

TRPS1 and YAP1 Regulate Cell Proliferation and Drug Resistance of Osteosarcoma via Competitively Binding to the Target of circTADA2A – miR-129-5p

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Introduction: The yes-associated protein (YAP) and trichorhinophalangeal syndrome 1 (TRPS1) have been reported to account for the pathogenesis of cancers and may play an important role in osteosarcoma (OS). This study intended to investigate the modulatory effect and relationship of TRPS1 and YAP1 in OS cells.

Methods: The expression difference of YAP1 and TRPS1 in OS cells was measured. Then, the effect of circTADA2A silence on YAP1 and TRPS1 expression as well as OS proliferation and drug resistance was estimated.

Results: TRPS1 and YAP1 were upregulated in OS cell lines, and TRPS1 and YAP1 were highly expressed in MG63 and U2OS cells, respectively. The cell proliferation of MG63 was lower than that of U2OS, but the opposite result was observed in the presence of cisplatin (DDP). CircTADA2A was upregulated while miR-129-5p was downregulated in MG63 and U2OS cells compared. Besides, circTADA2A knockdown inhibited cell proliferation and reduced DDP resistance in both MG63 and U2OS. MiR-129-5p was increased but TRPS1 and YAP1 were decreased by circTADA2A knockdown. Meanwhile, circTADA2A knockdown reduced TRPS1 protein expression but enhanced phosphorylated (p)-YAP1. In xenograft OS tumor mice, circTADA2A knockdown inhibited tumor growth in the absence or presence of DDP. Finally, miR-129-5p could bind to circTADA2A, TRPS1 and YAP1.

Discussion: CircRNA TADA2A could target miR-129-5p, which was competitively bound by TRPS1 and YAP1, thereby regulating OS cell proliferation and drug resistance.

Keywords: circRNA, drug resistance, osteosarcoma, trichorhinophalangeal syndrome 1, yes-associated protein 1

Introduction

Osteosarcoma (OS) is a malignant tumor caused by the differentiation of mesenchymal stem cells into osteoblasts, mediated by the genetic factors and epigenetic modifications.¹ OS is the most common primary bone malignant tumor and mainly occurs in children and adolescents. Environmental and genetic factors have been involved in the occurrence or progression of OS; however, the main cause of OS has not been fully understood. Although advanced surgical techniques have been combined with different types of chemotherapy in clinical practice, the survival rate of OS remains unsatisfactory, and many patients suffer from disease recurrence due to existing or potential distant metastasis.² Patients with OS may benefit from the

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application of adjuvant chemotherapy, such as cisplatin (DDP), doxorubicin, and methotrexate. However, these treatment strategies often lead to severe side effects and failure in clinical trials due to drug resistance.^{3,4} Therefore, exploring the mechanisms underlying OS progression and identifying its therapeutic targets is of great significance in order to improve the prognosis of OS patients.

The Hippo signaling pathway is an evolutionarily highly conserved signal transduction pathway involved in the regulation of organ size and the maintenance of tissue homeostasis via controlling cell proliferation and apoptosis. It has been extensively demonstrated that the Hippo signaling pathway exerts a tumor-suppressive role in various cancers. Yes-associated protein (YAP) is a major downstream effector of the Hippo pathway. It has been reported that YAP is upregulated and involved in the tumorigenesis of various types of cancer such as liver cancer,⁵ breast cancer,⁶ and pancreatic cancer.⁷ Previous studies have indicated that the aberrant activation of YAP is considered one of the most important mechanisms underlying the pathogenesis of cancer. In many tissues, YAP regulates key cellular processes, including cell proliferation, inhibition of apoptosis, and promotion of metastasis. By negatively regulating the oncogenic YAP activities, the kinases of the Hippo pathway act as important tumor-suppressive molecules. Furthermore, in several types of cancers, the Hippo signaling is dysfunctional and YAP is activated, with increased levels of nuclear proteins.⁸ Human tissue microarray analysis confirmed that the expression of YAP1 was higher in OS compared with the adjacent normal tissues.⁹

Trichorhinophalangeal syndrome 1 (TRPS1) serves an important role in regulating chondrocyte proliferation and apoptosis.¹⁰ Studies have shown that TRPS1 is generally overexpressed in multiple types of cancer and it is possibly associated with tumor cell invasion, metastasis and multidrug-resistance during chemotherapy.^{11–13} Our previous study illustrated that TRPS1 enhanced drug resistance of OS cells via upregulating the expression of multidrug resistance member 1 (MDR1) in OS. In addition, inhibition of TRPS1 expression could attenuate the resistance of OS cells to chemotherapeutic drugs.¹⁴ In breast cancer, TRPS1 was commonly amplified, and high TRPS1 activity was associated with decreased YAP1 activity, thus leading to decreased density of tumor-infiltrating immune cells.¹⁵ However, considering the high expression of YAP1 and the enhanced effect of TRPS1 on multidrug-resistance in

OS, whether TRPS1 may act as an epigenetic regulator of YAP activity in OS remains to be investigated.

It has been showed that circular RNA transcriptional adaptor 2A (circTADA2A) was highly expressed in OS, thereby promoting tumor cell proliferation and metastasis.¹⁶ Several RNA targets of circTADA2A have been identified. Among them, miR-129-5p was predicated to exert a high circTADA2A-binding probability by searching the ENCORI database (<http://starbase.sysu.edu.cn/>). Furthermore, circTADA2A could sponge miR-129-5p to inhibit its effect on the expression of downstream target molecules. Importantly, miR-129-5p was predicated to bind to both YAP1 and TRPS1 by searching the ENCORI database (<http://starbase.sysu.edu.cn/>). Besides, miR-129-5p was found to inhibit cell proliferation, migration and invasion during the development of OS.¹⁷ Therefore, the present study aimed to clarify the potential role of TRPS1 and YAP1 in OS, as well as to uncover the possible modulatory association between them in OS cell proliferation and drug resistance.

Materials and Methods

Cell Culture and Treatment

HEK293T cells, human osteoblast Hfob1.19 and human OS cells, including the MHM, Saos-2, U2OS and MG-63 cell lines, were obtained from the American Type Culture Collection (ATCC, Manassas, USA). All the cell lines, except for the Hfob1.19 cell line, were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, USA), 100 U/mL penicillin, and 100 U/mL streptomycin (Invitrogen, Carlsbad, USA). The Hfob1.19 cells were maintained in DMEM/F-12 supplemented with 10% FBS (Gibco) and 0.3 mg/mL G418 (Invitrogen). All the cells were cultured in an incubator at 37°C with 5% CO₂.

