) to induce cell differentiation. Real-time PCR results showed that the expression of DRR1 was increased in the RA-treated TNB1 cells when compared with that of non-treated TNB1 cells (Fig. 1e, f). Similarly, the expression of DRR1 was also increased in RA-treated N2A cells (Fig. 1g, h).

DRR1 regulates neuroblastoma cell differentiation

To evaluate the role of DRR1 in neuroblastoma cell differentiation, we infected N2A cells with DRR1 shRNA- or control shRNA-containing lentivirus. The cells were then cultured in 0.1% FBS for 48 h, and we found the DRR1 shRNA-treated cells shows shorter neuritis than control cells (Fig. 2a). Moreover, we treated TNB1 cells with DRR1 shRNA-containing lentivirus, and we found that DRR1 shRNA-treated cells showed shorter neurites (Fig. 2b) and lower expression of TH and GAP43 (Fig. 2c), than those of control shRNA-treated TNB1 cells. To further confirm the result, we infected TNB1 cells with DRR1 shRNA- or control shRNA-containing lentivirus, and then the cells were treated with or without RA. Forty eight hours later, the cells were fixed and stained with TH, GAP43 and DAPI, respectively. The results showed that the expression of TH and GAP43 was lower in DRR1 shRNA groups when compared with according control shRNA groups (Fig. 2d, e).

Furthermore, we overexpressed DRR1 in SH-SY5Y cells. The results showed that overexpression of DRR1 enhanced the expression of TH and GAP43 in SH-SY5Y cells (Fig. 3a, b), when compared to that in the control cells.

Interaction of DRR1 and actin is necessary for DRR1-mediated differentiation induction in neuroblastoma cells

DRR1 is a novel actin binding protein, and actin has been reported to regulate cell differentiation in several kinds of cells.^{24,25} To investigate whether or not DRR1 regulates neuroblastoma differentiation by interacting with actin, we expressed different fragments of DRR1 in SH-SY5Y cells (Fig. 3c). Real-time PCR results showed that only the DRR1 (1–120) fragment and the full length DRR1 (1–144) induced the expression of differentiation markers (Fig. 3d). We then investigated the interaction of different fragments of DRR1 with actin by immunoprecipitation. The results showed that only the DRR1 (1–120) fragment and the full length of DRR1 were co-precipitated with actin (Fig. 3e), which suggested

