LOXL1 modulates the malignant progression of colorectal cancer by inhibiting the transcriptional activity of YAP

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Abstract

Background

LOX-like 1 (LOXL1), as a lysyl oxidase, emerging evidences revealed the effect in cancer malignant progression. However, its role in colorectal cancer (CRC) and the underlying molecular mechanisms have not yet been elucidated.

Methods

LOXL1 expression in colorectal cancer was detected by immunohistochemistry, western blot and real-time PCR. In vitro , colony formation assay, wound healing assay, migration and invasion experiment were performed to investigate the effects of LOXL1 in cell proliferation, migration and invasion, respectively. In vivo , metastasis models and mouse xenograft were used to determine tumorigenicity and metastasis ability. Molecular biology experiments were utilized to reveal the underlying mechanisms of LOXL1 modulating Hippo pathway.

Results

LOXL1 is highly expressed in normal colon tissues compared with cancer tissues. In vitro, Silencing LOXL1 in CRC cell lines dramatically enhanced migration, invasion, and colony formation, while overexpression of LOXL1 manifested the opposite effects. Results of the in vivo experiments demonstrated that the enforced expression of LOXL1 in CRC cell lines had drastically inhibited the progression of metastasis and tumour growth. Mechanistically, LOXL1 inhibited the transcriptional activity of Yes-associated protein (YAP) was through interaction with MST1/2 and increasing the phosphorylation of MST1/2.

Conclusions

LOXL1 may function as an important tumour suppressor in regulating tumour growth, invasion and metastasis via negative regulating of YAP activity.

Background

Colorectal cancer (CRC) is ranked as the third most frequently diagnosed cancer and the second leading cause of cancer-related death worldwide [1]. Moreover, the five-year relative survival rate for surgical patients in the late stages of colon cancer is only around 10% [2] and over 50% of patients with colon cancer are clinically diagnosed at the late stages [3]. Invasion and distant metastasis of the tumours are considered to be the reasons leading most frequently to the mortalities associated with CRC. However, the mechanisms underlying this malignant progression have not been fully understood. Therefore, investigation of the associated mechanisms is very important for deploying strategies to treat patients with CRC.

The Hippo pathway is an evolutionarily conserved tumour suppressor pathway best known for its role in modulating the organ size, tissue homeostasis, and tumour progression [4]. Recent studies have revealed that dysregulation of the Hippo signalling pathway is associated with the progression of CRC [5–7]. In mammals, MST1/2, SAV1, LATS1/2, and MOB1 have been reported as the core kinase components [8]. When Hippo signalling is activated, MST1/2 utilises SAV1 and other scaffolds to phosphorylate and activate LATS1/2, which results in the phosphorylation of YAP at multiple sites and its transcriptional activity is inhibited through cytoplasmic confiscation or ubiquitin-mediated degradation. While the signalling is inhibited, unphosphorylated YAP and TAZ are transferred to the nucleus, where they are combined with TEAD. Then, the complex of YAP/TAZ/TEAD induces the expression of target genes associated with Hippo-YAP, which include CDC20, CDX2A, CTGF, and CYR61 [9]. Previous reports have suggested that Hippo signalling plays a critical role in the growth, invasion and metastasis of colon tumours [10, 11]. Therefore, understanding the regulatory mechanism of Hippo-YAP signalling is essential to determine the progression of CRC.

The lysyl oxidase (LOX) family of copper-dependent ε-amine lysine oxidases were first identified in mammalian cells and yeast [12]; these were found to contain five identified paralogs, which are as follows: LOX, LOX-like 1 (LOXL1), LOX-like 2 (LOXL2), LOX-like 3 (LOXL3), and LOX-like 4 (LOXL4). Extracellular forms of LOX oxidise lysine spontaneously, which along with unmodified lysine forms covalent cross-linkages through the Schiff base reaction [13]. Csiszar et al. (2002) had reported for the first time that LOX could be considered as a tumour suppressor in CRC [14]. Furthermore, Wu et al. (2007) have reported that LOXL1 suppresses the growth of bladder cancer [15]. To date, few studies on the role of LOXL1 in the progression of CRC are available. In our previous studies, it has been reported that LOXL3 lacking the signal peptide (SP) can function as a deacetylase in the nuclei facilitating Th17 cell differentiation through the regulation of STAT3 deacetylation [16]. Hence, our aim was to determine the exact effects and mechanisms underlying the involvement of LOXL1 in CRC.

Here, we have demonstrated that the overexpression of LOXL1 had repressed cell migration, invasion, and tumorigenesis in vitro and in vivo. Contrarily, knockdown of LOXL1 in CRC cells resulted in an opposite effect. Results of the luciferase reporter assays have revealed that LOXL1 could inhibit the transcriptional activity of YAP. Moreover, SP deletion in LOXL1 inhibits cellular secretions and the activity of YAP strongly. We also determined that LOXL1 could induce the activity of MST1/2 kinase. Therefore, we hypothesised that intracellular LOXL1 had restrained the malignancy of CRC through a p-YAP-dependent signalling pathway. Consistent with our hypothesis, the overexpression of LOXL1 with deleted SP had significantly suppressed the migration and invasive abilities of CRC cells. Overall, our results revealed the novel molecular mechanisms by which LOXL1 could inhibit the malignant progression of CRC in a YAP-dependent manner.

Methods:

Immunohistochemistry (IHC)

The LOXL1 expression levels were assessed using IHC on the paraffin-preserved tissue sections of 30 pairs CRC patients and 15 pairs CRC with liver metastasis patients. Primary LOXL1 antibody were obtained from sigma (HPA042111, anti-LOXL1 diluted 1:50). Each specimen was scored according to the proportion of positive cancer cell as: 1, 0–25%; 2, 25–50%; 3, 50–75%; and 4, > 75%. Specimens were also scored according to the staining intensity of cancer cells as follows: 0, negative; 1, light yellow; 2, dark yellow; 3, brown. The IHC staining score was calculated by multiplying the proportion of positive cancer cells by the staining intensity of cancer cells. All samples were obtained with approval from the Institutional Ethics Committee of The First Affiliated Hospital of Soochow University (authorisation number ECSU-2019000212)

Cell Culture

Human colorectal cancer cell lines such as DLD1, HCT116, HCT8, HT29, LoVo, SW480, SW620, and RKO were purchased from American Type Culture Collection (ATCC). All CRC cell lines were maintained in RPMI 1640 supplemented with 10% foetal bovine serum and 1% antibiotic (penicillin and streptomycin) at 37^{o}C in an atmosphere of 5% CO_2 and 95% air.

Lentiviral Vector Construction And Packaging

Lentiviral constructs of LOXL1 encoding pLent-EF1a-FH-CMV-GFP-P2A-puromycin were prepared as described previously [17]. Lentivirus expressing LOXL1 was produced in HEK293T cells and then packaged using pMD2.G and psPAX2. The HCT8 and SW480 cell lines were infected with the viral supernatant using 8 μg/mL polybrene (Sigma) and the infected cells were incubated for 48 h. Single colonies were obtained through puromycin selection (8 μg/mL, 2 m), which were detected using western blotting.

Wound Healing Assay

First, 1×10^6 cells were cultured in six-well plates and incubated for 24 h. The cultured cells were rinsed thrice using phosphate buffered saline (PBS) and three wounds (scratches) were created in parallel using a sterile 200-μL pipette tip. The wells were washed thrice with PBS to discard any floating cells. Representative images of their migration were captured immediately using a microscope, 24 h and 48 h after scratching.

In vitro transwell migration and invasion

Cell migration and invasion experiments were performed using 24-well plates with 8 μm-polycarbonate filter inserts (CORNING). HCT8-N/HCT8-LOXL1, SW480-N/SW480-LOXL1 and HT29-N/HT29-LOXL1 (KO), and RKO-N/RKO-LOXL1 (KO) were seeded at densities of 2×10^5 cells/200 μL and 1×10^5 cells/200 μL per well, respectively, in serum free RPMI 1640. All cells were either uncoated or Matrigel-coated (Biocoat) and incubated in chambers containing 600 μL of RPMI 1640 with 10% foetal serum as a chemoattractant. The cells were imaged and their migration and invasion was captured using a

microscope. The migrating and invading cells were eluted using acetic acid and quantified by measuring their absorbance at 570 nm. All experiments were performed thrice, independently.

