Transfer RNA demethylase ALKBH3 promotes cancer progression via induction of tRNA-derived small RNAs

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ABSTRACT

Transfer RNA is heavily modified and plays a central role in protein synthesis and cellular functions. Here we demonstrate that ALKBH3 is a 1-methyladenosine (m1A) and 3-methylcytidine (m3C) demethylase of tRNA. ALKBH3 can promote cancer cell proliferation, migration and invasion. In vivo study confirms the regulation effects of ALKBH3 on growth of tumor xenograft. The m1A demethylated tRNA is more sensitive to angiogenin (ANG) cleavage, followed by generating tRNA-derived small RNAs (tDRs) around the anticodon regions. tDRs are conserved among species, which strengthen the ribosome assembly and prevent apoptosis triggered by cytochrome c (Cyt c). Our discovery opens a potential and novel paradigm of tRNA demethylase, which regulates biological functions via generation of tDRs.

INTRODUCTION

Nowadays, more than 100 forms of modifications have been identified to regulate the biological functions of RNA (1). Transfer tRNA (tRNA), which functions as amino acid carrier during protein synthesis, are heavily modified post-transcriptionally (2). It has been reported that there are 13 modifications, on average, in one human tRNA (3). These modifications can influence the structure, stability and translation accuracy of tRNA (4,5). As reported, a large subset of tRNA modification enzymes are linked to human diseases (6).

Methylation is the most frequently happening post-transcriptional modification of tRNA (7,8). In cytoplasmic tRNA, 1-methyladenosine (m1A) at position 58 (m1A58) is the predominant m1A modification in eukaryotic cells (9). The 3-methylcytidine (m3C) modification occurs at position 32 in the anticodon loop of cytoplasmic tRNAThr, tRNASer and tRNAArg, and also at other positions (20 or 47) of tRNALeu and tRNAMet-e (10). Modifications of tRNA are crucial for its stability and biological functions (5). For instance, lack of m1A58 impairs the hydrogen bonds from A58 to A54 and A60 in initiator tRNAMet of yeast, leading to the degradation of tRNAMet (11). Since tRNA is essential to cellular functions (12), it will be of interest to query which enzyme participates in the dynamic variation of tRNA methylation.

It is well known that tRNA methyltransferases (MTases) such as rossmann-fold MTases (RFM) and SpoU–TrmD MTases (SPOUT) are responsible for tRNA methylation (13). However, demethylase which targets tRNA to remove the methyl group is not well illustrated. FTO and ALKBH5 have been identified as demethylases that catalyze the removal of m6A modifications in mRNA through an iron-dependent oxidative demethylation mechanism (14). Both FTO and ALKBH5 influence mouse fertility (15), human body weight (16) and cancer progression (17). Recently, m1A in tRNA was firstly identified that could be demethylated by ALKBH1 in vitro and in vivo (18). The dynamic methylation of tRNA affects the cellular level of tRNAMet and regulates translation initiation (18). Together, it indicates that other AlkB family members might be also involved in the dynamic methylation of tRNA.

Human ALKBH3 belongs to AlkB family that utilizes nonheme iron (II) to catalyze biological oxidation (19). Previous report showed that ALKBH3 prefers to demethylate single-stranded DNA (ssDNA) and RNA (19). Although ALKBH3 has been suggested to be involved in single strand RNA demethylation (20), studies tended to focus its demethylase activity on DNA, such as DNA repair

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and genomic stability (21,22). It has been reported that expression of ALKBH3 is elevated in pancreatic (23), lung (24) and urothelial (25) cancer as compared to the normal tissue. Also, ALKBH3 is suggested to benefit the growth and progression of colorectal (26) and lung (24) cancer cells. However, it remained to be elusive whether ALKBH3-modulated DNA/RNA demethylation is involved in its promotion effects on cancer progression.

Here, we found that ALKBH3 acted as tRNA demethylase to remove the methyl group of m1A and m3Ci nt RNA motion effects on cancer progression. Also, it is suggested to benefit the growth of modulated DNA cells. However, it remained to be elusive whether ALKBH3-modulated DNA/RNA demethylation is involved in its promotion effects on cancer progression.

MATERIALS AND METHODS

Cross-linking immunoprecipitation (CLIP) and sequencing

The CLIP procedures were conducted using HeLa cells according to the previous study (27) with slight modifications. Briefly, four plates of HeLa cells in 15 cm dishes were transfected with pPB/ALKBH3 plasmid for 48 h to reach about 90% cell confluency. After washing twice with ice-cold PBS, cells were irradiated twice with 400 mJ/cm² at 254 nm by Stratalinker on ice. Cells were lysed in high salt lysis buffer (300 mM NaCl, 0.2% NP-40, 20 mM Tris–HCl pH 7.6, 0.5 mM DTT, protease inhibitor cocktail (1 tablet/50 ml), and RNase inhibitor (1:200)) at 4°C for 30 min. Supernatant was collected at 17 000 g at 4°C for 15 min and further treated with 1 U RNase T1 for 15 min at 24°C. After centrifugation and filtration through 0.22 µM filter, 10% supernatant was collected as input for further sequencing analysis. Anti-Flag M2 beads (Sigma-Aldrich, St. Louis, MO, USA, 80 µl) were washed three times with lysis buffer and incubated with the filtered supernatant at 4°C for 4 h. After removing the flow through by magnetic rack, beads were washed with for three times with 1 ml of high salt buffer (5% PBS supplemented with 0.1% SDS, 1% Nonidet P-40 and 0.5% sodium deoxycholate) and another three times with 1 ml of wash buffer (20 mM Tris–HCl, pH 7.4, 10 mM MgCl₂ and 0.2% Tween-20). Beads and input were further incubated with 1 U RNase T1 exactly for 8 h. Then, bead samples were treated with 10 U RNase T1 exactly for 8 h. After treatment, the CLIP procedures were conducted using HeLa cells according to the previous study (27) with slight modifications. Generally, the 50 µl reaction system contained the following components: RNAs (probes), wt or mutant ALKBH3 protein, KCl (100 mM), MgCl₂ (2 mM), RNasin (0.2 U µl⁻¹, Invitrogen), L-ascorbic acid (2 mM), α-ketoglutarate (300 µM), (NH₄)₂Fe(SO₄)₂·6H₂O (150 µM) and 50 mM of HEPES buffer (pH 7.0). The reactions were incubated at 37°C for 1.5 h, then quenched by addition of 5 mM EDTA and heated at 95°C for 10 min. Samples were then centrifuged at 13 000 g for 30 min at room temperature and supernatant was collected for LC–MS/MS analysis. The probes were synthesized in our lab and purified by HPLC. The sequences of the probes were listed as follows:

