ALKBH5 suppresses malignancy of hepatocellular carcinoma via m^6^A-guided epigenetic inhibition of LYPD1

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Abstract

**Background:** N6-methyladenosine (m^6^A) modification is an emerging layer of epigenetic regulation which is widely implicated in the tumorigenicity of hepatocellular carcinoma (HCC), offering a novel perspective for investigating molecular pathogenesis of this disease. The role of AlkB homolog 5 (ALKBH5), one of the m^6^A demethylases, has not been fully explored in HCC. Here we clarify the biological profile and potential mechanisms of ALKBH5 in HCC.

**Methods:** Expression of ALKBH5 and its correlation with clinicopathological characteristics of HCC were evaluated using tissue microarrays and online datasets. And biological effects of ALKBH5 in HCC were determined in vitro and in vivo. Subsequently, methylated RNA immunoprecipitation sequencing (MeRIP-seq) combined with RNA sequencing (RNA-seq), and following m^6^A dot blot, MeRIP-qPCR, RIP-qPCR or dual luciferase reporter assays were employed to screen and validate the candidate targets of ALKBH5.

**Results:** We demonstrated that ALKBH5 was down-regulated in HCC, and decreased ALKBH5 expression was an independent prognostic factor of worse survival in HCC patients. Functionally, ALKBH5 suppressed the proliferation and invasion capabilities of HCC cells in vitro and in vivo. Mechanistically, ALKBH5-mediated m^6^A demethylation led to a post-transcriptional inhibition of LY6/PLAUR Domain Containing 1 (LYPD1), which could be recognized and stabilized by the m^6^A effector IGF2BP1. In addition, we identified that LYPD1 induced oncogenic behaviors of tumors in contrast to ALKBH5. Dysregulation of ALKBH5/LYPD1 axis impelled the progression of HCC.

**Conclusion:** Our study reveals that ALKBH5, characterized as a tumor suppressor, attenuates the expression of LYPD1 via an m^6^A-dependent manner in HCC cells. Our findings enrich the landscape of m^6^A-modulated tumor malignancy, and provide new insights into potential biomarkers and therapeutic targets of HCC treatment.

**Keywords:** N6-methyladenosine (m^6^A), Hepatocellular carcinoma (HCC), ALKBH5, LYPD1

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Background

Hepatocellular carcinoma (HCC) is one of the most prevailing malignancies with poor long-term prognosis and high mortality [1]. Although diagnosis and treatment of HCC have considerably improved, the frequent recurrence or metastasis of HCC can hardly be prevented owing to the inadequate understanding of its sophisticated molecular pathogenesis [2, 3]. Therefore, it is quite essential to further explicate the biological mechanisms of HCC malignancy aiming to develop more effective therapeutic strategies.

Aberrations in epigenetic regulations such as DNA methylation, histone acetylation and RNA methylation, are crucial hallmarks of HCC carcinogenesis [4]. Emerging as the most common type of mRNA methylation in eukaryotes, N6-methyladenosine (m6A) modification attracted increasingly more attention nowadays [5]. The process of m6A methylation is reversible and dynamic regulated by methyltransferases (writers), demethylases (erasers) and effectors proteins (readers) [6]. The canonical complex of writers called “WMM” is comprised of methyltransferase-like 3 (METTL3), methyltransferase-like 14 (METTL14) and Wilms tumor 1-associated protein (WTAP) [7], while identified erasers consist of fat-mass and obesity-associated protein (FTO) and AlkB homolog 5 (ALKBH5) [8, 9]. And readers are m6A-binding proteins including YT521-B homology (YTH) domain-containing family proteins (YTHDF1/2/3), YTH domain-containing proteins (YTHDC1/2), the insulin-like growth factor 2 mRNA-binding proteins family (IGF2BP1/2/3) and the heterogeneous nuclear ribonucleoprotein family (HNRNPs), which determine diverse comprehensive effects [10–12]. m6A modification accounts for far-ranging biological processes containing RNA metabolism, protein translation efficiency, transcription splicing, cell fate determination, immunologic homeostasis and tumorigenesis [13, 14].

Actually, it has been demonstrated that m6A modulation is extensively involved in the development of HCC [15]. For example, METTL14 is identified as a tumor suppressor via manipulating the m6A-mediated processing of pri-miR126 [16], while METTL3 enhances m6A-modification of SOCS2 to promote the evolution of HCC [17]. Besides, our previous work also emphasized the significance of WTAP in HCC through HuR-dependent post-transcriptional silencing of ETS1 [18]. And KIAA1429, a non-canonical writer, accelerates HCC pathogenesis via epigenetic regulation of GATA3 [19]. Moreover, Hou et al. substantiate that YTHDF2 suppresses tumor vasculature of HCC by facilitating the degradation of m6A-marked IL11 and SERPINE2 mRNA [20]. For m6A erasers, FTO has been reported to participate in HCC progression with controversial roles. Li et al. illustrate that FTO facilitates the tumorigenesis of HCC via modulating PKM2 demethylation [21]. However, a most recent study delineates that FTO, which is regulated by SIRT1-induced SUMOylation, functions as a tumor suppressor in HCC [22]. These outcomes underscore the complexity of m6A-mediated effects in HCC.

Nevertheless, few studies have investigated the role of another demethylase ALKBH5 in HCC tumorigenesis [23]. In our current study, we found that ALKBH5 was down-regulated in HCC, and lower ALKBH5 expression predicted poorer survival. Functionally, ALKBH5 inhibited the proliferation and invasiveness of HCC cells in vitro and in vivo. In addition, we verified that ALKBH5-modulated m6A modification, which is recognized by IGF2BP1, contributed to the post-transcriptional inactivation of LYG/PLAUR Domain Containing 1 (LYPD1). Furthermore, LYPD1 was subsequently identified as a novel oncoprotein in HCC. Thus ALKBH5-LYPD1 axis was closely involved in the malignancy of HCC. Our findings extend the understanding of m6A-driven machinery in HCC oncogenesis and highlight the significance of ALKBH5 in epitranscriptomic regulation.

Materials and methods

Patients and samples

Two HCC cohorts were included in this study, which was approved by Institutional Ethics Committee in First Affiliated Hospital of Zhejiang University. Cohort one contained 80 HCC patients who had undergone curative surgery from 2015 to 2018 in our hospital. Specimens of tumor and adjacent tissues were collected from these patients. RNA (70 pairs) or proteins (10 pairs) were isolated from frozen tissues for quantitative real-time PCR (qPCR) or western blotting assay to assess the expression of ALKBH5 in HCC. Cohort two consisted of 90 HCC patients which were the source of commercial tissue microarrays (TMA) supplied by Shanghai Outdo Biotech (LivH180Su07, Shanghai, China) together with integrated follow-up and clinical information data (illustrated in Table S1). This TMA cohort was employed to evaluate the role of ALKBH5 in HCC prognosis and construct the correlation of ALKBH5 and LYPD1 expression. Written informed consents were acquired from each patient relying on guidelines of the Declaration of Helsinki.