Cell Transfection

Human lentivirus-sh-circTADA2A, miR-129-5p mimic or NC (negative control) were purchased from GeneChem (Shanghai, China). The lentiviruses were ultracentrifuged, concentrated, validated and added to the cell culture medium. After infection, the cells were selected with puromycin (Gibco) for 1 week, and the surviving cells were continuously cultured for subsequent experiments. The miR-129-5p mimic or NC were transfected into cells using lipofectamine 2000 according to the manufacturer's instruction (Thermo Fisher, USA).

Cell Counting Kit-8 (CCK-8)

For the assessment of cell viability, the U2OS and MG63 cells were seeded in a 96-well plate at a cell density of 200 cells per well. The cells were incubated with 10 μ L of WST-8 working solution (Beyotime, China) for 2 h under normal cell culture condition, and then the absorbance increase at 460nm was measured with a microplate reader.

Xenograft Tumor Models

All animal studies were performed in accordance with the Care and Use Guide of Laboratory Animals of the National Institutes of Health. U2OS and MG63 cell lines that transfected with sh-circTADA2A or not were injected subcutaneously into the six-week-old male BALB/C nude mice. For DDP ($\geq 99.9\%$, Sigma-Aldrich, USA) treatment, animals were treated by intraperitoneal injection of cisplatin (30 μ g/kg) on the third day after xenograft, at 3 day interval for four times. The body weights of animals and the two perpendicular diameters of each tumor were measured every three days. And the tumor volume was calculated by this formula: tumor volume (mm^3) = (length, mm) \times (width, mm)² / 2. At the 21 days after the first injection, all mice were euthanized and the tumors were excised and weighed.

RT-qPCR

Total RNA was extracted from cells or tumor tissue samples. Cells were lysed with TRIzol reagent (Thermo Fisher, USA) and then RNA was extracted in accordance with instructions of the manufacturer, quantified by photometrical measurement. A total of 1 μ g total RNA was reversely transcribed to cDNA using PrimeScriptTM kit (Takara, Dalian, China). The relative expression levels of each sample were measured using the $2^{-\Delta\Delta\text{CT}}$ method. The sequences of specific primers were designed as follows: *TRPS1*, forward 5'-AAGTTTTCTGGGGGCTCGTG-3', reverse 5'-GTTTCCTCCCTTA CTG-GGGC-3'; *YAP1*, forward 5'-CCCTCGTTTTGCC ATGAACC-3', reverse 5'-GTTGC-TGCTGGTTGGAGT TG-3'; *circTADA2A*, forward 5'-TGTGCACCAAGACC AAGGA-G-3', reverse 5'-AGGAAAATCTGAAGTAGT GA-3'; *miR-129-5p*, forward 5'-CGGCGGTTTTTTGCGG TCTGGGCT-3', reverse 5'-AGCCAGACCGCAAAAA- ACCGCCG-3'; *U6*, forward 5'-CTCGCTTCGGCAGCA CA-3', reverse 5'-AACGCTTCACGAATTTGCGT-3'; *GAPDH*, forward 5'-GAAAGCCTGCCGGTG-ACTAA-3', reverse 5'-TTCCCGTTCTCAGCCTTGAC-3'. CircRNA and microRNA expression was normalized to U6 and mRNA expression was normalized to GAPDH, respectively.

Western Blot

The total proteins from cells or animal samples were extracted using RIPA buffer containing phenylmethanesulfonyl fluoride. The concentration and purity of each sample were determined by BCA kit (Solarbio, Beijing, China). Next, 30 mg of protein was transferred onto PVDF membranes (Millipore, USA) and blocked with non-fat milk at room temperature for 2 h. The membrane was then incubated with primary antibodies against TRPS1, total (t)-YAP1, phosphorylated (p)-YAP1 and GAPDH (Abcam, Cambridge, UK). The secondary antibodies were conjugated with horseradish peroxidase (Santa Cruz) and incubated for immunoblotting analysis. Signal detection was measured using an ECL Western blotting detection kit (Amersham Biosciences, USA). GAPDH was used as the internal control.

Immunohistochemical Staining

The tumors from nude mice were collected for preservation and immunohistochemistry (IHC) analysis according to the manufacturer's instructions. After formalin fixation and paraffin embedding, tumor tissue sections were incubated with antibody against CD3 (Santa Cruz Biotechnology, Santa Cruz, USA). Pictures ($\times 200$) were taken under optical microscope (Olympus).

Luciferase Reporter Assay

HEK293T cell lines were co-transfected with miR-129-5p mimic or NC mimic (RiboBio, Guangzhou, China) and reporter plasmids containing the wild-type (WT) or mutant (MUT) circTADA2A, TRPS1 and YAP1 promoters (GeneChem), respectively. Luciferase reporter assay was performed using Dual-Luciferase Reporter Assay System (Promega, USA). For comparisons, firefly luciferase activity was normalized to Renilla luciferase activity. The effect of a miR-129-5p on the luciferase reporter with the circTADA2A 3'-UTR, TRPS1 3'-UTR, YAP1 3'-UTR or the corresponding mutant was calculated by comparing the reporter with the control. Each luciferase reporter assay was conducted in triple replicates.

Statistical Analysis

Statistical analyses were performed with GraphPad 6.0 statistical software (GraphPad, USA) and results are presented as the mean \pm SD. Differences among groups were assessed using one-way analysis of variance (ANOVA) followed by Tukey's test. $P < 0.05$ was considered statistically significant.