m1A in loop RNA: 5’-CCCGGUUCG5UUCCGG (5 = m1A, T-loop of tRNAHisGTG, loop size 8)

m1A in linear RNA: 5’-CACGGGUUCG5UUCAAAG (5 = m1A, no second structure)

Cell proliferation, colony formation, invasion and apoptosis

Cell proliferation was tested by CCK-8 kit (Dojindo, Kumamoto, Japan). Colony formation was detected by CytoSelect 96-well Cell Transformation Assay (Cell Biolabs, USA). Cell invasion was examined by the CytoSelect™ 24-well extracellular matrix (ECM) array (Cell Biolabs, USA). The apoptosis was detected by FITC annexin V apoptosis detection kit with PI (Biolegend, San Diego, CA, USA).

Polysome profiling

Polysome profiling was performed according to previously stated method (29). The fractions were categorized and used to isolate total RNA by Trizol reagent for RT-PCR, tRNA purification and LC-MS/MS measurement.

tRNA fragment purification and sequencing

Total RNA (100 µg) were denatured at 70°C for 5 min and separated by a 15% TBE–urea gel with 20/100 or 10/60
ALKBH3 catalyzes demethylation of m^1A and m^3C in tRNA

Since CLIP-seq suggested that ALKBH3 was associated to tRNA in vivo, we hypothesized that the known tRNA methylations such as m^3G, m^3G, m^1A, m^5C and m^3C, might be the target of ALKBH3. In vitro biochemical assay showed that ALKBH3 significantly decreased the levels of m^1A and m^3C, but not others, in tRNAs from both HeLa (Figure 2A) and 293T (Supplementary Figure S2A) cells. Similar observations were obtained using the commercial tRNA from bakers yeast (Sigma, Cat. 10109495001) (Supplementary Figure S2B). ALKBH3 had no effect on the modifications including f^6A, m^6Am, Am, Cm, Gm, Um or Ca^3C in tRNAs from HeLa cells (data not shown). Besides, enzymatic kinetic studies using purified tRNA and synthesized RNA probes showed that ALKBH3 rapidly (2 min) demethylated m^1A and m^3C of tRNA in vitro, effect of which is comparable to the m^1A demethylation activity of ALKBH1 (18). Moreover, m^3C demethylation activity of ALKBH3 tended to be higher on tRNA and loop RNA probes than that of linear RNA probe (Figure 2B).

To evaluate the demethylation effects of ALKBH3 in vivo, HeLa cells were transfected with pcDNA/ALKBH3 or si-ALKBH3 1~3 (Supplementary Figure S2C). Overexpression of ALKBH3 led to a significant decrease in m^1A (16%) and m^3C (26%) of total tRNA after 24 h transfection (Figure 2C and D). Meanwhile, knockdown of ALKBH3 by si-ALKBH3-1~3 remarkably increased m^1A (17~24%) and m^3C (20~45%) of total tRNA (Figure 2C and D). Similar observations were obtained in 293T cells (Supplementary Figure S2D). Furthermore, 16% and 21% reductions of m^1A and m^3C levels in total tRNAs, respectively, were detected in HeLa cells stably overexpressing ALKBH3 (Supplementary Figure S2E). Consistently, increase of m^1A (15%) and m^3C (12%) levels in tRNAs from Alkbh3^-/- cells were observed when compared to that from wild-type HeLa cells (Supplementary Figure S2E).

We further tested the functions of ALKBH3 in wild type (WT) and Alkbh3^-/- mice. The high to low expression levels of ALKBH3 in organs of wide type mice was lung, testis, epididymis, liver, kidney and heart (Supplementary Figure S2F). The levels of m^1A (Figure 2E) and m^3C (Figure 2F) in tRNAs isolated from lung, testis, epididymis of Alkbh3^-/- mice were significantly (P < 0.05) enhanced. However, there was no statistical difference for m^1A or m^3C in liver, kidney or heart between WT or Alkbh3^-/- mice (Supplementary Figure S2G). We next examined the potential effects of ALKBH3 on mRNA, rRNA or mtRNA (mitochondria RNA). Our data showed that ALKBH3 was independent to the m^1A of mRNA (Supplementary Figure S2H), rRNA (Supplementary Figure S2I) or mtRNA (data now shown) in tested cells.
Characterization and catalytic properties of ALKBH3 on specific tRNA

ALKBH3 acting as demethylase on tRNA was further characterized. In vitro pulldown assays confirmed that ALKBH3 showed favoritism to m1A and m3C than A or C of ssRNA probe (Supplementary Figure S3A). To better understand the demethylation activity, two ALKBH3 mutants were constructed, which were defective in its DNA/RNA binding groove residue Arg122 or central position at the active site Leu177, respectively, via Ala substitutions (Supplementary Figure S3B). Results showed that both R122A and L177A mutants lost their demethylation activity on m1A o ft R N A in vitro (Figure 3A) and in vivo (Supplementary Figure S3C). This was consistent with previous data that Arg122 and Leu177 were essential to the ALKBH3 activity (33).

