HELZ2 Promotes K63-Linked Polyubiquitination of c-Myc to Induce Retinoblastoma Tumorigenesis

Hanjun Dai (✉ daihanjun@whu.edu.cn)

Wuhan University Zhongnan Hospital <https://orcid.org/0000-0002-5533-6615>

wen ZENG

Wuhan University Zhongnan Hospital

WEIJUAN ZENG

Wuhan University Zhongnan Hospital

MING YAN

Wuhan University Zhongnan Hospital

ping jiang

Wuhan University Zhongnan Hospital

ying LI

Wuhan University Zhongnan Hospital

wei zhang

Wuhan University Zhongnan Hospital

min ke

Wuhan University Zhongnan Hospital

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Abstract

Retinoblastoma is a rare ocular tumor in children that originates in the retina. Several core transcriptional regulators maintain the expansion of retinoblastoma tumors, including c-Myc. Here, we demonstrated that Helicase with zinc finger domain 2 (HELZ2) promoted retinoblastoma tumorigenesis by targeting c-Myc. HELZ2-deficient inhibited retinoblastoma cell proliferation, whereas overexpression of HELZ2 promoted retinoblastoma cell proliferation. In addition, high levels of HELZ2 promoted xenograft retinoblastoma tumorigenesis and inhibited animal survival. Mechanistically, HELZ2 interacted with c-Myc and promoted its K63-linked polyubiquitination. We indicated that HELZ2 promoted the interaction between E3 ubiquitin ligase HUWE1 and c-Myc, and HELZ2-mediated K63-linked polyubiquitination and activation of c-Myc were dependent on HUWE1. Taken together, HELZ2 plays a critical role in the regulation of retinoblastoma tumorigenesis by enhancing the activity of c-Myc.

Introduction

Retinoblastoma was first described by Pawius in the sixteenth century [1]. Retinoblastoma is the most common intraocular malignancy in children, accounting for 3% of all childhood cancers [2]. Tumor formation usually begins with mutation in both alleles of the retinoblastoma tumor suppressor gene RB1, followed by a series of other genetic alterations that correlate with the clinical stage and pathologic findings of the tumor [3]. The annual incidence rate of newborns worldwide is around 1 in 12000 to 1 in 20000, and the incidence rate of retinoblastoma continues to increase [4]. Meanwhile, the incidence is higher in underdeveloped countries, which are often accompanied by metastasis, recurrence and poor prognosis [5]. According to the different conditions observed by the patient, retinoblastoma can be treated in many methods, such as enucleation, radiotherapy, chemotherapy and cryotherapy [6]. Nevertheless, there are still many shortcomings in the treatment of high-risk retinoblastoma recurrence. Therefore, in-depth exploration of the intracellular signal transmission mechanism of retinoblastoma is essential to discover potential therapeutic targets.

c-Myc oncoprotein is a transcription factor that can regulate a variety of genes, such as cell proliferation [7]. Proper c-Myc activity and stability are essential for controlled cell proliferation. Ubiquitination-associated post-translational modification plays an important role in c-Myc protein activity and stability regulation. c-Myc can be ubiquitinated by a variety of E3 ubiquitin ligases, such as SCF^{FBX028}, SCF^{b-TRCP}, TRIM32 and SCF^{Fbw7} [8]. Depending on the specific E3 ligase, ubiquitination can positively or negatively regulate the activity and stability of c-Myc. HUWE1 is a member of the HECT-domain containing ubiquitin E3s, that enhances c-Myc activity by promoting its K63-linked polyubiquitination [9, 10]. Our previous studies have demonstrated that ZCCHC2 suppresses retinoblastoma tumorigenesis by inhibiting HUWE1-mediated K63-linked polyubiquitination of c-Myc [11]. In this study, we demonstrate that HELZ2 positively regulates retinoblastoma cell proliferation and tumorigenesis. HELZ2 promotes c-Myc K63-linked polyubiquitination by promoting the interaction of HUWE1 with c-Myc.

