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ORIGINAL ARTICLE

Crosstalk of lysyl oxidase-like 1 and lysyl oxidase prolongs their half-lives and regulates liver fibrosis through Notch signal

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Abstract

Background: Lysyl oxidase (LOX) family members (LOX and LOXL1 to 4) are crucial copper-dependent enzymes responsible for cross-linking collagen and elastin. Previous studies have revealed that LOX and LOXL1 are the most dramatically dysregulated LOX isoforms during liver fibrosis. However, the crosstalk between them and the underlying mechanisms involved in the profibrotic behaviors of HSCs, as well as the progression of liver fibrosis, remain unclear.

Methods: pCol9GFP-HS4,5^{Tg} mice, *Loxl1*$^{fl/fl}$*Gfap*^{Cre} mice, human HSC line, and primary HSCs were enrolled to study the dysregulation pattern, profibrotic roles, and the potential mechanisms of LOX and LOXL1 interaction involved in the myofibroblast-like transition of HSCs and liver fibrogenesis.

Results: LOX and LOXL1 were synergistically upregulated during liver fibrogenesis, irrespective of etiology, together orchestrating the profibrotic behaviors of HSCs. LOX and LOXL1 coregulated in HSCs, whereas LOXL1 dominated in the coregulation loop. Interestingly, the interaction between LOXL1 and LOX prolonged their half-lives, specifically enhancing the Notch signal-mediated myofibroblast-like transition of HSCs. Selective disruption of *Loxl1* in *Gfap*$^+$ HSCs deactivated the Notch signal, inhibited HSC activation, and relieved carbon tetrachloride-induced liver fibrosis.

Conclusions: Our current study confirmed the synergistic roles and the underlying mechanisms of LOXL1 and LOX crosstalk in the profibrotic behaviors of HSCs and liver fibrosis progression, providing experimental

Abbreviations: CCl4, carbon tetrachloride; CHX, cycloheximide; ECM, extracellular matrix; Gfap, glial fibrillary acidic protein; GFP, green fluorescent protein; HES1, hes family bHLH transcription factor 1; LOX, lysyl oxidase; LOXL1, lysyl oxidase-like 1; rhTGF-β1, recombinant human transforming growth factor β1.

Ning Zhang and Aiting Yang are co-first authors.

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evidence for further clear mechanism-based anti-LOXL1 strategy development in the therapy of liver fibrosis.

INTRODUCTION

Excessive deposition of extracellular matrix (ECM) components is the prime hallmark of liver fibrosis. There are 10-fold increased collagenous and noncollagenous ECM components in cirrhotic over normal livers.[1] Stabilizing these ECM components through cross-linking contributes to liver fibrosis progression and the prevention of matrix metalloproteinases-induced proteolytic degradation.[2,3] In decades years, lysyl oxidase family members (lysyl oxidase [LOX] and lysyl oxidase-like 1 [LOXL1] to 4) have been well recognized as extracellular copper–dependent enzymes responsible for catalyzing the cross-linking of collagens and elastin.[2] Among them, LOX, LOXL1, and LOXL2 have been reported to be pathologically upregulated in fibrotic livers.[2,4–7] Inhibition of any of their expressions or activities in experimental mouse models disrupts ECM stabilization during liver fibrosis progression and accelerates spontaneous reversal.[4–7] However, several clinical trials of a monoclonal antibody against LOXL2 have shown no clinical benefits in liver fibrosis treatment,[8–10] dampening the enthusiasm for LOX family member targeting in the field of liver fibrosis therapy.

Because LOX isoforms have the potential to interact with each other,[2] the compensatory effects cannot be ignored when a single LOX family member is selected as a therapeutic target. Recent studies have investigated the mutual regulations among the LOX family members in physiological or pathological lungs; there exist direct or indirect regulatory relationships among LOX members in lung fibrosis but the conclusions are inconsistent.[11–13] Up to now, the interacting relationships among LOX members in fibrotic liver are still less clear. During liver fibrogenesis, HSCs-derived LOX and LOXL1 rather than others are more critical since their expressions are markedly upregulated than others.[2,14] In addition, LOX and LOXL1 possess propeptides in the N-terminal regions, which drastically differs from other LOX family members.[2] Therefore, the crosstalk or perturbation within LOX isoforms in liver fibrosis, especially between LOX and LOXL1, deserves to be elucidated.

Previous studies have demonstrated LOX usually cooperates with LOXL1, assisting extracellular tropoelastin cross-linking by deaminating their lysine residues under the guidance of fibulin-4 and fibulin-5, respectively.[15,16] Fibulin-4 binds to LOX pro-region through its N-terminal region, then directs LOX to tropoelastin; the C-terminal domain of fibulin-5 recognizes and activates LOXL1 after tethering it to elastic fibers.[15,16] Except for the canonical role in catalyzing covalent cross-linking of ECM structural proteins, LOX and LOXL1 exhibit noncanonical biological roles. By comparison, in silico analysis shows most of the interactive partners of LOX belong to the matrisome, while partners of LOXL1 are mostly intracellular.[17] Indeed, specific knockout of *Loxl1* in HSCs inhibits focal adhesion kinase/phosphoinositide 3-kinase/protein kinase B/hypoxia-inducible factor 1-alpha signals in carbon tetrachloride (CCl_4)-treated mice,[6] and reverses the metabolic disorder in the choline-deficient L-amino acid defined diet-fed mice.[7] These findings strengthen the noncanonical role of LOXL1 involved in metabolism or signal transduction.

Our current study aims to shed light on the crosstalk or perturbation between LOX and LOXL1 in HSCs and explore their underlying effects and mechanisms in liver fibrogenesis, providing a rationale for developing therapeutic opportunities.

METHODS

Mice

pCol9GFP-HS4,5^{Tg} mice were donated by Dr David A. Brenner (University of California San Diego). The pCol9GFP transgene includes the collagen gene promoter (−3122 to +111) linked to the green fluorescent protein (GFP) reporter gene, whereas the pCol9GFP-HS4,5 transgene contains deoxyribonuclease I-hypersensitive sites 4,5 located upstream of the collagen promoter in pCol9GFP.[18] *Loxl1*$^{fl/fl}$ mice were obtained from Shanghai Model Organisms Center, Inc (NM-CKO-2118121). The *Loxl1*^{loxP} allele was created by inserting *loxP* sites flanking exon 2 of *Loxl1.* Floxed mice were bred with glial fibrillary acidic protein (*Gfap*).*Cre mice* (JAX024098, Jackson Laboratories) to generate HSC-specific *Loxl1* knockout (*Loxl1*$^{\Delta HSC}$) mice. Floxed (*Loxl1*$^{fl/fl}$) mice were used as control. Sprague-Dawley rats were commercially purchased from Beijing HFK Bioscience Co., Ltd. All rodents were housed and bred in a specific pathogen-free grade animal facility with appropriate temperature (23 ± 2°C), 12-hour light-dark cycle, and standard chow and water ad libitum. The Animal Care and Use Committee of Beijing Friendship Hospital, Capital Medical University, approved the rodent-related studies.

Statistical analysis

Data are expressed as mean ± SD. Differences between 2 groups were compared using the Student *t* test or Mann-Whitney test; differences among 3 or more groups were determined by a one-way ANOVA followed by the least significant difference test or Kruskal-Wallis test. A $p < 0.05$ was considered statistically significant.

Additional methodological details

For further information on animal models and other materials and methods, please refer to Supplemental Information, http://links.lww.com/HC9/A817. Antibodies, reagents, and full-length immunoblots for all the related figures are also included in the Supplemental Information, http://links.lww.com/HC9/A817.

