

# LSD1 regulates Notch and PI3K/Akt/mTOR pathways through binding the promoter regions of Notch target genes in esophageal squamous cell carcinoma

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**Background:** The aberrant activation of Lysine-specific demethylase 1 (LSD1), Notch and PI3K/Akt/mTOR signaling pathways were frequently happened in many cancers, including esophageal squamous cell carcinoma (ESCC). However, the regulatory relationship between LSD1 and Notch as well as PI3K/Akt/mTOR pathways is still unclear.

**Purpose:** This study aimed to explore the regulatory effects and mechanisms of LSD1 on Notch and PI3K/Akt/mTOR pathway in ESCC.

**Results:** Firstly, we demonstrated that LSD1 and proteins in Notch and PI3K/Akt/mTOR pathway were expressed in ESCC cells. Secondly, inhibition of LSD1 by tranylcypromine (TCP) or shRNA could decrease the expressions of related proteins in Notch and PI3K/Akt/mTOR signaling pathways in ESCC cells. Finally, we found that LSD1 could bind to the promoter regions of Notch3, Hes1 and CR2, and the combinations between them were reduced by TCP in ESCC.

**Conclusion:** Summarily, this study indicated that LSD1 might positively regulate Notch and PI3K/Akt/mTOR pathways through binding the promoter regions of related genes in Notch pathway in ESCC.

**Keywords:** LSD1, Notch, PI3K/Akt/mTOR, esophageal squamous cell carcinoma

## Introduction

Esophageal carcinoma is one of the most common tumors worldwide. It is classified as esophageal squamous cell carcinoma (ESCC) or esophageal adenocarcinoma (EAC) depending on the clinicopathological features. EAC is now the most common cancer type in Western countries, while ESCC is the predominant type in China.<sup>1,2</sup> Due to its high mortality rate and poor prognosis, the 5-year survival rate of ESCC patients in China is only about 20%; although this has improved with the progress of treatment strategies in recent years, it is still very low.<sup>3</sup> Therefore, further exploration of the molecular mechanisms of tumorigenesis in ESCC is warranted to develop novel targeted therapeutic strategies.

Epigenetic changes, such as abnormal DNA methylation and modification of histone, were reported to be associated with the development of various cancers.<sup>4-6</sup> Lysine-specific demethylase 1 (LSD1, KDM1A), the first identified histone demethylase, could specifically catalyze the demethylation of monomethyl and dimethyl H3K4 (H3K4me1/2) and H3K9 (H3K9me1/2).<sup>7</sup> The overexpression of LSD1 was

correlated with tumorigenesis, and numerous LSD1 inhibitors have been developed so far.<sup>8</sup> Tranylcypromine (TCP), which irreversibly inhibits LSD1 by forming a covalent adduct with the flavin adenine dinucleotide co-factor, has been shown to inhibit tumor growth in xenograft models of breast cancer and oral squamous cell carcinoma.<sup>9,10</sup>

The Notch pathway is involved in various cellular processes such as cell growth and differentiation.<sup>11</sup> In mammals, the Notch family consists of four Notch receptors (Notch1–4) and five ligands (Delta-like 1, 3 and 4; Jagged 1 and 2).<sup>12</sup> The binding of the ligand with the Notch receptor triggers proteolytic cleavage to release Notch intracellular domain (NICD), which is then translocated into the nucleus and forms a transcriptional activation complex with proteins in the Mastermind-like family and CSL, and thus transcriptionally activates target genes of Notch such as hairy/enhancer of split 1 (Hes1), Deltex1 (DTX1), c-Myc, CR2 and nuclear factor-kappa B (NF- $\kappa$ B).<sup>13,14</sup> It is reported that Notch pathway interacts with other signaling pathways such as PI3K/Akt/mTOR, Wnt and Ras/MAPK to regulate tumorigenesis in many types of cancers,<sup>15,16</sup> and activated PI3K (phosphoinositide 3-kinase) regulates cell survival and proliferation by stimulating its downstream factors such as Akt and mTOR.<sup>17</sup>

Several studies have revealed potential regulatory effects of LSD1 on the Notch pathway;<sup>18–21</sup> for example, LSD1 modulates the Notch/ASCL1 axis by binding to the Notch1 locus in small-cell lung cancer.<sup>21</sup> Some studies also reported that LSD1 could affect the mTOR pathway through regulating autophagy.<sup>22,23</sup> However, the regulating effects of LSD1 on the Notch and mTOR pathways remain poorly understood in ESCC, which was just the problem we explored in this study.

## Materials and methods

### Reagents and antibodies

TCP was purchased from MedChemExpress (USA). Primary antibodies recognizing LSD1 (ab17721) and Hes1 (ab71559) were obtained from Abcam (UK), DTX1 (GTX112367) were obtained from GeneTex (USA), and H3 (9728S), Notch 1 (3608S), Notch 3 (3889S), H3K4me2 (3889S), PI3K (4228S), Rictor (9476S), p-Akt (Ser473) (4060S), Akt (2920S), p-mTOR (Ser2448) (5536S), p-mTOR (Ser2481) (2974S), mTOR (2983S), Raptor (2280S), p-p70S6K (Thr389) (9206S) and GAPDH (5174S) as well as the secondary antibodies were obtained from Cell Signaling Technology (USA).

LSD1 shRNA and control shRNA vector were obtained from GenePharma Company (Shanghai, China).