Plate Colony Formation Assay

HCT8-N/HCT8-LOXL1, SW480-N/SW480-LOXL1, RKO-N/RKO-LOXL1 (KO), and HT29-N/HT29-LOXL1 (KO) cells were independently seeded in six-well plates at densities of 5000 cells/well at 37^{o}C. The medium, RPMI 1640 containing 10% foetal serum, was changed every alternate day. After 10 d, the cells forming colonies were immersed in 4% paraformaldehyde for 20–30 min, stained using crystal violet for 2 h, and rinsed thrice with PBS to remove the excess crystal violet. Finally, images were captured using a microscope and the number of colony-forming units were counted.

Luciferase Reporter Assay

The promoter of 3 × GTIIC was subcloned into the XhoI/HindIII site of the pGL4.2 vector (Promega). HEK293T cells were transiently co-transfected with the pGL4.2-3 × GTIIC, pcDNA3.1-YAP, and LOXL1/LOXL1 ΔSP/LOXL1 mutants. The pRL-TK vector was co-transfected in each experimental sub-hole as an internal control. After 24 h of transfection, the cells were collected and analysed using the Dual-Luciferase Reporter Assay Kit (Promega).

Immunoprecipitation And Western Blotting

HEK293T cells were transfected with FL LOXL1 and its mutants. After 24 h, the medium was collected and centrifuged at 1000 g for 5 min. The supernatant was subjected to immunoprecipitation using M2-conjugated magnetic beads (Sigma) by rotating for 4 h at 4^{o}C. The immunoprecipitates were washed thrice using PBS and subjected to western blot analysis. Additionally, the cells were lysed using a lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM DTT, and the complete protease inhibitor cocktail) for 10 min on ice and centrifuged at 20,000 g for 30 min. The cell lysates were analysed by subjecting them to SDS-PAGE and immunoblotting with antibodies as indicated in the figures.

Total RNA isolation and Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Expressions of the genes CYR61, CDC20, CDX2A, and CTGF were detected using q-PCR and normalized to that of GAPDH. Total RNAs were extracted using TRIzol (TIANGEN), according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed using the PrimeScript RT reagent Kit (TaKaRa). SYBR green (Bimake) and an ABI Step One Plus real-time PCR system (Applied Biosystems) were used to conduct q-PCR. The primers used in this study are mentioned in Table 1.

Table 1
Primers used for real-time quantitative PCR (QPCR)

Target mRNA		Sequences 5'—3'
GAPDH	F	5’- GGAGCGAGATCCCTCCAAAAT-3’
	R	5’- GGCTGTTGTCATACTTCTCATGG-3
CDX2	F	5’-CCAATGACAACGCCTCCTG-3’
	R	5’-TGGTGCAGCCAGAAAGCTC-3’
CTGF	F	5’-AAAAGTGCATCCGTACTCCCA-3’
	R	5’-CCGTCGGTACATACTCCACAG- 3’
CYR61	F	5’- AGCCTCGCATCCTATACAACC- 3’
	R	5’- TTCTTTCACAAGGCGGCACTC-3’
CDC20	F	5’-GACCACTCCTAGCAAACCTGG-3’
	R	5’- GGGCGTCTGGCTGTTTTCA-3’

Animal Experiments

To carry out the xenograft tumorigenesis assays, 1×10^7 cells of HCT8-N/HCT8-LOXL1 were subcutaneously injected into 4-week-old male nude BALB/c mice. The tumour sizes were monitored every 3 d and their volumes were determined using the following formula: volume (mm^3) = (length × $width^2$)/2. Subsequently, they were subjected to H-E staining and immunohistochemistry (IHC).

To carry out the tail vein metastasis assay, cells were injected into the lateral tail veins of 4 w old male nude BALB/c mice. Eight weeks later, the mice were anesthetised using nembutal (pentobarbital, TRC). The mice were sacrificed and examined at necropsy for the presence of metastases. Their lungs, livers, and bones were fixed in formalin. Subsequently, the samples were subjected to H-E staining and IHC.

To conduct the liver metastasis assay, cells were harvested using 0.25% trypsin, washed thrice with PBS, and suspended in PBS at a final concentration of 1.5×10^7 cells/mL. The 6-week-old BALB/c nude mice were anesthetised through an intraperitoneal injection of nembutal at a dose of 75 mg/kg. Then, a small incision, approximately 10 mm in length, was made through the skin over the spleen. Using a 27 gauge needle, 100 µL of the tumour cell suspension was slowly injected into the spleen, after which it was placed back in the abdominal cavity. The incision was closed through simple continuous suturing. The mice were sacrificed after 20 d and liver metastasis was confirmed pathologically [18].

All animal experiments were approved by the Animal Care and Use Committee as well as the Ethical Committee of Soochow University (SYXK2017–0043). All surgery was performed under sodium pentobarbital anesthesia with minimum fear, anxiety and pain.

Statistical analysis

The data obtained was statistically analysed using SPSS (version 20.0; IBM, New York) and represented as the mean ± SD. A t-test (for two groups) was used to determine differences between the groups, which were considered statistically significant at $P < 0.05$.

Results

LOXL1 expression is significantly downregulated in CRC and CRC liver metastasis tissues

To illustrate the expression pattern of LOXL1 in CRC, we evaluated the protein expression level of LOXL1 in 30 paired CRC and their adjacent normal tissues by immunohistochemistry (IHC). We observed a significant lower expression of LOXL1 in CRC samples compared with their adjacent non-tumourous samples (Fig. 1a), and that IHC staining score was statistically significant ($P < 0.001$) (Fig. 1b). Consistently, western blot in 5 paried CRC and adjacent normal tissues also revealed that LOXL1 dramatically lower expression in CRC than paired normal tissues (Fig. 1c). In another independent 15 CRC with liver metastasis patients, We also observed a significantly lower expression of LOXL1 in CRC and CRC with liver metastasis tissues than normal colorectal tissues(Fig. 1d), and the IHC staining score was statistically significant ($P < 0.001$) (Fig. 1e). We further examined the expression of LOXL1 in 15 pairs of CRC and adjacent normal tissues, the result also demonstrated that LOXL1 is down-regulaed in CRC, and the result was statistically significant ($P < 0.05$) (Fig. 1f). All of these data indicated that LOXL1 lower expressed in CRC and CRC with liver metastasis compared with normal tissues.

Overexpression of LOXL1 suppresses the migration and invasion abilities of CRC cells in vitro

We evaluated the expression of LOXL1 in CRC cell lines such as DLD1, HCT116, HCT8, HT29, LoVo, SW480, SW620, and RKO. Because lower levels of LOXL1 is expressed in HCT8 and SW480 cells compared to those of the other CRC cell lines, these were selected for conducting the experiments (Fig. 2a). Then, lentiviral constructs expressing LOXL1 were used to overexpress LOXL1 in HCT8 and SW480 cell lines to investigate its role in the malignant progression of CRC. The transfection efficiency was analysed through western blotting and the results revealed that LOXL1 was markedly overexpressed in HCT8 and SW480 cells (Fig. 2b). The induction of cancer metastasis is dependent on the migration and invasive properties of cancerous cells. Wound healing is a well-established methodology, which is evaluated to determine their migration potential. We conducted the wound healing assay to investigate the effect of LOXL1 on the migration of CRC cells in models involving HCT8 and SW480 cells. The results revealed that the wound healing was slower in the presence of LOXL1 than that observed in the negative control group ($P < 0.05$, Fig. 2c and f). To assess the contribution of LOXL1 in the development of migratory and invasive phenotypes of CRC cells, migration and invasion experiments were conducted

using both HCT8 and SW480 cells, in which, the expression of LOXL1 and functions of the control vector were found to be stable. The data indicated that the overexpression of LOXL1 had significantly decreased the migration and invasion of HCT8 and SW480 cells ($P < 0.001$, Fig. 2d and g). Colony formation assays were carried out to investigate the effect of LOXL1 on the proliferation of CRC cells. Overexpression of LOXL1 was found to significantly inhibit the clonogenicity ($P < 0.001$, Fig. 2e and h) of HCT8 and SW480 cells compared to those of the control. Collectively, these observations suggested that LOXL1 is a negative regulator of migration, invasion, and tumorigenesis in CRC cells.

