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Hepatocyte Nuclear Factor 4α Transcriptionally Activates *TM4SF5* through the DR1 Motif*

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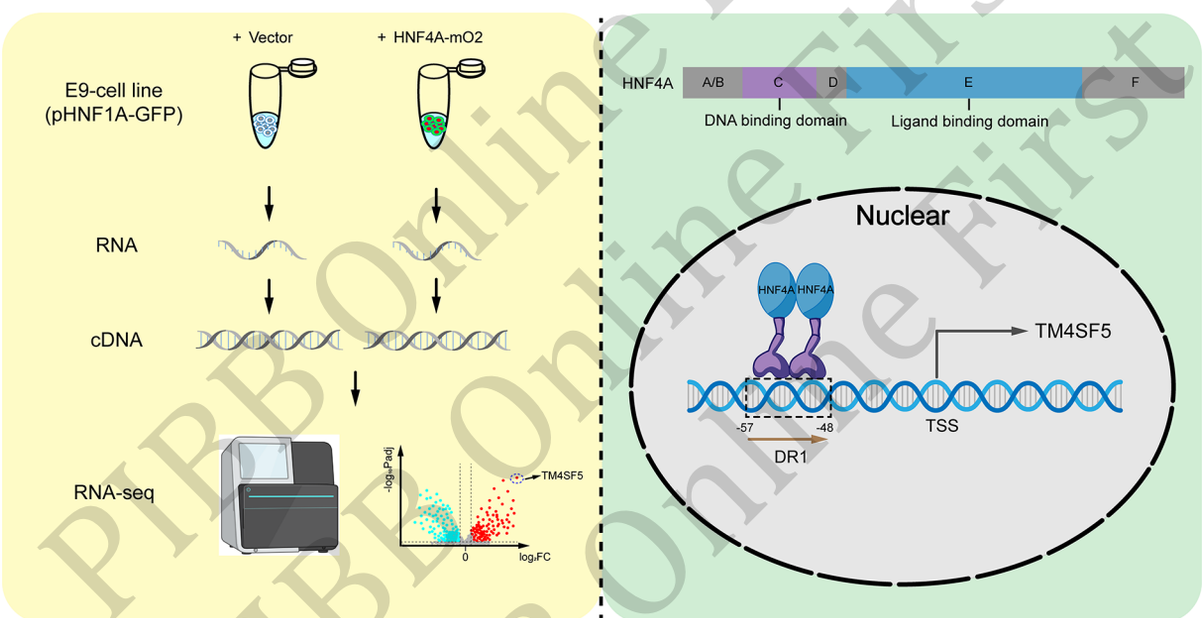
Graphical abstract



Abstract **Objective** Hepatocyte nuclear factor 4-alpha (HNF4A) is a critical transcription factor in the liver and pancreas. Dysfunctions of HNF4A lead to maturity onset diabetes of the young (MODY1). Notably, MODY1 patients with *HNF4A* pathogenic mutations exhibit decreased responses to arginine and reduced plasma triglyceride levels, but the mechanisms remain unclear. This study aims to investigate the potential target genes transcriptionally regulated by HNF4A and explore its role in these metabolic pathways. **Methods** A stable 293T cell line expressing the *HNF1A* reporter was overexpressed with HNF4A. RNA sequencing (RNA-seq) was performed to analyze transcriptional differences. Transcription factor binding site prediction was then conducted to identify HNF4A binding motifs in the promoter regions of relevant target genes. **Results** RNA-seq results revealed a significant upregulation of transmembrane 4 L six family member 5 (TM4SF5) mRNA in HNF4A-overexpressing cells. Transcription factor

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binding predictions suggested the presence of five potential HNF4A binding motifs in the *TM4SF5* promoter. Finally, we confirmed that the DR1 site in the (−57 to −48) region of the *TM4SF5* promoter is the key binding motif for HNF4A. **Conclusion** This study is the first to identify *TM4SF5* as a target gene of HNF4A and to determine the key binding motif involved in its regulation. Given the role of TM4SF5 as an arginine sensor in mTOR signaling activation and triglyceride secretion, which closely aligns with phenotypes observed in MODY1 patients, our findings provide novel insights into how HNF4A regulates triglyceride secretion in the liver and arginine-stimulated insulin secretion in the pancreas.