Results

TRPS1 and YAP1 are Relatively Upregulated in MG63 and U2OS OS Cells, Respectively

The mRNA level of TRPS1 and YAP1 in human osteoblast Hfob1.19 and human OS cell lines including the MHM, Saos-2, U2OS and MG-63, was determined. We found that the expression level of TRPS1 and YAP1 was significantly increased in various OS cell lines compared with Hfob1.19 cells, a normal osteoblast cell line (Figure 1A). Moreover, TRPS1 level was relatively higher in MG63 cells and YAP1 expression was higher in U2OS cells, when compared with other OS cell lines (Figure 1A). Intriguingly, MG63 showed a lowest level of YAP1 and U2OS exhibited a lowest expression of TRPS1. Therefore, U2OS and MG63 cell lines were chosen for the following experiments. Results from Figure 1B revealed that, after culturing for 24 h, the cell viability of MG63 was significantly lower than that of U2OS cells, indicating that U2OS cells had a higher proliferation capacity than MG63 cells. In addition, to investigate the drug resistance of U2OS and MG63 cells, different concentrations of DDP were utilized

to treat cells that have been incubated for 24 h and the cell viability inhibition was then measured at 24 h post-treatment. As shown in Figure 1C, the cell viability inhibition of DDP on U2OS cells was obviously larger than MG63 cells from the concentrations of 4 μ g/mL, suggesting that MG63 cells exhibited a stronger drug resistance. These data implicated that U2OS cell line, which highly expressed YAP1, showed a higher proliferation ability but a lower drug resistance. On the contrary, MG63 cell line that highly expressed TRPS1 exerted a weaker proliferation ability but a stronger drug resistance. Our results suggested that in OS, the expression of TRPS1 and YAP1 may also be negatively correlated, and they regulated tumor proliferation and drug resistance through mutual restriction.

Silencing of circTADA2A Inhibits U2OS and MG63 Cell Proliferation and Resistance to DDP

The mRNA level of circTADA2A and miR-129-5p in Hfob1.19, MG63 and U2OS cells was evaluated. We found that circTADA2A was remarkably upregulated while

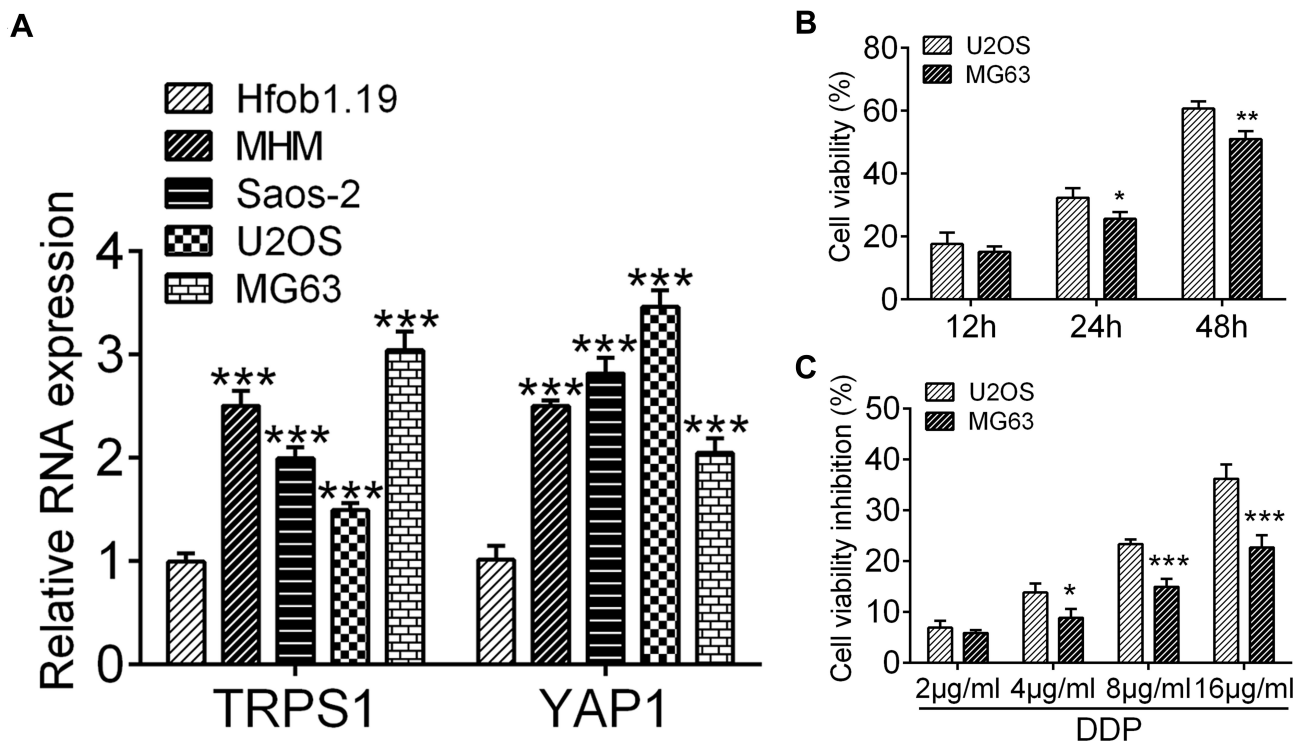


Figure 1 Expression of YAP1 and TRPS1 in OS cell lines. (A) mRNA expression levels of TRPS1 and YAP1 in the human osteoblasts Hfob1.19 and OS cell lines, MHM, Saos-2, U2OS, and MG63. *** $P < 0.001$ vs Hfob1.19 cells. (B) U2OS and MG63 cell viability at 12, 24 and 48 h post-cultivation. * $P < 0.05$, and ** $P < 0.01$ vs U2OS cells. (C) Inhibition of cell viability of U2OS and MG63 cells treated with different concentrations of DDP. * $P < 0.05$, and *** $P < 0.001$ vs U2OS.

Abbreviations: YAP1, yes-associated protein 1; TRPS1, trichorhinophalangeal syndrome 1; OS, osteosarcoma; DDP, cisplatin.

miR-129-5p was downregulated in OS cell lines MG63 and U2OS (Figure 2A). These data revealed that TADA2A may play a role in OS via sponging to miR-129-5p. Then we knock-down circTADA2A expression in both MG63 and U2OS cells. Two pairs of chemically synthesized shRNAs (shRNA1 and shRNA2) targeting circTADA2A and negative controls (NC group) were transfected into MG63 and U2OS cells, respectively. The results of RT-qPCR showed that compared with the NC group, the expression of circTADA2A was obviously inhibited by both shRNAs and the inhibition efficiency of shRNA-1 was higher than that of shRNA-2 (Figure 2B). Therefore, shRNA-circTADA2A-1 was transfected into cells for subsequent experiments. Figure 2C and D showed that cells that transfected with shRNA-circTADA2A exhibited obviously lower cell viability than cells that transfected with negative control. Meanwhile, the inhibitory effect of circTADA2A knockdown on U2OS cell proliferation was relatively stronger than that on MG63 cell proliferation. As shown in Figure 2E and F, knockdown of circTADA2A increased the inhibitory effect of both 4 $\mu\text{g/mL}$ and 8 $\mu\text{g/mL}$ DDP on U2OS and MG63, indicating that knockdown of circTADA2A could blunt the drug resistance of U2OS and MG63 to DDP. These results suggested that circTADA2A could promote both proliferation and drug resistance of OS.

