$N^6$-methyladenosine modification-mediated mRNA metabolism is essential for human pancreatic lineage specification and islet organogenesis

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Pancreatic differentiation from human pluripotent stem cells (hPSCs) provides promising avenues for investigating development and treating diseases. $N^6$-methyladenosine (m6A) is the most prevalent internal messenger RNA (mRNA) modification and plays pivotal roles in regulation of mRNA metabolism, while its functions remain elusive. Here, we profile the dynamic landscapes of m6A transcriptome-wide during pancreatic differentiation. Next, we generate knockout hPSC lines of the major m6A demethylase ALKBH5, and find that ALKBH5 plays significant regulatory roles in pancreatic organogenesis. Mechanistic studies reveal that ALKBH5 deficiency reduces the mRNA stability of key pancreatic transcription factors in an m6A and YTHDF2-dependent manner. We further identify that ALKBH5 cofactor α-ketoglutarate can be applied to enhance differentiation. Collectively, our findings identify ALKBH5 as an essential regulator of pancreatic differentiation and highlight that m6A modification-mediated mRNA metabolism presents an important layer of regulation during cell-fate specification and holds great potentials for translational applications.
In the last decades, model organisms, such as zebrafish, frog, and mouse, have been used to describe the normal development of the pancreas. These studies have identified an intricate regulatory network containing transcription factors, epigenetic regulators, and signaling pathways that can orchestrate pancreatic differentiation from hPSCs. It is necessary to understand how human pancreatic lineages are specified and use that information to make functional human pancreatic cells that can be applied to interpret and treat metabolic diseases, including diabetes. However, the technical and ethical challenges remain daunting in the study of early human development. In recent years, tremendous efforts have been made to differentiate hPSCs into insulin-secreting pancreatic β-like cells. Briefly, stepwise differentiation protocols have been developed to guide the directed differentiation of hPSCs into definitive endoderm (DE), posterior foregut (PF), pancreatic progenitors (PPs), and finally human islet-like organoids (hILOs). Now, the hPSC-based pancreatic differentiation represents a feasible platform to study human pancreatic biology. In addition, precise genome editing and high-throughput sequencing approaches have provided researchers powerful tools to investigate pancreatic differentiation and development. Recently, such approaches have been successfully applied to study the roles of many key pancreatic development and diabetic susceptibility genes, including PDX1, NGN3, RFX6, GATA6, GLIS3, HNF1A, and MAFB. These studies demonstrate that hPSCs can offer a unique opportunity for investigating human development and disease phenotypes.

N6-methyadenosine (m6A) is the most prevalent internal messenger RNA (mRNA) modification in mammals. The mRNA m6A modification is installed by a large mRNA methyltransferase complex composed of METTL3, METTL14, WTAP, and auxiliary or scaffold proteins like VIRMA, ZC3H13, and HAKAI. The m6A mark can be dynamically removed by the demethylases FTO and ALKBH5. Multiple mRNA reader proteins, including YTHDF1-3, YTHDC1-2, and IGF2BP1-3, can specifically bind to m6A sites and implement their biological functions. The mRNA m6A modification is highly dynamic, and influences mRNA metabolism, including their processing, export, stability, and translation. Therefore, mRNA m6A modification plays a very important physiological roles. Recent studies suggest that m6A modification of mRNAs regulates various biological processes, including circadian rhythms, spermatogenesis, neurogenesis, pluripotency, and immunity. Furthermore, increasing evidence supports the pathological roles of perturbed m6A metabolism in several diseases, such as tumorigenesis and inflammation. However, the roles of m6A modification-mediated mRNA metabolism in pancreatic differentiation and development remain elusive.

ALKBH5 belongs to the AlkB family of α-ketoglutarate-dependent dioxygenases and is a major m6A demethylase. Zheng et al. identified ALKBH5 as an m6A eraser and demonstrated that Alkbh5 deficiency led to aberrant spermatogenesis and apoptosis in mouse testes. Subsequently, Zhang et al. found that ALKBH5 was responsible for the hypoxia-induced breast cancer stem cell phenotype. Similarly, Zhang et al. showed that ALKBH5 maintained tumorigenicity of glioblastoma stem-like cells by sustaining FoxM1 expression and cell proliferation. Liu et al. identified that ALKBH5 modulated cellular metabolic rewiring and inhibited viral replication. Later, Zhang et al. demonstrated that ALKBH5 promoted the cell proliferation of renal cell carcinoma by regulating AURK expression. Recently, Shen et al. and Wang et al. independently found that ALKBH5 played important roles in acute myeloid leukemia and could be harnessed for effective therapy. In addition, Li et al. found that ALKBH5 regulated anti-PD-1 therapy response by modulating tumor microenvironment. Besides tumorigenesis, the functions of ALKBH5 in other biological processes are still largely unknown. Until now, the roles of ALKBH5 in pancreatic differentiation have not been studied.

In this work, we employ hPSC-based pancreatic differentiation platform to profile the mRNA m6A dynamics during pancreatic differentiation, and find that m6A demethylase ALKBH5 plays essential roles in pancreatic differentiation and islet organogenesis by modulating m6A and YTHDF2-dependent mRNA metabolism.

**Results**

**mRNA m6A landscape during human pancreatic differentiation.** To investigate mRNA m6A dynamics during human pancreatic differentiation, we step-wisely differentiated hPSCs into human islet-like organoids (hILOs). Later, Zhang found that ALKBH5 regulated anti-PD-1 therapy response by posterior foregut (PF), pancreatic progenitors (PPs), and increasing evidence supports the pathological roles of perturbed metabolism in several diseases, such as tumorigenesis and inhibited viral replication. In this work, we employ hPSC-based pancreatic differentiation platform to profile the mRNA m6A dynamics during pancreatic differentiation, and find that m6A demethylase ALKBH5 plays essential roles in pancreatic differentiation and islet organogenesis by modulating m6A and YTHDF2-dependent mRNA metabolism.

