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Livin promotes colon cancer progression by regulation of H2A.X^{Y39ph} via JMJD6

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ABSTRACT

Livin is an important member of the human inhibitor of apoptosis proteins (IAPs) family. IAPs are proteins with antiapoptotic abilities, and their functions are different from the Bcl-2 (B-cell lymphoma-2) family proteins. However, the precise role of Livin in colon cancer progression remains unclear. The purpose of this study is to assess the effect of overexpression Livin in colon cancer cells and to examine its molecular mechanism. We demonstrated that Livin induced a colon cancer phenotype, including proliferation and migration, by regulating H2A.X^{Y39ph} (histone family 2A variant (H2AX) phosphorylated on the 39th serine site). We elucidated that Livin degraded Jumonji-C domain-containing 6 protein (JMJD6), which was mediated by the proteasome murine double minute 2 (MDM2), thereby regulating H2A.X^{Y39ph}. Above all, the overexpression of JMJD6 recovered H2A.X^{Y39ph} in colon cancer cells with a high level of Livin, thus inhibiting colon cancer malignancy progression. These results reveal a previously unrecognized role for Livin in regulating the tumor-initiating capacity in colon cancer and provide a novel treatment strategy in cancer via the interruption of H2A.X^{Y39ph} function and the interaction between H2A.X^{Y39ph} and JMJD6.

1. Introduction

Livin, also named ML-IAP (melanoma inhibitor of apoptosis protein) or KIAP (kidney inhibitor of apoptosis protein), is known as an important member of the human inhibitor of apoptosis proteins (IAPs) family [1]. IAPs are proteins with antiapoptotic abilities, and their functions are different from the Bcl-2 (B-cell lymphoma-2) family proteins [2]. Numerous studies have reported that increased Livin in colorectal tumor correlates with a more aggressive behavior, such as proliferation and migration, leading to a shorter overall survival or a shorter disease-free survival. For example, siRNA-mediated decreased gene expression of Livin dramatically suppresses colon tumor growth [3]. Liu et al. demonstrated that silencing Livin improved the sensitivity of colon cancer cells to 5-fluorouracil by regulating the crosstalk between apoptosis and autophagy [4]. These studies help us to further understand the underlying mechanisms of how Livin drives tumor progression potential and the factors that contribute to this process.

Cancer epigenetics explains a large amount of heritable alterations in gene expression, without changing the DNA sequence. Histone modifications, aberrant DNA methylation and the expression of long non-coding RNAs (lncRNAs) play vital roles in epigenetic mechanisms, which are associated with cancer initiation, cancer progression and cancer metastasis. Histone family 2A variant (H2AX) is a vital element in the DNA damage repair process, among which γ-H2AX is a neoteric biomarker for double-stranded DNA breaks. H2A.X^{Y39ph} (H2A.X phosphorylated on the 39th serine site) is newly reported and is known for its complexity and diversity of modifications or functions about H2A.X. As reported, the level of H2A.X^{Y39ph} is higher in several tumors tissues than in the adjacent tissues, especially with the highest difference in colon cancer, but the role of H2A.X^{Y39ph} is unclear [2]. Jumonji-C domain-containing 6 protein (JMJD6) has intrinsic tyrosine kinase activity and utilizes ATP and GTP as phosphate donors to phosphorylate H2A.X^{Y39ph} [5]. Thus, JMJD6 might be an important molecule regulating H2A.X^{Y39ph} during colon cancer progression.

Nevertheless, although these early studies have reported that Livin might be aggressive in colorectal tumors, the direct role of Livin in the H2A.X^{Y39ph} signal pathway via JMJD6 has not been illustrated. Consequently, we investigated the role of Livin, H2A.X^{Y39ph}, and JMJD6 in colon cancer cells or a colon cancer phenotype. Firstly, we found that Livin induced a colon cancer phenotype, including proliferation and migration, by regulating H2A.X^{Y39ph}. We elucidated that Livin degraded JMJD6, which was mediated via the proteasome, thereby regulating H2A.X^{Y39ph}. Above all, the overexpression of JMJD6 recovered H2A.X^{Y39ph} in colon cancer cells with high levels of Livin,

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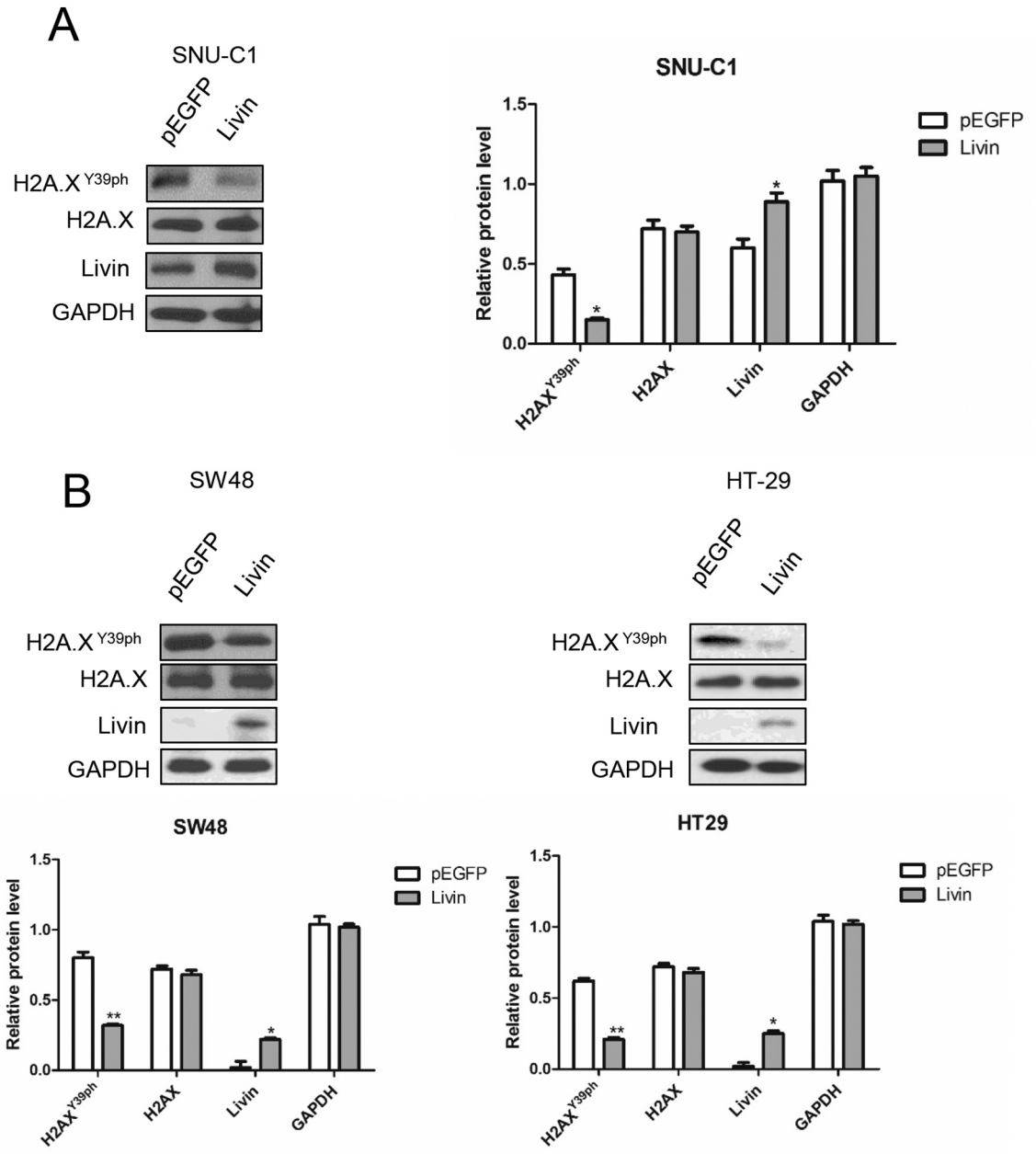