Silencing of circTADA2A Upregulates miR-129-5p and Downregulates TRPS1 and YAP1 Expression in U2OS and MG63 Cells

Then, the mRNA expression of miR-129-5p, TRPS1 and YAP1 together with protein expression of TRPS1, t-YAP1 and p-YAP1, in U2OS and MG63 cells that knockdown with circTADA2A or no, was assessed. As demonstrated in Figure 3A and B, knockdown of circTADA2A enhanced miR-129-5p expression while reduced TRPS1 and YAP1 expression in both U2OS and MG63 cells. Moreover, silence of circTADA2A significantly inhibited TRPS1 and t-YAP1 protein expression, but promoted p-YAP1 protein expression. The ratio of p-YAP1 was also considerably enhanced (Figure 3C and D).

Silencing of circTADA2A Attenuates Tumor Growth and DDP Resistance in U2OS and MG63 Xenograft Tumor Models

Next, to explore the effect of circTADA2A silence on OS tumor growth and drug resistance in vivo, nude mice were

xenografted with U2OS and MG63 cell lines that transfected with sh-circTADA2A or not (Figure 4A). Results from Figure 4B–G revealed that silence of circTADA2A significantly repressed tumor growth of both U2OS and MG63 xenograft. In addition, circTADA2A silence inhibited U2OS tumor growth more remarkably. The data from Figure 5 were utilized to investigate the effect of circTADA2A knockdown on drug resistance of U2OS and MG63-like tumor. We found that circTADA2A knockdown enhanced the sensitivity of OS tumor derived from both U2OS and MG63 to DDP. At the same time, the inhibitory effect of circTADA2A silence on MG63 tumor was relatively stronger than that on U2OS tumor.

MiR-129-5p is Sponged by circTADA2A and Directly Binds to TRPS1 and YAP1

As shown in Figure 6A, the expression of T lymphocyte CD3 in tumor tissues that derived from both U2OS and MG63 was remarkably decreased by circTADA2A silence. In accordance with in vitro results, the mRNA level of circTADA2A in tumor tissues was also significantly down-regulated in the presence of sh-circTADA2A (Figure 6B). Furthermore, the protein expression levels of TRPS1 and relative p-YAP1 in tumor tissues derived from both U2OS and MG63 were also increased (Figure 6C and D). Finally, the interaction between miR-129-5p and circTADA2A, TRPS1 and YAP1 was determined. Results from Figure 6E–G illustrated that, consistent with our speculation, miR-129-5p could directly bind to circTADA2A, TRPS1 and YAP1, indicating that miR-129-5p may be sponged by circTADA2A and then directly target TRPS1 and YAP1, to regulate OS cell proliferation and drug resistance.

Discussion

YAP1 is a candidate oncogene, involved in tumorigenesis and progression of many malignant tumors.¹⁸ A large number of studies have indicated that YAP1 is highly expressed and contributes to the progression of human OS. For example, YAP1 knockdown inhibited OS cell proliferation in vitro and in vivo.^{19–22} In addition, upregulation of YAP1 promoted OS cell proliferation, migration, and invasion, thereby leading to tumorigenesis.²³ TRPS1 is also generally overexpressed in multiple types of cancer^{11–13,24,25} and our previous study has confirmed its role in enhancing drug resistance in OS cells.¹⁴ Consistent with previous studies, the results of the current study showed that YAP1 and TRPS1 were upregulated in