RESULTS

LOX and LOXL1 were synergistically elevated during liver fibrogenesis regardless of etiology

Pathological upregulation of LOX and LOXL1 transcripts has been observed in CCl_4-induced mouse models.[4,14] Herein, to ask whether the upregulation pattern of LOX and LOXL1 in liver fibrosis was independent of etiological factors, we first analyzed the publicly available gene expression profiles from the Gene Expression Omnibus database. As shown in Figure 1A, both *LOX* and *LOXL1* transcripts were significantly exacerbated in human fibrotic livers with metabolic disorder (GSE49541), chronic HCV (GSE14323), or HBV (GSE84044) infection compared to nonfibrotic livers; besides, *LOX* and *LOXL1* gene expression depended on histological severity in HBV-related liver fibrosis. Moreover, the expression of *LOXL1* transcript was highly correlated with *LOX* in fibrotic or nonfibrotic patients from GSE49541 ($r = 0.672$, $p < 0.01$), GSE14323 ($r = 0.525$, $p < 0.01$), and GSE84044 ($r = 0.631$, $p < 0.01$), respectively (Supplemental Figure S1, http://links.lww.com/HC9/A817).

We next established chemical damage and cholestasis-associated liver fibrosis models to affirm their protein expression pattern. As shown in Figure 1B, CCl_4 intoxication for 12 weeks notably increased the protein expression of LOX and LOXL1 (fold change = 2.3 and 5.5, $p < 0.01$) along with αSMA in mouse livers; bile duct ligation operation also upregulated both LOX and LOXL1 protein levels, especially in the advanced stage of fibrosis. LOX and LOX1 proteins were mainly recruited in the septal and portal areas, colocalizing with αSMA and GFP-labeled collagen I in 12-week CCl_4-treated pCol9GFP-HS4,5^{Tg} mouse livers (Figure 1C). These results suggest that LOX and LOXL1 are mainly upregulated in activated HSCs and exhibit comparable expression and location patterns in fibrotic livers, independent of any specific pathogenic factor.

LOX and LOXL1 synchronously orchestrated the profibrotic behaviors of HSCs

As HSCs are the primary source of LOX and LOXL1 dysregulation, we tested their canonical covalent cross-linking roles through *LOX* or *LOXL1* intervention in HSCs. As shown in Figure 2A, *LOX* or *LOXL1* transfection in HSCs accelerated the accumulation of extracellular collagen fibers and the aggregation of tropoelastin monomers into larger insoluble elastin globules, whereas the knockdown of *LOX* or *LOXL1* counteracted recombinant human TGF-β1 (rhTGF-β1)-stimulated deposition of collagen fibers and the formation of elastin globules (Figure 2A).

We next evaluated and compared the noncanonical effects of *LOX* or *LOXL1* intervention on HSCs myofibroblast-like characteristics. As shown in Figures 2B–K, the overexpression of *LOX* or *LOXL1* significantly provoked the activation, proliferation, migration, and contraction of HSCs; oppositely, the silence of either of them counteracted the activated, proliferative, migratory, and contractile properties of HSCs under rhTGF-β1 stimulation. Relatively, the *LOXL1* intervention had observably more substantial effects on profibrotic behaviors of HSCs over the *LOX* intervention (Figures 2B–K). Taken together, except for the extracellular canonical roles, LOX and LOXL1 are also synchronously involved in orchestrating the profibrotic behaviors of HSCs.

LOXL1 dominated in LOX-LOXL1 coregulation in HSCs

As aforementioned, LOX and LOXL1 are synergistically upregulated during liver fibrogenesis and synchronously involved in the profibrotic behaviors of HSCs, highlighting their potent pathogenic roles in liver fibrogenesis. To inquire whether there is a compensatory effect on mutual regulation when either of them is selected as a therapeutic target, we tested the reciprocal regulation across LOX and LOXL1 in HSCs. The overexpression of *LOX* in HSCs only induced a slight increase in LOXL1 protein expression, while the overexpression of *LOXL1* evoked a marked upregulation of LOX protein (Figure 3A). The coregulation relationship was also verified in HEK293 cells (Supplemental Figure S2, http://links.lww.com/HC9/A817). Moreover, confocal immunofluorescence further confirmed that the overexpression of *LOX* in HSCs had