Key words *HNF4A*, RNA-seq, *TM4SF5*, DR1 motif, MODY1
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Hepatocyte nuclear factor 4-alpha (HNF4A) is a transcription factor that belongs to the steroid/thyroid hormone receptor superfamily. Initially identified as a liver-enriched transcription factor, HNF4A is also expressed in the kidney, pancreas, gastrointestinal tract, and epithelia tissues[1-3]. Dysfunction in HNF4A has been reported to be associated with diabetes, including maturity onset diabetes of the young (MODY1), which is characterized by impaired glucose-dependent insulin secretion[4]. Unlike diabetes mellitus type 2, patients with MODY1 also exhibit diminished responses to arginine and decreased plasma triglyceride levels[5-6]. While the relationship between HNF4A and diabetes is well-established, further research is necessary to fully elucidate the underlying mechanisms involved.

HNF4A primarily binds to the promoter region of target genes and activates their transcription, thus playing a key regulatory role in diabetes. The transactivation of *HNF1A* by HNF4A is crucial for glucose transport, insulin production, and insulin secretion[7-8], with abnormalities leading to hyperglycemia in MODY1 patients. The ATP-sensitive K+-ATP channel protein Kir6.2 is a transcriptional target of HNF4A, with deficiencies impacting insulin secretion in MODY1 patients[4, 9]. Additionally, HNF4A directly transactivates the transcription of *APOC3*[10], and its down-regulation leads to triglyceride accumulation in the liver, inducing non-alcoholic fatty liver disease (NAFLD) in MODY1 patients[11]. In summary, HNF4A exerts its regulatory effects in diabetes by transcriptionally activating the expression of target genes, with many potential target genes yet to be discovered.

The transcriptional activation of target genes by HNF4A relies on the presence of DR1-like motifs (AGGTCAxAGGTCA) within the promoter region[12]. RNA interference of *HNF4A* in HepG2 cells, coupled with ChIP-seq, revealed that many genes contain at least one predicted DR1 site[13]. Moreover, HNF4A also binds to the HNF4-specific binding motif (H4-SBM, xxxxCAAAGTCCA), which operates independently of other transcription factors[12]. The binding motif of novel target genes of HNF4A are likely complex and require further investigation.

Here, we identified *TM4SF5*, as a new target gene of HNF4A by analyzing RNA-seq data from HNF4A-overexpressing cells. Transient transfection of truncated and mutated *TM4SF5* promoters determined that the DR1 site located in the proximal region (− 57 to − 48) is the key binding motif for HNF4A-dependent activation of *TM4SF5*. These findings provide evidence for the direct regulation of *TM4SF5* by HNF4A, and offer novel insights into HNF4A regulation of triglyceride secretion in the liver and arginine-stimulated insulin secretion in the pancreas.

1 Material and methods

1.1 Cell culture and transfection

Human embryonic kidney (HEK) -293T cells were cultured in DMEM containing L-glutamine, supplemented with 10% FBS, and 1% penicillin/streptomycin. The stable cell line E9 (integrated pHNF1A-GFP) was constructed in a previous study [14]. Cells were washed with PBS (pH 7.4) and dissociated with TrypLE Express. For transient transfection, cells were plated in 24 well plate and were transfected with plasmids using Lipofectamine 3000 (Thermo).

1.2 Cloning and construction of plasmids

Human *HNF4A* isoform2 cDNA (GenBank: NM_000457) was PCR amplified from pcDNA3.1-Flag-HNF4A. *HNF4A* pathogenic mutations R85W and M373R were introduced by site-directed mutagenesis, cDNA fragments were inserted directly into the *Not*I

and *BamH*I sites of pQCXIP-mOrange2 (mO2).

Human *TM4SF5* promoters were designed according to the Eukaryotic Promoter Database (EPD), and was PCR amplified from human genomic DNA using the primers listed in Table S1. The resulting *TM4SF5* promoter PCR product (− 2 000 to +100) was digested with *Xho*I and *Hind* III and then ligated into pGL3-GFP plasmid in which the luciferase was substituted to GFP in pGL3-Basic background. The truncated promoters (− 1 000 to +100 and -100 to +100) were amplified from full-length promoter (− 2 000 to +100). Disruption of the DR1 site in the *TM4SF5* (− 100 to +100) promoter were done by site-directed mutagenesis using the primers listed in Table S1.

1.3 Live−cell imaging and fluorescent quantification

Cells were imaged with the Opera Phenix High Content Screening System in confocal mode with the ×20 water NA 1.0 objectives at 37°C and 5% CO_2 at the indicated time. The fluorophores were detected with the following excitation and emission (Ex/Em) wavelengths: Hoechst 33342 (405/435 − 480), mOrange2 (561/570 − 630) and GFP (488/500 − 550). Quantification analyses were performed using the Harmony software. All experiments were conducted three times independently, data was shown in the Table S2.