### Cell lines and cell culture

KYSE450, KYSE790, ECa109, EC9706 and TE-1 cells were obtained from Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). As described before,<sup>24</sup> cells were cultured in RPMI-1640 medium (Biological Industries, Israel) containing 10% fetal bovine serum (FBS) (Biological Industries, Israel) with a CO<sub>2</sub> incubator at the condition of 5% CO<sub>2</sub> and 37 °C.

### Western blot

Total proteins and histone proteins of KYSE450, KYSE790, ECa109, EC9706 and TE-1 cells treated with 10 or 50  $\mu$ M TCP at for 48 h were extracted, respectively, using total protein or histone protein extraction kit (Epigentek, USA). 30  $\mu$ g of total proteins or 2  $\mu$ g of histone proteins were separated on 8–10% SDS-PAGE gels and then electro-transferred to supported nitrocellulose membranes using a wet transferor. After the membranes were blocked with 5% skimmed milk for 2 h, then incubated with primary antibodies recognizing LSD1 (1:1000), Hes1 (1:1000), DTX1 (1:1000), H3 (1:1000), Notch1 (1:1000), Notch3 (1:1000), H3K4me2 (1:1000), PI3K (1:500), Rictor (1:1000), p-Akt (Ser473) (1:1000), Akt (1:1000), p-mTOR (Ser2448) (1:1000), p-mTOR (Ser2481) (1:1000), mTOR (1:1000), Raptor (1:500), p-p70S6K (Thr389) (1:500) and GAPDH (1:1000) diluted in 5% skimmed milk in PBST at 4 °C overnight, followed by incubation with the HRP-linked secondary antibodies at room temperature (RT) for 2 h. The membranes were rinsed three times with TBST between the incubations described above. Finally, the bands of specific proteins on the membranes were visualized with enhanced chemiluminescence (ECL) reagent (Thermo Scientific, USA) according to the manufacturer's instructions. All measures of protein expression were repeated at least three times independently and the blots were analyzed using Image J software (NIH, USA).

### Cell transfection and screening

ECa109 and EC9706 cells seeded in six-well plates were transfected with LSD1 shRNA or control shRNA vector, respectively, using Lipofectamine<sup>®</sup> 3000 reagent (Invitrogen, USA) according to the manufacturer's protocols and cells stably expressing LSD1 shRNA or control shRNA were screened as described earlier.<sup>25</sup> In brief, after

ECa109 and EC9706 cells ( $2 \times 10^5$  cells per well) were cultured in a six-well plate for 24 h, the culture medium was changed as fresh serum-free medium (2 mL per well). Meanwhile, 5  $\mu$ g LSD1 shRNA or control shRNA vector, respectively, was mixed with a mixture of 125  $\mu$ L serum-free medium and 5  $\mu$ L Lipofectamine™ 3000 reagent for 5 min at RT. Cells continued to be cultured for 8 h after the mixture was added into the wells, then the medium was replaced with RPMI-1640 medium containing 10% FBS for another 24 h. Subsequently, 2.5  $\mu$ g/mL of puromycin was added into the wells for screening cells with LSD1 shRNA or control shRNA vector, and the untreated cells were as control. When all the control cells were dead, the screened cells were cultured with medium containing 2.5  $\mu$ g/mL puromycin for another 2 d, and continued to be cultured and passaged using medium with 1.25  $\mu$ g/mL of puromycin, the total protein of which was extracted for analyzing the expression of LSD1 and proteins in the Notch and PI3K/Akt/mTOR pathways by Western blot.

## Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed using chromatin immunoprecipitation kit (Millipore, Germany) according to the manufacturer's instructions. In brief, ECa109 cells treated with 50  $\mu$ M of TCP were fixed in 1% formaldehyde at RT for 10 min, and 125 mM of glycine was added for another 5 min. Subsequently, the cells were washed with cold PBS containing protease inhibitors and resuspended in lysate buffer containing 1% SDS and protease inhibitors. After the cell lysates were placed on ice for 8 min and centrifuged at 12,000 rpm for 10 min, the protein G-agarose magnetic beads were added into the supernatant and incubated at 4 °C for 1 h, then centrifuged at 1000 rpm for 1 min. One percent of the supernatant was retained at 4 °C as input solution, and the remaining supernatant for ChIP assay was incubated at 4 °C overnight with antibodies against RNA polymerase II, normal mouse IgG and LSD1, respectively, followed by the introduction of 60  $\mu$ L protein G-agarose and incubation at 4 °C for 1 h. After being centrifuged at 4 °C, 1000 rpm for 1 min, the sediment obtained was washed successively with low-salt, high-salt, LiCl and TE buffer solutions, being incubated for 5 min and centrifuged 1000 rpm for 1 min during each washing to obtain the DNA-protein complex. Subsequently, 200  $\mu$ L of elution buffer consisting of 10  $\mu$ L of 20% SDS, 20  $\mu$ L of 1 M NaHCO<sub>3</sub> and 170  $\mu$ L of sterile distilled water was added into the DNA-protein

complex at RT for 30 min. At the same time, the same volume of elution buffer was added to the input solution and kept at 4 °C. Then, the DNA-protein complex with elution buffer was incubated with 8  $\mu$ L of 5 M NaCl at 65 °C for 4 h and 1  $\mu$ L of RNase A at 37 °C for 30 min, followed by the addition of 1  $\mu$ L of proteinase K, 4  $\mu$ L of 0.5 M EDTA and 8  $\mu$ L of 1 M Tris-HCl and incubation at 45 °C for 1 h. Finally, phenol and ethanol were used for extracting DNA following the template of quantitative real-time PCR (qRT-PCR) described below.