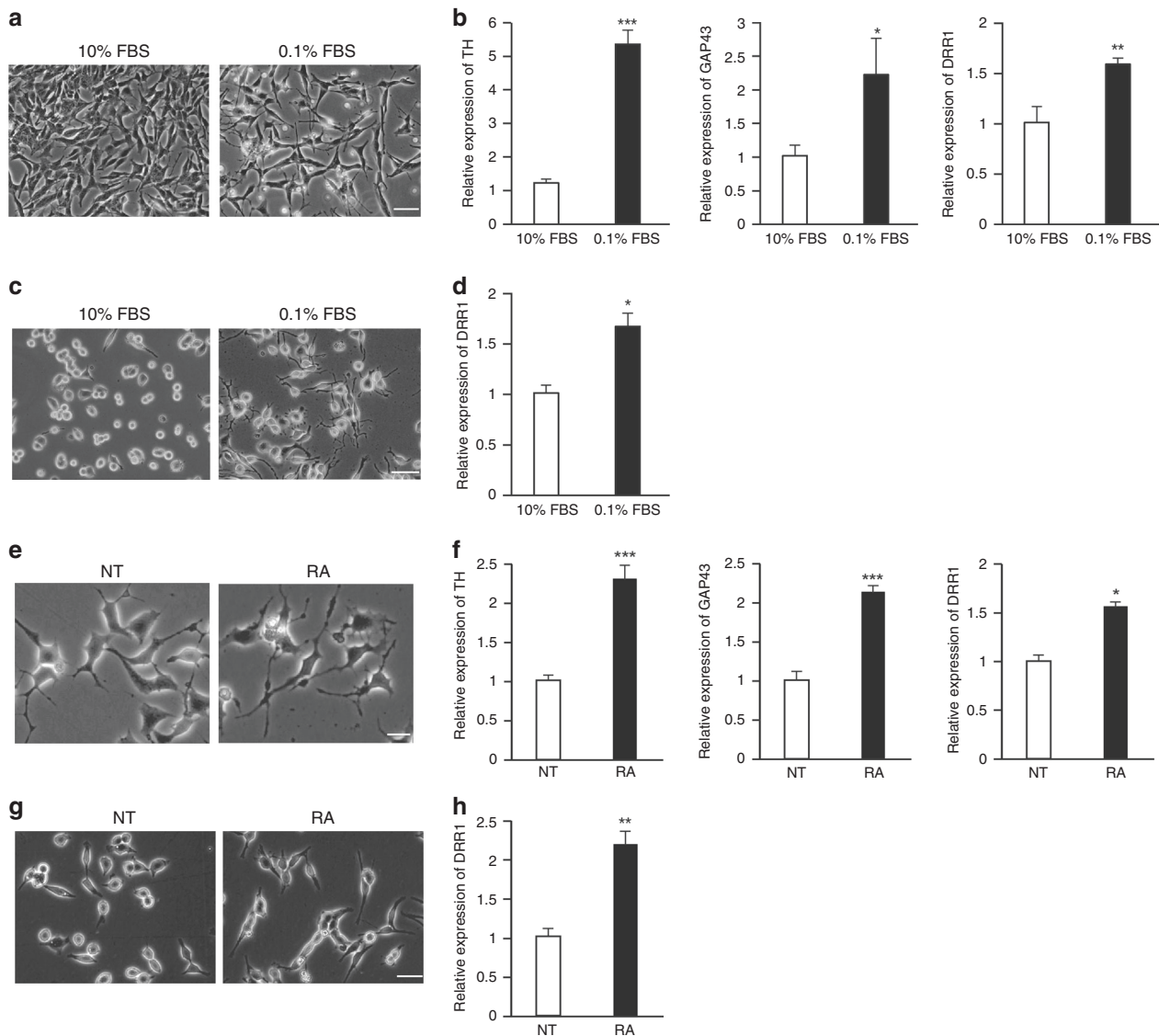


Fig. 1 The expression of DRR1 was increased after neuroblastoma cell differentiation. **a** SH-SY5Y cells were cultured with 10% FBS or 0.1% FBS for 72 h. Scale bar: 50 μ m. **b** The mRNA level of TH, GAP43, and DRR1 in SH-SY5Y cells was examined 24 h after treatment with 10% FBS or 0.1% FBS using real-time PCR. $n = 3$. * $p < 0.05$ versus 10% FBS group. ** $p < 0.01$ versus 10% FBS group. *** $p < 0.005$ versus 10% FBS group. **c** N2A cells were cultured with 10% FBS or 0.1% FBS for 24 h. Scale bar: 50 μ m. **d** The mRNA level of DRR1 in N2A cells was examined 24 h after treatment with 10% FBS or 0.1% FBS using real-time PCR. $n = 3$. * $p < 0.05$ versus 10% FBS group. **e** TNB1 cells were treated with or without 5 μ M of retinoic acid (RA) for 24 h. Scale bar: 20 μ m. **f** The mRNA level of TH, GAP43 and DRR1 in TNB1 cells was examined 24 h after RA treatment using real-time PCR. $n = 3$. * $p < 0.05$ versus NT group; *** $p < 0.005$ versus NT group. **g** N2A cells were treated with or without 1 μ M of RA for 24 h. Scale bar: 50 μ m. **h** The mRNA level of DRR1 in N2A cells was examined 24 h after RA treatment using real-time PCR. $n = 3$. ** $p < 0.01$ versus NT group.

that binding with actin is essential for DRR1 to regulate cell differentiation. Our previous study found that two mutants of DRR1 (DRR1 Δ R74-76A, DRR1 Δ K81-84A) showed decreased nuclear localization.⁶ To investigate whether nuclear localization of DRR1 is essential for its cell differentiation induction, we expressed these two mutants in SH-SY5Y cells (Supplementary Fig. 1a). The results showed that the expression of DRR1 Δ R74-76A and DRR1 Δ K81-84A did not increase the expression of TH and GAP43 in SH-SY5Y cells significantly (Supplementary Fig. 1b).

CREB is a downstream gene of DRR1 in neuroblastoma cells

Nuclear actin and its binding proteins has been identified to play important roles in gene transcription regulation, which indicated that DRR1 may also be involved in gene transcription regulation. We performed ChIP-seq in SH-SY5Y cells, and the expression of

the candidate genes from the ChIP-seq data was checked after DRR1 downregulation and cell differentiation in neuroblastoma cells. Moreover, the correlation between the expression of candidate genes and the expression of DRR1, or the correlation between the expression of candidate genes and the patients' survival probability was examined by analyzing clinic neuroblastoma patients' database. Finally, we found that CREB is a candidate downstream gene of DRR1. To confirm whether DRR1 binds to the promoter region of CREB, we performed ChIP and found that DRR1 bound to -1000 bp promoter region of the CREB gene (Fig. 4a). Furthermore, we overexpressed DRR1 in SH-SY5Y cells or knocked down the expression of DRR1 in TNB1 cells, and then examined the expression levels of CREB in these cells. The result showed that the expression of CREB was increased in DRR1-overexpressed SH-SY5Y cells (Fig. 4b) and was decreased in DRR1