1.4 Total RNA isolation and RNA−seq analysis

Total RNA of cells overexpressing wild-type *HNF4A* (OE-HNF4A) or pathogenic variants were extracted using the TRIzol reagent (Invitrogen), according to the manufacturer's protocol. The concentration of the RNAs was evaluated by NanoDrop (Thermo Fisher Scientific, Waltham, United States) and RNA integrity was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The samples were then used for subsequent library preparation. Sequencing of the library was performed using NovaSeq 6000 (Illumina, San Diego).

Raw sequencing reads were subjected to quality control using FastQC (v0.11.9). Trimmomatic (v0.39) was used to trim adapters and filter low-quality reads. Clean reads were aligned to the reference genome (GRCh38) using HISAT2 (v2.2.1) with default settings. The DESeq2 software (v1.30.1) was used to calculate the differential expression of transcripts and genes for OE-HNF4A compared with the control sample, with a false discovery rate (FDR) adjusted *P*-value <0.05 considered significant. Differential expression and enrichment results were visualized using ggplot2 (version 3.2.1) and heatmaps generated with pheatmap (version 1.0.12). The Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology (GO) term analysis were performed by the enrichKEGG and enrichGO function in clusterProfiler package respectively. Data was shown in the Table S3.

1.5 Reverse transcription and quantitative PCR amplification

Two μg of isolated RNA was reverse transcribed by Quant Reverse Transcriptase (TIANGEN) using primer mix (oligo-dT and random primers). Specific primers for the selected differential expressed genes (DEGs) and the *GAPDH* were designed using Primer3 software (https://www. primer3plus. com) and synthesized. Primer sequences were listed in Supplementary Table S4. The cDNAs product were analyzed by quantitative real-time PCR (RT-PCR) on QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, United States) using the UltraSYBR Mixture (CWBIO). Relative gene expression levels were calculated using the 2^-ΔΔCt method, with *GAPDH* serving as the internal control for normalization. Each sample was analyzed in triplicate, and data were presented as mean ± standard deviation (SD). Statistical significance was determined using Student's *t*-test, with *P*-values <0.05 considered significant.

1.6 Statistical analysis

All statistical analyses were performed using R software (version 4.1.0). Data were presented as mean ± standard deviation (SD) for continuous variables. Significant difference was assessed by a two-tail Student's *t* test, and a cutoff of *P*-value < 0.05 was employed to determine significance, *P** <0.05, *P*** < 0.01, *P**** <0.001.

2 Results

2.1 RNA−seq Revealed Potential Targets of HNF4A

To investigate potential target genes of HNF4A, wild-type HNF4A (WT-HNF4A-mOrange2) or pQCXIP-mOrange2 (mO2) control plasmids were transfected into E9-cells, a 293T cell line stably

expressing the pHNF1A-GFP reporter which indicated the transcriptional activity of HNF4A (Figure 1a). RNA-seq analysis was performed to explore potential HNF4A targets in E9-cells. A total of 431 genes were differentially expressed (P<0.05), with 399 genes upregulated (fold change (FC) >1.5) and 32 genes downregulated (FC<0.67) (Figure 1b). To confirm the RNA-seq results, the expression of known HNF4A target genes, including *HNF1A*, *OTC*, *SOAT2*, and *APOA1*, was analyzed and showed strong activation upon HNF4A expression (Figure 1c and Table S5).

To further validate RNA-seq results, we compared our data with published transcriptome data from colon, liver, and pancreas tissues[15-17]. A total of 175 genes that were significantly upregulated in our transcriptome were consistent with those in the reported studies (Figure 1d and Table S6). Among the eight genes upregulated across all four tissues, only *SLCO2B1* has been reported as a direct HNF4A target gene[18]. Further investigation using published ChIP-X data (extracted from 87 publications) from the Harmonizome portal[19-20] revealed that 73% (291/399) of our upregulated genes are predicated to be HNF4A targets in the ChIP Enrichment Analysis (CHEA) Transcription Factor Targets dataset. GO enrichment analysis of overlapping upregulated genes revealed that overexpressing of HNF4A significantly impacts various biological processes related to lipid metabolism, including lipid absorption, transport, and homeostasis. The enrichment of processes such as intestinal absorption, digestive system process, fatty acid beta-oxidation, cholesterol homeostasis, and triglyceride levels is consistent with HNF4A function in nutritional metabolism (Figure 1e).

