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FULL LENGTH ARTICLE

IL-13/IL-13RA2 signaling promotes colorectal cancer stem cell tumorigenesis by inducing ubiquitinated degradation of p53

Baoyu He [a,b,1], Jing Liang [a,1], Qianqian Qin [c], Yuqin Zhang [a], Shuo Shi [a], Jinghe Cao [c], Zhixin Zhang [d], Qingli Bie [a,b], Rou Zhao [a], Li Wei [a], Baogui Zhang [d,**], Bin Zhang [a,b,e,*]

[a] *Department of Laboratory Medicine, Affiliated Hospital of Jining Medical University, Jining Medical University, Jining, Shandong 272000, China*
[b] *Postdoctoral Mobile Station of Shandong University of Traditional Chinese Medicine, Jinan, Shandong 250355, China*
[c] *Department of Reproductive Medicine, Affiliated Hospital of Jining Medical University, Jining Medical University, Jining, Shandong 272000, China*
[d] *Department of Gastrointestinal Surgery, Affiliated Hospital of Jining Medical University, Jining Medical University, Jining, Shandong 272000, China*
[e] *Institute of Forensic Medicine and Laboratory Medicine, Jining Medical University, Jining, Shandong 272067, China*

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Abstract Cancer stem cells (CSCs) are considered tumor-initiating cells and the main drivers of disease progression. Targeting these rare cancer cells, however, remains challenging with respect to therapeutic benefit. Here, we report the up-regulation of IL-13RA2 expression in colorectal cancer (CRC) tissues and spheroid cells. The expression of IL-13RA2 was positively correlated with canonical stemness markers in CRC. We further demonstrated that the level of IL-13 was up-regulated in the serum of CRC patients. Biologically, recombinant IL-13 (rIL-13) stimulation promoted the sphere formation, proliferation, and migration of CRC cells *in vitro* and enhanced tumorigenesis *in vivo*. This phenotype could be reversed by knocking down

Abbreviations: Co-IP, Co-immunoprecipitation; CRC, Colorectal cancer; CSC, Cancer stem cell; rIL-13, Recombinant IL-13; UPS, Ubiquitin-proteasome system.

* Corresponding author. Department of Laboratory Medicine, Affiliated Hospital of Jining Medical University, 89 Guhuai Road, Jining, Shandong 272000, China.
** Corresponding author. Department of Gastrointestinal Surgery, Affiliated Hospital of Jining Medical University, Jining Medical University, 89 Guhuai Road, Jining, Shandong 272000, China.
E-mail addresses: zbg2019@mail.jnmc.edu.cn (B. Zhang), zhangbin@mail.jnmc.edu.cn (B. Zhang).
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[1] These authors contributed equally to this work.

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Ubiquitinated degradation

IL-13RA2. Mechanistically, IL-13 activated autophagy by inducing LC3I/LC3II transformation in CRC-CSCs, which was crucial for the biological functions of IL-13. We further demonstrated that IL-13RA2 acted as a modular link of the E3 ligase UBE3C and the substrate p53 protein, enhancing the interaction of UBE3C and p53, thereby inducing the K48-linked ubiquitination of p53. In conclusion, the IL-13/IL-13RA2 signaling cascade promotes CRC-CSC self-renewal and tumorigenesis by inducing p53 ubiquitination, adding an important layer to the connection between IL-13 and p53, which can be translated into novel targeted therapies.

Introduction

Colorectal cancer (CRC) is the third most common cause of cancer mortality worldwide, with approximately 1.8 million new cancer cases and nearly 1.0 million cancer deaths annually.[1] Similar to most other human cancers, CRC initiation and development are multistep processes that involve various genetic changes and epigenetic modifications.[2,3] However, the precise molecular mechanisms underlying CRC development and progression remain unclear. Although great strides have been made in surgery and new drug applications, the prognosis of CRC patients remains suboptimal. Therefore, new therapeutic strategies based on a deeper understanding of the molecular mechanisms of CRC are urgently needed.

Cancer stem cells (CSCs) are characterized as tumor-initiating cells in many types of cancer, including CRC.[4,5] CSCs are defined as a small subpopulation of tumor cells that possess a large capacity for self-renewal, the potential to propagate diverse cell types that support tumor onset, tumor metastasis, and recurrence, and confer resistance to conventional treatment.[6–8] Recently, increasing evidence suggests that CSCs are a dynamic population that is continuously formed by the fusion of genetic, epigenetic and microenvironmental factors.[5] Therefore, investigating key molecules and the underlying mechanism of sustaining CSC characteristics may provide new strategies for CRC therapy.

IL-13, a 17-kDa cytokine mainly secreted by activated T cells, NK cells, and tumor cells, is involved in allergic inflammation and fibrosis and contributes to carcinogenesis in renal cell carcinomas and chronic lymphocytic leukemia.[9] Additionally, IL-13 promotes the biosynthesis of cytidine deaminase in colonic epithelial cells to confer the potential of activating the inflammatory—cancer transition in the gut.[10] Canonical IL-13 signaling transduction is mediated by two receptor complexes, including a stimulating responder and an inhibitory receptor.[11,12] IL-13RA2 is an inhibitory decoy receptor with a high affinity for IL-13 and diminishes its certain effector functions. IL-13/IL-13RA2 signaling has been repeatedly reported as an excellent therapeutic target for multiple types of cancers.[13] Although many physiological and pathological roles of IL-13/IL-13RA2 signaling transduction have been described, its biological roles and regulatory mechanisms in CSCs remain largely unknown.

Here, we report that recombinant IL-13 (rIL-13) promotes the spheroid-forming ability of CRC-CSCs *in vitro* and tumor formation *in vivo* via IL-13/IL-13RA2 signaling. Furthermore, autophagy activation is closely associated with IL-13/IL-13RA2-mediated regulation of CSC function. Therefore, the findings reveal a previously unappreciated biological role of IL-13 in CSC function regulation and highlight the importance of IL-13/IL-13RA2 signaling in maintaining the homeostasis of CSCs, suggesting that this pathway is a new therapeutic target for CRC.

Materials and methods

Patient specimens

Primary tumor tissues ($n = 28$) and adjacent noncancerous tissues ($n = 28$, more than 5 cm away from the primary tissue site) were obtained from CRC patients who underwent surgery at the Affiliated Hospital of Jining Medical University from January 2018 to January 2022. All patients were pathologically diagnosed with primary CRC without receiving any antineoplastic therapy prior to the biopsy. Venous blood samples were collected from patients prior to any radiotherapy or chemotherapy treatment. The study protocol was approved by the ethics committee of the Affiliated Hospital of Jining Medical University (No. 2021B006). Written informed consent was obtained from all participants in this study. All the research was carried out in accordance with the provisions of the Declaration of Helsinki of 1975.

Cell culture

Human colon cancer HCT116 and DLD1 cells and human embryonic kidney HEK293T cells were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Gibco, Grand Island, NY), 100 U/mL penicillin, and 100 mg/L. The cells were grown at 37 °C in a 5% CO_2 (Thermo, MA, USA) atmosphere in a fully humidified incubator. Cells were tested routinely for Mycoplasma contamination (Yeasen).

Colony formation assay

The colony formation assay was used to detect the passage and proliferation ability of the cells in each group. The cells in the logarithmic growth phase were trypsinized and

counted. Then, they were evenly seeded into a six-well plate (1000 cells per well). After the cells adhered, PBS or rIL-13 (50 and 100 ng/mL, RD) was added. The cells were incubated for 2 weeks under conventional culture conditions. Finally, the cell colonies were successively fixed, stained, and counted. The cell colony number was calculated using ImageJ software (National Institutes of Health) after obtaining images with an inverted light microscope (CKX3-SLP, Olympus Corporation).

Migration assay

The migration assay was performed using transwell chambers as described previously with minor modifications.[14] In brief, cells were serum-starved for 24 h and treated with rIL-13. Then, cells were trypsinized and resuspended in a serum-free medium. A 100-μL cell suspension was added to the upper chamber (8.0 mM pore size; Corning), and medium supplemented with 10% FBS was added to the bottom chamber. Cells on the upper surface of filters were removed after incubation for 36 h. Afterward, cells on the undersurface were fixed with 4% paraformaldehyde for 30 min, stained with 5% crystal violet and counted. Images of each membrane were captured, and the number of migratory cells was counted under a microscope.

