

## USP16 Regulates the Stability and Function of LDL receptor by Deubiquitination

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### Summary

Low-density lipoprotein (LDL) particles are known to be atherogenic agents in coronary artery diseases. They adjust to other electronegative forms and can be the subject for the enhancement of inflammatory events in vessel subendothelial spaces. The LDL uptake is related to the membrane scavenger receptors, including LDL receptor (LDLR). The LDLR expression is closely associated with LDL uptake and occurrence of diseases, such as atherosclerotic cardiovascular diseases. Our findings identified USP16 as a novel regulator of LDLR due to its ability to prevent ubiquitylation-dependent LDLR degradation, further promoting the uptake of LDL. The enhancement of USP16-mediated deubiquitination and the suppressive degradation of the LDLR cause the presentation of a potential strategy to increase LDL cholesterol clearance.

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**Key words:** Degradation, Coronary artery diseases

Low-density lipoprotein (LDL) is a microscopic blob composed of an outer rim of lipoprotein and a cholesterol center.<sup>1)</sup> The oxidative modification hypothesis of atherosclerosis, which is related to oxidized LDLs (oxLDLs) and plays a crucial role in the initiation and progression of atherosclerosis, remains controversial.<sup>2,3)</sup> Atherosclerotic cardiovascular disease is the main cause of death among patients with diabetes mellitus and metabolic syndrome. One of the mechanisms involved in such an increased risk is the high incidence of lipoprotein modification in these pathologies. Increased glycosylation, oxidative stress, and high non-esterified fatty acid levels in the blood, among other factors, promote modification and subsequent alteration of properties of lipoproteins. Since LDL modification is a triggering factor in the development of atherosclerosis, quantification of modified LDLs in blood as biomarkers for cardiovascular risk has significantly gained scholars' attention.<sup>4)</sup>

One of the challenges in endocrinology is the prevention of high incidence of cardiovascular disease. Validation of new biomarkers that may contribute to a better evaluation of cardiovascular risk and enhance treatment strategies is of great clinical significance in the prevention and reduction of cardiovascular risk. LDL modification is a key element in the development of atherosclerotic lesions.<sup>5)</sup> Notably, cardiovascular risk is closely associated with a high level of LDL cholesterol, leading to lipid peroxidation and formation of foam cells in atheromatous

plaques.

There is increasing evidence that LDL cholesterol plays a role in the pathology of osteoarthritis.<sup>6)</sup> Additionally, oxLDL, which has been shown to play a pivotal role in the development of atherosclerosis, could be involved in synovial inflammation, cartilage destruction, and bone deformities.<sup>6,7)</sup> OxLDL can activate synovial cells, e.g., macrophages, endothelial cells, and synovial fibroblasts, causing the release of growth factors, matrix metalloproteinase, and pro-inflammatory cytokines.<sup>8)</sup> It has been reported that lectin-like oxidized LDL receptor-1 is a pattern recognition receptor that plays a critical role in vascular diseases and host immune responses.<sup>9)</sup>

The existence of the LDL receptor (LDLR) was formally demonstrated in 1974.<sup>10)</sup> Furthermore, LDLR was found as a genetic cause of heart attacks, which led to the appearance of a new insight for cholesterol metabolism. The discovery of LDLR also introduced three general concepts to cell biology: receptor-mediated endocytosis, receptor recycling, and feedback regulation of receptors. The latter concept provides the mechanism, in which statins selectively lower plasma LDL, reduce heart attacks, and prolong human's life.<sup>11)</sup>

Recently, the ubiquitin-proteasome system has been recognized as a novel regulator in the LDLR pathway through ubiquitylation-dependent degradation of the LDLR mediated by the E3-ubiquitin ligase inducible degrader of the LDLR (IDOL).<sup>12)</sup> Simultaneously, ubiquitin-

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specific protease (USP) 2, one of the deubiquitinating enzyme (DUB), regulates the LDLR pathway by counteracting the E3-ubiquitin ligase IDOL.<sup>13-16)</sup> However, regulation of deubiquitylases in cholesterol metabolism has remained elusive. The widespread pathogenicity of LDL is urgently required for a better understanding of this mechanism, as well as exploring new therapeutic approaches.