2.2 *TM4SF5* might be a new target of HNF4A

To further investigate the effects of HNF4A, hierarchical clustering and KEGG pathway analysis of 399 upregulated genes were conducted (Figure 2a and 2b). The pathway analysis of upregulated genes in OE-

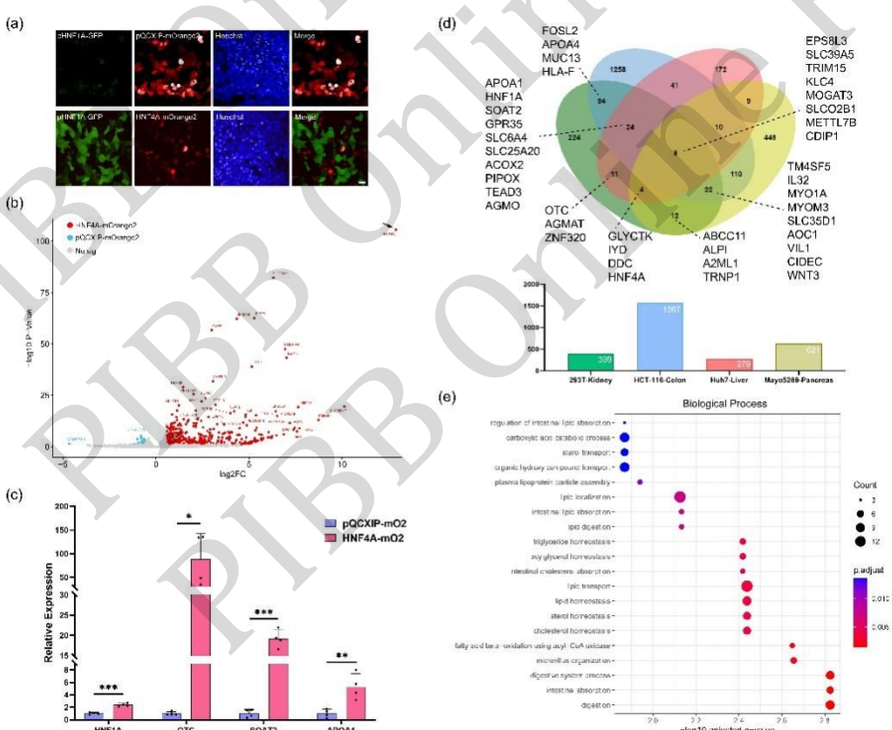

Fig. 1　Transcriptomics analysis of HNF4A overexpression cells by RNA−seq

(a) Representative confocal microscopy images of E9-cell line (integrated with pHNF1A-GFP) transfected with HNF4A-mOrange2 plasmid or control vector pQCXIP-mOrange2, 488-nm channel for pHNF1A-GFP (green); 561-nm channel for HNF4A-mO2 (red); 405-nm channel for Hoechst. (b) Volcano plots of differential gene expression in OE-HNF4A cells *vs* pQCXIP, adjusted P-value < 0.05. (c) Individual analysis of known HNF4A target genes. RPKM expression values for the *HNF1A*, *OTC*, *SOAT2*, and *APOA1* are shown, P*<0.05, P**<0.01, P***<0.001. (d) Venn diagram comparing specifically identified upregulated genes in different tissues with overexpressed or knock-down *HNF4A*. A minimum enrichment fold change of 1.5 was used. 293-Kindy: our overexpressed HNF4A RNA-seq; HCT-116-Colon: overexpressed HNF4A-isoform2 RNA-seq, Huh7-Liver: knock-down *HNF4A* RNA-seq; Mayo5289-Pancreas: knock-down *HNF4A* RNA-seq. (e) Significantly enriched gene ontology biological processes of upregulated genes in (d) were analyzed using the enrichGO clusterProfiler package.

HNF4A cells indicated that the most significantly enriched metabolic pathway was fat digestion and absorption. Additionally, other significantly enriched metabolic pathways included glycine, serine, threonine, and tryptophan metabolism, complement and coagulation cascades, and cholesterol and glycerolipid metabolism.

Among the top ten upregulated genes in OE-HNF4A cells, we identified *TM4SF5*, which had not previously been reported as an essential HNF4A target (Figure 2c and Table S4). To validate the RNA-seq data, quantitative real-time PCR was performed on cells overexpressing either wild-type HNF4A or the pathogenic variants R85W and M373R. There was a 6-fold increase in *TM4SF5* mRNA levels in the Wild type group, whereas the pathogenic mutation groups showed levels comparable to the control group (Figure 2d and Table S4). These results suggest that *TM4SF5* may be a target gene of HNF4A.