## qRT-PCR analysis

Quantity binding with LSD1 of Notch target gene promoters was detected by qRT-PCR analysis using the SYBR Green PCR Kit (Roche, USA). The DNA above was subjected to qRT-PCR analysis in 20  $\mu$ L system with 10  $\mu$ L SYBR Green Master Mix, 2  $\mu$ L DNA template, 1  $\mu$ L forward primer, 1  $\mu$ L reverse primer and 6  $\mu$ L double distilled water. Primers for different regions of Notch target gene promoter used in the present study were synthesized by Sangon Biotech (Shanghai, China) as shown in Table 1. The PCR condition was as follows: 95 °C for 10 min; followed by 45 cycles at 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s; finally 95 °C for 10 s, 65 °C for 60 s and 97 °C for 1 s. Relative quantity of mRNA was calculated using the comparative cycle threshold method ( $2^{-\Delta\Delta C_t}$ ) as previously described.<sup>26,27</sup> The  $C_t$  value of each ChIP DNA sample ( $\Delta C_{t[\text{correction ChIP}]}$ ) was adjusted according to the formula:  $\Delta C_{t[\text{correction ChIP}]} = (C_{t[\text{ChIP}]} - (C_{t[\text{Input}]} - \text{Log}_2(\text{Input dilution fraction})))$ , and the %input value for each ChIP DNA sample was calculated according to the formula:  $\% \text{Input} = 2^{(-\Delta C_{t[\text{ChIP}]})}$ .

## Statistical analysis

All experiments in this study were performed at least three times and the results were analyzed by one-way analysis of variance (ANOVA) or Student's *t*-test using SPSS 19.0 software. The results were presented as the mean  $\pm$  standard deviation, with  $P < 0.05$  considered statistically significant.

## Results

### Expression of LSD1, proteins involved in Notch and mTOR pathway in ESCC cells

The expression levels of LSD1, related proteins in Notch and PI3K/Akt/mTOR signaling pathways in KYSE450, KYSE790, ECa109, EC9706 and TE-1 cell lines were detected by Western blot. As shown in Figure 1A, LSD1

**Table 1** Primer sequences of Notch target genes promoter evaluated by qRT-PCR analysis

Primer			Primer sequences 5'-3'
Hes1	-2200	Forward	AGG TCA CCC AGA GTC AGG AA
	-2200	Reverse	CCA GCG TCT TGT TTG ATG TG
Hes1	TSS	Forward	CGT GTC TCC TCC TCC CAT T
	TSS	Reverse	GAG AGG TAG ACG GGG GAT TC
Hes1	+500	Forward	TCA ACA CGA CAC CGG ATA AA
	+500	Reverse	TCA GCT GGC TCA GAC TTT CA
Hes1	+2600	Forward	GGC TTT TGG TGG AAT TTG AA
	+2600	Reverse	TCA TGG AGG ATT GGT GAA AAG
Notch3	-5000	Forward	TAG CCC CTG GTC AGT CAT TC
	-5000	Reverse	GGT GCA TCG TAT CAG GAG GT
Notch3	TSS	Forward	TGG CCT CAG TTT CCA GAG TT
	TSS	Reverse	CAC ACC CAA CCT CGT GAA C
Notch3	+3000	Forward	GTC TCA GCA CAC CCC ATT CT
	+3000	Reverse	AAC CAC AAA GCA GGG GAA G
Notch3	+28600	Forward	GGG GGC TAA AGA CAC AAA CA
	+28600	Reverse	GTT CCT TCT CTC CCC ACT CC
DTX1	-1700	Forward	TGT GAA TGA CAT GGC AGA GG
	-1700	Reverse	TGA ATC TCC TGC CAG TAC CC
DTX1	+30000	Forward	ACA TGC CAG ACA GCA GAA CA
	+30000	Reverse	AAC CTT CCA GAC CCT GTG TG
CR2	+5000	Forward	GCC GGA AGG ATG TTC TTG TA
	+5000	Reverse	CAG GGA AGG CCA TGA AAA TA
CR2	+21000	Forward	CCC CAC AGT GCT TAC GAT CT
	+21000	Reverse	AAG CCA GGA TTG CAG TCA AC

**Note:** Primer sequences above were designed according to the gene sequence from the GenBank of PubMed, and the ID number: 3280 for Hes1, 4854 for Notch3, 1840 for DTX1 and 1380 for CR2.

was expressed in all the five ESCC cell lines, especially in ECa109 and EC9706 cell lines. The expressions of related proteins in the Notch pathway, such as Notch1 and Hes1, as well as in the mTOR pathway, such as Rictor and p-Akt (Ser473), were also observed in the five ESCC cell lines (Figure 1B), while the regulation relationship between LSD1 and the Notch and mTOR pathways warrants further exploration.