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results highlight the dynamics of m6A modification in mRNAs during pancreatic specification and suggest that the rewiring of m6A modification modulates gene expression upon transition of hPSCs to hILOs.

mRNA m6A dynamics across human pancreatic differentiation. To further investigate the dynamics of m6A modifications during pancreatic differentiation, we analyzed the origin of m6A modifications, which can inherit from earlier differentiation stages or yield de novo. Intriguingly, the percentage of inherited peaks gradually decreased and the percentage of de novo peaks gradually increased during pancreatic differentiation (Fig. 2a). In PP and hILO stage, most of the inherited peaks originated from hPSCs (Fig. 2b). The Kyoto encyclopedia of genes and genomes (KEGG) analysis showed that genes with inherited m6A peaks were more related to fundamental biological functions, such as spliceosome, ubiquitin-mediated proteolysis, autophagy, and cell cycle (Fig. 2c). On the other hand, genes contained de novo m6A peaks were more dynamic, and related to signaling pathways and stage-specific functions (Fig. 2c). Next, we clustered mRNAs into three categories according to m6A-tagged peak number (0–1 peak, 2–4 peaks, and ≥5 peaks) and observed similar distribution pattern among stages (Fig. 2d). GO and KEGG analyses showed that transcripts with more m6A peaks were also more dynamic and enriched in various signaling pathways (e.g., Wnt, TGFβ, and Notch) (Fig. 2e; Supplementary Fig. 2a). These results indicate that mRNA m6A modification becomes stage-specific and more diverse, contributing to pancreatic specification.
To evaluate the relationship between m6A modification and mRNA expression level, we next performed an integrated stepwise analysis of the m6A-seq and RNA-seq datasets. Globally, the negative correlations between m6A abundance and mRNA levels were observed among the transitions from hPSCs to hILOs (Fig. 2f, g; Supplementary Fig. 2b). Of note, we observed the highest percentage of upregulated genes with m6A downregulation during the DE-to-PP transition, which is consistent with the significant reduction of m6A level at PP stage. These data further suggest that m6A modifications play a critical role in regulating mRNA stability and degradation.

These results led us to investigate how m6A modification functionally participate in pancreatic differentiation. Interestingly, among the m6A core regulators, the expression of ALKBH5 was upregulated from PP stage, which was inversely correlated with the m6A level change during pancreatic differentiation (Fig. 1b; Supplementary Figs. 1d, e, 2c, d). The protein level of ALKBH5 also increased at PP stage (Fig. 3a). So far, ALKBH5 knockout (KO) hPSCs and the roles of ALKBH5 in hPSC differentiation have not been reported. Inspired by the above intriguing observations, we therefore decided to explore the roles of ALKBH5 in human pancreatic differentiation.
**Generation of ALKBH5 knockout hPSC lines.** Using our recently established CRISPR-Cpf1 system for precise genome editing of hPSCs\(^{13}\), we generated ALKBH5 KO hPSCs using CRISPR-Cpf1. We designated ALKBH5 KO hPSCs as A5-KO, and wild type hPSCs as WT. The PCR genotyping and Sanger sequencing results suggested the success in the generation of ALKBH5 KO cell lines (A5-KO and A5-KO\(^2\)) (Supplementary Figs. 3b, c, 4a). Next, western blotting results verified the absence of ALKBH5 proteins in the homozygous A5-KO lines (Fig. 3c; Supplementary Fig. 4b). A5-KO hPSCs have typical hPSC morphology and proliferate normally during long-term in vitro culture. Immunostaining results showed that both WT and A5-KO hPSCs almost homogenously expressed pluripotent stem cell markers OCT4 and NANOG (Supplementary Figs. 3d, 4c). Using RT-qPCR, we did not detect the significant differences of the marker genes OCT4, SOX2, and NANOG expression levels between WT and A5-KO hPSCs (Supplementary Fig. 3e). These results suggest that ALKBH5 is not required for hPSC survival and self-renewal.

**ALKBH5 is required for pancreatic lineage specification.** We checked the differentiation capacity of WT and A5-KO hPSCs towards pancreatic lineage (Fig. 3d). Firstly, we differentiated hPSCs into DE via treatment with Wnt signaling activator.
CHIR99021 and Activin A with a high concentration. Both WT and A5-KO hPSCs could be efficiently differentiated into DE cells co-expressing SOX17 and FOXA2 (Supplementary Figs. 3d, 4c). Fluorescence-activated cell sorting (FACS) analysis based on DE markers, SOX17, FOXA2, and CXC4, showed that there were more than 80% DE cells in both samples (Supplementary Figs. 3f, g, 4d, e). We could not observe difference between WT and A5-KO at DE stage, indicating that deficiency of ALKBH5 does not affect DE specification.

Next, we checked the differentiation capacity of WT and A5-KO DE towards PF and found that both WT and A5-KO lines could generate comparable PDX1-positive PF cells (about 80% for both samples) as analyzed by immunostaining and FACS (Supplementary Fig. 3d, h). Thus, ALKBH5 is not required for PF specification.