Fig. 1. H2A.X^{Y39ph} is regulated by Livin.
A, Peritoneal metastasis of colon cancer cells (SNU-C1) was transfected with empty-pEGFP-N1 (vector) or pEGFP-Livin expressing plasmids. The H2A.X^{Y39ph} and Livin levels were then measured by a western blot after 48 h.
B, Colon adenocarcinoma cells (SW48 and HT-29) were transfected with empty-pEGFP-N1 (vector) or pEGFP-Livin expressing plasmids. The H2A.X^{Y39ph} and Livin levels were then measured by a western blot after 48 h.

thus inhibiting colon cancer malignancy progression.

2. Materials and methods

2.1. Plasmid and siRNA construction

The products for the coding genes for human H2AX, JMJD6, MDM2 (murine double minute 2), pCMV-HA and wild-type K-Livin were amplified from colon cancer cell cDNA using a PCR test. These PCR products were subsequently cloned into an HA-tag vector and were then checked by sequencing. The pEGFP-K-LivinG12V plasmid was mutated by site-directed mutagenesis. pEGFP-K-LivinG12V T35S was mutated using the pEGFP-K-LivinG12V plasmid. The siRNAs targeting MDM2 were purchased from Shanghai GenePharma Co., Ltd. The pEGFP-H2A.X^{Y39E} plasmid was constructed by the TaKaRa MutanBEST Kit (Code No.: D401), which was purchased from TaKaRa Bio-technology Co., Ltd.

2.2. Cell culture and treatments

The peritoneal metastasis of colon cancer cells (SNU-C1) and the colon adenocarcinoma cells (SW48 and HT-29) were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). These cells were cultured in complete DMEM, which was supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin, at 37 °C in a humidified atmosphere with 5% CO_2.

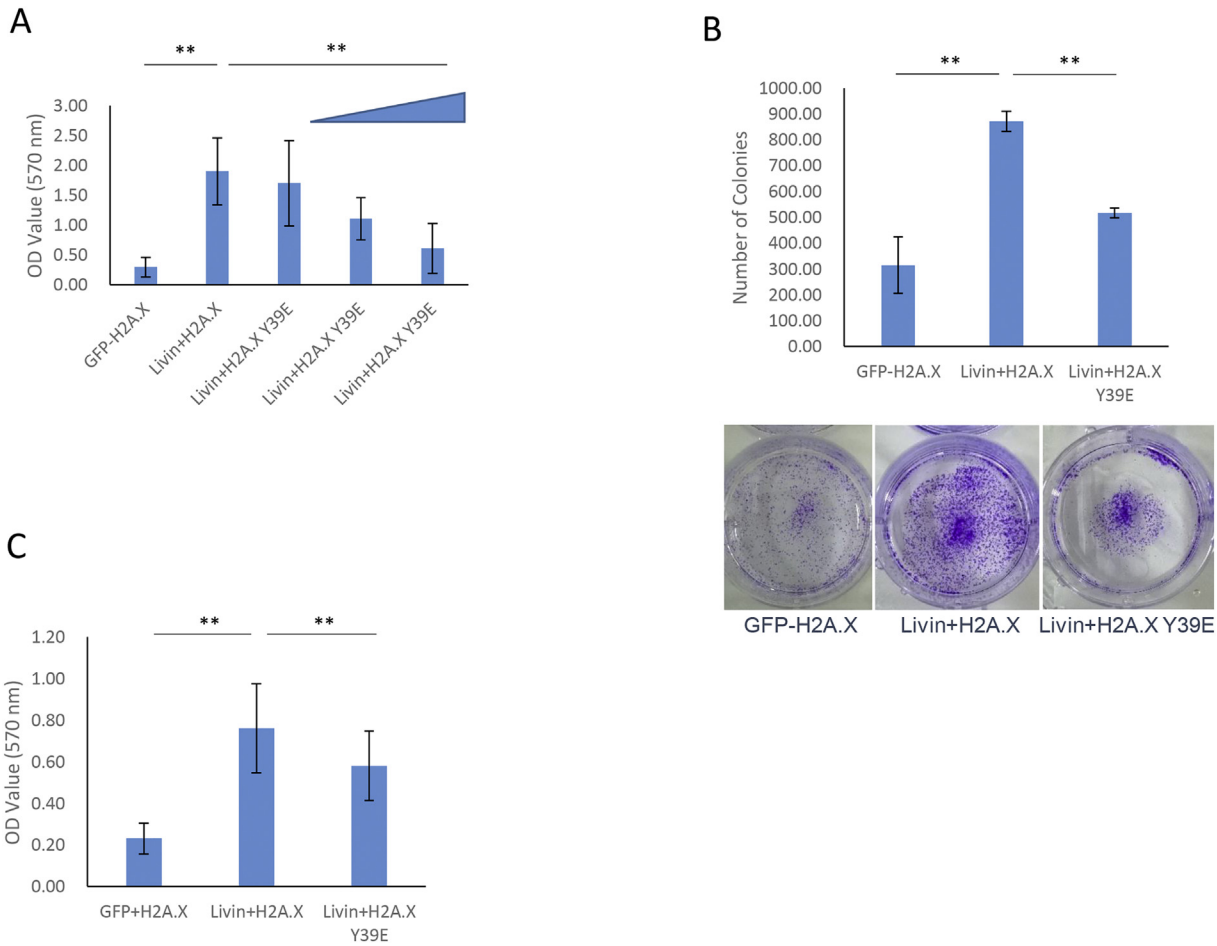

Fig. 2. H2A.X^{Y39ph} is functionally involved in the oncogenic Livin signaling pathway.
The SW48 cells were transfected with the pEGFP-N1, pEGFP-H1.2, pEGFP-Livin, or pEGFP-H2A.X Y39E (indicated as GFP, H1.2, Livin and H2A.X Y39E, respectively) plasmids. The presence of H2A.X^{Y39ph}, which was mimicked by H2A.X^{Y39ph} expression, reduced Livin-induced cell viability (A), colony formation (B), and cell migration (C). For (A), increasing amounts of the H2A.X Y39E (0.5, 1, and 2 mg) expression plasmid was co-transfected with 0.6 mg of the pEGFP-Livin plasmid. The bar graphs represent the mean ± S.E of the optical density (OD) values (A and C) or the number colonies (B). $^{**}p < 0.01$.