2.3 *TM4SF5* Proximal Promoter Region Contains HNF4A Binding Site

To elucidate the molecular mechanism of *TM4SF5* regulated by HNF4A, it was examined whether HNF4A directly regulates *TM4SF5* transcription. Five putative DR1-type consensus motifs as HNF4A binding sites in the *TM4SF5* promoter region were predicted using the JASPAR website (Figure 3a and 3b). Transient transfection with a series of truncated reporters ranging from －2 000 to +100 bp, along with WT-HNF4A expression plasmid, showed that the longest *TM4SF5* reporter (－2 000 bp) displayed 3.8-fold higher transcriptional activation (Figure 3c and Table S2). Further deletion to －100 bp retained a similar activation level to longer promoters. The fact that deletion to －1 000 bp did not significantly reduce HNF4A activation indicates that the putative motif 1 and motif 2 were not key binding sites of HNF4A. Additionally, the pathogenic *HNF4A* mutations R85W and M373R showed reduced or complete loss of transcriptional

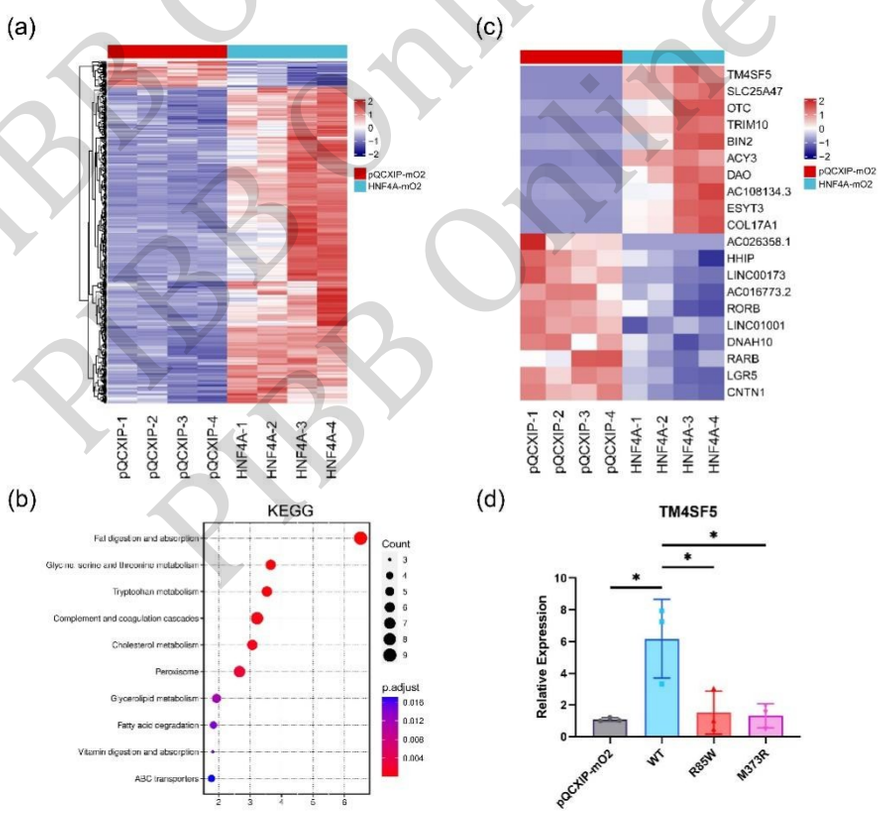

Fig. 2 *TM4SF5* is the top upregulated gene in HNF4A overexpression cells

(a) Hierarchical clustering analysis of the E9-cell lines expressing HNF4A or control, based on their RNA expression profiles using the fold change of 1.5 (*P*-value <0.05). (b) Top pathways altered in OE-HNF4A cells were analyzed using the enrichKEGG clusterProfiler package. (c) Top upregulated and downregulated genes in OE-HNF4A vs pQCXIP-mO2 groups. (d) qPCR analysis of E9-cells overexpressing WT or known pathogenic HNF4A variants, *P**<0.05.

activity for the (– 100 to +100) reporter (Figure 3d and Table S2). These results indicate that the critical binding motif for HNF4A in the *TM4SF5* promoter region lies between – 100 to +100.