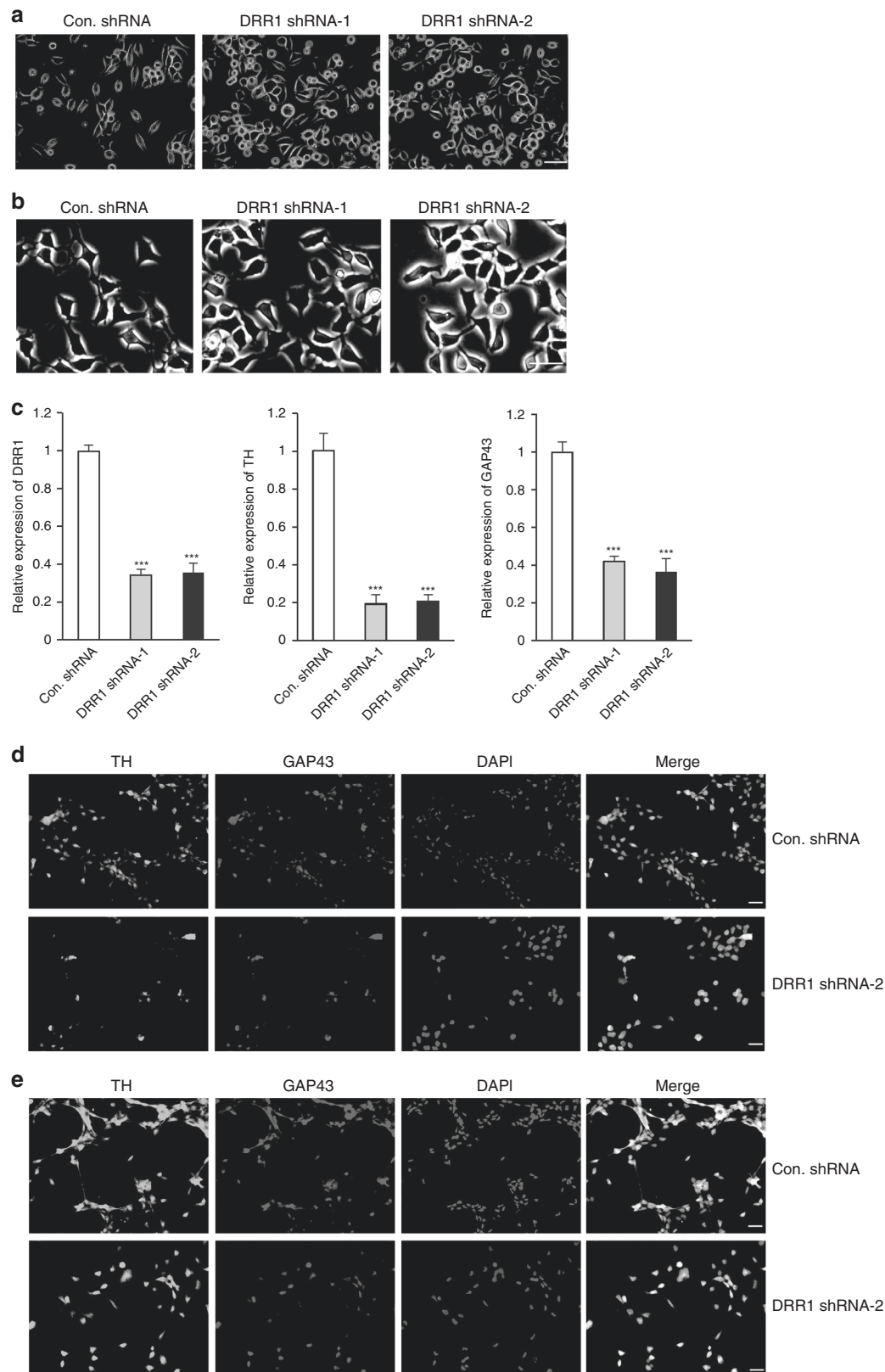


Fig. 2 Downregulation of DRR1 expression suppressed neuroblastoma cell differentiation. **a** N2A cells were infected with DRR1 shRNA- or control shRNA-containing lentivirus. After infection, the cells were cultured with 0.1% FBS for 48 h. Scale bar: 50 μ m. **b** TNB1 cells were infected with DRR1 shRNA- or control shRNA-containing lentivirus. The images were taken 72 h later after infection. Scale bar: 50 μ m. **c** The mRNA levels of DRR1, TH, and GAP43 in TNB1 cells infected with DRR1 shRNA- or control shRNA-containing lentivirus were examined using real-time PCR. $n = 3$. *** $p < 0.005$ versus control shRNA group. **d, e** TNB1 cells were infected with DRR1 shRNA- or control shRNA-containing lentivirus. After infection, the cells were treated with **(e)** or without **(d)** 1 μ M of RA, and the cells were stained with TH (green), GAP43 (red) and DAPI (blue) 48 h later after RA treatment. Scale bar: 50 μ m.