Knockdown of LOXL1 promotes the migratory and invasive abilities of CRC cells in vitro

Next, knockdown of LOXL1 in RKO and HT29 expressing high amounts of endogenous LOXL1 was carried out, as described in Fig. 1A, followed by the western blot analysis, which enabled determination of the transient transfection efficiency of siRNA (Fig. 3a). A wound healing assay was performed to explore the effects of LOXL1 on the ability of migration of RKO and HT29 cells. The results revealed that wound healing was highly regulated in the absence of LOXL1 and the potential of migration of these cells was high compared to that of the control ($P < 0.05$, Fig. 3b and c). Using the transwell migration assays, we could demonstrate that the knockdown of LOXL1 had significantly increased the migration and invasion of RKO and HT29 cells compared to those belonging to the negative control group ($P < 0.01$, Fig. 3d and e). We also carried out the colony formation assay in the absence of LOXL1 to determine its effect on the tumorigenesis of RKO and HT29 cells. The results revealed that a reduction in the expression levels of LOXL1 allows significant promotion of clonogenicity compared to that observed in the control cells ($P < 0.05$, Fig. 3f and g). Taken together, these observations (Fig. 2 and Fig. 3) suggest that LOXL1 acts as a tumour suppressor and facilitates migration, invasion, and tumorigenesis in CRC cells.

Intracellular LOXL1 Inhibits The Transcriptional Activity Of YAP

The Hippo-YAP signalling pathway is one of the most important pathways involved in epithelial-mesenchymal transition (EMT) and metastasis [19–21]. We measured the transcriptional activity of YAP by co-transfecting 3 × GTIIC and LOXL1 constructs into HEK293T cells to investigate whether LOXL1 could modulate the activities of the Hippo-YAP signalling pathway. Results of the luciferase reporter assay revealed that the activity of 3 × GTIIC was drastically suppressed by LOXL1 in a dose-dependent manner. Results of the western blot analysis suggested that the overexpression of LOXL1 did not change the total expression levels of YAP (Fig. 4a). Additionally, mRNA studies revealed that LOXL1 had repressed the expressions of CDC20, CDX2A, CTGF, and CYR61 in a dose-dependent manner, which have been reported as the genes downstream to YAP, after its transient expression in HEK293T cells (Fig. 4b). LOXL1 contains a signal peptide, pro-sequence, and proline-rich and catalytic domains. Among these, the signal peptide and catalytic domains are responsible for the secretion of LOXL1 and mediating its enzyme activity, respectively. We identified the domain of LOXL1 responsible for the inhibition of YAP. We constructed various expression plasmids, which included those with deleted signal peptide (LOXL1 ΔSP), deleted signal peptide and the C-terminal (LOXL1 ΔSP & ΔC), and mutated amino acids (H449 to Q449, H451 to Q451) to facilitate the loss of the enzyme activity of LOXL1. Initially, the supernatant of cultured

cells was harvested and used to conduct the immunoprecipitation assay. Its results demonstrated that LOXL1 FL was extracellularly secreted in significant amounts by two short isoforms. However, their presence was not detected in the LOXL1 ΔSP and LOXL1 ΔSP & ΔC groups (Fig. 4c). Based on these observations, we further determined that LOXL1 ΔSP had inhibited activities of the 3 × GTIIC reporter more than those of LOXL1 FL in a dose-dependent manner but had not affected the expressions of total YAP, for which, LAST2 was used as a positive control (Fig. 4d). However, in the absence of its enzyme activity, LOXL1 could still inhibit the transcriptional activity of YAP (Fig. 4e). Collectively, these results indicated that LOXL1 had negatively regulated the transcriptional activity of YAP, while deletion of SP in LOXL1 resulted in its intracellular retention and demonstrated stronger inhibition. Furthermore, it was determined that this function did not depend on the enzyme activity of the lysine oxidases of LOXL1.

LOXL1 Activates The Hippo Pathway Through Interaction With MST1/2

YAP is regulated by a myriad of extrinsic and intrinsic signals including soluble extracellular factors, stress signals, cell-cell contact, mechanotransduction, and cell polarity [8]. These signals mainly regulate the phosphorylation events of the core MST–LATS kinase cascade and lead to the phosphorylation of YAP. Here, we have investigated the mechanism by which LOXL1 and LOXL1 ΔSP had suppressed the activity of YAP, but not its expression. We had speculated that LOXL1 affects the phosphorylation of YAP. Interestingly, the western blot analysis revealed that the level of p-YAP (S127) had increased significantly in HEK293T cells overexpressing LOXL1 (Fig. 5a). Simultaneously, the phosphorylation of p-YAP (S127) was found to be elevated LOXL1 ΔSP compared to that observed in LOXL1 FL after their transient expressions in HEK293T and CRC cells (HCT8 and SW480, Fig. 5b and c). We further detected the activities of the MST-LATS kinase cascade, which was mainly upstream. The results showed that the overexpression of LOXL1 and LOXL1 ΔSP in HEK293T cells had activated the MST-LATS kinase cascade. Compare to LOXL1 FL, intracellular LOXL1 has much better ability to activate the Hippo signalling pathway (Fig. 5d). The activation of MST1/2 kinases is considered to be the initial event in Hippo signalling [22]. Co-immunoprecipitation results have shown that LOXL1 could interact with MST1 and MST2 (Fig. 5e), based on which we elucidated the effect of LOXL1 on the activation of MST kinase. These results indicated that intracellular LOXL1 could interact with MST kinase and then activate it to restrain the transcriptional activity of YAP.

LOXL1 △SP inhibits migration and invasion of CRC cells more than LOXL1 FL

LOXL1 is a secreted lysine oxidase, which can be cleaved by morphogenetic protein-1 (BMP-1) leading to enzyme activation [23]. Three variants of LOXL1 are known and we found that intracellular LOXL1 functions as an MST kinase activator. To further explore the criticality of the role of intracellular LOXL1 in CRC, we expressed N/LOXL1/LOXL1 ΔSP ectopically in HCT8 and SW480 cells using lentiviral constructs. Wound healing and transwell assays were performed using HCT8 and SW480 cells to investigate the effect of LOXL1 and LOXL1 ΔSP on their abilities of migration and invasion, both of which were stably overexpressed in these cells. The results showed that LOXL1 ΔSP could significantly suppress the migration ability of HCT8 and SW480 cells compared to LOXL1 ($P < 0.05$, Fig. 6a, b, d and e).

The results of colony formation assays revealed that in HCT8 and SW480 cells, the overexpression of LOXL1 had resulted in the significant inhibition of clonogenicity compared to that observed in the control cells, while that of LOXL1 ΔSP was inhibited more than LOXL1 FL ($P < 0.001$, Fig. 6c and f). These observations suggested that LOXL1 is a negative regulator of migration ability, invasiveness, and tumorigenesis in CRC cells. We also found that LOXL1 ΔSP had suppressed the secretion of LOXL1 and this truncation played a major role in inhibiting the malignant progression in CRC.

Overexpression LOXL1 inhibits tumorigenesis in vivo

To further explore the effect of LOXL1 on tumorigenesis in vivo, cells overexpressing LOXL1 (HCT8-LOXL1, SW480-LOXL1) and their corresponding controls (HCT8-N, SW480-N) were subcutaneously injected into nude mice in the form of xenografts. The ectopic expression of LOXL1 was found to significantly decrease the size of the xenograft tumours in mice injected with the HCT8 and SW480 cells, and corresponding results were observed upon haematoxylin-eosin (H-E) staining (Fig. 7a and d). Furthermore, tumour growth was also inhibited by the overexpression of LOXL1, as observed from the tumour growth curve (Fig. 7b and e). The western blot analysis showed that the overexpression of LOXL1 had increased the expression of p-YAP (S127; the content of total YAP had not changed; Fig. 7c and f).