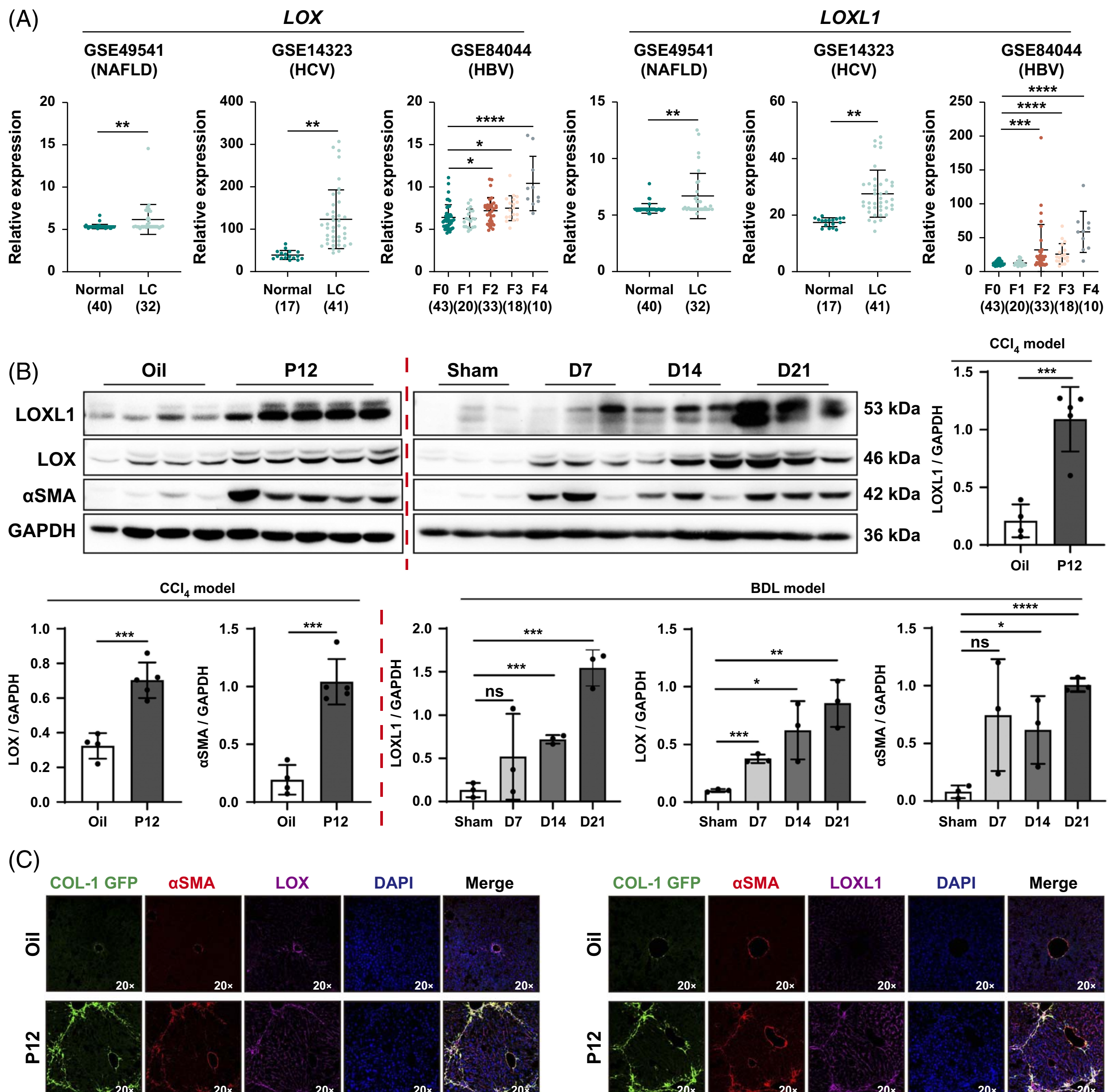

FIGURE 1 LOX and LOXL1 were synergistically elevated during liver fibrogenesis regardless of etiology. (A) *LOX* or *LOXL1* gene expression in human nonfibrotic and fibrotic livers with metabolic disorder (GSE49541), chronic HCV infection (GSE14323), or HBV infection (GSE84044) from GEO database. The sample size in each group was marked below the bar. (B) Immunoblotting analyses of LOX, LOXL1, and αSMA protein expression in chemical damage and cholestasis-associated liver fibrosis models. Relative integrated density was adjusted by GAPDH protein. N = 3–5 for each group. (C) Immunofluorescent staining of LOX, LOXL1, and αSMA protein in olive oil (Oil) and CCl_4 (P12)-treated mice. Images were acquired with a 20× objective. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns represents not significant. Abbreviations: αSMA, α-smooth muscle actin; D7, 7 days; D14, 14 days; D21, 21 days; BDL, bile duct ligation; CCl_4, carbon tetrachloride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEO, Gene Expression Omnibus; LC, liver cirrhosis; LOX, lysyl oxidase; NAFLD, nonalcoholic fatty liver disease; Oil, olive oil; P12, CCl_4 treatment for 12 weeks; Sham, sham operation.

a negligible effect on intracellular LOXL1 protein upregulation. In contrast, the overexpression of *LOXL1* in HSCs markedly enhanced the expression of intracellular LOX protein (Figure 3B and Supplemental Figure S3, http://links.lww.com/HC9/A817). On the other hand, under the stimulation of rhTGF-β1, a 3.2-fold inhibition of LOX protein expression in HSCs caused a 1.8-fold decrease in LOXL1 protein expression. Still, a 3.0-fold silence of LOXL1 protein expression resulted in a 3.0-fold downregulation of LOX protein expression (Figure 3C).

To ask if the coregulation happens at the transcriptional or translational level, we also determined the mRNA expression of *LOX* or *LOXL1* after inhibiting either of them. As shown in Figure 3D, *LOX* knockdown in HSCs did not affect *LOXL1* mRNA expression and

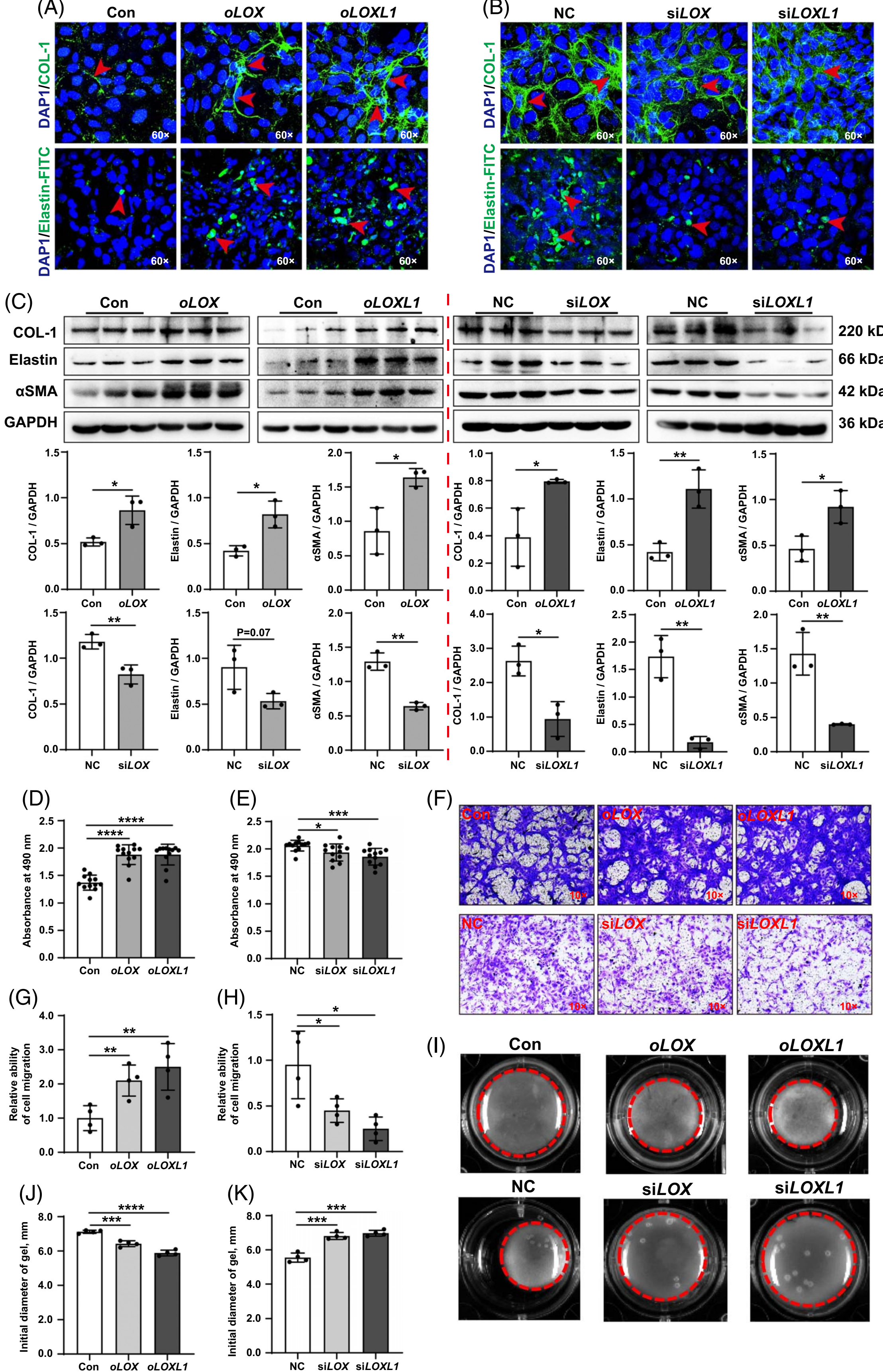

FIGURE 2 LOX and LOXL1 synchronously orchestrated the profibrotic behaviors of HSCs. (A, B) Immunofluorescent staining and visualization of COL-I and elastin in the cell layers of HSCs after *LOX* or *LOXL1* intervention with or without FITC-labeled tropoelastin (1 μg/mL, 48 h) treatment. Red arrows mark green collagenous fibers and elastin globules. Images were acquired with a 60× objective. (C) Immunoblotting

analyses of COL-1, elastin, and αSMA expression in HSCs after *LOX* or *LOXL1* perturbation (n = 3 for each group). Relative integrated density was adjusted by GAPDH protein. (D, E) Proliferative alterations of HSCs in response to *LOX* or *LOXL1* intervention were measured using the MTT assay (n = 12 for each group). (F–H) HSC migration was tested after *LOX* or *LOXL1* intervention using a transwell migration assay (n = 4 for each group). Images were acquired with a 10× objective. (I–K) Collagen gel contraction assay using HSCs after *LOX* or *LOXL1* intervention (n = 4 for each group). 3×FLAG-tagged *LOX* or *LOXL1* plasmids: 2.0 μg/mL; specific *LOX* or *LOXL1* siRNAs: 50 nM; rhTGF-β1: 10 ng/mL; all the reagents were treated for 48 hours except as specified; rhTGF-β1 was pretreated when silence experiments were performed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Abbreviations: Con, control; FITC, fluorescein isothiocyanate; LOX, lysyl oxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NC, negative control; o*LOX*, *LOX* overexpression; o*LOXL1*, *LOXL1* overexpression; rhTGF-β1, recombinant human transforming growth factor β1; si*LOX*, *LOX* inhibition; si*LOXL1*, *LOXL1* inhibition.

vice versa. Further immunofluorescent staining showed that both LOX and LOXL1 proteins were mainly upregulated and colocalized in the cytoplasm of rhTGF-β1-treated human primary HSCs (Figure 3E); in addition, LOX protein coimmunoprecipitated with LOXL1 protein in LX-2 cells (Figure 3F). Collectively, LOX and LOXL1 coregulate with each other at the posttranscriptional level, whereas LOXL1 dominates in the coregulation loop in HSCs.