Spheroid formation assay

Stem cell spheroidization medium consisted of DMEM basic medium, 20 ng/mL EGF, 20 ng/mL bFGF, and 2% B27 (Gibco, New York, USA) was prepared. Differently treated HCT116 cells were trypsinized, centrifuged, resuspended in a spheroidization medium, and seeded with 1000 cells per well into low-attachment 6-well plates. After 7—10 days of incubation, spheroids were obtained and counted under a light microscope at 4× and 20× magnification.

SiRNA, overexpression plasmids, and transfection

The siRNAs targeting IL-13RA2 and UBE3C and the scrambled control siRNA (NC) were purchased from RiboBio (Shanghai, China). N-Terminal FLAG-tagged IL-13RA2 and HA-tagged p53 expression vectors in mammalian cells (pCMV backbones) were obtained from GeneCopoeia (Guangzhou, China). The sequences of siRNAs and primers are listed in Table S1. SiRNAs and plasmids were transfected using Lipofectamine 3000 reagent (Invitrogen). Cells were harvested 72 h post-transfection for various assays.

RNA extraction, reverse transcription, and quantitative PCR

RNA Easy Reagent (Vazyme, Nanjing, China) was utilized to isolate total RNA from CRC tissues and cells. MRNA was reverse transcribed into cDNA using HiScript III RTSuperMix (Vazyme, Nanjing, China). Quantitative real-time PCR was performed on a LightCycler 96 (Roche Diagnostics) using FastStart Essential DNA Probes Master Mix (Roche Diagnostics). Primer sequences are listed in Table S2. GAPDH was used as the reference gene for normalization.

Coimmunoprecipitation and Western blotting assay

Coimmunoprecipitation (CoIP) was performed as previously described.[15] Briefly, cells were lysed in 1 mL of IP lysis buffer (Beyotime Bio). Cell lysates were immunoprecipitated with the indicated primary antibodies overnight at 4 °C and then incubated with Protein A/G agarose beads for 2 h. The beads were washed three times with lysis buffer and eluted in SDS sample buffer. The eluted immunocomplexes were resolved by SDS-PAGE, followed by Western blotting analyses.

In vivo tumor xenograft model

For subcutaneous injection models, serial dilutions (10^3, 10^4, and 10^5) of control and knockdown cells were implanted into the posterior dorsal flank region of mice (male BALB/c nude mice; aged 4—6 weeks; $n = 4$ per group) with a Matrigel scaffold (BD Matrigel matrix, BD Biosciences). Mice were maintained under standard conditions according to the institutional guidelines for animal care. Tumor volume was measured at the indicated time points and calculated as length × width^2/2. All experimental protocols were approved by the Animal Ethics Committee of the Affiliated Hospital of Jining Medical University (2021B006).

Immunofluorescence

Immunofluorescence staining was performed as described previously.[16] Briefly, fresh subcutaneous tumor tissues were fixed in 4% paraformaldehyde for 48 h. Then, tissues were successively dehydrated, paraffin-embedded and sectioned (thickness: 4—8 μm). Afterward, paraffin-embedded tissues were deparaffinized in xylene and blocked in PBS containing 5% BSA for 1 h at room temperature. The cells were then incubated with primary antibodies in 10% goat serum at 4 °C overnight, followed by incubation with secondary fluorochrome-labeled antibodies for 40 min at 37 °C. After incubation with DAPI for 3—5 min at room temperature to stain the nucleus, the cells were washed three times with PBS and imaged with a confocal laser scanning microscope. Detailed information for the primary antibodies and secondary antibodies is listed in Tables S3 and S4.

Flow cytometry analyses

The cells that had undergone different treatments were trypsinized and resuspended in PBS and incubated with fluorescently labeled anti-human CD133-PE, anti-human LGR5-PE or anti-human CD44-PE antibodies in the dark for 30 min at 4 °C. The data were analyzed using FlowJo software.

Enzyme-linked immunosorbent assay (ELISA)

Serum samples from CRC patients and healthy control individuals were collected and stored at −80 °C until testing. Serum IL-13 levels were measured using a protein-specific ELISA kit (Solarbio, Beijing) following the manufacturer's

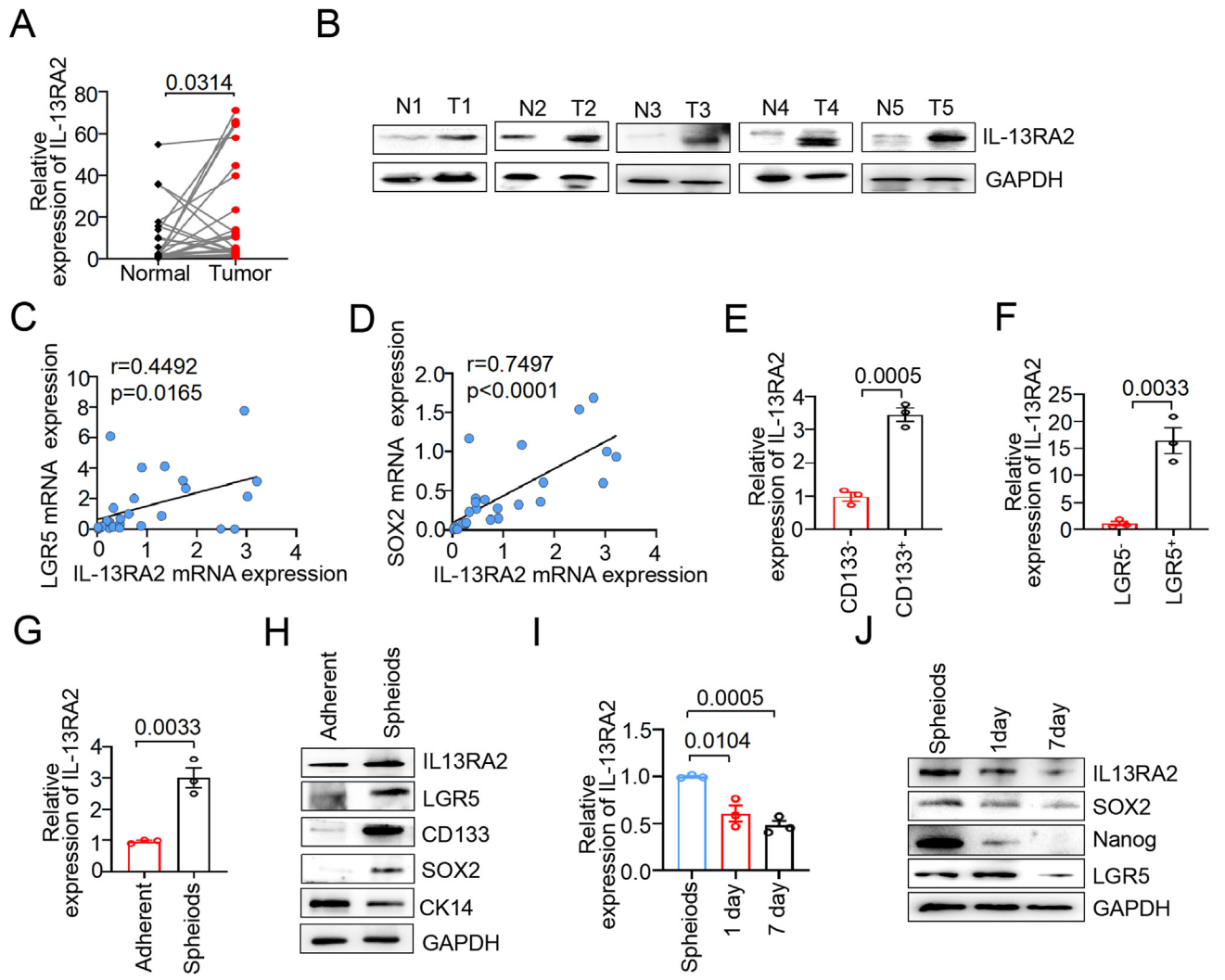

Figure 1 IL-13RA2 is highly expressed and is a CSC stemness marker in colorectal cancer (CRC). **(A)** mRNA expression ofIL-13RA2 was measured in CRC tissues and their adjacent tissues using qPCR analysis ($n = 28$, Wilcoxon test). **(B)** Protein expression of IL-13RA2 was measured in CRC tissues and adjacent tissues using Western blotting analysis ($n = 5$). **(C, D)** The correlation between the mRNA level of IL-13RA2 with that of LGR5 or SOX2 was measured in CRC tissues ($n = 28$, Spearman rank-correlation analysis). **(E)** Expression level of IL-13RA2 in CD133-positive ($CD133^+$) and CD133-negative ($CD133^-$) cells sorted from HCT116 cells. **(F)** Expression level of IL-13RA2 in LGR5-positive ($LGR5^+$) and LGR5-negative ($LGR5^-$) cells sorted from HCT116 cells. **(G)** The mRNA expression levels of IL-13RA2 were evaluated *in vitro* and in adherent cultured HCT116 cells. **(H)** The expression levels of IL-13RA2, LGR5, CD133, SOX2, and CK14 were measured in spheroids and adherent cultured HCT116 cells. **(I)** The mRNA expression levels of IL-13RA2 were evaluated in spheroids and readherent cultured HCT116 cells. **(J)** Protein expression of IL-13RA2, SOX2, Nanog, and LGR5 was detected by Western blotting in spheroids and readherent cultured HCT116 cells. The *P* values in E–G & I were determined by a two-tailed unpaired Student's *t*-test. Data are presented as the mean ± SEM of three independent experiments.

instructions. All measurements were performed in duplicate, and the average value was calculated from the standard curve analyses.

