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ORIGINAL ARTICLE

Natural product mediated mesenchymal–epithelial remodeling by covalently binding ENO1 to degrade m6A modified β -catenin mRNA



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Abstract The transition of cancer cells from epithelial state to mesenchymal state awarded hepatocellular carcinoma (HCC) stem cell properties and induced tumorigenicity, drug resistance, and high recurrence rate. Reversing the mesenchymal state to epithelial state by inducing mesenchymal–epithelial remodeling could inhibit the progression of HCC. Using high-throughput screening, chrysin was selected from natural products to reverse epithelial–mesenchymal transition (EMT) by selectively increasing CDH1 expression. The target identification suggested chrysin exerted its anti-HCC effect through covalently and specifically binding threonine 205 (Thr205) of alpha-enolase (ENO1). For the first time, we revealed that ENO1 bound β -catenin mRNA, and recruited YTHDF2 to identify the m6A modified β -catenin in the 3′-UTR region to degrade β -catenin mRNA. Eventually, the CDH1 gene expression was improved through the regulation of β -catenin mRNA. ENO1/ β -catenin mRNA interaction might be a promising target for cellular plasticity reprogramming. Moreover, chrysin could mediate mesenchymal–epithelial remodeling through increasing degradation of β -catenin mRNA by promoting the binding of ENO1 and β -catenin mRNA. To the best of our knowledge, chrysin is the first reported small molecule inducing β -catenin mRNA degradation through binding to ENO1. The water-soluble derivative of chrysin may be a natural product-derived lead compound for circumventing metastasis, recurrence, and drug resistance of HCC by mediating mesenchymal–epithelial remodeling.

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1. Introduction

Hepatocellular carcinoma (HCC), the major type of primary liver cancer, is the third leading cause of cancer death¹. Metastasis is the ultimate and most lethal manifestation of HCC². EMT (epithelial–mesenchymal transition) is an important phenotypic plasticity process to metastasis in which epithelial state cancer cells lose polarity, cell–cell adhesion and eventually acquire mesenchymal state features, including increased invasiveness and motility³. Cancer cells in mesenchymal state acquired increased plasticity, exhibited chemotherapy resistance, constituted a pool of metastatic initiating cells, and induced cancer stem cell-like properties^{4–6}. Therefore, the identification of drugs that can reprogram the plasticity of cancer cells from mesenchymal state to epithelial state may provide new therapeutic strategies in the field of cancer therapy.

Cadherin-1 (CDH1), which encodes E-cadherin protein⁷, plays a pivotal role in maintaining the mesenchymal state of cancer cells and its loss or inactivation associated with the progression of multiple types of cancer^{8,9}. In addition, CDH1 silence increased the stemness and chemotherapy resistance of cancer cells to promote tumor progression^{10–12}. The E-cadherin protein was a potent tumor suppressor and maintained the epithelial phenotype through regulating various signaling pathways¹³. Cell surface localization of E-cadherin resulted in the mesenchymal state by disrupting cell–cell adhesion and intracellular signaling; while cell nuclear localization of E-cadherin resulted in inversely CD133 expression regulation¹⁴. Moreover, the subcellular localization of E-cadherin (nuclear/membranous ratio) was considered as a potential cancer diagnosis biomarker¹⁵. The down-regulation, heterozygosity, mutation, and cell surface localization inhibition of E-cadherin in clinical were all strongly associated with cancer progression, overall survival, and poor prognosis¹⁶. As a result, chemotherapy targeting CDH1 gene expression or E-cadherin subcellular localization was an important option in drug discovery. Traditional chemotherapeutics often cause severe systemic side effects, and metastasis, recurrence, and drug resistance are still severe problems to be solved. The current treatment regimens are far from satisfactory. To circumvent the existing therapeutic difficulties, novel therapeutic strategies with unique modes of action targeting plasticity by reversal mesenchymal state to epithelial state are urgently needed.

ENO1, also known as 2-phospho-D-glycerate hydrolase, is a traditional metabolic enzyme that dehydrates 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP) in glycolysis¹⁷. Besides the catalytic function in glycolysis, ENO1 is also multifunctional in cancer progression. ENO1 bound to plasminogen to promote extracellular matrix degradation and enhance metastasis when located on cell surface¹⁸. Moreover, ENO1 was associated with mitochondrial membrane stability and multiple intracellular signaling pathways regulation in cytoplasm¹⁹. In addition, Myc-binding protein (MBP1), another splicing transcription form of ENO1, is located in the nucleus and performed as the transcriptional suppressor of c-myc to inhibit tumor progression²⁰. ENO1 was overexpressed in over 70% of human cancer cases and was considered as a potential cancer biomarker^{17,20–22}.

We discovered that the natural product chrysin induced the reversal of EMT reprogram by increasing CDH1 gene expression. Chrysin could covalently and specifically bind to Thr205 of ENO1 and regulate its RNA binding effect with no obvious effect on the protein expression and the metabolic enzyme activity of ENO1. The results suggested that chrysin can be used as a natural lead compound for reprogramming mesenchymal–epithelial remodeling, and ENO1/β-catenin mRNA interaction facilitation may be a promising therapeutic target for cancer treatment.

2. Results

2.1. CDH1 mediated mesenchymal–epithelial remodeling

CDH1, an important tumor suppressor gene related to cancer cell mesenchymal state, was downregulated in HCC tissues compared to normal tissues in GSE database and TCGA database (Fig. 1a–d). CDH1 gene level was negatively correlated with HCC stage and overall survival. The higher HCC stage, the lower CDH1 gene expression (Fig. 1e). Moreover, the high expression CDH1 group exhibited significantly longer overall survival (Fig. 1f). The acquisition of mesenchymal cell state by CDH1 knockdown in HCC was often associated with more aggressive characteristic. We knocked down CDH1 by shRNA and verified by Western blot assay (Fig. 1g). Then the metastasis ability, cancer stemness, and chemotherapy resistance which were characterized with mesenchymal state were significantly potentiated. The tumorsphere formation ability, metastasis ability, percentage of CD133⁺ HCC stem cells, and associated stemness protein markers (CD133, ALDH1A1) were obviously increased after CDH1 knockdown (Fig. 1h–j, l). What's more, epithelial state-related protein ZO-1 decreased significantly while mesenchymal state-related protein including N-cadherin and vimentin correspondingly increased after CDH1 knockdown (Fig. 1i). Furthermore, the chemotherapy resistance of VP16, ADR, GEM and Taxol was increased after CDH1 knockdown which illustrated cancer cells in mesenchymal state exhibited chemotherapy resistance (Fig. 1k).

2.2. Chrysin reprogramed cellular plasticity from mesenchymal state to epithelial state

CDH1 knockdown induced the acquisition of mesenchymal state characterization which suggested CDH1 gene expression promoters could reverse cancer cells in an epithelial state. To find natural products induced cancer cells from mesenchymal cell state to epithelial state, we aimed to identify the compound that could selectively increase CDH1 gene expression. Firstly, we inserted the CDH1 promoter sequence (–250 to +250) into the pGL3-basic plasmid to construct dual fluorescence reporting system and compounds induced CDH1 gene expression would increase the luciferase fluorescence (Fig. 2a). Then we verified the CDH1 promoter activity and the results showed that the fluorescence intensity of CDH1 increased 112 times compared to empty vector which proved that reporting system was working and the sequence was the core promoter region of CDH1 (Fig. 2b). An in-house screening of natural products was performed. The results

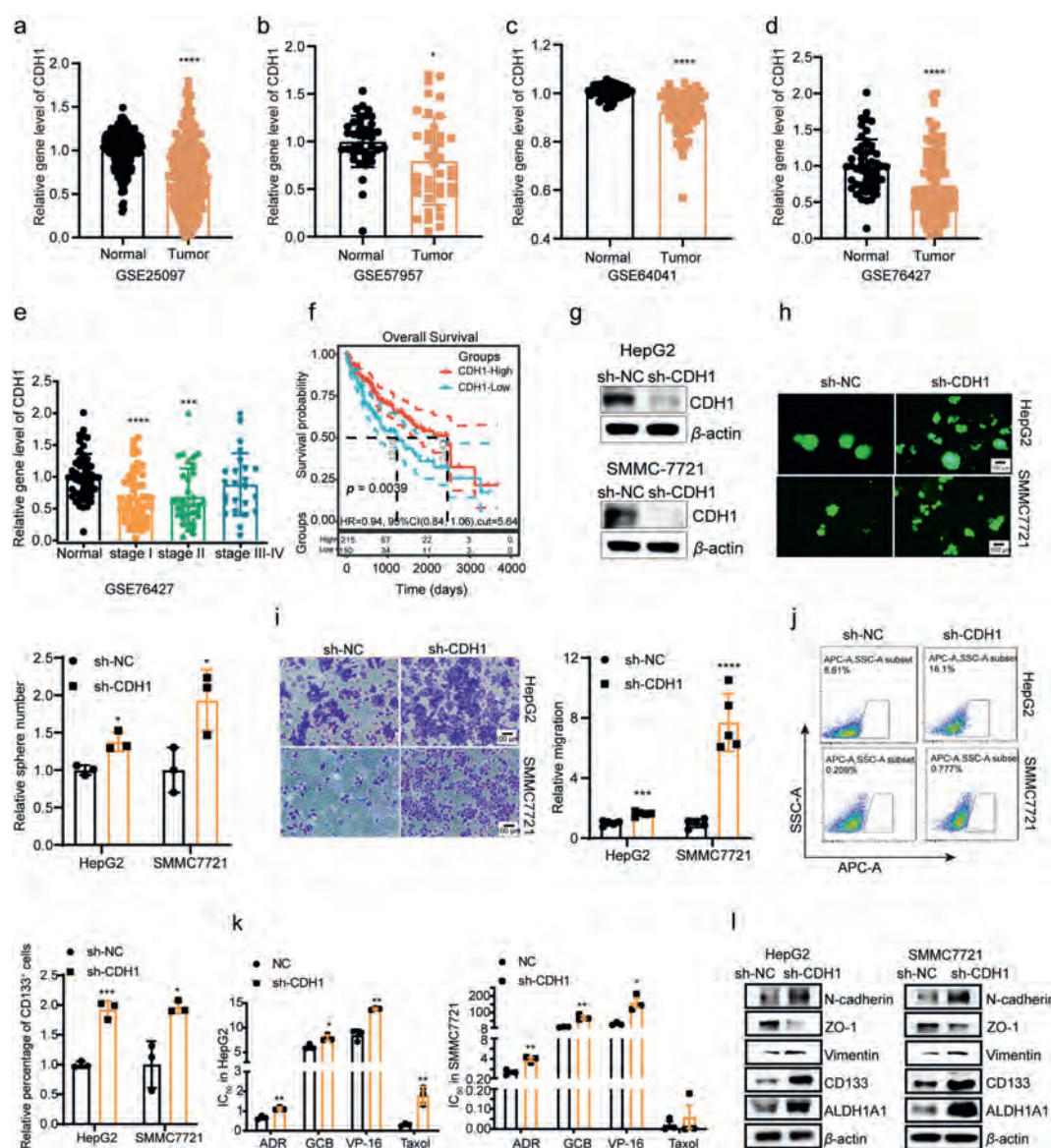


Figure 1 CDH1 mediated mesenchymal-epithelial remodeling. (a–d) The expression of CDH1 in normal and HCC tissues examined by GEO database (GSE 25097, GSE 57957, GSE 64041, GSE 76427). (e) The relationship between CDH1 expression and HCC stage based on GEO database (GSE 76427). (f) The relationship between CDH1 expression and HCC patient overall survival analyzed by SangerBox. (g) Knockdown of CDH1 in HepG2 and SMMC771 cells. (h–j) The effect of CDH1 knockdown on tumorsphere formation, cell migration and HCC stem biomarker CD133. Original magnification, 40×; scale bar: 100 μm. (k) IC_{50} of adriamycin (ADR), gemcitabine (GCB), Vepeside (VP-16), paclitaxel (Taxol) in sh-NC and sh-CDH1 group. (l) Immunoblotting analysis of mesenchymal and epithelial markers (N-cadherin, ZO-1, Vimentin) and HCC stemness biomarkers (CD133, ALDH1A1) after CDH1 knockdown.

suggested natural products apigenin, resveratrol and chrysin could significantly increase CDH1 promoter activity up to five times (Fig. 2c). And half activation concentration (AC_{50}) of apigenin, resveratrol, and chrysin were 2.8, 0.8, 1.2 μmol/L, respectively (Fig. 2d–f). Resveratrol and chrysin were selected due to the lower AC_{50} value. Moreover, chrysin was eventually selected for further evaluation with higher anticancer activity compared with resveratrol (Fig. 2g). And the CDH1 mRNA expression was obviously increased after being treated with chrysin in HepG2 and SMMC7721 cells (Fig. 2h–i).