Subsequently, at the PP stage, we clearly observed that PDX1 and NKX6.1 double-positive cells were less in PPs from A5-KO than in PPs from WT as determined by immunostaining (Fig. 3e; Supplementary Fig. 4c). In addition, FACS results demonstrated that the percentage of PDX1 and NKX6.1 double-positive cells were significantly reduced in A5-KO (Fig. 3f; Supplementary Fig. 4f). RT-qPCR results confirmed that the expression levels of pancreatic progenitor marker genes PDX1, NKX6.1, HNF6, and SOX9 in A5-KO were lower than those in WT (Fig. 3g; Supplementary Fig. 4g). The protein level of NKKX6.1 was also significantly reduced in A5-KO (Fig. 3h and Supplementary Fig. 3i). Next, we checked cell proliferation at PP stage by Ki67 staining, and did not find significant difference between WT and A5-KO lines (Supplementary Fig. 3d). In order to get insights into the transcriptome changes after ALKBH5 KO, we performed RNA-seq with PP samples. The results demonstrated that 965 significantly up- and downregulated, respectively, upon ALKBH5 depletion (Fig. 3i). These downregulated genes included PDX1, SOX9, HNF6, MNX1, and NKX6.1, all of which were critical for pancreatic lineage specification; while other endodermal lineage marker genes, such as hepatocyte genes ALB and AFP, were upregulated (Fig. 3j). We checked hepatic marker ALBUMIN (ALB), and observed the increase of ALB+ cells in A5-KO at PP stage, indicating that ALKBH5 deletion may disturb pancreatic specification (Supplementary Fig. 5a). GO analysis identified that these downregulated genes were related to multiple biological processes, including RNA modification, regulation of cell morphogenesis, and pancreas development (Fig. 3k). On the other hand, upregulated genes were enriched for digestive system process and lipid homeostasis, which may be caused by the specification towards other endodermal lineages (Fig. 3k). Collectively, these data demonstrate that ALKBH5 is required for the efficient formation of PDX1 and NKX6.1 double-positive PPs.

m^4A demethylation activity of ALKBH5 is required. To rule out off-target possibility and determine whether enzymatic activity of ALKBH5 is required, we restored ALKBH5 expression in A5-KO hPSCs for rescue experiments. Previous studies have already shown that the H204A mutation can cause the loss of demethylation activity of ALKBH528,47,48. We generated A5-KO + A5WT and A5-KO + A5Mut hPSC lines by infecting A5-KO hPSCs with A5WT and A5Mut lentiviruses. In addition, we infected WT and A5-KO hPSCs with a GFPN lentivirus, and used them as controls. These four hPSC lines were applied for the pancreatic differentiation experiments (Fig. 5a). At first, we checked the ALKBH5 expression level in these cell lines, and observed the restoration of ALKBH5 expression in A5-KO + A5WT and A5-KO + A5Mut hPSC lines (Fig. 5b). Western blotting results also showed the recovery of ALKBH5 on the protein level (Fig. 5c). By UHPLC-QQQ-MS/MS, we confirmed that the m^4A level was also rescued after ALKBH5 restoration (Fig. 5d). Next, we differentiated these hPSC lines into pancreatic lineage as previously described. We checked the formation of PPs by immunostaining and FACS, and found that A5WT could recover the differentiation capacity of A5-KO hPSCs, suggesting that the deficiency of pancreatic differentiation capacity of A5-KO hPSCs was caused by ALKBH5 KO rather than the off-target effects (Fig. 5e, f). On the other hand, A5Mut failed to rescue the defects in pancreatic differentiation caused by ALKBH5 deficiency, indicating that enzymatic activity of ALKBH5 is required for pancreatic differentiation (Fig. 5e, f). The RT-qPCR results further supported the phenotypic observations. The gene expression levels of pancreatic progenitor cell markers, including PDX1, NKX6.1, SOX9, and HNF6, significantly increased after A5WT expression in the A5-KO cells (Fig. 5g). Together, these results indicate that ALKBH5, along with an intact demethylase activity, is necessary for hPSC-to-PF specification.

Identification of potential targets of ALKBH5. To decipher the underlying mechanisms of ALKBH5 involved in human pancreatic differentiation, we conducted m^4A-seq for samples at PP stage, starting from which step we could obviously detect the phenotypic differences between WT and A5-KO. The m^4A-seq data demonstrated that a vast majority of m^4A peaks were distributed in the CDS and 3'UTR of transcripts (Supplementary Fig. 6a–c). Particularly, the m^4A peak density in 3'UTR and near
stop codon increased after ALKBH5 KO, which was consistent with its functional role as an m6A eraser. Next, we took integrative analysis of m6A-seq and input RNA-seq data (Fig. 6a). We found that 7903 m6A peaks located on 4343 genes revealed hypermethylation in A5-KO versus WT PPs (Fig. 6a, b). Especially, 281 genes among them were downregulated according to the RNA-seq results, which might imply the role of m6A modification for mediating mRNA decay (Fig. 6b). GO analysis suggested that these potential targets were involved in the regulation of cell morphogenesis, stem cell development, and pancreas development (Fig. 6c).

Interestingly, several transcription factors that are important for pancreatic lineage specification, such as MNX1, SOX9, HNF6, and NKX6.1, were identified as candidate targets of ALKBH5 (Fig. 6a). We analyzed other published pancreatic differentiation datasets, and found that the expression of ALKBH5 was positively correlated with pancreatic progenitor maker genes PDX1, NKX6.1, SOX9, MNX1, while HNF6 only showing a weakly and insignificantly positive correlation (Supplementary Fig. 6d). Next, we detected the m6A enrichment on the mRNAs of these five transcription factors. The m6A-RIP-qPCR results confirmed that the m6A modification levels of PDX1, NKX6.1, MNX1, and SOX9 were indeed upregulated after ALKBH5 deletion (Fig. 6d and Supplementary Fig. 6e, f). Further, the ALKBH5-RIP-qPCR experiment validated that ALKBH5 could bind with transcripts of PDX1, NKX6.1, MNX1, and SOX9 (Fig. 6e). Taken together, we have identified key pancreatic differentiation maker genes as ALKBH5 targets, including PDX1, NKX6.1, SOX9, MNX1.