The subcellular targets of ALKBH3 were further investigated. Our data showed that in Alkbh3−/− HeLa cells, m1A level of tRNA in cytoplasm, but not in nucleus, was remarkably higher than that of wild type cells (Figure 3B). Similar results were observed in HeLa cells stably overexpressing ALKBH3 (Supplementary Figure S3D). This might be due to that ALKBH3 predominantly located in the cytoplasm. Next, we divided tRNA into two fractions, translation initiation fraction (containing 40S ribosomes, 60S ribosomes, 80S monosomes, <80S) and translation activating fraction (containing polysomes, >80S), from HeLa cells by use of ribosome profiling (Figure 4F). The tRNA content in <80S fraction was much greater than that of >80S fraction (Supplementary Figure S3E). LC–MS/MS (Figure 3C) and northern blot analysis (Supplementary Figure S3F) showed that m1A level of tRNA in <80S fraction, but not > 80S fraction, was significantly increased in Alkbh3−/− HeLa cells. In addition, northern blot analysis indicated that m1A modification mainly existed in tRNA (Supplementary Figure S3F).

We further validated the demethylation activity of ALKBH3 on selected tRNA. Gel-images showed that individual tRNAs were successfully purified and identical in wild type and Alkbh3−/− HeLa cells (Supplementary Figure S3G). LC/MS/MS showed that deletion of ALKBH3 significantly increased the m1A levels of most target tRNAs such as tRNA\textsubscript{GluCTC}, tRNA\textsubscript{SeCTCA}, tRNA\textsubscript{GlyGCC}, tRNA\textsubscript{HisGTG}, tRNA\textsubscript{SerGCT}, tRNA\textsubscript{GlaCTG}, tRNA\textsubscript{ValCAC}, tRNA\textsubscript{ArgCCT}, tRNA\textsubscript{LeuCAA}, tRNA\textsubscript{GlyGCC}, tRNA\textsubscript{AlaGGC} and tRNA\textsubscript{MetCAT}. However, m1A levels of some targets, such as tRNA\textsubscript{LysCTT} and tRNA\textsubscript{PheGAA} were not affected by ALKBH3. tRNA\textsubscript{Met} or tRNA\textsubscript{PheGAA} was not the target of ALKBH3. As expected, no significant effect of ALKBH3 on their m1A levels was detected (Figure 3D). In vitro biochemical assay showed ALKBH3 reduced the m1A levels of its targets such as tRNA\textsubscript{GluCTC}, tRNA\textsubscript{GlyGCC}, tRNA\textsubscript{HisGTG}, tRNA\textsubscript{GlaCTG}, tRNA\textsubscript{ValCAC}, tRNA\textsubscript{ArgCCT}, tRNA\textsubscript{LeuCAA} and tRNA\textsubscript{AlaGGC} (Supplementary Figure S3H).

Besides, m3C levels of target tRNA were significantly up-regulated in Alkbh3−/− HeLa cells, including tRNA\textsubscript{SerGCT}, tRNA\textsubscript{ArgCCT}, tRNA\textsubscript{LeuCAA} and tRNA\textsubscript{MetCAT} (Figure 3E). We conducted primer extension analysis for
Figure 2. ALKBH3 catalyzes demethylation of m1A and m3C in tRNA. (A) Total tRNAs were purified from HeLa cells and incubated with ALKBH3 protein or ALKBH3 protein + EDTA for 1 h (EDTA chelates cofactor iron and inactivates ALKBH3); (B) Michaelis–Menten plot of the steady-state kinetics of ALKBH3-catalyzed demethylation in stem-loop structured RNA probes that mimic the TΨC loops of tRNAHis(GUG), linear RNA probe and tRNA purified from HeLa cells at pH 7.5 at 37°C; (C, D) HeLa cells transfected with pcDNA (Vector) or pcDNA/ALKBH3 for 24 h, or siRNA negative control (si-NC) and si-ALKBH3–1∼3 for 48 h. Total tRNAs were then purified and digested for HPLC-MS/MS measurements of m1A (C) and m3C (D); (E, F) The relative levels of m1A (E) and m3C (F) of total tRNAs from lung, testis and epididymis of wild type or Alkbh3−/− mice. Data were presented as means ± SD from three independent experiments. *P < 0.05 compared with control; **P < 0.01 compared with control. See also Supplementary Figure S2.

The expression levels of ALKBH3-regulated tRNA were verified by northern blot analysis, showing that deletion of ALKBH3 had no obvious effect on the expression of most target tRNAs expect tRNAGlyGCC, which showed a slight up-regulation. Notably, deletion of ALKBH3 was independent to the expression of tRNA^GlyGCC, which is crucial to translation initiation (Supplementary Figure S3J).

The promotion effects of ALKBH3 on cancer progression

ALKBH3 contributed to survival and growth of cancer cells via yet-to-be illustrated mechanisms (21,25,35). We found that knockdown of ALKBH3 significantly inhibited the proliferation of HeLa, PC3, DU145 and HepG2 cells (Supplementary Figure S4A). Moreover, the Alkbh3−/− HeLa cells showed a significant reduction in cell proliferation (Figure 4A), colony formation (Figure 4B) and invasion capability (Figure 4C) when compared with the wild type cells. Consistently, HeLa cells stably overexpressing ALKBH3 showed remarkable promotion effects on cell proliferation, colony formation and invasion (Supplementary Figure S4B−D). The Alkbh3−/− HeLa cells were more sensitive to chemotherapy drug doxorubicin (Dox) than that of wild type cells (Supplementary Figure S4E). The IC50 values of Dox in wild type and Alkbh3−/− HeLa cells were 2.51 and 1.22 μM, respectively. Similarly, Alkbh3−/− cells were more susceptible to cell stress induced by sodium arsenite (Supplementary Figure S4F). Taken together, ALKBH3 positively regulated proliferation, colony formation, and invasion of cancer cells.