Materials And Methods

Animals

Eight- to ten-week-old and age-matched BALB/c nude mice have free access to water and food in a temperature-controlled room (23-25 °C) and relative humidity of 40-70%, the light/dark cycle is 12 hours, and there are 5 mice in each cage. Allow the animals to acclimate to the environment for at least 7 days before experiment. All animals were randomly and injected subcutaneously on both flanks with 1×10^6 Y79 and HXO-RB44 cells expressing the NC, g*HELZ2*, g*HELZ2* + *HELZ2*. All animal use and experimental protocols were carried out in compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines and approved by the Animal Care and Ethics Committee of Wuhan University of Medicine.

Cell culture

Y79 cells, HXO-RB44 cells and HEK293T cells were obtained from ATCC. All cells were cultured in DMEM (Gibco) supplemented with 10% FBS (Biological Industries) and 1% penicillin-streptomycin (Thermo Fisher Scientific) at 37°C with 5% CO₂.

Transfection

Y79 cells were transfected by Lipofectamine 2000. HEK293T cells were transfected by standard calcium phosphate precipitation.

CRISPR-Cas9 knockout

The double-stranded oligonucleotides corresponding to the target sequences were cloned into the Lenti-CRISPR-V2 vector, and HEK293T cells were co-transfected with the packaging plasmid. Two days after transfection, the viruses were harvested, ultra-filtrated (0.45 mm filter, Millipore) and used to infect Y79 or HXO-RB44 cells in the presence of polybrene (8 mg/mL). Use puromycin (1 mg/mL) to select infected cells for at least 6 days.

Cell viability

Cell viability was assessed by the ability of cells to absorb Thiazole Blue Tetrazolium Bromide (MTT) (Bio-Tek, USA). The method is carried out in accordance with the manufacturer's instructions.

Immunoprecipitation and immunoblotting analysis

Cells were lysed in 1 ml NP-40 lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% Triton X-100, 10 µg/ml aprotinin, 10 µg/ ml leupeptin and 1 mM phenylmethylsulfonyl fluoride). For each immunoprecipitation reaction, incubate 0.4 ml of lysate with 0.5-2 µg of the designated antibody or control IgG and 35 µL of 1:1 protein-G Sepharose (GE Healthcare) slurry at 4°C for 3 hours. Sepharose beads use 1 ml of lysis buffer containing 500 mM NaCl wash 3 times [12]. The precipitates were fractionated by SDS-PAGE, and immunoblotting analysis was performed with the indicated antibodies. Antibodies used in this study were purchased from the following indicated companies: HA-Tag (#3724),

Flag-Tag (#14793), c-Myc (#5605), or β -actin (#8457) from Cell Signaling Technology (MA, USA), or HELZ2 (#PA5-101743) from Invitrogen.

Statistics

All data are expressed as the mean $\pm$ SE. Newman-Keuls tests were used for performing ad hoc comparisons when appropriate. Student's t-tests and variance analyses were used to determine differences among groups. $p < 0.05$ was considered statistically significant.

Results

HELZ2 promotes retinoblastoma cell proliferation

To explore the potential therapeutic targets of retinoblastoma, we performed a genome-wide CRISPR/Cas9 knockout screen to identify the genes that are required for retinoblastoma Y79 cell proliferation, and HELZ2 was identified as a candidate protein (**Fig. 1a**). Knockout of HELZ2 inhibited Y79 cell proliferation (**Fig. 1b**). Overexpression of HELZ2 promoted Y79 cell proliferation (**Fig. 1c**). In addition, we further determined the function of HELZ2 in retinoblastoma cell proliferation. We created HELZ2-deficient Y79 and HXO-RB44 cells (**Fig. 1d and e**). We found that HELZ2 deficiency inhibited retinoblastoma cell proliferation, while cell growth was restored after rescuing HELZ2 (**Fig. 1d and e**). Together, these results demonstrate that HELZ2 is potential positively regulator of retinoblastoma cell proliferation

HELZ2 promotes retinoblastoma tumor development

We further subcutaneously injected the NC, g*HELZ2* or g*HELZ2* + HELZ2 retinoblastoma cells into BALB/c nude mice, and showed that cells with HELZ2-deficient performed lower retinoblastoma growth and greater animal survival (**Fig. 2a-d**). These results suggested that HELZ2 promoted retinoblastoma growth tumorigenesis.

