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BIRC6 Modulates the Protein Stability of Axin to Regulate the Growth, Stemness, and Resistance of Renal Cancer Cells via the β-Catenin Pathway

Kaihua Zhong,[#] Xiaohong Wang,[#] Heyuan Zhang, Nanhui Chen, Yang Mai, Sipin Dai, Lawei Yang, Dong Chen,[*] and Weifeng Zhong[*]

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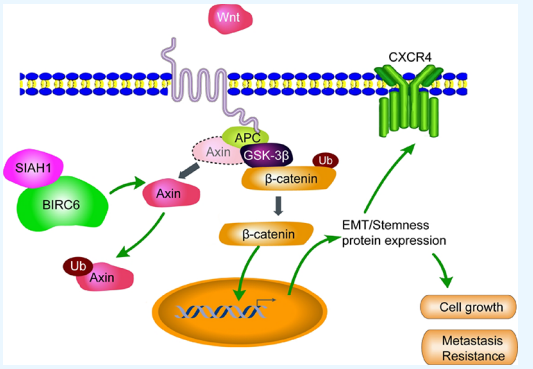
ABSTRACT: The mechanism underlying the development of renal cell carcinoma (RCC) remains unclear, and effective prevention and therapeutic measures are lacking. BIRC6, a protein inhibitor of apoptosis, has attracted great interest. Our data indicated that overexpression of BIRC6 elevated cell growth, colony formation, migration, and invasion of cultured RCC cells, while siRNA knockdown of BIRC6 suppressed these processes. Additionally, BIRC6 was highly expressed in RCC clinical samples along with a downregulated level of Axin. Immunoprecipitation assays found that BIRC6 interacted with Axin and the two proteins colocalized within the cytoplasm of RCC cells. Overexpression of BIRC6 promoted the ubiquitination modification of Axin, while genetic knockdown of BIRC6 suppressed it. Furthermore, overexpression of BIRC6 significantly promoted the turnover of Axin, suggesting BIRC6's inhibitory effect on Axin protein stability. BIRC6 was also upregulated in cancer stem-like cells of RCC and increased the drug resistance of RCC cells against sunitinib. Western blotting assays showed that the overexpression of BIRC6 upregulated CXCR4 protein expression and activated the β-catenin pathway. Two cell lines were then constructed with BIRC6 overexpressed by lentiviruses. Pharmacological administration of a Wnt/β-catenin inhibitor, XAV-939, or genetic knockdown of β-catenin inhibited cell growth, tumor sphere formation, colony formation, migration, and invasion of BIRC6-overexpressed cells. *In vivo* administration of XAV-939 markedly suppressed the tumorigenesis of BIRC6-overexpressed RCC cells in nude mice. In conclusion, we propose that BIRC6 activates the β-catenin signaling pathway via mediating the ubiquitination and degradation of Axin, promoting the growth, stemness, and drug resistance of RCC cells. This project aims to elucidate the role of BIRC6 as a potential therapeutic target and provide new insights into the clinical treatment of RCC.



INTRODUCTION

Renal cell carcinoma (RCC) is a life-threatening tumor that arises from the epithelial part of the renal parenchymal tubules, caused by known or unknown reasons.[1] The etiology and pathogenesis of this disease remain unclear, making it a challenging issue in the diagnosis and treatment of genito-urinary system diseases. At the time of the initial diagnosis, about 20% of patients with kidney cancer already exhibit metastases, and during follow-up after treatment, 20%−40% of patients experience tumor metastasis.[2] The treatment of metastatic renal cancer is currently a challenge, with drugs such as sunitinib being the main therapy, but its efficacy is not significant.[3,4] Therefore, studying the molecular mechanisms of RCC and drug resistance is of great significance for targeted treatment.

Baculoviral IAP repeat-containing 6 (BIRC6) is a member of the inhibitor of apoptosis proteins family and contains a ubiquitin-conjugating enzyme E2 structure domain.[5] BIRC6 possesses both ubiquitin-conjugating enzyme activity and apoptosis-inhibiting function, impeding cell apoptosis by facilitating the ubiquitination and degradation of targeted apoptotic proteins.[6] As an apoptosis inhibitor, BIRC6 plays a significant role in the development of malignancies.[7] BIRC6 regulates the formation and movement of the spindle from the intermediate microtubules to the intermediate ring, participating in the process of cell mitosis.[8] BIRC6 interacts with and promotes p53 degradation, suppressing apoptotic signaling and promoting the occurrence of liver cancer.[9] Recent studies have

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found that BIRC6, as an apoptosis inhibitor, is involved in the initiation and prognosis of nonsmall cell lung, colorectal, and prostate cancer.[10−12] Studies report that BIRC6 can also regulate the process of cell autophagy, promoting cell autophagy by bridging lysosomes and autophagosomes through interaction with the lysosomal protein LAMP2.[13] As a ubiquitin-conjugating enzyme, the discovery of BIRC6's function was relatively late, and research on the mechanisms by which BIRC6 functions in tumor metastasis and drug resistance is currently very limited. In particular, the mechanism of BIRC6 in the RCC is still unknown.

The canonical β-catenin signaling is a critical pathway that modulates cell differentiation, maintains the self-renewal of stem cells, and regulates organ formation.[14] When the ligand binds to Wnt, the APC/Axin/GSK3β degradation complex loses its activity, resulting in the nuclear accumulation of β-catenin and subsequent translation of numerous targeted genes.[15] Many of the gene products induced by the pathway have the capability to induce epithelial-mesenchymal transition (EMT) and promote cell regeneration.[16,17] Cancer stem-like cells (CSCs) enable tumor cells to self-renew and undergo unlimited proliferation and differentiation.[18] Studies have shown the presence of rare subpopulations of stem cells within solid tumors that exhibit resistance to chemotherapy or radiotherapy.[19] The high self-renewal ability of CSCs enables them to contribute to tumor recurrence following treatment.[20] One of the primary challenge in this field is to identify specific markers that can effectively recognize CSCs. Currently, there are no universally applicable markers for the RCC, and it remains unclear whether BIRC6 is involved in the development of CSCs.

The objective of this study is to elucidate the molecular mechanism by which BIRC6 regulates Axin protein stability, leading to activation of the β-catenin pathway and contributing to the development, stemness, and drug resistance of RCC.

MATERIALS AND METHODS

Ethics Statement. Clinical samples of renal cell carcinoma and three adjacent control samples were collected from Guangzhou 12th People's Hospital. Prior to commencing the study, written informed consent was obtained from all patients. All experiments involving human samples were performed following the guidelines outlined in the Declaration of Helsinki and were approved by the committee of Guangzhou 12th People's Hospital. BALB/c nude mice, weighing 20−22 g and aged 6−8 weeks, were obtained from the Guangdong Medical Laboratory Animal Center (No. SCXK[Yue] 2009−0023). Animal experiments were performed in accordance with the Animal Committee of Guangzhou 12th People's Hospital.