LOXL1-mediated myofibroblast-like transition of HSCs through Notch signal

We next measured the transcriptomic variations in HSCs after *LOX* or *LOXL1* knockdown under rhTGF-β1 incubation to explore the potential downstream intracellular effectors. Gene set enrichment analysis revealed *LOX* or *LOXL1* inhibition downregulated the Kyoto Encyclopedia of Genes and Genomes pathways, including metabolism, immune, intracellular signal transduction, and ECM receptor interaction (Figures 4A, B). We noticed that the most significantly downregulated Kyoto Encyclopedia of Genes and Genomes pathway after *LOXL1* silence was the Notch signaling pathway ($p < 0.001$); gene signature in the leading-edge subsets of Notch signal tended to decrease in response to *LOXL1* siRNA treatment (Figure 4C). Increasing evidence has confirmed that the Notch cascade is a fundamental pathway capable of controlling cell fates, and dramatically participating in HSC activation and liver fibrosis.[19] Hence, we experimentally tested the LOXL1-Notch axis in HSC activation. As shown in Figure 4D, *LOXL1* overexpression in HSCs accelerated Notch signal activation; however, *LOXL1* inhibition reversed rhTGF-β1-evoked Notch signal activation. Increased expressions of collagen I, αSMA, and hes family bHLH transcription factor 1 (HES1, a key member downstream of the Notch signal) in HSCs through *LOXL1* overexpression were blocked by LY411575, a specific Notch signal inhibitor (Figure 4E), indicating LOXL1-Notch axis is essential in LOXL1-mediated HSC activation.

To affirm how LOXL1 interplays with NOTCH1 on HSCs, we first studied the colocalization of LOXL1 and NOTCH1 in LX-2 cells grown in conditioned media from LX-2 cells following *LOXL1* overexpression. We observed a strongly positive colocalization of LOXL1 and NOTCH1 at the cell surface of LX-2 cells (Figure 4F), suggesting the secreted LOXL1 has the potential to interact with NOTCH1 on HSCs. Next, we performed the coimmunoprecipitation assay using LX-2 cells to determine whether there is a physical interaction between LOXL1 and NOTH1. As shown in Figure 4G, LOXL1 but not LOX protein could physically bind to NOTCH1 protein. In short, these results suggest LOXL1 affects the myofibroblast-like transition of HSCs, at least in part, through the Notch signal.

LOXL1 interaction with LOX prolonged their half-lives, specifically enhancing the Notch signal-mediated myofibroblast-like transition of HSCs

To investigate the effect of the crosstalk of LOX and LOXL1 in HSC activation and Notch signal, first, *LOX* (2 μg/mL) or *LOXL1* (2 μg/mL)-overexpressed HSCs were treated for various periods (0–10 h) with cycloheximide (CHX) that is a protein synthesis inhibitor and can be used to evaluate the protein stability in eukaryotic cells.[20] In *LOX*-overexpressed LX-2 cells, treatment with CHX for ≥2 hours caused a drastic degradation of HSC activation markers (collagen I and αSMA), Notch signal activation marker (HES1), and LOX itself; in *LOXL1*-overexpressed LX-2 cells, incubation with CHX until 4 hours caused a relatively rapid decrease in collagen I, αSMA, HES1, and LOXL1; however, interestingly, co-overexpression of *LOX* (1 μg/mL) and *LOXL1* (1 μg/mL) plasmids in HSCs significantly inhibited CHX treatment-induced degradation of collagen I, αSMA, HES1, LOX and LOXL1 (Figure 5A). Further immunofluorescent staining validated that compared to overexpression of single *LOX* or *LOXL1* plasmid, co-overexpression of both *LOX* and *LOXL1* plasmids could delay the degradation of HES1 protein in Notch signal when CHX was incubated for up to 10 hours (Figure 5B).

Furthermore, without CHX treatment, co-overexpressing *LOX* (1 μg/mL) and *LOXL1* (1 μg/mL) plasmids in HSCs boosted NOTCH1 and HES1 protein expression compared to single overexpressing *LOX* (2 μg/mL) or *LOXL1* (2 μg/mL) plasmid. At the same time, preincubation with the Notch signaling inhibitor (LY411575) abolished the effects on the Notch signal