To explore if LOXL1 could promote the metastasis of CRC cells in vivo, cells overexpressing LOXL1 (HCT8-LOXL1, SW480-LOXL1) and their corresponding controls (HCT8-N, SW480-N) were injected into the lateral tail veins of nude mice. The results were observed after 8 w, which revealed that LOXL1 had repressed tumour metastasis in the lungs of mice compared to that in case of mice injected with the control vector-containing HCT8 cell lines. Picric acid was used to visualise and fix the samples of mouse lung to observe the metastases (Fig. 7g). However, no metastases were observed in the lungs of mice injected with the SW480 cell lines, but were present in the livers of mice injected with the SW480-N cells. No metastases were also observed for the SW480-LOXL1 group (Fig. 7h).

We further investigated the functional relevance of LOXL1 with the metastasis observed in the liver. As HCT8-LOXL1, SW480-LOXL1 and their corresponding control cells were slowly injected into the spleen, the overexpression of LOXL1 not only drastically decreased the number of metastatic tumours in the livers of mice, but also their size (Fig. 7i and j). Collectively, the in vivo results demonstrated the criticality of the role of LOXL1 as a tumour suppressor in the metastasis of CRC cells.

Discussion

Increasing evidences have revealed that the lysyl oxidase, LOXL1 is involved in the malignant progression of cancer [15, 24, 25]. However, the underlying molecular role of LOXL1 in CRC has not been elucidated. Here, we aimed at evaluating the molecular mechanisms involved in the cell migration, invasion, and tumorigenesis mediated by LOXL1 in CRC. Our present study has revealed novel mechanisms through which LOXL1 was found to suppress the metastasis of CRC. We detected that the overexpression of LOXL1 had inhibited migration ability, invasiveness, and tumorigenesis of CRC cells in vivo and in vitro, whereas opposite effects were observed upon its downregulation. Previous studies have reported that

LOX is a tumour suppressor, the expression of which was found to be reduced in the tumour tissue, and its downregulation was controlled through methylation [26, 27]. However, evidence has indicated that the tumour suppressor activity of LOX is dependent on the pro-peptide domain and not within the active enzyme itself [28–31]. Since LOXL1 is highly homologous to LOX, we hypothesised that its tumour suppressor activity was also dependent on its intracellular function. Our results proved that when SP was deleted from LOXL1 (LOXL1 ΔSP), the extracellular secretion of LOXL1 was inhibited. Furthermore, the suppression of tumour metastasis was more pronounced after the cytoplasmic retention of LOXL1 ΔSP than that observed with LOXL1 in vitro.

Previous studies have reported that YAP could regulate the expression of members belonging to the LOX family [32–34]. We found that LOXL1 was involved in the progression of the Hippo pathway. The core kinase signalling cassette and components of the Hippo pathway are highly conserved [35]. Overall, activated MST1/2 interacts with SAV1 through the SARAH domains, leading to phosphorylation and activation of LATS1/2, which suppresses the carcinogenicity of YAP by promoting its phosphorylation at Ser 127 and cytoplasmic retention [36]. As a central component of the Hippo signalling pathway, the critical role of YAP has been wildly reported in CRC. However, the modulators of YAP have not been well described previously [6, 7, 37]. We identified for the first time that LOXL1 is a novel regulator of YAP involved in CRC tumorigenesis. In our study, we have revealed that LOXL1 could inhibit the malignant progression of cells by inducing the activity of MST kinase, which leads to the inhibition of transcriptional activity of YAP in CRC. Consistent with our hypothesis, we observed that the LOXL1 enzyme mutants had also inhibited the transcriptional activity of YAP, which may have occurred due to the interaction of intracellular LOXL1 with MST kinase. However, the molecular mechanisms through which LOXL1, particularly intracellular LOXL1, activates the Hippo signalling pathway are still unclear and need to be studied further.

Furthermore, our results have established the major role played by LOXL1 in the molecular mechanism of CRC development, since it was found to inhibit the transcription of YAP, a classical gene involved in the Hippo signalling pathway, to inhibit the development of CRC. It is well known that tumours are developed as a result of multi-gene, multi-stage altering processes and emerging evidence has suggested the involvement of numerous oncogenes in the process of tumorigenesis and malignant progression of CRC. However, little information is available on the role of tumour suppressor genes associated with CRC. Here, we believed that tumour suppressor genes, such as LOXL1, could provide potential drug targets for intervening with the malignant progression of CRC. We have revealed that LOXL1 could inhibit the development of CRC by inhibiting the transcriptional gene YAP.

Conclusions

In conclusion, our results revealed evidence about the contributions of LOXL1 to inhibit the malignant progression of CRC, including the suppression migration, invasion and proliferation. All together, our studies encourage further efforts to uncover and evaluate LOXL1-relevant drug targets mediating the

malignant progression of CRC, to provide new theoretical basis for the molecular mechanisms which advances the translational medicine of clinical treatments

List Of Abbreviations

CRC, Colorectal cancer;

LOXL1, Lysyl oxidase-like 1;

IHC, immunohistochemistry;

HE, hematoxylin and eosin;

IP, immunoprecipitation

Declarations

Ethics approval and consent to participate

All study participants provided informed consent, and the study design was approved by the Institutional Ethics Committee of Soochow University.

Consent for publication

Not applicable.

Availability of data and materials

Source data and reagents are available from the corresponding author upon reasonable request.

Competing of interests

The authors declare no conflict of interest.

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Authors' contributions

LH, YH-W and Y-EC conceptualized and designed the study; LH and YW written, reviewed and revised the manuscript; LH, JW and YL-W developed the methodology; LH, LW, CW and BM analysed and interpreted

the data; YH-W and Y-EC supervised the study. All authors read and approved the final manuscript.