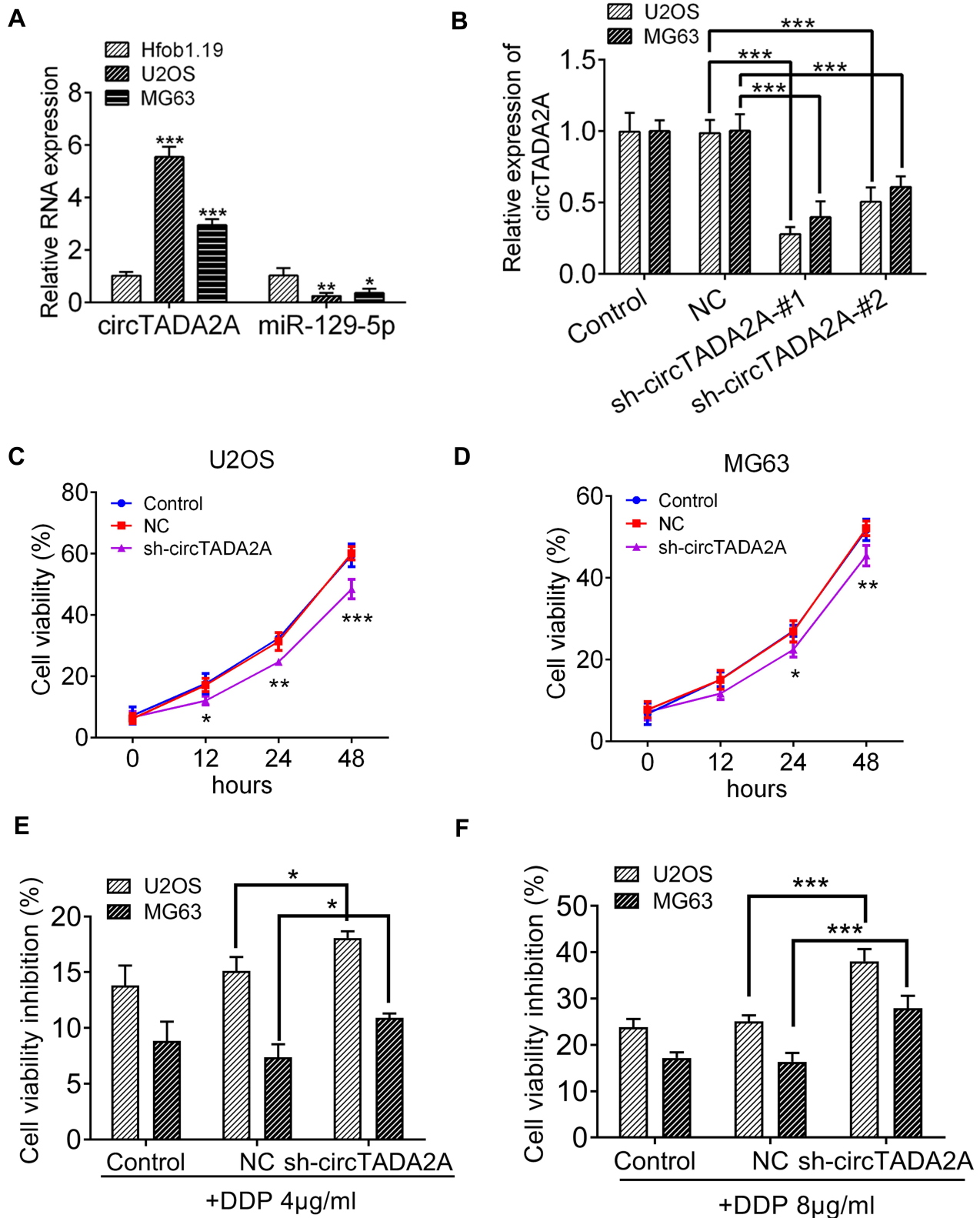


Figure 2 Effect of circTADA2A knockdown on U2OS and MG63 cell viability and resistance to DDP. **(A)** RNA expression levels of circTADA2A and miR-129-5p in Hfob1.19, and U2OS and MG63 OS cells. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs Hfob1.19. **(B)** RNA expression levels of circTADA2A in U2OS and MG63 cells treated or not with sh-circTADA2A. *** $P < 0.001$ vs NC. **(C)** U2OS and **(D)** MG63 cell viability at 12, 24 and 48 h post-cultivation. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs NC. **(E)** Inhibition of cell viability of U2OS and MG63 cells treated with 4 $\mu\text{g}/\text{mL}$ DDP for 24h. * $P < 0.05$ vs NC. **(F)** Inhibition of cell viability of U2OS and MG63 cells treated with 8 $\mu\text{g}/\text{mL}$ DDP for 24h. *** $P < 0.001$ vs NC.

Abbreviations: circTADA2A, circular RNA transcriptional adaptor 2A; DDP, cisplatin; miR-129-5p, microRNA-129-5p; OS, osteosarcoma; NC, negative control.

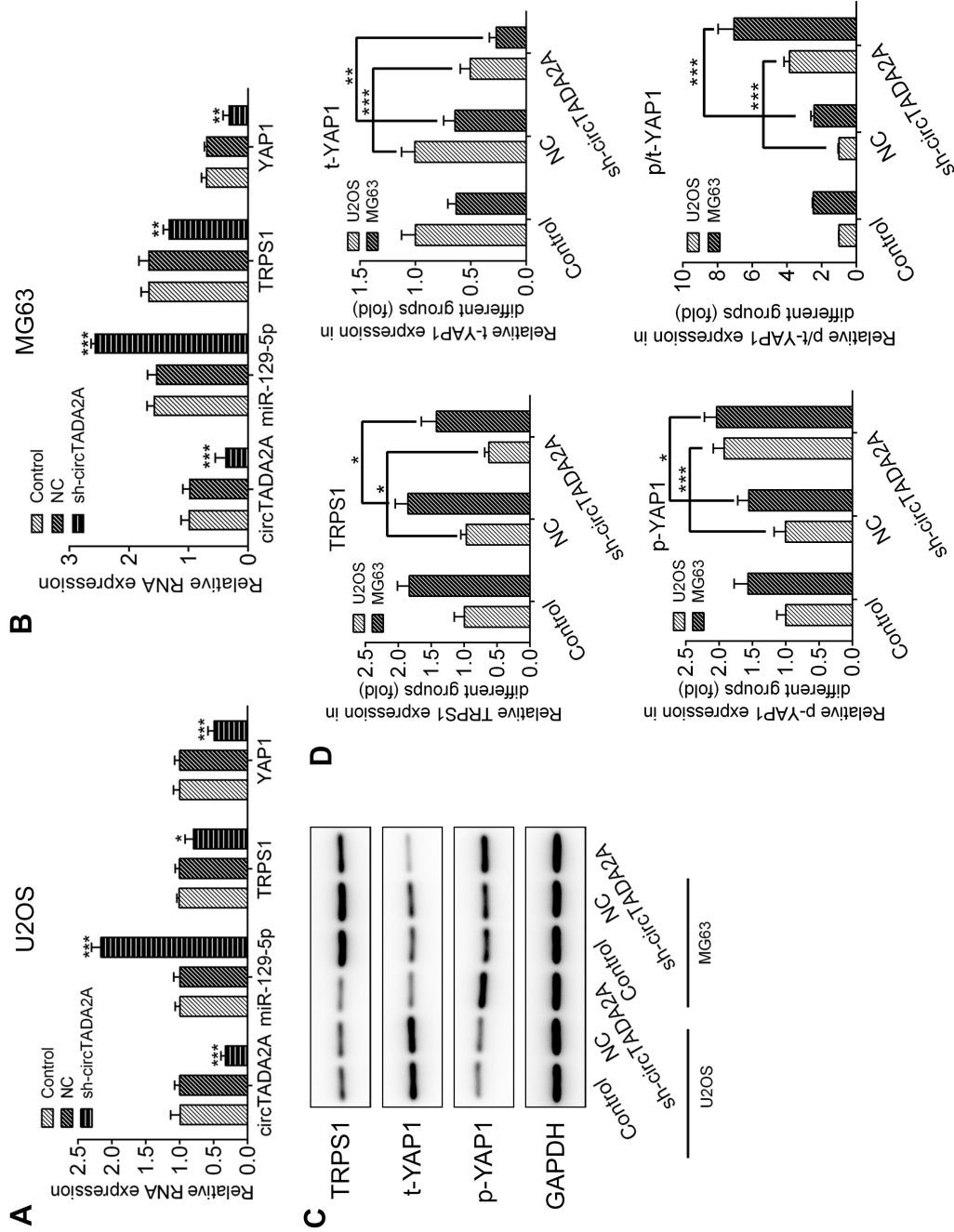


Figure 3 Effect of circTADA2A knockdown on miR-129-5p, TRPS1 and YAP1 expression. **(A and B)** RNA expression levels of circTADA2A, miR-129-5p, TRPS1 and YAP1 in U2OS and MG63 cells treated or not with sh-circTADA2A. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs NC. **(C and D)** Protein expression of TRPS1, total (t)-YAP1, phosphorylated (p)-YAP1, in U2OS and MG63 cells treated with or without sh-circTADA2A. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs NC.