m6A-mediated mRNA metabolism regulates differentiation. Next, we wanted to uncover how ALKBH5 regulated its targets during pancreatic differentiation. As the m6A modification has been implicated in the control of mRNA metabolism, including stability and degradation30, we firstly analyzed the RNA-seq data...
of PP-WT and PP-A5-KO, and found that mRNA transcripts without m^6^A modification were generally more stable than m^6^A mRNAs with m^6^A modification (Fig. 6f). Therefore, we explored the mRNA degradation in WT and A5-KO PPs. WT and A5-KO PPs were treated with transcription inhibitor actinomycin D (ActD), and collected at 0, 1, 2, and 3 h, respectively. Then we evaluated the half-life of mRNAs by RNA-seq. Interestingly, we observed reduced global mRNA stability in A5-KO cells (median of mRNA half-lives in A5-KO cells: 3.11 h; median of mRNA half-lives in WT cells: 7.81 h, \( p = 0 \), Mann–Whitney U-test) (Fig. 6g). A further analysis showed that mRNAs without m^6^A modification were generally more stable compared to those with m^6^A modification transcripts in A5-KO samples (\( p = 0 \), Mann–Whitney U-test) (Fig. 6h). Moreover, ALKBH5 deletion led to shorter (~38% in average) lifetimes of mRNAs harboring m^6^A modification in comparison with mRNAs without m^6^A modification (\( p = 1.377 \times 10^{-11} \), Mann–Whitney U-test) (Fig. 6i).

Notably, 1555 transcripts showed significantly decreased half-lives after ALKBH5 deletion, whereas only a few (\( n = 22 \)) transcripts had increased half-lives (Fig. 6j). GO analysis demonstrated that genes with shortened half-life were enriched for stem cell population maintenance, Notch signaling, and pancreatic development (Fig. 6k). Among these transcripts, we further confirmed that mRNA stabilities of key pancreatic differentiation marker genes, including PDX1, NKX6.1, MNX1, and SOX9, indeed significantly decreased in A5-KO cells (Fig. 6l and Supplementary Fig. 6g).

The effects of m^6^A modification are mediated by cell-type-specific m^6^A reader proteins. For example, YTHDF1 and YTHDF3 promote translation, and IGF2BP1-3 can stabilize m^6^A modification in comparison with mRNAs without m^6^A modification.
m^6^A methylated transcripts. Notably, YTHDF2 has been known to specifically recognize m^6^A and trigger the rapid degradation of m^6^A-containing mRNAs. We found that the expression of YTHDF2 significantly increased in PP stage during differentiation, and was much higher than those of other readers in PPs (Supplementary Fig. 7a–c). Based on these facts and observations, we thought that YTHDF2 played the major role to perform the function, and thus tested the effects of YTHDF2. First, we applied YTHDF2-RIP-qPCR, and validated the binding of PDX1, NKX6.1, MNX1, and SOX9 transcripts with YTHDF2 (Fig. 7a). Then, we knocked down YTHDF2 by shRNAs (shDF2-1 and shDF2-2) and evaluated the effects on pancreatic differentiation (Fig. 7b, c). As shown in Fig. 7d, shDF2s could partially rescue the defect of pancreatic differentiation caused by A5-KO (Supplementary Fig. 8a). In addition, shDF2 partially restored the expressions of PDX1, NKX6.1, MNX1, and SOX9 (Fig. 7e and Supplementary Fig. 8b).

Collectively, these results support that ALKBH5 regulates human pancreatic differentiation through manipulating mRNA m^6^A modification and stabilities of critical genes involved in human pancreatic differentiation and development.

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Fig. 6 Molecular mechanism of ALKBH5 in regulating human pancreatic lineage specification. a Flowchart of ALKBH5 downstream target screening. b Venn diagram showing the integrative analysis to identify potential ALKBH5 targets. AS-KO-down: significantly downregulated genes in AS-KO PPs. AS-KO-m6A-hyper: genes with significantly higher m6A abundance in AS-KO PPs. c GO categories of the positive targets of ALKBH5, p-values were calculated by one-tailed hypergeometric test. d RIP-qPCR showing the m6A enrichment increased on indicated mRNA transcripts in AS-KO PPs compared with WT PPs (n = 3 biological replicates). e RIP-qPCR analysis of the indicated mRNA transcripts bound with ALKBH5 in PPs (n = 3 biological replicates). f Violin plots showing expression changes between AS-KO and WT PPs for methylated (with m6A) and not-methylated (without m6A) transcripts. The upper and lower quartiles and the median are indicated for each group. Dots, the average value of fold changes. p-value was calculated using two-tailed Wilcoxon test. g Cumulative distributions of global transcript half-life changes in WT and AS-KO PPs. h Cumulative distributions of mRNA input changes (AS-KO/WT, log2FC) of methylated (with m6A) and not-methylated (without m6A) transcripts. i Cumulative distributions of mRNA lifetime changes (AS-KO/WT, log2FC) of methylated (with m6A) and not-methylated (without m6A) transcripts. j Volcano plot of transcripts with significant half-life change in AS-KO PPs compared to WT PPs. Red dots: transcripts with increased half-life; blue dots: transcripts with shortened half-life. k GO categories of genes with shortened half-life, p-values were calculated by one-tailed hypergeometric test. l The decay curves for PDX1, NKX6.1, MNX1, and SOX9 in WT and AS-KO PPs (n = 3 biological replicates). All data are presented as mean ± s.d. Statistical significance calculated using two-tailed Student’s t-test, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. Source data are provided as a Source Data file.

improve human pancreatic differentiation through modulating m6A modification. However, to date, specific and potent activators of ALKBH5 remain very limited. α-ketoglutarate (αKG) is the cofactor of ALKBH5, and therefore we tested the effects of αKG on human pancreatic differentiation. Notably, we observed that cell-permeable dimethyl-αKG (dm-αKG) significantly increased the percentage of PDX1 and NKX6.1 double-positive PPs (Fig. 7f and Supplementary Fig. 8c). RT-qPCR showed that dm-αKG upregulated the expression of PDX1, NKX6.1, SOX9, and MNX1 (Fig. 7g). Consistently, the m6A levels of PDX1, NKX6.1, SOX9, and MNX1 mRNAs decreased after dm-αKG administration (Fig. 7h). Therefore, αKG-AlKBH5-m6A axis can be harnessed to promote human pancreatic differentiation and will facilitate its translational applications.