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Figures

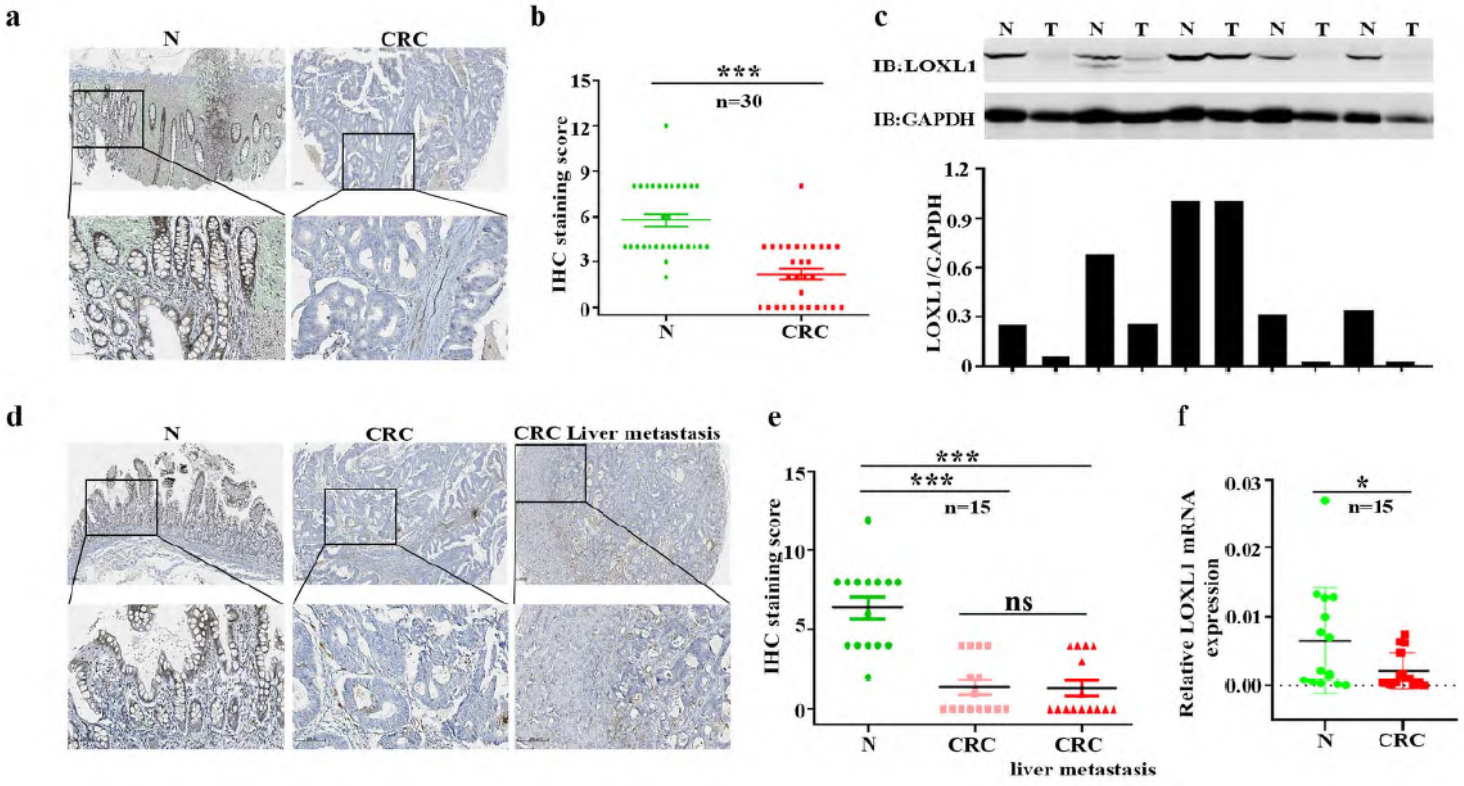

Figure 1

LOXL1 expression is significantly downregulated in CRC and CRC liver metastasis tissues. a IHC staining performed using an antibody against LOXL1 and representative photographs of the LOXL1 in normal and CRC tissues. (Scale bar: 100μm). b IHC staining score of LOXL1 in the 30 pair of CRC patients, *p < 0.05, **p < 0.01, ***p < 0.001. c Western blot performed using an antibody against LOXL1 in 5 pair CRC patients and protein quantification against GAPDH. d IHC staining performed using an antibody against LOXL1 and representative photographs of the LOXL1 in 15 pair of CRC with liver metastasis patients (Scale bar: 100μm). e IHC staining score of LOXL1 in the 15 pair of CRC with liver metastasis patients, *p < 0.05, **p < 0.01, ***p < 0.001. f mRNA expression of LOXL1 in 15 pair of CRC patients, *p < 0.05, **p < 0.01, ***p < 0.001.

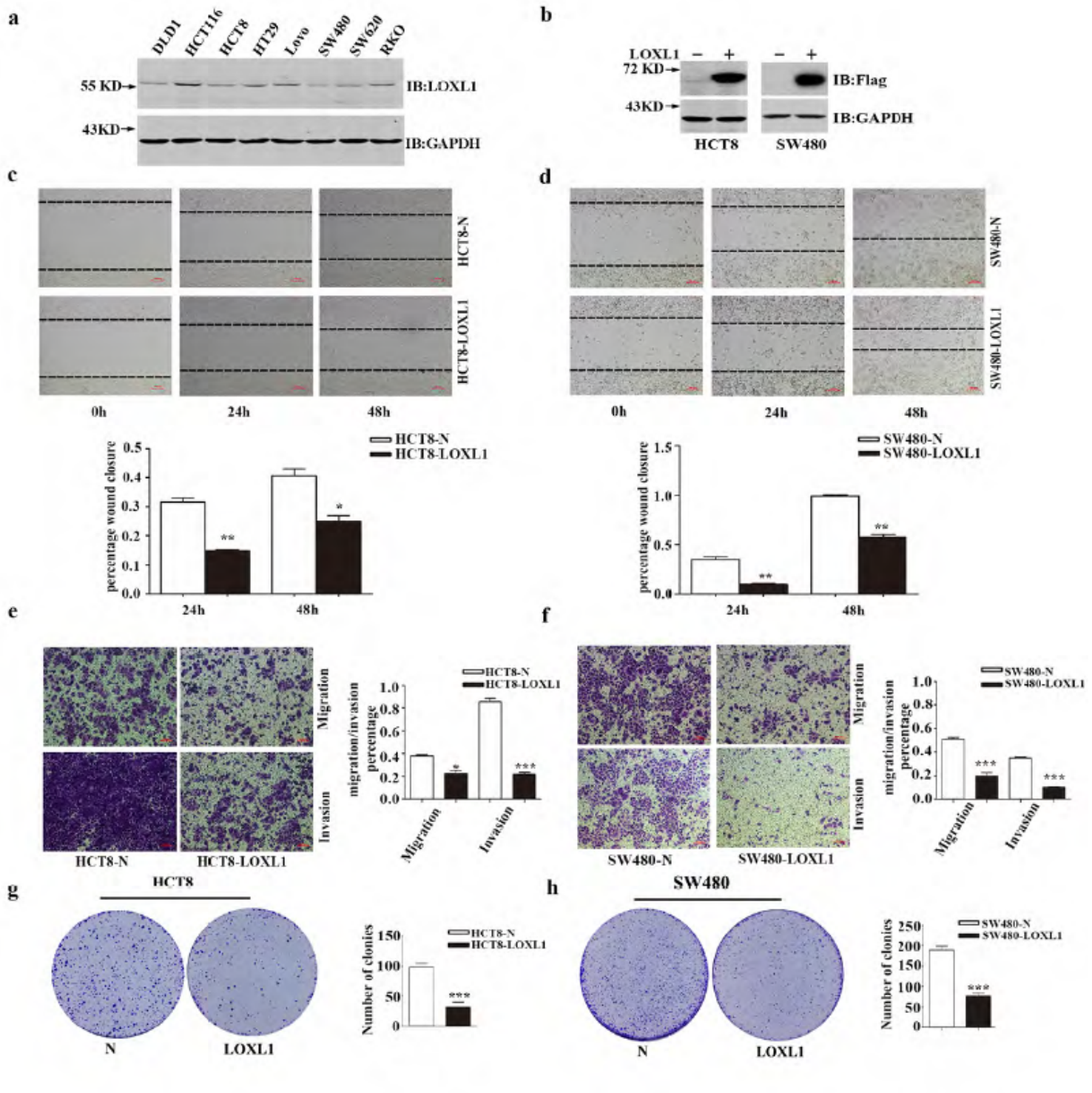

Figure 2

Effect of the expression of LOXL1 on the migration and invasion of HCT8 and SW480 cells in vitro. a Western blot analysis demonstrating the expression of LOXL1 in CRC cell lines. b Western blot analysis determining the expression of LOXL1 in stable HCT8/SW480 cells. c Wound healing analysis: effect of the overexpression of LOXL1 in stable HCT8 cells at 0 h, 24 h, and 48 h, and calculation of the percentage of wound healing. d Transwell migration and Matrigel invasion assays using stable HCT8-LOXL1 and HCT8-N cells and calculation of the rate of migration/invasion in relevant stable HCT8 cells. e The

clonogenic assay was performed in HCT8 cells with or without LOXL1. Left panel: representative images, right panel: quantification analysis. f Wound healing analysis to determine the effect of LOXL1 overexpression in stable SW480 cells at 0 h, 24 h, and 48 h, and calculation of their wound healing percentages.g Transwell migration and Matrigel invasion assays using stable SW480-LOXL1 and SW480-N cell lines and calculation of the rate of migration/invasion in corresponding stable SW480 cells. h A clonogenic assay was performed using SW480 cells with or without LOXL1. Left panel: representative images, right panel: quantification analysis. Data are of three independent experiments are presented as the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