Cells and Treatment. Human RCC cell lines 786-O and ACHN were obtained from the Shanghai Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Both cell lines were cultured in DMEM medium (Gibico, Grand Island, NY, USA) with 10% FBS (Gibco) in a humidified atmosphere incubator at 37 °C with 5% CO2. When the cells reached over 80% confluence, they were treated with XAV-939 (purchased from Selleckchem, Houston, TX, USA; 10 μM dissolved in DMSO) or cycloheximide (CHX) obtained from MedChemExpress (Shanghai, China; 20 μg/mL).

Plasmid, siRNA, and Lentiviral Infection. BIRC6 cDNA was cloned and inserted into the GFP-C1 or Flag vector to construct BIRC6-GFP or Flag-BIRC6 encoding plasmids, respectively. Axin was inserted into a Myc vector, and SIAH was inserted into a HA vector to generate Myc-Axin and HA-SIAH encoding plasmids. All constructed plasmids were confirmed by sequencing. The lentiviruses overexpressing BIRC6 were obtained from GeneCopoeia (Guangzhou, China). To establish stable RCC cell lines with BIRC6 overexpression, cells were infected with the lentiviruses in the presence of puromycin (10 μg/mL), following the manufacturer's instructions. For siRNA experiments, siRNA fragments against BIRC6 were purchased from GenePharma (Shanghai, China). Cells were transfected with Lipofectamine 2000 (Invitrogene, Carlsbad, CA, USA) at a concentration of 100 pmol/well of siRNA fragments. The specific siRNA sequence for si-BIRC6 was 5′-CUCAGGAGAGUACUGCUCAdTdT-3′, which was previously validated in other reports[8] and in our previous study,[21] and the si-β-catenin sequence was 5′-CATGUGUTGGUAAGCUCUA-3′.[22] A scramble sequence was used as a negative control (NC).

Western Blotting. The Western blotting assay was conducted as previously reported.[23] Cell collection was performed using RIPA lysis buffer (Beyotime, Shanghai, China) followed by protein concentration determination using a BCA protein assay kit (Beyotime). Subsequently, 30 μg of proteins was loaded for electrophoresis on a 10% SDS-polyacrylamide gel and transferred onto PVDF membranes. After blocking, membranes were cut according to the appropriate molecular range and incubated with primary antibodies overnight at 4 °C as follows: anti-BIRC6 (#ab291072; 1:1000), anti-SIAH1 (#ab305249; 1:1000), anti-Axin (#ab115205; 1:500), anti-CD105 (#ab252345, 1:1000), anti-CD44 (#ab254530; 1:1000), and anti-MMP-7 (#ab207299; 1:1000) were purchased from Abcam (Cambridge, MA, USA), anti-Myc (#AF2867; 1:1000), anti-HA (#AF0039; 1:1000), anti-Flag (#AF0036; 1:1000), anti-β-catenin (#AF0066; 1:1000), anti-CXCR4 (#AF6621; 1:1000), and anti-GAPDH (#AF2819; 1:1000) were purchased from Beyotime. Horseradish peroxidase-conjugated secondary antibodies (mouse, rabbit, or rat; purchased from Beyotime) were then applied to the membranes, which were processed by using the ECL kit (Beyotime) accordingly. Then, the chemiluminescence was exposed to films by manual exposure of the developer and fixer solution in a dark room. The intensity was measured by ImageJ (NIH, USA) and normalized with the loading control and compared with the control group as 100%.

Cycloheximide (CHX) Chase Assay. The turnover rates of Axin protein were determined by using cycloheximide to inhibit protein synthesis. RCC cells were first transfected with BIRC6 or the vector control. Twenty-4 h later, cells treated with 50 μg/mL cycloheximide (Sigma) were harvested at different time points after removing the cycloheximide inhibition. Then, the sample lysates were collected and subjected to Western blotting with an Axin antibody. Band densities were normalized to the one at the 0 time point, which was set at 100%.

MTT Analysis. An MTT assay kit (Sigma-Aldrich, MO, USA) was used to assess cell viability. Cells that were transfected or treated with drugs were cultured in 96-well plates at a density of 5×10^3 cells/well and incubated for 24, 48, and 72 h. After incubation, the cells were exposed to MTT solution (10 μL/well) and then incubated for 4 h at 37 °C. To stop the reaction, 100 μL of DMSO was added to each well at room temperature for 10 min. The optical density (OD) values

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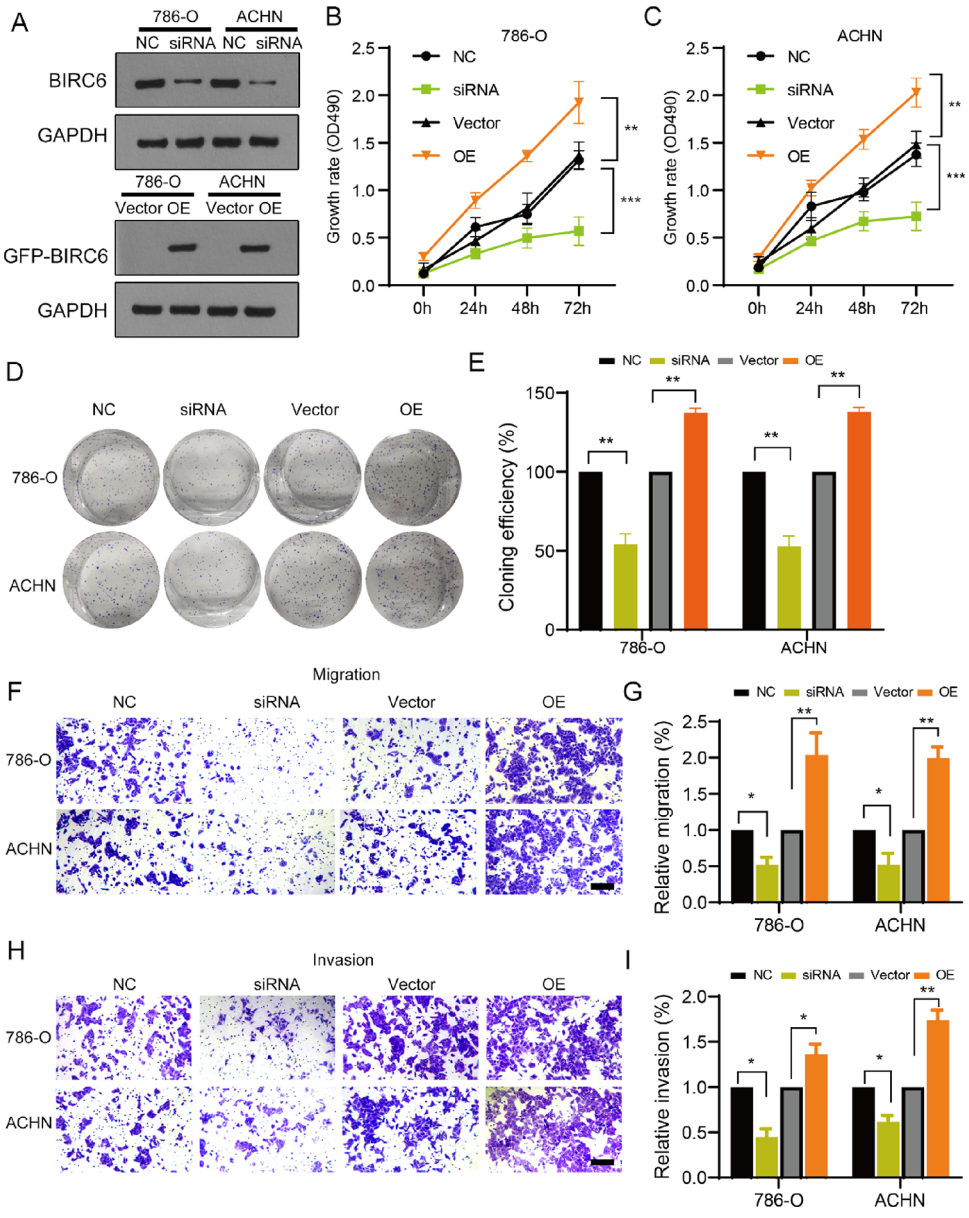