In DUB family, USP and ubiquitin C-terminal hydrolase enzymes are well characterized. USP proteins have the largest family with more than 60 members. It has been reported that USP2 can regulate the deubiquitination of LDLR.<sup>13)</sup> However, the regulation of LDLR by other members of the USP family has remained obscure. We attempted to find out a new regulation mechanism for LDLR ubiquitination by siRNA screening and found that USP16, as a novel regulator of LDLR, can prevent ubiquitylation-dependent degradation of the LDLR, further promoting the uptake of LDL. It is therefore helpful to study the mechanism of LDL uptake and provide data to conduct further investigation.

## Methods

**Cell culture and treatments:** Human cervical cancer (Hela) cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in a medium containing 10% fetal bovine serum (HyClone Laboratories Inc., Logan, UT, USA) and 1% pen/strep (Invitrogen, Carlsbad, CA, USA) at 37°C in the presence of 5% CO<sub>2</sub>. Media containing cells were passaged every 2-3 days. Four independent series of treatments were conducted to obtain the two technical repeats that were used for all studies.

**siRNA-knockdown of USPs and overexpression plasmids transfection:** In the present research, siRNAs targeting USP16 (Table I) were transfected to Hela cells using Lipofectamine 2000 Reagent (11668019; Life Technologies Inc., Carlsbad, CA, USA) according to the manufacturer's instructions.

The LDLR was cloned into pLVX-IRES vectors with Myc-tag, and USP16 was cloned into pLVX-IRES vectors with HA-tag. The Hela cells were infected with LDLR-Myc lentivirus and screened with purinomycin.

**Immunofluorescence microscopy:** Hela cells were seeded into 24-well plates at 1 day before the experi-

ments. Cells were fixed with 4% paraformaldehyde at 4°C for 60 minutes and then extracted with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 10 minutes. Nuclei were visualized by staining with DyLight 594. Images were acquired using an Olympus FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan).

**Co-immunoprecipitation (Co-IP) assay:** Cells were lysed in NP-40 lysis buffer supplemented with 1 µg/mL leupeptin, 1 µg/mL pepstatin, and 1 µg/mL phenylmethanesulfonyl fluoride. The lysates were then centrifuged and incubated with anti-LDLR (1:50, ab52818; Abcam, Cambridge, UK), anti-USP16 (1:50, ab121650; Abcam, Cambridge, UK), or IgG (as a negative control, 1:1000, ab172730; Abcam, Cambridge, UK) at 4°C overnight. Then, the protein-antibody complex was incubated with protein A/G magnetic beads for 5 hours at 4°C. The Co-IP product was then collected by centrifugation at 1000 × g for 5 minutes at 4°C, and the beads complex were washed four times with PBS. After final washing, protein A/G magnetic beads were eluted by boiling in 5× concentration of sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer before analyzed with Western blotting.

**Western blot analysis:** Protein extracts were prepared by lysis in a buffer containing 50 mM Tris-HCl (pH 7.4), 0.1% Triton X-100, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, and protease inhibitors (Roche, Basel, Switzerland). Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Blots were probed with the following antibodies: anti-human LDLR rabbit polyclonal (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-Myc mouse monoclonal (Invitrogen, Carlsbad, CA, USA), anti-Apoe (Abcam, Cambridge, UK), anti-USP16 (Abcam, Cambridge, UK), anti-TfR (Abcam, Cambridge, UK), and mouse monoclonal anti-β-actin antibody (Cell Signaling Technology, Inc., Danvers, MA, USA).

**RNA extraction and quantitative reverse transcription-polymerase chain reaction (RT-qPCR):** RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA was synthesized using Invitrogen SuperScript III Reverse Transcriptase kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Besides, SYBR Green-Based RT-qPCR was conducted using Mx3000P qPCR System (Stratagene California, San Diego, CA, USA) according to the manufacturer's instructions. Four independent amplifications were conducted for each sample in each technique. Additionally, β-actin was considered as an internal reference. The primer sequences used for RT-qPCR are shown in Table II.