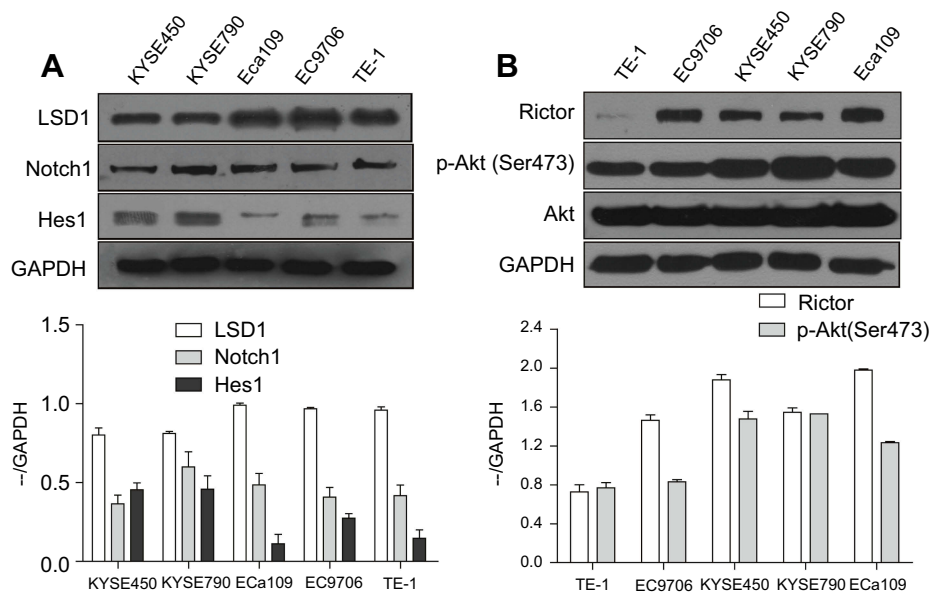
### TCP inhibited the expression and function of LSD1 in ESCC cells

We determined firstly the effects of LSD1 inhibitor TCP on expression and function of LSD1 in KYSE450, KYSE790, ECa109, EC9706 and TE-1 cells by Western blot through treating cells with different concentrations of TCP (0, 10 and 50  $\mu$ M) for 48 h. As shown in Figure 2A

and B, after cells were treated with increasing doses of TCP, the expression of LSD1 clearly decreased while H3K4me2 increased. Compared to their control cells the decreasing ratios of LSD1 were 38.8%, 36.9%, 34.3%, 60.9% and 56.8%, respectively, and the increased ratios of H3K4me2 were 31.0%, 57.2%, 11.9%, 9.0% and 10.7%, respectively, in KYSE450, KYSE790, ECa109, EC9706 and TE-1 cells treated with 50  $\mu$ M of TCP ( $P < 0.05$ ). These results suggest that TCP can not only inhibit the expression of LSD1, but also suppress the demethylating function of LSD1 in ESCC cells.

### Downregulation of LSD1 inhibited Notch signaling pathway in ESCC cells

ECa109 and EC9706 cells, which had higher expression of LSD1 than other ESCC cell lines, were chosen to study the



**Figure 1** Expressions of LSD1 and proteins in Notch and mTOR pathway in ESCC cells. Total proteins of KYSE450, KYSE790, Eca109, EC9706 and TE-1 cells were extracted and the expressions of LSD1. Related proteins in Notch pathway such as Notch1 and Hes1 (A) as well as in mTOR pathway such as Rictor and p-Akt (Ser473) (B) were detected by Western blot.

regulatory effects of LSD1 on the Notch signaling pathway in this study. After Eca109 and EC9706 cells were treated with different concentrations of TCP (0, 10 and 50  $\mu$ M) for 48 h, the expressions of related proteins in the Notch signaling pathway were measured by Western blot. As shown in Figure 3A, TCP decreased the expressions of Notch3, Notch1, DTX1 and Hes1 in a dose-dependent manner in both cells ( $P < 0.05$ ). Compared with untreated cells, the decreasing ratios of Notch3, Notch1, DTX1 and Hes1 were 36.9%, 43.3%, 52.6% and 62.5%, respectively, in Eca109 cells, and 50.4%, 43.1%, 79.3% and 88.4%, respectively, in EC9706 cells at 50  $\mu$ M of TCP, indicating that TCP could effectively inhibit the Notch signaling pathway.