We further investigated the potential in vivo effects of ALKBH3 on cancer progression. Both wild type and Alkbh3−/− HeLa cells were injected subcutaneously into nude female mice. At the end of the experiments, tumor growth and volumes were significantly lower in mice injected with Alkbh3−/− HeLa cells compared to those injected with wild type cells (Figure 4D). IHC results also suggested that ALKBH3 depletion led to a lower level of Ki67 in xenograft tumor tissues (Figure 4E). Significant in-
Figure 3. Characterization and catalytic properties of ALKBH3 on specific tRNA. (A) In vitro biochemical assays of ALKBH3 wild type (WT), mutant R122A or mutant L177A in total tRNA of HeLa cells at 37°C for 1 h (EDTA chelates cofactor iron and inactivates ALKBH3); (B) the m^1A levels of tRNA in cytoplasm and nucleus isolated from wild type (WT) and Alkbh3^−/− HeLa cells, respectively; (C) the m^1A levels of tRNA isolated from the translation initiation non-polysome fractions (<80S, containing 40S ribosome; 60S ribosome and 80S monosome) and translation activating fraction (>80S, containing polysomes) from wild type (WT) and Alkbh3^−/− HeLa cells, respectively; (D) levels of m^1A in specific tRNA from wild type (WT) and Alkbh3^−/− HeLa cells, pulled down by corresponding cDNA probes; (E) levels of m^3C in specific tRNA from wild type (WT) and Alkbh3^−/− HeLa cells, pulled down by corresponding cDNA probes; Data were presented as means ± SD from three independent experiments. *P < 0.05 compared with control. **P < 0.01 compared with control. See also Supplementary Figure S3.

increased expression of ALKBH3 in cervical cancer versus normal tissues has been observed in Biewenga (Supplementary Figure S4G) and Pyeon (Supplementary Figure S4H) data from Oncomine database.

We evaluated effects of ALKBH3 on protein synthesis and translation. Confocal showed a lower synthesis rate of nascent proteins in Alkbh3^−/− cells as compared with that in wild type cells (Figure 4F). Consistently, stable overexpression of ALKBH3 enhanced the protein synthesis rate (Supplementary Figure S4I). Polysome profiling showed that deletion of ALKBH3 decreased the 80S monosome and polysome content, suggesting that protein synthesis process was delayed (Figure 4G). Since tRNA^M^et was not the target of ALKBH3 according to the results of CLIP-seq and mass spectrum, we investigated the roles of ALKBH3 in tRNA-mediated elongation during protein translation. It is achieved by a reporter that six repeated specific codon sequences (6×CAC for tRNA^His^G^G^T^G^C, 6×GGC for tRNA^Gly^G^G^C^C^C and 6×GAC for tRNA^Gln^G^C^C^T^G^C) were added in the front of 5' end of firefly luciferase (F-luc) (18), so that the elongation rate was detected as the fluorescence signal of F-luc (Figure 4H). However, there was no significant difference in the luciferase translation of 6×CAC(His), 6×GGC(Gly) or 6×CAG(Gln) between wild type and Alkbh3^−/− cells (Figure 4I). Considering that upregulation of tRNA^Gly^G^G^C^C was observed in Alkbh3^−/− HeLa cells, we blocked its function in cells by transfection a reverse complementary RNA which can target the anti-codon region of tRNA^Gly^G^G^C^C according to the previous study (36). However, blocking of tRNA^Gly^G^G^C^C had no effect on growth inhibition of Alkbh3^−/− cells (Supplementary Figure S4J). Data above indicated that ALKBH3-triggered cancer progression might be independent to the translational functions of tRNA.

ALKBH3 catalyzes generation of tDRs via an ANG-dependent manner

Demethylation of tRNA can suppress tRNA stability and trigger the generation of tDRs (37,38). tDRs are associated to cancer progression via increasing cell proliferation in breast and prostate cancers (32). Thus, we hypothesized that ALKBH3 may regulate the generation of tDRs in cancer cells. Small RNAs (smRNA) ranging from 15 to 50 bp from wild type and Alkbh3^−/− mice epididymis was recovered by TBE–urea gel (Supplementary Figure S5A) and subjected to Illumina ultra-high–throughput sequencing (Supplementary Table S2). Data showed that tDRs was the predominant RNA type in smRNA pool from both wild type and Alkbh3^−/− mice epididymis, however, percentage of tDRs decreased from 44.4% in wild type to 37.2% in Alkbh3^−/− mice (Figure 5A). Similar composition profiles of tDR were observed in both wild type and
Figure 4. The promotion effects of ALKBH3 on cancer progression. (A) The relative cell proliferation of wild type (WT) vs Alkbh3−/− HeLa cells were measured by CCK-8 kit; (B) cells were seeded in soft agar in a 96-well plate, grown for a week and soft agar colony formation was measured; (C) in vitro invasion assay of wild type (WT) versus Alkbh3−/− HeLa cells for 24 h; (D) tumor growth curves of wild type (WT) versus Alkbh3−/− HeLa cells in xenograft models at the indicated time interval; (E) IHC (Ki67)-stained paraffin-embedded sections obtained from xenografts. Red bar = 50 μm. (F) The wild type (WT) or Alkbh3−/− HeLa cells were replaced with methionine-free medium supplemented with methionine analog HPG and incubated for 1 h. HPG incorporation was measured by confocal microscope, quantitatively analyzed the intensity of fluorescence, and normalized to nucleus (NuclearMask); (G) The polysome profiling of wild type (WT) or Alkbh3−/− HeLa cells were analyzed; (H) Representative scheme of the reporter assay: the RNA reporter vector encodes firefly luciferase (F-luc) as the primary reporter and Renilla luciferase (R-luc) on the same plasmid as the internal transfection control. 6×CAC (His), 6×GGC (Gly) or 6×CAG (Gln)-coding sequences (recognized by tRNAHisGTG, tRNA GlyGCC and tRNA GlnCTG, respectively) were inserted after the PLK promoter region. (I) Positive and control reporter were transfected into wild type (WT) or Alkbh3−/− HeLa cells for 24 h. The control reporter without any insertion was used to normalize the translation differences between the two cell lines. Data were presented as means ± SD from three independent experiments. The cell proliferation was replicated six times for each group. * P < 0.05, ** P < 0.01 compared with control. See also Supplementary Figure S4