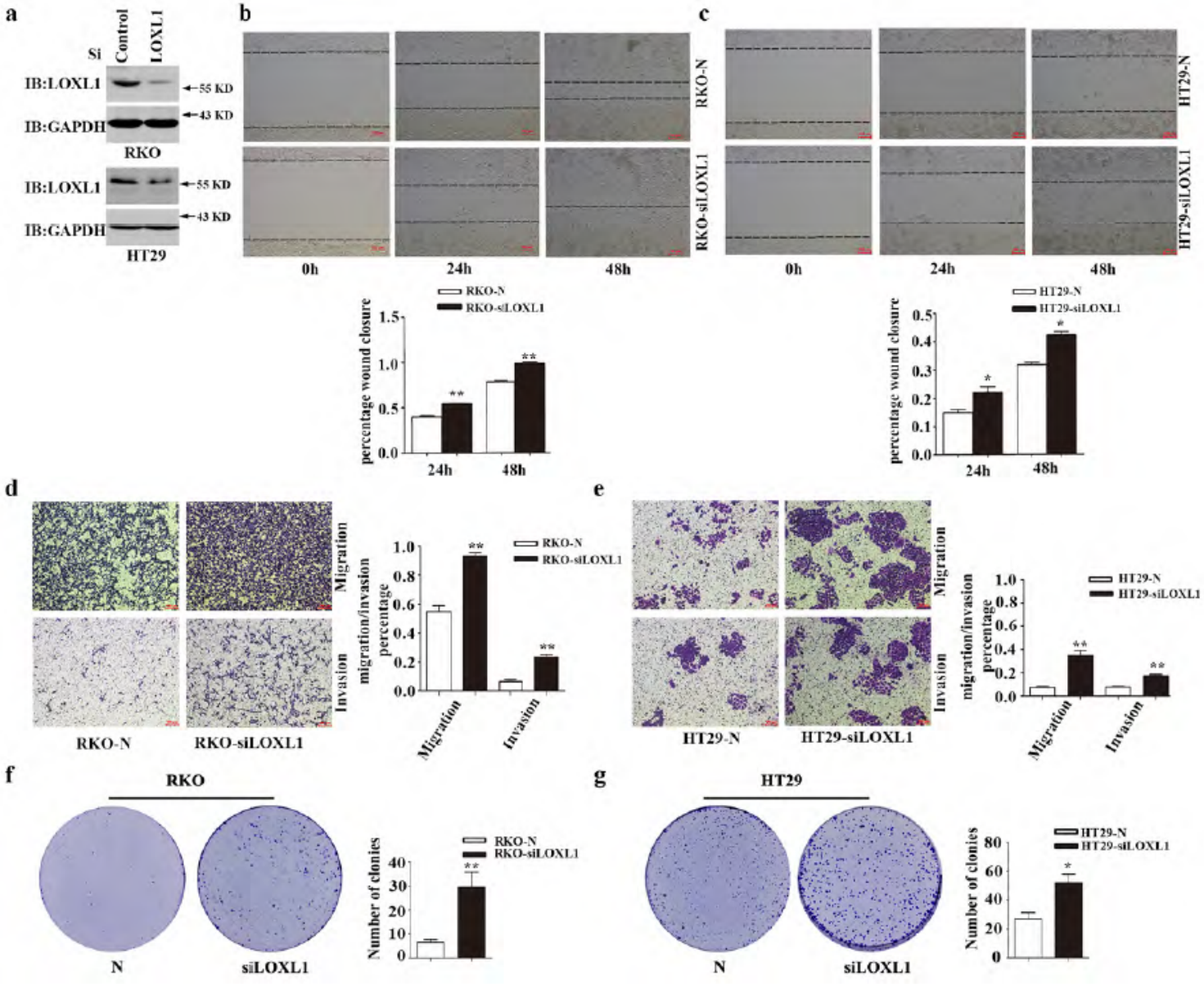

Figure 3

Knockdown of LOXL1 in RKO and HT29 promotes their migration and invasion abilities in vitro. a Detection of LOXL1 in RKO and HT29 cells through western blot using the LOXL1 siRNA. b Wound healing

analysis upon LOXL1 knockdown in RKO cells transiently transfected with an si-LOXL1 vector or a control vector at 0 h, 24 h, and 48 h, and calculation of their wound healing percentages. c Wound healing analysis conducted after reducing the expression of LOXL1 in HT29 cells transiently transfected with an si-LOXL1 expression vector or a control vector at 0 h, 24 h, and 48 h, and calculation of their wound healing percentages. d Transwell migration and Matrigel invasion assays using RKO-siLOXL1 and RKO-N cells and calculation of the number of cells undergoing migration/invasion in these cell lines. e Transwell migration and Matrigel invasion assays using HT29-siLOXL1 and HT29-N cells and calculation of the number of cells undergoing migration/invasion in these cell lines. f The clonogenic assay was performed using RKO cells with or without LOXL1. Left panel: representative images, right panel: quantification analysis. g The clonogenic assay was performed using HT29 cells with or without LOXL1. Left panel: representative images, right panel: quantification analysis. Data of three independent experiments are presented as the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

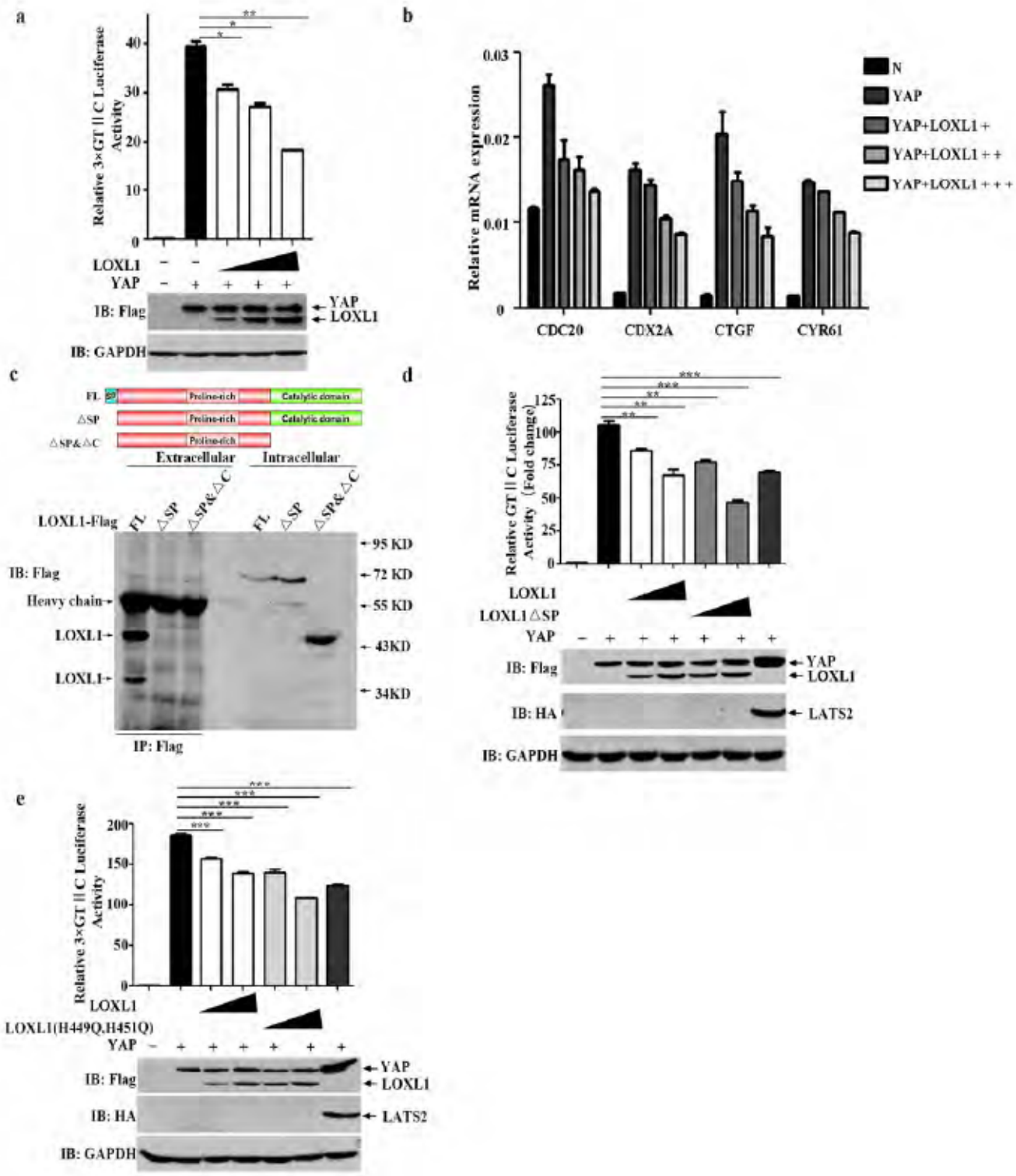

Figure 4

Intracellular LOXL1 inhibits the transcriptional activity of YAP. a Overexpression of LOXL1 suppresses the activity of YAP in HEK293T cells, as detected by the reporter assay. b q-PCR analysis of CDC20, CDX2A, CTGF, and CYR61 in HEK293T cells overexpressing LOXL1 and YAP. c Western blot analysis to determine the localisation of LOXL1 and its truncations. d Reporter assay to analyse the activity of YAP resulting

from the overexpression of LOXL1 and its truncations. e Reporter assay to detect the activity of YAP by overexpressing LOXL1 and its mutants.