2.4 The – 57 to – 48 Region of the *TM4SF5* Promoter is the Key Motif for HNF4A Activation

Given that the – 100 to +100 region was significantly transcriptionally activated in OE-HNF4A E9-cells, the key binding motif of HNF4A was determined. Among the two predicted binding sites in the – 100 to +100 region, mutations were conducted on the DR1 motif 4, which displayed a higher score than motif 5 (Figure 3b). The key base pairs of the DR1 site were mutated at the 2nd, 7th—8th, 5th—10th, as well as 2nd—11th nucleotide acids, named pMUT1-pMUT4 (Figure 4a). The reporter assay showed that pMUT1 reduced transcriptional activity by HNF4A by 50%, while the other three mutants led to the thorough loss of transcriptional activity (Figure 4b and Table S2). These results further indicated that the – 57 to – 48 region of the *TM4SF5* promoter is the key binding motif for HNF4A.

3 Discussion

In this study, we performed transcriptome analysis following the overexpression of HNF4A in E9 cells and identified 399 upregulated and 32 downregulated genes. Among the upregulated genes, classical HNF4A target genes such as *HNF1A*, *OTC*, and *APOA4* were identified. By comparing our results with existing transcriptomic data from liver, pancreatic, and intestinal cells, we observed a 43% (175/399) overlap of upregulated genes, while 224 genes were novel. KEGG pathway analysis revealed that these differentially expressed genes are enriched in lipid and amino acid metabolism pathways, consistent with the reported transcriptomic results and functions of HNF4A [15, 21-24]. Among the novel genes,

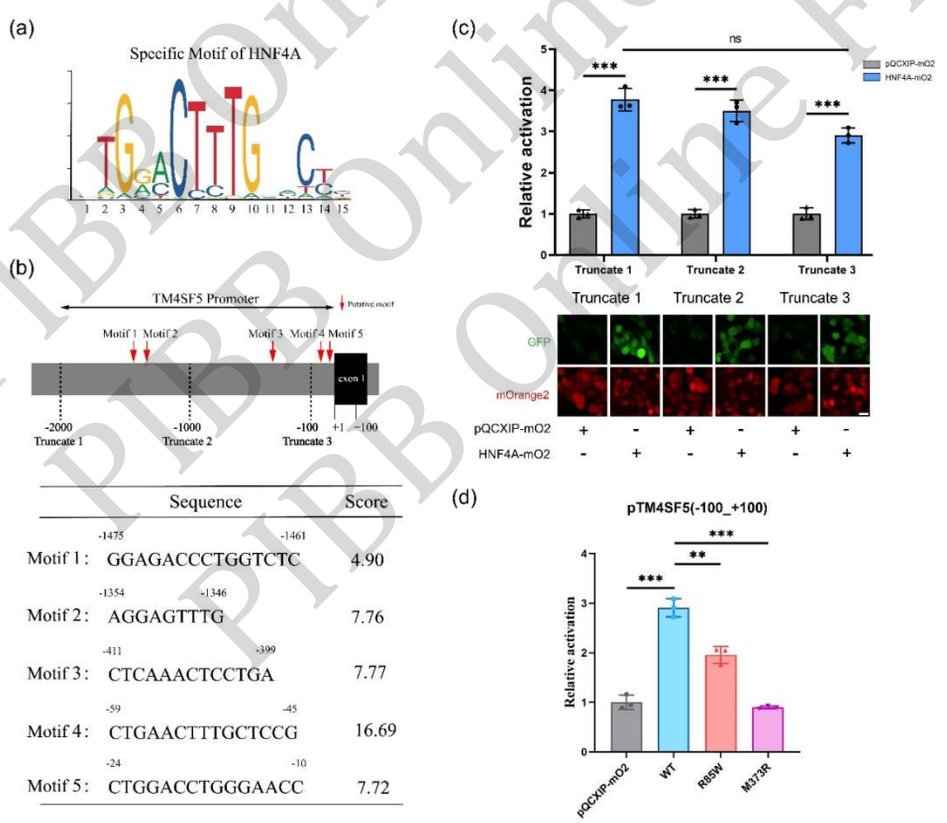

Fig. 3　A functional HNF4A−binding motif in the proximal region of the *TM4SF5* promoter

(a) HNF4A DNA motif from JASPAR was displayed. (b) Schematic diagram of HNF4A-binding motifs in *TM4SF5* promoter region from – 2 000 to +100 bp. HNF4A putative binding motifs 1—5 are shown in red arrows, and the corresponding sequences and scores are listed in the table. (c) Proximal region of – 100 to +100 mediates the activation of *TM4SF5* by HNF4A. As shown in Figure 3b, truncated *TM4SF5* promoters (truncate 1 to truncate 3) were separately constructed into the pGL3-GFP plasmid (see materials and methods), then cotransfected into 293T cells with HNF4A-mO2 or control vector pQCXIP-mO2 and imaged using HCS. Experiments were conducted triple independently, $P^{***}<0.001$. (d) 293T cells were co-transfected with the pTM4SF5(– 100 to +100) and WT or pathogenic HNF4A variants, $P^{**}<0.01$; $P^{***}<0.001$.