2.3. Transfection

After the colon cancer cells were cultured and entered the logarithmic growth phase, they were seeded into 6-well plates (5×10^5 per well) until they reach 80% confluency. Then, the plasmids or siRNAs were transfected into the colon cancer cells alone or together using Lipofectamine 2000 (Invitrogen) following the recommended protocol. Forty-eight hours later, the transfected colon cancer cells were collected for western blot or RT-PCR analyses.

2.4. Cell viability

After the colon cancer cells were cultured and entered the logarithmic growth phase, the cells were seeded into 96-well plates (5×10^3 per well) for transfection, and untreated cultures were seeded for the negative control. Then, the cells were cultured for 48 h. At detection, 20 μL of MTT (5 mg/mL) was added to each well and was incubated at 37 °C for 4 h. Subsequently, 100 μL dimethyl sulfoxide (DMSO) was added to terminate the reaction. The absorbance was measured at 450 nm using a microplate reader. The A450 value reflected the number of surviving cells.

2.5. RNA extraction and RT-PCR

When the SW48 cells were grown to the logarithmic growth phase, the total RNA was isolated using the TRIzol reagent (Invitrogen, USA). DNase-I was firstly added to the total RNA, and then, M-MuLV reverse transcriptase (Fermentas, Canada) and oligo-dT primers (Invitrogen, USA) were added to total RNA for obtaining first-strand cDNA. GAPDH was used as the internal control, and the analysis of every sample was repeated at least five times in triplicate.

2.6. Real-time PCR

The cDNA was also used for real-time PCR using the QuantiTect SYBR Green PCR Kit (Qiagen, Germany). This assay was conducted in a 7500 PCR machine (ABI, USA). For relative quantification, GAPDH was used as the internal control, and every sample was repeated at least five times in triplicate.

2.7. Soft agar assay

The SW48 colon cancer cells were mixed with 0.35% low-melting agarose and were seeded into 6-well plates (1×10^3 cells/well), and the bottom of the 6-well plates was solidified with 0.6% agarose. Then, the SW48 cells were cultured in DMEM for three weeks. The colonies were then stained with a 0.005% crystal violet solution, and the number of colonies was counted by a light microscope at ×20 magnification.

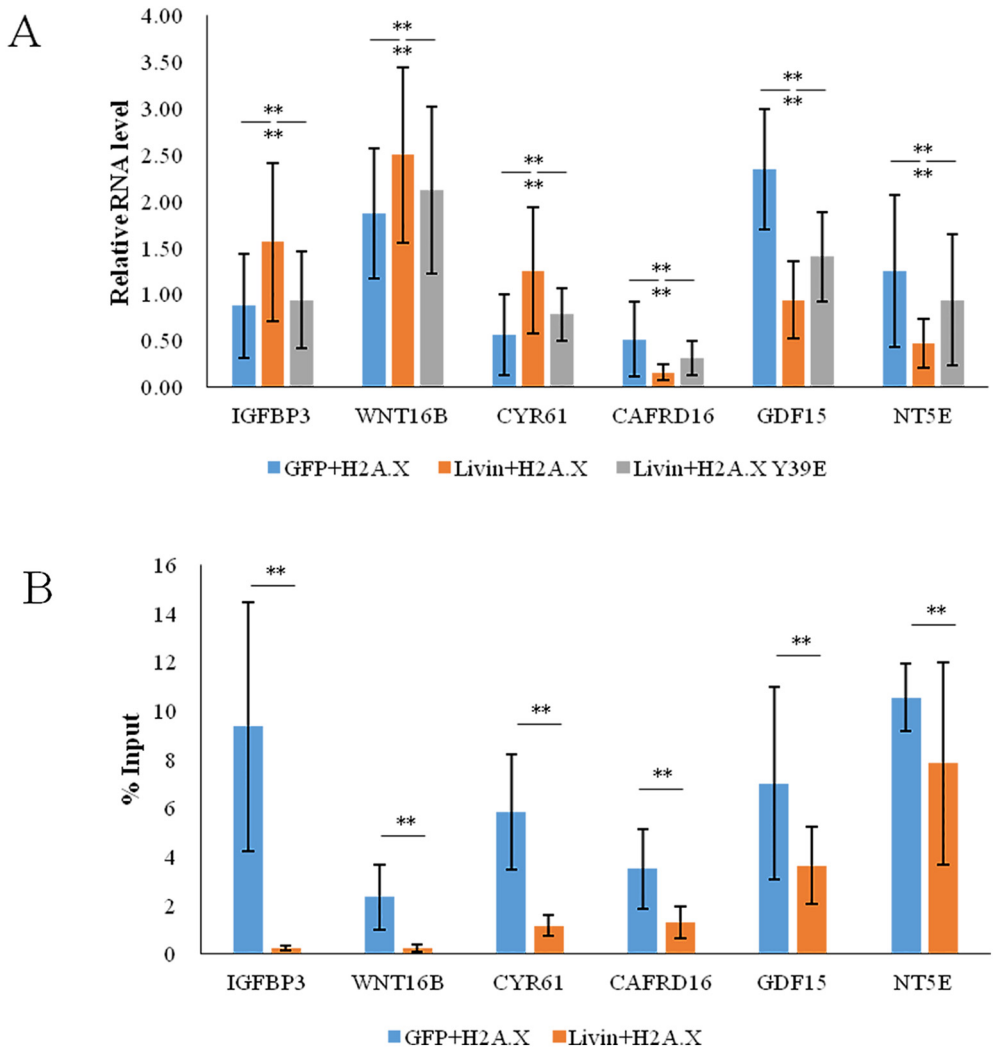

Fig. 3. The transcription of Livin-targeted genes is partially rescued by increased H2A.X^{Y39ph}.
A, The transcription of several genes was tested using real time PCR. The values are presented as the mean ± S.E. percentage relative to GAPDH expression.
B, As detected using ChIP, reduced levels of H2A.X^{Y39ph} were present on the differentially expressed genes. The values are presented as the means ± S.E. from three independent experiments.