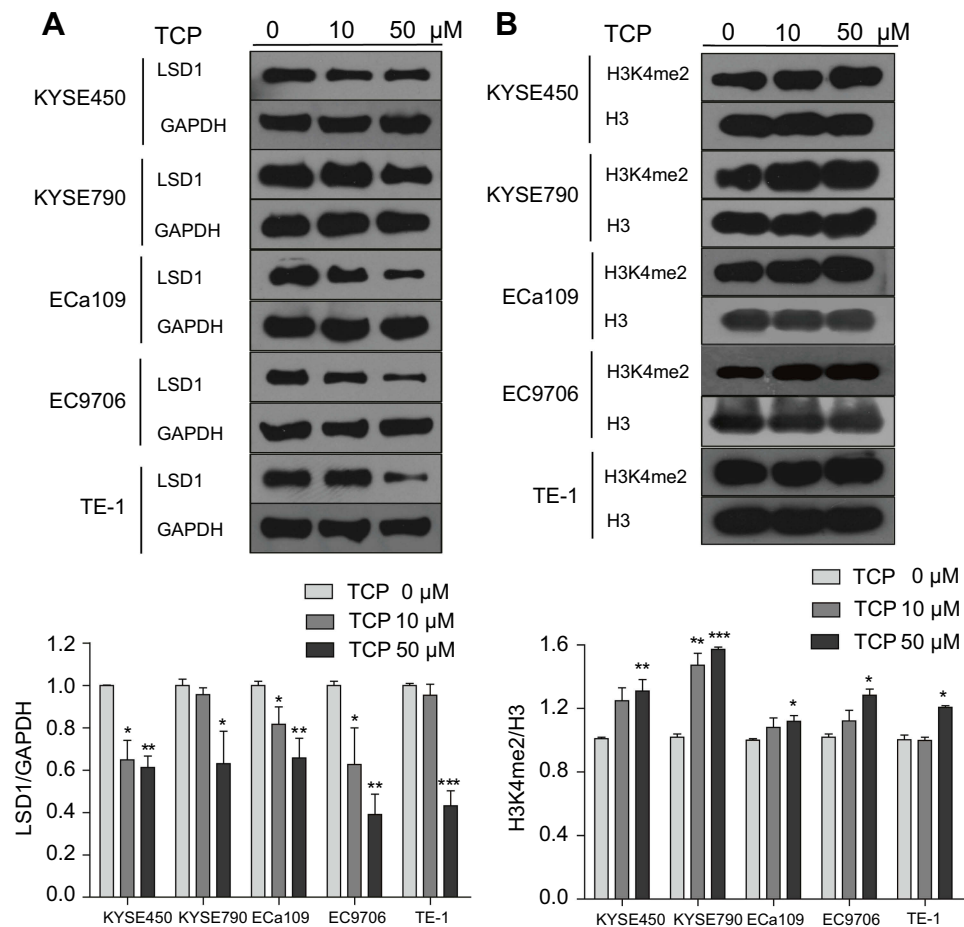
In addition, to further determine the regulation effects of LSD1 on the Notch signaling pathway, after the expression of LSD1 was downregulated by LSD1 shRNA, the expressions of related proteins in the Notch signaling pathway were investigated by Western blot. As shown in Figure 3B, compared with the control shRNA group, the expression of LSD1 in the LSD1 shRNA group clearly decreased ( $P < 0.05$ ); and the decrease ratios were 43.8% and 46.4% in EC9706 and Eca109 cells, respectively, indicating that LSD1 shRNA was transfected successfully into cells and expressed stably in cells. After the expression of LSD1 in ESCC cells was knocked down, the expressions of Notch3, Notch1, DTX1 and Hes1 also decreased at ratios of 68.2%, 38.2%, 54.9% and 39.6%,

respectively in Eca109 cells, and 53.7%, 41.8%, 53.3% and 30.2%, respectively in EC9706 cells ( $P < 0.05$ ). These findings demonstrate that inhibition of LSD1 by either TCP or shRNA decreased the expressions of related proteins in the Notch signaling pathway, suggesting that LSD1 might promote activation of the Notch signaling pathway in ESCC cells.

### Downregulation of LSD1 inhibited the PI3K/Akt/mTOR signaling pathway in ESCC cells

We also investigated protein expression in the PI3K/Akt/mTOR signaling pathway by Western blot after Eca109 and EC9706 cells were treated with different concentrations of TCP (0, 10 and 50  $\mu$ M) for 48 h. As shown in Figure 4A, along with the concentration increase of TCP, the expressions of PI3K, p-Akt (Ser473), p-mTOR (Ser2448), p-mTOR (Ser2481) and Rictor were decreased at ratios of 80.2%, 67.1%, 85.8%, 47.6%, 81.5% in Eca109 cells and 78.1%, 53.9%, 43.6%, 56.9%, 71.1% in EC9706 cells at 50  $\mu$ M of TCP ( $P < 0.05$ ) compared with untreated cells, indicating that TCP could inhibit the PI3K/Akt/mTOR signaling pathway.

Moreover, downregulation of LSD1 by shRNA had a similar effect with TCP: the expressions of PI3K, p-Akt (Ser473), p-mTOR (Ser2448), p-mTOR (Ser2481) and Rictor decreased in the LSD1 shRNA group ( $P < 0.05$ )



**Figure 2** TCP decreased LSD1 but increased H3K4me2 expression in ESCC cells. After KYSE450, KYSE790, Eca109, EC9706 and TE-1 cells were treated with different concentration of TCP (0, 10 and 50 μM) for 48 h, total and histone proteins were extracted for analysis the expressions of LSD1 (A) and histone H3K4me2 (B) by Western blot. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  versus untreated cells.

compared with the control shRNA group, a ratios of 80.5%, 41.4%, 30.4%, 28.9% and 44.7% in Eca109 cells and 55.9%, 54.1%, 24.1%, 33.7% and 41.3% in EC9706 cells, respectively (Figure 4B;  $P < 0.05$ ). These show that inhibition of LSD1 by either TCP or shRNA could inhibit the expressions of related proteins in the PI3K/Akt/mTOR signaling pathway, suggesting that LSD1 might contribute to activation of the PI3K/Akt/mTOR signaling pathway.