Alkbh3−/− mice epididymis (Figure 5B). Since the RPM (Reads per million total reads) of 5′-tDR in epididymis of wild type mice was 24-fold greater than that of 3′-tDR, we only focused on the potential roles of 5′-tDRs in the next study. Among them, 5′-tDR-GlyGCC was the most abundant tDRs in Alkbh3−/− mice epididymis, followed by 5′-tDR-GlyCCC, 5′-tDR-ValCAC and 5′-tDR-HisGTG and 5′-tDR-GlnCTG (Supplementary Figure S5B). Norther blot analysis (Figure 5C) and TaqMan qRT-PCR (Supplementary Figure S5C) showed that the expression of 5′-tDR-GlyGCC was decreased in epididymis, testis and lung of Alkbh3−/− mice than that of WT mice. As shown in Figure 5D, 5′-tDR-GlyGCC was derived from np 1–30–32 of mature tRNA GlyGCC. Similarly, other ALKBH3-regulated tDRs, including 5′-tDR-GlyCCC, 5′-tDR-ValCAC and 5′-tDR-HisGTG, were also derived from np 30–33 of their corresponding mature tRNAs (Supplementary Figure S5D). Since overexpression of ALKBH3 had no significant effect on the expression of tDRs from m3C-containing tRNAs including tRNA SerGCT, tRNA ArgCCT, tRNA LeuCAT and tRNA MetCAT, m1A demethylation is the most likely reason for ALKBH3-induced generation of tDRs.
Figure 5. ALKBH3 catalyzes generation of tDRs via an ANG-dependent manner. (A) All smRNAs from WT or Alkbh3<sup>−/−</sup> mice epididymis were purified and subjected to Illumina ultra-high-throughput sequencing (n = 2). The smRNAs were mapped to miRNAs, tRNAs, rRNAs or mRNA transcripts and normalized to the total reads; (B) pie chart showing the identified top 12 individual tDRs in WT or Alkbh3<sup>−/−</sup> mice epididymis; (C) the 5′tDR-GlyGCC of lung, testis and epididymis from WT or Alkbh3<sup>−/−</sup> mice was detected by northern blot analysis using biotin labeled probe (left) and quantitatively analyzed (right); (D) major cleavage sites and their corresponding percentages of cleaved tRNA<sup>GlyGCC</sup> in wild type mice epididymis; (E) after transfected with si-ANG or negative control (si-NC) for 24 h, the 5′tDR-GlyGCC from stably overexpressing ALKBH3 and control HeLa cells were detected by northern blot analysis using biotin labeled probe (left) and quantitatively analyzed (right); (F) the purified tRNA (500 ng) of WT or Alkbh3<sup>−/−</sup> mice epididymis was further treated with 100 ng ANG for 2 h. The production of 5′tDR-GlyGCC was detected by northern blot analysis using biotin labeled probe (left) and quantitatively analyzed (right). Data were presented as means ± SD from three independent experiments. **P < 0.01 compared with control. NS, no significant. See also Supplementary Figure S5.

We also sequenced the tDRs in HeLa cells stably overexpressing ALKBH3 and control cells (Supplementary Table S3). Consistently, overexpression of ALKBH3 enhanced the generation of tDRs in HeLa cells. The order of abundance from high to low was: 5′tDR-GlyGCC, 5′tDR-HisGTG, 5′tDR-GlyCCC and 5′tDR-GlnCTG (Supplementary Figure S5E). Notably, the top 10 abundant tDRs in HeLa cells (Supplementary Figure S5F) were consistent to those in mouse epididymis. Northern blot analysis (Figure 5E) and TaqMan qRT-PCR (Supplementary Figure S5G) confirmed overexpression of ALKBH3 can induce the up-regulation of 5′tDR-GlyGCC in HeLa cells. Furthermore, overexpression of ALKBH3 increased the expression of 5′tDR-GlyCCC, 5′tDR-GlnCTG and 5′tDR-HisGCT according to TaqMan qRT-PCR assay (Supplementary Figure S5H).