Statistical analysis

GraphPad Prism software was used to create the graphs. Data are plotted as the mean ± SEM. Statistically significant differences between groups were assessed using analysis of variance and Student's *t*-test with SPSS 17.0 software version (SPSS Inc.). Spearman rank-correlation analysis was performed to analyze the correlation between two molecules. $P < 0.05$ was considered statistically significant.

Results

IL-13RA2 is highly expressed and is a potential CSC stemness marker in colorectal cancer

To explore the role of IL-13RA2 in CRC, we analyzed the mRNA expression levels of IL-13RA2 in 28 pairs of CRC tissues and matched paracancerous tissues using qRT-PCR. The results showed that IL-13RA2 was significantly

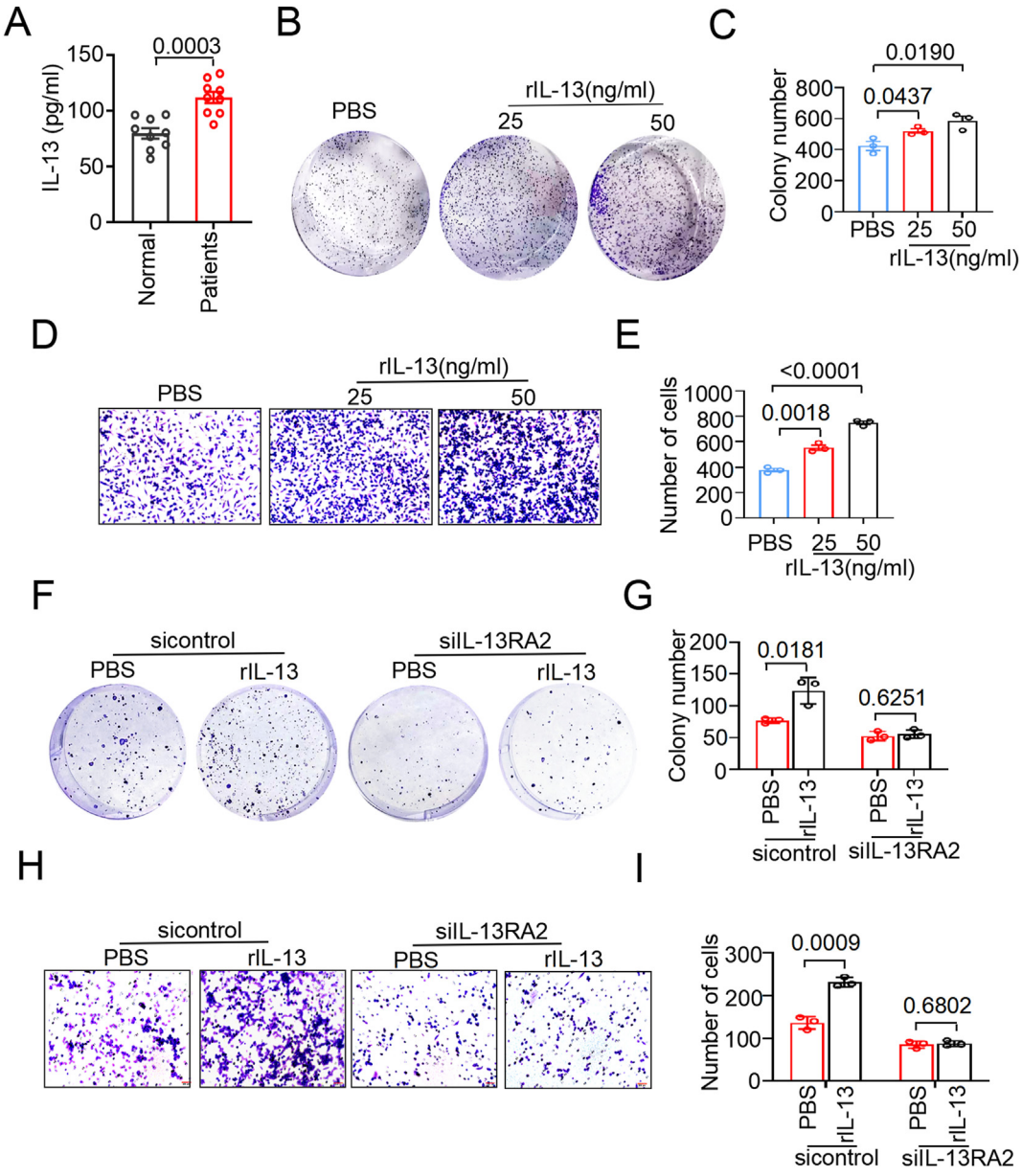

Figure 2 IL-13/IL-RA2 signaling enhances the proliferation and migration of colorectal cancer (CRC) cells. **(A)** Serum IL-13 levels were measured in CRC patients and healthy controls using ELISA ($n = 9$). **(B, C)** HCT116 cell colony-forming capacity was evaluated after treatment with rIL-13 or PBS. **(D, E)** HCT116 cell migratory ability was assessed after treatment with rIL-13 or PBS. **(F, G)** Colony formation was assessed after transfection of IL-13RA2 siRNA in IL-13-treated HCT116 cells. **(H, I)** Cell migratory ability was analyzed after transfection of IL-13RA2 siRNA in IL-13-treated HCT116 cells. Student's *t*-test (two-tailed) was used for C, E, G & I. Data are presented as the mean ± SEM of three independent experiments.

increased in cancer tissues (Fig. 1A). Consistently, IL-13RA2 expression was higher in CRC tissues compared to that in paired paracancerous tissues at the protein level (Fig. 1B). Growing evidence suggests that CSCs play a key role in various tumors.[4,5] To clarify whether IL-13RA2 is involved in CSC formation and stemness maintenance, we first detected and analyzed the relationship between IL-13RA2 and two well-known CSC markers, LGR5 and SOX2. Correlation analysis showed that IL-13RA2 was positively correlated with the mRNA expression of LGR5 and SOX2 in CRC tissues (Fig. 1C, D). We next analyzed the expression and correlation between IL-13RA2 and CSC markers based on an external database. First, normalized expression level of IL-13RA2 was significantly increased in cancer tissues compared to adjacent non-tumor tissues from the TCGA COAD dataset (Fig. S1A). Next, a positive correlation between IL-13RA2 and LGR5 (or SOX2) transcript levels was found in the TCGA dataset ($r = 0.3509$ for IL-13RA2 *vs.* LGR5; $r = 0.3007$ for IL-13RA2 *vs.* SOX2; both $P < 0.001$; Fig. S1B).