Figure 1. BIRC6 plays a critical role in the growth, colony formation, migration, and invasion of RCC cells. (A) BIRC6 siRNA transfection significantly suppresses endogenous levels of BIRC6 in 786-O and ACHN RCC cell lines. 786-O and ACHN RCC cell were transfected with BIRC6 siRNA fragments or the negative control (NC), or BIRC6 overexpression plasmid or the control vector (GFP). Transfected cells were subjected to western blotting with the staining of BIRC6 and GFP antibodies. 786-O and ACHN RCC cell were treated as in (A), then cells were subjected to MTT assay (B−C) to evaluate the cell growth rate, and to colony formation assay (D, E), and to Transwell assay to show the migration and invasion ability in both cell lines (E−I). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; scale bar, 10 μm.

were obtained, and absorbance data were collected at 490 nm by using a microplate reader.

Flow Cytometry. To isolate a cell group of CSCs from renal carcinoma tissues, the tissue specimens were minced into small cube chunks and enzymatically dissociated into single cells. Subsequently, the cells were incubated with CD105-FITC antibody (BioLegend, CA, USA; clone 43A3; 5 μg/test) and CXCR4-APC antibodies (Thermo Fisher Scientific; clone 2B11; 5 μg/test). The cells were then analyzed using a FACSCalibur system to isolate the desired cell type, with a minimum of 10,000 cells measured per analysis.

Co-immunoprecipitation Assay. For immunoprecipitation, RCC cells were lysed with an RIPA buffer (Beyotime) containing a protease inhibitor cocktail. Then, the supernatants were incubated with 2 μg of anti-BIRC6 antibody (Abcam), using IgG antibody as a negative control, overnight at 4 °C with rotation. The supernatants were then added to Pierce Protein A/G Beads (10 μL; Thermo Fisher) and rotated at 4 °C for an additional 6 h. Later, the beads were washed, and protein loading buffer was added, followed by denaturation at 100 °C for 10 min. Subsequently, the lysates were subjected to western blotting analysis. For the overexpression immunoprecipitation assay, RCC cells overexpressed with Myc-Axin, HA-SIAH, and Flag-BIRC6 were incubated with 2 μg of Flag antibody (Beyotime) followed by staining with anti-Myc, anti-HA, and anti-Flag antibodies (1:1000; all from Beyotime) for western blotting.

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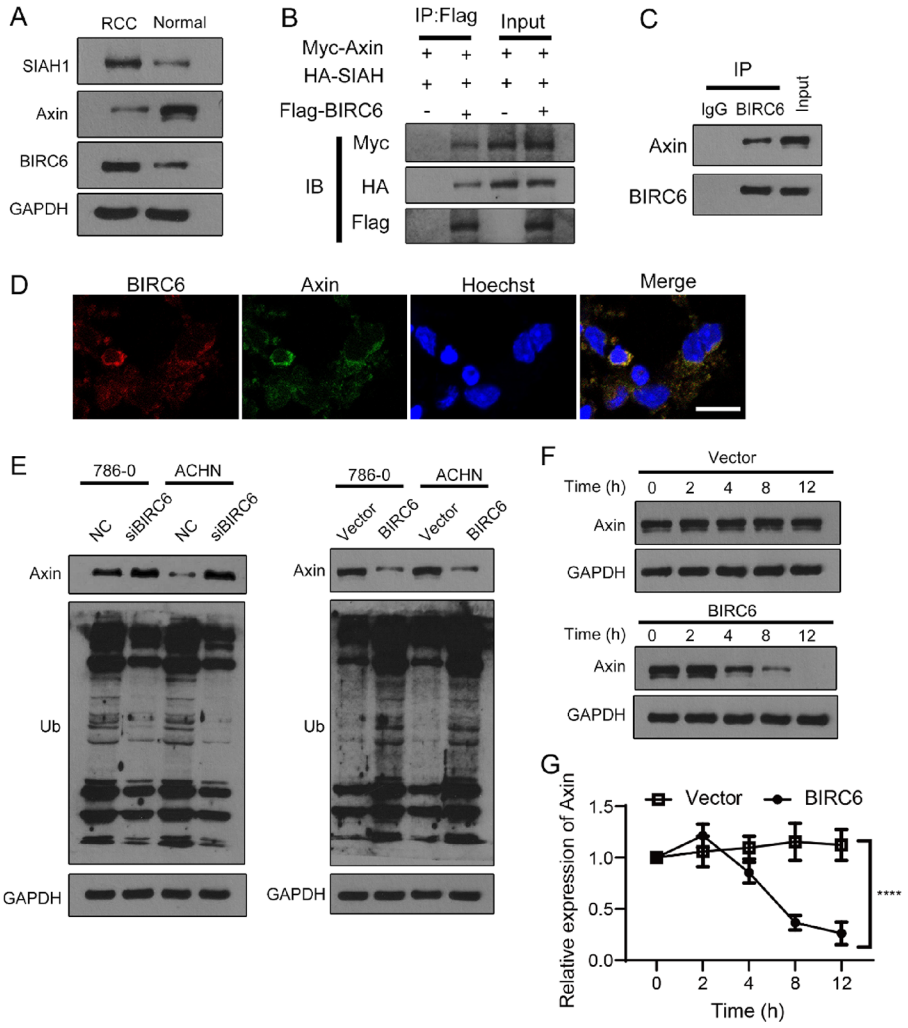