2.8. Transwell migration assay

The transwell migration assay was performed in 24-well transwell plates, which contained inserts with an 8-μm pore size that were polycarbonate membranes. After transfection, 100 μL of the cell suspension was loaded into the upper chamber of the transwell plates (1×10^5 cells/well), and complete media was added into the bottom of the wells without cells. Then, the cells were incubated at 37 °C for 12 h. The cells that migrated into the bottom surface of the membrane were fixed and were observed by microscopy. The cells remaining on the upper surface of the membrane were scrubbed off gently using cotton-tipped swab and were then assessed by measuring the OD570.

2.9. Western blot analysis

After the SW48 cells were transfected for 48 h, the cells were collected and washed with PBS twice. The whole-cell lysates were prepared using radio immunoprecipitation assay (RIPA; Cwebio, Beijing) lysis buffer with a protease inhibitor (Cwebio, Beijing). The protein was quantified using the BCA kit (Cwebio, Beijing). The denatured proteins were separated by SDS-PAGE and transferred were onto nitrocellulose membranes (NC, Thermo, USA). After blocking with 5% BSA (pH 7.4) in PBST at room temperature (RT, 24–26 °C) for 1 h, the membranes were then incubated with the appropriate primary antibody at 4 °C overnight and were subsequently incubated with an HRP-conjugated anti-mouse or anti-rabbit secondary antibody at RT for 1 h. The blots were developed using an ECL reagent (PTG, USA) and were quantitated by QUANTITY ONE as recommended by the manufacturer. The western blots were performed using the following antibodies: rabbit anti-hum Livin (1:1000, PTG, USA); rabbit anti-hum H2AX (1:1000, PTG, USA); rabbit anti-hum H2AX^{Y39ph} (1:1000, PTG, USA); rabbit anti-hum JMJD6 (1:1000, CST, USA); rabbit anti-hum GAPDH (1:5000, PTG, USA); rabbit anti-hum actin (1:5000, CST, USA); and a polyclonal HRP-conjugated goat anti- rabbit antibody (1:5000, PTG, USA).

Flow Cytometric Analysis of the Cell Cycle.

After the SW48 colon cancer cells were transfected for 24 h, the cells were washed with ice-cold PBS twice and were fixed with 1% (v/v) paraformaldehyde at RT for 1 h. The cells were rehydrated with PBS and were then stained with a propidium iodide (PI) staining solution at RT for 30 min. Then, the cells were resuspended in 1 × binding buffer at a concentration of 1–5 × 10^6/ml. A total of 100 μL cell suspension was detected by the Flow cytometer (Beckman, USA) for each sample. Then, the percentages of cells in the G0/G1, S and G2/M phases of the cell cycle were determined based on the FlowJo software.

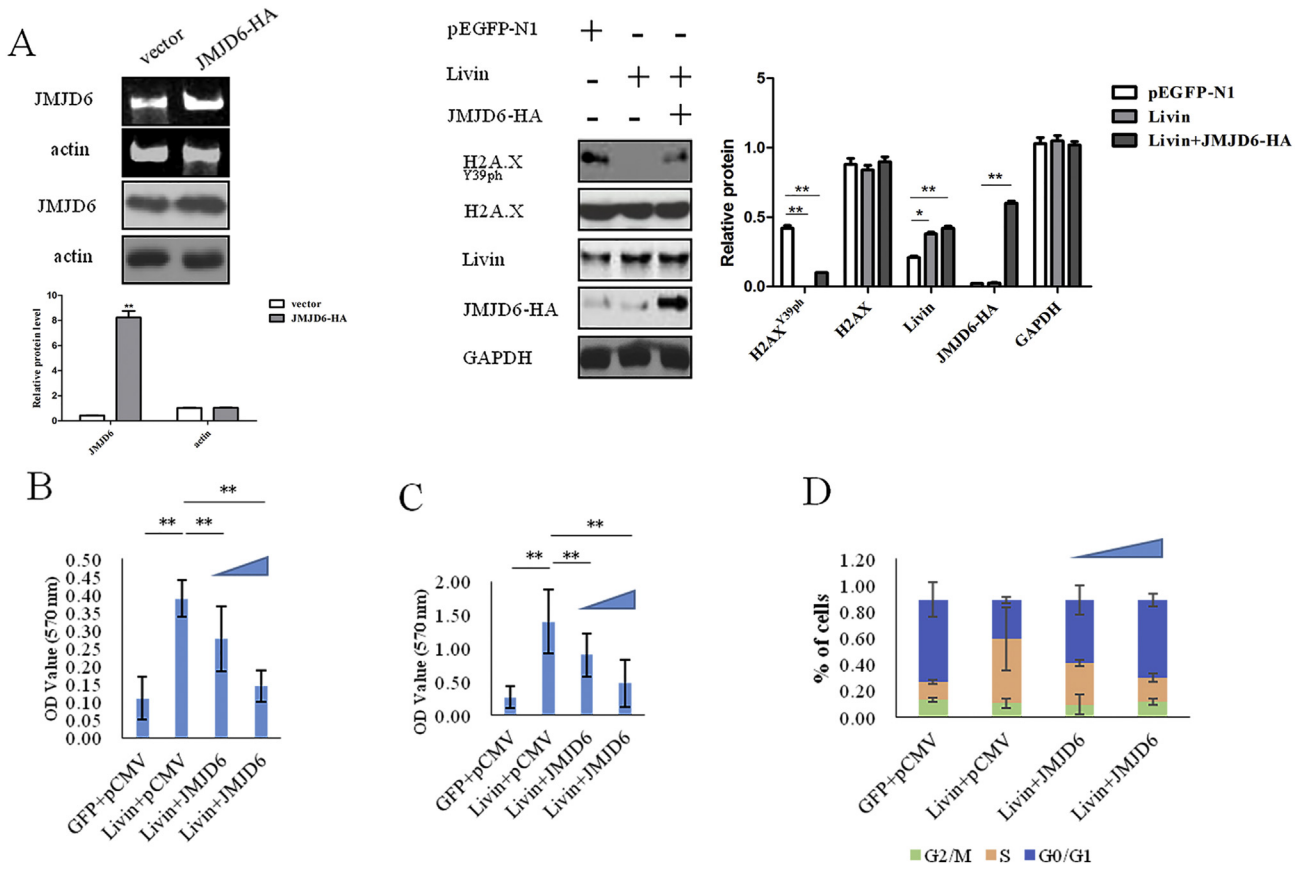

Fig. 4. JMJD6 overexpression prevents Livin overexpression-induced decreases in H2A.X^{Y39ph}.
A, The efficiency of JMJD6 overexpression was determined (left panel). The overexpression of JMJD6 prevented the Livin activation-induced decrease in the H2A.X^{Y39ph}. SW48 cells were co-transfected as indicated. Whole cell lysates were assayed by a western blot.
B–D, SW48 cells were co-transfected with the pEGFP-Livin or pEGFP-N1 plasmids and the JMJD6-expressing or pCMV-HA plasmids, as indicated (Livin, GFP, JMJD6, or pCMV, respectively). As detected using the Transwell and cell viability assays, JMJD6 overexpression resulted in reduced cell migration and cell viability (B and C). Cell cycle progression was measured using flow cytometry (D). The overexpressed plasmids of JMJD6 in B and C were transfected with gradient increasing transfection, and the two groups, respectively, were 0.5 mg and 1 mg.