HELZ2 promotes c-Myc K63-linked polyubiquitination

c-Myc is a well-known transcription factor that controls most actively transcribed genes and regulates many tumorigenesis. To identify whether HELZ2-regulated cell proliferation is associated with c-Myc, we performed coimmunoprecipitation assays. Results showed that HELZ2 interacted with c-Myc (**Fig. 3a**). The focus of c-Myc research is its stability and activity. Immunoblot analysis of c-Myc protein levels in HELZ2-deficient Y79 and HXO-RB44 cells, and showed that c-Myc protein levels did not show significant difference after HELZ2-deficient (**Fig. 3b**). Therefore, we tested the c-Myc activity after overexpression of HELZ2. Results showed that overexpression of HELZ2 promoted c-Myc K63-linked polyubiquitination (**Fig. 3c**). Meanwhile, endogenous HELZ2-deficient inhibited c-Myc K63-linked polyubiquitination (**Fig. 3d**). Taken together, our data indicated that HELZ2 promotes K63-linked polyubiquitination of c-Myc.

HELZ2-mediated K63-linked polyubiquitination of c-Myc is dependent on HUWE1

HUWE1, an E3 ubiquitin ligase, positively regulates c-Myc activity by promoting its K63-linked polyubiquitination. Coimmunoprecipitation assays showed that HELZ2 interacted with HUWE1 (**Fig. 4a**). Overexpression of HELZ2 promoted the interaction of c-Myc with HUWE1 (**Fig. 4b**). Meanwhile, HELZ2-deficient performed the opposite results (**Fig. 4c**). Furthermore, we created a HUWE1 knockout cell line and showed that HELZ2-mediated K63-linked polyubiquitination of c-Myc was blocked after HUWE1-deficient (**Fig. 4d**). Together, these results suggest that HELZ2 promotes c-Myc K63-linked polyubiquitination by promoting the interaction between c-Myc and HUWE1.

Discussion

In this study, we demonstrated that HELZ2 plays a critical role in c-Myc-associated retinoblastoma tumorigenesis. HELZ2 positively regulated retinoblastoma cell proliferation and tumor development. HELZ2 interacted with c-Myc and promoted its K63-linked polyubiquitination. We further indicated that HELZ2-mediated K63-linked polyubiquitination of c-Myc was dependent on HUWE1.

We performed a genome-wide CRISPR/Cas9 knockout screen to identify the genes that are required for retinoblastoma cell proliferation. Our results identified HELZ2 was a positively candidate protein for retinoblastoma cell proliferation. HELZ2-deficient inhibited retinoblastoma cell proliferation, whereas overexpression of HELZ2 promoted. Meanwhile, HELZ2-deficient inhibited subcutaneously retinoblastoma growth and promoted animal survival. Taken together, we indicated that HELZ2 promotes retinoblastoma cell proliferation and tumorigenesis.

In addition, we found that HELZ2 interacted with c-Myc, HELZ2-deficient did not affect c-Myc stability. However, overexpression of HELZ2 promoted c-Myc K63-linked polyubiquitination, while loss of HELZ2 inhibited. HUWE1 is a c-Myc ubiquitin E3 ligase that enhances c-Myc activity by promoting its K63-linked polyubiquitination. We found that HELZ2 was associated with HUWE1 and overexpression of HELZ2 promoted interaction of c-Myc with HUWE1, meanwhile, HELZ2-deficient had the opposite effect. In addition, HUWE1-deficient blocked HELZ2-mediated regulation of K63-linked polyubiquitination of c-Myc. Our findings suggest that HELZ2 positively regulates c-Myc activity and HELZ2 promotes HUWE1-mediated c-Myc K63-linked polyubiquitination.

In conclusion, we indicated that HELZ2 was a positive regulator of retinoblastoma cell proliferation and tumorigenesis. HELZ2 interacted with c-Myc and promoted HUWE1-mediated c-Myc K63-linked polyubiquitination. HELZ2 may be a potential therapeutic target for retinoblastoma.

Declarations

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Authors' contribution

DHJ and KM participated in the design of the study, performed the measurements and the statistical analysis, and wrote the manuscript. ZW, ZWJ, YM, JP, LY and ZW helped in data collection and the interpretation of data. All authors read and approved the manuscript.