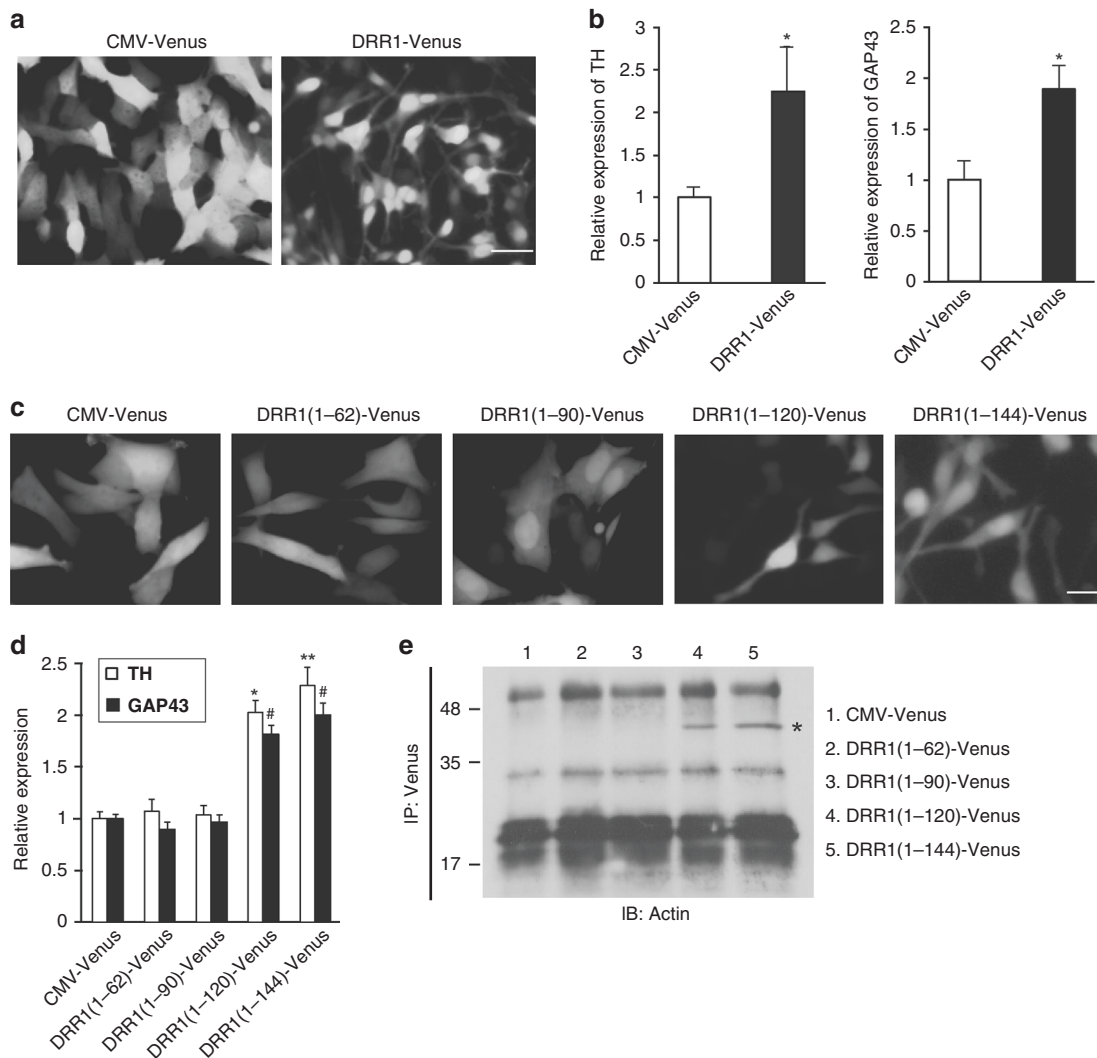


Fig. 3 Overexpression of DRR1 promoted differentiation of neuroblastoma cells. **a** SH-SY5Y cells were infected with CMV-Venus or CMV-DRR1-Venus-containing lentivirus for 72 h. Scale bar: 50 μ m. **b** The mRNA levels of TH and GAP43 in CMV-Venus or CMV-DRR1-Venus-treated TNB1 cells were examined using real-time PCR. $n = 3$. * $p < 0.05$ versus CMV-Venus group. **c** SH-SY5Y cells were infected with the indicated vector-expressing lentivirus, and fluorescent images of Venus and Venus fusion proteins were taken 72 h later. Scale bar: 20 μ m. **d** The mRNA levels of TH and GAP43 in different DRR1 fragment-expressing SH-SY5Y cells were examined using real-time PCR. $n = 3$. * $p < 0.05$, ** $p < 0.01$ versus CMV-Venus group (TH); # $p < 0.05$ versus CMV-Venus group (GAP43). **e** HeLa cells transfected with different DRR1 fragments were lysed and subjected to immunoprecipitation and Western blotting with the anti-actin antibody. The asterisk indicated the position of the bands of actin.

shRNAs-treated TNB1 cells (Fig. 4c). Analysis of a R2 clinic patient database (<http://hgserver1.amc.nl/cgi-bin/r2/main.cgi>) showed a positive correlation between the expression of DRR1 and CREB in the tumors of neuroblastoma patients (Fig. 4d). Moreover, low CREB expression was associated with poor prognosis in neuroblastoma patients (Fig. 4e), which is consistent to the result of DRR1 in neuroblastoma patients.⁶

CREB promotes neuroblastoma cell differentiation

To understand the role of CREB in neuroblastoma, we performed knockdown and overexpression experiments in neuroblastoma cell lines. Our results showed that the downregulation of CREB expression in TNB1 cells inhibited the expression of differentiation markers of neuroblastoma cells (Fig. 5a, b). Alternatively, overexpression of CREB enhanced the expression of differentiation markers in SH-SY5Y cells (Fig. 5c, d). Data from the R2 clinic patient database showed positive correlation between CREB expression and the expression of differentiation markers in neuroblastoma patients (Fig. 5e, f).