2.10. Chromatin immunoprecipitation (ChIP)

After the SW48 cells were transfected for 24 h, about 3×10^6 SW48 cells were cross-linked in 1% formaldehyde for 10 min at RT for every sample. Then, the cells were washed twice with cold PBS and were lysed in SDS Lysis Buffer (Solarbio, Beijing, China). Then, the lysate products were sonicated in an ultrasonic bath (Aipu, China) to obtaining DNAs of 200–800 bp in length on average. After sonication, the products with the DNAs were centrifuged at 10,000 $\times g$ for 4 min at 4 °C. The supernatants were diluted with ChIP Dilution Buffer (Solarbio, Beijing, China) and were immunoprecipitated overnight with rabbit anti-H2A.X^{Y39ph} (2 μg) adhered to beads at 4 °C. The control immunoprecipitation was 2 μg of normal anti-GFP antibody. Then, the beads were washed sequentially in low-salt, high-salt and LiCl buffers at 4 °C each for 5 min. Lastly, the beads were washed twice in 1 × TE at RT for 2 min. The DNA was washed for 15 min at RT from the beads by 1% SDS/100 mM $NaHCO_3$. In a final concentration of 200 mM of NaCl, the crosslinks were incubated at 65 °C for 7 h. Then, the eluted DNA was precipitated at −20 °C with ethanol overnight and mixed with 20 μg of proteinase K. Subsequently, the eluted DNA was purified using QIAquick PCR Purification Columns (Solarbio, Beijing, China) as recommended by the manufacturer. The immunoprecipitated DNA (1.5 μL), with serial dilutions of the 10% input DNA (1:4, 1:20, 1:100 and 1:500), were analyzed by real-time qPCR.

2.11. Statistical analysis

The statistical analyses were performed using SPSS 18.0 (SPSS, USA). The statistical differences among the groups were tested using a one-way analysis of variance (ANOVA). The follow-up analysis used the least significant difference (LSD) test. The results are expressed as the mean ± SD. Results with $p < 0.05$ were considered statistically significant.

3. Result

3.1. H2A.X^{Y39ph} is specifically regulated by the Livin pathway

Firstly, we determine whether Livin affected the level of H2A.X^{Y39ph}. The pEGFP-Livin plasmid was transfected into the colon cancer cells, and thus, Livin was specifically over-expressed. Then, we measured the level of H2A.X^{Y39ph} in the transfected cells. In the SNU-C1 peritoneal metastasis of colon cancer cells, we found that the level of H2A.X^{Y39ph} in the cells transfected with the pEGFP-Livin plasmid was reduced compared with the cells transfected with the empty-pEGFP-N1 plasmid (Fig. 1A). This decrease in H2A.X^{Y39ph} was also observed in the colon adenocarcinoma cells (SW48 and HT-29) (Fig. 1B). These results showed that when Livin was over-expressed, the level of H2A.X^{Y39ph} was reduced accordingly in colon cancer cells. This suggested that H2A.X^{Y39ph} was regulated by the Livin pathway.

H2A.X^{Y39ph} down-regulation is involved in the regulation of Livin

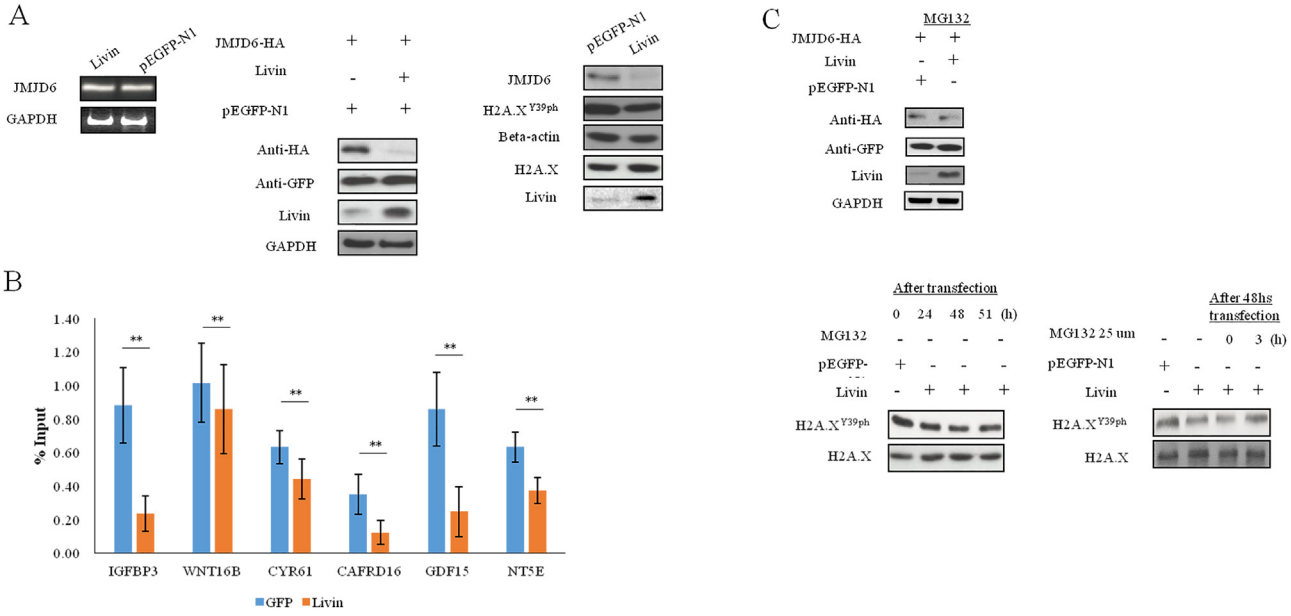

Fig. 5. Livin overexpression is associated with JMJD6 degradation.
A, SW48 cells were transfected with 0.6 mg of pEGFP-Livin, 0.6 mg of pEGFP-N1, or 3 mg of JMJD6-HA plasmids. Then, 24 h later, the nucleic acids were extracted for PCR, and 48 h later, the cell lysates were analyzed by a western blot.
B, The recruitment of JMJD6 to the genes that exhibited changes in H2A.X^{Y39ph} was analyzed using ChIP analysis.
C, Whole cell extracts from the SW48 cells that were treated with MG132 were prepared, and the JMJD6 protein levels were analyzed by a western blot with antibodies against HA and GFP. Whole cell extracts from SW48 cells that were untreated (the samples were collected at 24, 48 and 51 h following transfection; upper panel) or treated with MG132 (the MG132 was added at 48 h following transfection, and the samples were collected at 24, 48, 51 and 54 h following transfection; lower panel) were analyzed by a western blot.

on the phenotype of colon cancer cells.