Figure 2. BIRC6 interacts with and regulates the protein stability of Axin. (A) Western blotting assay of clinical RCC samples or the adjacent non-RCC samples (Normal group) shows upregulation of BIRC6 expression level in RCC tissues, along with upregulated levels of SIAH1 and downregulated levels of Axin. (B) Immunoprecipitation assay with Flag antibody shows physical interaction between Myc-Axin, HA-SIAH, and Flag-BIRC6 in RCC cells. Sediments after immunoprecipitation were subjected to western blotting with antibodies against Myc, HA and Flag. (C) Endogenous immunoprecipitation assay with BIRC6 antibody, the sediments were subjected with Western blotting with antibody of Axin, showing Axin protein detected in the sediments. (D) Immunocytochemistry assay with BIRC6 (Red) and Axin (Green) antibodies confirms the interaction between BIRC6 and Axin in the cytoplasm of RCC cells. Blue indicates the nucleus. Scale bar, 10 μm. (E) RCC cells were transfected with BIRC6 siRNA fragments or overexpression plasmid, the cell lysates were subjected to western blotting with Axin and Ub antibodies. (F, G) CHX-chase assay shows overexpression of BIRC6 markedly promotes the degradation of Axin protein. ***$P < 0.001$.

Immunocytochemistry. To conduct immunocytochemistry, treated cells were fixed with 4% paraformaldehyde (PFA) for 40 min at 4 °C. Subsequently, the coverslips were blocked and then exposed to primary antibodies, specifically anti-BIRC6 (#ab291072; 1:1000; from Abcam) and anti-Axin (#ab115205; 1:500; from Abcam), and incubated overnight at 4 °C. Then, the cells were treated with secondary antibodies (1:5000 dilution): antirabbit Alexa 488 (#A-11008; Thermo Fisher, MA, USA) and donkey anti-Goat IgG Alexa 555 (#A-21432; from Thermo Fisher) for 1 h at room temperature. Finally, the cells were covered with Fluoro-Gel II containing DAPI (from Electron Microscopy Sciences, PA, USA). Images were captured, and colocalization analysis was carried out using a Carl Zeiss LSM 780 confocal microscope (Zeiss, Germany).

Colony Formation Assay. RCC cells under different treatments were seeded into 6-well plates at approximately 1000 cells/well. The cells were then cultured for an additional 10 days until visible colonies could be seen with the naked eye. Afterward, the colonies were gently washed and fixed with ethanol for 30 min. Following another round of washing, the colonies were stained for 20 min with a 0.1% crystal violet solution (Beyotime). The colony numbers in each group were counted, and the control group was set to 100% for normalization.

Tumor Sphere Formation Assay. The RCC cells under different treatments were cultured in a 6-well low-attachment plate at a density of 5,000 cells/well, in a serum-free DMEM medium (Gibco) supplemented with 20 ng/mL EGF, 4 mg/mL insulin, B27 (1:50 dilution), 20 ng/mL bFGF, and 0.4% BSA. The medium was half-replaced every other day. Tumor sphere formation was detected, and images were captured under a light microscope (Zeiss). The size of the tumor sphere was measured for each group.

Migration and Invasion Assay. RCC cells were processed by using a Transwell system (Corning, Cambridge,

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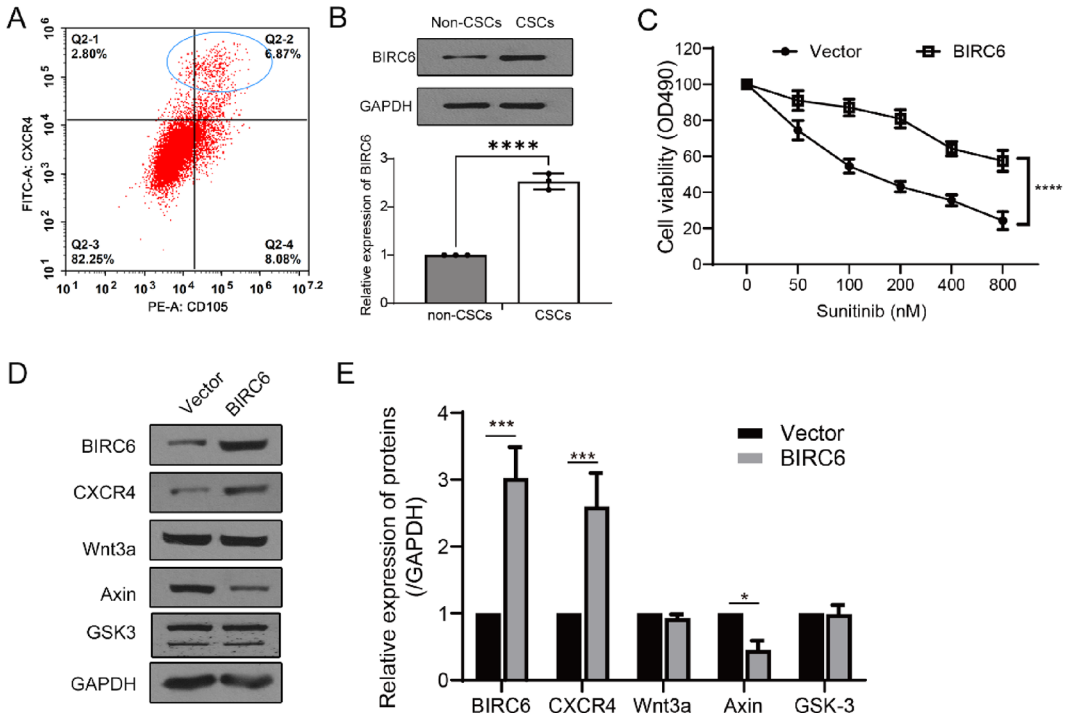

Figure 3. BIRC6 enhances stemness and drug-resistance of renal cell carcinoma cells by regulating CSCs. (A) RCC tissues were digested and subjected to flow cytometry with the antibody of CD105 and CXCR5, a population of CD105+ and CXCR4+ cells were successfully isolated from RCC cells, which retained the characteristics of CSCs. (B) Western blotting assay of CSC lysates showed that BIRC6 was overexpressed in CSCs. (C) Enhanced overexpression of BIRC6 in CSCs significantly further promoted cell viability. (D, E) CSCs were transfected with BIRC6 plasmid, and the cell lysates were subjected to Western blotting. Quantified data of (D) showed that overexpression of BIRC6 markedly upregulated the levels of CXCR4 and downregulated the level of Axin protein in CSCs.