Abbreviations: circTADA2A, circular RNA transcriptional adaptor 2A; miR-129-5p, microRNA-129-5p; TRPS1, trichorhinophalangeal syndrome 1; YAP1, yes-associated protein 1; NC, negative control.

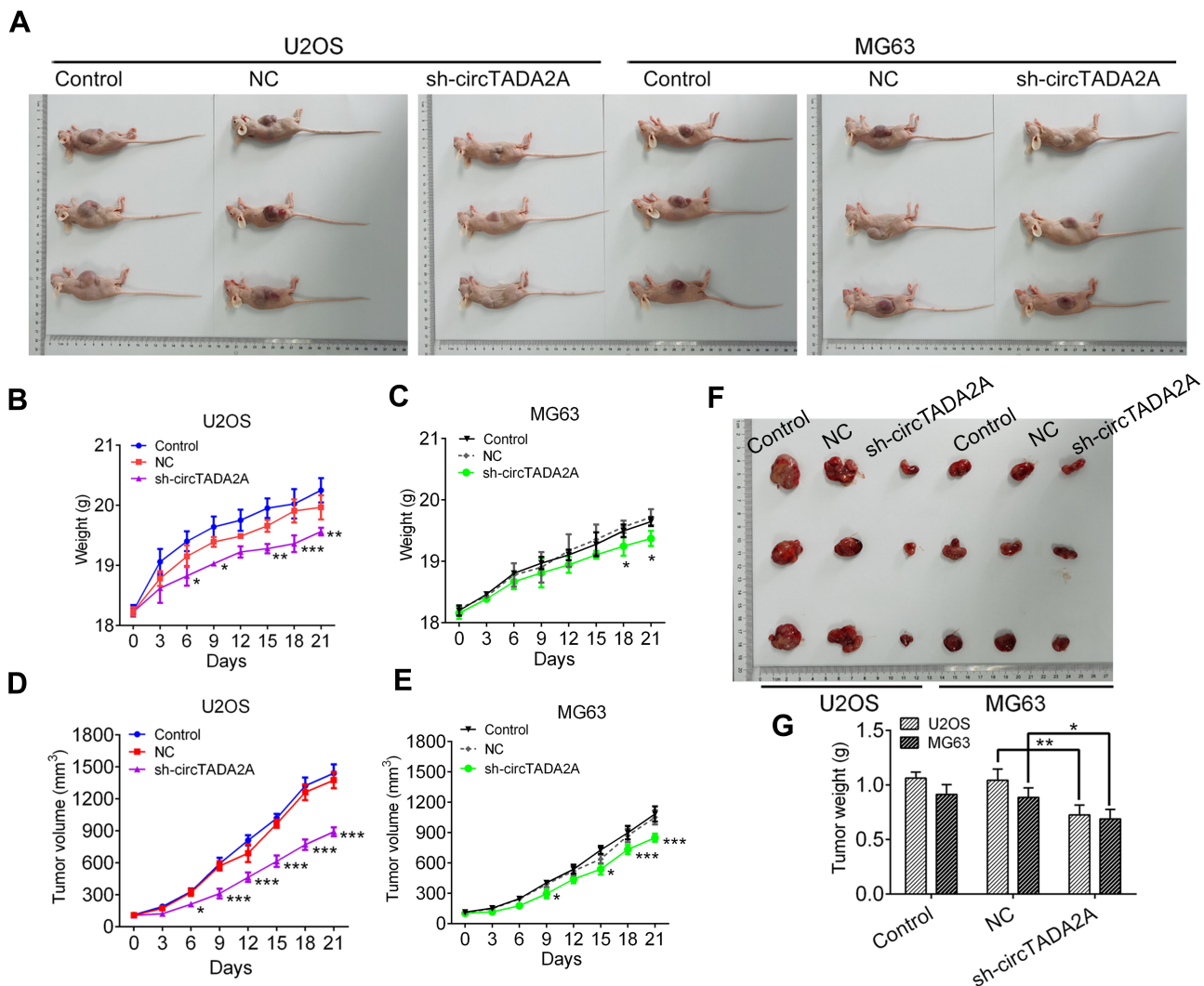


Figure 4 Knockdown of circTADA2A inhibited tumor growth in U2OS and MG63 xenograft models. **(A)** Representative images showing euthanized nude mice xenografted with U2OS and MG63 cells. **(B and C)** The body weight and **(D and E)** tumor volume were measured every three days following xenograft model establishment. The values were plotted as the mean \pm standard deviation ($n=7$). **(F and G)** At the 21st day following xenograft model establishment, the tumors were isolated and their weights were recorded. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ vs NC.

Abbreviations: circTADA2A, circular RNA transcriptional adaptor 2A; NC, negative control.

various OS cell lines and their expression was higher in U2OS and MG63 cells, respectively. Noteworthy, U2OS cells, where YAP1 was highly expressed, showed the lowest expression of TRPS1 and enhanced proliferation ability. Additionally, MG63 cells, highly expressing TRPS1, exhibited decreased expression of YAP1 and increased resistance to DDP. These findings further confirmed our previous results suggesting that TRPS1 was involved in enhanced drug resistance in OS. The association between YAP1 and TRPS1 was primarily clarified by Elster et al, who suggested that YAP1 was downregulated, while TRPS1 was commonly amplified in breast cancer, and high TRPS1 activity was associated with decreased YAP1 activity, leading to decreased frequency

of tumor-infiltrating immune cells.¹⁵ Therefore, the present study hypothesized that YAP1 and TRPS1 could also negatively correlate with each other in OS cells and contribute to OS cell proliferation and drug resistance, respectively.