Next, we investigated the effect of H2A.X^{Y39ph} on the colon cancer phenotype when Livin is overexpressed. The Livin plasmid was constructed, and H2A.X^{Y39E} plasmid was constructed to simulate the phosphorylated state of H2A.X^{Y39ph}. Firstly, we only tested the phenotype (viability, colony formation and cell migration) of the colon cancer cells transfected with the Livin plasmid at a concentration of 0.6 μg. To further validate whether the colon cancer cell phenotype was affected by H2A.X^{Y39ph} with Livin, the H2A.X^{Y39E} and Livin plasmid were co-transfected into the colon cancer cells, and the H2A.X^{Y39E} plasmid was transfected at three different concentrations (0.5, 1 and 2 μg). We found that when only Livin was overexpressed in the colon cancer cells was the cell viability increased remarkably ($p < 0.01$) (Fig. 2A), and the same was true for the number of colonies ($p < 0.01$) (Fig. 2B) and cell migration ($p < 0.01$) (Fig. 2C). When the Livin plasmid was transfected together with the H2A.X^{Y39E} plasmid into colon cancer cells, the proliferation was decreased remarkably compared with the cells only transfected with the Livin plasmid ($p < 0.01$) (Fig. 2A), and the same was true for the number of colonies ($p < 0.01$) (Fig. 2B) and cell migration ($p < 0.01$) (Fig. 2C). Altogether, these results demonstrated that the phenotype of the colon cancer cells was recovered by increasing the phosphorylated state of H2A.X^{Y39ph}.

H2A.X^{Y39ph} is involved in the regulation of the transcription of target genes downstream of the Livin pathway.

The H2A.X^{Y39E} plasmid was constructed to mimic the phosphorylation status of this site and was co-transfected with the Livin expression plasmid to verify whether the restoration of the phosphorylation at the H2A.X^{Y39} site, in the context of Livin overexpression, restored the downstream target gene transcription. The real time PCR analysis revealed the relative RNA levels of the downstream target genes, such as IGFBP3, WNT168, CAR61, YRD16, GDF15, and NT5E in the cells with phosphorylated H2A.X^{Y39ph} were significantly reduced compared with the cells with H2A.X (Fig. 3A). The genes shown in the figure were all altered after Livin overexpression and are associated with tumor cell proliferation and migration activity [2]. Furthermore, it was confirmed by ChIP that H2A.X^{Y39ph} was directly regulated by the promoter region of the above genes (Fig. 3B). Together, these results suggested us that H2A.X^{Y39ph} is involved in the regulation of the transcription of target genes downstream of the Livin pathway.

Overexpression of JMJD6 recovers the colon cancer cell phenotype.

To determine whether the regulation of JMJD6 on H2A.X^{Y39ph} regulates the colon cancer cell phenotype, we constructed a JMJD6-overexpression system. Firstly, we measured the mRNA and proteins level of JMJD6 after the JMJD6 plasmid was transfected into the SW48 colon cancer cells. The result showed that the efficiency of JMJD6 was increased (left panel of Fig. 4A). Subsequently, we tested whether the overexpression of JMJD6 recovered the reduction of H2A.X^{Y39ph} caused by Livin. We found that H2A.X^{Y39ph} increased after JMJD6 overexpression (right panel of Fig. 4A). Furthermore, we detected whether JMJD6 played a similar role on the colon cancer cell phenotype, by assessing proliferation, migration and cell cycle progression. The JMJD6 plasmid was transfected into SW48 colon cancer cells at 0.5 g and 1 g. These results demonstrated that cell migration was significantly reduced after the pEGFP-Livin plasmid and the JMJD6-expressing plasmid were co-transfected into the SW48 cells compared with the cells only transfected with the pEGFP-Livin plasmid in a concentration-dependent manner (Fig. 4B). Similarly, cell viability was significantly reduced (Fig. 4C). As shown in Fig. 4D, the reduced G0/G1 phase and G2/M phase was obviously increased, and the increased S phase was obviously reduced in the SW48 cells co-transfected with the pEGFP-Livin plasmid and the JMJD6-expressing plasmid compared with the cells only transfected with the pEGFP-Livin plasmid, and this also occurred in a concentration-dependent manner. Together, these data indicated that the overexpression of JMJD6 recovered the colon cancer cell phenotype induced by Livin.