Data availability

Data sharing are applicable to this article without restriction.

Conflicts of interest The authors declare no conflicts of interest.

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Figures

Figure 1

Figure 1

HELZ2 promotes retinoblastoma cell proliferation (a) Schematic diagram of CRISPR screening for suitable genes. Cas9 expressing cells are transduced using a mixed lentivirus guide library, targeting thousands of genes, each with several guide sequences. The cells then multiply for several weeks. Over

time, the guide sequences that target adaptive genes that cause cell death will gradually run out, while the guide sequences that target growth-suppressing genes become more abundant. Then prepare genomic DNA (gDNA) from the samples (day 14 after transduction) and control samples (day 3 after transduction). The leader sequence is amplified by PCR, and the relative frequency of the leader in the two populations is determined by next-generation sequencing (NGS). Assess the enrichment or consumption of the guide sequence relative to the control. sgRNA, single guide RNA. (b) HELZ2-deficient inhibited Y79 cell proliferation. Cell count analysis of Y79 cells after HELZ2-deficient at the indicated times (n=6, *p < 0.05 vs NC). (c) Overexpression of HELZ2 promoted Y79 cell proliferation. Cell viability analysis of Y79 cells at 5 days after transfection of HELZ2 (0, 5, 10, 20 ng) (n=6, *p < 0.05 vs Vec). (d and e) HELZ2-deficient inhibited Y79 and HXO-RB44 cell proliferation. Cell count analysis of Y79 (d) and HXO-RB44 (e) cells after HELZ2-deficient or HELZ2 expression at the indicated times (Left). (n=6, *p < 0.05 vs NC, #p < 0.05 vs gHELZ2). Data are expressed as the mean $\pm$ SE. Statistical analysis was performed by Student's t-test and variance analysis.

Figure 2

Figure 2

HELZ2 promotes retinoblastoma tumorigenesis (a and b) Xenograft tumor growth (a) and survival (b) after injection of 1×10^6 Y79 cells. ($n=6$, $*p < 0.05$ vs NC, $\#p < 0.05$ vs gHELZ2). (c and d) Xenograft tumor growth (c) and survival (d) after injection of 1×10^6 HXO-RB44 cells. ($n=6$, $*p < 0.05$ vs NC, $\#p < 0.05$ vs gHELZ2). Data are expressed as the mean $\pm$ SE. Statistical analysis was performed by Student's t-test and variance analysis.

Figure 3

Figure 3

HELZ2 promotes c-Myc K63-linked polyubiquitination (a) HELZ2 interacts with c-Myc. HEK293T cells were transfected with indicated plasmids for 20 h before coimmunoprecipitation and immunoblotting analysis. (b) Immunoblot analysis of c-Myc levels in Y79 cells or HXO-RB44 after HELZ2-deficient. (c) Effects of HELZ2 on polyubiquitination of c-Myc. HEK293T cells were transfected with indicated plasmids for 20 h followed by coimmunoprecipitation and immunoblotting analysis. (d) Effects of HELZ2

deficiency on the polyubiquitination of c-Myc. The NC or HELZ2-deficient HEK293T cells were transfected with indicated plasmids for 20 h followed by coimmunoprecipitation and immunoblotting analysis.

Figure 4

Figure 4

HELZ2 promotes HUWE1-mediated c-Myc K63-linked polyubiquitination (a) HELZ2 interacts with HUWE1. HEK293T cells were transfected with indicated plasmids for 20 h followed by coimmunoprecipitation and immunoblotting analysis. (b) HELZ2 promotes the association of c-Myc with HUWE1. HEK293T cells

were transfected with indicated plasmids for 20 h followed by coimmunoprecipitation and immunoblotting analysis. (c) HELZ2-deficient inhibits the association of c-Myc with HUWE1. HEK293T cells were transfected with indicated plasmids for 20 h followed by coimmunoprecipitation and immunoblotting analysis. (d) HELZ2-mediated polyubiquitination of c-Myc is dependent on HUWE1. Control or HUWE1-deficient HEK293T cells were transfected with indicated plasmids for 20 h followed by coimmunoprecipitation and immunoblotting analysis.