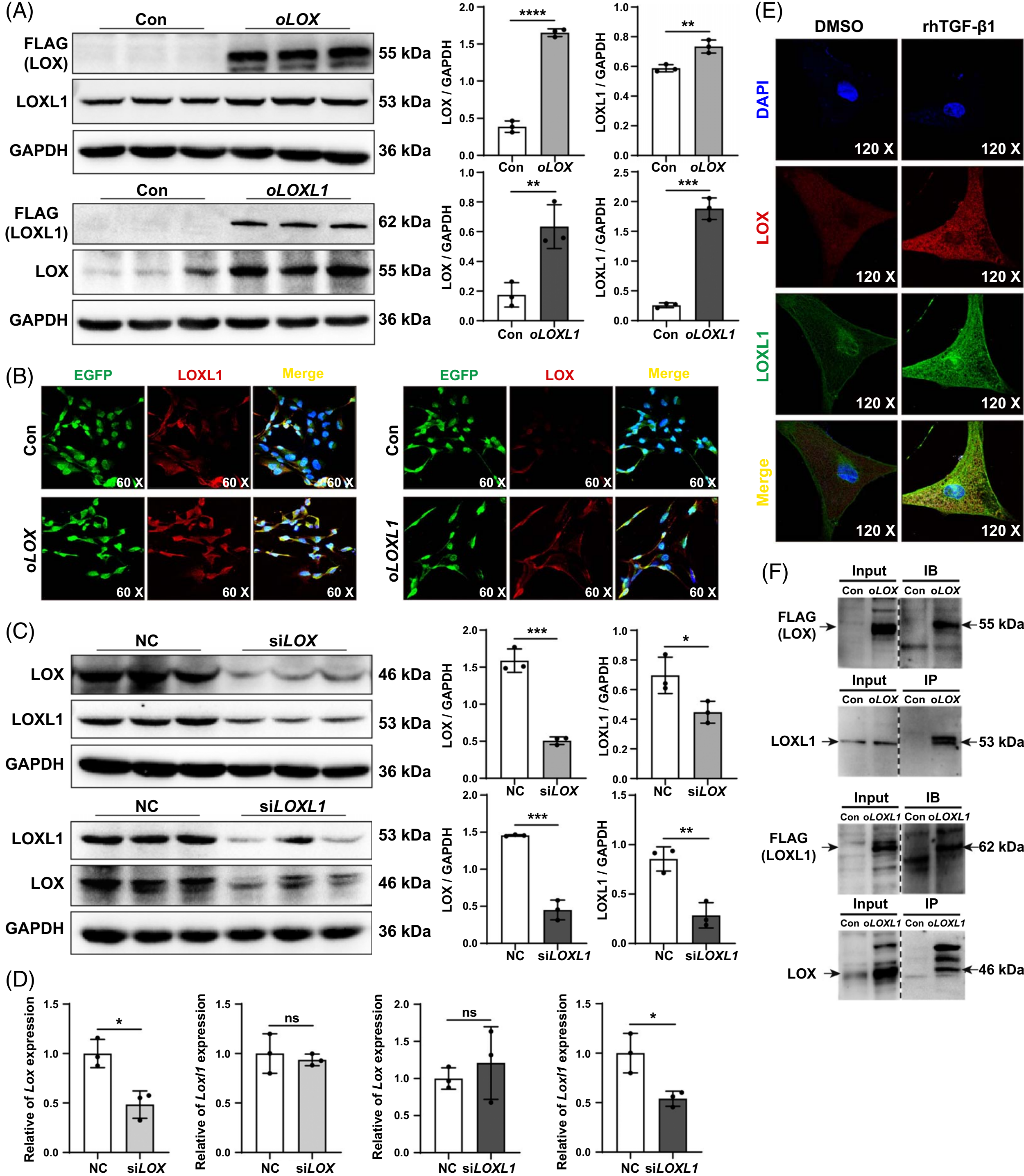

FIGURE 3 LOXL1 dominated in LOX-LOXL1 coregulation loop in HSCs. (A) Immunoblotting analyses of LOX or LOXL1 protein expression after *LOX* or *LOXL1* plasmids transfection or *LOX* or *LOXL1* siRNA inhibition (n = 3 for each group). Relative integrated density was adjusted by GAPDH protein. The molecular weight of 3×FLAG-tagged LOX (LOX-3×FLAG) is 55 kDa, while the molecular weight of 3×FLAG-tagged LOXL1 (LOXL1-3×FLAG) is 62 kDa. (B) Immunofluorescent visualization of LOX (red) or LOXL1 (red) in HSCs after the transfection of 3×FLAG-tagged *LOX*, *LOXL1*, or null plasmids with EGFP tag (green). Images were acquired with a 60× objective. (C) Immunoblotting analyses of LOX or LOXL1 protein expression after *LOX* or *LOXL1* inhibition in HSCs under rhTGF-β1 stimulation (n = 3 for each group). Relative integrated density was adjusted by GAPDH protein. (D) qPCR analyses of *LOX* and *LOXL1* genes after *LOX* or *LOXL1* inhibition in HSCs under rhTGF-β1 stimulation (n = 3 for each group). (E) Immunofluorescent visualization of LOX (red) and LOXL1 (green) in primary human HSCs in the presence or absence of rhTGF-β1 treatment. Images were acquired with a 120× objective. (F) Coimmunoprecipitation of LOX and LOXL1 in HSCs in the setting of *LOX* or *LOXL1* plasmids overexpression. 3×FLAG-tagged *LOX* or *LOXL1* plasmids: 2.0 μg/mL; specific *LOX* or *LOXL1* siRNAs: 50 nM; rhTGF-β1: 10 ng/mL; all the reagents were treated for 48 hours except as specified; rhTGF-β1 was pretreated when silence experiments were performed. FLAG antibody was used to specifically detect LOX-3×FLAG or LOXL1-3×FLAG fusion protein after *LOX* or *LOXL1* plasmids overexpression, which was shown as FLAG (LOX) or FLAG (LOXL1). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns represents not significant. Abbreviations: Con, control; EGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IB, immunoblotting; IP, immunoprecipitation; LOX, lysyl oxidase; NC, negative control; o*LOX*, *LOX* overexpression; o*LOXL1*, *LOXL1* overexpression; si*LOX*, *LOX* inhibition; si*LOXL1*, *LOXL1* inhibition.

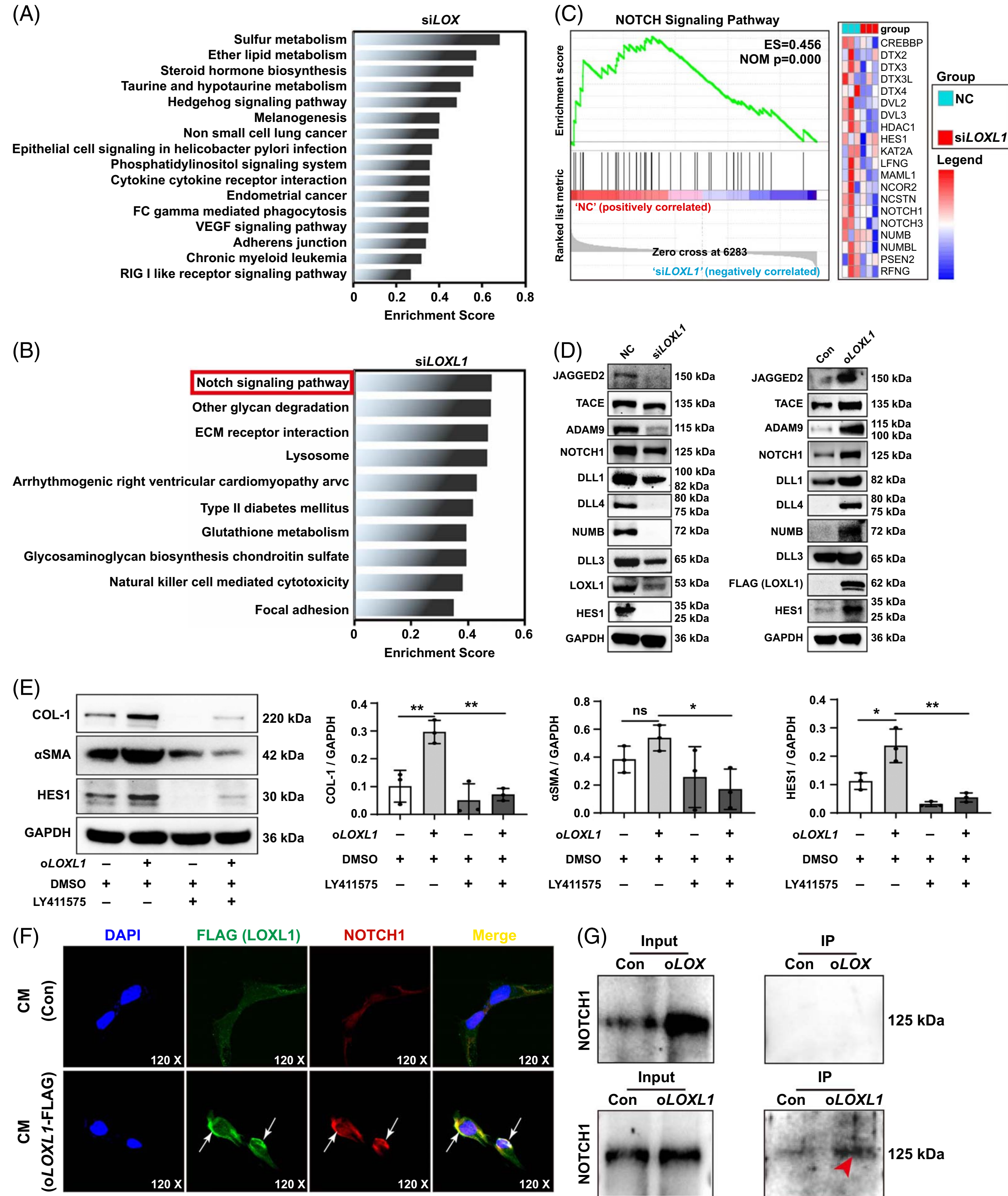

FIGURE 4 LOXL1 mediated myofibroblast-like transition of HSCs through Notch signal. (A, B) GSEA analysis based on the transcriptomic gene expression profiles from HSCs with or without *LOX* or *LOXL1* silence. All the KEGG pathways presented were significantly enriched ($p < 0.05$). The x-axis represents the ES. (C) Enrichment plot of Notch signaling pathway from GSEA analysis after *LOXL1* inhibition in HSCs. The right heatmap near to enrichment plot shows the expression levels of a gene signature in the leading-edge subset of the Notch signal. Expression levels are color-coded. The higher the red, the higher the expression; the higher the blue, the lower the expression. (D) Immunoblotting analyses of Notch cascade in HSCs after *LOXL1* overexpression or inhibition. (E) Effects of *LOXL1* plasmid overexpression on COL-1, αSMA, and HES1 expression in HSCs in the presence or absence of LY411575 (1 μm/mL, 48 h) treatment. (F) LX-2 cells with or without incubation (48 h) of CM from *LOXL1*-3×FLAG plasmid-transfected LX-2 cells (2 μg/mL, 48 h). Colocalization (yellow) of LOXL1 (green) and NOTCH1 (red) on the cell plasma membrane is marked by a white arrow. Objective magnification, 60×. (G) Coimmunoprecipitation of NOTCH1 and LOX or LOXL1 in HSCs after *LOX* or *LOXL1* overexpression. 3×FLAG-tagged *LOX* or *LOXL1* plasmids: 2.0 μg/mL; specific *LOX* or *LOXL1* siRNAs: 50 nM; rhTGF-β1: 10 ng/mL; all the reagents were treated for 48 hours except as specified; rhTGF-β1 was pretreated when silence experiments were performed. FLAG