MA, USA). Following treatment, cells were added to the upper chamber of a 24-well Transwell plate and cultured for 24 h at 37 °C. Only the lower chamber contained 10% FBS, while the upper chamber was serum-free. For invasion assays, the Transwell chamber was coated with 100 μL of Matrigel (BD Biosciences, Bedford, MA). After the assay, we removed the Matrigel and noninvaded cells from the upper surface of the filters. The upper surface of the membrane was cleared, and the remaining cells that had migrated or invaded into the lower surface were then fixed and stained before being counted under a microscope.

Xenograft Mouse Model. Cells were prepared for injection into mice by mixing control cells or stable 786-O cells with BIRC6 cells with 50 μL of matrix gel and 50 μL of PBS solution. The resulting mixture was then injected subcutaneously into the lower limb of mice at a concentration of 1×10^6 cells. Following injection, mice were treated with XAV-939 (2.5 mg/kg) or a control solution via intraperitoneal injection every other day. Tumor volume data were collected every 5 days, measured by the following formula: volume = $(L \times W^2)/2$, where L and W represent the longest and shortest diameters. After 25 days, mice were euthanized, and tumor samples were collected for quantification of the tumor weight and western blotting analysis.

Statistical Analyses. The data are presented as mean ± SD, collected from a minimum of three independent experiments. Statistical analyses were performed using SPSS 27.0, and the normality of the data was tested using the Shapiro−Wilk W method. Single comparisons were analyzed with a t test, while multiple comparisons were analyzed using one-way analysis of variance. A p value < 0.05 was considered statistically significant.

RESULTS

BIRC6 Is Necessary and Sufficient for the Development of RCC Cells. To investigate the impact of BIRC6 on RCC cell development, we constructed an overexpression plasmid for BIRC6 and synthesized a verified siRNA fragment against BIRC6. Following the transfection of the siRNA fragment and the BIRC6 overexpression encoding plasmids into two RCC cell lines, we assessed the cell development. As illustrated in Figure 1A, the endogenous levels of BIRC6 in the 786-O and ACHN cell lines were significantly suppressed with the siRNA fragment, and the western blotting assay confirmed the successful transfection of the BIRC6 overexpression encoding plasmids. The growth rate, as revealed by the MTT assay (Figure 1B,C), demonstrated that overexpression promoted cell growth, while genetic knockdown of BIRC6 suppressed the growth of both cell lines. The colony formation assay (Figure 1D,E) indicated that the siRNA against BIRC6 inhibited colony formation, whereas the overexpression of BIRC6 promoted colony formation in both cell lines. A similar trend was also observed in the cell migration and invasion assays, as revealed by the Transwell assay (Figure 1E−I). All of these data collectively suggest that BIRC6 plays a crucial role in the growth, colony formation, migration, and invasion of RCC cells, highlighting its significance in these cellular processes.

BIRC6 Interacts with Axin and Regulates Its Protein Stability. To delve into the detailed mechanism of BIRC6 involvement in RCC cell development, clinical RCC samples

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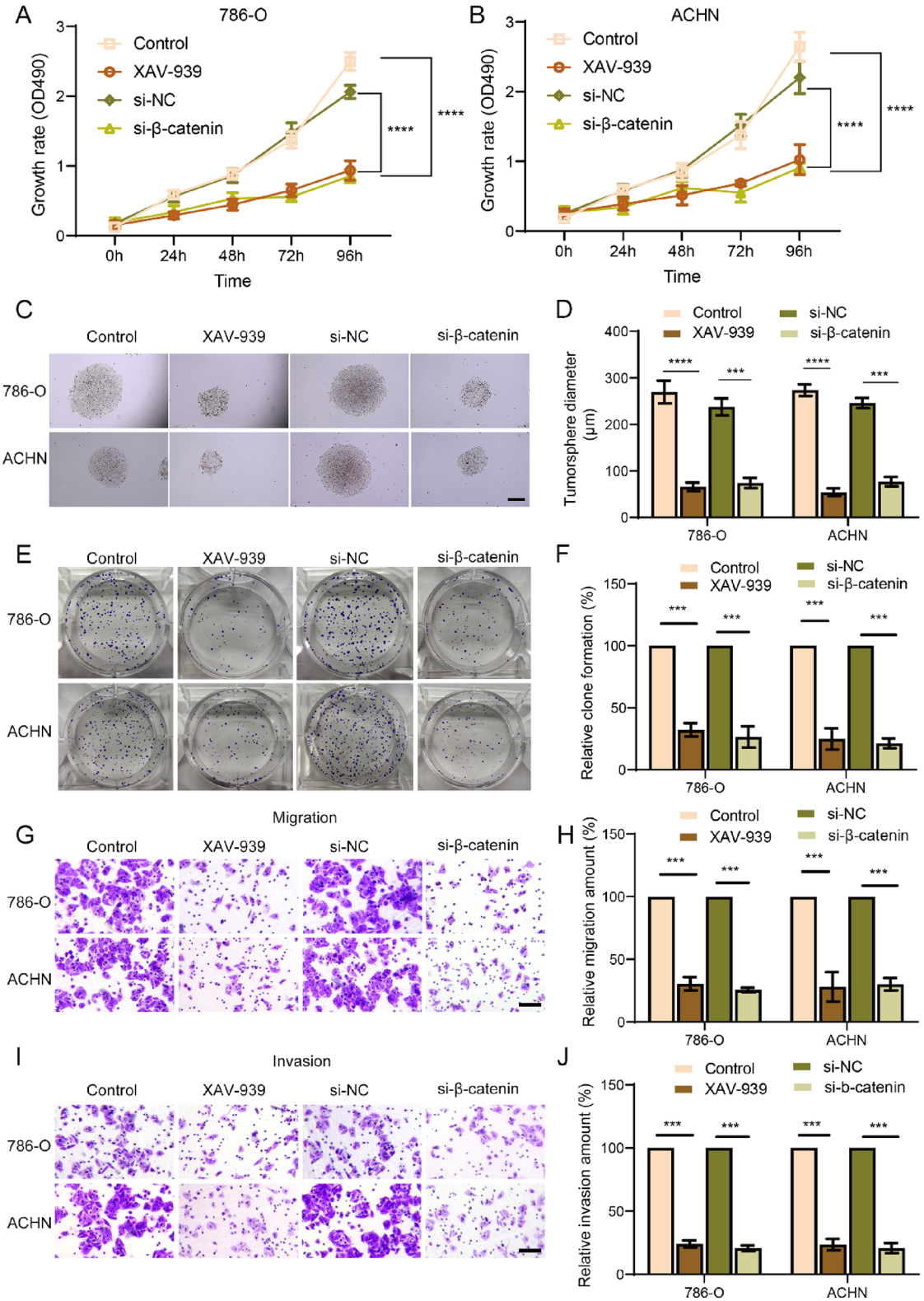