Cell culture

The human HCC cell lines Huh7, MHCC97H, HCCLM3, HepG2, Hep3B, PLC/PRF/5, SMCC7721 and BEL7402 were obtained from the Shanghai Institutes of Biological Sciences (Shanghai, China). STR fingerprinting authentications of all employed HCC cells are available upon request. And here we present STR certificates for three of them (Huh7, MHCC97H and HCCLM3), which are mainly investigated in our study (Additional file 10). These cells were incubated at 37 °C in a 5% CO2 incubator (ThermoFisher, USA) with the humidified environment. And they were cultured with Minimum Essential Media (MEM, BI, Israel), which were routinely supplemented
with 10% fetal bovine serum (FBS, BI), penicillin (100 units/ml) and streptomycin (100 μg/ml).

m⁶A dot blot assay
Total RNA isolated from HCC cells or subcutaneous tumors was mixed in three times volume of incubation buffer and denatured at 65 °C for 5 min. Samples (400 ng, 200 ng or 100 ng) dissolved in SSC buffer (Sigma-Aldrich, Germany) were deposited on an Amersham Hybond-N+ membrane (GE Healthcare, USA) which was settled on the Bio-Dot Apparatus (Bio-Rad, USA). Then the membrane was crosslinked by UV light for 5 min, followed by the staining with 0.02% Methylene blue (Sangon Biotech, China). Scanning of blue dots were performed to show the input RNA content. And the membrane was hatched with m⁶A antibody (1:5000, Synaptic System, #202003) overnight at 4 °C. Dot blots were visualized by the imaging system after incubation with secondary antibody.

Methylated RNA immunoprecipitation sequencing (MeRIP-seq) and data analysis
MeRIP-sequencing and following data analyses were mainly supported by Genesky Biotechnologies Inc. (Shanghai, China). HCCLM3 cells with stable overexpression of ALKBH5 and control cells transfected with an empty vector were collected (two replications; labeled as ALKBH5 and Vector, respectively). More than 250 μg of total RNA was extracted from each group, and mRNA was further purified with the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB#E7490) using oligo (dT) beads. Concentration and integrity of RNA were evaluated using NanoDrop Spectrophotometer and Agilent 2100 Bioanalyzer (Agilent, USA). The mRNA was then chemically fragmented into ~150 nts nucleotides. Based on MeRIP-seq results, we focused on the sites of LYPD1 transcript where differential m⁶A modification on individual transcripts. In brief, 150 μg total RNA was isolated from pretreated cells and randomly fragmented into a size of 100 or less nucleotides. RNA samples were then immunoprecipitated with magnetic beads pre-coated by 10 μg anti-m⁶A antibody (Millipore, Germany) or anti-rabbit IgG (Millipore). And N6-methyladenosine 5'-monophosphate sodium salt (6.7 mM) were applied to elute the m⁶A-modified RNA fragments. Based on MeRIP-seq results, we focused on the sites of LYPD1 transcript where differential m⁶A peak was identified between ALKBH5-overexpressing cells and empty control cells (Fig. 5a). Specific primers were designed for MeRIP-qPCR analysis according to the information from MeRIP-seq and a motif-dependent m⁶A site predictor SRAMP (http://www.cuilab.cn/sramp) (Forward: AGCAGAATTGGCTGGTTTCG; reverse: AGGCCCATCCTAAGTCCCA). Relative enrichment of m⁶A was normalized to the input: %Input =1/10^10 × 2^(-ΔCt [IP] – ΔCt [input]).

Statistical analysis
Statistical analysis was performed with the GraphPad Prism 8.0 (GraphPad, Inc., USA) and SPSS 22.0 (SPSS, Inc., USA) software. Experiments were independently repeated for at least three times. Representative data was exhibited as the means ± SD. Quantitative data was compared using two-tail Student t test, while qualitative data was evaluated by Chi-Square test. The overall and recurrence-free survival were analyzed with Kaplan–Meier method and log-rank test. And univariate and multivariate Cox regression models were employed to determine differentially expressed genes [26]. And major R codes during analyses were provided in Additional file 11.
Fig. 1 Down-regulated ALKBH5 expression correlates with poor outcomes of HCC patients. a The mRNA expression of ALKBH5 in tumor and normal tissues was measured based on 70 pairs of HCC samples (from cohort one); b Ten pairs of HCC samples (from cohort one) were subject to western blotting analysis of ALKBH5; c Grayscale analysis of ALKBH5 expression in b was conducted (calculated by log2 ratio of "adjacent/tumor pair", normalized to GAPDH); d IHC scores of matched HCC and normal tissues (n = 90) were computed based on ALKBH5 staining (cohort two); e Representative images of ALKBH5 IHC staining in HCC samples were shown (scale bars, 100 μm; magnification, 100x and 200x); f Kaplan-Meier analysis of overall survival (left) and recurrence-free survival (right) of HCC patients based on ALKBH5 expression (n = 90). Cutoffs for grouping were determined by the median of IHC scores; g Multivariate analysis was employed for HCC patients using COX regression model based on those factors which were statistically significant in univariate analysis. Symbols and bars in forest plots correspond to HR and 95% CIs, respectively. T: tumor; P: para-tumor; HR: hazard rate; CI: confidence interval.
Fig. 2 (See legend on next page.)
labeled on figures, and $P < 0.05$ was reckoned as statistically significant ($^* P < 0.05$, $^{**} P < 0.01$, $^{***} P < 0.001$, $^{****} P < 0.0001$).

More detailed methodology could be obtained in Supplementary Materials and Methods.

**Results**

**Down-regulation of ALKBH5 is associated with poor prognosis of HCC**

To investigate the expression profile of ALKBH5 in HCC, we analyzed the mRNA and protein levels of ALKBH5 in HCC and matched adjacent tissues, and found that ALKBH5 was significantly down-regulated in HCC (Fig. 1a-c). And subsequent immunohistochemistry (IHC) staining with TMA from an independent HCC cohort confirmed these results (Fig. 1d, e). Besides, HCC patients with lower ALKBH5 expression obtained shorter overall survival (OS) and recurrence-free survival (RFS) (Fig. 1f). This finding was further validated by results from the Cancer Genome Atlas (TCGA) database (Additional file 5: Figure S1a). The expression of ALKBH5 also seemed to perform well in survival prediction of early-stage HCC patients (Additional file 5: Figure S1b). Moreover, loss of ALKBH5 was identified as an independent prognostic factor for HCC patients (HR = 2.24, $P = 0.007$) (Fig. 1g). It implies that dysregulation of ALKBH5 may be involved in the progression of HCC.

