

Supplementary Materials and Methods

Western Blotting

Trophoblasts were washed twice with ice-cold PBS and proteins were isolated using 1% Triton X-100 reagent (Sigma-Aldrich; Merck, Germany) on ice. The lysate was centrifuged at 12000 rpm/min at 4 °C for 25 min. The supernatant was obtained, and the protein concentration was detected using a BCA kit (Pierce, Rockford, IL, USA). Equal amounts of protein were subjected to 10% SDS polyacrylamide gel electrophoresis, followed by electrotransferring onto PVDF membranes (Pall Corporation, Ann Arbor, MI, USA). Then, 5% fat-free milk powder in TBS with 0.1% tween-20 (Sigma-Aldrich; Merck, Germany) was used to block the PVDF membranes for 1 h. Antibodies recognizing ALKBH5 (Novus, NBP1-82188) and CYR61 (Abcam, ab24448) were used to perform western blot analysis using standard techniques. GAPDH (Abcam, ab181602) was detected as loading control.

Total RNA methylation quantification

Total RNA methylation were quantified using the EpiGentek (EpiQuik m⁶A RNA Methylation Quantification Kit; #P-9005) according to the manufacturer's instructions. In this experiment, abundance of m⁶A marks was quantified by absorbance and reported as relative values to the negative and positive controls, an RNA containing no m⁶A and an m⁶A oligo normalized to have 100% of m⁶A. The global m⁶A level in RNA was determined in 200 ng aliquots of total RNA extracted from recurrent miscarriage patients and healthy controls. Total RNA samples were then normalized against the total amount (ng) of RNA loaded. All assays were performed in triplicate and biological replicates were completed at least three times.

Immunohistochemistry

Immunohistochemical staining was performed as previously described [1]. In brief, tissue slides were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 20 min. Slides were blocked with 5% FBS for 30 min and incubated with rabbit anti-ALKBH5 (dilution 1:100, Novus, NBP1-82188) at 4°C overnight.

After washing in PBS, the sections were incubated with biotinylated secondary antibody and stained by using a Mouse and Rabbit Specific HRP/DAB IHC Detection Kit (ab236466, Abcam, Cambridge, MA, USA). Meyer's hematoxylin (Sigma-Aldrich, St. Louis, MO) was used as a counterstain dye. A negative control was obtained by replacing the primary antibody with PBS. Images were captured with the Leica microscope (Leica, Buffalo Grove, IL).

Luciferase reporter assay

Luciferase reporters were generated by cloning 3'UTR of CYR61 or 3'UTR of CYR61 (mutant A to T) RNA into psiCHECK2 (Promega Corporation, USA). Then, HTR-8 cells (2.5×10^4 in each well of 24-well plate) were cotransfected with 150 ng of renilla reporters and siALKBH5 or siCtrl, with 1.5 μ L of the jetPRIME reagent. HTR-8 cells lysates were harvested 24 h after transfection. Luciferase activities were detected using the Dual-Luciferase® Reporter Assay System (Promega) according to manufacturer's protocol.

Invasion Assay

We evaluated the invasive ability of trophoblasts objectively across the extracellular matrix (ECM) using the Transwell Matrigel invasion assay, as previously described for trophoblasts [2]. In brief, cell culture inserts (pore size, 8 μ m; diameter, 6.5 mm; Corning) were coated with 25 μ L of Matrigel™ (Corning, New York, USA) and placed in a 24-well plate. Two sets of invasion assays were performed: HTR-8 cells were transfected with siCtrl, siALKBH5, siCYR61, control vector, CYR61 or the ALKBH5 expressing-vector and cultured for 48 hour. Then, 1.2×10^5 cells/200 μ L of DMEM were placed into the upper chamber of each insert. The lower chambers were filled with 800 μ L of DMEM containing 15% FBS, and the cells were incubated at 37°C for 48 hour. The inserts were removed, washed in ice-cold PBS, and the non-invading cells, together with the ECM, were removed from the upper surface of the filter by wiping with a cotton bud. The cells on the lower surface of the inserts were fixed in 4% paraformaldehyde, stained with crystal violet, and observed using an inverted phase-contrast microscope (Leica). The number of cells that had invaded the lower surface was counted at a magnification of $\times 200$. To eliminate individual variability, the results were assessed by two independent researchers, and the invasive index was calculated as the proportion of the

invading cells in each experimental group expressed relative to the appropriate control cells. Each experiment was performed in duplicate and the experiments were independently repeated three times.