Discussion

During cell-fate transitions, transcriptome changes are dynamically regulated and highly coordinated at multiple levels. Besides epigenetic modifications, such as DNA and histone methylation, mRNA m6A methylation has recently been recognized as an important layer of controls over cellular differentiation and organ development24. In this report, using hPSC-based pancreatic differentiation platform, we investigated mRNA m6A dynamics during pancreatic lineage specification and further explored the roles of m6A modification and its demethylase ALKBH5 in pancreatic differentiation.

For technical issues, it is challenging to collect large amounts of samples for m6A-seq, and there is so far no report for describing mRNA m6A dynamics during pancreatic lineage specification30. Based on the directed differentiation platform of hPSCs to hILOs, we found that the pancreatic lineage progression was accompanied by changes in m6A modification on numerous transcripts. This study of mRNA m6A dynamics during pancreatic differentiation presents a valuable resource for further exploration.

Using CRISPR-based precise genome editing tool, we generated ALKBH5 KO hPSC lines. Then, we investigated the roles of ALKBH5 during human pancreatic differentiation in more details. Unexpectedly, we found that ALKBH5 played critical roles on pancreatic lineage specification. Previously, Alkbh5 KO mice were reported to be normal only with impaired fertility28,31 and no abnormal phenotype was reported on pancreatic development. Our study supports that hPSCs can offer a unique opportunity for studying human disease phenotypes that are not readily recapitulated in model organisms.

Mechanistically, we found that m6A modifications of many essential transcription factors like PDX1, NKX6.1, SOX9, and MNX1 involved in pancreatic development were altered after ALKBH5 depletion (Fig. 7i). m6A modification influences mRNA metabolism in almost every step of its lifecycle, including mRNA splicing, export, stability, and translation efficiency23,35. We further identified PDX1, NKX6.1, MNX1, and SOX9 as the direct targets of ALKBH5, and showed that m6A modification could regulate the stability of these mRNA transcripts through YTHDF2-mediated mRNA decay pathway. In addition, we found that ALKBH5 cofactor αKG could decrease the m6A modification of these mRNA transcripts and significantly promote human pancreatic differentiation. In the future, more potent molecules can be developed for specifically modulating ALKBH5 and YTHDF2, which will facilitate better understanding of the molecular mechanisms of m6A modification.

In sum, our study demonstrates that m6A mRNA modification is highly dynamic during pancreatic differentiation. Accordingly, ALKBH5 plays important regulatory roles in regulating essential gene expression in pancreatic progenitors and islet endocrine cells. Therefore, harnessing mRNA m6A regulation would provide strategies for controlling human pancreatic differentiation and developing effective approaches for generating large amounts of functional islets for the study of pancreatic biology and treatment of various metabolic diseases.

Methods

Statement. This work complies with all relevant ethical regulations and was reviewed by the Internal Review Committee of Zhejiang University.

Plasmid construction. The wild-type type ALKBH5-CDS was cloned into the pSiN-BSD vector to generate pSiN-ALKBH5. For pSiN-ALKBH5 plasmid, ALKBH5 H204A mutation was introduced using the Gibson Assembly kit (New England Biolabs). For pSiN-GFP plasmid, the sequence of GFP N-terminal domain was linked with pSiN-BSD backbone by using Gibson Assembly kit. For shRNA plasmid construction, single-strand DNA was synthesized from Sangon Biotech. After annealing, the insert DNA was ligated with pKO.1 vector. All vectors were checked by Sanger sequencing.

Cell culture. Human embryonic kidney 293T (HEK293T) cells were cultured in Dulbecco’s Eagle Medium (DMEM) (Life Technologies), 10% fetal bovine serum (FBS) (Gibco), and 1× Penicillin/Streptomycin (Life Technologies). HEK293T cells were from ATCC (CRL-3216).

Undifferentiated hPSCs were cultured in hPSC medium: DMEM/F12 (Life Technologies), 20% KnockOut Serum Replacement (KSR) (Life Technologies), 1× Non-Essential Amino Acids (NEAA) (Life Technologies), 0.055 mM 2-mercaptoethanol (Sigma), 1× Penicillin/Streptomycin, and 10 ng ml−1 bFGF (Peprotech). hPSCs were maintained on CFE feeder cells at 37°C and 5% CO2. hPSCs were isolated by Accutase (Life Technologies) as 1:3 to 1:6 every 3–6 days. 0.5 mM biazoxyvin (TargetMed) were used during the first 24 h when passing or thawing cells. MEL1 INSGFP/W hESC line46 was a kind gift from Drs. E. G. Stanley and Andrew Elefanty. Mycoplasma contamination was routinely detected using TransDetect PCR Mycoplasma Detection Kit (TransGen Biotech).