The effect of DRR1-CREB axis on cell differentiation, cell cycle distribution and tumor growth in neuroblastoma

To further verify the role of DRR1-CREB axis in neuroblastoma cells, we overexpressed CREB in DRR1 shRNA-treated TNB1 cells. The results showed that expression of CREB rescued the effect of DRR1 shRNA-2 on cell differentiation (Fig. 6a, b). In our previous reports, we found that DRR1 suppressed G1/S phase transition in neuroblastoma cells.⁶ To check whether or not CREB is associated with DRR1 induced cell cycle arrest, we infected TNB1 cells with CMV-Venus or CREB-IRES-Venus expressing vectors plus DRR1 shRNA-2, and the cell cycle distribution was analyzed. The results showed that DRR1 knockdown promoted G1/S phase transition, whereas overexpression of CREB inhibited the G1/S phase transition induced with DRR1 shRNA (Fig. 6c, d). Finally, we performed in vivo xenograft experiments. The tumor volume and the tumor weight in DRR1 shRNA and CMV-Venus group were increased when compared to that in control shRNA and CMV-Venus group. The tumor volume and the tumor weight in DRR1 shRNA and CREB-IRES-Venus group was decreased when compared to DRR1 shRNA and CMV-Venus group (Fig. 6e, f).

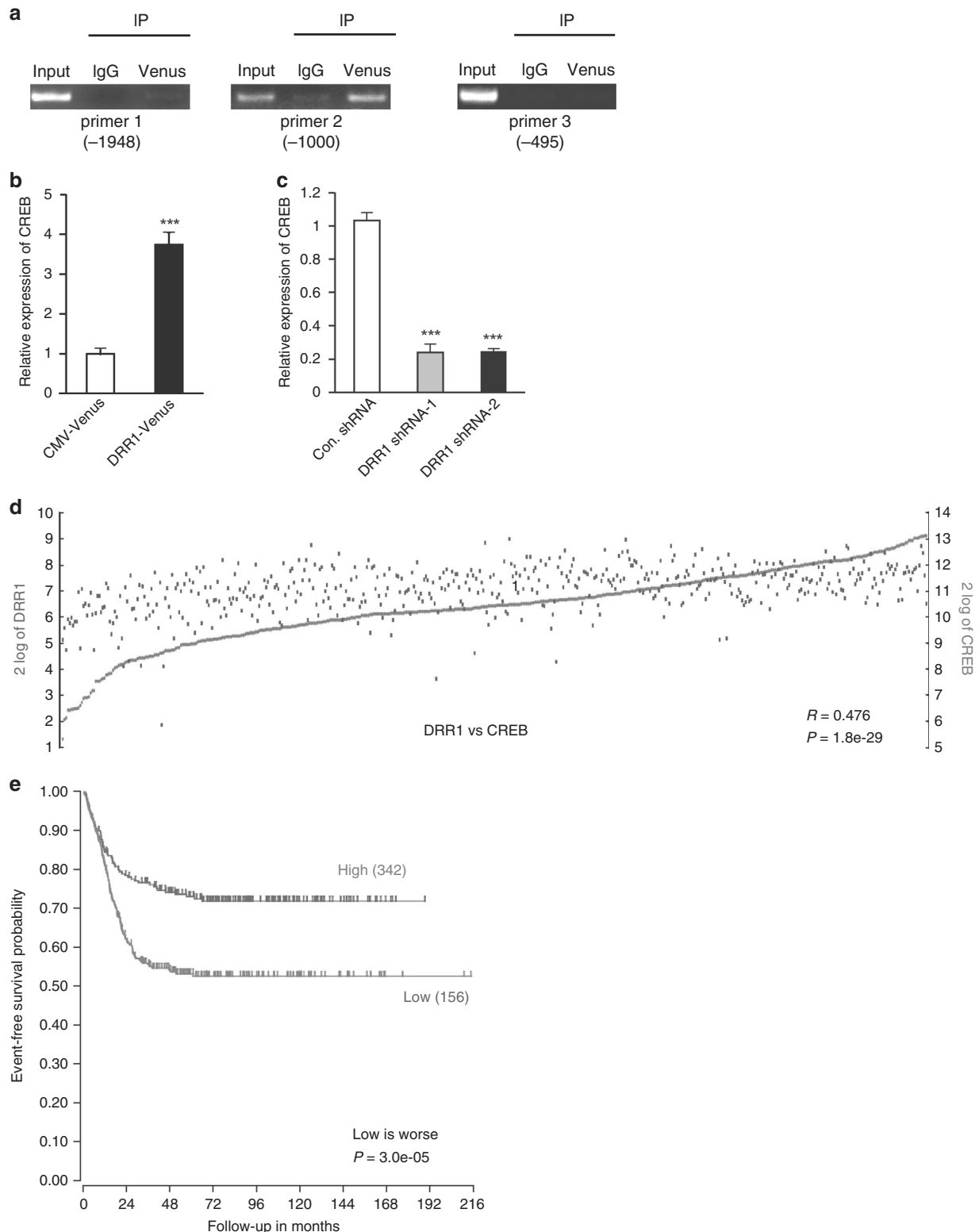


Fig. 4 CREB is a candidate downstream gene of DRR1. **a** SH-SY5Y cells treated with CMV-DRR1-Venus-containing lentivirus were lysed and subjected to ChIP with anti-GFP (Venus) or control IgG antibody. The DNA sample obtained from ChIP was used as the PCR template (Input: genomic DNA). **b** SH-SY5Y cells were infected with CMV-Venus or CMV-DRR1-Venus-containing lentivirus, the mRNA level of CREB was examined at 48 h after infection using real-time PCR. $n = 3$. *** $p < 0.005$ versus CMV-Venus group. **c** TNB1 cells were infected with DRR1 shRNA- or control shRNA-containing lentivirus. The mRNA levels of CREB were examined at 48 h after infection using real-time PCR. $n = 3$. *** $p < 0.005$ versus control shRNA group. **d** Correlation analysis of DRR1 expression (probe: UKv4_A_24_P208703) and CREB expression (probe: UKv4_A_23_P79231) in 498 neuroblastoma patients from a clinical database (SEQC-498-custom-ag44kcwlf). **e** Kaplan–Meier analysis of neuroblastoma prognosis in relation to CREB expression (probe: UKv4_A_23_P79231) in 498 patients from a clinical database (SEQC-498-custom-ag44kcwlf).