**Cycloheximide (CHX) degradation experiment:** LDLR stably transferred cell lines were secondly transfected with

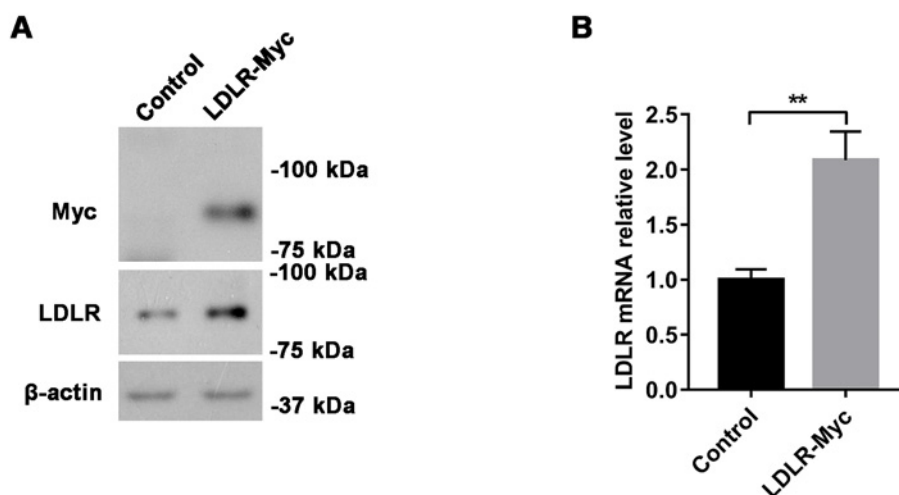
**Table I.** USP16 siRNA Sequence

Name	Sequence
USP16 siRNA1	CCAAGACTGTAAGACTGACAATAAA
USP16 siRNA2	AGCCTTCAGTTTGGCTGTGTCTTAA
USP2 siRNA	CGGGAAGAGACGGCATGAATTCTAA

**Table II.** Primer Sequences Used for RT-qPCR

Gene	Forward	Reverse
LDLR	AACCAGGACGGCTACAGCTA	ACTCCAGGCAGATGTTACAG
β-actin	AGCGAGCATCCCCAAAGTT	GGGCACGAAGGCTCATCATT

RT-qPCR indicates RNA extraction and quantitative reverse transcription polymerase chain reaction, and LDLR, low-density lipoprotein receptor.



**Figure 1.** Construction of LDLR-Myc stable cell lines and identification of their expression. **A:** HeLa cells were transfected with the indicated expression for LDL-Myc, or control, and cell lysates were immunoblotted as indicated. The HeLa cell lines were infected with LDLR-Myc lentivirus, screened with puromycin, and harvested for Western blot analysis. **B:** RT-qPCR was used to identify mRNA levels of LDLR in LDLR-Myc overexpressing HeLa cell lines. The experiments were repeated for four times. Non-parametric *t*-test, \**P* < 0.05, \*\**P* < 0.01. HA indicates hemagglutinin; LDLR, low-density lipoprotein receptor; mRNA, messenger ribonucleic acid; RT-qPCR, reverse transcription-polymerase chain reaction; and USP, ubiquitin-specific protease.

control siRNA or USP16 siRNA, respectively. After 48 hours, CHX was added at different time points as indicated, and then, cells were harvested, and the expression of LDLR was detected by Western blot analysis and quantification analysis of gray scanning.

**LDL Uptake Assay:** HeLa cells were incubated with a DyLight 594-conjugated secondary antibody (#46402; Thermo Fisher Scientific, Waltham, MA, USA) for 1 hour followed by overnight dialysis against PBS and stored at 4°C in the dark. HeLa cells were incubated in a fresh sterol-depletion medium for 16 hours before addition of LDL to increase LDLR abundance. To initiate LDL uptake, cells were incubated with 5 µg/mL DyLight 594-labeled LDL in Dulbecco's modified Eagle's medium supplemented with 0.5% bovine serum albumin at 37°C. Subsequently, cells were washed twice with PBS supplemented with excess sterols (PBS with 10% fetal calf serum), followed by additional washing with PBS. Endocytosis of LDL was conducted by lysing cells in radioimmunoprecipitation assay buffer, and quantification of the fluorescence signals was conducted using ImageJ software. Relative LDL uptake was calculated by measuring fluorescence values corrected for specific uptake and presented as mean ± standard deviation (SD).

**Statistical analysis:** All data were presented as mean ± SD. These data were evaluated by one-way analysis of variance (ANOVA) test using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). *P* < 0.05 was considered statistically significant.