The effect of chrysin on metastasis ability and cancer stemness characterized with a mesenchymal state was evaluated. The

results suggested the wound healing ability and migration ability were significantly decreased after chrysin treatment in a dose-dependent manner which suggested chrysin inhibited cancer cell metastasis (Fig. 2j–m). The tumorsphere formation ability and $CD133^{+}$ percentage were clearly declined after chrysin treatment which illustrated chrysin inhibited cancer cell stemness (Fig. 2n–o). Additionally, cancer cell stemness-related proteins including CDH1, CD133, ALDH1A1 and SOX2 were obviously reduced after the treatment of chrysin (Fig. 2p). These results indicated chrysin reprogramed cellular remodeling from mesenchymal state to epithelial state through inducing CDH1 gene expression.

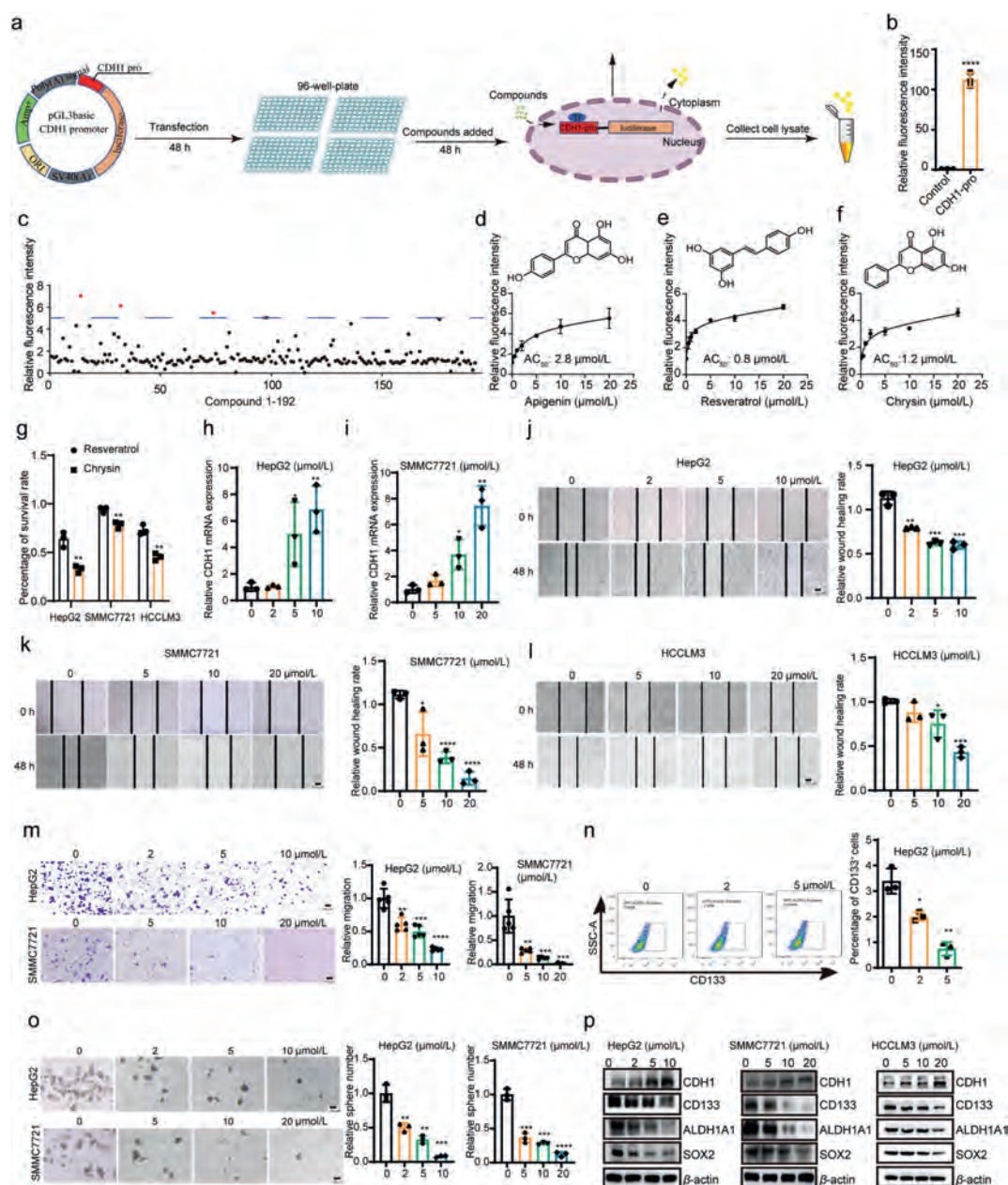


Figure 2 Chrysin reprogrammed cellular plasticity from mesenchymal state to epithelial state. (a) Schematic diagram for screening compounds. (b) The relative fluorescence intensity of CDH1 promoter after transfection with plasmid pGL3-CDH1 and Relia for 48 h in HepG2 cells by dual-luciferase reporter assay system. (c) The fluorescence intensity after transfection of plasmid pGL3-CDH1 and Relia for 48 h, incubation with 192 natural products at 5 μ mol/L. (d–f) The fluorescence intensity of CDH1 promoter after the treatment of apigenin, resveratrol, chrysin for 48 h. (g) The survival rate of HepG2, SMMC7721 and HCCLM3 after incubation with resveratrol and chrysin for 72 h at a concentration of 20 μ mol/L for HepG2, SMMC7721 and 100 μ mol/L for HCCLM3. (h, i) CDH1 gene level analyzed by RT-qPCR after incubation with chrysin for 48 h. (j–l) The representative pictures and statistical results of cell wound healing assay, (m) Transwell assay, (n) flow cytometry assay, (o) tumorsphere assay, after treatment of chrysin at different concentrations. Original magnification, 40 $\times$; scale bar: 100 μ m. (p) Immunoblotting analysis of CDH1, and HCC cell stem markers including CD133, ALDH1A1, SOX2 after treatment of chrysin for 48 h at different concentrations.

2.3. Water-soluble derivative of chrysin exhibited excellent anti-cancer effect

The activity of chrysin *in vitro* prompted us to evaluate its anti-HCC potency *in vivo*. However, the water solubility of chrysin was very poor. We designed and synthesized the water-soluble

chrysin analogue **3** with retained activity, which also indicated that the hydroxyl group at C-7 is not essential for its activity (Fig. 3a). We firstly evaluated the effect of chrysin analogue **3** at the cellular level. The result suggested analogue **3** significantly increased CDH1 promoter activity, CDH1 gene and protein level (Fig. 3b–d). And cancer cell mesenchymal state-related proteins

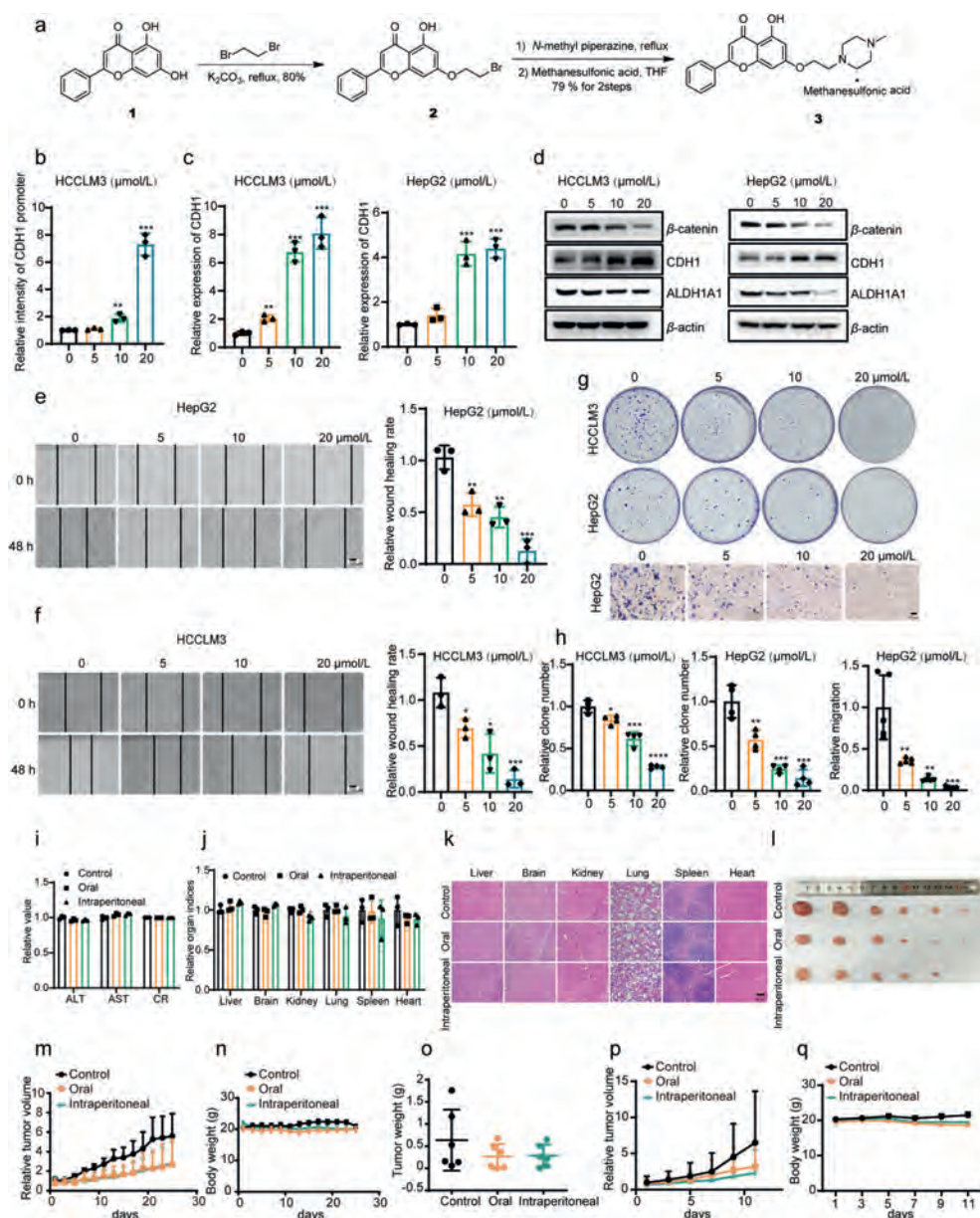


Figure 3 Water-soluble derivative of chrysin exhibited excellent anti-cancer effect. (a) Synthesis of the water-soluble chrysin analogue compound **3**. K_2CO_3 : potassium carbonate; THF: tetrahydrofuran. (b) The fluorescence intensity of CDH1 promoter after incubation with **3** at different concentrations. (c) CDH1 gene level analyzed by RT-qPCR after incubation with **3**. (d) Immunoblotting analysis of β -catenin, CDH1, ALDH1A1 expression after cells treated with **3**. (e, f) The representative pictures and statistical results of wound healing assay, and (g, h) colony formation assay, Transwell assay after cells treated with **3**. Original magnification, 40 $\times$; scale bar: 100 μm . (i) The level of ALT, AST and CR after oral or intraperitoneal administration with **3** at 200 mg/kg for 24 h. (j, k) The organ indices and HE staining analysis of liver, brain, kidney, lung, spleen and heart after oral or intraperitoneal administration with **3** at 200 mg/kg for 24 h. Original magnification, 100 $\times$; scale bar: 50 μm . (l) The picture, (m) tumor volume, (n) body weight, (o) tumor weight of HCC CDX tumors after orally or intraperitoneally administration with compound **3** at 100 mg/kg. (p) The tumor volume and (q) body weight of HCC PDX model after oral or intraperitoneal administration with compound **3** at 100 mg/kg.

including β -catenin and ALDH1A1 were obviously reduced after the treatment of compound **3** which suggested compound **3** was the water solubility alternative of chrysin with a similar activity (Fig. 3d). To clarify the effect of compound **3** on metastasis ability and cancer stemness, wound healing, Transwell and colony formation assay were performed. The results indicated compound **3** dose-dependently inhibited the wound healing ability, migration ability and colony formation ability (Fig. 3e–h), which further

indicated compound **3** exhibited similar anti-HCC activity as chrysin *in vitro*. Then we evaluated the safety of compound **3** *in vivo*. The results indicated that the glutamic oxalacetic transaminase (AST) level, glutamic–pyruvic transaminase (ALT) and creatinine (CR) were not obviously changed after the administration of compound **3** intraperitoneally/orally at 200 mg/kg in BALB/c mice which indicated compound **3** exhibited no effect on normal liver function (Fig. 3i). Moreover, no apparent changes

were observed in the histomorphology including liver, brain, kidney, lung, spleen, heart and corresponding organ indices compared with vehicle control group which suggested compound **3** showed no significant organ toxicity (Fig. 3j–k).