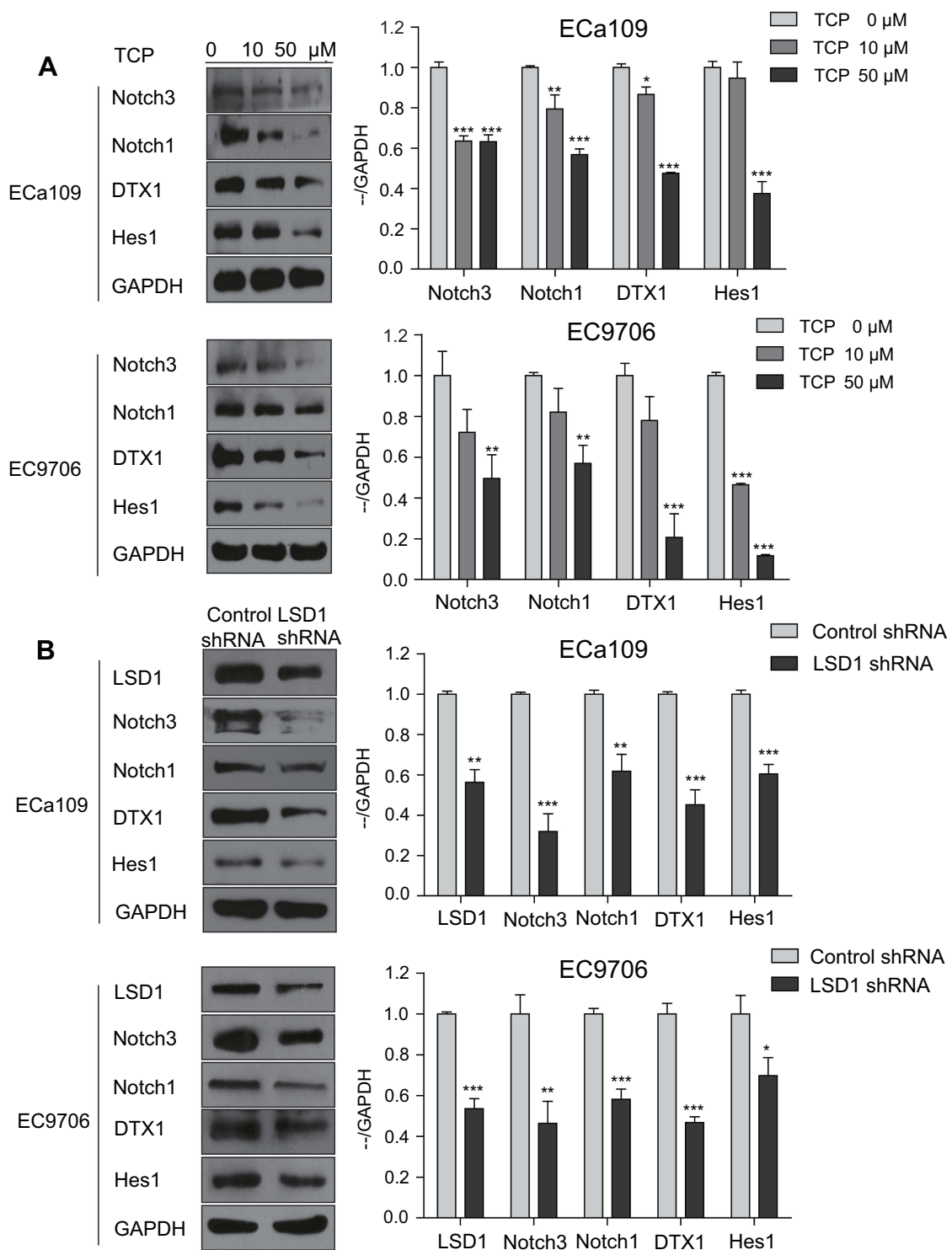
### LSD1 binding the promoter regions of Notch target genes in Eca109 cells

The regulatory mechanisms of LSD1 to Notch signaling pathway were explored by ChIP analysis. As shown in Figure 5, after Eca109 cells were treated with 50 μM TCP for 48 h, the combined ability of LSD1 with the promoters of Notch3, HES1 and CR2 obviously decreased ( $P < 0.05$ ), but not DTX1. The combining site was at downstream 3000 bp of transcription start site

(TSS) for Notch3, upstream 2200 bp and the downstream 2600 bp of TSS for Hes1, and downstream 21000 bp of TSS for CR2. These results indicated that LSD1 might regulate the Notch signaling pathway through binding the promoter regions of Notch target genes in ESCC cells.