Library preparation for ALKBH5 knockdown Transcriptome sequencing

A total amount of 3 µg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEB Next First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3'ends of DNA fragments, NEB Next Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 250~300 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 µl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

Clustering and sequencing

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform and 125 bp/150 bp paired-end reads were generated.

Quality control

Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. At the same time, Q20, Q30 and GC content the clean data were calculated. All the downstream analyses were based on the clean data with high quality.

Reads mapping to the reference genome

Reference genome and gene model annotation files were downloaded from genome website directly. Index of the reference genome was built using Hisat2 (v2.0.5) and paired-end clean reads were aligned to the reference genome using Hisat2 (v2.0.5). We selected Hisat2 as the mapping tool for that Hisat2 can generate a database of splice junctions based on the gene model annotation file and thus a better mapping result than other non-splice mapping tools.

Quantification of gene expression level

FeatureCounts v1.5.0-p3 was used to count the reads numbers mapped to each gene. And then FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. FPKM, expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced, considers the effect of sequencing depth and gene length for the reads count at the same time, and is currently the most commonly used method for estimating gene expression levels.

Differential expression analysis

Differential expression analysis of two conditions/groups (two biological replicates per condition) was performed using the DESeq² R package (1.16.1). DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted *P*-value < 0.05 found by DESeq² were assigned as differentially expressed.

Prior to differential gene expression analysis, for each sequenced library, the read counts were adjusted by edgeR program package through one scaling normalized factor. Differential expression analysis of two conditions was performed using the edgeR R package (3.18.1).

The *P* values were adjusted using the Benjamini & Hochberg method. Corrected *P*-value of 0.05 and absolute fold change of 2 were set as the threshold for significantly differential expression.

References:

1. Tian FJ, He XY, Wang J, Li X, Ma XL, Wu F, et al. Elevated Tristetraprolin Impairs Trophoblast Invasion in Women with Recurrent Miscarriage by Destabilization of HOTAIR. *Mol Ther Nucleic Acids*. 2018;12:600-609.
2. Zhang Y, Jin F, Li XC, Shen FJ, Ma XL, Wu F, et al. The YY1-HOTAIR-MMP2 Signaling Axis Controls Trophoblast Invasion at the Maternal-Fetal Interface. *Mol Ther*. 2017;25:2394-2403.

Supplementary data

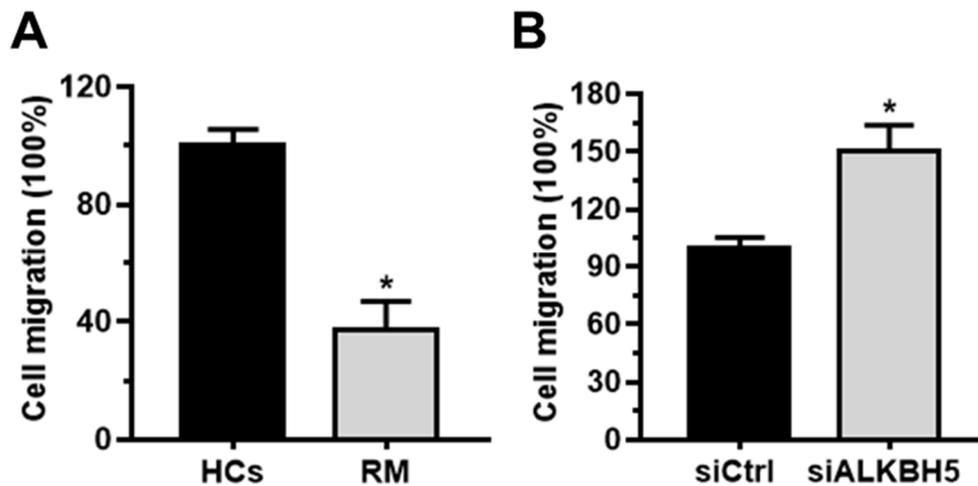


Figure S1: (A) Extravillous explants were obtained from HCs and RM patients at 6-10 weeks of gestation and cultured on Matrigel. Serial pictures of the explants were taken under a light microscope after 24 h and 72 h of culture in vitro. Statistical assay of the migration distance of villous tips (%). Data are presented as means \pm SD of three independent experiments. (B) Extravillous explants from RM patients at 6-10 weeks of gestation were maintained in culture on Matrigel. Serial pictures of the explants transfected with siALKBH5 or siCtrl were taken under a light microscope after 24 h and 72 h of culture in vitro. Statistical assay of the migration distance of villous tips (%). Data are presented as means \pm SD of three independent experiments.