antibody was used to specifically detect LOXL1-3×FLAG fusion protein after *LOXL1* plasmid overexpression, which was shown as FLAG (LOXL1). $*p < 0.05$, $**p < 0.01$, ns represents not significant. Abbreviations: CM, conditioned media; Con, control; ES, enrichment score; GSEA, gene set enrichment analysis; IP, immunoprecipitation; KEGG, Kyoto Encyclopedia of Genes and Genomes; LOX, lysyl oxidase; NC, negative control; o*LOX*, *LOX* overexpression; o*LOXL1*, *LOXL1* overexpression; si*LOX*, *LOX* inhibition; si*LOXL1*, *LOXL1* inhibition.

but did not affect the expression of both LOX and LOXL1 (Figure 5C). Meanwhile, we also found the crosstalk of LOX and LOXL1 amplified the marker gene expression of HSC activation (*Col1a1* and *Acta2*), proliferation (*Mki67* and *Pcna*), migration (*Arhgef4* and *Actr3*),[21,22] and contraction (*Rhoa*),[23] which was counteracted by LY411575 pretreatment (Figure 5D). To sum up, synergistically elevated LOX and LOXL1 and their crosstalk in HSCs during liver fibrogenesis enhanced the stabilities of LOX and LOXL1, specifically prolonging the stimulation on their downstream Notch signal.

Specific ablation of Loxl1 in Gfap$^+$ HSCs inhibited Notch signal activation and alleviated CCl_4-induced liver fibrosis

Notch cascade is a fundamental pathway controlling cell fate and dramatically participates in the myofibroblast-like transition of HSCs and liver fibrosis progression.[19] To determine whether the Notch signal is the potential downstream effector of LOXL1 in HSC activation and liver fibrogenesis in vivo, *Loxl1*$^{fl/fl}$ and *Loxl1* $^{fl/fl}$*Gfap*^{Cre} (*Loxl1*$^{\Delta HSC}$) mice were intraperitoneally injected with CCl_4 for 6 weeks. Histological assessments (Figure 6A) and downregulation of collagen I and tropoelastin (Figure 6B) showed a specific deficiency of *Loxl1* in *Gfap*$^+$ HSCs alleviated CCl_4-induced liver fibrosis. Also, *Gfap*$^+$ HSCs-derived *Loxl1* ablation in vivo significantly alleviated both LOX and LOXL1 protein expression in the total liver (Figure 6B). Further, immunoblotting and immunofluorescent analyses revealed that the activation of liver Notch signal was decreased in CCl_4-treated *Loxl1*$^{\Delta HSC}$ mice compared to *Loxl1*$^{fl/fl}$ mice, as the key elements of Notch signal in the total liver including JAGGED2, TACE, ADAM9, NOTCH1, DLL1, and HES1 were downregulated in *Loxl1*$^{\Delta HSC}$ mice (Figures 6C, D), suggesting a selective depletion of *Loxl1* in *Gfap*$^+$ HSCs protected against the activation of Notch signal during CCl_4 intoxication.

DISCUSSION

ECM stabilization and the subsequent attenuation of its turnover during liver fibrosis are usually attributable to the covalent cross-linking occurring among ECM collagenous and noncollagenous components.[2] Tissue transglutaminase and LOX family members are 2 types of cross-linking enzymes that have been reported to be upregulated in liver fibrosis.[2,4,5,14,24] Nevertheless, tissue transglutaminase knockout in mice has been experimentally confirmed with no effects on toxin-induced ECM stability and liver fibrosis regression.[25] In contrast, LOX, LOXL1, and LOXL2 inhibition can retard ECM cross-linking in toxin-induced liver fibrosis mouse models,[4–7] certifying their pathogenic roles in fibrogenesis. However, in advanced liver fibrosis, the expression of LOX and LOXL1 but not LOXL2 is robustly enhanced.[2,14] Based on the chemical damage and cholestasis-associated liver fibrosis models and publicly available gene expression profiles from patients with varying etiologies, our current study found the dysregulations of LOX and LOXL1 were regardless of etiology, depended on histological severity, and correlated with each other. Hence, although the anti-LOXL2 strategy with simtuzumab has failed in growing clinical trials, it will still be promising to try the anti-LOX or LOXL1 strategy in liver fibrosis treatment.

Our current study, consistent with previous reports,[2,4,6,7,14] showed activated HSCs are the primary cellular source for LOX and LOXL1 dysregulation. Hence, we first validated the traditional role of HSCs-derived LOX and LOXL1 in the covalent cross-linking and assembles of ECM collagen fibers and elastin globules. Lysine oxidation of tropoelastin by LOX or LOXL1 often requires the assistance of other ECM molecules. Generally, fibulin-4 binds with LOX, and fibulin-5 wraps with LOXL1, guiding them onto tropoelastin monomers and covalently cross-linking them into globules.[15,16] Our current study identified the ECM glycoprotein fibronectin as another guide for LOXL1 catalyzing ECM cross-linking (Supplemental Figure S3, http://links.lww.com/HC9/A817). Fibronectin has been recently reported to interact with collagens reciprocally, thus accelerating and enhancing nascent ECM assembly,[26] and promoting elastin deposition, elasticity, and mechanical strength.[27] Therefore, the interaction between fibronectin and LOXL1 may contribute to the physical targeting of LOXL1 to tropoelastin. Except for the canonical role, our in vitro experiments also demonstrated that LOX or LOXL1 is involved in the perturbation of activated, proliferative, migratory, and contractile properties of HSCs. LOX or LOXL1 may reprogram the myofibroblast-like transition of HSCs probably through the transcription factor Snail since Snail has been reported to be involved in fibroblast-to-myofibroblast transition[28] and it can physically interact with both LOX and LOXL1.[29] Our coimmunoprecipitation assay also found both LOX and LOXL1 can physically interact with another LOX isoform—LOXL2 (Supplemental Figure S3, http://links.lww.com/HC9/A817). Growing studies have implicated LOXL2 acts in multiple intracellular roles on cell proliferation, differentiation, migration, adhesion, and angiogenesis.[30] Therefore, LOX-mediated or