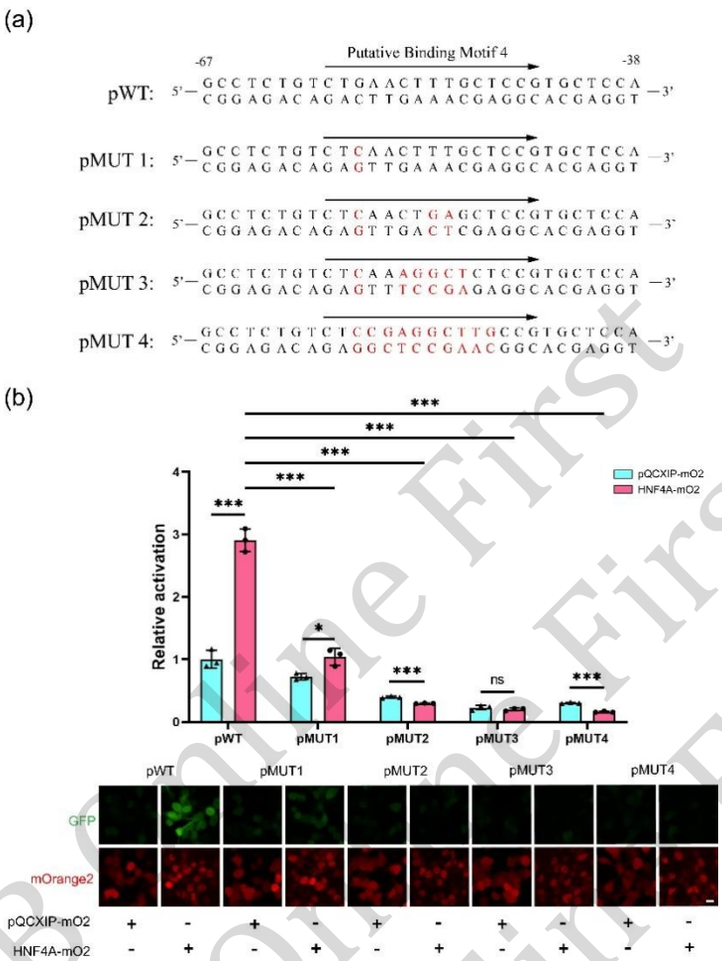

Fig. 4 The putative binding motif 4 is the key functional HNF4A response element in the *TM4SF5* promoter region

(a) Schematic diagram of the mutated motif 4 promoters. Numbers (in base pairs) refer to the promoter site of *TM4SF5*. The mutations introduced into the motif 4 to generate the indicated mutated pMUT1-pMUT4 promoter constructs are shown in red. (b) Cell imaging results and relative activation of WT or mutated pTM4SF5(－100 to +100)-GFP plasmids. 293T cells were co-transfected with the WT pTM4SF5(－100 to +100)-GFP plasmid or pMUT1-pMUT4 promoter constructs and WT HNF4A-mO2 plasmid or control vector pQCXIP-mO2, $P^{*}<0.05$; $P^{***}<0.001$.

24% (53/224) are predicted HNF4A targets in the CHEA dataset, including *PCSK9*, which is known to be regulated by HNF1A[25]. Notably, *TM4SF5* was identified as the most significantly upregulated gene. Investigation of the *TM4SF5* promoter region using a GFP reporter assay revealed HNF4A binding sites in the proximal promoter, with further analysis identifying the DR1 motif at the －57 to －48 region as the direct binding site of HNF4A. These findings confirm *TM4SF5* as a novel target gene of HNF4A and expand the known profiling of its regulatory network.

To validate the role of HNF4A in regulating *TM4SF5*, we examined two pathogenic variants, R85W and M373R, located in critical functional domains of HNF4A. The mutation R85W is located in the DNA-binding domain (DBD) and significantly impairs DNA-binding ability, while M373R is located in the ligand-binding domain (LBD) [26-27]. Both mutations are strongly associated with MODY1 and abolished the ability of HNF4A to activate *TM4SF5* transcription, highlighting the essential role of functional HNF4A in *TM4SF5* regulation.