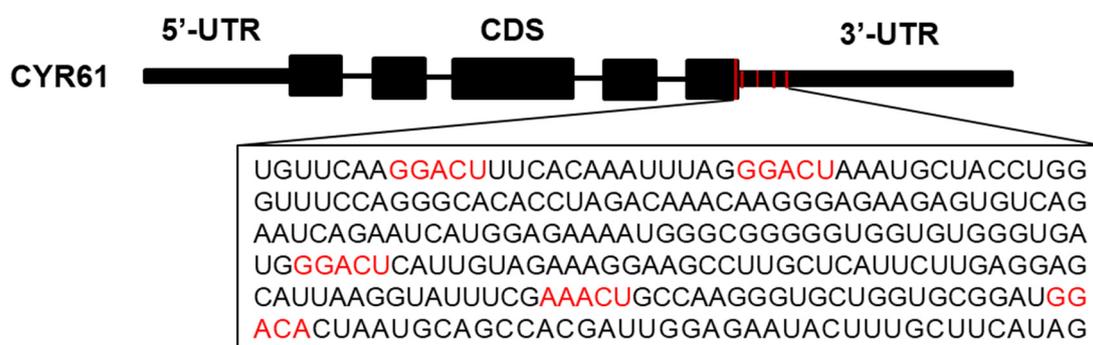


Figure S2: Schematic representation of the position of m⁶A motifs (RRACH) within *CYR61* mRNA.

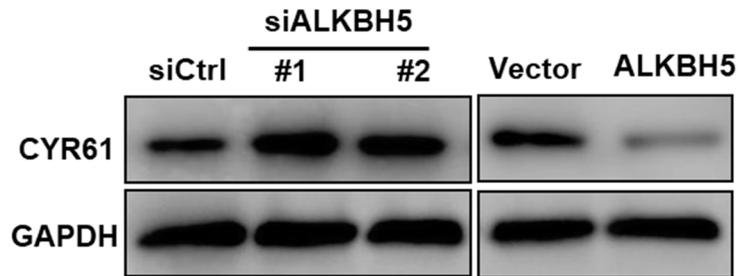


Figure S3: Western blotting analysis of CYR61 expression in JEG-3 cells transfected with siCtrl, siALKBH5 #1, siALKBH5 #2, control vector, or ALKBH5-expressing plasmid after 48 h.