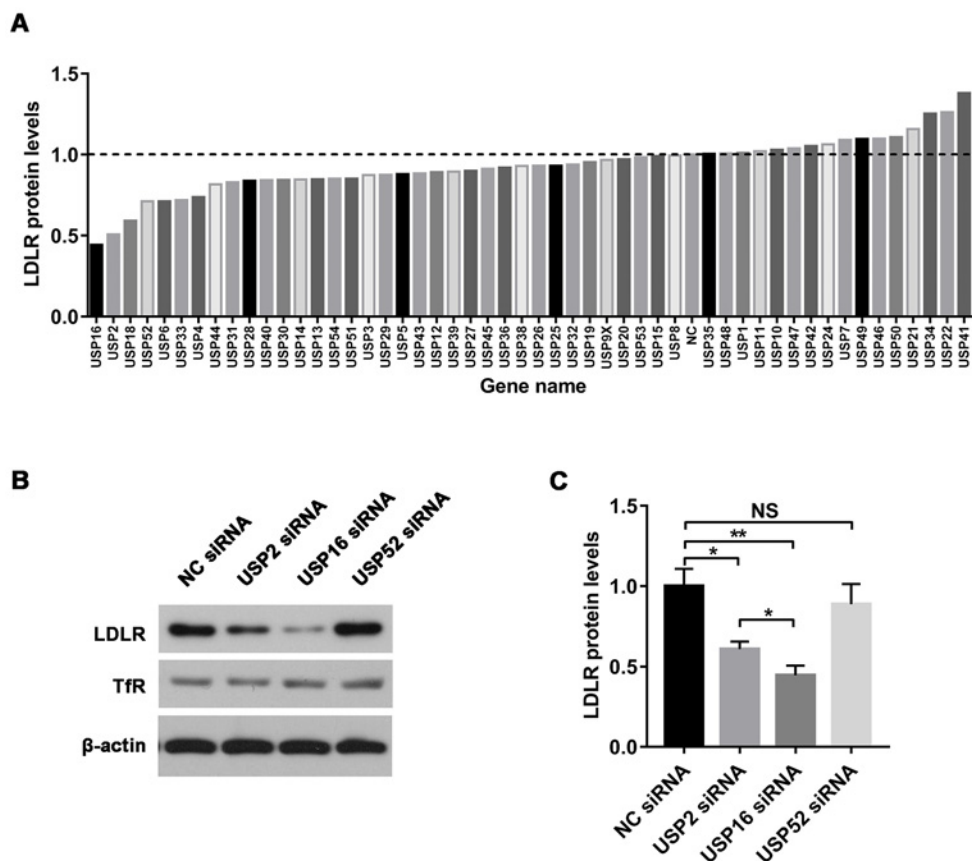
## Results

**Construction of overexpressed LDLR in HeLa cell lines:** First, we attempted to investigate the regulation of LDLR

in HeLa cells. In order to investigate the biological function of LDLR and regulatory mechanism of LDL uptake by LDLR in HeLa cells, the overexpression of LDLR in HeLa cell lines was constructed. Furthermore, the overexpression of LDLR was successfully verified in both protein and mRNA expression levels (Figure 1A, B).

**USP16 increases the stability of LDLR:** To find out potential DUBs that increased LDLR stability, we have screened a USP cDNA expression library. In the present study, through the USP siRNA library screening, it was found that the USP16, USP2, USP18, and USP52 had the greatest effect on the level of LDLR protein (Figure 2A), which was consistent with findings of a previous research,<sup>13</sup> and we also found that the cell viability was decreased in the group of USP18 siRNA (data not are shown). In the repeated check experiments, we found the same changes in USP16 and USP2 siRNA, but there is no statistical change in LDLR protein level after USP52 interference (Figure 2B, C).

To date, remarkably, the regulation of LDLR by USP 16 has not been reported. Therefore, we further verified the regulation of LDLR protein by USP16 and compared it with USP2, which was noted to have significant regulation of LDLR, and USP52 with no noticeable regulation of LDLR, as detected in the present study (Figure 2B, C). These results revealed that USP2 could significantly regulate LDLR protein, which was consistent with the outcomes of a previous research.<sup>13</sup> There was no remarkable influence on LDLR after USP52 interference (Figure 2A-C). By contrast, USP16 markedly regulated LDLR protein levels compared with USP2. Collectively, the above-mentioned data demonstrated that through siRNA library screening, USP16 has the most significant effect on LDLR.