Angiogenin (ANG) has been reported to mediate tRNA cleavage via a highly regulated process (39), while methylation of tRNA can protect tRNA against this cleavage (40). We then knocked down the expression of ANG via specific siRNAs (Supplementary Figure S5I) to investigate whether ANG is involved in ALKBH3-induced tDRs generation. Northern blot analysis (Figure 5E) and TaqMan qRT-PCR (Supplementary Figure S5G) showed si-ANG attenuated the ALKBH3-induced up-regulation of 5′tDR-GlyGCC in HeLa cells. Meanwhile, ANG treatment synergistically increased the expression of 5′tDR-GlyGCC in HeLa cells stably overexpressing ALKBH3 (Supplementary
Figure S5J). Further, in vitro biochemical assay revealed that tRNA^{GlyGCC} isolated from Alkbh3^{−/−} mice epididymis was more resistant to ANG cleavage than that from wild-type mice (Figure 5F and Supplementary Figure S5K). Together, our results indicated that ALKBH3-induced m1A demethylation was involved in the generation of tDRs via an ANG-dependent manner.

tDRs are involved in ALKBH3-induced cancer progression

tDRs are functionally diverse and associated to the regulation of gene expression, RNA processing and cancer cell proliferation (32,41,42). The synthesized 5′tDR-GlyGCC, which is the most abundant tDR induced by ALKBH3, promoted the proliferation of wild-type HeLa cells and attenuated the suppression effects of Alkbh3^{−/−} cells on proliferation (Figure 6A). Consistently, knockdown of 5′tDR-GlyGCC by its specific siRNAs according to the previous study (32) (Supplementary Figure S6A) suppressed the proliferation of HeLa, PC3 and DU145 cells (Supplementary Figure S6B). Besides, synthesized 5′tDR-GlyGCC promoted protein synthesis in HeLa cells (Figure 6B and Supplementary Figure S6C). These data suggested that 5′tDR-GlyGCC was involved in ALKBH3-induced cancer progression.

To investigate the potential mechanisms of how tDRs regulate cancer progression, we evaluated the subcellular distribution of tDRs in cells. TaqMan qRT-PCR results showed that the expression of 5′tDR-GlyGCC in cytoplasm was much higher than those in nucleus (Supplementary Figure S6D). Polysome profiling combining with TaqMan qRT-PCR showed that 5′tDR-GlyGCC tended to be distributed in <80S fraction rather than >80S fraction (Supplementary Figure S6E). Similarly, the expression of 5′tDR-GlyGCC in the <80S fraction, rather than >80S fraction, in HeLa cells stably overexpressing ALKBH3 was significantly greater than that in control cells (Supplementary Figure S6F). These results were in line with the finding that ALKBH3 mainly demethylated tRNAs in <80S fraction (Figure 4C). Moreover, the expression of 5′tDR-GlyGCC in 40S portion was significantly greater than that in 60S or 80S portion of ribosome (Figure 6C).

Proteins interacting with tDRs were isolated using biotin-labeled 5′tDR-GlyGCC, 5′tDR-GlnCTG and analyzed by protein mass spectrometry. The main reasons for chosen 5′tDR-GlnCTG were as following: (i) it showed promotion effect on cell proliferation (Supplementary Figure S6B&G); (ii) it was generated at the other side of anti-codon regions within 40−50 nt length (41 nt) (Supplementary Figure S5D). By using a non-functional RNA as control, we confirmed that both biotin-labeled 5′tDR-GlyGCC and 5′tDR-GlnCTG can trigger cell proliferation (Supplementary Figure S6H). Next, 18 extra proteins were precipitated by both 5′tDR-GlyGCC and 5′tDR-GlnCTG (Figure 6D, Supplementary Figure S6I), compared to the control. Among them, 12 proteins were related to RNA binding, including 40S ribosomal protein S21 (RPS21), nucleophosmin (NPM1), 60S ribosomal protein L26 (RPL26), HACA ribonucleoprotein complex subunit 4 (DKC1) and elongation factor 1-alpha 1 (EEF1A1). According to the STRING database (Supplementary Figure S6J), RPS21 and RPL26 are two compositions of eukaryotic ribosome, while Box H/A CA small nucleolar ribonucleoprotein (snorNP) is involved in tRNA folding via site-specific pseudouridylation.

RPS21 immunoprecipitation (IP) and TaqMan qRT-PCR confirmed the direct interaction between tDRs and RPS21 in vivo (Figure 6E), which showed significant enrichments of RPS21 in IP-groups of both 5′tDR-GlyGCC and 5′tDR-GlnCTG. It indicated that tDRs may interact with RNA binding proteins or ribosome to regulate translation process.

We employed IRES reporter assay to investigate the mechanisms of how tDRs participate in protein translation (29,43). Three commonly used IRES reporters (HCV IRES, EMCV IRES and CrPV IRES) were introduced individually in the front of F-Luciferase to report the protein translation. HCV IRES bypasses the eIF4 complex and eIF4G-induced loop formation by directly recruiting 40S and eIF3; EMCV IRES directly binds to eIF4G subunit of the eIF4 complex; while CrPV IRES recruits the ribosome completely independent of initiation factors (eIFs) (29,43). Our found that translation of HCV IRES reporter showed a 57% drop in Alkbh3^{−/−} cells compared to that in wild-type cells (Figure 6F), while the EMCV or CrPV IRES reporter was not affected. Further, both 5′tDR-GlyGCC and 5′tDR-GlnCTG increased HCV IRES-dependent translation (Figure 6G). Together with the results that assembly of 80S monosome was suppressed in Alkbh3^{−/−} cells (Figure 4G), our data indicated that ALKBH3-induced tDRs can promote the translation, which likely relies on the direct recruitment of 40S ribosome.

Interestingly, mass spectrometry indicated that both 5′tDR-GlyGCC and 5′tDR-GlnCTG interacted with cytochrome c (Cyt c), which protects cells from apoptosis (44). Interactions between tDRs and Cyt c were further verified by immunoprecipitation and TaqMan qRT-PCR assay (Supplementary Figure S6K). Moreover, flow cytometry showed that cells transfected with 5′tDR-GlyGCC were more resistant to stress-induced apoptosis (Figure 6H). Western blot analysis also confirmed that stress-induced caspase-3 cleavage was attenuated by transfection of 5′tDR-GlyGCC (Figure 6I). Collectively, these data indicated that the interaction between tDRs and Cyt c, which prevents cell apoptosis, was involved in ALKBH3-induced cancer progression.