Then, we sorted cells positive or negative for CD133 and LGR5 ($CD133^{+}/LGR5^{+}$ or $CD133^{-}/LGR5^{-}$) and observed that

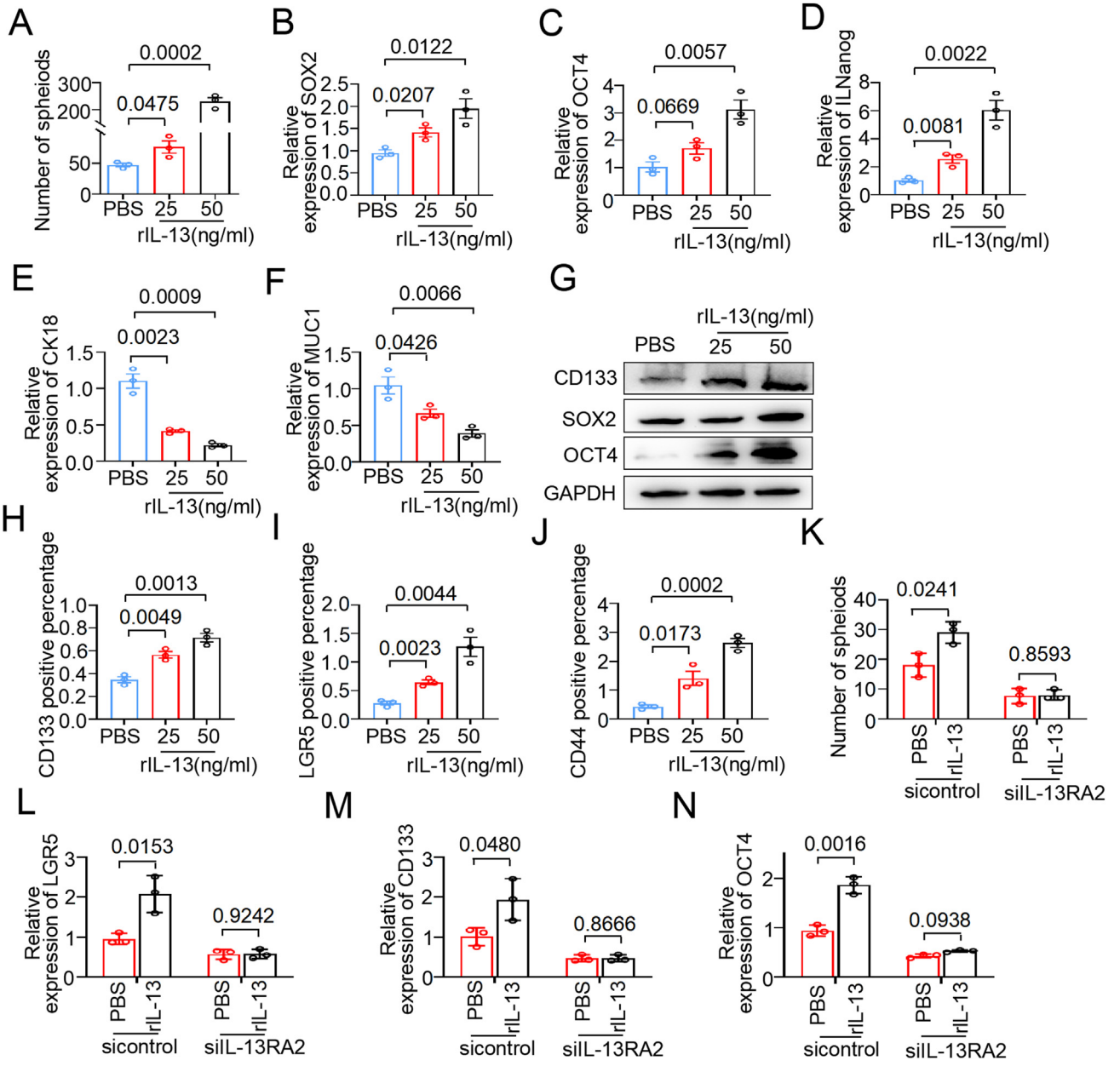

Figure 3 IL-13/IL-13RA2 signaling promotes CSC self-renewal capacity. **(A)** The spheroid number of HCT116 cells treated with rIL-13 or PBS cultured in serum-free medium for 7 days. **(B—F)** The mRNA expression levels of SOX2, OCT4, Nanog, CK18, and MUC1 were measured in HCT116 cells treated with different concentrations of rIL-13. **(G)** Protein levels of CD133, SOX2, and OCT4 were detected in HCT116 cells treated with different concentrations of rIL-13. **(H—J)** The $CD133^{+}$, $LGR5^{+}$, or $CD44^{+}$ cell percentages sorted from HCT116 cells treated with different concentrations of rIL-13. **(K)** Spheroid-forming ability was assessed after transfection of IL-13RA2 siRNA in IL-13-treated HCT116 cells. **(L—N)** The mRNA expression levels of LGR5, CD133, and OCT4 were measured after transfection of IL-13RA2 siRNA in IL-13-treated HCT116 cells. Student's *t*-test (two-tailed) was used for A—F & H—N. Data are presented as the mean ± SEM of three independent experiments.

IL-13RA2 was highly expressed in $CD133^{+}/LGR5^{+}$ colon cancer cells (Fig. S1C, D; Fig. 1E, F). Next, HCT116 cells were cultured in low-adherence plates with serum-free spheroidization medium to generate spheroid cells. Spheroid cells were validated by measuring stemness markers, including Nanog, SOX2, and CD133, and the epithelial differentiation marker CK18 (Fig. S1E). Consistently, we observed significantly higher expression of IL-13RA2 in spheroid cells compared to that in matched adherent cells, which was consistent with the expression pattern of classical CSC markers (Fig. 1G, H). Similar results were observed in DLD1 cells (Fig. S1F, G).

To further verify the effect of IL-13RA2 on the stemness of CRC-CSCs, we performed a spheroid redifferentiation test by reseeding spheroid cells for 1 or 7 days. With the prolongation of differentiation time, the expression of stemness markers was significantly decreased, while the expression of differentiation markers was significantly

increased (Fig. S1H). As expected, the mRNA and protein expression of IL-13RA2 were reduced, which was consistent with the trends of changes in the expression of the stemness markers (Fig. 1I, J; Fig. S1I, J). Together, these results suggest that IL-13RA2 is a potential stemness marker for CRC-CSCs.

IL-13/IL-RA2 signaling enhances the colony forming ability and migratory capacity of colon cancer cells

IL-13 is a ligand for IL-13RA2. IL-13/IL-13RA2 plays a crucial role in promoting early intestinal tumorigenesis. First, the level of IL-13 in the preoperative serum of colon cancer patients was tested by ELISA. The results demonstrated that IL-13 was significantly higher in the serum of colon cancer patients compared to healthy subjects (Fig. 2A). To confirm the biological function of IL-13 in CRC, we treated HCT116 and DLD1 cells with gradient concentrations of rIL-13. We observed that rIL-13 promoted the colony forming ability and migratory capacity in a dose-dependent manner (Fig. 2B—E; Fig. S2A, B). To further verify the role of IL-13/IL-13RA2 signaling in CRC, we performed rIL-13 stimulation in IL-13RA2 knockdown CRC cells. The results showed that the oncogenic effect of rIL-13, including the colony forming ability and migratory capacity, was blocked after knockdown of IL-13RA2 in HCT116 and DLD1 cells (Fig. 2F—I; Fig. S2C, D). Collectively, these findings suggest that IL-13/IL-13RA2 signaling enhances the colony forming ability and migratory capacity of colon cancer cells.

IL-13/IL-13RA2 signaling promotes CRC-CSC self-renewal capacity

To investigate the effect of IL-13/IL-13RA2 signaling on the biological functions of CRC-CSCs, a spheroid formation assay was performed. As expected, rIL-13 remarkably promoted spheroid formation (Fig. S3A, B; Fig. 3A). To further verify the effect of IL-13 on the stemness of CRC-