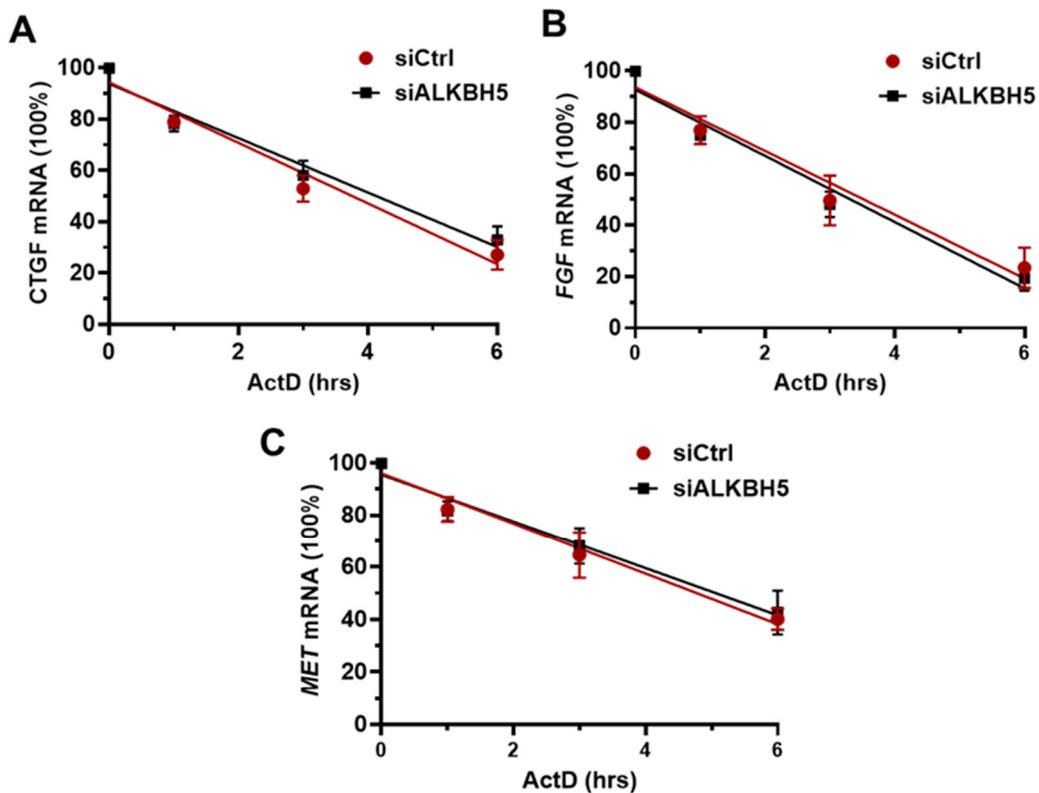


Figure S4: HTR-8 cells were transfected with siCtrl or siALKBH5 for 36 h; a time course for RNA stability was initiated by adding an RNA-Polymerase II inhibitor [actinomycin D (5

$\mu\text{g/mL}$)]. Cells were harvested at the indicated time points. Gene expression levels were normalized to that at “0 h”, and *GAPDH* was used as the reference gene. The results are shown as the mean of at least three independent experiments. $*P < 0.05$ compared with the vector or siCtrl.

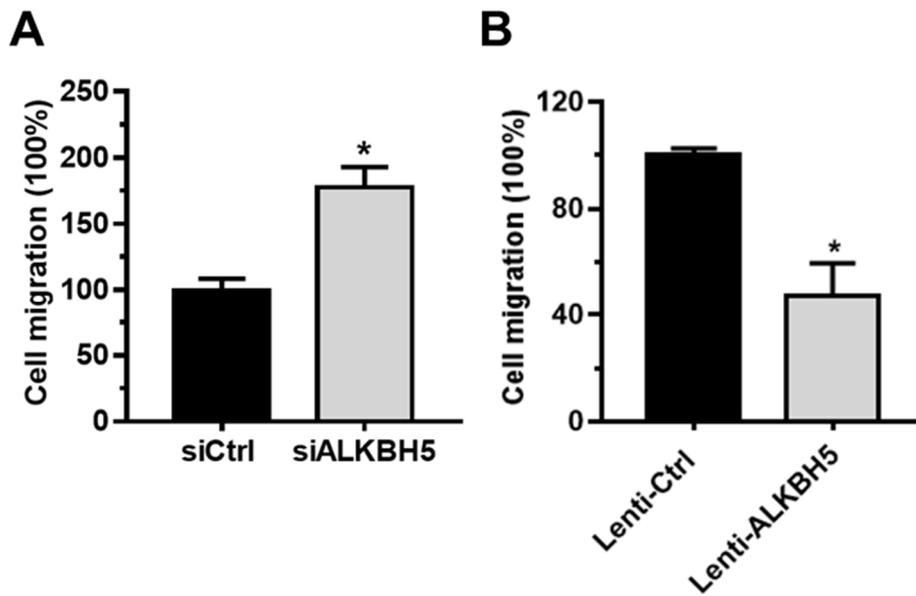


Figure S5: (A) Extravillous explants from HC at 6-10 weeks of gestation were maintained in culture on Matrigel. Serial pictures of the explants transfected with siALKBH5 or siCtrl were taken under a light microscope after 24 h and 72 h of culture in vitro. Statistical assay of the migration distance of villous tips (%). Data are presented as means \pm SD of three independent experiments. (B) Extravillous explants were obtained from HCs at 6-10 weeks of gestation and cultured on Matrigel. Serial pictures of the explants transfected with lenti-Ctrl or lenti-ALKBH5 lentivirus were taken under a light microscope after 24 h and 72 h of culture in vitro. Statistical assay of the migration distance of villous tips (%). Data are presented as means \pm SD of three independent experiments.