**ALKBH5 inhibits HCC proliferation in vitro and in vivo**

To evaluate the functional roles of ALKBH5 in HCC, we firstly examined the expression of ALKBH5 in HCC cell lines (Additional file 5: Figure S1c, d). Huh7 or MHCC97H and HCCLM3 cells were chosen to establish ALKBH5-silencing and ALKBH5-overexpressing models, respectively. And the transfection efficiency was validated by qPCR and western blotting (Additional file 5: Figure S1e-h). As were indicated by CCK-8 and colony formation assays, knockdown of ALKBH5 enhanced the proliferation capability of HCC cells, while up-regulation of ALKBH5 showed the opposite effect (Fig. 2a). Similarly, EdU assay also demonstrated that ALKBH5 could suppress cell growth in vitro (Fig. 2b-e). Moreover, the re-expression of ALKBH5 was introduced into stable ALKBH5-knockdown cells (Additional file 6: Figure S2a, b). As expected, the restoration of ALKBH5 could rescue the enhanced proliferation capabilities induced by ALKBH5 loss in both Huh7 and MHCC97H cells (Additional file 6: Figure S2c, d), which was supported by EdU results as well (Additional file 6: Figure S2e-g).

To further address the anti-oncogenic role of ALKBH5 in HCC, we conducted in vivo experiments with subcutaneous tumor models. When ALKBH5 was silenced (Additional file 5: Figure S1i, j), volumes and weights of xenografted tumors increased compared with control group (Fig. 2f). On the contrary, ALKBH5 overexpression retarded tumor growth with considerably diminished tumor volumes and weights (Fig. 2g). And attenuation of PCNA, a marker of proliferation, was also detected along with the elevated expression of ALKBH5 (Additional file 6: Figure S2h). These results suggest that ALKBH5 exerts an inhibitory effect on HCC tumor growth in vitro and in vivo.

**ALKBH5 restrains migration/invasion abilities of HCC cells and inhibits metastasis in vivo**

We performed transwell assays and noticed that inhibition of ALKBH5 promoted both migration and invasion abilities of HCC cells, while overexpression of ALKBH5 impairs these phenotypes (Fig. 3a). Then the wound healing assay also indicated that ALKBH5 tended to attenuate migration of HCC cells (Fig. 3b). Interestingly, we always observed the altered cell morphology under microscope when ALKBH5 was silenced (data not shown). To verify whether this phenomenon was due to the reshaping of the cytoskeleton, phalloidin staining was performed subsequently. As expected, knockdown of ALKBH5 led to a looser and more divergent pattern of cytoskeleton through the rearrangement of microtubules and microfilaments (Fig. 3c, d), which denoted a more active migrating form.

To clarify the effects of ALKBH5 on HCC metastasis in vivo, ALKBH5-overexpressing and negative control HCCLM3-luc cells were implanted into BALB/c mice via tail vein injection, followed by the bioluminescence imaging. It seemed that activation of ALKBH5 damaged the metastatic potential of HCC cells with lower luciferase activity and less pulmonary metastasis (Fig. 3e, f), which was confirmed by HE staining results (Fig. 3g). In contrast, silencing of ALKBH5 promoted the metastasis of HCC (Additional file 6: Figure S2i). Therefore,
Fig. 3 (See legend on next page.)
ALKBH5 suppresses the migration/invasion abilities of HCC cells in vitro and their metastatic capabilities in vivo.

**MeRIP-seq combined with RNA-seq reveals LYPD1 as a target of ALKBH5**

We firstly applied dot blot assays to examine the role of ALKBH5 in modulating m\(^6\)A modification. Loss of ALKBH5 led to a convincingly increased m\(^6\)A level in both Huh7 and MHCC97H cells, while ALKBH5 overexpression generated the opposite result (Fig. 4a). And the same conclusion could be obtained from the analysis of subcutaneous tumors (Additional file 6: Figure S2i).

To find out the precise mechanisms underpinning the observed ALKBH5-dependent phenotypes, an integrated approach combining MeRIP-seq and RNA-seq was employed using stable ALKBH5-overexpressing and vector-transfected HCCLM3 cells. MeRIP-seq revealed 1538 differential m\(^6\)A peaks with reduced abundance (1344 corresponding transcripts) when ALKBH5 was up-regulated. Meanwhile, RNA-seq uncovered 481 down-regulated transcripts upon ALKBH5 overexpression.

We attached more importance to oncogenes whose methylation patterns and expression levels were regulated by ALKBH5. Therefore, merely those transcripts owning both hypo-m\(^6\)A-peaks and decreased expression upon ALKBH5 overexpression were selected for following investigations (Fig. 4b). To further narrow down the scope of candidates, we focused on the top 10 genes from the overlap, namely COCH, LYPD1, ADAMTS14, ABCA4, TP53I11, COLCA2, TMED7, CYP4F3, IL17RB and VCAN, listed in ascending order of expression fold change. They were subject to preliminary validation in ALKBH5-silencing or -overexpressing cells by qPCR (Fig. 4c). Intriguingly, only LYPD1 was consistently found to be inversely regulated by ALKBH5 in all three HCC cells (Fig. 4d-g; Additional file 7: Figure S3a-e), which was further confirmed by western blotting results (Fig. 4h). Taken together, LYPD1 may be the direct downstream target of ALKBH5.