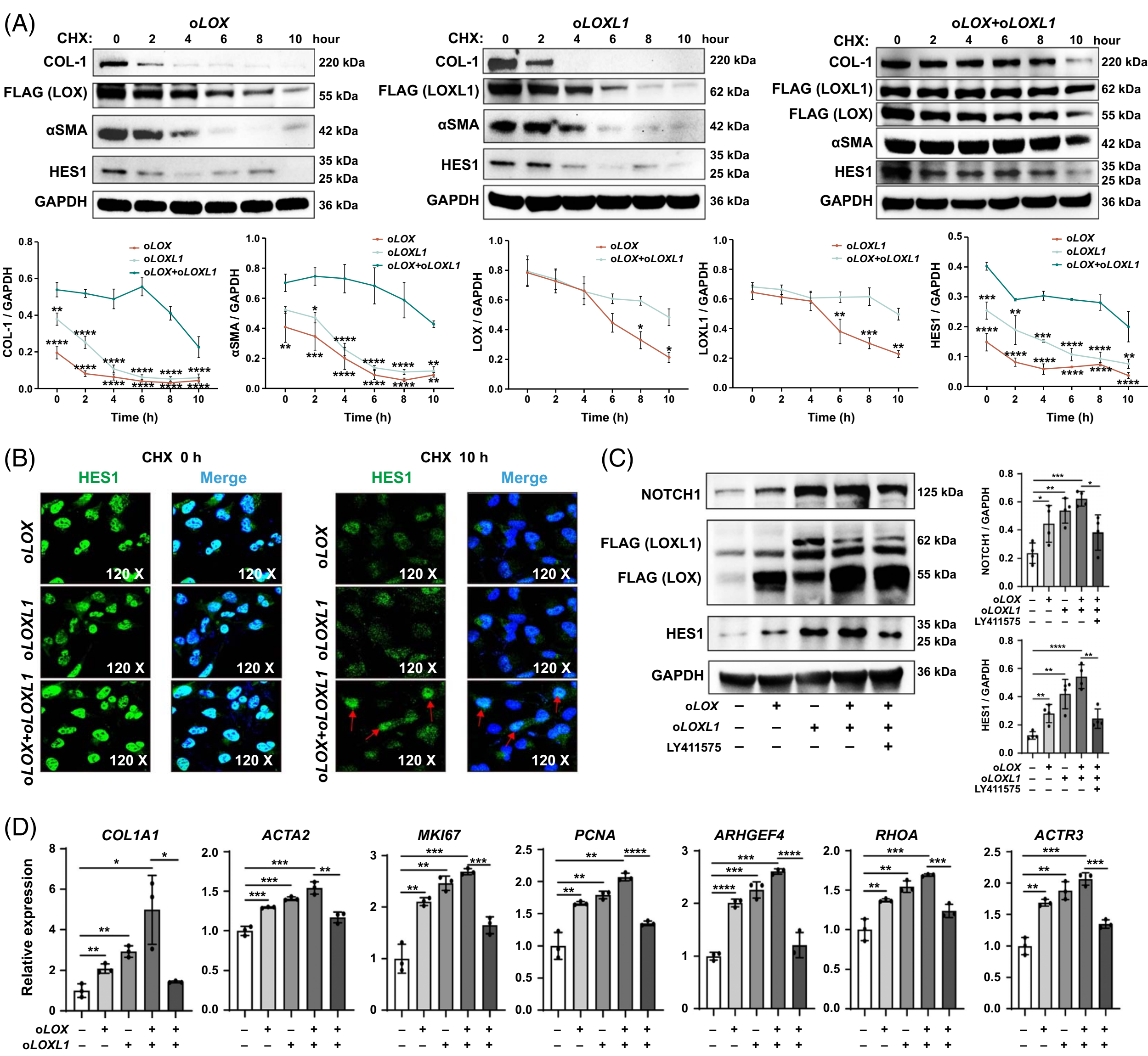

FIGURE 5 LOXL1 interaction with LOX prolonged their half-life and enhanced the Notch signal in HSCs. (A) Immunoblotting analyses of LOX, LOXL1, COL-1, αSMA, and HES1 levels in *LOX* and/or *LOXL1* plasmids preoverexpressed HSCs (48 h) in the presence of CHX (200 μg/mL) at 0, 2, 4, 6, 8, and 10 hours (n = 3 for each group). Quantitation of protein levels was adjusted by GAPDH expression. The molecular weight of 3×FLAG-tagged LOX (LOX-3×FLAG) is 55 kDa, while the molecular weight of 3×FLAG-tagged LOXL1 (LOXL1-3×FLAG) is 62 kDa. (B) Immunofluorescent analyses of HES1 (green) in *LOX* and/or *LOXL1*-overexpressed HSCs treated with CHX (200 μg/mL) at 0 and 10 hours. Images were acquired with a 120× objective. (C) Effects of *LOXL1* and *LOX* plasmids co-overexpression on NOTCH1, LOXL1-3×FLAG, LOX-3×FLAG, and HES1 expression in HSCs in the presence or absence of LY411575 (1 μm/mL, 48 h) treatment. (D) Effects of *LOXL1* and *LOX* plasmids co-overexpression on *COL1A1*, *ACTA2*, *MKI67*, *PCNA*, *ARHGEF4*, *RHOA*, and *ACTR3* gene expression in HSCs in the presence or absence of LY411575 (1 μm/mL, 48 h) treatment. 3×FLAG-tagged *LOX* or *LOXL1* plasmids: 2.0 μg/mL for single transfection and 1.0 μg/mL for combined transfection. FLAG antibody was used to specifically detect LOX-3×FLAG or LOXL1-3×FLAG fusion protein after *LOX* or *LOXL1* plasmids overexpression, which was shown as FLAG (LOX) or FLAG (LOXL1). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Abbreviations: αSMA, α-smooth muscle actin; CHX, cycloheximide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LOX, lysyl oxidase; o*LOX*, *LOX* overexpression; o*LOXL1*, *LOXL1* overexpression.

LOXL1-mediated HSC behavior perturbation could also be through LOXL2 in an indirect way, which deserves to be elucidated in the future.

As mentioned, LOX and LOXL1 are promising therapeutic targets for liver fibrosis since they are pathogenically upregulated in fibrotic liver and involved in ECM stability and HSC activation. However, a combined strategy should be considered if the compensatory effect between LOX and LOXL1 dysregulation exists. Our present study observed a synergistic other than compensatory effect between LOX and LOXL1 dysregulation when either intervened. Interestingly, LOXL1 dominates in the LOX and LOXL1 coregulation loop in HSCs. Up to the present, intracellular TGF-β1 and