DISCUSSION

We presented herein a systematic study that ALKBH3 acted as a tRNA demethylase to catalyze the demethylation of m1A and m3C in tRNA, followed by triggering the generation of tDRs. ALKBH3 was widely expressed by cancer cells and mainly located in cytoplasm with strong binding capability to tRNA. It potently and selectively processed demethylation of m1A and m3C of tRNA as a tRNA demethylase to catalyze the demethylation of m1A and m3C in tRNA, followed by triggering the generation of tDRs. ALKBH3 was widely expressed by cancer cells and mainly located in cytoplasm with strong binding capability to tRNA. It potently and selectively processed demethylation of m1A and m3C of tRNA both in vitro and in vivo. We found that ALKBH3 triggered cancer progression, including cell proliferation, migration, protein synthesis and drug sensitivity. Results showed that m1A demethylated tRNAs are more sensitive to ANG treatment and tended to generate tDRs around their anticodon regions. These tDRs can strengthen the ribosome assembly and prevent apoptosis of cancer cells (Figure 7). Our discov-
Figure 6. tDRs are involved in ALKBH3-induced cancer progression. (A) Wild type (wt) or Alkbh3<sup>−/−</sup> HeLa cells were transfected with scrambled control RNA or synthesized 5′tDR-GlyGCC (31 nt) with the final concentration of 50 nM for the indicated times, and the cell proliferation was detected by CCK-8 kit; (B) HeLa cells transfected with scrambled control RNA or synthesized 5′tDR-GlyGCC (31 nt) for 24 h were replaced with methionine-free medium supplemented with methionine-free HPG and incubated for 1 h. HPG incorporation was measured by confocal microscope, quantitatively analyzed the intensity of fluorescence, and normalized to nucleus (NuclearMask); (C) the ribosome fractions of HeLa cells were grouped to non-ribosomal mRNPs, 40S, 60S, 80S and polysome. The expression of 5′tDR-GlyGCC in 40S, 60S and 80S were measured by TaqMan qRT-PCR; (D) compared to the scrambled control RNA group, 18 extra proteins were precipitated by both 5′tDR-GlyGCC and 5′tDR-GlnCTG in HeLa cells; (E) after crosslinking, the RPS21-binding RNAs were pulled down by the specific antibody. The 5′tDRs in input and RPS-binding RNAs were measured by TaqMan qRT-PCR and normalized to the total RNA amount; (F) both cap-dependent translation (R-luc) and IRES-dependent translation (F-luc) were normalized with LacZ activity as transfection control. The IRES-dependent translation (F-luc) were compared between wild type (WT) and Alkbh3<sup>−/−</sup> cells for all the three groups; (G) both 5′tDR-GlyGCC and 5′tDR-GlnCTG had statistically significant effects on the HCV IRES reporter in HeLa cells; (H) HeLa cells transfected with control RNA or 5′tDR-GlyGCC for 24 h were further treated with NaAsO<sub>2</sub> for 6 h, stained with annexin V and propidium iodide (PI), and analyzed by flow cytometry (left). The percentages of apoptotic cells (annexin V and PI double positive) were quantified (right); (I) the caspase 3 and cleaved caspase 3 in cells treated as (I) were measured by western blot analysis. Data were presented as means ± SD from three independent experiments. The reporter assays were replicated for six times for each group. *<i>P</i> < 0.05, **<i>P</i> < 0.01 compared with control. See also Supplementary Figure S6.

ery opens a potential new paradigm of tRNA demethylase which regulates cellular biological functions via induction of tDRs.

ALKBH3 has been suggested to function as a DNA-repair protein to protect the genomic integrity (21,45). Analogously to ALKBH2, ALKBH3 contains a flexible hairpin which is involved in base flipping to distinguish single-stranded substrates from double-stranded substrates (19). Our findings revealed that ALKBH3 mainly located in cytoplasm and functioned as a demethylase in tRNA. In vitro and in vivo studies showed that ALKBH3 specifically demethylated m<sub>1</sub>A and m<sub>3</sub>C, but not other modifications, in tRNA, which was in line with previous biochemical assay that recombinant ALKBH3 demethylated m<sub>1</sub>A and m<sub>3</sub>C of purified tRNA in vitro (46). Ueda et al (46) reported that ALKBH3 can demethylate m<sub>6</sub>A of tRNA, while this effect was not observed in our present study. The exist of m<sub>6</sub>A in mammalian tRNA is still a controversial issue (47).

Modifications of tRNA are crucial for its structural maintenance, biological functions and stability. Our data showed that ALKBH3 catalyzed m<sub>1</sub>A and m<sub>3</sub>C demethylation of tRNA mainly in the monosome of cytoplasm, m<sub>1</sub>A<sub>58</sub>, which is essential to the tRNA stability (9,10), is the predominant substrate of ALKBH3 in our present study.
Figure 7. Proposed model of tDRs-mediated cancer progression triggered by ALKBH3. ALKBH3 catalyzed m1A and m3C demethylation, which increases sensitivity of tRNA to ANG cleavage, leading to the formation of tDRs. ALKBH3-generated tDRs triggers the ribosome assembly and interacts with Cyt c to prevent cell apoptosis.