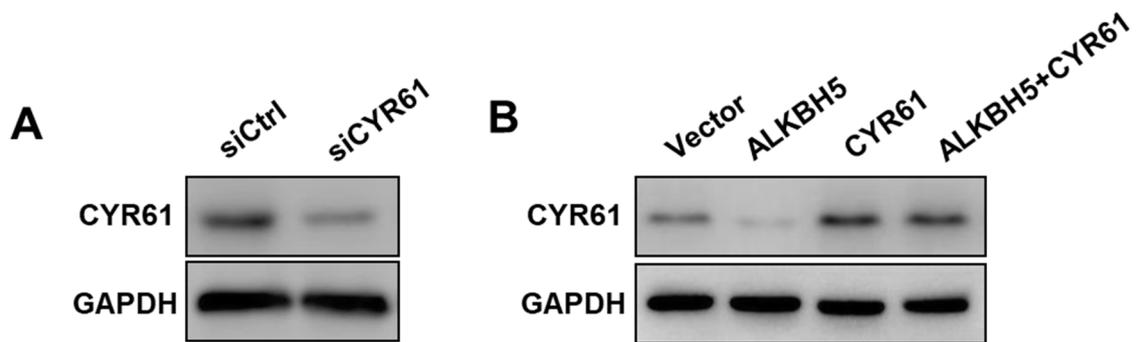


Figure S6: (A) Western blotting analysis of CYR61 expression in HTR-8 cells were transfected with siCtrl or siCYR61 after 48 h. (B) Western blotting analysis of CYR61 expression in HTR-8 cells transfected with control vector, ALKBH5-, CYR61 or ALKBH5+CYR61-expressing plasmid after 48 h.

Supplementary Primer:

Real-time PCR primer:

METTL3 F: GCAGGCTCAACATACCCGTA
METTL3 R: GATGCGTTGCAGTTGATTTGTCT
METTL14 F: ACTCTACCATTGAGGAATGCAGAT
METTL14 R: CCCATCTGGCTTGATGTAGTCT
WTAP F: GGAAGGTGAACTGGAACAGACT
WTAP R: GCCTTCCAAGCTCTTGATTCT
YTHDF2 F: GGATCTGTACATCAAAGGATGGAT
YTHDF2 R: GCTCAGCTGTCCATAAGAAGTTAAGT
YTHDC1 F: AGATGGGTCTGTCAGATCTGGT
YTHDC1 R: TCTGAACCTGCATATGACTCTGAT
FTO F: GCTGGCATCATGATGAAAATCT
FTO R: TGTGTCTCTATGTCCCATGAGATCT
ALKBH5 F: TCACTGCATACGGCCTCAGGACAT
ALKBH5 R: TTAGAGCAGGGTCCCTGTTGT
RBM15 F: CAGGGACCTTTATCCTGACTCT
RBM15 R: GCAGATCTCGATCACCTCTGT
ZC3H13 F: ATGCAACAGGGATCGAGATAGAT
ZC3H13 R: TCTGTCTCTGTCTCTGTCCCTGT
YTHDF1F: CAAGCACACAACCTCCATCTTCG
YTHDF1R: GTAAGAACTGGTTCGCCCTCAT
WTAP F: GTACAAGCTTTGGAGGGCAAGT
WTAP R: TGGACTTGCTTGAGGTAAGTGA
METTL16 F: ACAGAAGACACTCCTGATGG
METTL16 R: TTAACAGAACTAGGCGGAGG
FN1 F: ATAGGTGAGGAAATCCAAATTGGT
FN1 R: CTGTACTCAGAAGTGTCTGGAAT
TPM4 F: ACGAGGAGGTAGCTCGTAAGCT
TPM4 R: GTTCTTCTTCCAGGTCACACAT
SUMO3: CGTCCTCTCTTTGATGTACTTCAAGT
SUMO3: GTACCCACAATATCTTGCAACTCAT
FGF1 F: AAAGAGTACCGAGACTGGCCAGT
FGF1 R: TGTAATGGTTCTCCTCCAGCCT
CTGF F: AAGCTGACCTGGAAGAGAACAT
CTGF R: CTCCACAGAATTTAGCTCGGTAT
MET F: TGCAAGAACTGTATGCTGGAT

MET R: ATTATCCACTTCACTGGCAGCT
CYR61 F: CTTACGCTGGATGTTTGAGTGT
CYR61 R: AGACTGGATCATCATGACGTTCT
COL5A2 F: GTGATGACCTAAAGCTTTGCCAT
COL5A2 R: CAGGTTTTACGTGGTACACTGGAT
CAV1 F: ACCACCTTCACTGTGACGAAAT
CAV1 R: GCATGGTACAACCTGCCAGAT
YY1 F: AGAATAAGAAGTGGGAGCAGAAGC
YY1 R: ACGAGGTGAGTTCTCTCCAATGAT
CGB F: AAAGGAGCGCCATGGATTAC
CGB R: CCATTACTGTGACCCTGTTA
GAPDH F: CACTGGGCTACACTGAGCAC
GAPDH R: AGTGGTCGTTGAGGGCAAT