Generation of ALKBH5 knockout hPSC lines. CRISPR-Cpf1 crRNAs were designed using an online software (http://chopchop.cshl.edu) to target the first exon of ALKBH5 locus. Two most efficient crRNAs (cr1: GAGTGGGTGCCAC-CAGCTGGTGATCAAAA; cr2: TTATTCCGACTCTGGCGTGGCTCGGCT) were used to produce indel mutations. Plasmids were transfected into hPSCs
according to our previous protocol. All plasmids (pcDNA3.1-hLbCpf1 (Addgene, Plasmid #31938), pCpfcr-cr1, and pCpfcr-cr2) were extracted by ZymoPURE Plasmid Maxiprep Kit (ZYMO). 1 × 10⁶ cells were resuspended with plasmid mixture and electroporation solution (Human Stem Cell Nucleofector Kit 1, Lonza) followed by electroporation. After electroporation, cells were cultured in one well of six-well plate for 2–3 days. Subsequently, 500–2000 cells were passaged onto 10 cm dishes and cultured for about one week. hPSC colonies were picked, expanded, genotyped, and banked for further studies. Primer sequences for genotyping are listed in Supplementary Table 3.

Human pancreatic differentiation from hPSCs. hPSCs were differentiated into pancreatic lineage by a previously described protocol. hPSCs were seeded into 12-well plates at a density of 5 × 10⁵ per well and the differentiation was initiated 48 h
after seeding. hPSCs were quickly washed by DPBS (Life Technologies) and exposed to differentiation media. Here is the detailed media: Day 1: RPMI (Life Technologies), 1% Penicillin/Streptomycin, and 100 ng ml⁻¹ activin A, and 3 μM CHIR99021 (TargetMol). Day 2: RPMI, 0.2% FBS, 1% Penicillin/Streptomycin, and 100 ng ml⁻¹ activin A. Day 3: RPMI, 2% FBS, 1% Penicillin/Streptomycin, and 100 ng ml⁻¹ activin A. Day 4–6: RPMI, 0.5x B27 (Gibco), 0.5x N2 (Gibco), 0.05% BSA (Yeasen), and 50 ng ml⁻¹ KGF (PeproTech). Day 7–8: DMEM, 1x B27, 0.05% BSA, 1% Penicillin/Streptomycin, 0.25 mM vitamin C (Sigma-Aldrich), 50 ng ml⁻¹ KGF, 0.1 μM LDN-193189 (Tocris), 0.1 μM GDC-0449 (Selleck), and 2 μM retinoic acid (Sigma). Day 9–14: DMEM, 1x B27, 0.05% BSA, 1% Penicillin/Streptomycin, 0.25 mM vitamin C, 0.1 μM LDN-193189, and 50 ng ml⁻¹ KGF (PeproTech). Then, cells were suspended to form aggregates, and differentiated into hiHSC stage using medium modified from our previous protocol. Day 15–22 (R6 medium): DMEM, 1:50 B27, 0.05% BSA, 1% Penicillin/Streptomycin, 10 μM zinc sulfate (Sigma), 10 μg ml⁻¹ heparin (Sigma), 10 μM 616452 (TargetMol), 1 μM T3 (Sigma), 0.1 μM LDN-193189, 0.1 μM compound E (Sigma), and 0.25 mM vitamin C.

**Rescue experiments.** Lentiviruses were produced in HEK293T cells using Lipo-fectamine™ 3000 Transfection Reagent (Invitrogen). For lentiviral infection, 60–70% confluent hPSCs were infected with viruses for 4 h. Then cells were cultured in hiHSC medium for about one week, and 10 μM -1 blastycin (YEASEN) was used to select positive cells from day 3.

**Generation of YTHDF2 knockdown hPSC lines.** hPSCs were transduced with lentiviruses expressing shRNAs (shYTHDF2-1: 5 ’-GAAA-3 ’, shYTHDF2-2: 5 ’-GAAA-3 ’). Generation of lentiviruses expressing shRNAs (shYTHDF2-1: 5 ’-GAAA-3 ’, shYTHDF2-2: 5 ’-GAAA-3 ’) was used to select positive cells from day 3.

**Immunostaining.** Cells were stained according to our previous protocol. In detail, cells were fixed with 4% paraformaldehyde (PFA) for 10–15 min at room temperature (RT). The cells were washed with PBS buffer (PBS: 0.3% Triton X-100) for three times, blocked in blocking buffer (PST: 5% BSA) for 1 h at RT followed by incubation with the primary antibody at 4 °C overnight. Then, secondary antibodies were used at 1:2000 dilution and incubated for 1 h at RT. Detailed primary and secondary antibodies are listed in Supplementary Table 1. Finally, cells were stained with Hoechst at 1:5000 dilution to mark nuclei.

**Western blotting.** Lysis buffer (Beyotime) was used to extract total protein from cells. Cell extracts were centrifuged at 12,000 × g for 15 min and the supernatants were collected. Cell lysates were resolved on 10% acrylamide gradient SDS-PAGE gels and transferred to PVDF membranes (Millipore). The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h, incubated with primary and secondary antibodies, and detected by immunoblotting with the High Sensitive ECL Chemiluminescence Detection Kit (Vazyme) or Super ECL Detection Reagent (Yeasen Biotech). Primary and secondary antibodies are outlined in Supplementary Table 1.

**Flow cytometry.** Cells were dissociated into single cells using Accutase and washed with PBS buffer. Then, cells were fixed by 4% PFA for 30 min and washed by PBS for 3–5 times followed by centrifuged at 200 × g, 5 min per time. Thereafter, cells were blocked by blocking buffer and incubated with primary antibodies at 4 °C overnight. After washing with PBS for 3 times, cells were incubated in secondary antibodies at RT for 1 h. All the antibodies are detailed in Supplementary Table 1. FACs data were acquired by Beckman CytoFlex (Beckman Culture) and analyzed by CytExpert software.