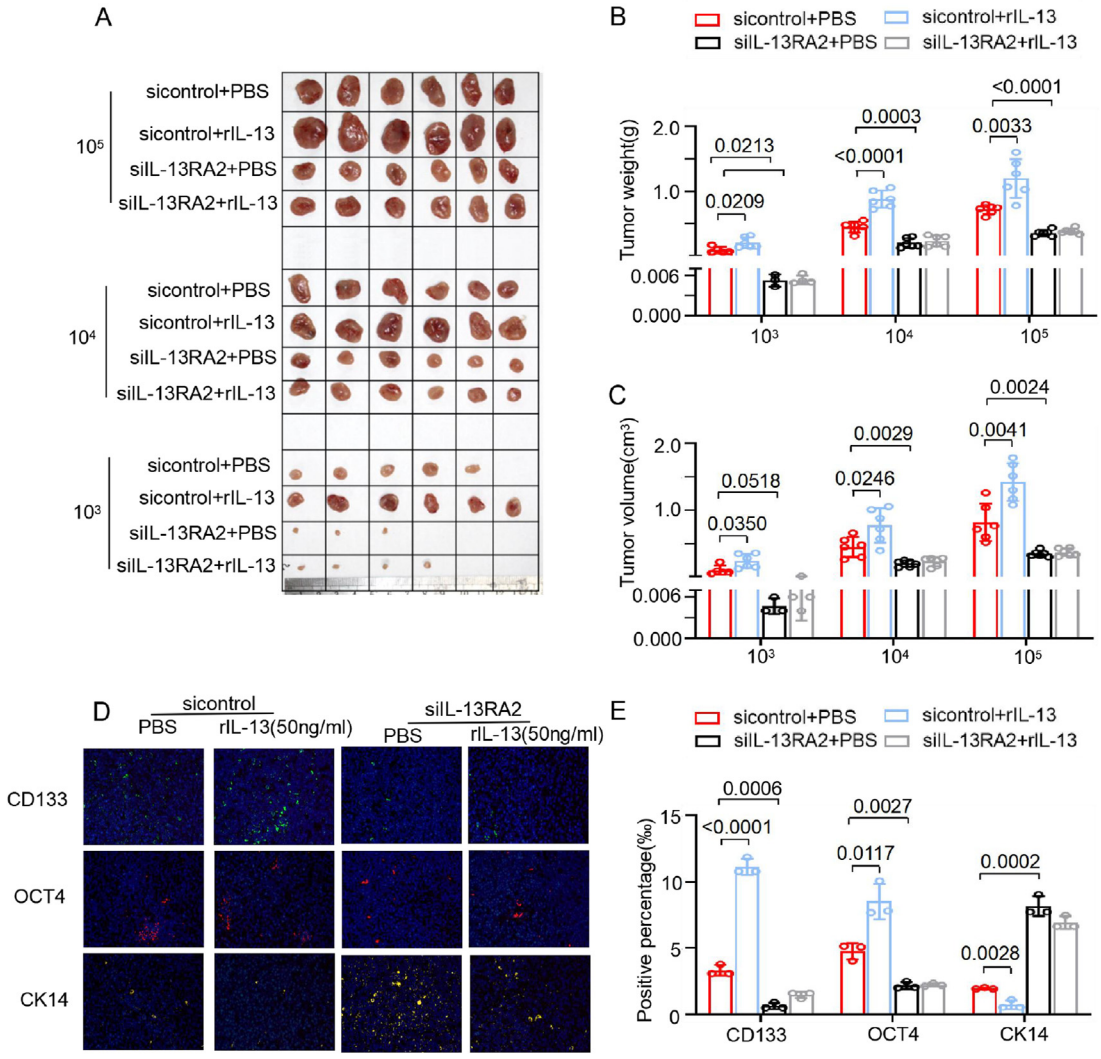

Figure 4 IL-13/IL-13RA2 signaling promotes tumor formation of CRC-CSCs. **(A—C)** HCT116 cells were transfected with sicontrol or siIL-13RA2 after stimulation with rIL-13 or PBS. The cells were then serially diluted and injected subcutaneously into nude mice ($n = 6$ for each group). The tumor weight and tumor volume were monitored for the indicated tumor. **(D, E)** Levels of CD133, OCT4, and CK1 in xenograft tissues were detected using immunofluorescence staining analysis.

CSCs, we measured the expression of stem cell markers. As shown in Figure 3B—G and Figure S3C—H, rIL-13 treatment markedly enhanced the expression of the stem cell markers SOX2 and OCT4 and inhibited the expression of the differentiation markers CK14 and MUC1 in a concentration-dependent manner at both the mRNA and protein levels in HCT116 and DLD1 cells. Flow cytometry analysis results demonstrated that rIL-13 treatment significantly increased the proportion of $CD133^{+}/LGR5^{+}/CD44^{+}$ cells (Fig. S4; Fig. 3H—J). We further determined the involvement of IL-13RA2 in IL-13-mediated CRC-CSCs function. The results showed that IL-13RA2 knockdown reversed the rIL-13-enhanced spheroid-forming ability and expression of stem cell markers (Fig. S3I, J; Fig. 3K—N). Taken together, these data indicate that IL-13/IL-13RA2 promotes the stemness of CRC-CSCs.

IL-13/IL-13RA2 signaling promotes tumor formation of CRC-CSCs

The oncogenic role of IL-13 was further confirmed in xenograft models. We established a xenograft tumor model by subcutaneously injecting HCT116 cells pre-treated with different concentrations of rIL-13. The results showed that rIL-13 promoted tumor growth in a concentration-dependent manner, as indicated by the xenograft tumor volume and tumor weight (Fig. S5A—D). Next, we determined the

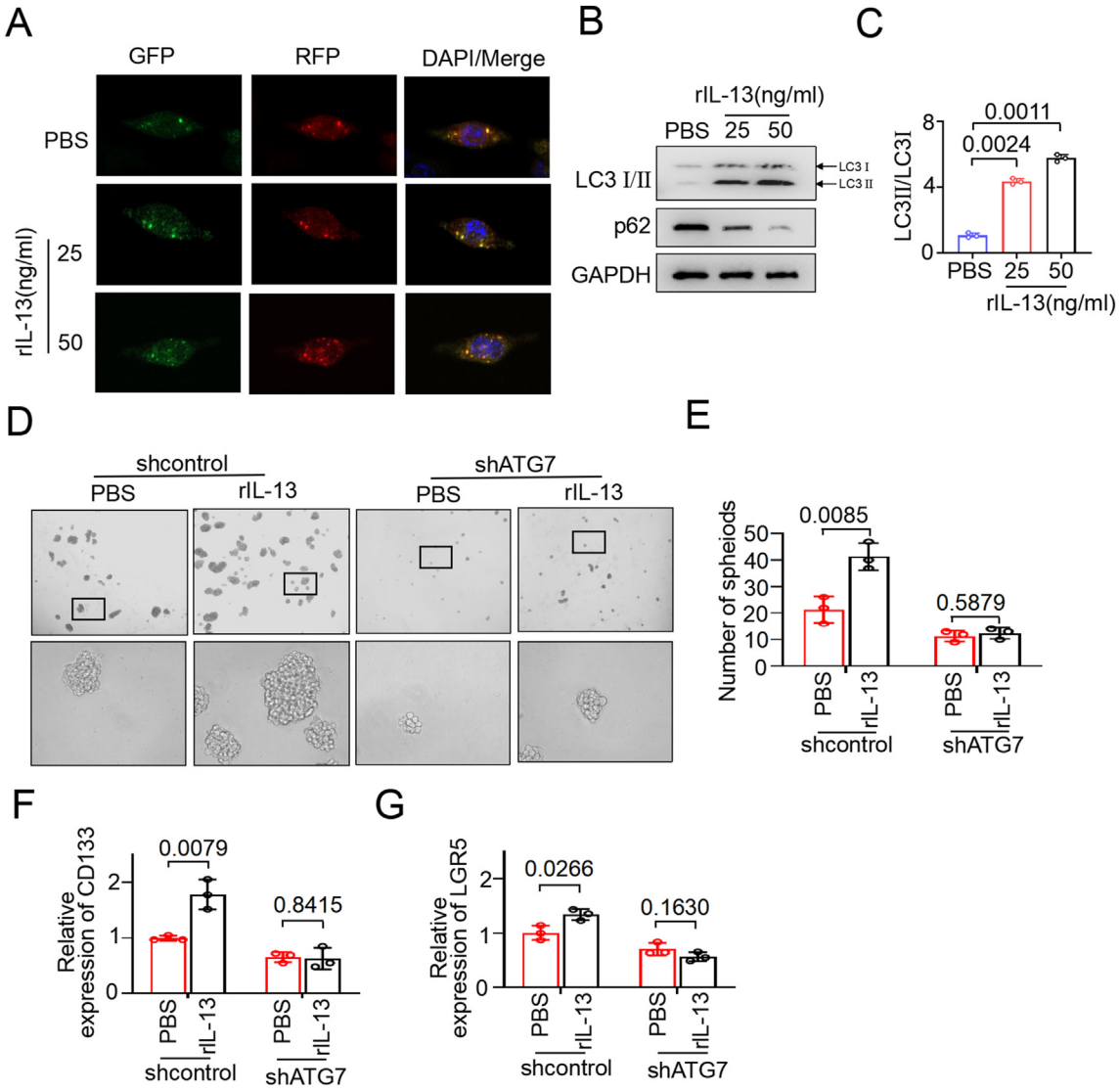

Figure 5 IL-13 activates CSC function by regulating autophagy activation. **(A)** HCT116 cells were transfected with an adenovirus that expressed GFP-linked LC3 (GFP-LC3) and then treated with rIL-13 or PBS. Confocal microscopy was used to obtain fluorescent images. **(B)** The expression levels of LC3I/II and P62 were evaluated in HCT116 cells treated with different concentrations of rIL-13. **(C)** Quantification of LC3II/LC3I shown in Figure 2B by ImageJ software. **(D, E)** Spheroid-forming ability was assessed after transfection of ATG7 siRNA in IL-13-treated HCT116 cells. **(F, G)** The mRNA expression levels of LGR5 and CD133 were measured after transfection with ATG7 siRNA in IL-13-treated HCT116 cells. Student's *t*-test (two-tailed) was used for C & E—G. Data are presented as the mean ± SEM of three independent experiments.