To further evaluate the anti-HCC effect *in vivo*, the cell-derived tumor xenograft model (CDX) was established. The results showed that compound **3** significantly inhibited tumor growth and tumor weight without affecting body weight by intraperitoneal or oral administration (Fig. 3l–o). Patient-derived xenograft model (PDX) was further established to analyze the effect of compound **3** *in vivo*. The results in PDX model also illustrated the excellent effect of compound **3** (Fig. 3p–q). These results indicated that the water solubility alternative of chrysin was an excellent potential candidate drug for further exploration to induce cancer cell mesenchymal cell state to epithelial state in cancer therapy.

2.4. Chrysin targeted ENO1 to mediate mesenchymal–epithelial remodeling

To investigate the direct target of chrysin, the biotin-coupled chrysin probe (compound **5**) was designed and synthesized (Fig. 4a). Then the pull-down assay was performed with compound **5** to tag the cellular targets of chrysin. The silver staining result showed a clear and specific band with a molecular mass of about 48 kDa was obviously precipitated (Fig. 4b). Then the specific band was picked out and identified by LC–MS/MS assay. After the LC–MS/MS performed, taking the number of peptides, peptide coverage, molecular weight, abundance, and non-specific exclusion into consideration, we speculated ENO1 might be the specific target of chrysin.

To verify the potency of chrysin targeting ENO1, we first performed Western blot assay to identify the interaction. The result suggested compound **5** bound ENO1 with a dose-dependent manner in HepG2 and SMMC7721 cells (Fig. 4c). Then the binding effect was further demonstrated with recombinant ENO1 (Fig. 4d). Cellular thermal shift assay (CETSA) showed an obvious enhanced thermal stability of ENO1 after treated with chrysin, implying a direct interaction of ENO1 and chrysin (Fig. 4e). In addition, compound **5** bound ENO1 in time-dependent saturation which was consistent with irreversible covalent binding mechanism (Fig. 4f). To further elucidate the binding ability of ENO1 and chrysin, the surface plasmon resonance (SPR) and microscale thermophoresis (MST) were conducted. The result of SPR assay showed chrysin could bind ENO1 with a dose-dependent manner (Fig. 4g). The result of MST assay showed the K_d value of ENO1 binding to chrysin was 15.05 $\mu\text{mol/L}$ (Fig. 4h). To evaluate whether the mesenchymal–epithelial remodeling of chrysin depended on the presence of ENO1, we constructed stable ENO1 gene knockdown cell lines (Fig. 4i) and performed wound healing assay and Transwell assay. The results indicated wound healing ability and migration ability in control group were obviously decreased while that in ENO1 knockdown cells were not dose-dependently affected by chrysin, which demonstrated when intracellular ENO1 levels decreased by shRNA, the effect of chrysin decreased correspondingly (Fig. 4j–v). Collectively, these results demonstrated ENO1 was the indispensable target of chrysin for reversing EMT in hepatocellular carcinoma.

2.5. Chrysin covalently bound Thr205 of ENO1

To confirm the specific amino acid site of chrysin covalently binding, LC–MS/MS analysis was performed using recombinant ENO1 protein treated with or without compound **3**. After incubation with recombinant ENO1 protein overnight, the ENO1 protein was digested with trypsin, the peptide was detected and analyzed by LC–MS/MS. The results indicated a specific Thr205-containing peptide DATNVGDEGGFAPNILENK ($m/z = 775.03$) with a calculated mass of 2323.08 Da was found in compound **3** treated group, which was 362.16 kD larger than that of control group ($m/z = 980.97$, MW = 1960.92 Da). An obvious mass shift of 362.16 Da was observed from fragment ion b3+ to b7+ and the increased mass of 362.16 Da exactly matched the molecular weight of unsalted compound **3**–H₂O (Fig. 5a and b). Accordingly, we speculated chrysin covalently bound ENO1 Thr205 through a dehydration reaction. To further confirm the specific binding between chrysin and Thr205, we mutated the Thr205 of ENO1 to serine and valine respectively. The results indicated the amount of chrysin-bound mutant protein was significantly reduced compared with wild-type ENO1 (Fig. 5c). These results indicated chrysin covalently bound Thr205 site of ENO1 and removed one molecule of water during the covalent reaction.

However, how the dehydration reaction occurred needed to be further clarification. We speculated the 5-hydroxyl of chrysin covalently bound the hydroxyl groups of ENO1 Thr205 through a dehydration condensation reaction (Fig. 5d). Based on this hypothesis, we inferred that the hydroxyl is an important active site for the biological function of chrysin. To verify this hypothesis, we designed and synthesized compound **7** to replace the hydroxyl group with hydroxymethyl groups (Fig. 5e). Interestingly, the anti-cancer activity of compound **7** dramatically reduced compared with compound **3** (Fig. 5f–i). Furthermore, we synthesized the biotin-coupled probe of hydroxymethyl group substituted chrysin, compound **9** (Fig. 5j). Pull-down assay revealed that the potency of compound **9** for binding ENO1 was obviously reduced compared with that of compound **5** (Fig. 5k). Therefore, the 5-hydroxyl of chrysin is essential for covalently binding with ENO1 Thr205 and necessary for its anti-HCC activity and the 5-hydroxyl of chrysin covalently bound the hydroxyl groups of ENO1 Thr205 through dehydration condensation reaction.

2.6. ENO1 mediated β -catenin mRNA dependent degradation by recruiting YTHDF2

β -Catenin is an important regulatory protein of the WNT signaling pathway and is associated with cancer cell mesenchymal–epithelial remodeling. ENO1 was newly discovered to maintain mRNA stability as a RNA-binding protein²³. As a result, we speculated ENO1 might bind β -catenin mRNA to regulate its stability. To verify our hypothesis, the RNA immunoprecipitation (RIP) assay was performed and the result indicated that ENO1 directly bound to β -catenin mRNA in HepG2 and HCCLM3 cells (Fig. 6a). To detect the binding sequence of ENO1 to β -catenin mRNA, the sanger sequencing was performed. The results suggested ENO1 bound to the 3'-UTR regions of β -catenin mRNA (Fig. 6b). Furthermore, we analyzed the 3'-UTR regions of β -catenin mRNA using GEO dataset (GSE149510) and the result

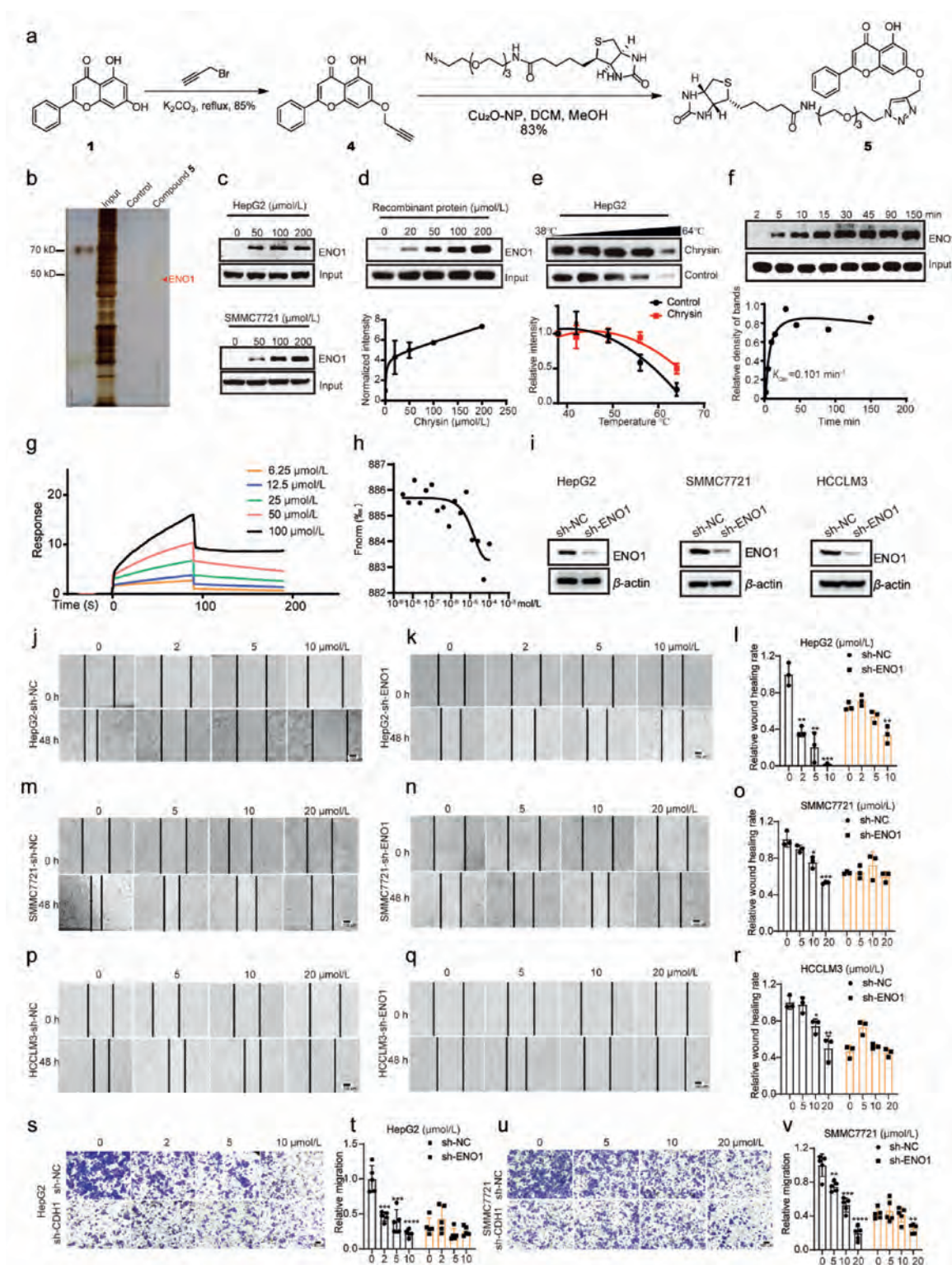


Figure 4 Chrysin targeted ENO1 to mediate mesenchymal-epithelial remodeling. (a) Synthesis of chrysin biotin coupled probe compound 5. Cu₂O-NP: nano-cuprous oxide; DCM: dichloromethane; MeOH: methanol. (b) Sliver staining of target protein after HepG2 lysate incubated with 5. (c) Immunoblotting analysis of the binding of chrysin with ENO1 using compound 5 in cell lysate and (d) recombinant ENO1 protein. (e) Thermal stability of ENO1 was measured after incubating with chrysin. (f) The K_{obs} determination between 5 and ENO1. (g) SPR analysis of the interaction between chrysin and ENO1. (h) MST analysis of the interaction intensity between chrysin and ENO1. (i) Knockdown of ENO1 in HepG2, SMMC771 and HCCLM3 cells. (j–l) The representative pictures and statistical results of wound healing rate after ENO1 knockdown in HepG2 cells. (m–o) The representative pictures and statistical results of wound healing rate after ENO1 knockdown in SMMC7721 cells. (p–r) The representative pictures and statistical results of wound healing rate after ENO1 knock down in HCCLM3 cells. (s, t) The representative pictures and statistical results of transwell assay after ENO1 knockdown in HepG2 and (u, v) SMMC7721 cells. Original magnification, 40 $\times$; scale bar: 100 μ m.