**Real-Time qPCR.** Total RNA was extracted and purified using Quick-RNA MiniPrep Kit (ZYM0) and converted into cDNA using PrimeScript RT Master Mix (TaKaRa). RT-qPCR was performed using TB Green Premix Ex Taq II Kit (Takara) on CFX Connect Real-Time system (Bio-Rad). Primer sequences are listed in Supplementary Table 2.

**RNA stability assays and mRNA stability profiling.** WT and AS-KO PPs were treated with actinomycin D (Sigma) at a final concentration of 1 μg ml⁻¹ and collected at indicated time points. Total RNA was extracted by Fast Pure Cell Total RNA Isolation Kit (Vazyme) and analyzed by RT-qPCR and RNA-seq. The half-life of mRNAs was calculated according to a previously published protocol. After inhibiting transcription by actinomycin D treatment, the change of mRNA concentration (C) as shown in the following equation:

\[
\frac{dc}{dt} = -k_{\text{decay}}C
\]

(1)

The minus symbol indicates that the mRNA is being degraded rather than synthesized. This relationship leads to the derivation of the equation:

\[
\ln(C/C_0) = -k_{\text{decay}}t
\]

(2)

When 50% of mRNAs is decayed (C/C₀ = 0.5), the mRNA half-life (t₁/₂) can be calculated by the equation:

\[
\ln(1/2) = -k_{\text{decay}}t_{1/2}
\]

(3)

From which:

\[
t_{1/2} = \ln(2)/k_{\text{decay}}
\]

(4)

For RNA-seq data, cDNA library construction and high-throughput sequencing were performed by Novogene, raw RNA-seq data were trimmed by fastq (v0.20.1) to remove adapter sequence and reads with low sequencing quality, paired-end sequencing was carried out with the Illumina HiSeq 2500 with paired-end 150 bp read length. Clean reads were aligned to the human genome (GRCh38) using HISAT2 (v2.1.0) with the default parameter settings. Transcript assembly was performed by stringtie (v2.0.3) and expression of transcripts sharing each gene_id was quantified as transcripts Per Million (TPM). TPM was converted to attooles by linear fitting of the RNA ERCC spike-ins. The degradation rates of mRNA and the mRNA half-life were calculated according to the aforementioned formula. The final half-life was calculated by using the average of 0.1, 2, and 3 h.

**Glucose-stimulated insulin-secretion (GSIS).** hiHLCs were washed twice with 1 ml KRKH buffer (128 mM NaCl, 5 mM KCl, 2.7 mM CaCl₂, 1.2 mM MgCl₂, 1 mM NaHPO₄, 1.2 mM KH₂PO₄, 5 mM NaHC03, 10 mM HEPES, 0.1% BSA). Then, cells were preincubated in 3 ml KRKH with 2 mM glucose for 30 min. hiHLCs were then incubated in KRKH with 16.8 mM glucone (low glucose KRKH) for 1 h to remove residual insulin. During incubation, all tube lids were left open for air exchange. Clusters were washed twice with KRKH buffer followed by incubated in 1 ml low glucose KRKH for 1 h. After incubation, 200 μl of the supernatant were collected for ELISA analysis (low glucose sample). Then, clusters were washed twice in KRKH and then incubated in KRKH with 16.8 mM glucose (high glucose KRKH) for 1 h, and 200 μl of supernatant were collected after incubation (high glucose sample). Finally, clusters were dispersed into single cells using Trypsin for cell counting. Supernatant samples were detected by Human insulin immunoassay kit (EZAssay).

**Quantification of m6A in RNA by UHPLC-QQQ-MS/MS.** Polyadenylated RNAs were purified from total RNA using a GenElute mRNA Miniprep Kit (Sigma). 200 ng of them were digested by nuclease P1 (1U) (Wako) in 30 μl reaction buffer containing 20 μM of CH3COONa (pH 5.3) at 42 °C for 2 h, followed by addition of 1 μl shrimp alkaline phosphatase (NEB) together with 3.5 μl 10x CutSmart buffer (NEB) and incubated at 37 °C for another 2 h. The samples were diluted to 60–70 μl with DEPC-treated water and filtered (0.22 μm pore size, Millipore), and 10 μl of the solution were injected into UHPLC-QQQ-MS/MS. The nucleosides were quantified by AB Sciex Qtrap 6500+ using the nucleoside to base ion mass transitions of 282.1–150.1 (m²A), and 268.2–136.1 (A). Quantification was performed in centroid chromatograms and standard curves obtained from pure nucleoside standards running on the same batch of samples. The ratio of m²A to A was calculated based on the calibration curves.

**m²A-seq.** For samples from hiPSC, DE, PP, and hiHLO stages, total RNAs were extracted with TRIzol® Reagent (Invitrogen), and were further enriched by GenElute mRNA Miniprep Kit (Sigma). m²A-seq experiments were performed following previously published protocol. Briefly, mRNAs were fragmented into about 100-nt fragments and immunoprecipitated (IP) with 5 μg m²A antibody (SYSY, Cat#20003), both input and IP products were subjected to library construction (Illumina). For samples from WT and A5-KO PPs, total RNAs (2–10 μg) were extracted, fragmented, and subjected to immunoprecipitation directly due to limited materials. RNAs in input fragments were first depleted by mRNA Depletion Kit (NEB), then both input and IP fragments were subjected to library preparation using SMARTer® Stranded Total RNA-Seq Kit v2 (Takara). All prepared libraries were then sequenced on Illumina Hiseq X10 system with paired-end 150 bp read length.