3.2. Livin overexpression induces the degradation of JMJD6 leading to the down-regulation of H2A.X^{Y39ph}

Firstly, the protein and mRNA levels of the protein kinase JMJD6

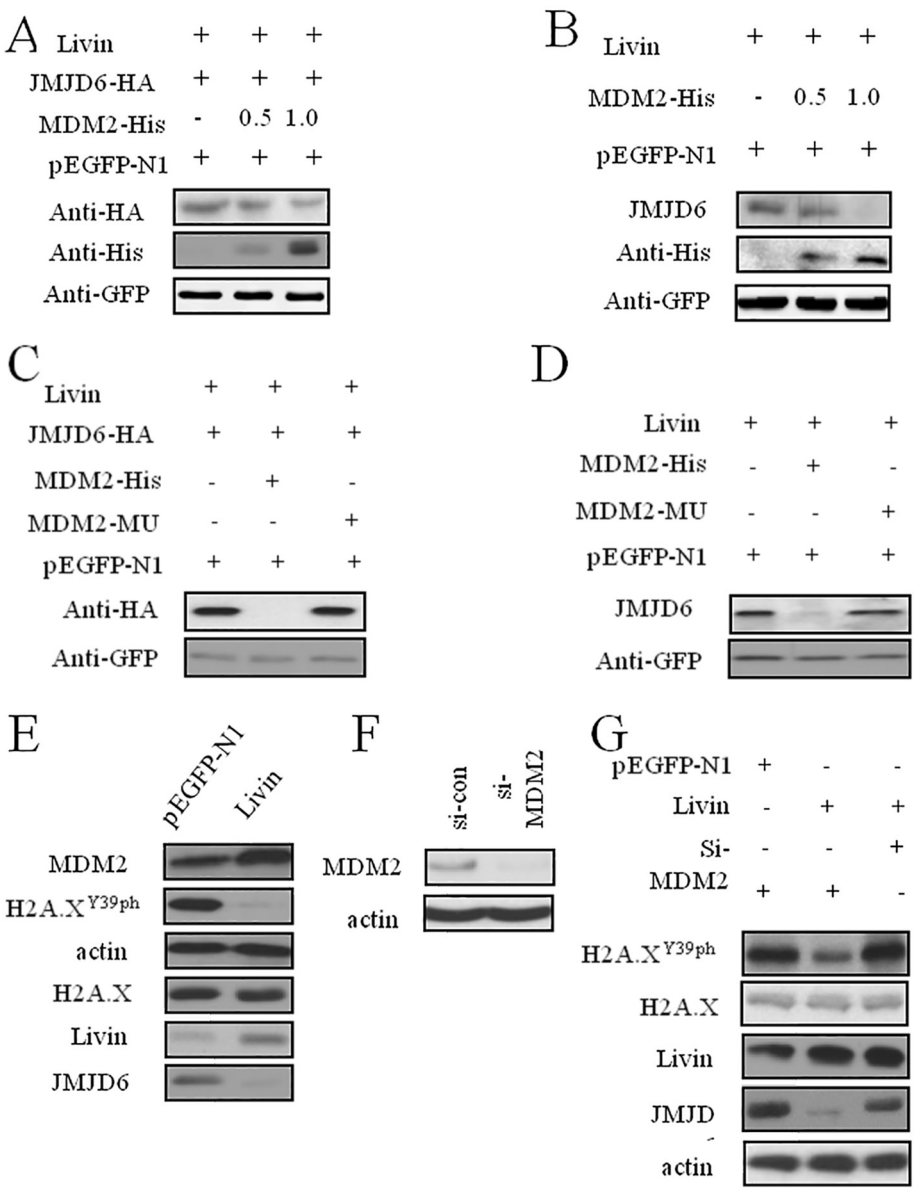

Fig. 6. The Livin signaling pathway-induced degradation of JMJD6 is mediated by MDM2.
A and B, MDM2 induces the degradation of JMJD6 (A exogenous and B endogenous). SW48 cells were co-transfected with 1 mg of JMJD6-HA, 0.6 mg of pEGFP-Livin, 0.1 mg of the pEGFP-N1 plasmids and increasing amounts of the MDM2-His plasmid (0.5 and 1 mg).
C and D, A mutation in MDM2 (MDM2-MU) that abolishes its ubiquitin ligase activity prevents JMJD6 degradation. The SW48 cells were co-transfected as indicated.
E, Whole cell extracts from SW48 cells that were transfected with pEGFP-N1 or pEGFP-Livin were analyzed by a western blot.
F, The efficiency of the siRNA-mediated MDM2 knockdown.
G, The co-transfection of pEGFP-Livin and MDM2-specific siRNA restored H2A.X^{Y39ph} to normal levels.

were tested in endogenous and exogenous experiments, before and after Livin was overexpressed in the SW48 colon cancer cells, respectively. The result showed that the exogenous and endogenous JMJD6 protein content was obviously down-regulated after Livin overexpression, but the mRNA level did not change (Fig. 5A). Subsequently, we performed ChIP to confirm whether JMJD6 was bound to the promoter region of the downstream target genes regulated by H2A.X^{Y39ph}. We found that JMJD6 did bind to the promoter region of the downstream target genes, such as IGFBP3, WNT168, CYR61, GAFRD16, GDF15 and NT5E, which are regulated by H2A.X^{Y39ph}, ad this was consistent with the down-regulation of H2A.X^{Y39ph} (Fig. 5B). By adding the proteasome inhibitor MG132 to the SW48 colon cancer cell culture medium, we confirmed that the degradation of JMJD6 was increased with the assistance of the proteasome pathway (Fig. 5C). To further confirm this result, we performed a time-dependent experiment, which indicated that it was indeed that the H2A.X^{Y39ph} level gradually returned to normal with time (Fig. 5C).

3.3. Livin overexpression-induced JMJD6 degradation is mediated by MDM2

Previous reports show that MDM2 plays an important role in promoting tumor cell cycle arrest, DNA repair and apoptosis. Firstly, we analyzed whether Livin overexpression regulated JMJD6 degradation by the E3 ubiquitin ligase MDM2. The Livin plasmid and the HA-tagged JMJD6 plasmid were co-transfected into SW48 colon cancer cell, which expressed MDM2-His. We found that the exogenous expression of HA-JMJD6 was significantly reduced in the cells with MDM2-His compared with the cells that only expressed -His (Fig. 6A), and the reduction of HA-JMJD6 was concentration dependent. A similar reduction of endogenous JMJD6 occurred in SW48 colon cancer cells that only expressed MDM2-His (Fig. 6B). The results showed that MDM2 significantly reduced the expression of HA-JMJD6. Moreover, when we transfected the MDM2^{C464A} mutant, which is the mutation site that is located in the RING-finger domain without ubiquitin ligase activity, into the SW48 colon cancer cells, there was no obvious alteration in the exogenous JMJD6 protein levels (Fig. 6C) and the endogenous levels (Fig. 6D). The above results indicated that the degradation of JMJD6 required the participation of MDM2. Furthermore, we analyzed

whether JMJD6 degradation, caused by Livin overexpression, was due to the up-regulation of MDM2 levels. We examined the endogenous MDM2 protein level by a western blot. This approach revealed that the amount of MDM2 protein actually increased several times after the overexpression of Livin (Fig. 6E). Subsequently, we knocked down MDM2 levels by siRNA in the SW48 colon cancer cells transfected with the Livin expression plasmid to test whether the up-regulation of MDM2 accelerated JMJD6 degradation. Before this test, we examined the efficiency of the siRNA-mediated MDM2 knockdown. The result showed that there was no MDM2 expression in the SW48 colon cancer cells transfected with the MDM2-specific siRNA (Fig. 6F). The co-transfection of the Livin expression plasmid and the MDM2-specific siRNA recovered the expression of H2A.XY39ph to normal levels (Fig. 6G), which illustrated that the Livin pathway actually regulated H2A.X^{Y39ph} by inducing JMJD6 degradation by MDM2.