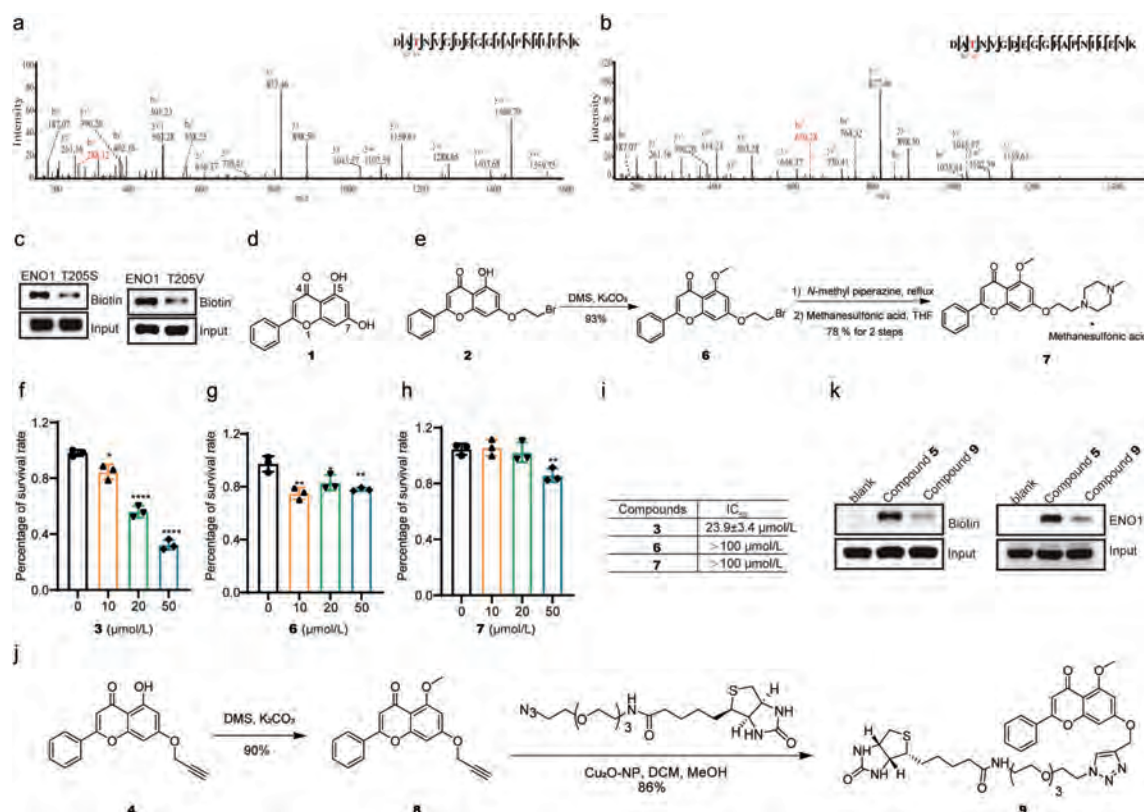


Figure 5 Chrysin covalently bound Thr205 of ENO1. (a, b) LC–MS/MS analysis of peptide fragment of recombinant ENO1 containing Thr205 after incubated with **3** (right) and without **3** (left). (c) Immunoblotting analysis of the interaction between chrysin probe **5** with ENO1 protein and threonine 205 mutant ENO1 protein. (d) The structural formula of chrysin. (e) The synthesis scheme of compound **7**. DMS: dimethyl sulfate; K₂CO₃: potassium carbonate; THF: tetrahydrofuran. (f–h) The survival rate of HepG2 cells after incubation with compounds **3**, **6**, **7** for 72 h. (i) The IC₅₀ of compounds **3**, **6**, **7**. (j) The synthesis of compound **9**. (k) Immunoblotting analysis of the binding between ENO1 with compound **5** and compound **9**.

suggested an obvious m6A methylated modification site in 3'-UTR of β -catenin mRNA (Fig. 6e). The RNA immunoprecipitation (RIP)-qPCR assay was performed to demonstrate the m6A methylation of β -catenin. The RIP-qPCR result revealed β -catenin mRNA was m6A methylated modification (Fig. 6c). Following that, RIP-qPCR assay specific to 3'-UTR of β -catenin was performed to prove the m6A methylated modification site. The result showed almost 3-fold m6A-enriched specific to the 3'-UTR of β -catenin mRNA which was clearly stronger than 1.5-fold m6A-enriched total β -catenin mRNA (Fig. 6d). This result indicated the 3'-UTR of β -catenin mRNA was the methylated modification site.

RNA N6-methyladenosine (m6A) methylation, a reversible dynamic RNA modification in eukaryotic cells, was involved in mRNA stability by recruiting m6A reader protein²⁴. Based on the above information, we hypothesized that ENO1 might bind to β -catenin mRNA, recruit m6A reader protein, and mediate m6A-dependent degradation of β -catenin. YTHDF2 was an indispensable m6A reader protein identifying m6A site to regulate RNA stability²⁵. We speculated YTHDF2 may mediate m6A-dependent degradation of β -catenin as a reader protein. To verify the hypothesis, the β -catenin gene and protein level were detected after YTHDF2 knock down. The results indicated β -catenin mRNA and protein levels increased significantly after YTHDF2 knockdown (Fig. 6f and g). Furthermore, the RIP-qPCR assay consistently suggested that YTHDF2 directly bound to β -catenin mRNA

(Fig. 6h). All these results suggested YTHDF2 could identify the m6A methylated modification site of β -catenin mRNA and mediated β -catenin mRNA degradation as a reader protein. Moreover, the interaction between ENO1 and YTHDF2 was analyzed by co-immunoprecipitation (Co-IP) assay and the results showed ENO1 interacted with YTHDF2 which suggested ENO1 could recruit YTHDF2 protein (Fig. 6i and j). The results indicated ENO1 recruits YTHDF2 to mediate m6A-dependent degradation of β -catenin.

Comprehensively, ENO1 bound to the 3'-UTR regions of β -catenin mRNA, and recruited YTHDF2 to identify the m6A methylated modification site in 3'-UTR regions and mediated m6A-dependent degradation of β -catenin.

2.7. β -Catenin reprogramed mesenchymal–epithelial remodeling through negatively regulating CDH1 transcription

To evaluate the potential relationship between β -catenin function and cancer mesenchymal state, β -catenin gene expression analysis was performed in the TCGA database and GEO database. The results showed that β -catenin gene expression was significantly increased in cancer tissues compared with normal tissue (Fig. 7a and b). The stage-specific analysis suggested higher β -catenin gene level was significantly associated with higher stage and grade in TCGA and GEO database (Fig. 7c–e). Moreover, higher β -catenin gene levels exhibited significantly shorter overall

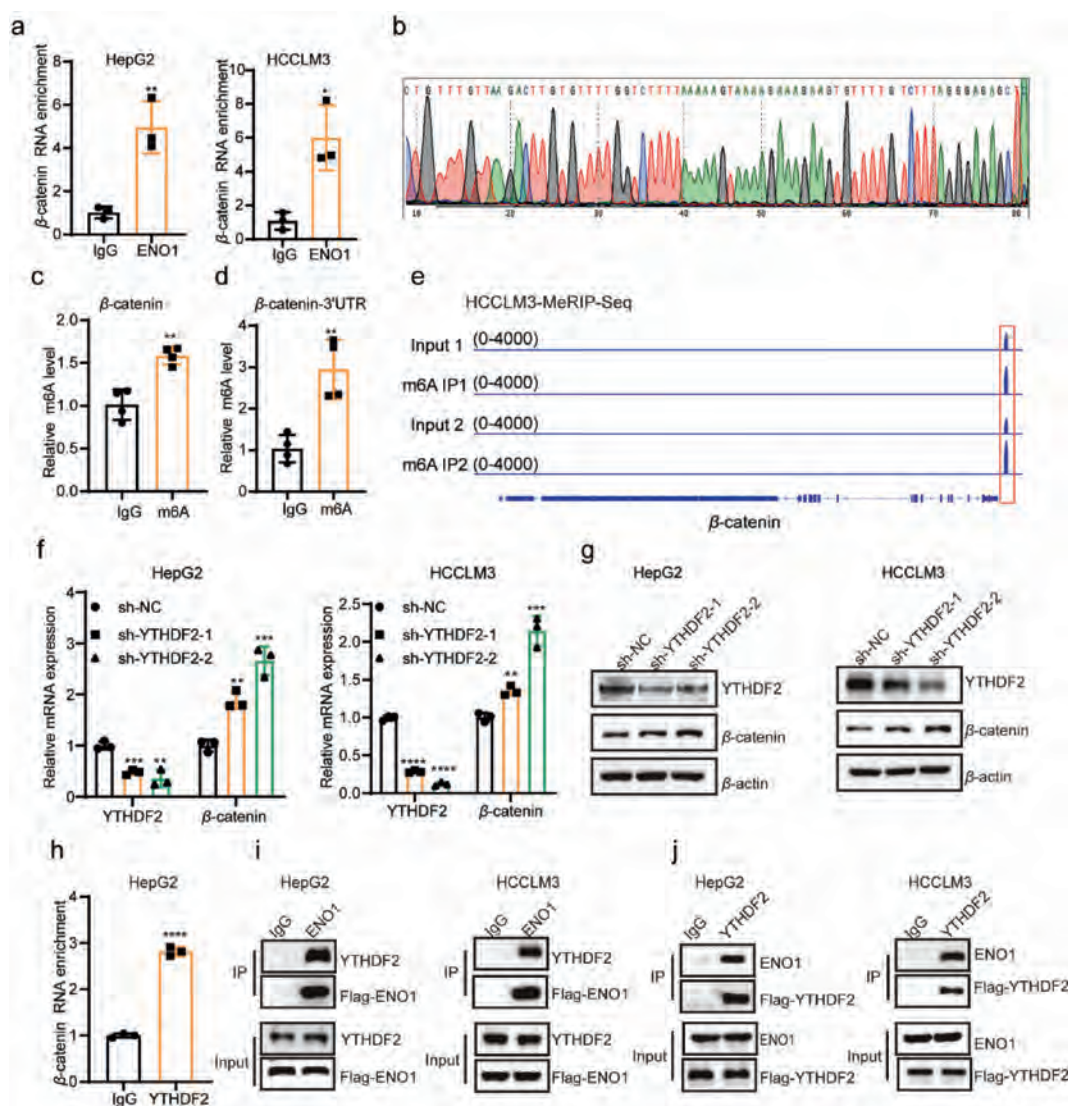


Figure 6 ENO1 mediated β -catenin m6A-dependent degradation by recruiting YTHDF2. (a) RIP analysis of the binding between β -catenin mRNA and ENO1 in HCC cells. (b) Sanger sequencing results containing the binding region of ENO1 and β -catenin. (c) The m6A level of β -catenin mRNA in HCCLM3 cells. (d) The m6A level of 3'-UTR regions in β -catenin mRNA. (e) m6A peak of β -catenin mRNA in 3'-UTR (GSE 149510). (f) The mRNA level and (g) protein level of β -catenin in YTHDF2 knockdown cells. (h) RIP analysis of the binding between β -catenin mRNA and YTHDF2. (i) The co-IP analysis of the interaction between ENO1 and YTHDF2 after overexpressing ENO1. (j) The co-IP analysis of the interaction between ENO1 and YTHDF2 after overexpressing YTHDF2.

survival (OS), disease special survival (DSS) and progression free interval (PFI) (Fig. 7f). These results suggested β -catenin positively associated with cancer progression.