**ALKBH5-regulated m\(^6\)A modification abolishes stability of LYPD1 via an IGF2BP1-dependent manner**

Our MeRIP-seq analysis suggested that m\(^6\)A peak of LYPD1 in 3’UTR shrunk remarkably with the overexpression of ALKBH5 (Fig. 5a). To substantiate this result, we first conducted RIP assays using the anti-ALKBH5 antibody in Huh7 and HCCLM3 cells. We observed that ALKBH5 could enrich LYPD1 mRNA (Fig. 5b), implying that LYPD1 may be regulated in RNA level upon interaction with ALKBH5. Then MeRIP-qPCR assays with specific primers aiming at potential m\(^6\)A sites revealed that knockdown of ALKBH5 could promote m\(^6\)A modification of LYPD1 in 3’UTR, while activation of ALKBH5 led to a decreased m\(^6\)A level in this site (Fig. 5c). To further demonstrate the essential role of m\(^6\)A in the regulation of LYPD1, we designed a luciferase reporter inserting a wild-type (WT) LYPD1–3’UTR sequence or mutant (Mut) counterpart whose putative m\(^6\)A sites were mutated (Fig. 5d). As expected, the luciferase activity of cells transfected with LYPD1-WT plasmid tended to increase when ALKBH5 was silenced, while that of mutant group seemed to be unaffected. And analogous results could be verified in ALKBH5-overexpressing cells (Fig. 5e). In addition, we found that ALKBH5 deficiency induced a slower degradation rate of LYPD1 mRNA, whereas ALKBH5 overexpression abolished the stability of LYPD1 (Fig. 5f).

Now that “readers” were crucially responsible for the direct effect on m\(^6\)A-modified transcripts, we investigated potential effectors participating in the process illustrated above. As YTHDFs and IGF2BPs were extensively involved in the modulation of RNA stability [11], YTHDF1–2 and IGF2BP1–3 were knockdown successively in two HCC cells to examine the alterations of LYPD1 expression. We noticed that intervene of YTHDF1/2 and IGF2BP2/3 hardly impacted LYPD1 expression (Additional file 7: Figure S3f-i). Nevertheless, LYPD1 was significantly inhibited when IGF2BP1 was impaired (Fig. 5g), which was consistent with the knowledge that IGF2BP1 intended to promote the transcription of its targets [10]. And the interaction between IGF2BP1 and LYPD1 via an IGF2BP1-dependent manner was further confirmed by western blotting results (Fig. 5h). Taken together, LYPD1 may be the direct downstream target of ALKBH5.
Fig. 4 (See legend on next page.)
protein and LYPD1 mRNA was confirmed by RIP assays (Fig. 5h). Moreover, knockdown of IGF2BP1 counteracted the accumulation of LYPD1 caused by ALKBH5 loss (Fig. 5i). In summary, LYPD1 is governed by ALKBH5-mediated m^6^A modification and recognized by IGF2BP1 which enhances its stability.

LYPD1 is identified as an oncogenic driver in HCC

To illustrate the role of LYPD1 in HCC, we established LYPD1-knockdown Huh7 and MHCC97H cell lines (Fig. 6a, b; Additional file 8: Figure S4a). CCK-8 and colony formation assays indicated that silencing of LYPD1 suppressed cell growth and viability (Fig. 6a, b), which was consistent with results of EdU (Fig. 6c, d). Moreover, loss of LYPD1 led to the inhibition of migration and invasion abilities of HCC cells (Fig. 6e, f). To evaluate the role of LYPD1 in vivo, lentiviruses carrying shRNA targeting at LYPD1 were transfected into Huh7 and MHCC97H cells with verified efficiency (Additional file 8: Figure S4b). Subsequently, subcutaneous implantation experiments were conducted in nude mice. As expected, knockdown of LYPD1 markedly impaired the growth of xenografted tumors (Fig. 6g, h).

Furthermore, bioinformatics analysis was carried out to explore the clinical association of LYPD1. The analysis of HCC cohort from TCGA and three other cohorts from Gene Expression Omnibus (GEO) datasets demonstrated that LYPD1 was up-regulated in tumorous tissues compared with normal tissues (Fig. 6i; Additional file 8: Figure S4c, d). Moreover, the up-regulation of LYPD1 was frequently detected in HCC patients who suffered nodal metastasis or belonged to higher tumor grades/stages (Additional file 8: Figure S4e-g). And pan-cancer analysis manifested that expression of LYPD1 was widely elevated across numerous cancers (Additional file 8: Figure S4h). Besides, Kaplan-Meier analysis implied higher LYPD1 expression correlated with poorer OS and disease-free survival (DFS) in HCC (Fig. 6j). Taken together, LYPD1 is activated during HCC development and promotes the oncogenesis of HCC.

The effects of ALKBH5 inhibition are reversed by loss of LYPD1

To confirm that the observed phenotypes were mediated by the dysregulation of ALKBH5-LYPD1 axis, we conducted several functional rescue assays. As CCK-8 and colony assays showed, knockdown of ALKBH5 led to the enhanced proliferation capacity in two HCC cells, which could be reverted by LYPD1 silencing (Fig. 7a-d). Knockdown of LYPD1 also significantly abolished the increased mobility ability induced by ALKBH5 loss (Fig. 7e-g). Besides, wound healing assays suggested that the inhibition of ALKBH5 failed to promote cell migration in LYPD1-silenced Huh7 and MHCC97H cells (Fig. 7h). To sum up, dysfunction of LYPD1 may account for the ALKBH5-mediated proliferation or mobility signatures of HCC cells.

Clinical relevance of the ALKBH5/LYPD1 axis in HCC

To further explore the correlation between expression of ALKBH5 and LYPD1 in HCC tissues, IHC staining of these two proteins were performed on TMA from the second cohort. As expected, approximately 62.2% of specimens with lower expression of ALKBH5 presented stronger LYPD1 staining, while nearly 66.7% of those with higher ALKBH5 expression exhibited weaker LYPD1 dyeing (Fig. 8a, b). Furthermore, the analysis of two independent GEO datasets revealed that ALKBH5 negatively interrelated with LYPD1 in RNA level (Fig. 8c). In conclusion, the expression of ALKBH5 and LYPD1 are inversely correlated in HCC samples.

Discussion

Accumulating evidence demonstrates that the aberration of m^6^A modification is dramatically involved in the pathogenesis of multiple diseases including HCC [27]. Actually, our previous work focuses on one of the m^6^A methyltransferases WTAP, which is identified to promote the progression of HCC in an m^6^A-dependent manner [18]. In addition, METTL3, METTL14 and KIAA1429 have been successively reported to impact
Fig. 5 (See legend on next page.)
the growth and invasion of HCC cells via diverse mechanisms [16, 17, 19]. And two studies have reported ambiguous results about FTO, which may serve as either an oncogene or a tumor suppressor in HCC [21, 22]. Meanwhile, our preliminary experiments also found the controversial effects of FTO on proliferation abilities in different HCC cells (Additional file 9: Figure S5a-k). It implies that roles of FTO in HCC may be momentous but perplexing. However, whether ALKBH5 contributes to the evolution of HCC still remains obscure. Therefore, our present study concentrated on the role of ALKBH5, and first addressed that the decreased ALKBH5 expression correlated with worse survival in HCC patients. We functionally confirmed that ALKBH5 suppressed growth and invasion abilities of HCC cells in vitro and in vivo. Mechanistically, LYPD1 was regulated by ALKBH5 via an m6A-mediated and IGF2BP1-associated pattern. And LYPD1 was subsequently verified as an oncogenic driver in HCC. Altogether, the dysregulation of ALKBH5/LYPD1 axis facilitated the progression of HCC (Fig. 8d).