Figure 4. BIRC6 regulates the development of RCC cells via Wnt/β-catenin pathway. Lentiviral constructed RCC cell lines with BIRC6 overexpressed were administrated with XAV-939 or transfected with siRNA fragments against β-catenin, then cells were subjected to MTT assay to evaluate the cell growth (A, B), tumor sphere formation assay (C, D), colony formation assay (E, F), Transwell assay for migration (G, H), and invasion (I, J). *P < 0.05, **P < 0.01, ***P < 0.001; scale bar, 10 μm.

were collected and subjected to a western blotting assay. As illustrated in Figure 2A, the staining results revealed a significant upregulation of BIRC6 expression in RCC tissues, accompanied by an upregulation of SIAH1 and a down-regulation of Axin. Considering BIRC6 as an E3 ligase, we aimed to investigate whether BIRC6 regulates the ubiquitination of the Axin protein. First, we sought to determine whether BIRC6 physically interacts with the Axin protein. RCC cells

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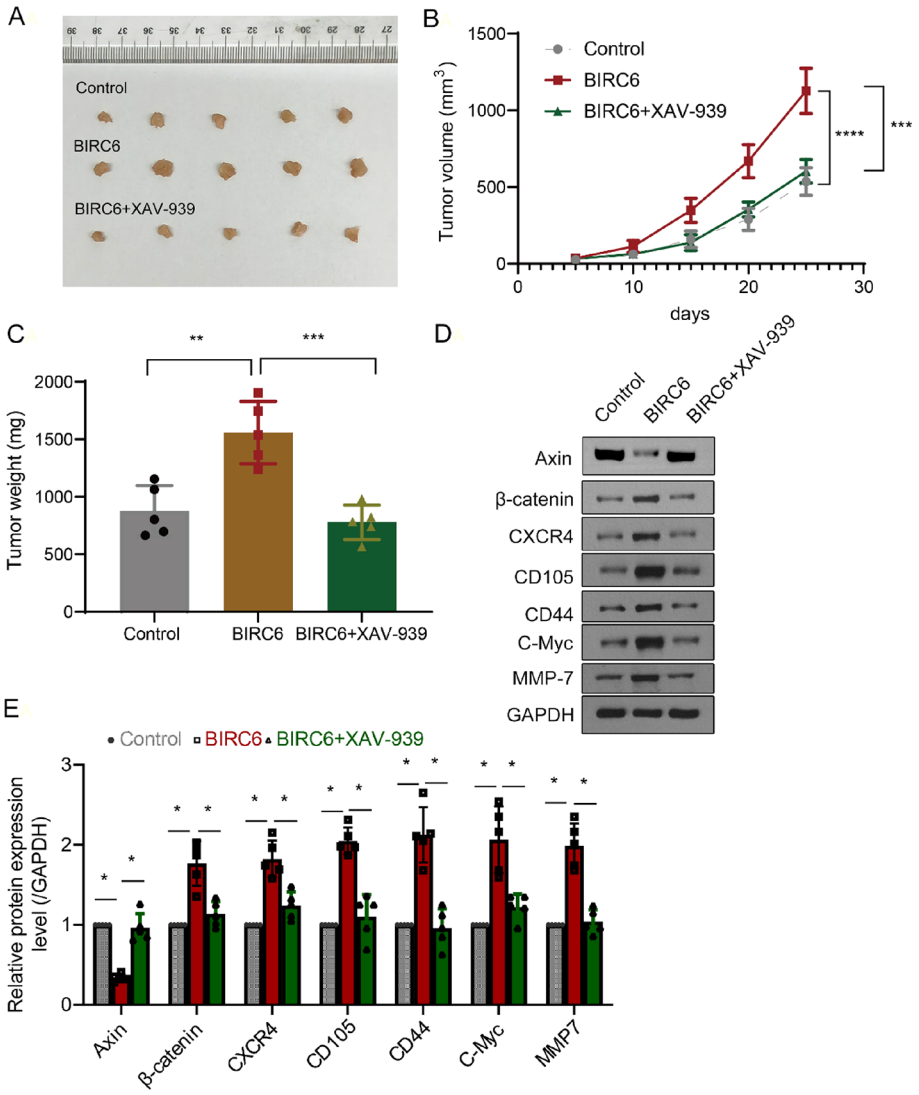

Figure 5. BIRC6 modulates the tumorigenesis of RCC cells *in vivo*. (A) 786-O with BIRC6 stable-overexpression cell lines or control cells were injected into the lower limbs of nude mice, and XAV-939 were administrated every other. Tumor tissues were dissected 25 days later. (B) Tumor volume at each time point was recorded. (C) Tumor weight was measured for each group. (D, E) We performed western blot analysis on the dissected tumor tissues using antibodies against specific proteins. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

were overexpressed with Myc-Axin, HA-SIAH, and Flag-BIRC6, and the lysates were subjected to an immunoprecipitation (IP) assay using Flag antibody. As depicted in Figure 2B, the Myc and HA signals were detected in the IP sediments using the Flag antibody. Additionally, we performed an endogenous IP using the BIRC6 antibody. Figure 2C shows that the Axin protein was detected in the IP sediments using the BIRC6 antibody. The immunocytochemistry assay of RCC cells revealed that endogenous BIRC6 interacts with Axin within the cytoplasm (Figure 2D). These results collectively indicate a direct interaction between BIRC6 and Axin.

Next, we investigated whether BIRC6 regulates the ubiquitination of Axin. Figure 2E demonstrates that the genetic knockdown of BIRC6 significantly upregulated the protein level of Axin by suppressing the ubiquitination level. Conversely, overexpression of BIRC6 downregulated Axin protein levels by upregulating the ubiquitination level. Furthermore, using the administration of CHX (cycloheximide), we examined the protein degradation rate of Axin. As shown in Figure 2F,G, the CHX-chase assay revealed that overexpression of BIRC6 markedly promoted the degradation of the Axin protein. These data collectively indicate that BIRC6 interacts with Axin and ubiquitinates Axin to regulate the protein stability of Axin.

BIRC6 Enhances the Stemness and Drug-Resistance of RCC Cells by Regulating CSCs. To determine the role of BIRC6 in Cancer Stem Cells (CSCs) derived from RCC cells, we initially attempted to isolate CSCs from RCC cells. As demonstrated in Figure 3A, using the markers CD105 and CXCR4, we successfully detected a distinct population of CD105+ and CXCR4+ cells that exhibited the characteristic features of CSCs. These isolated CSCs were then cultured to establish a cell line. Subsequently, the lysates of the CSCs were subjected to a western blotting assay, revealing the overexpression of BIRC6 in CSCs. Notably, the enhanced overexpression of BIRC6 in CSCs significantly promoted cell viability, as illustrated in Figure 3C. Additionally, BIRC6 overexpression led to a marked upregulation of CXCR4 levels and a downregulation of Axin protein levels but without any effect on the levels of Wnt3a and GSK-3 proteins (Figure

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3D,E). These data strongly suggest that BIRC6 is upregulated in CSCs and enhances the stemness and drug resistance of RCC cells.