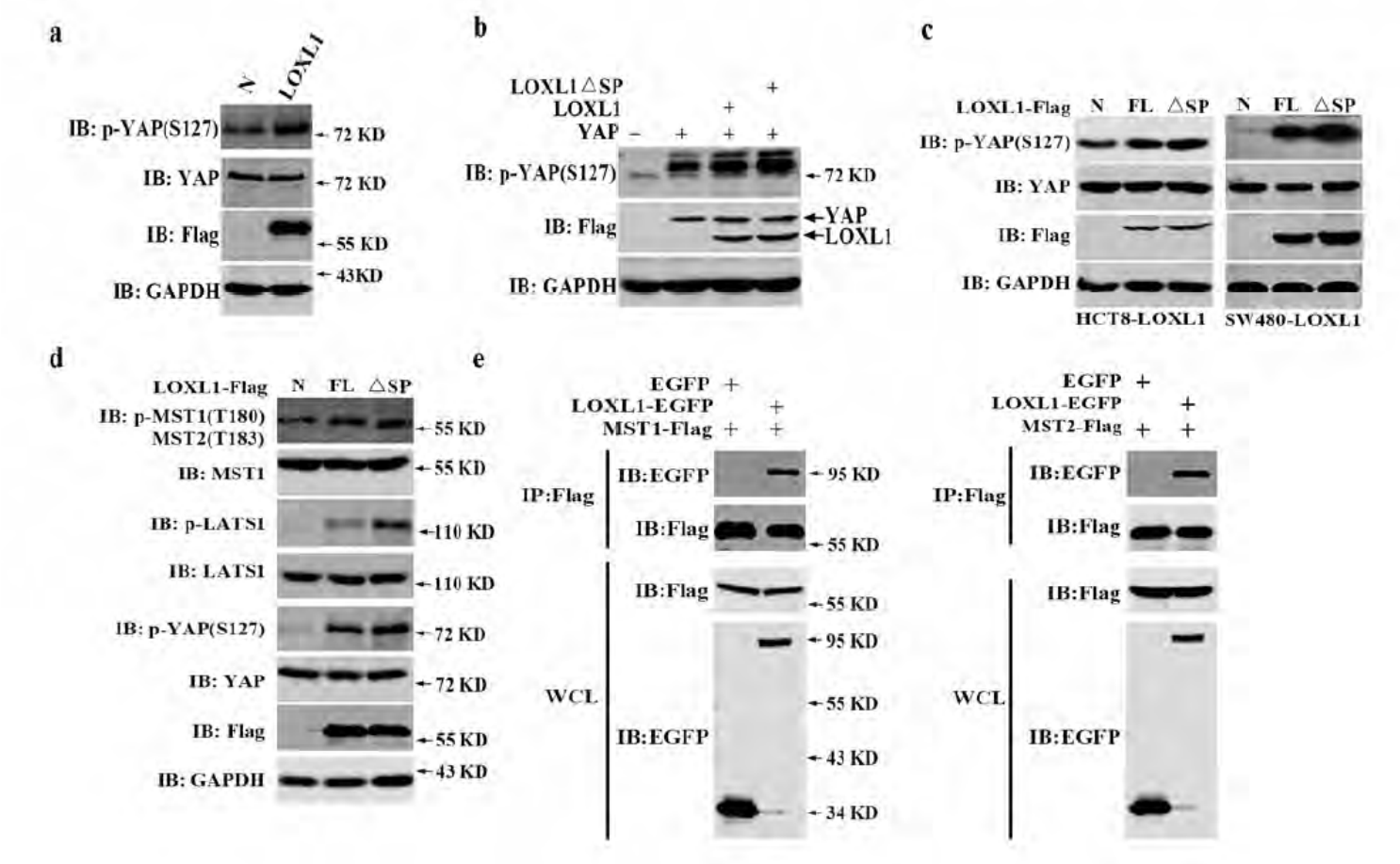

Figure 5

LOXL1 activates the Hippo pathway through interaction with MST1/2 a Western blot analysis to determine the extent of phosphorylation of YAP in HEK293T cells overexpressing LOXL1. b Western blot to determine the extent of phosphorylation of YAP through overexpression of LOXL1 and its truncation in HEK293T cells. c Western blot analysis determining the expressions of LOXL1, YAP, and p-YAP (S127) in HCT8/SW480 cells transfected with N/LOXL1/LOXL1 △SP. d LOXL1 and LOXL1 △SP plasmids were transfected into HEK293T cells and their lysates were analysed using the indicated antibodies. e Flag tagged MST1 or 2 co-transfected with EGFP tagged LOXL1 in HEK293T cells. FLAG immunoprecipitated proteins were detected using western blot analysis.