**Figure 2.** The expression of LDLR was down-regulated after USP16 interference. **A:** USP siRNA library screening. The siRNAs of USP family were transfected into LDLR-Myc overexpressing Hela cell lines, and the abundance of LDLR was detected by Western blotting. The influence of different USP siRNAs on abundance of LDLR was calculated with  $\beta$ -actin as the internal reference. **B:** Western blot analysis was used to verify the abundance of LDLR after USP2, USP16, and USP52 siRNAs. Transferrin (TfR) was detected as negative control. **C:** The relative abundance of LDLR was quantified by gray scanning after USP2, USP16, and USP52 siRNAs. The experiments were repeated for four times. One-way ANOVA test, \* $P < 0.05$ , \*\* $P < 0.01$ . LDLR indicates low-density lipoprotein receptor; NS, not significant; TfR, transferrin receptor; and USP, ubiquitin-specific protease.

**USP16 positively regulates LDLR via post-translational modification:** In the present study, we attempted to explore how USP16 can regulate the abundance of LDLR. First, we constructed an overexpressed USP16 in Hela cell lines, and the mRNA expression of LDLR was detected. Compared with the control group and the overexpressed LDLR group, the results showed that the overexpressed USP16 did not influence mRNA levels of LDLR (Figure 3A). Furthermore, we noted that the mRNA level of LDLR was not down-regulated after USP16 interference (Figure 3B).

These results demonstrated that regulation of LDLR protein by USP16 was independent of transcriptional regulation while may be associated with post-transcriptional regulation.

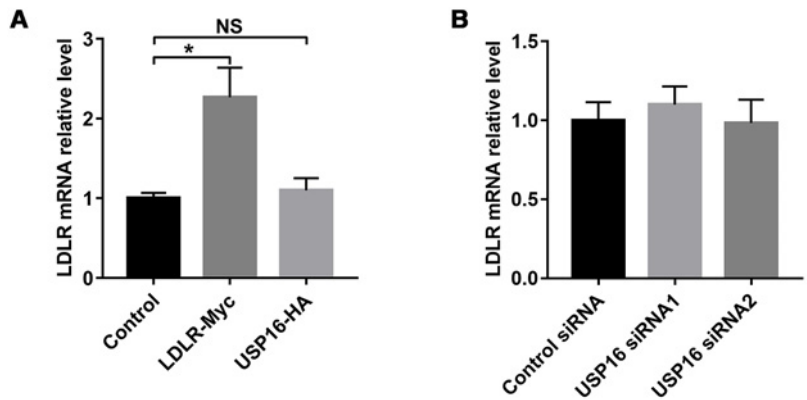
**USP16 stabilizes LDLR through deubiquitination:** In order to investigate the up-regulatory mechanism of USP16 on LDLR protein, we co-transfected Myc-LDLR and HA-USP16 to Hela cells and conducted co-IP assay. The results showed that LDLR, but not IDOL, was co-immunoprecipitated by USP16 (Figure 4A, B), reflecting

an interaction between USP16 and LDLR.

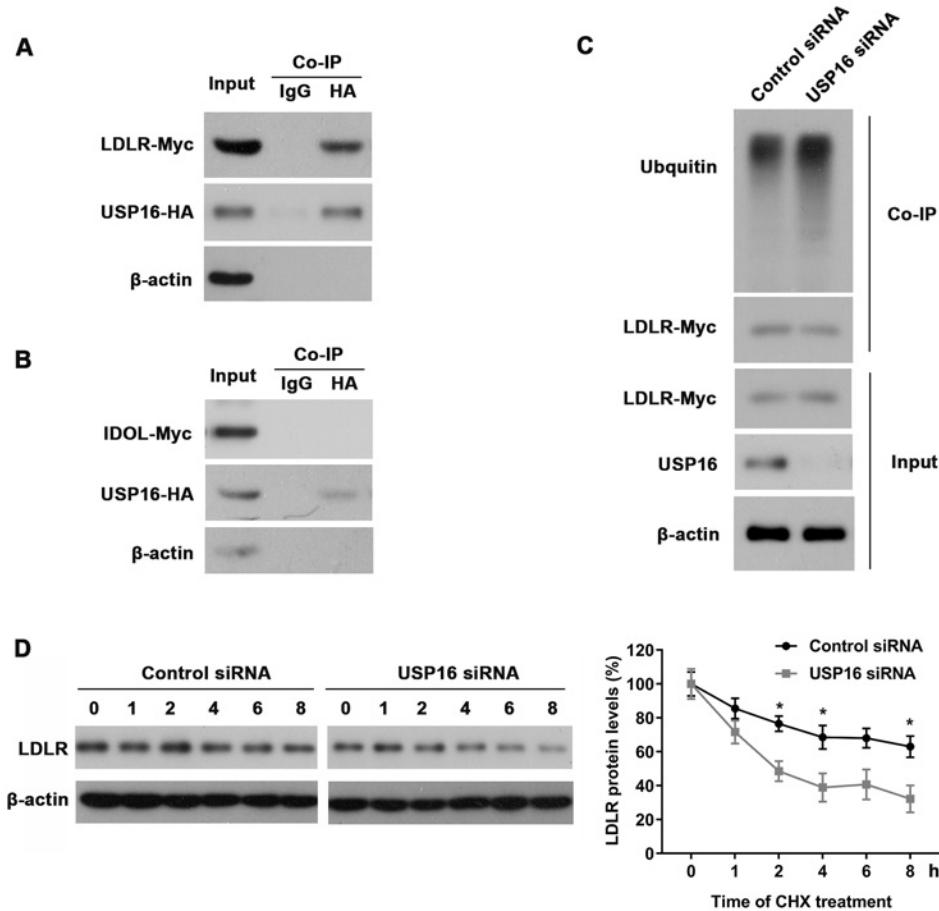
Since USP16 is a DUB, we attempted to indicate whether USP16 can regulate the LDLR protein level by its deubiquitinase activity. The ubiquitin of LDLR was detected, and it was noted that the ubiquitin level of LDLR was significantly higher with USP16 interference (Figure 4C). The above-mentioned result suggests that USP16 regulates the stability of LDLR protein through the ubiquitin-proteasome pathway.