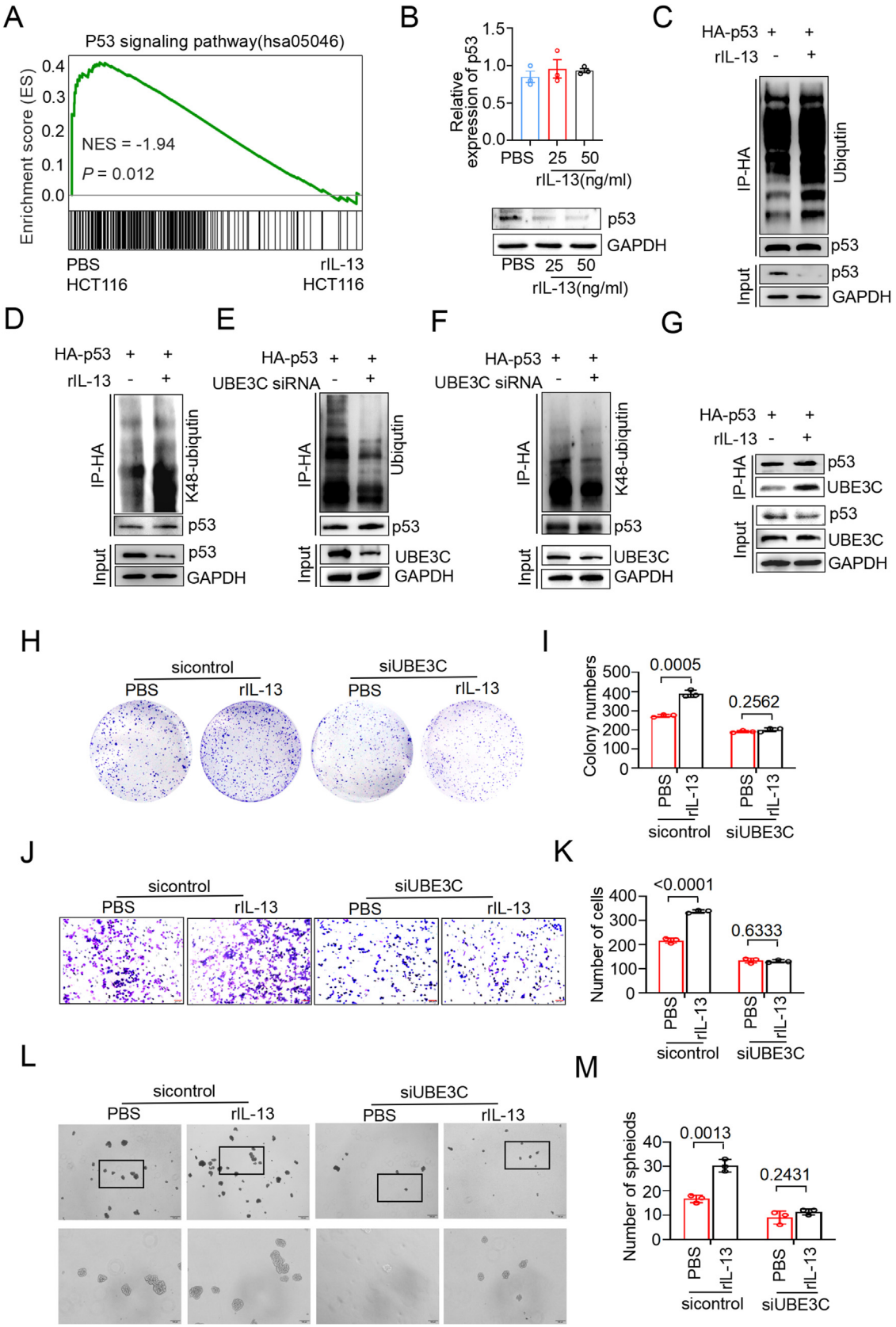

Figure 6 IL-13 promotes the stemness and autophagy of CRC-CSCs by regulating the K48-linked ubiquitination of p53. **(A)** The correlation between p53 signaling pathway gene sets and rIL-13 treatment is assessed via GSEA. **(B)** The mRNA and protein level of p53 in HCT116 cells treated with different concentrations of rIL-13. **(C, D)** HEK293T cells were first transfected with HA-p53 vector and then treated with rIL-13 or PBS. The cell lysates were subjected to an IP assay with anti-HA agarose and immunoblotted with

effect of IL-13/IL-13RA2 signaling on the tumorigenicity of CRC-CSCs. Different concentrations of cells (10^3, 10^4, and 10^5) pre-treated with IL-13RA2 siRNA and rIL-13 were subcutaneously injected into severely immunodeficient mice. First, rIL-13 treatment significantly promoted the tumor incidence of CRC cells and increased the tumor weight and volume; while IL-13RA2 knockdown substantially attenuated the tumor-promoting capacity induced by IL-13 (Fig. 4A–C and Table S5).

Next, we analyzed the expression of the stem cell markers OCT4 and CD133 and the differentiation marker CK14 in xenograft tissues. As shown in Figure 4D and E, up-regulated OCT4 and CD133 and down-regulated CK14 were observed in xenografts originating from rIL-13-treated HCT116 cells; whereas IL-13RA2 knockdown remarkably weakened IL-13-induced OCT4 and CD133 up-regulation and CK14 down-regulation. Taken together, the data indicate that IL-13/IL-13RA2 signaling promotes tumorigenesis of CRC-CSCs *in vivo*.

IL-13 activates CSC function by regulating autophagy activation

Next, we aimed to elucidate the molecular mechanisms by which IL-13 regulates CSC stemness. Autophagy is necessary to maintain the stemness of CSCs in various tumor types.[6] Moreover, several members of the interleukin family have been reported to be involved in the regulation of autophagy, which prompted us to hypothesize that IL-13 may exert biological effects in CRC-CSCs through autophagy. To this end, we first examined the impacts of IL-13-mediated autophagy activation and observed that rIL-13 treatment indeed induced the formation of autophagosomes in a concentration-dependent manner (Fig. 5A). Furthermore, rIL-13 treatment promoted cleavage-mediated conversion of LC3 protein and enhanced the ratio of LC3 II/LC3I (Fig. 5B, C). In addition, autophagy-related marker P62 was remarkably reduced after rIL-13 stimulation (Fig. 5B). Altogether, the data suggest IL-13 activates autophagy in CRC.

To further consolidate the role of IL-13 in regulating autophagy activation of CSCs, we first blocked autophagy activation by knocking down ATG7 expression, which drives the fundamental stages of degradative autophagy (Fig. S6). The results demonstrated that ATG7 silencing significantly blocked the spheroid-forming ability enhanced by rIL-13 treatment (Fig. 5D, E). Additionally, the knockdown of ATG7 attenuated the IL13-induced up-regulation of stemness markers, including CD133 and LGR5 (Fig. 5F, G). Taken together, these results suggest that IL-13 promotes stemness by impacting autophagy in CRC-CSCs.

IL-13 promotes the stemness and autophagy of CRC-CSCs by regulating the K48-linked ubiquitination of p53

Next, we investigated the molecular mechanisms by which IL-13 promotes the stemness and autophagy of CRC-CSCs. We performed transcriptome sequencing analysis of HCT116 cells stimulated with rIL-13 or PBS. A total of 2233 down-regulated genes and 1745 up-regulated genes (≥1.2-fold) were detected after rIL-13 stimulation (Table S6). Gene set enrichment analysis (GSEA) revealed that the gene sets related to cell proliferation, colon cancer chemosensitivity, tumor early initiation, and metastasis positively correlated with rIL-13 stimulation in colon cancer cells. The RNA-sequencing data bioinformatically validates the oncogenic role of IL-13 in colon cancer (Fig. S7A–D).

Excitingly, we found that the p53 signaling pathway was significantly inhibited after rIL-13 stimulation (Fig. 6A). Considering the key role of this tumor suppressor in autophagy and tumorigenesis, we speculated that IL-13 may regulate CSC function and autophagy through p53 signaling pathway. We first analyzed the mRNA and protein expression of p53 and observed IL-13 stimulation dramatically reduced the p53 protein level but had no effect on the p53 mRNA level (Fig. 6B), indicating IL-13 may impact p53 protein post-translational modification.