**m²A-RIP-qPCR.** Total RNAs were extracted, fragmented and immunoprecipitated by m²A-antibody. Both input and IP fragments were re-sequenced (Takara) and RT-qPCR experiments were performed using Taq Universal SYBR Green Supermix (Bio-Rad) on Bio-Rad CFX96 Connect Real-Time system. GAPDH was chosen as negative control for calculation of m²A enrichment level as following: the expression levels of selected genes in both input and m²A-IP samples were first normalized to GAPDH, and the m²A enrichment was calculated as m²A IP/input. Primer sequences are listed in Supplementary Table 2.
0.5 mM DTT, 50 mM Hepes pH 7.5, 1:100 protease inhibitor cocktail, 200 U ml⁻¹ RRI (Takara), pipetted up and down several times and incubated on ice for 15 min and treated with ultraviolet for 1 min then centrifuged at 14,000 × g membrane syringe (1% protease inhibitor cocktail and 200 U ml⁻¹ RRI added) and incubated with cell lysate-saturated protein mixture at 4 °C for another 4 h, protein A beads were collected with magnetic stand and washed with binding buffer (100 mM KCl, 1.5 mM MgCl₂, 0.05% NP-40, 2 mM EDTA, 0.5 mM DTT, 50 mM Hepes, pH 7.5) for three times, resuspended in 500 μl binding buffer (1% protease inhibitor cocktail and 200 U ml⁻¹ RRI added) and incubated with cell lysate-saturated protein mixture at 4 °C overnight, 30 μl protein A beads were collected with magnetic stand and washed with binding buffer (1% protease inhibitor cocktail and 200 U ml⁻¹ RRI added) for three times and then mixed with 500 μl trizol to get RNA and saved as IP. Equal amount of input and IP products were reversely transcribed with random hexamer and subjected to qPCR as described using primers listed in Supplementary Table 2. The enrichment level of selected targets was calculated as 2^[Ct(IP)-Ct(input)]. For RIP of ALKBH5, PPs expressing flag tagged ALKBH5 were used, and 30 μl anti-flag magnetic beads (Sigma) were used for the enrichment of ALKBH5 following the same procedures.

**m6A-seq analysis** All libraries were sequenced on Illumina HiSeq X10 with paired-end 150 bp read length. The deep sequencing data were first trimmed by fastp (v0.20.1) to remove adapter sequence and reads with low sequencing quality. Clean data were then aligned to human reference genome version 38 (GRCh38) using HISAT2 (v2.1.0) with parameters: -ma-strandness = FR - k 1. The m6A peaks were called using R package exonPeak2 (IP/input ≥ 1.5, p value < 10⁻⁵) from the Bioconductor project [http://www.bioconductor.org/]. The longest isoform was retained if a gene has more than one isoform. The differential m6A peaks were calculated using R package exonPeak with parameters: log2FC ≥ 1, adjusted p value < 0.05. Motif enrichment was done using HOMER (v4.11) selecting a motif length of 5, 6, and 7 nucleotides.

**RNA-seq analysis** Total RNA was isolated using Quick-RNA MiniPrep Kit (ZYMOS). cDNA library construction and high-throughput sequencing were performed by Novogene, raw RNA-seq data were trimmed by fastp (v0.20.1) to remove adapter sequence and reads with low sequencing quality. Clean data were then aligned to the human genome (GRCh38) using HISAT2 (v2.1.0) with the default parameter settings. Transcript assembly was performed by stringtie (v2.0) and expression of transcripts sharing each gene_id was calculated as 2^[Ct(IP)-Ct(input)]. For RIP of ALKBH5, PPs expressing flag tagged ALKBH5 were used, and 30 μl anti-flag magnetic beads (Sigma) were used for the enrichment of ALKBH5 following the same procedures.

**GO and KEGG analysis** The visualization of GO and KEGG enrichment analysis was performed by stringtoolkit from the Bioconductor project, and adjusted p-value < 0.05 was considered as statistically significant.

**Inheritance and origin analyses of m6A peaks.** For m6A peak’s inheritance and origin analyses in hPSCs to hiLLEOs, we defined the m6A peaks with location overlap >50% between a stage and the previous stage as m6A peaks inherited from the previous stage, and the remaining peaks in this stage were stage-specific peaks. The m6A peak would be used for analysis when the TPM of this m6A tagged expression analysis was performed by R package DESeq2 (v1.20.1) from the Bioconductor project, the threshold of significantly differentially expressed genes: FC > 1.5 or <0.67 and adjusted p value < 0.05. Heatmaps were generated by R package heatmap2 from CRAN [https://cran.r-project.org/].

**Correlation analysis between gene expression and m6A modification.** For calculating the relative m6A level for each gene, we used the R package DESeq2 (v1.20.1) to compare the read counts of genes between IP and input samples on a transcriptome-wide scale. Next, we obtained the adjusted fold changes between IP and input samples in each stage and these adjusted fold changes represent the relative m6A level. We defined high confidence m6A-tagged genes with the thresholds the TPM of gene in input samples > 5, the fold change in IP/input > 1.5, and adjusted p value < 0.05. To measure the correlation between m6A level and gene expression, we calculated the Pearson correlation coefficient between m6A level and gene expression, and the absolute value of the correlation coefficient > 0.5 and p value < 0.05 was defined as m6A level significantly correlated with gene expression.

**Statistics and reproducibility.** All experiments were carried out with at least three biological replicates and showed successful reproducibility. All graphs were generated using GraphPad Prism 8 V.8.0.3.538 (64-bit). All data are shown as the mean with error bars representing the s.d. Two-tailed unpaired t-tests (Student’s t-test) were used to obtain the p-values. The following convention was used for indicating the level of significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Exact p-values are provided in Source Data. The sample size (n) indicates the total number of independent biological replicates.

**Data availability** Source data are provided with this paper in the Source Data file. The RNA-seq and m6A-RIP-seq data generated in this study have been deposited in the Gene Expression Omnibus (GEO) database under accession code GSE16986. Source data are provided with this paper.

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