To further verify the effect of β -catenin in cancer mesenchymal state, the metastasis ability and cancer stemness-related protein expression which is characterized with mesenchymal state were analyzed after β -catenin knockdown. The results suggested the wound healing ability and migration ability were significantly decreased after β -catenin knockdown which suggested β -catenin regulated cancer cell metastasis (Fig. 7g-j). More importantly, β -catenin gene was negatively associated with CDH1 gene expression (Fig. 7k). CDH1 promoter activity and gene expression levels were clearly increased after β -catenin knockdown which suggested β -catenin negatively regulated CDH1 transcriptionally (Fig. 7l and m). Additionally, cancer cell mesenchymal state and

stemness-related protein ALDH1A1 was reduced while CDH1 was increased obviously after β -catenin knockdown which indicated β -catenin regulated cancer cell mesenchymal state maintenance (Fig. 7n).

After chrysin treatment, β -catenin protein was strongly declined in a dose-dependent manner which revealed chrysin covalently bound ENO1 to induce CDH1 gene expression through reducing β -catenin expression level (Fig. 7o).

2.8. Chrysin promoted the RNA binding potential of ENO1 to reprogram mesenchymal-epithelial remodeling through meditating β -catenin mRNA degradation

To explore how chrysin reprogramed mesenchymal-epithelial remodeling by binding ENO1, we first detected the protein

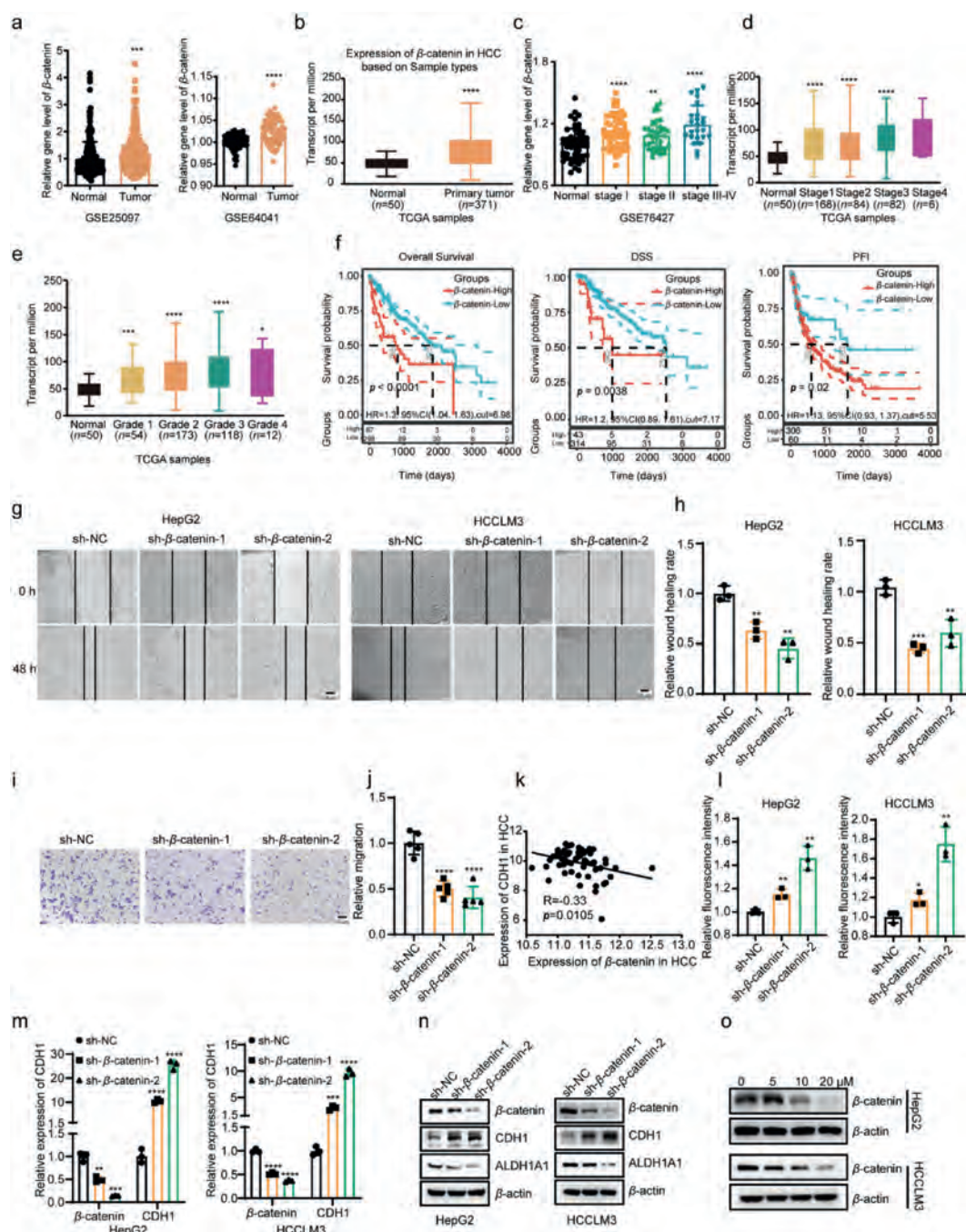


Figure 7 β-Catenin reprogrammed mesenchymal-epithelial remodeling through negatively regulating CDH1 transcription. (a) The expression of β-catenin in normal and HCC tissues examined by GEO database (GSE 25097, GSE 64041) and (b) TCGA database. (c) The relationship between β-catenin expression and HCC stage and grade analyzed by GEO database (GSE 76427) and (d, e) TCGA database. (f) Survival analysis including overall survival (OS), disease specific survival (DSS), progression-free interval (PFI) of β-catenin in HCC analyzed by SangerBox. (g, h) The representative pictures and statistical results of wound healing rate and (i, j) migration after β-catenin knockdown. Original magnification, 40 ×; scale bar: 100 μm. (k) The correlation between β-catenin and CDH1 expression in HCC tissues in GEO database (GSE 64041). (l) The relative fluorescence intensity of CDH1 promoter and (m) CDH1 gene expression after β-catenin knockdown. (n) Analysis of β-catenin, CDH1, ALDH1A1 protein level in β-catenin knockdown cells. (o) Analysis of β-catenin protein level after treated with chrysin.

level of ENO1 after treatment with chrysin at different concentrations and the results showed ENO1 protein level was not obviously changed (Fig. 8a). As an important enzyme to catalyze glycolysis, the metabolic enzyme activity of ENO1 was measured and the result exhibited chrysin could not

significantly affect ENO1 enzyme activity (Fig. 8b). Then we inferred chrysin might promote the ENO1/β-catenin mRNA interaction or promote ENO1/YTHDF2 protein-protein interaction by binding ENO1 to induce CDH1 gene expression to reprogram cellular plasticity. To confirm the hypothesis, the

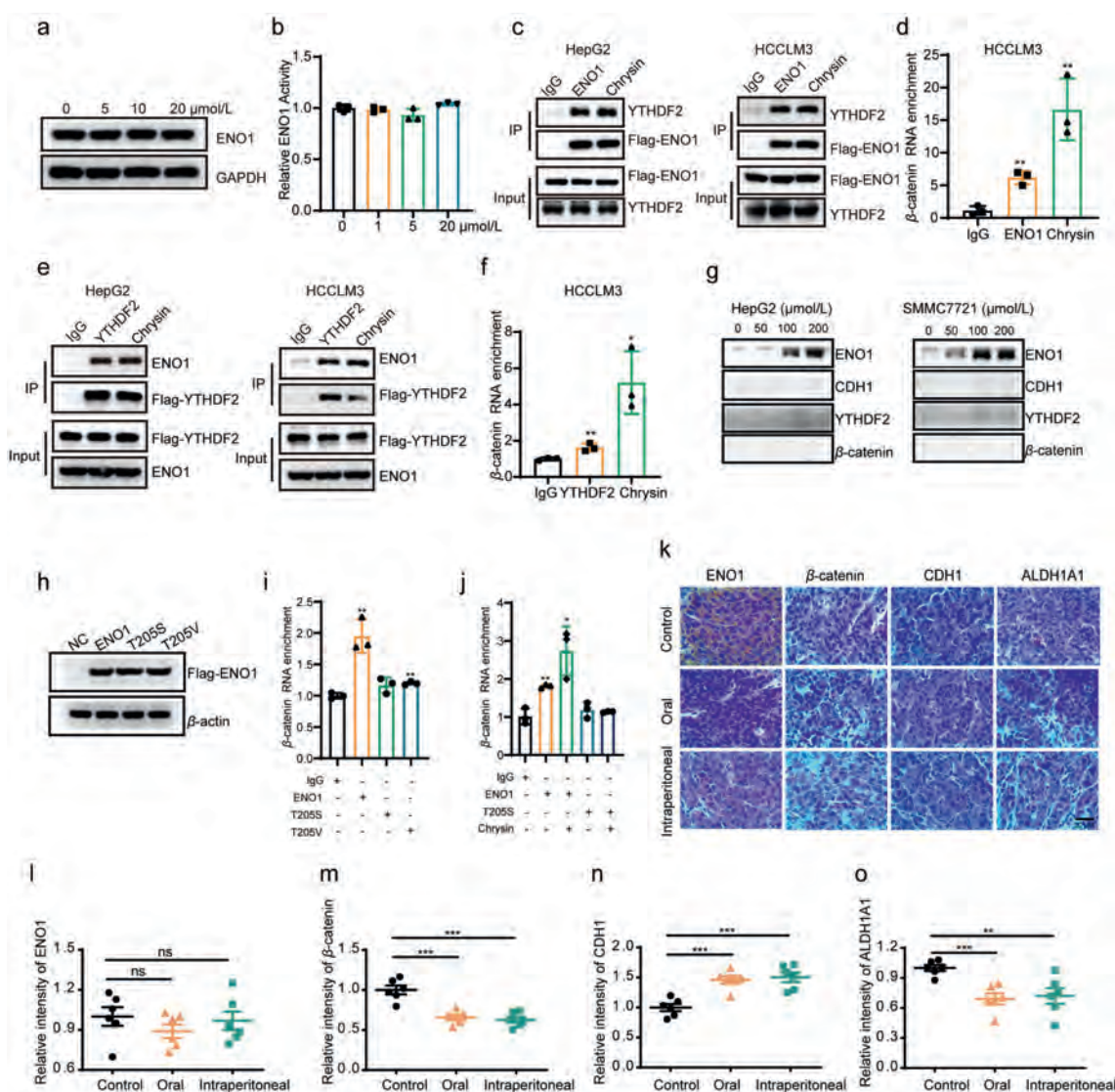


Figure 8 Chrysin promoted the RNA binding potential of ENO1 to reprogram mesenchymal-epithelial remodeling through β -catenin mRNA degradation. (a) The protein level and (b) enzyme activity of ENO1 after cells incubated with chrysin. (c) The co-IP analysis of the interaction between ENO1 and YTHDF2 after overexpressing ENO1 followed by chrysin treatment for 48 h. (d) RIP analysis of the binding between β -catenin mRNA and ENO1 after overexpressing ENO1 followed by chrysin treatment. (e) The co-IP analysis of the interaction between ENO1 and YTHDF2 after overexpressing YTHDF2 followed by chrysin treatment for 48 h. (f) RIP analysis of the binding between β -catenin mRNA and ENO1 after overexpressing YTHDF2 followed by chrysin treatment. (g) Immunoblotting analysis the specific binding of chrysin with ENO1, CDH1, YTHDF2 and β -catenin using compound **5** in cell lysate. (h) Immunoblotting analysis of the expression of wide type ENO1 and threonine 205 mutant ENO1 in HCCLM3 cells. (i) RIP analysis of the binding between β -catenin mRNA and ENO1 wide type, ENO1 mutant T205S and T205V in HCCLM3. (j) RIP analysis of the binding between β -catenin mRNA and ENO1 wide type or T205S after treated with chrysin for 48 h (k-o) The IHC assay of ENO1, β -catenin, CDH1, ALDH1A1 and corresponding statistical results. Original magnification, 400 $\times$; scale bar: 20 μ m.

interaction between ENO1 and YTHDF2 was performed by Co-IP assay. The result confirmed chrysin exhibited no effect on the association between ENO1 and YTHDF2 which suggested chrysin was not an ENO1/YTHDF2 protein-protein interaction promoter to reprogram mesenchymal-epithelial remodeling when using ENO1 to enrich YTHDF2 protein (Fig. 8c). Then the ENO1/ β -catenin mRNA interaction potential was analyzed after chrysin treatment by RIP-qPCR assay. The result revealed chrysin promoted the RNA binding potential of ENO1 to bind

more β -catenin mRNA (Fig. 8d). Moreover, the effect of chrysin on the association between ENO1 and YTHDF2 was further demonstrated using YTHDF2 to enrich ENO1 protein and the result also demonstrated chrysin exhibited no effect on the association between ENO1 and YTHDF2 (Fig. 8e). Then the RIP assay was performed after chrysin treatment and results suggested more YTHDF2 was recruited to identify the m6A methylated modification site to degrade β -catenin mRNA (Fig. 8f).