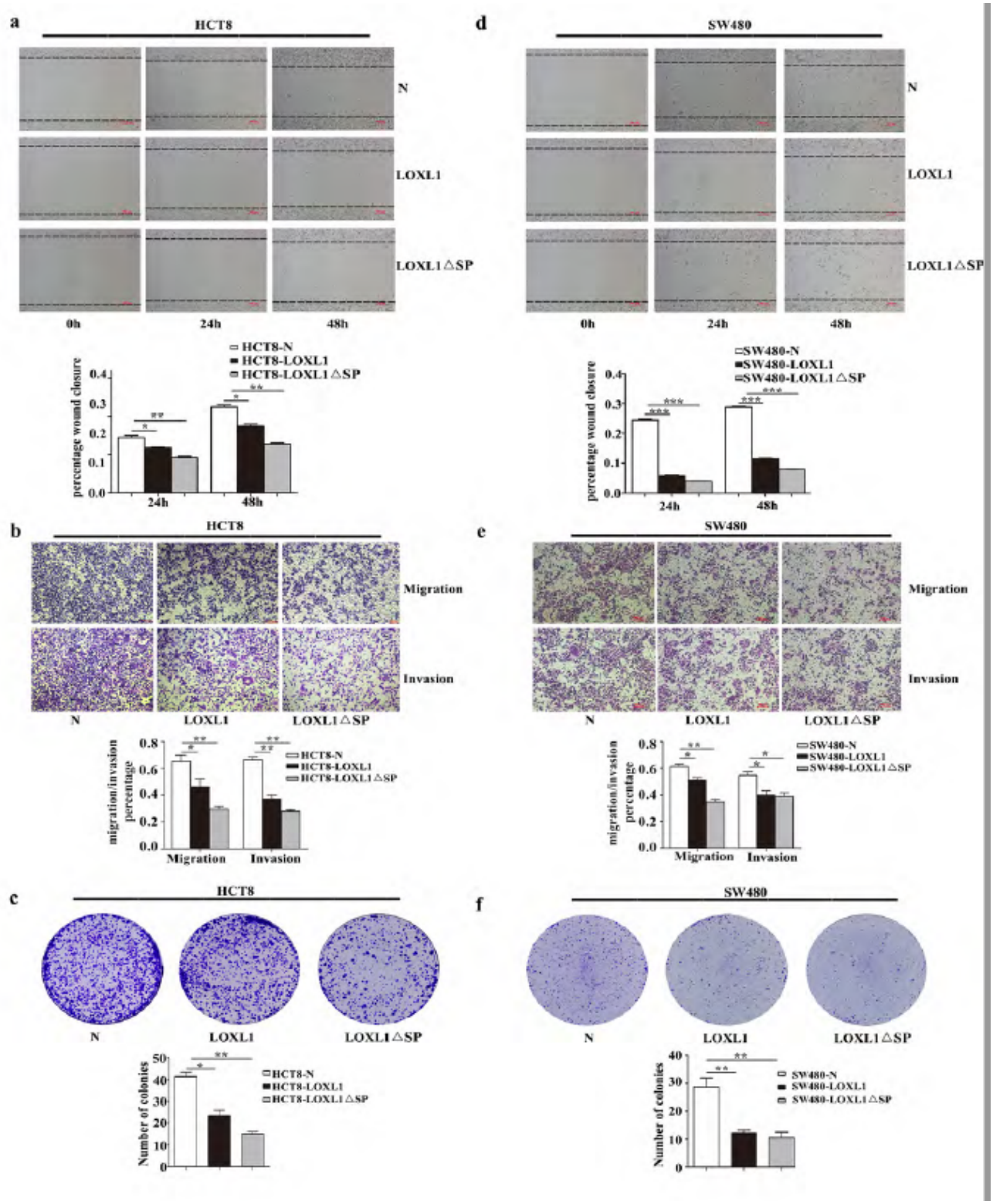

Figure 6

Effect of LOXL1 △SP expression on the migration and tumorigenesis of HCT8 and SW480 cells in vitro. a Wound healing analysis carried out in HCT8 cells transfected with the control vector, and LOXL1 and LOXL1 △SP expression vectors at 0 h, 24 h, and 48 h, and the calculation of their wound healing percentages. b Transwell migration and Matrigel invasion assays conducted using the overexpressed cell lines HCT8-N, HCT8-LOXL1 and HCT8-LOXL1 △SP and calculation of the rate of migration/invasion in

relevant stable HCT8 cell lines. c The clonogenic assay was performed using HCT8 cells after transfecting with LOXL1 or LOXL1 △SP. An empty vector was used as the negative control. Left panel: representative images, right panel: quantification analysis. d Wound healing analysis carried out on SW480 cells transfected with the LOXL1 expression vector, control vector, and LOXL1 △SP vector at 0 h, 24 h, and 48 h, and calculation of their wound healing percentages. e Transwell migration and Matrigel invasion assays using the overexpressed cell lines SW480-N, SW480-LOXL1, SW480-LOXL1 △SP and calculation of the rate of migration/invasion for relevant SW480 cell lines. f The clonogenic assay was carried out using SW480 cells after transfecting with LOXL1 or LOXL1 △SP. An empty vector was used as the negative control. Left panel: representative images, right panel: quantification analysis. Data of three independent experiments are presented as the mean ± SD. *P < 0.05, **P < 0.01, ***P <0.001.

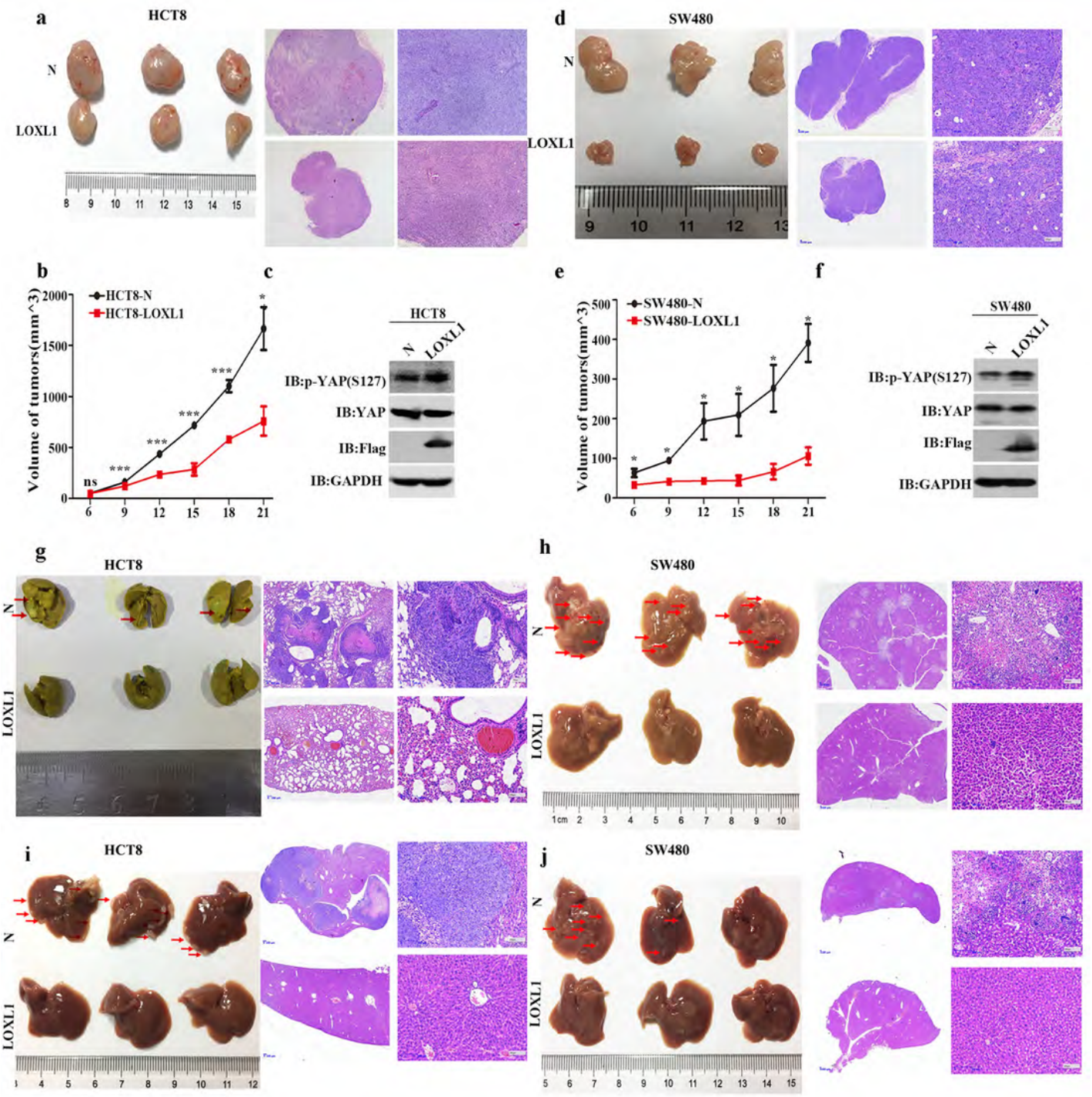

Figure 7

Effect of the overexpression of LOXL1 on CRC tumorigenesis in vivo. a Left: images of the xenograft tumours of HCT8-N and HCT8-LOXL1 in nude mice, right: corresponding images of H-E staining at magnifications of 25× and 100×. b Growth curve analyses of tumour volumes in HCT8-N/HCT8-LOXL1 measured from day 7 to day 19. Bars represent the mean ± SD. c Western blot analyses of p-YAP, YAP, LOXL1 and GAPDH in HCT8-N/HCT8-LOXL1 xenograft tumours. d Left: images of xenograft tumours of

SW480-N and SW480-LOXL1 in nude mice, right: corresponding images of H-E staining at magnifications of 25× and 100×. e Growth curve analyses of tumour volumes in SW480-N/SW480-LOXL1 measured from day 7 to day 19. Bars represent the mean ± SD. f Western blot analyses of p-YAP S127, YAP, LOXL1, and GAPDH in SW480-N/SW480-LOXL1 xenograft tumours. g Left: corresponding images of the lungs of nude BALB/c mice after injection of HCT8-N and HCT8-LOXL1 cells into their tail veins; right: representative H-E images of metastases observed in the lungs of mice. h Left: SW480-N and SW480-LOXL1 cells were injected into tail vein of nude BALB/c mice and relevant images of metastatic tumours in their livers; right: representative H-E image of metastases observed in the livers of mice. i-j left: HCT8-N/HCT-LOXL1 and SW480-N/SW480-LOXL1 were injected into the spleens and metastatic tumours in the livers and relevant images of their livers have been captured; right: representative H-E image of metastases observed in the livers of mice.