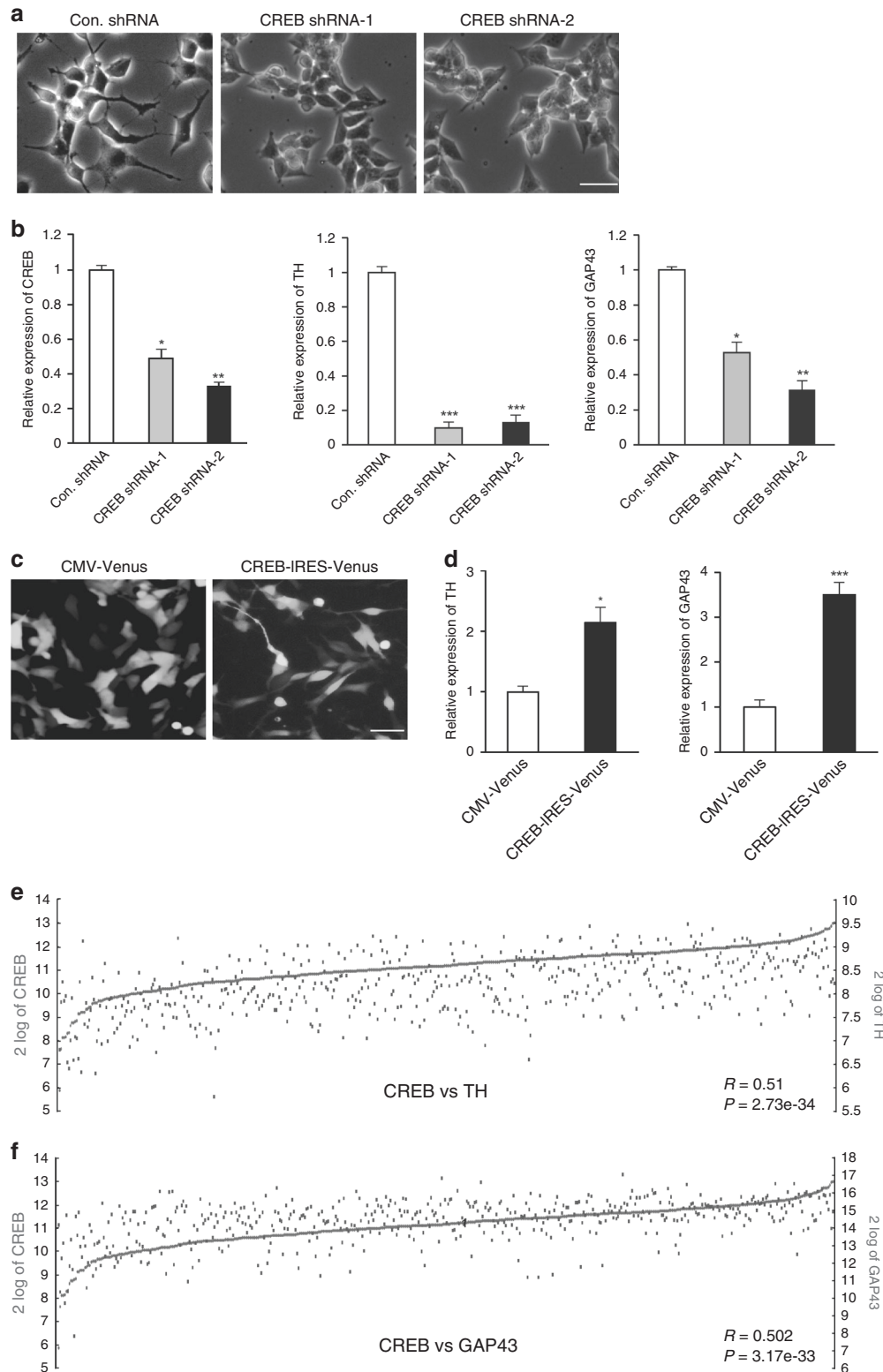


Fig. 5 CREB mediates cell differentiation of neuroblastoma cells. **a** TNB1 cells were infected with CREB shRNA- or control shRNA-containing lentivirus. The images were taken 72 h later after infection. Scale bar: 50 μ m. **b** The mRNA levels of CREB, TH and GAP43 in the TNB1 cells infected with CREB shRNA- or control shRNA-containing lentivirus were examined using real-time PCR. $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ versus control shRNA group. **c** SH-SY5Y cells were infected with CMV-Venus or CMV-CREB-IRES-Venus-containing lentivirus. The images were taken 72 h later after infection. Scale bar: 50 μ m. **d** The mRNA levels of TH and GAP43 in CMV-Venus or CMV-CREB-IRES-Venus-treated cells were examined using real-time PCR. $n = 3$. * $p < 0.05$, *** $p < 0.005$ versus CMV-Venus group. **e** Correlation analysis of CREB expression (probe: UKv4_A_23_P79231) and TH expression (probe: UKv4_A_23_P924602) in 498 neuroblastoma patients from a clinical database (SEQC-498-custom-ag44kewolf). **f** Correlation analysis of CREB expression (probe: UKv4_A_23_P79231) and GAP43 expression (probe: UKv4_A_23_P253446) in 498 neuroblastoma patients from a clinical database (SEQC-498-custom-ag44kewolf).