Bioinformatic analysis revealed that that miR-129-5p could directly bind to both YAP1 and TRPS1. It has been reported that miR-129-5p is downregulated in OS cells and suppresses OS cell proliferation, migration and invasion.¹⁷ Furthermore, circTADA2A was predicted to sponge miR-129-5p and promote OS progression and metastasis.¹⁶ Based on the above evidence, the current study hypothesized that circTADA2A could sponge miR-129-5p, which in turn could target both YAP1 and TRPS1, thereby

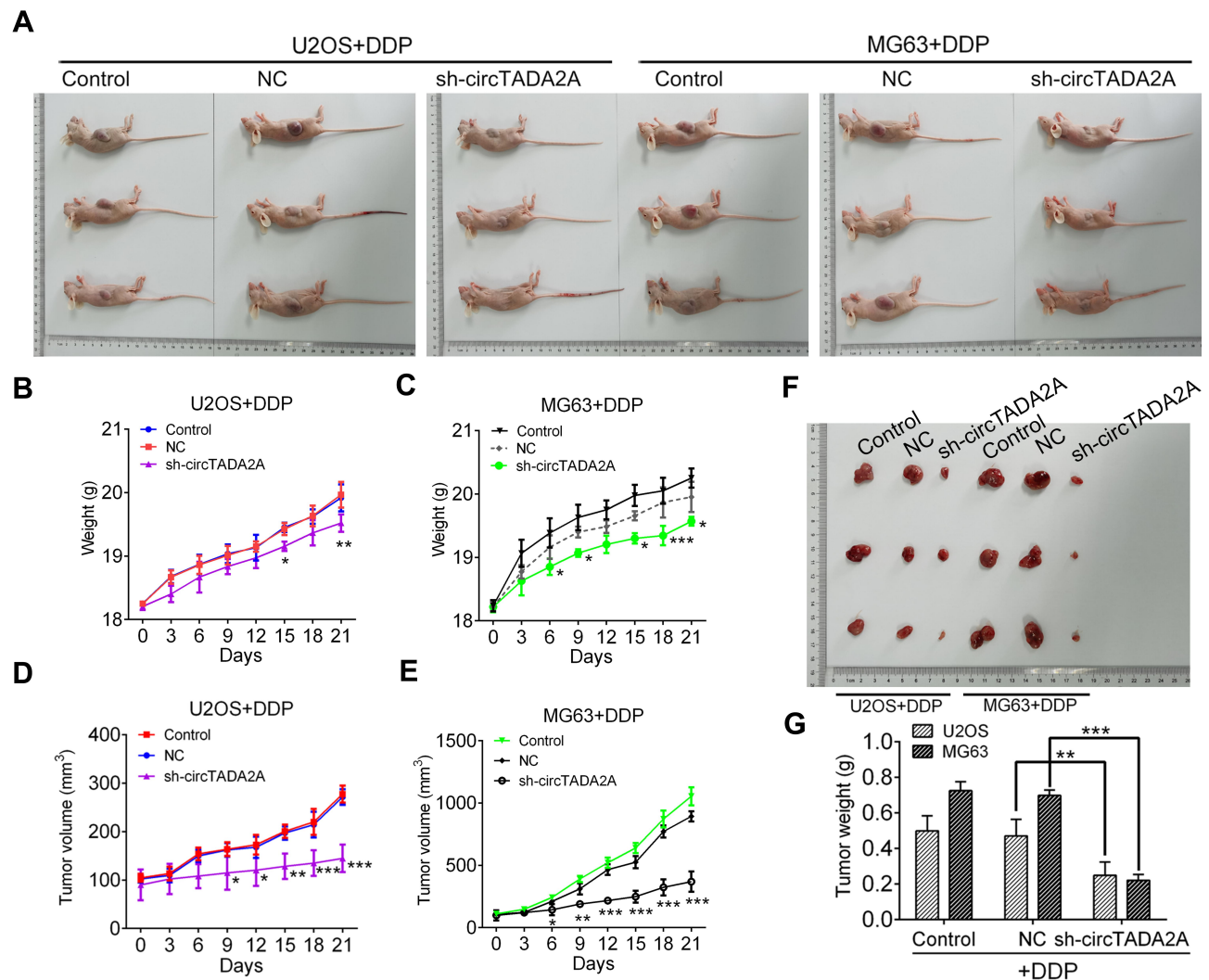


Figure 5 Knockdown of circTADA2A enhanced the sensitivity of U2OS and MG63 xenograft tumors to DDP. **(A)** Representative images showing euthanized nude mice xenografted with U2OS and MG63 cells treated with DDP. **(B and C)** The body weight and **(D and E)** tumor volume were measured every three days following xenograft model establishment. The values are expressed as the mean \pm standard deviation ($n=7$). **(F and G)** At the 21st day following xenograft model establishment, the tumors were isolated and their weights were recorded. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ vs NC.

Abbreviations: circTADA2A, circular RNA transcriptional adaptor 2A; DDP, cisplatin; NC, negative control.

promoting OS cell proliferation and drug resistance. In accordance with previous evidence and our hypothesis, circTADA2A was remarkably upregulated and miR-129-5p was considerably downregulated in U2OS and MG63 cells compared with Hfob1.19 cells. Subsequently, circTADA2A knockdown experiments demonstrated that circTADA2A silencing could inhibit OS cell proliferation and drug resistance. Additionally, circTADA2A knockdown enhanced miR-129-5p expression and reduced that of TRPS1 and YAP1. At the same time, the ratio of p-YAP1 was notably increased following circTADA2A knockdown. The activation of the Hippo signaling pathway is characterized by the upstream kinase-induced phosphorylation of its downstream effectors, including YAP1,

resulting in the nuclear exclusion of YAP1, and ultimately, its cytoplasmic degradation.^{26,27} Taken together, the aforementioned findings indicated that circTADA2A could downregulate miR-129-5p, thereby enhancing YAP1 and TRPS1 expression. However, miR-129-5p downregulation prevented the phosphorylation of YAP1 and its cytoplasmic degradation, thus contributing to OS cell proliferation and drug resistance.