To better confirm whether chrysin produced off-target effects during mesenchymal–epithelial remodeling, IP assay using biotin coupled chrysin probe was performed. The results showed chrysin directly bound only ENO1, rather than β -catenin, YTHDF2, or CDH1 by Western blot assay (Fig. 8g). To clarify whether mutation of ENO1 at Thr205 affected the ability to bind to β -catenin mRNA, we overexpressed ENO1 and ENO1 mutant T205S, T205V in HCCLM3 and performed RNA immunoprecipitation (RIP) assay. The results showed ENO1 Thr205 point-mutation resulted in a significant decrease of β -catenin mRNA binding ability, which further illustrated the crucial site of ENO1 Thr205 in exerting RNA binding function (Fig. 8h–i). Moreover, chrysin enhanced the ability of wild-type ENO1 to recruit β -catenin mRNA, but the function of chrysin to promote β -catenin recruitment by ENO1 significantly decreased when Thr205 of ENO1 is mutated to valine (Fig. 8j). The results further demonstrated chrysin binding ENO1 Thr205 was the decisive event to reprogram mesenchymal–epithelial remodeling through mediating β -catenin mRNA degradation.

All these results demonstrated that chrysin promoted the ENO1/ β -catenin mRNA interaction to bind more β -catenin mRNA, recruited more YTHDF2 to degrade β -catenin mRNA, induced CDH1 gene expression to reverse cancer cell mesenchymal cell state to epithelial state. We further investigated the

effect of chrysin on ENO1, β -catenin, CDH1 and ALDH1A1 *in vivo*. The IHC assay indicated β -catenin and ALDH1A1 protein levels were significantly decreased while CDH1 was increased after the administration of the water solubility alternative of chrysin (Fig. 8k–o). The result further proved the conclusion chrysin promoted β -catenin mRNA degradation, induced *CDH1* gene expression and reprogrammed mesenchymal–epithelial remodeling.

In conclusion, chrysin covalently bound ENO1 Thr205 through a dehydration reaction, promoted ENO1/ β -catenin mRNA interaction, then recruited YTHDF2 protein to selectively degrade m6A modified β -catenin mRNA, and eventually reprogrammed cancer cell plasticity from mesenchymal state to epithelial state by increasing CDH1 gene expression (Fig. 9).

3. Discussion

Mesenchymal-epithelial remodeling for driving metastasis is an important cellular progress associated with the loss of epithelial features of cell–cell adhesion and acquirement of mesenchymal features of invasiveness and motility, which played a key role in regulating tumor metastasis and induced complicated tumor treatment progression like cancer stem cell acquisition, chemotherapy resistance, and adaptation to the tumor microenvironment^{5,26}.

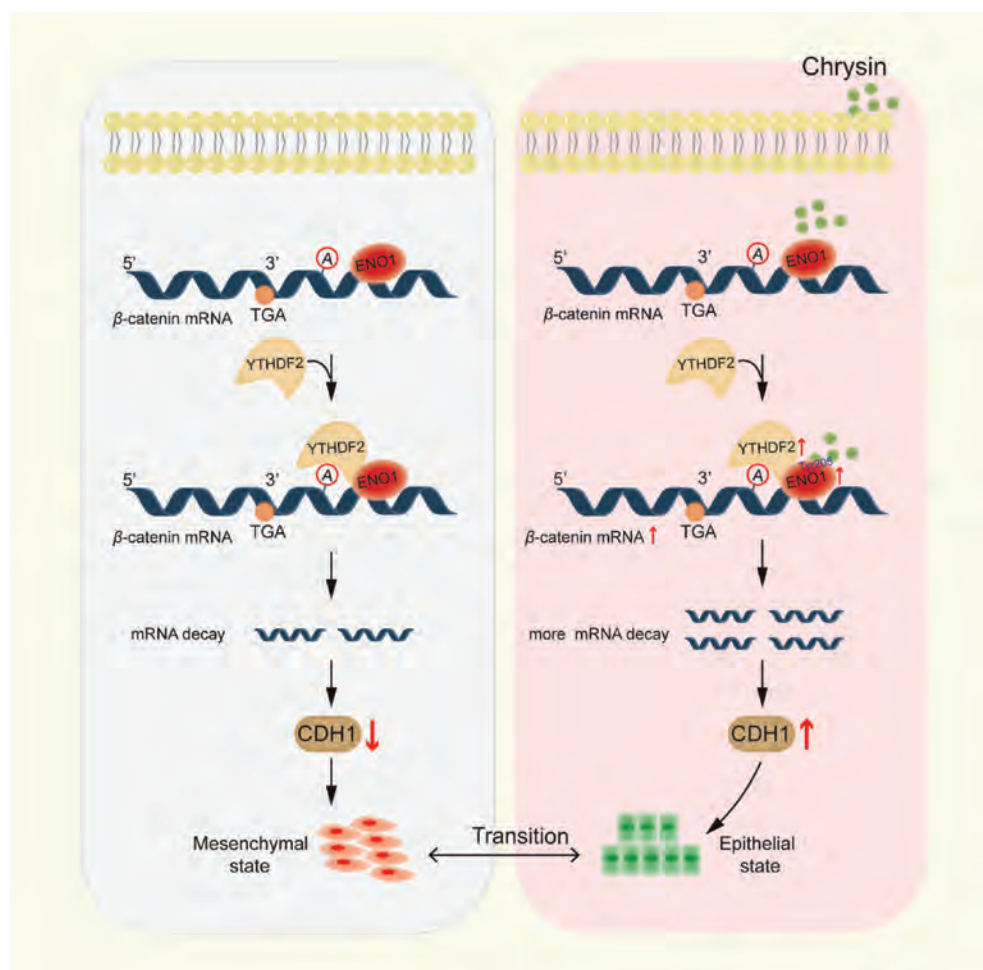


Figure 9 Schematic diagram of our proposed model of mechanism for how chrysin reprogrammed mesenchymal–epithelial remodeling by increasing *CDH1* gene expression.

CDH1 gene was a key tumor suppressor gene closely correlating the transition of cell epithelial state to mesenchymal state⁹. Chemotherapy increased *CDH1* gene expression and was considered as an important option in drug discovery. However, compounds specifically upregulating the *CDH1* gene expression to regulate cellular plasticity remain limited. And the monoclonal antibody targeting multiple cadherins including E-cadherin and OB-cadherin showed no effect on the formation of primary tumors within tissues²⁷. As a result, new candidate drugs targeting E-cadherin were urgently needed and should be developed. Hence, based on *CDH1* gene, we established a high-throughput screening system to identify natural product reprogramming cells from the mesenchymal state to epithelial state. Chrysin was then selected and exerted the ability of regulating cellular plasticity through regulating *CDH1* expression. Animal experiments including the CDX model and PDX model suggested chrysin showed excellent tumor-inhibiting effect without obvious toxicity.

Chrysin was a dietary phytochemical found in large quantities in a variety of plant extracts such as propolis, blue passionflower (*Passiflora edulis*), and honey²⁸. And chrysin showed multiple pharmacological activities as a promising bioactive flavonoid including cancer prevention, inflammatory disorders, oxidative stress, diabetes mellitus and so on²⁹. The mechanisms by which chrysin functions including NF- κ B signal pathway, ROS/RNS inducing, ERK/Nrf-2 signal pathway, TNF- α regulated signal pathway and DGK α /FAK interaction inhibition were revealed^{30,31}. However, the exact direct target of chrysin in mesenchymal-epithelial remodeling and other multiple pharmacological effects was currently unclear. To discover and identify the direct target of chrysin, we synthesized biotin coupled chrysin and revealed chrysin covalently and specifically bound the Thr205 of ENO1. The binding ability of ENO1 and chrysin was apparently decreased in Thr205-mutant ENO1 and 5-hydroxyl-covered chrysin. The result suggested chrysin formed a covalent complex with ENO1 via ether bonds and the hydroxyl of chrysin covalently bound the hydroxyl groups of ENO1 Thr205 through a dehydration condensation reaction. However, the exact mechanism of the dehydration reaction occurring remains unclear and we speculated the amino acids surrounding the active site of threonine 205 may promote the formation of reactive transition states and play important roles in the occurrence of the reaction. Until now, most covalent inhibitors have focused on targeting cysteine residues and have exhibited clinical benefits which included EGFR inhibitors and BTK inhibitors. The development of covalent inhibitors targeting lysine, serine, threonine and tyrosine has broadened the range of targeted amino acids³². Furthermore, the combination model that chrysin non-canonical covalently and specifically bound ENO1 Thr205 was different from other covalent inhibitors and threonine covalent binding agents which provided a new perspective on covalent targeting.

ENO1 was reported to be a potential cancer biomarker as a key glycolytic enzyme and plasminogen receptor. Besides these functions, ENO1 was also a candidate for maintaining mRNA stability as an RNA-binding protein^{23,33}. In the research, we discovered ENO1 regulated β -catenin mRNA stability as a RNA-binding protein. Moreover, we revealed ENO1 bound the 3'-UTR region of β -catenin mRNA which contained m6A methylation, and recruited the reader protein YTHDF2 to mediate β -catenin mRNA degradation. YTHDF family including YTHDF1, YTHDF2, YTHDF3 and YTHDC1 were the most studied "readers" proteins which bound and read m6A sites on mRNA to regulate diverse downstream signaling pathways³⁴. YTHDF1 promoted mRNA translation in a m6A dependent manner³⁵, YTHDF2 mediated the

mRNA degradation³⁶, YTHDF3 affects translation and degradation of m6A-modified mRNAs by interacting with YTHDF1 and YTHDF2³⁷, and YTHDC1 promoted exon inclusion in the nucleus³⁸. ENO1 could bind β -catenin mRNA and recruit YTHDF2 to recognize m6A sites to degrade β -catenin mRNA, and then the *CDH1* gene expression was improved through β -catenin mRNA stability regulation. As a result, ENO1/ β -catenin mRNA interaction, and ENO1/YTHDF2 protein-protein interaction might be promising targets for mesenchymal-epithelial remodeling.

After treated with chrysin, ENO1 and YTHDF2 protein-protein interaction showed no obvious changes, while ENO1 and β -catenin mRNA interaction was significantly enhanced. These results suggested chrysin covalently bound Thr205 of ENO1, promoted ENO1 and β -catenin mRNA 3'-UTR region interaction, then recruited YTHDF2 to recognize and degrade β -catenin mRNA and eventually reprogrammed mesenchymal-epithelial remodeling. To the best of our knowledge, chrysin is the first reported small molecule inducing mRNA degradation through binding to ENO1. Chrysin may be used as a natural lead compound for circumventing metastasis, recurrence, and drug resistance of HCC by reprogramming cellular plasticity which laid a solid material foundation for the further application of chrysin in clinical.