ALKBH5 is a nucleic acid oxygenase which can catalyze the demethylation of m6A-labelled RNA [9]. Initially, it is reported that ALKBH5 deficiency leads to impaired fertility by controlling splicing of long 3'UTR mRNA in germ cells [9, 28]. And following researches in the field of viral infection reveal that ALKBH5 is involved in antiviral processes via m6A-guided regulation on cellular metabolism and innate immunity [29, 30]. Besides, Song et al. establish the link between ALKBH5 and autophagy in ischemic heart diseases [31]. Furthermore, ALKBH5 plays an essential part in the tumorigenesis as well. ALKBH5 enhances self-renewal and oncogenesis of glioblastoma by sustaining FOXM1 expression [32], and it also mediates the hypoxia-induced stem cell phenotypes of breast cancer [33]. However, Tang et al. uncover the tumor suppressive function of ALKBH5 through m6A-dependent inactivation of Wnt signaling [34]. In addition, ALKBH5 inhibits tumor growth and metastasis via abolishing expression and activity of YAP in non-small cell lung cancer [35]. And our findings also clarify that ALKBH5 inhibits HCC malignancy through impairing the expression of LYPD1. It implies that effects of ALKBH5 on carcinogenesis depend on the specific tissue context and different downstream molecules.

It is noteworthy that although downstream targets of ALKBH5 have been frequently explored as mentioned above, upstream masters of ALKBH5 are still vague. Hence, the factors which may induce ALKBH5 deficiency in HCC merit a debate. Based on TCGA pan-cancer data, Li et al. evaluate the alteration frequency of copy number variations (CNVs) for all m6A regulators and demonstrate that ALKBH5 gains prevalent CNV deletions in HCC (frequency of 0.432) [36]. CNV loss may be partly responsible for down-regulation of ALKBH5. In addition, microRNAs (miRNA) have been shown to inhibit several m6A enzymes [37]. We find miR-17-3p, which promotes the growth and invasion of HCC cells [38], is the potential miRNA targeting at ALKBH5 as predicted by TarBase and TargetScan tools. It should be further validated whether miR-17-3p or other miRNAs can regulate the level of ALKBH5. And it is reported that cigarette smoke condensate may induce the hypomethylation of ALKBH5 CpG island in esophageal squamous cell carcinoma [39]. That suggests that ALKBH5 can also be regulated by DNA methylation, which is widely involved in HCC pathogenesis. Besides, histone acetylation and methylation actively participate in the modulation of m6A enzymes as well [40]. Whether ALKBH5 is controlled by suppressive histone modifications such as H3K9me3 or H3K27me3 requires further investigations.
Fig. 6 (See legend on next page.)
Although LYPD1 is predicted as a glycosylphosphatidylinositol (GPI)-anchored and membrane-bound protein [41], it was originally identified from central nervous systems [42] and it may govern anxiety by binding to neuronal nicotinic acetylcholine receptors (nAChRs) [43]. Albeit LYPD1 was judged as a tumor suppressor in HeLaHF cells [44], few other investigations offered information about its role in cancer. In our study, loss of LYPD1 disrupted the proliferation ability and invasion potential of HCC cells (Fig. 6a-h), while LYPD1 expression was elevated in tumor tissues and high level of LYPD1 indicated a poorer prognosis of HCC (Fig. 6i, j and Additional file 8: Figure S4c-g). Here we have systematically illustrated the cancer-related behavior of LYPD1 and its upstream partners within the m^6^A-based modulation, providing novel insights into functions of LYPD1 in tumorigenesis. Perhaps we should further exploit how to make it feasible to abolish LYPD1 activity clinically.

Although we have supplied abundant evidence to support the significant role of m^6^A-regulated ALKBH5/LYPD1 axis in HCC progression, there are still several drawbacks in our work. For example, the results of ALKBH5/IGF2BP1 rescue assays showed a little difference between two HCC cells, which implies that IGF2BP1 may not be the only m^6^A reader downstream of ALKBH5 loss (Fig. 5i). Although we have screened YTHDFs and IGF2BPs family which are closely related to RNA stability modulation (Additional file 7: Figure S3f-i), other effector proteins such as YTHDCs (involved in alternative splicing or nuclear export) [45, 46], HNRNPs (related to molecular structure) [47] and other non-canonical readers (like HuR, etc.) have not been investigated. Maybe some of them can also participate in the regulation of LYPD1, which deserves a further exploration.

Besides, the underlying mechanisms of LYPD1-mediated modulation of downstream pathways is not fully characterized. To hunt for some clues, we have ever re-analyzed the results of transcriptome sequencing. Gene ontology analysis demonstrated that ALKBH5 expression was tightly correlated with cell motility and proliferation (Additional file 10: Figure S6j), which is consistent with our functional results. In addition, ALKBH5 may be involved in the regulation of PI3K and GTPases pathways (Additional file 10: Figure S6k). The PI3K/akt/mTOR cascade is one of the most crucial signaling in tumor, which controls various cellular activities including cell growth and migration [48]. And Rho GTPases are responsible for the domination of cytoskeleton organization and cell mobility [49]. Our western blotting outcomes revealed that knockdown of ALKBH5 efficiently triggered the PI3K/AKT/mTOR and Rho GTPases pathways, while suppression of LYPD1 retrieved these activated machineries (Additional file 10: Figure S6l). To ensure that these two pathways were explicitly involved in ALKBH5/LYPD1-dependent modulation, further work was required including exploring direct link between LYPD1 and downstream signaling and assessing whether ALKBH5/LYPD1 axis could remodel the sensitivity of HCC cells to inhibitors of PI3K/AKT/mTOR or GTPases pathways in vitro and in vivo.