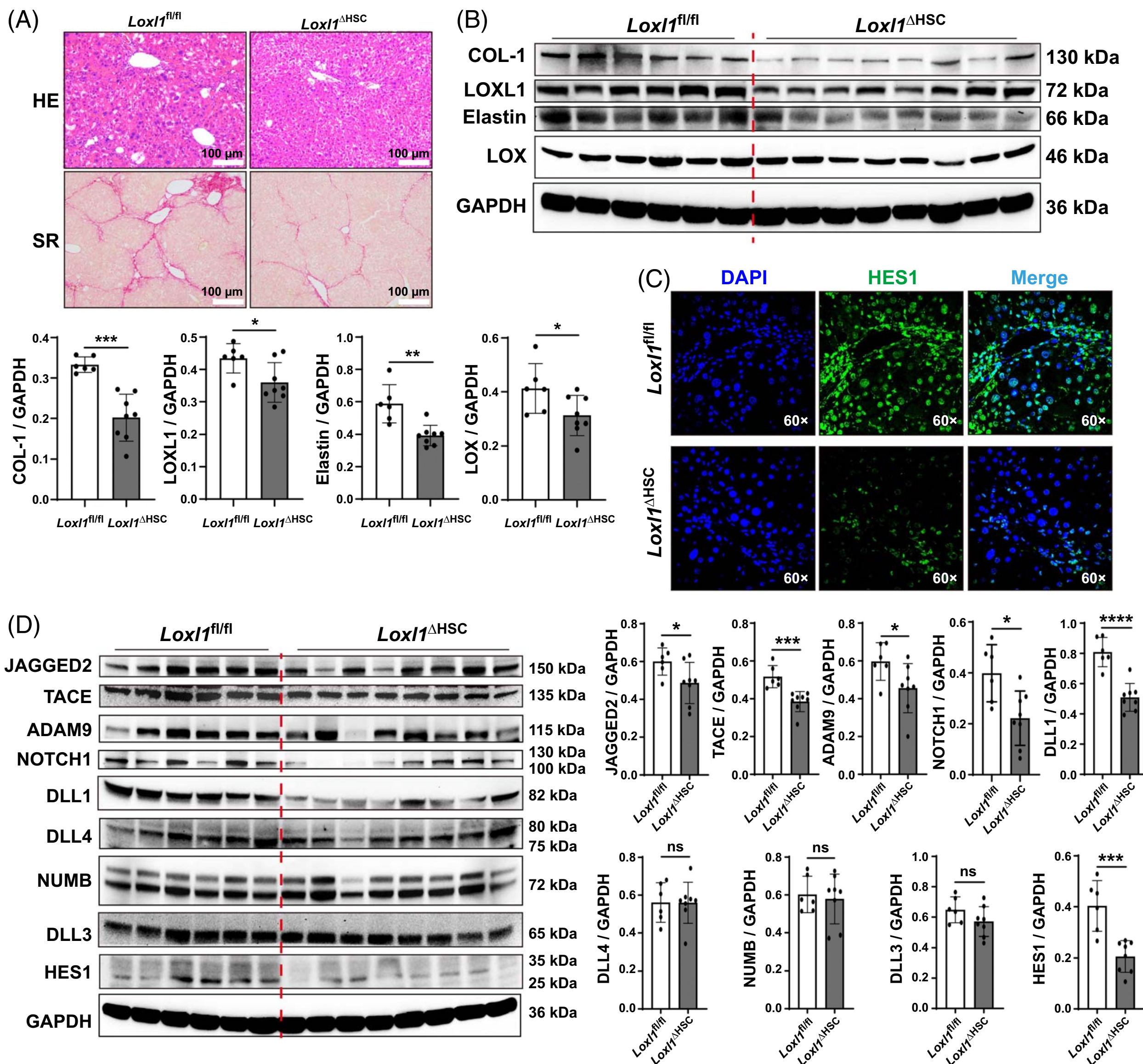

FIGURE 6 Specific ablation of *Loxl1* in *Gfap*⁺ HSCs inhibited Notch signal activation and alleviated CCl_4-induced liver fibrosis. (A) Liver histological assessments (HE and SR staining) of CCl_4-treated *Loxl1*$^{fl/fl}$ and *Loxl1*$^{\Delta HSC}$ mice. Scale bar: 100 μm. (B) Immunoblotting analyses of liver COL-1, elastin, LOX and LOXL1 between CCl_4-treated *Loxl1*$^{fl/fl}$ (n = 6), and *Loxl1*$^{\Delta HSC}$ (n = 8) mice. Relative integrated density was adjusted by GAPDH protein. (C) Immunoblotting analyses of members involved in Notch signal in livers from CCl_4-treated *Loxl1*$^{fl/fl}$ (n = 6) and *Loxl1*$^{\Delta HSC}$ (n = 8) mice. Relative integrated density was adjusted by GAPDH protein. (D) Repressive immunofluorescent visualization of HES1 in liver slices from CCl_4-treated *Loxl1*$^{fl/fl}$ and *Loxl1*$^{\Delta HSC}$ mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns represents not significant. Abbreviations: CCl4, carbon tetrachloride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HE, hematoxylin eosin staining, LOX, lysyl oxidase; *Loxl1*$^{fl/fl}$, *Loxl1*$^{flox/flox}$; *Loxl1*$^{\Delta HSC}$, *Loxl1* $^{flox/flox}$*Gfap*^{Cre}; SR, Sirius red staining.

focal adhesion kinase signals have been confirmed as the downstream effectors of LOXL1 in HSCs.[6,31] To thoroughly understand the nonclassical functions of LOXL1, we next explored the transcriptomic alteration in HSCs in response to *LOXL1* inhibition under rhTGF-β1 stimulation. Notch signal was the most evidently down-regulated pathway after *LOXL1* silence, which was not observed after *LOX* silence in HSCs. Moreover, the coimmunoprecipitation assay confirmed a direct interaction between NOTCH1 and LOXL1 but not LOX. A selective intervention of HSCs-derived LOXL1 in vivo and in vitro perturbs HSC activation by regulating the Notch signal. Over the past decade, the role and mechanism of Notch signaling in HSC activation and liver fibrosis progression has been well recognized.[19,32] Therefore, the disclosure of HSC-derived LOXL1-Notch axis will help further understand LOXL1 in HSCs and liver fibrosis and assist mechanism-based drug development.

Next, we explored the role of LOX in LOXL1-Notch axis-mediated HSC activation, as LOX and LOXL1 are synergistically elevated in fibrotic livers and synchronously orchestrate the profibrotic behaviors of HSCs. Early studies have revealed that protein stability appears to be governed by noncovalent interactions,

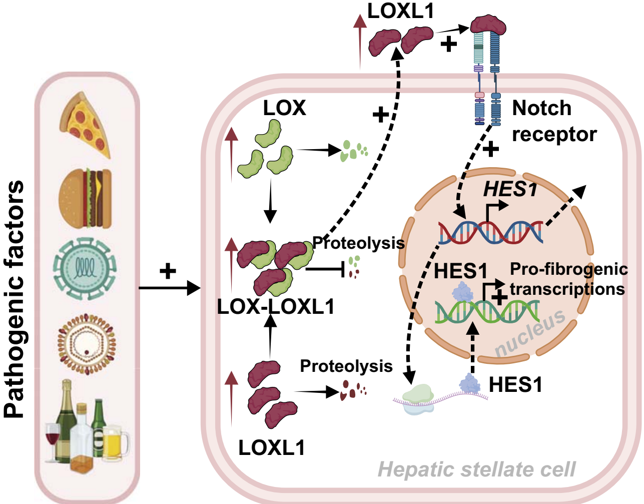

FIGURE 7 Schematic diagram of the crosstalk between LOX and LOXL1 isoforms and their noncanonical role and mechanisms involved in the myofibroblast-like transition of HSCs. The red up arrow represents an increase, and the blue down arrow represents a decrease. The plus sign indicates acceleration. Abbreviation: LOX, lysyl oxidase.

and unstable proteins are expected to receive significant additional stability from the interaction.[33,34] Our coimmunoprecipitation assay found that LOX physically interacts with LOXL1; accordingly, we questioned whether LOX-LOXL1 interaction could enhance their intrinsic stabilities. Because CHX can prevent translational elongation and is regarded as an inhibitor of protein biosynthesis,[20] we employed it to assess the stabilities of LOX and LOXL1 in our current study. With the CHX translation shut-off assay, we found cotransfection of *LOX* and *LOXL1* plasmids significantly delayed their degradation rates compared to single transfection of either of them, explicitly prolonging the stimulation on Notch signal and its downstream myofibroblast-like transition of HSCs. However, our current study still needs to unveil how LOX and LOXL1 interaction increases their half-lives, which requires further exploration.

In summary, our current study found LOX and LOXL1 were synergistically increased in fibrotic livers regardless of pathogenic factors and synchronously orchestrated the profibrotic behaviors of HSCs; LOXL1 and LOX interacted and coregulated with each other in HSCs; however, LOXL1 dominated in the LOX-LOXL1 coregulation loop; LOXL1 interaction with LOX prolonged their half-lives, specifically enhancing Notch signal-mediated myofibroblast-like transition of HSCs (Figure 7). The deficiency of HSC-derived *Loxl1* could retard liver Notch signal and liver fibrosis progression induced by CCl_4 intoxication. Our study systematically unmasked the potential mechanisms of LOXL1 involved in liver fibrosis exacerbation, providing the rationale for developing therapeutic opportunities for liver fibrosis based on the LOXL1 target.

AUTHOR CONTRIBUTIONS

Hong You and Wei Chen designed and supervised this study; Aiting Yang performed the in vivo experiments regarding *Loxl1*$^{\Delta HSC}$ mice with the support of Xuzhen Yan and Qi Han; Ning Zhang performed the other experiments with the support of Wei Chen, Wen Zhang, Hong Li, Anjian Xu, and Bingqiong Wang; Ning Zhang analyzed the data with the support of Wei Chen and Hong You; Wei Chen and Ning Zhang drafted the manuscript; Hong You critically revised the manuscript; and all the authors edited the manuscript.

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CONFLICTS OF INTEREST

The authors have no conflicts to report.

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