4. Experimental

4.1. Chemistry

Reagents were purchased at the highest commercial quality and used without purification. Solvents for chromatography were used as supplied by Tianjin Reagents chemical. Reactions were monitored by thin layer chromatography (TLC) carried out on silica gel plates using UV light as a visualizing agent and aqueous phosphomolybdic acid as a developing agent. 200–300 mesh silica gel purchased from Qingdao Haiyang Chemical Co., China and was used for column chromatography. ¹H NMR and ¹³C NMR were recorded on Bruker AV 400 and calibrated using internal references and solvent signals CHCl₃ (δ_H = 7.26 ppm, δ_C = 77.16 ppm), DMSO-*d*₆ (δ_H = 2.50 ppm, δ_C = 39.52 ppm) and MeOH-*d*₄ (δ_H = 3.34 ppm, δ_C = 49.86 ppm). ¹H NMR data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants and integration. High-resolution mass spectra (HRMS) were detected by Varian 7.0T FTMS.

Synthesis of compound 2. To a solution of chrysin **1** (2 g, 7.87 mmol, in 80.0 mL of acetone) was added K₂CO₃ (4.35 g, 31.5 mmol) and 1,2-dibromoethane (1.34 mL, 15.72 mmol). The mixture was refluxed for 4 h and then concentrated under reduced pressure. The crude product was purified by silica gel chromatography (CH₂Cl₂) to obtain **2** (2.27 g, 80%) as a colorless oil. Compound **2**: ¹H NMR (400 MHz, CDCl₃) δ 12.72 (s, 1H), 7.85 (d, *J* = 7.3 Hz, 2H), 7.51 (q, *J* = 7.8, 7.2 Hz, 3H), 6.64 (s, 1H), 6.49 (s, 1H), 6.34 (s, 1H), 4.34 (t, *J* = 6.2 Hz, 2H), 3.66 (t, *J* = 6.2 Hz, 2H) (Supporting Information Fig. S1); ¹³C NMR (100 MHz, CDCl₃) δ 182.5, 164.2, 164.0, 162.4, 157.8, 132.0, 131.3, 129.2, 126.4, 106.2, 106.0, 98.7, 93.4, 68.2, 28.4 (Supporting Information Fig. S2); HRMS (ESI) calculated for C₁₇H₁₄BrO₄⁺ [*M* + *H*]⁺: 361.0070, found 361.0069.

Synthesis of compound 3. To a solution of **2** (1 g, 2.78 mmol, in 50.0 mL of MeCN) was added *N*-methyl piperazine (0.68 mL, 6.11 mmol). The mixture was refluxed for 4 h, then concentrated

under reduced pressure. The crude product was purified by silica gel chromatography (5%–10% MeOH in CH₂Cl₂) to obtain amine (835.5 mg) as a colorless oil. Then the amine (835.5 mg, 2.2 mmol) was dissolved in THF (22 mL), and methane sulfonic acid (143 μ L, 2.20 mmol) was added. The reaction mixture was filtered and the filter cake was washed with 5 mL of THF, and dried in vacuum to give the compound **3** (1.04 g) in yield of 79% for two steps. Compound **3**: ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.76 (s, 1H), 9.58 (s, 1H), 8.05 (d, *J* = 7.3 Hz, 2H), 7.63–7.52 (m, 3H), 6.98 (s, 1H), 6.77 (d, *J* = 2.4 Hz, 1H), 6.38–6.33 (m, 1H), 4.22 (t, *J* = 5.3 Hz, 2H), 3.41 (s, 4H), 3.09 (s, 4H), 2.91–2.86 (m, 2H), 2.79 (s, 3H), 2.41 (s, 3H) (Supporting Information Fig. S3); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 182.0, 164.3, 163.4, 161.2, 157.3, 132.2, 130.5, 129.2, 126.4, 105.3, 105.0, 98.6, 93.3, 66.0, 55.1, 52.4, 49.6, 42.3, 39.8 (Supporting Information Fig. S4); HRMS (ESI) calculated for C₂₃H₂₈N₂O₇SNa⁺ [M + Na]⁺: 499.1510, found 499.1507.

Synthesis of compound 4. The synthetic procedures of compound **4** referred to those of **2**. Compound **4**: Yield, 85%. ¹H NMR (400 MHz, CDCl₃) δ 12.73 (s, 1H), 8.21–7.72 (m, 2H), 7.62–7.45 (m, 3H), 6.67 (s, 1H), 6.59 (d, *J* = 2.3 Hz, 1H), 6.45 (d, *J* = 2.2 Hz, 1H), 4.77 (d, *J* = 2.4 Hz, 2H), 2.60 (t, *J* = 2.4 Hz, 1H) (Supporting Information Fig. S5); ¹³C NMR (100 MHz, CDCl₃) δ 182.7, 164.3, 163.5, 162.4, 157.8, 132.0, 131.4, 129.3, 126.5, 106.4, 106.1, 99.1, 93.7, 77.6, 77.4, 76.7, 56.3, 29.84 (Supporting Information Fig. S6); HRMS (ESI) calculated for C₁₈H₁₃O₄⁺ [M + H]⁺: 293.0809, found 293.0805.

Synthesis of compound 5. To a solution of compound **4** (79 mg, 0.270 mmol, in DCM: MeOH = 2:1, 3.0 mL in total) were added commercially available **10** (72 mg, 0.162 mmol) and Cu₂O-NP (4.0 mg; the synthesis was followed by our previously reported procedure³⁹). The reaction mixture was stirred at room temperature for 24 h. The mixture was filtered through a pad of celite and the filtrate was concentrated under reduced pressure to obtain a residue. The residue was purified by silica gel chromatography (5%–15% MeOH in CH₂Cl₂) to obtain **5** (165.1 mg, 83%) as a white powder. Compound **5**: ¹H NMR (400 MHz, CDCl₃) δ 12.73 (d, *J* = 3.4 Hz, 1H), 7.88 (dd, *J* = 9.8, 4.5 Hz, 3H), 7.52 (d, *J* = 7.3 Hz, 3H), 6.77 (d, *J* = 4.9 Hz, 1H), 6.68 (d, *J* = 3.2 Hz, 1H), 6.63 (s, 1H), 6.61 (s, 1H), 6.43 (d, *J* = 3.6 Hz, 1H), 5.61 (s, 1H), 5.28 (t, *J* = 3.0 Hz, 2H), 4.57 (d, *J* = 5.2 Hz, 2H), 4.46 (d, *J* = 6.5 Hz, 1H), 4.27 (d, *J* = 7.1 Hz, 1H), 3.88 (d, *J* = 5.4 Hz, 2H), 3.58 (d, *J* = 11.6 Hz, 8H), 3.54–3.50 (m, 2H), 3.40 (d, *J* = 5.4 Hz, 2H), 3.10 (d, *J* = 6.4 Hz, 1H), 2.91–2.81 (m, 1H), 2.70 (d, *J* = 12.8 Hz, 1H), 2.18 (s, 2H), 1.75–1.56 (m, 4H), 1.40 (q, *J* = 8.3, 7.8 Hz, 2H) (Supporting Information Fig. S7); ¹³C NMR (100 MHz, CDCl₃) δ 182.6, 173.5, 164.3, 164.2, 164.1, 162.1, 157.9, 142.9, 132.1, 131.2, 129.2, 126.5, 124.6, 106.1, 106.0, 99.2, 93.4, 70.6, 70.4, 70.1, 70.1, 69.5, 62.5, 61.9, 60.3, 55.7, 50.5, 40.6, 39.2, 36.1, 28.3, 28.2, 25.8 (Supporting Information Fig. S8). HRMS (ESI) calculated for C₃₆H₄₅N₆O₉S⁺ [M + H]⁺: 737.2964, found 737.2965.

Synthesis of compound 6. To a solution of **2** (68 mg, 0.188 mmol, in 2.0 mL of acetone) was added K₂CO₃ (78 mg, 0.565 mmol) and dimethyl sulfate (71 μ L, 0.753 mmol). The mixture was refluxed for 4 h, then quenched the mixture with NH₄OH (1 mL of 10% solution in water) and remove acetone under vacuum. The crude product was purified by silica gel chromatography (5%–10% MeOH in CH₂Cl₂) to obtain **6** (65.6 mg, 93%) as a white solid. Compound **6**: ¹H NMR (400 MHz, CDCl₃) δ 7.89–7.81 (m, 2H), 7.50 (dd, *J* = 5.3, 1.9 Hz, 3H), 6.68 (s, 1H), 6.55 (d, *J* = 2.3 Hz, 1H), 6.41 (d,

J = 2.3 Hz, 1H), 4.39 (t, *J* = 6.1 Hz, 2H), 3.96 (s, 3H), 3.69 (t, *J* = 6.1 Hz, 2H) (Supporting Information Fig. S9); ¹³C NMR (100 MHz, CDCl₃) δ 177.6, 162.5, 161.2, 160.9, 159.9, 131.6, 131.4, 129.1, 126.1, 109.9, 109.3, 96.6, 93.5, 68.2, 56.7, 28.5 (Supporting Information Fig. S10); HRMS (ESI) calculated for C₁₈H₁₆BrO₄⁺ [M + H]⁺: 375.0227, found 375.0226.

Synthesis of compound 7. The synthetic procedure for the synthesis of compound **7** referred to those of **3**. Compound **7**: ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.44 (s, 1H), 8.03 (dt, *J* = 8.1, 2.6 Hz, 2H), 7.56 (h, *J* = 5.5, 4.4 Hz, 3H), 6.87 (d, *J* = 2.3 Hz, 1H), 6.77 (s, 1H), 6.48 (d, *J* = 2.3 Hz, 1H), 4.25 (d, *J* = 5.4 Hz, 2H), 3.82 (s, 3H), 3.40 (s, 4H), 3.09 (s, 4H), 2.86 (s, 2H), 2.79 (s, 3H), 2.39 (s, 3H) (Supporting Information Fig. S11); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 175.7, 162.8, 160.3, 159.6, 159.2, 131.5, 130.9, 129.1, 125.9, 108.4, 108.3, 96.6, 94.0, 66.0, 56.2, 55.3, 52.6, 50.2, 49.8, 42.3 (Supporting Information Fig. S12); HRMS (ESI) calculated for C₂₄H₃₀N₂O₇S⁺ [M + H]⁺: 513.1666, found 513.1668.

Synthesis of compound 8. The synthetic procedure for synthesis of compound **8** referred to those of **6**. Compound **8**: ¹H NMR (400 MHz, CDCl₃) δ 7.91–7.84 (m, 2H), 7.50 (dd, *J* = 5.1, 2.0 Hz, 3H), 6.69 (s, 1H), 6.67 (d, *J* = 2.3 Hz, 1H), 6.45 (d, *J* = 2.3 Hz, 1H), 4.80 (d, *J* = 2.4 Hz, 2H), 3.96 (s, 3H), 2.62 (t, *J* = 2.4 Hz, 1H) (Supporting Information Fig. S13); ¹³C NMR (100 MHz, CDCl₃) δ 177.7, 162.0, 161.2, 160.9, 159.8, 131.7, 131.4, 129.1, 126.1, 110.0, 109.3, 96.8, 94.1, 76.8, 56.7, 56.3 (Supporting Information Fig. S14); HRMS (ESI) calculated for C₁₉H₁₅O₄⁺ [M + H]⁺: 307.0970, found 307.0967.