BIRC6 Regulates the Development of RCC Cells via the β-Catenin Pathway. Given that Axin is a critical component of the β-catenin pathway, we investigated whether BIRC6 affects this pathway to regulate the development of RCC cells. Using lentiviruses, we stably overexpressed BIRC6 in the two RCC cell lines. Subsequently, the cells were treated with an inhibitor of the β-catenin XAV-939,[24] or cotransfected with si-β-catenin fragments. As depicted in Figure 4A,B, cells treated with XAV-939 or cotransfected with si-β-catenin fragments exhibited a decreased growth rate. Furthermore, the tumor sphere formation assay demonstrated that the administration of XAV-939 or genetic knockdown of β-catenin significantly suppressed the tumor sphere size in both cell lines (Figure 4C,D), indicating suppression of tumor stemness upon manipulation of β-catenin. The colony formation assay (Figure 4E,F), migration assay (Figure 4G,H), and invasion assay (Figure 4I,J) exhibited similar trends, wherein treatment with XAV-939 or genetic knockdown of β-catenin resulted in the suppression of colony formation, migration, and invasion of BIRC6-overexpressed RCC cells. In summary, these findings indicate that inhibition of the β-catenin pathway counteracts the promoting effect of BIRC6 on the development of RCC cells.

BIRC6 Modulates the Tumorigenesis of RCC Cells *In Vivo*. To assess the impact of BIRC6 *in vivo*, a tumorigenesis assay was performed in nude mice. 786-O cells with stable overexpression of BIRC6 were injected into the lower limbs of the nude mice. Subsequently, the mice were treated with or without XAV-939 every other day for a duration of 25 days. Then, the mice were sacrificed, and tumor samples were subjected to western blotting analysis. As depicted in Figure 5A, cells with BIRC6 overexpression significantly promoted tumorigenesis, resulting in increased tumor volume and tumor weight (Figure 5B,C). Administration of XAV-939 significantly suppressed the tumorigenic ability of these cells, leading to a decreased tumor volume and size (Figure 5A−C). Moreover, the Western blotting analysis revealed that the expression levels of β-catenin, CXCR4, CD105, CD44, c-Myc, and MMP-7 were all significantly upregulated in tumor tissues with BIRC6 overexpression. However, the inhibition of β-catenin by the administration of XAV-939 could effectively suppress the expression levels of these proteins (Figure 5D,E). These data suggest that BIRC6 regulates the tumorigenesis of RCC cells in vivo by modulating the β-catenin pathway.

DISCUSSION

The complex diagnosis of renal cell carcinoma (RCC), coupled with its asymptomatic nature, contributes to its status as the second most dangerous disease among urinary tract tumors.[25] However, the mechanism underlying the involvement of BIRC6 in the RCC development remains unclear. Here, we aimed to investigate the potential role of BIRC6 in promoting the growth, stemness, and drug resistance of RCC cells. Our findings revealed that BIRC6 overexpression promoted cell growth, colony formation, and the metastatic properties of RCC cancer cells, while genetic knockdown of BIRC6 suppressed these processes. Furthermore, a highly expressed level of BIRC6 in clinical samples of RCC and its upregulation in cancer stem-like cells of RCC were observed. Importantly, we uncovered that BIRC6 activates the β-catenin pathway by mediating the ubiquitination and degradation of Axin, which consequently enhances the growth and stemming of RCC cells. The summarized process is depicted in Figure 6.

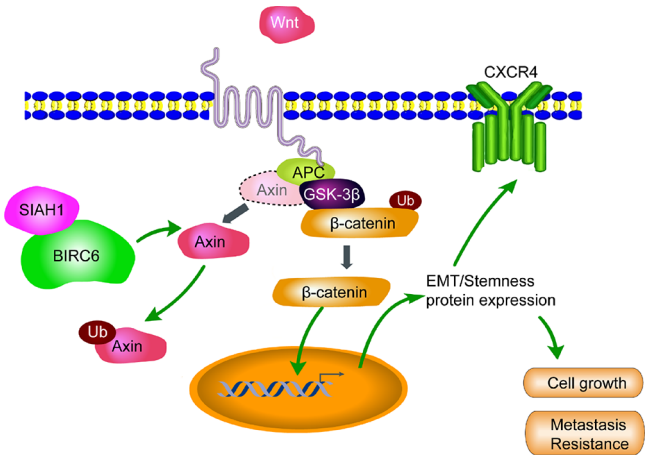

Figure 6. Schematic summary presents that BIRC6 modulates protein stability of Axin to promote the growth and stemness of RCC cells via the Wnt/β-catenin pathway. Upregulation of BIRC6 leads to the ubiquitination and degradation of Axin, resulting in the accumulation of β-catenin and the activation of targeted gene translation. This process ultimately leads to cancer stemness through the overexpression of CXCR4, as well as cancer growth, stemness, and resistance.

The apoptosis inhibitory protein family (IAPs), also known as baculoviral inhibitors of apoptosis repeat-containing (BIRC) proteins, is a crucial group of antiapoptotic proteins found in a variety of organisms such as viruses, yeast, nematodes, and mammals.[26] There are eight identified BIRC proteins, most of which provide protection against or delay in cell death induced by apoptotic stimuli when expressed at high levels.[5] Additionally, most of these proteins can bind to specific caspases to mediate the antiapoptosis effects.[6] BIRC6 is a member with the largest molecular weight and is the only one known to be linked to the membrane so far.[27] In addition to the BIRC region that is specific to the family, BIRC6 also possesses a ubiquitin-conjugating enzyme E2 (UBC) domain, indicating its involvement in ubiquitin ligase activity.[7] Studies have demonstrated that the UBC region within the BIRC6 structure has the capability to function as both E2 and E3 enzymes in the ubiquitination pathway.[28] It can also interact with various proteins within the caspase family, including caspase-3 and caspase-9 as well as Smac among others.[28] These interactions facilitate degradation via the ubiquitin−proteasomal system.[29] BIRC6 is critical for various types of cancers. It is highly expressed in different tumor cells and tissues, and its antiapoptotic function has been confirmed in glioma,[30,31] lung cancer,[32] cervical cancer,[33] and breast cancer.[34] The down-regulated expression of BIRC6 in breast and lung cancer cells increases the stability of p53 and activates caspase-3 in the mitochondrial apoptosis pathway, indicating that p53 is a downstream gene of BIRC6.[32,35] Increased BIRC6 expression has been observed in colon cancer[36,37] and primary acute lymphoblastic leukemia in children.[38,39] Upregulated mRNA levels of BIRC6 were related to poor prognosis, including increased lymphocyte counts, extramedullary disease, adverse reactions to chemotherapy, and a higher three-year recurrence rate.[38,39] Increased BIRC6 expression in nonsmall cell lung