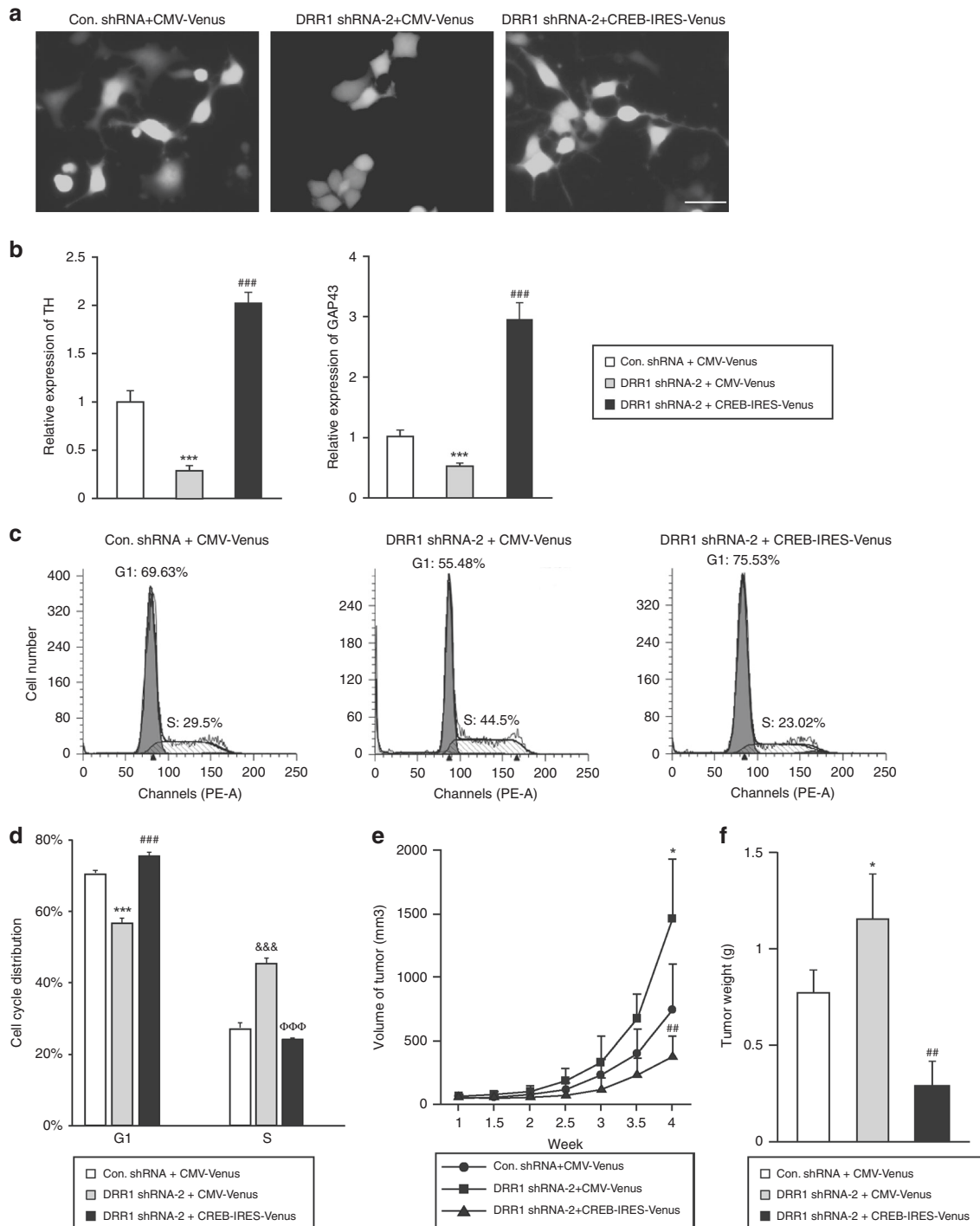


Fig. 6 CREB rescued the effect of DRR1 shRNA on cell differentiation, cell cycle distribution and tumor growth in neuroblastoma cells. **a** TNB1 cells were infected with indicated vectors-containing lentivirus. Fluorescent images of Venus and Venus fusion proteins were taken 72 h later. Scale bar: 50 μ m. **b** The mRNA levels of TH and GAP43 in the TNB1 cells were examined using real-time PCR. $n = 3$. *** $p < 0.005$ versus control shRNA and CMV-Venus group; ### $p < 0.005$ versus DRR1 shRNA-2 and CMV-Venus group. **c** TNB1 cells infected with the indicated lentivirus were collected and subjected to flow cytometry 72 h after infection. **d** The cell cycle distribution was calculated. The results represent the means \pm SD ($n = 3$). *** $p < 0.005$ versus the control shRNA and CMV-Venus group in G1 phase; ### $p < 0.005$ versus the DRR1 shRNA-2 and CMV-Venus group in G1 phase. &&& $p < 0.005$ versus the control shRNA and CMV-Venus group in S phase; $\Phi\Phi\Phi p < 0.005$ versus the DRR1 shRNA-2 and CMV-Venus group in S phase. **e** Tumor growth curve. TNB1 cells (5×10^6 cells in 50% Matrigel) infected with the indicated vector-expressing lentivirus were subcutaneously inoculated into nude mice. Tumor size was measured every week. The results represent the means \pm SD ($n = 6$). * $p < 0.05$ versus the control shRNA and CMV-Venus group, ### $p < 0.01$ versus DRR1 shRNA-2 and CMV-Venus group. **f** Tumor weight at 4 weeks. The results represent the means \pm SD ($n = 6$). * $p < 0.05$ versus the control shRNA and CMV-Venus group, ## $p < 0.01$ versus DRR1 shRNA-2 and CMV-Venus group.

DISCUSSION

The expression level of DRR1 was first found to be reduced in renal cancer cells, and overexpression of DRR1 inhibited renal cancer cell proliferation.⁷ DRR1 expression was gradually reported to be downregulated in diverse types of carcinoma, including neuroblastoma,^{5,6} Hodgkin lymphoma,⁸ astrocytoma,⁹ non-small cell lung cancer,¹⁰ and hepatocellular carcinoma,¹¹ later leading to its definition as a tumor suppressor gene. However, the underlying mechanism of DRR1 is still unclear.

In the present report, we found only full length DRR1 and DRR1 (1–120) fragment enhanced the expression of differentiation markers in neuroblastoma cells, and at the same time, only these two fragments could bind to actin. Actin and actin-binding proteins have also been reported to be involved in the differentiation of different cells.^{24,25} Accordingly, we hypothesized that DRR1 regulates neuroblastoma cell differentiation by interacting with actin.