The ubiquitin-proteasome system is the most important mechanism of p53 protein posttranslational modification. We thus postulated that IL-13 might impact p53 ubiquitination. As expected, IL-13 stimulation indeed induced total ubiquitination the K48-linked ubiquitination of p53 protein (Fig. 6C, D). The E3 ubiquitin ligase UBE3C was reported to regulate autophagy by mediating ubiquitination at K48. Moreover, we predicted the ubiquitination substrate of UBE3C through the UbiBrowser website (http://ubibrowser.ncpsb.org/) and identified p53 as the only high-confidence interacting protein of UBE3C (Fig. S8A). Therefore, we postulated that IL-13 impacts the interaction of the E3 ubiquitinase UBE3C and p53, thereby promoting p53 protein ubiquitination and subsequent autophagy activation, consequently promoting the self-renewal and tumorigenesis of CRC-CSCs. This notion was supported by the following pieces of evidence: (i) knockdown of UBE3C significantly reduced the total ubiquitination and K48-linked ubiquitination of p53 (Fig. S8B; Fig. 6E, F); (ii) coimmunoprecipitation (Co-IP) results confirmed that IL-13 stimulation greatly enhanced the interaction between UBE3C and p53 (Fig. 6G); (iii) UBE3C knockdown indeed reversed the oncogenic function induced by rIL-13 stimulation, including cell viability, migratory ability, and spheroidization (Fig. 6H–M). In the end, UBE3C knockdown

ubiquitin and K48-ubiqutin antibodies. **(E, F)** HEK293T cells were first transfected with HA-p53 vector and then cotransfected with UBE3C siRNA. The cell lysates were subjected to an IP assay with anti-HA agarose and immunoblotted with ubiquitin and K48-ubiqutin antibodies. **(G)** Interaction between UBE3C and p53 was assessed upon IL-13 stimulation using Co-IP analysis. **(H, I)** Colony formation was assessed after transfection of UBE3C siRNA in IL-13-treated HCT116 cells. **(J, K)** Cell migratory ability was analyzed after transfection of UBE3C siRNA in IL-13-treated HCT116 cells. **(L, M)** Spheroid-forming ability was assessed after transfection of UBE3C siRNA in IL-13-treated HCT116 cells. Student's *t*-test (two-tailed) was used for I, K & M. Data are presented as the mean ± SEM of three independent experiments.

reversed autophagy-related markers LC3II/LC3I ratio and P62 levels induced by rIL-13 stimulation (Fig. S8C, D), highlighting the important function of UBE3C in IL-13-mediated stemness and autophagy. Collectively, the data suggest that IL-13 promotes the stemness and autophagy of CRC-CSCs by regulating the K48-linked ubiquitination of p53.

IL-13RA2 is a molecular link between UBE3C and p53

In light of the above findings, we set out to determine how IL-13RA2 involved in IL-13-mediated p53 ubiquitination. First, we found that rIL-13 stimulation induced the expression of IL-13RA2 in HCT116 cells (Fig. 7A). Second, we observed that IL-13RA2 silencing inhibited the total ubiquitination and K48-linked ubiquitination of p53 (Fig. 7B, C). Third, Co-IP analyses showed that IL-13RA2, UBE3C, and p53 physically interacted with each other (Fig. 7D, E). In addition, IL-13RA2 knockdown attenuated the binding of UBE3C to p53; and UBE3C silencing weakened the interaction between IL-13RA2 and p53 (Fig. 7D, E). Together, the data suggest that IL-13RA2 serves as a molecular link between UBE3C and p53.

To further consolidate the regulation of the UBE3C-p53 interaction by IL-13RA2, we overexpressed IL-13RA2 in HEK293T cells. As indicated in Figure 7F and G, elevated IL-13RA2 expression significantly promoted UBE3C-p53 interaction and K48-linked ubiquitination of p53. Taken together, our data suggest that IL-13RA2 acts as a modular scaffold of UBE3C E3 ligase and p53 substrate and enhances the interaction of UBE3C and p53, which results in the activation of K48-linked ubiquitination of p53 (Fig. 8).

Discussion

CSCs are a self-renewing cell type found in various tumors that promote tumorigenesis, expansion, drug resistance, recurrence and metastasis.[4,17,18] Effective tumor treatment strategies rely on targeting CSCs and non-CSC to eliminate possible tumorigenesis and recurrence opportunities.[4,19] At present, several surface markers of CSCs have been found to facilitate the identification of CSCs, such as CD133, LGR5, SOX2, OCT4, and Nanog.[20] In addition, WNT/β-catenin, TGF-β, Hedgehog, and Notch are important signals to maintain the self-renewal of CSCs.[21–23] Nonetheless, the molecular mechanisms underlying the homeostatic regulation of CSCs in cancer remain incompletely

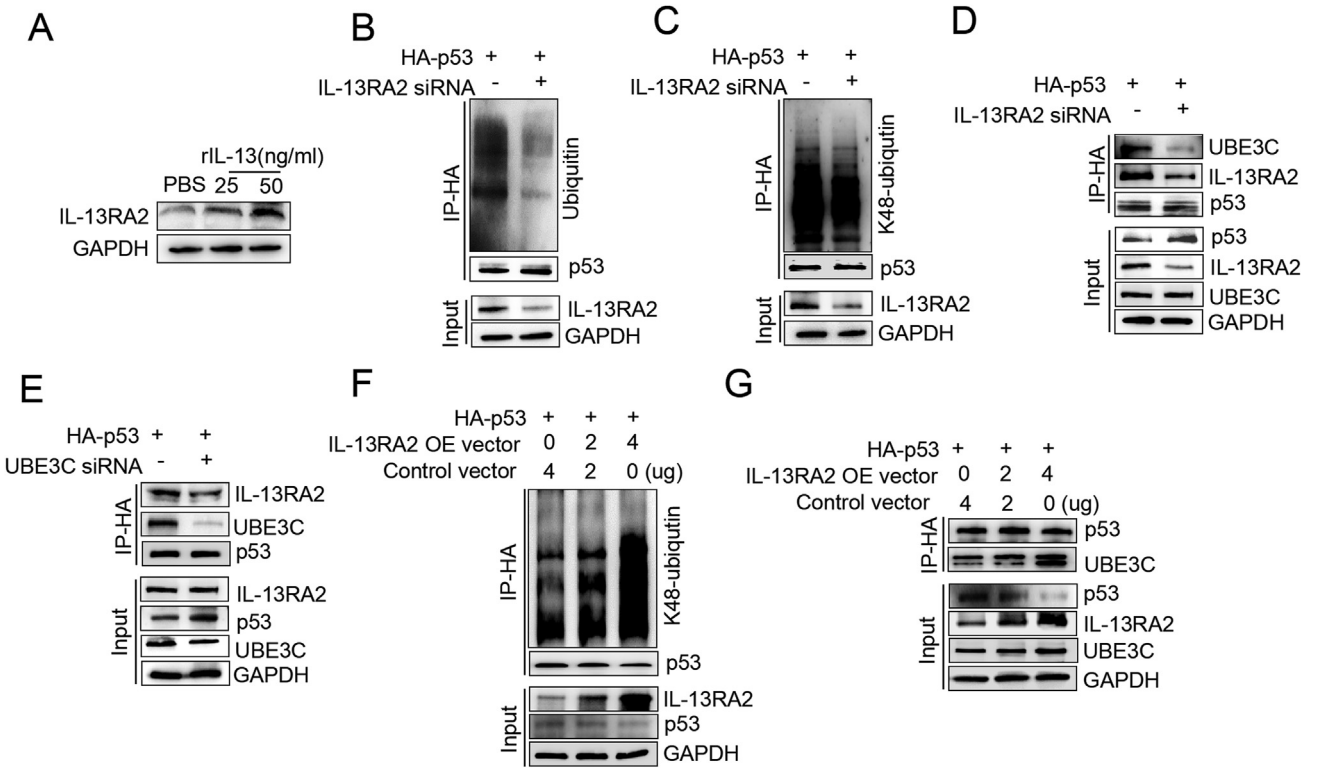

Figure 7 IL-13RA2 is a molecular link between UBE3C and p53. **(A)** The protein level of IL-13RA2 was assayed in HCT116 cells treated with different concentrations of rIL-13. **(B, C)** HEK293T cells were first transfected with HA-p53 vector, and the cells were cotransfected with IL-13RA2 siRNA. The cell lysates were subjected to an IP assay with anti-HA agarose and immunoblotted with ubiquitin and K48-ubiqutin antibodies. **(D)** HEK293T cells were cotransfected with HA-p53 vector and IL-13RA2 siRNA. The interaction between UBE3C and p53 was assessed by Co-IP analysis. **(E)** HEK293T cells were cotransfected with HA-p53 vector and UBE3C siRNA. The interaction between IL-13RA2 and p53 was assessed by Co-IP analysis. **(F)** HEK293T cells were cotransfected with HA-p53 vector and gradient IL-13RA2 plasmids. The cell lysates were subjected to an IP assay with anti-HA agarose and immunoblotted with the K48-ubiqutin antibody. **(G)** HEK293T cells were cotransfected with HA-p53 vector and gradient IL-13RA2 plasmids. The interaction between UBE3C and p53 was assessed using Co-IP analyses.