In order to enlarge the differences and eliminate the fake positive results, the cells were treated with CHX at different time points before cultivation (Figure 4D). Compared with the control group, when the synthesis of protein was inhibited, Western blot assay revealed that USP16 interference resulted in a remarkable decrease of LDLR protein in Hela cells (Figure 4D), indicating that USP16 significantly increased stability of LDLR.

Taken together, these results were consistent with the notion that USP16 removes ubiquitin from LDLR to prevent its degradation, and it was demonstrated that USP16 regulated the protein stability of LDLR via the deubiquiti-

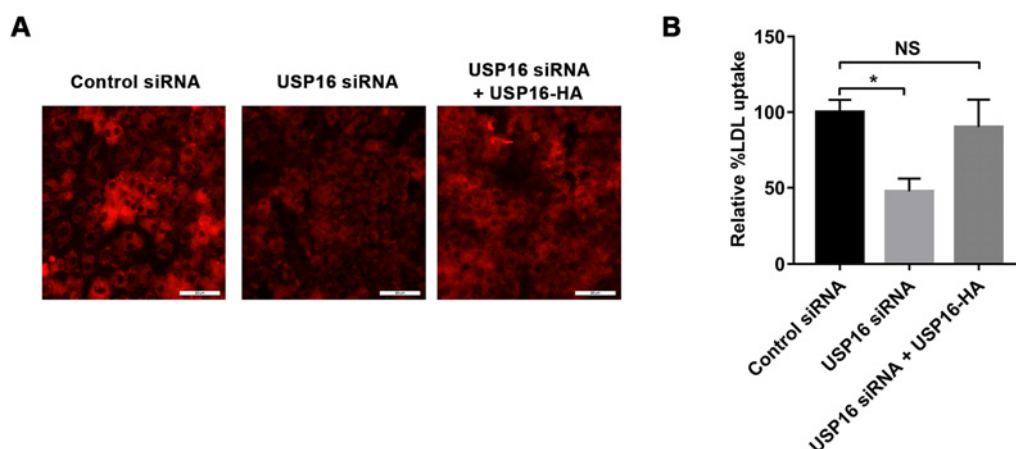


**Figure 3.** USP16 had no significant effect on the LDLR mRNA expression. **A:** RT-qPCR was used to detect the mRNA levels of LDLR after USP16 overexpression. **B:** RT-qPCR was used to detect the mRNA levels of LDLR after USP16 siRNA. SiRNA1 and siRNA2 indicated two distinct USP16 siRNAs. The experiments were repeated for four times. One-way ANOVA test,  $*P < 0.05$ . HA indicates hemagglutinin; LDLR, low-density lipoprotein receptor; mRNA, messenger ribonucleic acid; NS, not significant; RT-qPCR, reverse transcription-polymerase chain reaction; and USP, ubiquitin-specific protease.