TM4SF5 is a member of tetraspanin family and is expressed in the kidney, small intestine, liver, and pancreas, where it plays crucial roles in cellular processes such as proliferation, adhesion, migration, and signal transduction[28-32]. Dysfunction of TM4SF5 leads to phenotypes resembling those seen in MODY1 patients, including impaired insulin secretion and

reduced serum triglyceride levels[5]. In metabolic pathways, TM4SF5 operates as an arginine sensor that modulates amino acid availability to regulate mTORC1 activity, driving protein synthesis and cellular growth in the liver [33]. Additionally, TM4SF5 knockout mice exhibit decreased serum triglyceride levels under both normal and high-fat diet conditions, as well as significantly reduced plasma insulin levels under high-fat diet conditions, highlighting its role in insulin secretion in the pancreas[34]. These observations suggest that TM4SF5 is critical for maintaining lipid metabolism and insulin secretion, providing preliminary evidence for the association between TM4SF5 dysfunction and MODY1. Given the strong evidence that HNF4A directly regulates *TM4SF5* in this study, we propose that HNF4A loss-of-function mutations in MODY1 may contribute to the disease pathology by disrupting TM4SF5 function. This connection provides new insights into the potential mechanisms linking HNF4A dysfunction to MODY1 pathophysiology, further supporting the critical role of HNF4A in metabolic regulation.

One limitation of our study is that the reporter assays were performed in 293T cells, which do not represent the physical tissues where HNF4A is active. Further DNA binding experiments and functional assays on lipid metabolism or insulin secretion are necessary to elucidate the potential mechanisms by which TM4SF5 may be involved in MODY1. Another limitation is the absence of detectable binding signals in our ChIP assay, which may be attributed to the low endogenous expression of TM4SF5 in kidney-derived 293T cells. However, the RNA-seq and GFP reporter assay data provide strong evidence supporting HNF4A regulation of *TM4SF5* via the DR1 motif. Further experiments in cell lines with higher TM4SF5 expression may provide additional evidence.

4 Conclusion

In this study, we performed transcriptional profiling to identify potential target genes transcriptionally regulated by HNF4A. Among the novel target genes, we identified an arginine receptor, *TM4SF5*, as a direct transcriptional target. Truncation and point mutations on the reporter screening revealed that HNF4A regulates the activation of *TM4SF5* through the DR1 motif in the proximal promoter region. Due to the phenotypic overlap between *HNF4A* and *TM4SF5* mutations, more functional studies are necessary to further uncover the mechanisms by which MODY1 may be involved.

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Web Resources EPD: http://www.epd.isb-sib.ch; JASPAR: http://jaspar.genereg.net/

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肝细胞核因子4A通过DR1基序激活TM4SF5的转录*

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摘要　目的　肝细胞核因子4A（HNF4A）是肝脏和胰腺中的关键转录因子，其功能障碍会导致青少年发病的成年型糖尿病（MODY1）。值得注意的是，携带HNF4A致病性突变的MODY1患者表现出对精氨酸的响应降低，并伴有血浆甘油三酯水平下降，但其机制尚不明确。本研究旨在通过转录分析，探讨HNF4A调控的潜在靶基因，并探索其在上述代谢途径中的作用机制。**方法**　在稳定表达HNF1A报告基因的肾源293T细胞系E9细胞中过表达HNF4A，并进行转录组测序（RNA-seq）分析转录谱差异。随后，通过转录因子结合预测分析，鉴定HNF4A在相关靶基因启动子区域的结合位点。**结果**　RNA-seq结果显示，在HNF4A过表达的细胞中，TM4SF5（跨膜4 L六家族成员5）的mRNA表达显著上调。转录因子结合预测表明，TM4SF5启动子中可能存在5个HNF4A结合基序。最终，我们确认了位于TM4SF5启动子（-57到-48）区域的DR1位点是HNF4A的关键结合基序。**结论**　本研究首次揭示了TM4SF5是HNF4A的一个靶基因，并确定了其调控的关键结合位点。鉴于TM4SF5作为精氨酸传感器在mTOR信号通路激活和甘油三酯分泌中的作用，与MODY1患者观察到的表型高度相关，本研究为HNF4A在肝脏甘油三酯分泌和胰腺精氨酸刺激胰岛素分泌途径中的调控机制提供了潜在的见解。

关键词　肝细胞核因子4A，RNA-seq，跨膜4 L六家族成员5，DR1基序，青少年发病的成年型糖尿病1型
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