**Conclusion**

In summary, our work has revealed the tumor suppressor properties of ALKBH5 in HCC development. Down-regulation of ALKBH5 activates the m^6^A machinery.
Fig. 7 Dysregulation of the ALKBH5-LYPD1 axis triggers HCC malignancy. 

a and b CCK-8 proliferation assays were conducted in either ALKBH5-knockdown or LYPD1-knockdown Huh7 (a) and MHCC97H (b) cells; c and d Colony formation assays were carried out in either ALKBH5-silenced or LYPD1-silenced Huh7 (c) and MHCC97H (d) cells. Column diagrams (right panel) showed colony numbers of each group; e, f, and g Representative images of transwell assays to examine the effects of LYPD1 knockdown on ALKBH5-silenced Huh7 (e) and MHCC97H (f) cells were shown (scale bars, 200 μm); Quantification data presented the relative count (refer to negative control group) of cells which passed through the chamber membrane (g); h Representative images of wound healing assays conducted in ALKBH5/LYPD1-rescued cells were shown (left panel). And percent area of wound healed in each group was quantified (right panel).
**Fig. 8** (See legend on next page.)
contributing to the epigenetic activation of LYPD1 which is recognized and stabilized by IGFBP1. Our findings highlight the attractive values of m^6^A demethylases and enrich the understanding of m^6^A epitranscriptional modification in cancer research, further providing novel insights into exploiting effective predictors and therapeutic strategies for HCC.

**Supplementary information**

**Supplementary information** accompanies this paper at https://doi.org/10.1186/s12943-020-01239-w.

**Additional file 1**: Table S1. Clinical characteristics of 90 HCC patients depending on ALKBH5 expression.

**Additional file 2**: Table S2. Target sequences of siRNAs and shRNAs utilized in this work.

**Additional file 3**: Table S3. Sequences of primers utilized in this study.

**Additional file 4**: Table S4. Antibodies utilized in this work.

**Additional file 5**: Figure S1. Clinical significance and transfection efficiency of ALKBH5. a Kaplan-Meier analysis of all HCC patients based on ALKBH5 expression (from TCGA cohort, analyzed with KM plotter, https://kmplot.com/analysis/). b Kaplan-Meier analysis of HCC patients with early stages (stage 1 and 2, from TCGA cohort) based on ALKBH5 expression, c and d Protein (c) and RNA (d) expression of ALKBH5 in HCC cell lines, e, f, g, and h The transient knockdown and stable overexpression efficiency of ALKBH5 in three HCC cells was determined by western blotting (e) and qPCR (f). i The stable knockdown efficiency of ALKBH5 was measured via western blotting (j) and qPCR (k). OS: overall survival; PFS: progression-free survival.

**Additional file 6**: Figure S2. Further in vitro and in vivo information about the roles of ALKBH5 in HCC cells. a and b The knockdown and re-expression efficiency of ALKBH5 in two HCC cells were determined via qPCR (a) and western blotting (b); c and d CCK-8 (upper panel) and colony assays (lower panel) were conducted to check the effects of ALKBH5 re-expression in ALKBH5-silenced in Huh7 (c) and MHCC97H (d); cells. e, f, and g EdU assays were employed to further determine the effects of ALKBH5 reactivation on ALKBH5-knockdown Huh7 (e) and MHCC97H (f) cells. And percentage of cells in S phase was exhibited (g). h Typical IHC images of subcutaneous tumors using ALKBH5-overexpressed or vector transfected HCCLM3 cells were shown (scale bars: 50 μm); Staining of ALKBH5 was applied to validate the transfection efficiency, while intensity of PCNA staining represented the proliferation capability of tumors. i Representative HE staining images of metastasis in lungs induced by tail vein injection of negative control or ALKBH5-silenced MHCC97H cells were presented; j Tumors of xenografted mice implanted with ALKBH5-overexpressed or control HCCLM3 cells were shown to be unrelated to RNA isolation. The m^6^A level of each group was measured using m^6^A dot blot assays. And the representative images of dot blots were shown.

**Additional file 7**: Figure S3. Screening of ALKBH5 targets and potential m^6^A effectors of LYPD1. a, b, c, d, and e Expression of COLCA2 (a), TMED7 (b), CPYF4F3 (c), IL17RB (d), and VCAN (e) were checked in ALKBH5-knockdown or -overexpressed cells, respectively. Expression of ABCA4 was too low to detect, thus its data was not shown; f LYPD1 was measured by qPCR after YTHDF1 was knocked down in Hep3B and MHCC97H cells; g YPD1 was determined by qPCR after YTHDF1 was knocked down in HCC cells; h LYPD1 was determined using qPCR when IGFBP2 was knocked down in HCC cells; i LYPD1 was measured using qPCR after IGFBP2 was knocked down in HCC cells.

**Additional file 8**: Figure S4. LYPD1 was up-regulated in HCC. a Knockdown efficiency of LYPD1 using siRNA was verified in Huh7 and MHCC97H cells by western blotting; b Knockdown efficiency of LYPD1 using shRNA was confirmed via qPCR; c and d Expression of LYPD1 in HCC patients from TCGA cohort (c) or GEO (d) data was shown; e, f, and g Expression of LYPD1 in HCC cohorts based on TCGA data stratified by nodal metastasis status (e), tumor grade (f) and tumor stage (g). h and i analyzed by UALCAN; g analyzed by GEPIA; h Pan-cancer atlas of LYPD1 expression in HCC samples (data from TCGA, analyzed by UALCAN; blue color represented normal group and red color represented tumor group).

**Additional file 9**: Figure S5. Controversial functional roles of FTO in different HCC cells. a, b and c Knockdown efficiency of FTO in Huh7 (a) and MHCC97H were measured by western blotting (a) and qPCR (b) and c; and d CCK-8 and colony formation assays were conducted in FTO-silenced Huh7 (c) and MHCC97H (d) cells. Column charts showed colony numbers of each group (right panel). Loss of FTO contributed little to the proliferation abilities of these two cells. e, f and g Negative control and FTO-silenced Huh7 (e) or MHCC97H (f) cells were subject to EdU assays. Percentage of cells in S phase was quantified in column charts (g). h and i Knockdown efficiency of FTO in HepG2 and Hep3B were determined by western blotting (h) and qPCR (i); j and k CCK-8 and colony formation assays were conducted in FTO-knockdown HepG2 (j) and Hep3B (k) cells. Surprisingly, inhibition of FTO suppressed the proliferation capabilities of these two cells.