### Discussion

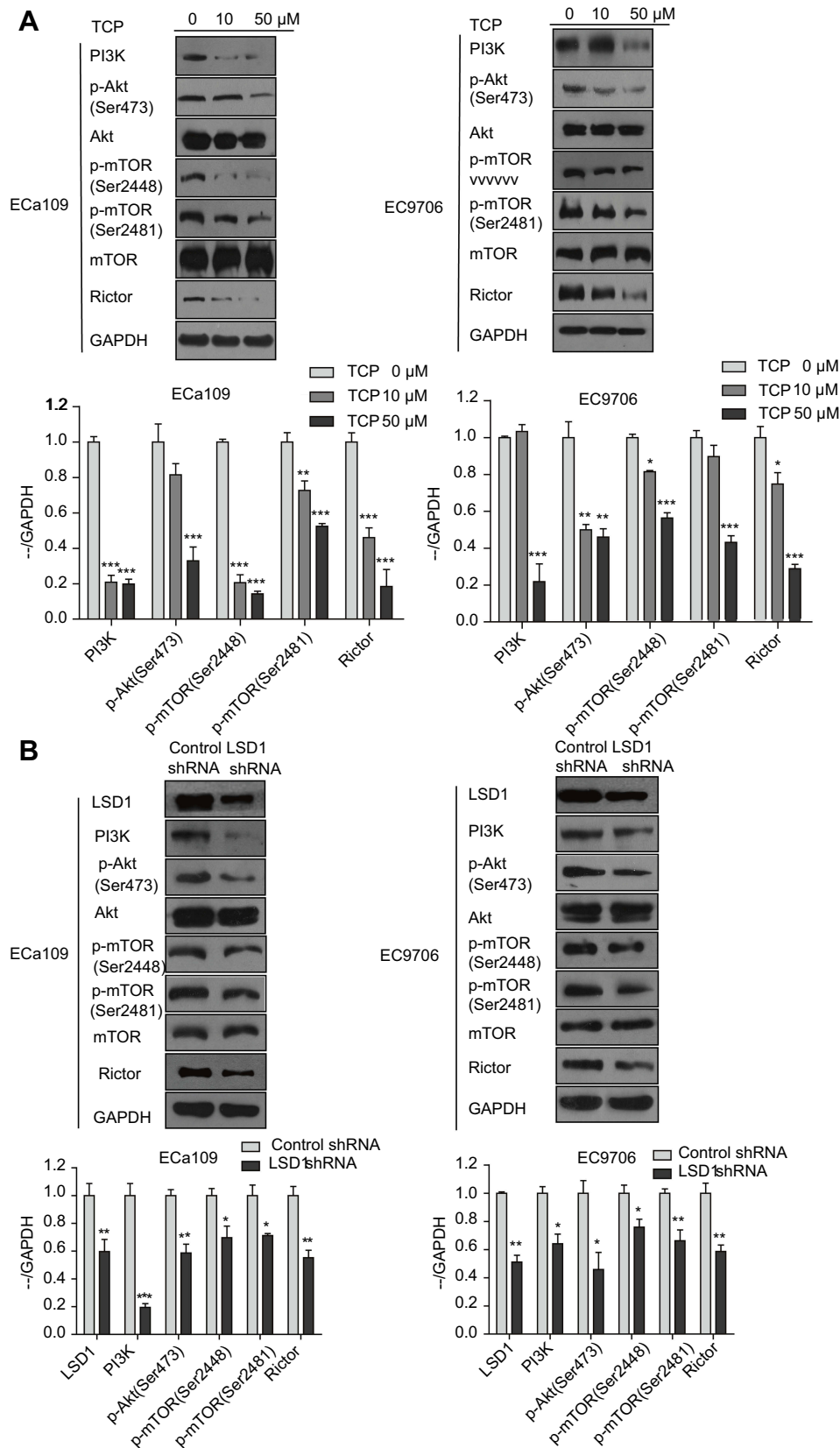
The unbalance of histone methylation and demethylation has been shown to be associated with the tumorigenesis of many cancers.<sup>6</sup> Overexpression of LSD1, the first discovered histone demethylase, was involved in human carcinogenesis by regulating chromatin.<sup>8</sup> The TCP-based LSD1 inhibitors have shown significant therapeutic effects in cancers and some of these have been tested in clinical trials, such as ORY1001 and GSK2879552, which indicated the potential of LSD1 as a therapeutic target for cancers.<sup>28</sup> Recent studies



**Figure 3** Downregulation of LSD1 by TCP or shRNA decreased the expressions of related proteins in the Notch pathway in ESCC cells. **(A)** ECa109 and EC9706 cells were treated with different concentrations of TCP (0, 10 and 50 μM) for 48 h, and total proteins were extracted for analysis the expressions of Notch3, Notch1, DTX1 and Hes1 by Western blot. **(B)** Total proteins of ECa109 and EC9706 cells with control shRNA or LSD1 shRNA were extracted, and the expressions of LSD1, Notch3, Notch1, DTX1 and Hes1 were analyzed by Western blot. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus control group.

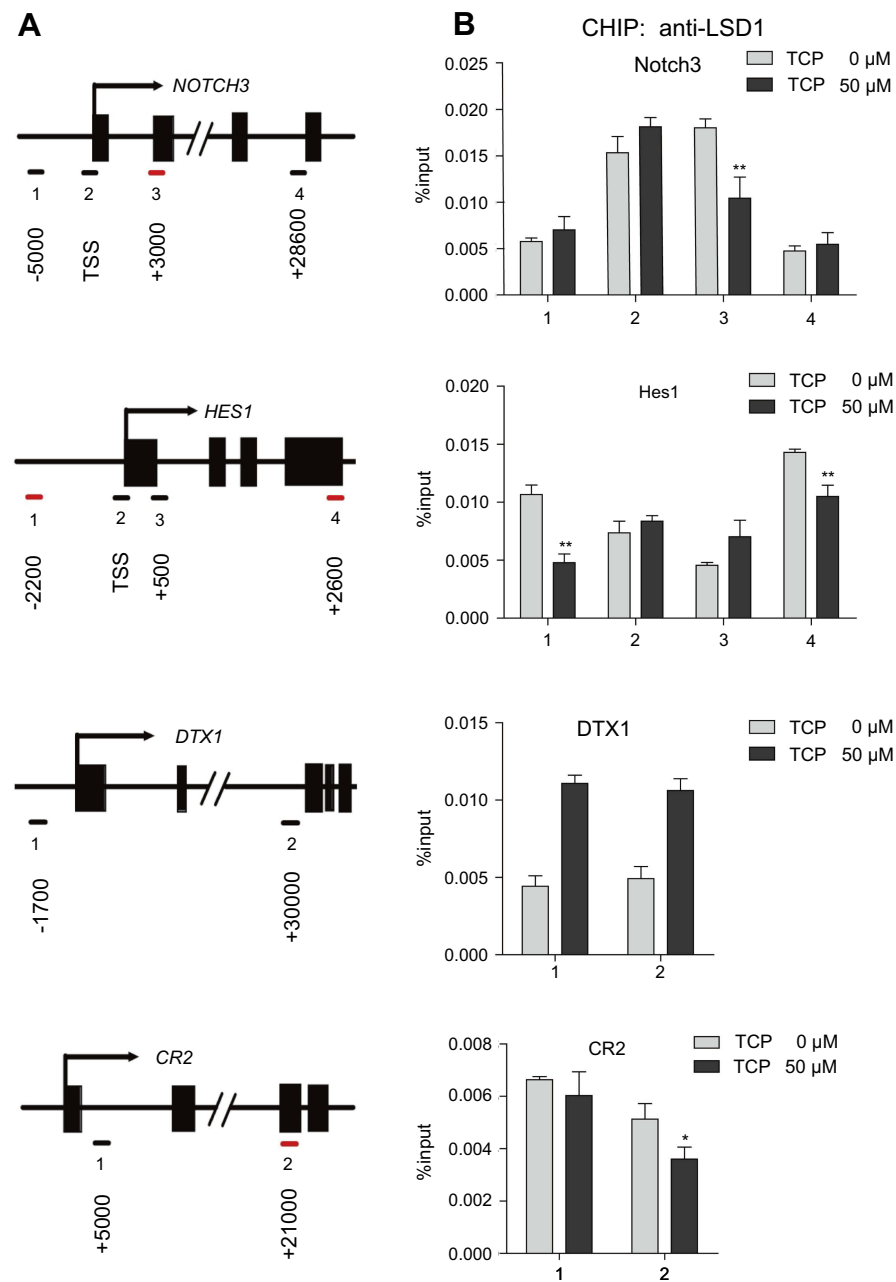
have also reported that LSD1 is overexpressed in ESCC specimens, suggesting that it is a potential therapeutic target for ESCC.<sup>5,29</sup> In this study, we also demonstrated