To further confirm the abovementioned findings in vivo, U2OS and MG63 xenograft models were established. Consistent with the in vitro results, circTADA2A silencing could attenuate U2OS and MG63 cell-derived tumor growth and drug resistance. Additionally, circTADA2A knockdown prevented the infiltration of

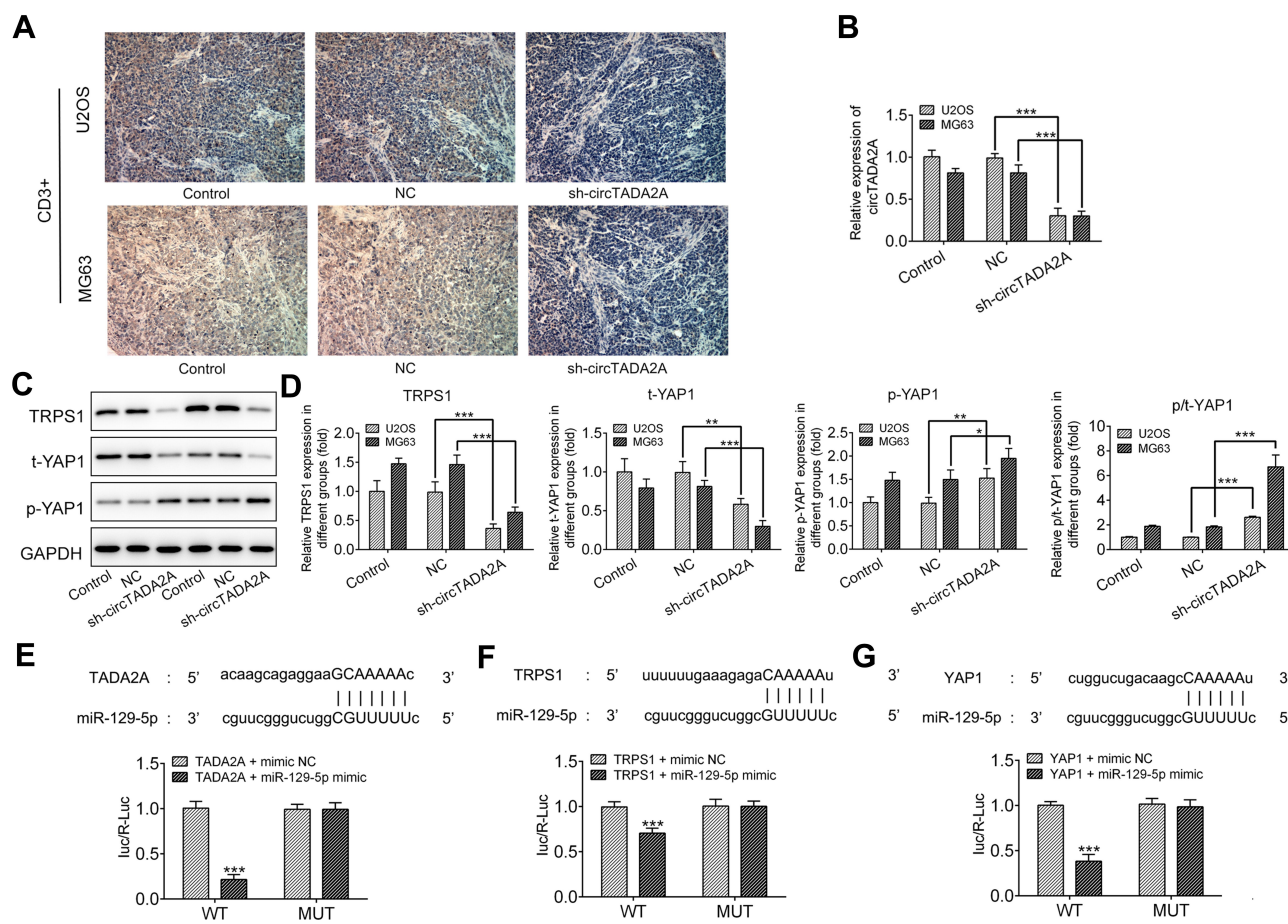


Figure 6 Effect of circTADA2A knockdown on miR-129-5p, TRPS1 and YAP1 expression in xenograft tumor tissues. **(A)** Representative images showing immunohistochemical staining against CD3 in xenograft tumor tissues. **(B)** RNA expression levels of circTADA2A in xenograft tumor tissues. $***P < 0.001$ vs NC. **(C and D)** Protein expression of TRPS1, total (t)-YAP1, phosphorylated (p)-YAP1 and p/t-YAP1 in xenograft tumor tissues. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs NC. **(E - G)** The effect of miR-129-5p on the expression of circTADA2A, TRPS1 and YAP1 was confirmed by luciferase reporter assays. $***P < 0.001$ vs TADA2A + mimic NC. **Abbreviations:** circTADA2A, circular RNA transcriptional adaptor 2A; miR-129-5p, microRNA-129-5p; TRPS1, trichorhinophalangeal syndrome 1; YAP1, yes-associated protein 1; NC, negative control.

CD3⁺ T lymphocytes in tumor tissues, thus confirming the stimulatory effect of circTADA2A on OS tolerance and immune escape.^{28,29} Finally, the direct interaction between miR-129-5p and circTADA2A, TRPS1 and YAP1 was also clarified. Furthermore, the expression of circTADA2A was relatively higher in U2OS cells compared with that in MG63 cells, whereas circTADA2A silencing exerted a relatively stronger inhibitory effect on OS cell proliferation compared with DDP, indicating that circTADA2A could upregulate YAP1 and promote OS cell proliferation. However, further experiments are needed to clarify the significance of YAP1 and TRPS1 expression and their different functions in OS, together with the specific modulatory effect of circTADA2A on the activities of TRPS1 and YAP1.

In summary, the current study uncovered the modulatory effect of YAP1 and TRPS1 on OS cell proliferation and drug

resistance, respectively. Furthermore, this study revealed that circTADA2A could upregulate TRPS1 and YAP1 via sponging miR-129-5p, thus promoting OS cell proliferation and drug resistance. These findings could provide potential therapeutic approaches targeting the circTADA2A/miR-129-5p/YAP1/TRPS1 axis for alleviating OS.

Ethics Statement

The animal experiments involved in this study were approved by the Ethics Committee of the Tumor Hospital of Yunnan Province (KY202018).

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Disclosure

The authors declare that they have no conflicts of interest.

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