4. Discussion

Along with Tyr142, H2A.X^{Y39ph} is a newly reported stable phosphorylated site on of H2A.X at Tyr39 [2]. H2A.X regulates newly synthesized histones, which is central to the DNA damage response as part of the restoration of chromatin at damaged sites. The overexpression of the phosphorylation of H2A.X at Tyr39 is reported in many types of cancer cells, and it is correlated with tumor histological grade, tumor node metastasis stage, tumor size and also with fraction surviving [2]. Consistent with this opinion, a high concentration of H2A.X is a main characteristic, indicating greater genome instability, in premalignant lesions and early stages of cancer [7–9]. At the present time, researchers are fighting for induce double-strand breaks and prevent efficient DNA repair in cancer cells, which would lead to apoptosis and cure cancers [10,11]. However, the role of H2A.X^{Y39ph} is rarely reported. In this report, we found that H2A.X^{Y39ph} regulated the colon cancer phenotype when Livin was overexpressed, and H2A.X^{Y39ph} was involved in the regulation of the transcription of target genes downstream of the Livin pathway in colon cancer cells. Recently, it was reported that H2A.X Tyr142 was phosphorylated by the WICH complex. WSTF (Williams–Beuren syndrome transcription factor), known as a sigma subunit of the WICH complex, exercises this novel phosphorylated kinase activity, which relies on its N-terminus targeting H2A.X Tyr142. The neoteric histone modification on this WSTF site recruits either pro-apoptotic factors or facilitates DNA repair to DNA damage sites [12]. Thus, the balance of phosphorylation/dephosphorylation on H2A.X Tyr142 may play an important role in administrating cell fate, especially related to cells with DNA damage. JMJD6 is the first identified kinase that phosphorylates H2A.X at Y39 [13]. Liu et al. demonstrated that JMJD6 was originally found to exercise tyrosine kinase activity, which phosphorylated Y39 on histone H2A.X by balancing ATP and GTP in phosphate activation (H2A.X^{Y39ph}) [13]. High levels of JMJD6 promote triple negative breast cancer cell autophagy by regulating the expression of genes related to autophagy via H2A.X^{Y39ph}. Thus, the JMJD6-H2A.X^{Y39ph} axis promotes negative breast cancer cell growth via the autophagy pathway. Similarly, in this report, we illustrated, for the first time, that JMJD6-H2A.XY39ph also restored the colon cancer cell phenotype mediated by MDM2.

In this report, JMJD6 was mainly degraded by the MDM2 proteasome pathway in the colon cancer cells induced by the overexpression of Livin, and then, H2AX^{Y39ph} was regulated, changing the cell phenotype. This result is consistent with reports indicating that JMJD6 is involved in several cancer proliferations. The dysregulation of JMJD6 participates in some human cancers because of its lysyl hydroxylase and arginine demethylase activities [14]. The effect of JMJD6 is in facilitating the progression of several cancers. For example, Wan et al. elucidated that JMJD6 promoted hepatocellular carcinoma carcinogenesis by targeting CDK4 [15]. Similarly, Liu et al. showed that JMJD6 promoted melanoma carcinogenesis through the regulation of the alternative splicing of PAK1, a key MAPK signaling component [16]. However, in this report, the overexpression of JMJD6 recovered the colon cancer cell phenotype, in which JMJD6 induced a different regulation compared with other reports. The reason for the difference requires intensive study, especially with regard to the molecules targeting JMJD6. The methylation of histone arginine plays an important role during cell development and cell proliferation, while research related to the reverse process of methylation-demethylation is limited. Importantly, research into the fundamental catalysis of JMJD6 has made great progress which paves the way for the follow-up work. JmjC containing JMJD6 demethylases are oxygenases that are dependent on Fe(II) and α-ketoglutarate (αKG) for their activity [17]. Whether other his specific enzymes are involved in the demethylation of histone arginine is the focus of following work, because other arginine sites on the histone cannot be removed by JMJD6. Beyond that, reports of the regulators that target JMJD6 are limited, such as ras-induced mir-146a and 193a, which target JMJD6 to regulate melanoma progression [18]. Also, a study demonstrated that JMJD6 is a target of miR-125 whose expression is negatively regulated by miR-125 [4]. In this study, the affinity between JMJD6 and the substrate was not detected after Livin overexpression. It is possible that the affinity between JMJD6 and the substrate was reduced, and this process may also coexist with quantitative change.

H2AX serves as a potential biomarker in the transformation of normal tissue to premalignant and thus to malignant tissues [19]. H2AX has already been discovered in several kinds of cancers, such as lung, breast, ovary, cervix, and colon cancers [19,20]. The prognostic value of H2AX is also reported in endometrial or breast cancer by Palla et al. [21]. As feedback to DNA double strand breaks, the phosphorylation at several sites of the histone variant H2AX at the break site is necessary for DNA damage sensor kinases. The phosphorylation of these sites recruits proteins for repairing double strand DNA breaks. In the repair process, the dephosphorylation of H2AX plays a vital role in stimulating cells to a prestress condition, and the dissolution of H2AX was associated with cell damage foci. The phosphatases PP2A and PP4 dephosphorylate H2AX. Recently, Moon et al. demonstrated that the wild-type p53 named WIP1 was also an important regulator of the dephosphorylation of H2AX. Moreover, they also found that the overexpression of WIP1 reduced the formation of gamma-H2AX foci in DNA damage foci by blocking the recruitment of MDC1 (mediator of DNA damage checkpoint 1) and 53BP1 (p53 binding protein 1) in the condition of ionizing and ultraviolet radiation [22]. Caspase-4 is also an upstream regulator of SSa-induced DNA damage and caspase activation in HCC cells [23]. The other causes of the decrease of H2AX, such as the localization of kinase molecules on H2AX, or the reason for the induced number of kinases stimulating the H2AX decrease remains to be further studied.

In addition, it has been reported in literature that JMJD6 promotes colon cancer carcinogenesis through the negative regulation of p53 by hydroxylation, which is highly expressed in colon cancer patients. In our report, the overexpression of JMJD6 recovered the colon cancer cell phenotype [24]. Therefore, some of analyses were requisite. The association of Livin-JMJD6-H2A.X^{Y39ph} was established in this report. However, in the complex human body environment, with colon cancer tissues and cells, when Livin is highly expressed, whether other factors simultaneously affect the expression of JMJD6 needs to be further studied. In Wang's paper, JMJD6 was only a hydroxylase targeting p53. How its other enzyme activities coordinate and regulate the development of colon cancer remains to be studied in-depth. JMJD6, p53, and H2A.X^{Y39ph} are senior chromatin structure control factors, and as such, their regulation of tumor cell biology behavior occurs by influencing the implementation of the related gene expression, and the influence of factors of the senior chromatin structure is very complex. Thus, in the human body, Livin, JMJD6, and how other factors, such as the adjustment factor of tumor cell proliferation and migration, affect this process, requires much more work.

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None.

Author contributions

Ge Y and Li SQ designed experiments; Liu BL carried out experiments; Cui JP analyzed experimental results. Ge Y wrote the manuscript; Li SQ approved the manuscript.

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