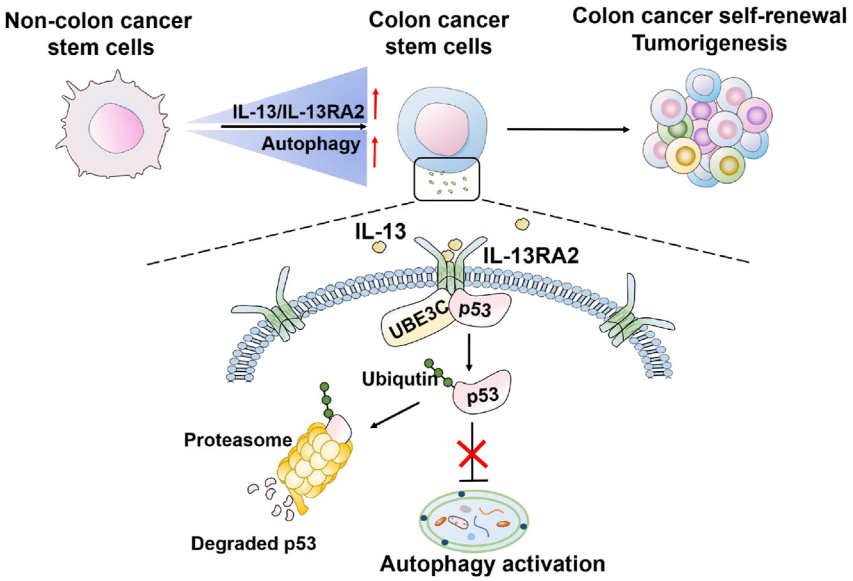

Figure 8 Schematic diagram of the study. IL-13 promotes IL-13RA2 expression and activation. Then, activated IL-13RA2 mediates and enhances the interaction of the E3 ubiquitinase UBE3C and the substrate p53 protein, resulting in p53 ubiquitination. p53 ubiquitination activates autophagy, which ultimately promotes the self-renewal and tumorigenesis of CRC-CSCs.

understood. In the present study, we demonstrated that the IL-13/IL-13RA2 signaling cascade plays a critical role in the homeostasis of CRC-CSCs. At the functional level, we demonstrated that IL-13/IL-13RA2 signaling promotes the tumorigenesis of CRC-CSCs, representing a viable therapeutic target for CRC.

Previous studies have shown that IL-13 is mainly involved in the regulation of inflammation and immune responses.[24,25] In recent years, IL-13 has been found to be involved in the occurrence and development of tumors, including breast cancer,[26] lung cancer,[27] pancreatic cancer,[28] bladder cancer[29] and brain tumor.[30] Many studies have found that IL-13 exerts its function through its cell surface receptors (IL-13RA1/IL-4RA, IL-13RA2).[31] The binding affinity of IL-13RA2 to IL-13 is higher than that of the IL-13RA1/IL-4RA complex. Commonly, IL-13RA2 is merely a decoy receptor that binds strongly to free IL-13 without eliciting a signal.[32] Recent studies have reported that IL-13RA2 is not just a decoy receptor. Fichtner-Feigl et al demonstrated a role for IL-13RA2-mediated signaling that requires the cytoplasmic tail of IL-13RA2 to participate in TGF-β1 production, providing evidence for IL-13RA2-mediated signaling.[12] Ko et al found that IL-13 can induce the expression of TGF-β through IL-13RA2 and exert its protumor activity in the early stage of intestinal tumorigenesis.[33] IL-13RA2 can also mediate the effect of IL-13 on colon cancer cell adhesion, and silencing IL-13RA2 reduced the promoting effect of IL-13 on colon cancer tumorigenesis and proliferation.[34] Here, we demonstrated that IL-13/IL-13RA2 signaling promotes the self-renewal and tumorigenicity of CRC-CSCs. Thus, other prior research and our present research consistently point to the notion that IL-13/IL-13RA2 signaling activation is an important tumor-initiating event.

Autophagy has been extensively studied in tumor biology and is generally thought to influence tumorigenesis, tumor metastasis, and drug resistance.[35–37] With the deepening of related research, the role and mechanism of autophagy in the regulation of CSC functions are constantly being discovered. The autophagy key protein LC3 was positively correlated with the expression of ALDH1, CD44 and CD133, which are important markers of CSCs.[38] Down-regulation of the expression of the autophagy-related proteins ATG5, ATG7, and Beclin-1 significantly reduced the self-renewal of CSCs.[39] Considering previous studies on the close links between autophagy and CSC function, we hypothesized that IL-13/IL-13RA2 may regulate CSC stemness by impacting autophagy. As expected, we demonstrated that IL-13 promoted autophagosome formation in CSCs. Knockdown of IL-13RA2 inhibited the activation of autophagy, and down-regulation of ATG7 abolished the self-renewal capacity induced by IL-13 in CRC-CSCs. More importantly, IL-13 enhanced the expression of IL-13RA2, suggesting that IL-13/IL-13RA2 forms a positive regulatory loop that controls CSC homeostasis. However, the way autophagy regulates CSC homeostasis remains unclear.

The principal tumor-suppressor protein, p53, accumulates in cells in response to DNA damage, oncogene activation, and other stresses.[40,41] Analysis of transcriptome sequencing data from rIL-13-treated cells showed that p53 signaling pathway was significantly inhibited after rIL-13 stimulation, indicating IL-13 might regulate CSC autophagy via p53 signaling pathway. Studies have revealed that p53 inhibits autophagy by interacting with FIP200, which is homologous to yeast ATG17.[42] Knockdown of p53 in CRC promoted the conversion of LC3I to LC3II and the expression of Beclin-1, resulting in the activation of autophagy.[43] By evaluating the alterations of mRNA and protein levels of p53 after rIL-13 stimulation, we demonstrated IL-13 impacts p53 protein post-translational modification. The ubiquitin–proteasome system (UPS) is the most important mechanism of p53 degradation.[44] Therefore, we

hypothesized that autophagy activation by IL-13/IL-13RA2 signaling may be mediated by p53 ubiquitination. Our results substantially confirmed that IL-13 stimulation promoted the K48-linked ubiquitination of p53. Furthermore, bioinformatics prediction combined with experimental validation confirmed that IL-13RA2 can link UBE3C and p53 by acting as a potential modular scaffold to promote the interaction of UBE3C and p53, augmenting the ubiquitination level of p53 and activating autophagy. Consistently, UBE3C knockdown indeed reversed the autophagy activation and oncogenic function induced by rIL-13 stimulation, including autophagy-related markers LC3 II/I ratio, P62 protein level, cell viability, migratory ability, and spheroidization. Together, IL-13 promotes the stemness and autophagy of CRC-CSCs by regulating the UBE3C-mediated ubiquitination of p53.

In conclusion, IL-13/IL-13RA2 signaling promotes the stemness of CRC-CSCs via autophagy activation. Furthermore, our study shows a link between IL-13 and p53 and highlights the necessity of the IL-13/IL-13RA2/p53 signaling pathway for maintaining CSC homeostasis. Targeting this pathway may be pivotal in the prevention or treatment of CRC.

Ethics declaration

The studies involving human participants were reviewed and approved by the ethics committee of the Affiliated Hospital of Jining Medical University (No. 2021B006). Written informed consent was obtained from all participants in this study. All the research was carried out in accordance with the provisions of the Declaration of Helsinki of 1975.

Author contributions

Bin. Z. and Baogui Z. conceived the experiments. B.H., J.L., Y.Z., S.S., J.C., Z.Z., Q.B., and R.Z. performed the experiments and analyzed the data. L.W. provided essential reagents. B.H., J.L., R.Z., and Bin. Z. wrote the manuscript. All authors agree to manuscript submission for publication in the journal.

Conflict of interests

The authors declare no competing financial interests.

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Data availability

The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.01.027.

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