For m3C modification, position 32 was the most frequently modified site in cytoplasmic tRNA, as well as the position 47 in tRNA^Ser and position 20 in tRNA^Met^e. Demethylation of m1A in tRNA catalyzed by ALKBH1 affected the levels of tRNA^Met^e and translation initiation (18). Although we showed that ALKBH3 had comparable demethylation activities with ALKBH1, CLIP-seq showed that tRNA^Met^e was not the target of ALKBH3. Further, neither m1A modification nor cellular level of tRNA^Met^e was modulated by ALKBH3. In addition, ALKBH3 had no effect on tRNA-mediated translation elongation. Our data showed that 55% of ALKBH3-bound tRNAs (GluCTC, GlyGCC, HisGTG, LysCCT, AlaAGC, AlaTGC, CysGCA, LysTTT, AsnGTT, ValCAC, LeuAAG) were also the targets of ALKBH1 in HeLa cells (18). While over expression of ALKBH3 had no effect on the expression of ALKBH1 in HeLa cells (data not shown). Thus, the potential reciprocal nature of tRNA demethylation between ALKBH1 and ALKBH3 remained to be elucidated.

Existence of high-abundant tDRs was supported by increasing literatures (30,32,38,41), while biogenesis of tDRs has not been clearly illustrated. Our data indicated that ALKBH3 triggered the production of tDRs, which mainly due to its function as m1A demethylase of tRNA. It has been reported that lack of m1A58 impairs the hydrogen bonds from A58 to A54 and A60 in initiator tRNA^Met^ of yeast, leading to the degradation of tRNA^Met^ (11). Yeast strains deficient in Trm6 (or Trm61) tRNA:m1A58-methyltransferases display a reduced level of tRNA ^m1A at elevated temperatures (49). Although there is no direct evidence about m1A58 and tRNA stability in human cells, our present study found that ALKBH3, which acted as a tRNA demethylase, induced the generation of tDRs in both human cells and mouse tissues. Science ALKBH3-induced tDRs were mainly originated from tRNAs without m3C modification, we hypothesized that m1A demethylation induced by ALKBH3 can reduce the stability of tRNA, resulting in the generation of tDRs. The detailed mechanisms underlying this process need further clarification. Molecular sizes of ALKBH3-induced tDRs are similar to those induced by stresses (30–40 nt) (44), which indicated that variations of m1A may also participate in the tDRs generation upon stress responses.

ALKBH3 had no significant effect on the expression of most its CLIP-targeted tRNA. Previous observations indicated that only a fraction of mature tRNAs are cleaved upon stress responses, causing a negligible change in the total tRNA pool (38). This might be due to that homeostasis of tRNA, which is essential for cellular functions and cell survival, is highly regulated by tRNA synthetases and related proteins (5). Inhibition of tRNA^Glu^GCC, the only up-regulated tRNA in Alkbh3−/− cells, had no obvious effect on growth inhibition triggered by deletion of ALKBH3. It suggested that the biological functions of ALKBH3, as tRNA demethylase, might be related to the byproducts of tRNA rather than mature tRNA themselves. Notably, it has been reported that there was a conserved methylation in D-loop of tRNA, which can sufficiently suppress the tRNA-mediated immune stimulations in human cells (50). Whether ALKBH3 is associated to this conserved tRNA methylation and therefore modulating immune stimulations, or even mediating other tRNA modifications to trigger novel biological functions need to be further investigated.

Our data showed that tDRs were involved in ALKBH3-promoted cancer progression. This was supported by evidences that: 1) both endogenous and synthesized tDRs can trigger the cancer progression and protein synthesis. It has been reported that tRNA halves can promote the progression of breast and prostate cancers via enhancing cell proliferation (32). Besides, transfection of tDR-Gln19 up-regulated 356 proteins while only down-regulated 43 proteins, particularly increasing the translation of ribosomal and poly(A)-binding protein (51); 2) ANG, which has been reported as the RNase that responsible for tRNA cleavage in mammalian cells (38), can support the proliferation of progenitor cells after radiation damages (52). Biological functions of tDRs and related mechanisms are far from well-illustrated. On one hand, it has been reported that tDRs suppresses the translation initiation via displacing eIF4G/eIF4A from uncapped > capped RNAs (53). On the other hand, cleavage of tRNA by ANG up-regulates the
translation of IRES-containing mRNAs such as encoding vascular endothelial growth factor (VEGF) (54) and promotes cell proliferation (32). Ueda et al. (46) reported that in vitro translation efficiency of ALKBH3-treated tRNA was higher than that of control tRNA. Our data showed that tDRs were enriched in 40S/60S monosomes and interacted with ribosomal proteins such as RPS21 and RPL26. Reporter assays indicated that tDRs regulated translation processes via recruiting 40S ribosome directly. Considering that biological functions of tDRs might be cell state/type-specific (52), the inconsistent results may due to the variations of tDR types, RNA modifications and cell lines.

Our study also revealed that ALKBH3-induced tDRs interacted with Cyt c to regulate cell apoptosis. It was evidenced by the results that tDRs bound to Cyt c and suppressed stress-induced apoptosis. The tDRs-Cyt c complex was formed in cytoplasm when Cyt c was released from the mitochondria, thus preventing the formation of apoptosis and activation of caspase, resulting in the inhibition of cell apoptosis (44). Involvement of tDRs in cell apoptosis was confirmed by the observations that Alkbh3−/− cells were more sensitive to stress-induced apoptosis. Considering that tDRs can target specific signaling pathways to regulate cellular functions, the roles of tDRs in ALKBH3-triggered cancer progression need to be further studied.

Collectively, we found that ALKBH3-mediated tRNA demethylation regulates cancer progression via induction of tDRs, which can modulate the translation by regulating ribosome assembly and prevent apoptosis through binding with Cyt c. Our present discovery here revealed a novel relationship between tDRs generation and tRNA modifications, which will stimulate the future studies about reversible tRNA methylation on human health and diseases.

DECLARATIONS

Consent for publication

The authors confirmed that we have obtained written consent from the patient to publish the manuscript.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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