**Additional file 10**: Figure S6. The explorations of whether FTO can regulate the m^6^A modification of LYPD1 and possible downstream pathways of ALKBH5/LYPD1 axis. a and b Expression of LYPD1 was measured when FTO was silenced in Huh7 and MHCC97H cells using western blotting (a) and qPCR (b) assays; c Transfection efficiency was measured by western blotting assays in two HCC cells with individual or double knockdown of FTO and ALKBH5; d and e Transfection efficiency was determined via qPCR assays in Huh7 (d) and MHCC97H (e) cells with individual or double knockdown of FTO and ALKBH5; f and g Relative m^6^A enrichment of LYPD1 in Huh7 (f) and MHCC97H (g) cells with single or double knockdown of FTO and ALKBH5 were determined by MeRIP-qPCR assays. h and i Relative luciferase activity of Huh7 (h) and MHCC97H (i) cells transfected with the LYPD1-wild type or LYPD1-m^6^A site-mutated construct were measured. For each group, FTO and ALKBH5 are individually or double knockdown. j and k GO functional categories containing BP and MF of RNA sequencing using ALKBH5-overexpression or control HCCLM3 cells. When ALKBH5 was overexpressed, those down-regulated transcripts were prominently enriched in BP including epithelial cell migration, cell proliferation or cell adhesion. Meanwhile, they were enriched in MF which mainly contains PI3K activity and GTPase regulator activity. l Western blotting analyses were performed in ALKBH5-silenced HCC cells to check the impacts of ALKBH5 on PI3K-AKT-mTOR and Rho GTPases signaling. Then effects of following LYPD1
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References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA Cancer J Clin. 2020; 70:7–30.
2. Yang JD, Hainaut P, Goes GJ, Amadou A, Plymoth A, Roberts L R. A global view of hepatocellular carcinoma: trends, risk and prevention management. Nat Rev Gastroenterol Hepatol. 2019;16:589–604.
3. Yegin EG, Oymaci E, Karataş E, Coker A. Progress in surgical and nonsurgical approaches for hepatocellular carcinoma treatment. Hepatobiliary Pancreat Dis Int. 2016;15:234–56.
4. Rebuissou S, Nault JC. Advances in molecular classification and precision oncology in hepatocellular carcinoma. J Hepatol. 2020;72:215–29.
5. Liu J, Harada BT, He C. Regulation of gene expression by N6(−)-methyladenosine in cancer. Trends Cell Biol. 2019;29:487–99.
6. Zuccaro S, Ries RJ, Jaffrey SR. Reading, writing and erasing mRNA methylation. Nat Rev Mol Cell Biol. 2019;20:608–24.
7. Ping XL, Sun BF, Wang L, Xiao W, Yang X, Wang WJ, Adhikari S, Shi Y, Lv Y, Chen YS, et al. Mammalian WTAP is a regulatory subunit of the RNA N6-methyladenosine methyltransferase. Cell Res. 2014;24:177–89.
8. Ja G, Fu Y, Zhao X, Dai Q, Zheng G, Yang Y, Yi C, Lindahl T, Pan T, Yang YG, He C. N6-methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. Nat Chem Biol. 2011;7:885–9.
9. Zheng G, Dahi JA, Niu Y, Federcock P, Huang CM, Li CJ, Vagbo CB, Shi Y, Wang WL, Song SH, et al. ALKBHS is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility. Mol Cell. 2013;49:18–29.
10. Huang H, Wang H, Sun W, Qin X, Shi H, Wu, Zhao BS, Mesquita A, Liu C, Yuan CL, et al. Recognition of RNA N6- methyladenosine by IGF2BP proteins enhances mRNA stability and translation. Nat Cell Biol. 2018;20:285–9.
11. Yang Y, Hsu PJ, Chen YS, Yang Y-G. Dynamic transcriptomic m6A: A decoration: writers, erasers, readers and functions in RNA metabolism. Cell Res. 2018;28:616–24.
12. Zhao Y, Shi Y, Shen H, Xie W: m(6)A-binding proteins: the emerging crucial performers in epigenetics. J Hepatol Oncol. 2020;13:35.
13. Deng X, Su R, Wang H, Huang H, Li Z, Chen J. RNA N6-methyladenosine modification in cancers: current status and perspectives. Cell Res. 2018;28:507–17.
14. Huang H, Wang H, Chen J. m(6)A modification in coding and non-coding RNAs: roles and therapeutic implications in cancer. Cancer Cell. 2020;37:270–88.
15. Zhou Z, Meng J, Su R, Zhang J, Chen J, Ma X, Xia Q, Epitranscriptomics in liver disease: basic concepts and therapeutic potential. J Hepatol. 2020. https://doi.org/10.1001/jhep.2020.04.009.
16. Ma JZ, Yang F, Zhou CC, Liu F, Yuan JH, Wang F, Wang TT, Xu QG, Zhou WP, Sun SH. METTL14 suppresses the metastatic potential of hepatocellular carcinoma by modulating N6-(methyladenosine)-dependent primary MicroRNA processing. Hepatology. 2017;65:529–43.
17. Chen M, Wei L, Law C-T, Tsang FH-C, Shen J, Cheng CL-H, Tsang L-H, Ho DW-H, Chiu DK-C, Lee JMF, et al. RNA N6-methyladenosine methyltransferase-like 3 promotes liver cancer progression through YTHDF2-dependent posttranscriptional silencing of SOCS2. Hepatology. 2018;67:2254–70.
18. Chen Y, Peng C, Chen J, Chen D, Yang B, He B, Hu W, Zhang Y, Liu H, Dai L, et al. WTAP facilitates progression of hepatocellular carcinoma via m6A-HuR-dependent epigenetic silencing of ET51. Mol Cancer. 2019;18:127.
19. Lan T, Li H, Zhang D, Xu L, Liu H, Hao X, Yan X, Liao H, Chen X, Xie K, et al. KIAA1429 contributes to liver cancer progression through N6-methyladenosine-dependent post-transcriptional modification of GATA3. Mol Cancer. 2019;18:163.
20. Liu J, Zhu L, Shi Y, Liu J, Lin C, Chen X. m6A demethylase FTO promotes hepatocellular carcinoma tumorigenesis via mediating PKM2 demethylation. Am J Transl Res. 2019;11:6048–44.
22. Liu X, Liu J, Xiao W, Zeng Q, Bo H, Zhu Y, Geng L, He D, Xing X, Li R, et al. SIRT1 regulates N6-methyladenosine RNA modification in hepatocarcinogenesis by inducing RANBP2-dependent FTO SUMOylation. Hepatology. 2020;13:817–89.

23. Chen M, Wong CM. The emerging roles of N6-methyladenosine (m6A) deregulation in liver carcinogenesis. Mol Cancer. 2020;19:44.

24. Dominissi D, Mositch-Moshkovitz S, Salmon-Dvon M, Amariglio N, Rechavi G. Transcriptome-wide mapping of N6(methyladenosine by m6A-seq based on immunocapturing and massively parallel sequencing. Nat Protoc. 2013;8:176–89.

25. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol. 2010;28:511–17.

26. Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L. Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat Biotechnol. 2013;31:46–54.

27. Hu BB, Wang XY, Gu XY, Zou C, Wu XY, Gao ZJ, Zhang H, Fan Y. N(6)-methyladenosine (m6A) RNA modification in gastrointestinal tract cancers: roles, mechanisms, and applications. Mol Cancer. 2019;18:178.

28. Tang C, Kukovich R, Peng H, Wang Z, Yu T, Zhang Y, Zheng H, Klungland A, Yan W. ALKBH5-dependent m6A demethylation controls splicing and stability of long 3′UTR mRNAs in male germ cells. Proc Natl Acad Sci U S A. 2018;115:E325–e333.

29. Zheng Q, Hou J, Zhou Y, Li Z, Cao X. The RNA helicase DDX46 inhibits innate immunity by entrapping m6A-demethylated antiviral transcripts in the nucleus. Nat Immunol. 2017;18:1094–103.

30. Liu Y, You Y, Lu Z, Yang J, Li P, Liu L, Hu H, Niu Y, Cao X. N6-methyladenosine RNA modification-mediated cellular metabolism rewiring inhibits viral replication. Science. 2019;365:1171–7.

31. Song H, Feng X, Zhang H, Luo Y, Huang J, Lin M, Jin J, Ding X, Wu S, Huang H, et al. METTL3 and ALKBH5 oppositely regulate m6A modification of TFE3 mRNA, which dictates the fate of hypoxia/reoxygenation-treated cardiomyocytes. Autophagy. 2019;15:1419–37.

32. Zhang S, Zhao B, Zhou A, Lin K, Zheng S, Lu Z, Chen Y, Sulman EP, Xie K, Bogler O, et al. A demethylase ALKBH5 maintains tumorigencity of glioblastoma stem-like cells by sustaining FOXM1 expression and cell proliferation program. Cancer Cell. 2017;31:591–606.e596.

33. Zhang C, Samanta D, Lu H, Bullen JW, Zhang H, Chen I, He X, Semenza GL. Hypoxia induces the breast cancer stem cell phenotype by HIF-dependent and ALKBH5-mediated m6A-demethylation of NANG mRNA. Proc Natl Acad Sci U S A. 2016;113:E247–56.

34. Tang B, Yang Y, Kang M, Wang Y, Wang Y, Bi Y, He S, Shimamoto F. m6A A demethylase ALKBH5 inhibits pancreatic cancer tumorigenesis by decreasing WIF-1 RNA methylation and mediating Wnt signaling. Mol Cancer. 2020;19:3.

35. Jin D, Guo J, Wu Y, Yang L, Wang X, Du J, Dai J, Chen W, Gong K, Mao S, et al. A demethylase ALKBH5 inhibits tumor growth and metastasis by reducing YTHDF3-mediated YAP expression and inhibiting miR-107/LATS2-mediated YAP activity in NSCLC. Mol Cancer. 2020;19:40.

36. Li Y, Xiao J, Bai J, Tian Y, Qu Y, Chen X, Wang Q, Li X, Zhang Y, Xu J. Molecular characterization and clinical relevance of m6A A regulators across 33 cancer types. Mol Cancer. 2019;18:137.

37. Cai X, Wang X, Cao C, Gao Y, Zhang S, Yang Z, Liu Y, Zhang X, Zhang W, Ye L. HBXIP-elevated methyltransferase METTL3 promotes the progression of breast cancer via inhibiting tumor suppressor let-7g. Cancer Lett. 2018;415:629–35.

38. Yu J, Xu Q, Wang Z, Yang Y, Zhang L, Ma J-z, Sun S-h, Yang F, Zhou W-p. Circular RNA circMARCS inhibits growth and metastasis in hepatocellular carcinoma. J Hepatol. 2018;68:1214–27.

39. Wu S, Zhang L, Deng J, Guo B, Li F, Wang Y, Wu R, Zhang S, Lu J, Zhou Y. A novel micropeptide encoded by Y-linked LINC00278 links cigarette smoking and AR signaling in male esophageal squamous cell carcinoma. Cancer Res. 2020;80:1790.

40. Wang Q, Chen C, Ding Q, Zhao Y, Wang Z, Chen J, Jiang Z, Zhang Y, Xu G, Zhang L, et al. METTL3-mediated m6A modification of HDGF mRNA promotes gastric cancer progression and has prognostic significance. Gut. 2020;69:1193–1205.

41. Masuda S, Matsura K, Shimizu T. Inhibition of LYPD1 is critical for endothelial network formation in bioengineered tissue with human cardiac fibroblasts. Biomaterials. 2018;166:109–21.

42. Dessau E, Salaun D, Gayet O, Chabbert M, de Lapeyriere O. Identification of lynv2, a novel member of the ly-6/lectin superfamily, expressed in neuronal subpopulations during mouse development. Mol Cell Neurosci. 2006;31:232–42.

43. Tekiray AB, Nong Y, Miwa JM, Lieberam I, Ibanez-Tallon I, Greengard P, Heintz N. A role for LYNX2 in anxiety-related behavior. Proc Natl Acad Sci U S A. 2009;106:4477–82.

44. Yu DH, Fan W, Liu G, Nguy V, Chatteeton JE, Long S, Ke N, Meyhack B, Brueggerer A, Brachat A, et al. PHTS, a novel putative tumor suppressor, is involved in the transformation reversion of HeLaH cells independently of the p53 pathway. Exp Cell Res. 2006;312:865–76.

45. Xiao W, Adhikari S, Dahal U, Chen Y-S, Hao Y-J, Sun B-F, Sun H-Y, Li A, Ping X-L, Lai W-Y, et al. Nuclear m6A-reader YTHDC1 regulates mRNA splicing. Mol Cell. 2016;61:507–19.

46. Roundtree IA, Luo G-Z, Zhang Z, Wang X, Zhou T, Cui Y, Sha J, Huang X, Guerrero L, Xie P, et al. YTHDC1 mediates nuclear export of N-6 methyladenosine methylated mRNAs. Elife. 2017;6:e31311.

47. Wu B, Su S, Patti DP, Liu H, Gan J, Jeffrey SR, Ma J. Molecular basis for the specific and multivariant recognition of RNA substrates by human hmNRP A2/B1. Nat Commun. 2018;9:420.

48. Manning BD, Toker A. AKT/PKB signaling: navigating the network. Cell. 2017;169:381–405.

49. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. Nature. 2002;419:353–62.