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cancer is also related to resistance to chemotherapy drugs.[40] Moreover, BIRC6 has been identified as a significantly increased peptide spectrum in the serum of liver cancer patients.[41] Large sample verification confirmed significant differences in BIRC6 expression between liver cancer and adjacent tissues.[42] These reports indicate that BIRC6 may be a potential diagnostic marker for cancers.[29] Here, BIRC6 was found to be highly expressed in RCC tissues and cell lines. Genetic inhibition suppressed, while overexpression of BIRC6 promoted the development of RCC cells. Our data are in line with the previous reports mentioned above, supporting the notion of BIRC6's oncogenic role. SIAH1 has been reported to promote the Wnt/β-catenin signaling,[43] and our results showed that upregulation of BIRC6 level was accompanied by the upregulation of SIAH and downregulation of Axin in RCC tissues. Thus, we asked whether SIAH should have a direct connection with the Axin protein. In the detailed mechanism, we found that BIRC6, by the affiliation of SIAH1, interacted with Axin and promoted the ubiquitination of Axin to restore the level of β-catenin. Overexpressed levels of BIRC6 promoted the stemness of RCC and drug resistance against sunitinib. BIRC6, as a giant E2/E3 ubiquitin ligase, could ubiquitinate many of its substrates, such as p53 in hepatocellular carcinogenesis.[9] Here, in our study, we did not determine the protein levels of other BIRC6 substrates, which is worth exploring in our future study.

The canonical Wnt signaling involves the disruption of the Axin/APC/GSK-3β complex and the cytoplasmic accumulation of β-catenin upon interaction between Wnt with its receptor.[44] Subsequently, β-catenin goes nucleic translocation, triggering the translation of downstream genes.[45] The regulation of ureteric bud development and the modulation of nephrogenesis in mesenchymal cells are key functions of this pathway, essential for kidney organogenesis.[46] Axin is an important negative regulator in the Wnt signaling pathway, possessing multiple functional domains that allow interaction with various proteins.[47] Besides, Axin may also function as a molecular partner of β-catenin, regulating the distribution of β-catenin between the cytoplasm and the nucleus.[48,49] Axin/APC/GSK-3β complex ensures that the concentration of β-catenin in the cell remains very low, guaranteeing the normal physiological and biochemical functions of the cell. Dysregulation of Axin, such as abnormal mutation, expression, or degradation of Axin, would result in tumorigenesis in many cancers. In hepatocellular carcinoma, mutations in Axin1 and Axin2 may also alter the Wnt signaling pathway, leading to the accumulation of β-catenin and thereby triggering hepatocellular carcinoma.[50] In colorectal cancer, mutations can result in the transcription of immature Axin protein, leading to the loss of the DIX domain on Axin. This loss, in turn, diminishes the negative regulatory role of Disheveled (DVL) on Axin.[51] Ubiquitination has widely reported to regulate the level of Axin, such as RNF146[52] and Smurf1.[53] And USP proteins such as USP7[54] and USP34[55] would stabilize the protein level of Axin to inhibit Wnt signaling. Here, in this study, we found that BIRC6, acting as an E3 ligase, interacted to regulate the ubiquitination level of Axin in RCC cells. Our study supplements the mechanism of Axin-regulated β-catenin signaling in RCC cells.

The Wnt/β-catenin aberrant modulation the has been related with many cancers, such as breast, colorectal, and renal cell carcinoma.[56] In RCC cells, the levels of Wnt, Wnt antagonists, and Wnt receptors have been shown to contribute to oncogenesis.[45] High Wnt1 expression in clear cell RCC (ccRCC) has been associated with aggressiveness and more advanced stage.[57] Similarly, Wnt10a expression is significantly increased in RCC cell lines and tissues, and is demonstrated as a risky factor for RCC development.[58] Cytoplasmic β-catenin is as a highly promising candidate linked to the clinicopathology and prognosis of patients with RCC.[57] High levels of cytoplasmic β-catenin in patients tends to have larger tumor diameters, more advanced disease stages, and vascular invasion.[57] The application of multilayer omics analysis in RCC has provided substantial evidence confirming the pivotal role of the β-catenin signaling in the pathogenesis of RCC.[59] Our data revealed that the β-catenin signal is regulated by the protein stability of Axin, which is influenced by BIRC6. The overexpression of BIRC6 enhanced the proliferation of RCC cells, but this effect could be mitigated by blocking the β-catenin pathway, through either pharmacological means or genetic knockdown approaches. Furthermore, this intervention also suppressed in vivo tumorigenesis in nude mice through administration of XAV-939.

In general, this study demonstrated the role of BIRC6 in regulating the protein stability of Axin and controlling the development of RCC cells. It was found that BIRC6 activates the β-catenin pathway by mediating the ubiquitination and degradation of Axin, thus promoting the growth, stemness, and drug-resistance of RCC cells. The study suggests that BIRC6 may be a potential therapeutic target for RCC.

■ ASSOCIATED CONTENT

Data Availability Statement

All data sets generated in this study are included in the article.

■ AUTHOR INFORMATION

Corresponding Authors

Dong Chen − *Sun Yat-sen Cancer Center, State Key Laboratory of Oncology in South China, Collaborative Innovation Center of Cancer Medicine, Guangzhou 510060, China*; Email: chendong1@sysucc.org.cn

Weifeng Zhong − *Department of Urology, Meizhou People's Hospital, Meizhou 514031, China; Department of Urology, Guangzhou Twelfth People's Hospital, Guangzhou 510630, China*; orcid.org/0000-0002-7453-9346; Email: zhongweifengzwf@126.com

Authors

Kaihua Zhong − *Department of Urology, Meizhou People's Hospital, Meizhou 514031, China*

Xiaohong Wang − *Department of Nephrology, Third Affiliated Hospital of Southern Medical University, Guangzhou 510500, China*

Heyuan Zhang − *Department of Urology, Meizhou People's Hospital, Meizhou 514031, China*

Nanhui Chen − *Department of Urology, Meizhou People's Hospital, Meizhou 514031, China*

Yang Mai − *Department of Urology, Guangzhou Twelfth People's Hospital, Guangzhou 510630, China*

Sipin Dai − *Department of Urology, Guangzhou Twelfth People's Hospital, Guangzhou 510630, China*

Lawei Yang − *Clinical Research Center, Affiliated Hospital of Guangdong Medical University, Zhanjiang 524001, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c07265

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[#]K.Z. and X.W. contributed equally to this work.

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Notes

The authors declare no competing financial interest.

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