Actin is a highly abundant and conserved protein. Actin in the cytoplasm regulates cell morphology, muscle contraction, cell motility, cell division, and so on.²⁶ Over the past 50 years, accumulating evidences indicated multiple roles of actin in the nucleus.²⁷ Nuclear actin is essential for various nuclear processes, and the role of actin and several actin-binding proteins in gene transcriptional regulation was also gradually established.^{28,29} To investigate whether DRR1 is involved in gene transcriptional regulation, we performed ChIP-seq, and found that DRR1 binds to the promoter region of CREB.

CREB is a basic-leucine zipper transcription factor regulating cell proliferation,³⁰ cell cycle,²¹ cell survival,³¹ and cell differentiation^{32,33} in a number of cell types. Moreover, the CREB signaling pathway was identified to be mainly changed in RA-induced differentiated neuroblastoma cells.³⁴ Our present study showed that CREB is a novel candidate downstream gene of DRR1 and that CREB promotes neuroblastoma cell differentiation. Moreover, the clinic patient database confirmed the association of CREB expression and the expression of cell differentiation markers in neuroblastoma cells. Our founding in differentiation regulation of neuroblastoma will provide the therapy targets for neuroblastoma patient therapy. Moreover, CREB was confirmed to be over-expressed and to serve as an oncogene in several kinds of cancers.³⁵ Several inhibitors of CREB were designed for the therapy of the cancers.^{36,37} Whereas, our current study indicated that CREB is a candidate tumor suppressor gene in neuroblastoma, which is different to the roles of CREB in other kinds of cancers. Our finding showed the diversity of the roles of CREB in cancers, and provides evidences for the rational use of CREB inhibitors in clinic treatment. Although the role of the DRR1-CREB axis was revealed, the actin dynamics and the downstream pathway of DRR1-CREB axis in neuroblastoma cell differentiation still need to be explored in future work.

DATA AVAILABILITY

The data during the study are available from the corresponding author by request.

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AUTHOR CONTRIBUTIONS

L.C., B.M., and Y.L. performed the experiments and analyzed the data. F.L. and P.M. conceived the study and wrote the manuscript. All authors read and approved the final manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The animal experiments were performed following the Animal Care and Use Guidelines of Shenyang Medical College. The experimental procedures with animals were approved by the Institutional Animal Care and Use Committee of Shenyang Medical College (approval no. SYXY-2019050801).

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