**Figure 4.** USP16 deubiquitylates inducible degrader of the LDLR and increases its stability. **A:** Immunoprecipitation (Co-IP) was used to study the interaction between USP16 and LDLR in Hela cells. **B:** Immunoprecipitation (Co-IP) was used to study the interaction between USP16 and IDOL in Hela cells. **C:** Western blot analysis was used to detect the effect of USP16 on LDLR levels. **D:** CHX degradation experiment was conducted to study the effect of USP16 on LDLR degradation. The experiments were repeated for four times. One-way ANOVA test,  $*P < 0.05$ . CHX indicates cycloheximide; HA, hemagglutinin; LDLR, low-density lipoprotein receptor; siRNA, small interfering RNA; IDOL, inducible degrader of the LDLR; and USP, ubiquitin-specific protease.





**Figure 5.** USP16 promotes the uptake of LDL. **A:** Immunofluorescence staining of DyLight 594 labeled LDL in different treatment groups. **B:** Quantification of fluorescence intensity is shown in **A**. The experiments were repeated for four times. One-way ANOVA test, \* $P < 0.05$ . HA indicates hemagglutinin; LDL, low-density lipoprotein; NS, not significant; siRNA, small interfering RNA; and USP, ubiquitin-specific protease.

nation manner.

**LDLR regulates the metabolism and uptake of LDL depending on USP16:** A number of scholars reported that LDLR is a major determinant of circulating levels of LDL.<sup>16-18)</sup> The above-mentioned studies demonstrated that USP16 can prevent LDLR degradation. Hence, we further explored the association between USP16 and LDL uptake. The uptake of LDL was detected by immunofluorescence staining, and the results showed that the uptake of LDL was significantly reduced with USP16 interference (Figure 5A, B). Conversely, there was no noticeable influence on LDL uptake even with USP16 interference in LDLR over-expressing Hela cells (Figure 5A, B). These results indicated that USP16 regulates the uptake of LDL through promoting the deubiquitination of LDLR. In summary, these data highlighted the pivotal role of DUB USP16 in the regulation of LDLR.

## Discussion

The LDLR family is composed of a class of single transmembrane glycoproteins and generally recognized as cell surface endocytic receptors, internalizing extracellular ligands for degradation by lysosomes.<sup>17)</sup> The LDLR provides the main entry portal for LDL into the cell, and the mechanisms characterizing its transcriptional regulation and endocytic network have been largely described.<sup>18,19)</sup>

A variety of malignant tumors express high levels of LDLRs on their membranes,<sup>20-22)</sup> including cervical cancer.<sup>23)</sup> Therefore, LDL may be used as a carrier to obtain selective delivery of antineoplastic drugs to tumors. Although the LDL uptake by the liver, spleen, and adrenals is higher than that by tumors, the LDLR-mediated uptake by these organs may be selectively down-regulated via methods that do not affect the expression of LDLRs on tumor cells.<sup>20)</sup>

The role of LDLR related protein as a tumor promoter in cervical cancer has been reported.<sup>24)</sup> The utilization of LDL to deliver cytotoxic drugs to tumor cells has been studied since the 1980s, when several cancer cell

lines were found to have higher LDLR activity than normal cells. Such differential uptake between tumor cells and normal cells may provide a special opportunity to use LDL as a tumor-specific carrier for the clinical treatment of cancer cells. Human cervical tumor cell lines, such as Hela and C-33A, were used to examine the cellular uptake of the [(125)I] DPIP-LDL conjugate. It was shown that the [(125)I] DPIP-LDL conjugate was specifically bound to and taken up by cervical tumor cells through an LDLR-mediated endocytosis pathway. The results indicated that LDL may be a potential carrier for delivery of hydrophobic radiopharmaceuticals to cancer cells and particularly for the diagnosis of cervical tumors.<sup>25)</sup>

Using a siRNA screening approach, in the present research, we identified USP16 as a post-transcriptional regulator of LDLR degradation. Our results revealed that USP16 could inhibit the ubiquitination of LDLR, promoting the uptake of LDL in human cervical tumor cell lines. However, further researches need to be conducted to enhance the expression of USP16, besides decreasing the LDL in the human body.

In summary, the present study identified deubiquitylase USP16 as a novel regulator of LDL uptake due to its ability to inhibit LDLR degradation. This finding can demonstrate that maintenance of USP16 activity may cause increased LDLR abundance and enhanced LDL uptake.

## Disclosure

**Conflicts of interest:** None.

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