clear expression of LSD1 in ESCC cell lines, and found that its expression and function could be inhibited by TCP.



**Figure 4** Downregulation of LSD1 by TCP or shRNA decreased the expressions of related proteins in the PI3K/Akt/mTOR signaling pathway in ESCC cells. **(A)** ECa109 and EC9706 cells were treated with different doses of TCP (0, 10 and 50 μM) for 48 h, and total proteins were extracted for analyzing the expressions of PI3K, p-Akt (Ser473), Akt, p-mTOR (Ser2448), p-mTOR (Ser2481), mTOR and Rictor by Western blot. **(B)** Total proteins of ECa109 and EC9706 cells with control shRNA or LSD1 shRNA were extracted, and the expressions of LSD1, PI3K, p-Akt, Akt, p-mTOR (Ser2448), p-mTOR (Ser2481), mTOR and Rictor were investigated by Western blot. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  versus control group.





**Figure 5** LSD1 bound to the promoter regions of Notch target genes in ECa109 cells. The chromatin from ECa109 cells treated with TCP (50  $\mu$ M) for 48 h was subjected to ChIP analysis. **(A)** The binding of LSD1 to the promoter sequences of Notch target genes is illustrated (red dash). **(B)** Occupancy of LSD1 at the promoter regions of Notch target genes was shown as %input. \* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001 versus control group.

**Abbreviation:** TSS: transcription start site.

Studies have shown that the Notch pathway is aberrantly activated in several human cancers, including ESCC,<sup>30</sup> and LSD1 could negatively regulate the Notch signaling pathway by interacting with CoREST in the developing cerebral cortex or SIRT1 in neurogenesis;<sup>18,19</sup> but LSD1 was also reported to be an integral component of the Notch-activating complex in the nucleus.<sup>20</sup> Further, some reports proved that LSD1 could regulate the PI3K/Akt/mTOR

pathway by combining the promoter region of tumor suppressor PTEN<sup>31</sup> and inducing autophagy in ovarian cancer cells.<sup>23</sup> In this study, we showed that inhibition of LSD1 decreased the expressions of related protein, not only in the Notch pathway (such as Notch3, Notch1, DTX1 and Hes1), but also in the PI3K/Akt/mTOR pathway such as PI3K, p-Akt (Ser473), p-mTOR (Ser2448), p-mTOR (Ser2481) and Rictor in ESCC cells, which indicated that LSD1 might

have positively regulating effects on the Notch and PI3K/Akt/mTOR signaling pathways in ESCC. However, the molecular mechanism for this remains to be elucidated.

To explore the molecular mechanism of the regulation effects of LSD1 on the Notch and PI3K/Akt/mTOR pathways, we detected the combination of LSD1 with the promoter regions of Notch pathway targets Notch3, Hes1, DTX1 and CR2 by ChIP assay. The results clearly demonstrated combinations of LSD1 with the promoter regions of Notch3, Hes1 and CR2; and TCP prevented the combinations of LSD1 with them, indicating that LSD1 might positively regulate the Notch pathway through interacting with the promoter of Notch pathway target genes in ESCC. Many studies have demonstrated that the Notch pathway might act as upstream signaling to positively regulate the PI3K/Akt/mTOR signaling pathway, and inhibition of the Notch pathway by a  $\gamma$ -secretase inhibitor (GSI) decreased the activation of both Notch and Akt/mTOR pathways;<sup>32–35</sup> thus we speculated that the molecular mechanism of LSD1 might positively regulate the PI3K/Akt/mTOR pathway through regulating the Notch pathway. Further investigation is needed.

## Conclusion

Taken together, this study found early evidence that LSD1 might positively regulate the Notch and PI3K/Akt/mTOR signaling pathways in ESCC cells through combining to the promoter regions of Notch target genes. These findings might provide new insight into the effects of LSD1 and the Notch and mTOR pathways in the tumorigenesis of ESCC.

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## Disclosure

The authors report no conflicts of interest in this work.

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