Synthesis of compound 9. The synthetic procedure for synthesis of compound **9** referred to those of **5**. Compound **9**: ¹H NMR (400 MHz, MeOD) δ 8.21 (s, 1H), 7.92 (d, *J* = 7.3 Hz, 2H), 7.52 (d, *J* = 7.2 Hz, 3H), 6.89 (s, 1H), 6.65 (s, 1H), 6.52 (s, 1H), 4.62 (t, *J* = 5.0 Hz, 2H), 4.45 (dd, *J* = 7.9, 4.9 Hz, 1H), 4.25 (dd, *J* = 8.0, 4.4 Hz, 1H), 3.91 (t, *J* = 5.0 Hz, 2H), 3.86 (s, 3H), 3.59 (d, *J* = 4.4 Hz, 2H), 3.55 (d, *J* = 10.3 Hz, 6H), 3.48 (t, *J* = 5.6 Hz, 2H), 3.35 (d, *J* = 1.6 Hz, 3H), 3.32 (d, *J* = 5.7 Hz, 3H), 3.18–3.10 (m, 1H), 2.92–2.84 (m, 1H), 2.67 (d, *J* = 12.7 Hz, 1H), 1.61 (m, 4H), 1.37 (p, *J* = 7.7 Hz, 2H) (Supporting Information Fig. S15); ¹³C NMR (100 MHz, MeOD) δ 179.7, 176.0, 166.0, 164.8, 163.1, 162.1, 161.1, 132.8, 132.3, 130.2, 127.2, 126.6, 109.7, 108.9, 97.9, 95.4, 71.5, 71.4, 71.4, 71.2, 70.6, 70.3, 63.3, 63.0, 61.6, 57.0, 56.7, 51.5, 41.1, 40.3, 36.7, 29.7, 29.5, 26.8 (Supporting Information Fig. S16). HRMS (ESI) calculated for C₃₇H₄₇N₆O₉S⁺ [M + H]⁺: 751.3120, found 751.3118.

4.2. Cell culture

Human cancer cells were cultured in a humidified incubator with 5% CO₂ at 37 °C with DMEM medium containing 10% fetal bovine serum, 100 U/mL of penicillin and 100 μ g/mL of streptomycin.

4.3. MTT assay

Cells were planted into 96-well plates (4000 cells/well) and then treated with compounds at different concentrations for 72 h. Then 20 μ L MTT (5 mg/mL) was added to the cell medium for another 4 h. Then the medium was discarded and the precipitate was dissolved with DMSO. The absorbance was measured at 570 nm using a microplate reader. All experiments were repeated in independent triplicate and the results were calculated by GraphPad Prism 8.

4.4. Transfection of shRNA

β -Catenin shRNA purchased from Beijing Tsingke Biotech was co-transfected with pMD2.G, psPAX2 into HEK293T cells using Lip3000. Then lentiviral particles in supernatant were collected and filtered through a 0.45 μ m filter and added into cells. Then 5 μ g/mL puromycin was used to select the infected cells.

4.5. Transwell assay

3×10^4 cells after treatment were collected and directly seeded into the top chamber with serum-free DMEM (300 μ L). The lower chambers were filled with 700 μ L DMEM medium containing 20% FBS. After incubation for 48 h, cells were fixed using 4% paraformaldehyde and stained with crystal violet. Then wiped off the upper part cells and observed the migrated cells.

4.6. Wound healing assay

Cells were seeded into 6-well plate at a density of 5×10^5 cells per well. When reached a confluency of about 80%, parallel scratches were made with a 20 μ L pipette tip. Then the wound healing rate was observed under a microscope.

4.7. Tumorsphere assay

Cells were seeded into 24-well plate with low adhesion at a density of 1000 cells per well. Different concentrations of compounds were added and incubated for 7–10 days in a humidified incubator with 5% CO₂ at 37 °C. The tumorsphere number was recorded and photographed under a microscope.

4.8. Luciferase assay

Cells transfected with plasmid (pGL3-basic, pGL3-CDH1, Relia) were seeded into 96-well plates (4000 cells/well). After incubated with compounds for 48 h, the cells were collected and washed with PBS buffer. The luciferase activity of cell lysates was determined by a luciferase assay system in accordance with the manufacturer's protocol. The fluorescence intensity was detected with a microplate reader.

4.9. Flow cytometry assay

Cells were seeded into 24-well plates (5×10^4 cells/well) and treated with chrysin for another 48 h. After collected and washed with PBS buffer, the cells were incubated with APC-coupled CD133 antibody for 30 min in the dark at 37 °C. Then the cells were washed and resuspended by PBS buffer. The samples were analyzed by flow cytometry.

4.10. Western blot assay

Cells were collected and lysed with RIPA buffer. 40 μ g protein of each sample was separated on SDS-PAGE gel and transferred onto a PVDF membrane. Then the membrane was then blocked with 5% nonfat milk and incubated with following primary antibodies including anti-E-cadherin (Cell Signaling Technology, 14472, dilution 1:1000), anti-CD133 (Cell Signaling Technology, 64326, dilution 1:1000), anti-SOX2 (Cell Signaling Technology, 3579, dilution 1:1000), anti- β -catenin (Cell Signaling Technology, 8480, dilution 1:1000), anti-ENO1 (Proteintech, 11204-1-AP, dilution 1:2000),

anti-Flag (Proteintech, 20543-1-AP, dilution 1:5000). Then corresponding secondary antibodies was used to incubate for 2 h. The Western blot bands were quantified by ImageJ software after examined with ECL detection.

4.11. Animal assay

The animal experiments were approved by Experimental Animal Ethics Committee of Nankai University and conformed to the legal mandates and national guidelines for the care and maintenance of laboratory animals. For the HCC CDX model, subcutaneous injection of HCCLM3 cells (5×10^6 cells) into the armpit of nude mice was conducted. After about two weeks, compound **3** was intraperitoneally or orally administrated at a dose of 100 mg/kg every two days. The control group was injected with an equal volume of water. Body weight and tumor volume were measured every two days. HCC PDX model mice was supplied by Changliang Shan laboratory. The tumors from PDX model were cut into small pieces and subcutaneously inoculated in nude mice. Then the mice were divided into groups and given compound **3** orally or intraperitoneally at 100 mg/kg.

4.12. Immunohistochemical assay

The tissues were collected and fixed in 4% paraformaldehyde overnight. After gradient dehydration, these tissues were made into paraffin blocks and sectioned to 4 μ m thickness. Sections were treated with primary antibodies including CD133, CDH1, β -catenin and ALDH1A1 at 4 °C overnight after being deparaffinized and hydrated and washed with PBST (50 rpm, 5 min each time, 3 times). Next, secondary antibodies were used to treat these sections for another 1 h. In addition, sections were developed by DAB and counterstained with hematoxylin. Neutral balsams were eventually used to cover these sections after gradient dehydration.

4.13. Real-time quantitative PCR

Total RNA was extracted with Trizol reagent (SparkZol Reagent, AC0101-B) and then reversed using BioRT Master HiSensi cDNA First Strand Synthesis kit (BSB40M1). RT-qPCR assay was conducted using 2 $\times$ SYBR qPCR Mix.

4.14. RNA immunoprecipitation (RIP) assay

Cells were collected and RNA immunoprecipitation (RIP) kit (BersinBio Bes5101) was used to perform the following assay. Cell lysate was collected and incubated with flag antibody (Proteintech, 20543-1-AP) and beads overnight at 4 °C. After washing the beads for three times, the RNA in the immune-precipitate was extracted using trizol reagent. The RT-qPCR was performed to analyze the β -catenin mRNA.

4.15. m6A methylated RNA immunoprecipitation-qPCR

Total mRNA in HCCLM3 was extracted by trizol reagent and Methylated RNA Immunoprecipitation Kit (BersinBio Bes5203-2) was used for experiments. Briefly, total mRNA was incubated with m6A antibody and beads with RNA inhibitor overnight at 4 °C. The beads were washed three times and the mRNA in the immune-precipitates were extracted by trizol reagent and analyzed by RT-qPCR.

4.16. Binding site assay

Recombinant ENO1 was incubated with compound **3** at room temperature overnight. Then SDS-PAGE was used to separate the mixture and a specific ENO1 band was excised. Then trypsin was used to digest the specific band and nano-LC-MS/MS experiments were conducted to determine the binding site using Triplequad Ion-trap and Orbitrap fusion (Thermo Fisher Scientific, USA).

4.17. Cellular thermal shift assay

Cell lysate or recombinant ENO1 was incubated with chrysin at room temperature for 3 h and heated at different temperatures individually for 3 min. Then mixture was centrifuged at $12,000\times g$ for 10 min and Western blot was performed to analyze the supernatants.

4.18. Surface plasmon resonance assay

The interaction between ENO1 and chrysin was examined by Biacore 8K at 25 °C. The recombinant ENO1 was immobilized on a COOH5 sensor chip through amine coupling. Then a series concentration of chrysin from 100 to 6.25 $\mu\text{mol/L}$ diluted in PBS buffer containing 5% DMSO was injected as analytes. The increased RU indicated the binding intensity between the immobilized ENO1 protein and chrysin.

4.19. Microscale thermophoresis assay

Chrysin was diluted in buffer containing 5% DMSO. The purified recombinant ENO1 labeled with NT-647-NHS was added to each dilution and incubated for 30 min. Then silica capillaries (NanoTemper Technologies, Germany) were used to load incubated chrysin-ENO1 samples and the results were measured with Monolith NT.115. The data was analyzed by MO. Affinity Analysis software.

4.20. Plasmids and transfections

We constructed ENO1 knockdown HCC cells using lentiviral which was collected from supernatant medium of HEK 293T cells after co-transfected with lentiviral shRNA and plasmids psPAX2, pMD2.G. The overexpression plasmid pLVX3-ENO1 was kindly provided by Changliang Shan group at Nankai University. And the pLVX3-YTHDF2 was constructed in our laboratory. For transient transfection, cells were transfected with plasmid or siRNA using Lipofectamine 3000 Transfection Reagent (Invitrogen, CA, USA).

4.21. Mutant plasmid construction

ENO1 gene was subcloned into pET-28a vector and the mutant plasmids were obtained using Fast site-directed mutagenesis kit according to the manufacturer's instructions.

4.22. Protein expression and purification

E. coli strain BL21 containing plasmid pET28a-ENO1, pET28a-T205S-ENO1, pET28a-T205V-ENO1 were cultured in LB medium (kanamycin 10 $\mu\text{g/mL}$). Isopropyl β -D-1-thiogalactopyranoside (IPTG) (0.5 mmol/L) was added into *E. coli* strain BL21 when the OD value is 0.5–0.8 at 600 nm. Then *E. coli* strain BL21 were cultured at 16 °C for 16–20 h. Precipitates of BL21 were then collected in lysis buffer (20 mmol/L PBS, pH = 7.5; 400 mmol/L

NaCl; 10 mmol/L imidazole) and cracked with ultrasonic crusher. Then Ni-beads column was used to fill the supernatants and washed with wash buffer (20 mmol/L PBS, pH = 7.5; 400 mmol/L NaCl; 20 mmol/L imidazole). Finally, the proteins were obtained after Ni-beads column eluted with elution buffer (20 mmol/L PBS, pH = 7.5; 400 mmol/L NaCl; 250 mmol/L imidazole).

4.23. Kinetic determination

Recombinant ENO1 was incubated with biotin-coupled chrysin for different times and the conjugate ability was detected by Western blot assay. The gray value of specific protein band was analyzed by Image J. And Kobs of chrysin binding ENO1 was calculated by Eq. (1):

$$[\text{ENO1}]_t = [\text{ENO1}]_0 \times E - k_{\text{obs}} \times t \quad (1)$$

$[\text{ENO1}]_0$ means the total concentration of input-ENO1, and $[\text{ENO1}]_t$ means the concentration of ENO1 at time t .

4.24. Acute toxicity assay

BALB/c mice were administrated with compound **3** at a dose of 200 mg/kg orally or intraperitoneally for 24 h. Then the behaviors, body and organ weights of mice were recorded and the levels of ALT, AST, CR was examined using ELISA. And we fixed the organs with 4% polyformaldehyde and the tissues were then dehydrated, waxed, and sliced to 4 μm . Then followed by dewaxed, hydrated and stained by HE. The morphological change was observed under microscope.

4.25. Statistical analysis

Data were analyzed using GraphPad Prism 8 and are presented as mean $\pm$ SD. T test was used to compare two groups. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$, $****P < 0.001$.

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Author contributions

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Conflicts of interest

The authors declare no competing financial interests.

Appendix A. Supporting information

Supporting information to this article can be found online at <https://doi.org/10.1016/j.apsb.2024.07.013>.

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