NSUN2-mediated RNA 5-methylcytosine promotes esophageal squamous cell carcinoma progression via LIN28B-dependent GRB2 mRNA stabilization

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5-Methylcytosine (m5C) is a posttranscriptional RNA modification participating in many critical bioprocesses, but its functions in human cancer remain unclear. Here, by detecting the transcriptome-wide m5C profiling in esophageal squamous cell carcinoma (ESCC), we showed increased m5C methylation in ESCC tumors due to the overexpressed m5C methyltransferase NSUN2. Aberrant expression of NSUN2 was positively regulated by E2F Transcription Factor 1 (E2F1). High NSUN2 levels predicted poor survival of ESCC patients. Moreover, silencing NSUN2 suppressed ESCC tumorigenesis and progression in NSun2 knockout mouse models. Mechanistically, NSUN2 induced m5C modification of growth factor receptor-bound protein 2 (GRB2) and stabilized its mRNA, which was meditated by a novel m5C mediator, protein lin-28 homolog B (LIN28B). Elevated GRB2 levels increased the activation of PI3K/AKT and ERK/MAPK signalling. These results demonstrate that NSUN2 enhances the initiation and progression of ESCC via m5C-LIN28B dependent stabilization of GRB2 transcript, providing a promising epitranscriptomic-targeted therapeutic strategy for ESCC.

Oncogene (2021) 40:5814–5828; https://doi.org/10.1038/s41388-021-01978-0

ARTICLE

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the most malignant cancers with only ~19% of 5-year survival [1, 2]. Most ESCC patients eventually die of cancer progression due to lack of effective treatment modalities [3]. Therefore, further elucidation of the comprehensive molecular mechanisms underlying ESCC is urgently needed to develop more effective diagnostic and therapeutic interventions for ESCC.

Recent discoveries have demonstrated that aberrations in epigenetic regulation, such as RNA methylation, are crucial hallmarks of tumor initiation, progression, and recurrence [4]. 5-Methylcytosine (m5C) is one of the most well-known and conserved RNA modifications extensively occurring on various types of eukaryotic RNA, including rRNA, IncRNA, tRNA, and mRNA [5–10]. To date, the known m5C methyltransferases include the NOP2/Sun RNA methyltransferase family member 1–7 (NSUN1−7) and the DNA methyltransferase 2 [11]. Aly/REF export factor (ALYREF) and Y-box binding protein 1 (YBX1) are two m5C readers respectively mediating RNA nuclear export and enhancing RNA stability [9, 12, 13]. Accumulating evidence confirms that m5C modification regulates multiple RNA metabolic and biological processes, such as RNA stability [12, 13], RNA export [9], RNA translation [14, 15], and RNA processing [16, 17]. Recently, with the advance in detecting and mapping techniques, distribution profiles of m5C sites on mRNAs have been discovered, suggesting that m5C sites are distributed in 5′ untranslated regions (5′UTR), coding sequences (CDS) and 3′ untranslated regions (3′UTR) of mRNAs and are especially prominent near translation start sites [8, 9]. NSUN2 and NSUN6 are two major methyltransferase catalyzing m5C modification of mammalian mRNAs [7, 9, 11, 18] and NSUN2 is currently the best-studied one. Emerging evidence has shown that NSUN2-mediated RNA m5C methylation plays a critical role in cell proliferation, development, and differentiation [16,19–21]. Mutation or aberrant expression of NSUN2 is involved in various diseases, such as cancer and developmental disorders [12, 21–23]. However, very little is known about the precise regulatory mechanism of NSUN2-mediated mRNA m5C modification in human diseases, especially human cancer.

In this study, we indicate an oncogenic role of NSUN2-mediated RNA m5C modification in human ESCC. Specifically, E2F Transcription Factor 1 (E2F1) binds to the promoter of NSUN2 and enhances its expression, which significantly increases m5C formation in growth factor receptor-bound protein 2 (GRB2). An RNA-binding protein lin-28 homolog B (LIN28B) acts as an m5C mediator preferentially binding to the m5C-modified GRB2 RNA and enhancing its stability. Upregulation of GRB2 evokes the oncogenic PI3K/AKT and ERK/MAPK signalling.

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Received: 2 March 2021 Revised: 18 July 2021 Accepted: 21 July 2021
Published online: 3 August 2021

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Oncogene (2021) 40:5814–5828; https://doi.org/10.1038/s41388-021-01978-0

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Oncogene (2021) 40:5814–5828; https://doi.org/10.1038/s41388-021-01978-0
We propose that the NSUN2-m$^5$C-GRB2-PI3K/AKT and ERK/MAPK signaling axes promote the initiation and the progression of ESCC.

RESULTS
Aberrant upregulation of NSUN2 plays an oncogenic role in ESCC
To determine the role of mRNA m$^5$C modification in ESCC, we evaluated the expression levels of two major mRNA m$^5$C methyltransferases NSUN2 and NSUN6 in an ESCC cohort ($n = 215$; Supplementary Table 1) from Sun Yat-sen University Cancer Center (SYSUCC) using qRT-PCR. We found aberrantly higher levels of NSUN2 RNA in tumors than in adjacent normal tissues (Fig. 1A). However, no obvious difference of NSUN6 RNA was observed (Fig. 1B). These findings were further verified in a public microarray dataset (GSE53625) consisting of 179 paired ESCC samples (Supplementary Fig. 1A, B). Furthermore, higher NSUN2 RNA was significantly associated with advanced ESCC tumor stage.

F

H

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NSUN2 enhances tumorigenesis and progression of ESCC

We then investigate the role of NSUN2 by changing its expression in ESCC cells. Overexpressing NSUN2 substantially promoted cell proliferation, migration and invasion, while silencing NSUN2 showed opposite effects (Fig. 3A – D; Supplementary Fig. 3A, B, E, F). However, overexpressing NSUN2 catalytic mutants (MUT1 or MUT2) [9, 12] had no obvious effects on malignant phenotypes of ESCC cells (Fig. 3E, G). Moreover, overexpressing wild-type but not mutant NSUN2 substantially reversed the inhibitory effects of NSUN2 knockdown on malignant phenotypes of ESCC cells (Fig. 3F, H; Supplementary Fig. 3D, H). These in vitro results suggest an oncogenic role of NSUN2 in ESCC cells.

We next generated Nsun2 knockout mice (Nsun2+/−, Supplementary Fig. 4A) to determine the oncogenic role of Nsun2 in vivo. A 14 bp shift due to the deletion mutation located on one allele of Nsun2 exon 4 was observed in the Nsun2+/− mice by Sanger sequencing (Supplementary Fig. 4B). Relative to those of Nsun2+/+ littermates, esophageal extracts of Nsun2+/− mice displayed significant reduction in NSUN2 protein levels (Supplementary Fig. 4C). We then used the Nsun2+/− mice and their wild-type (Nsun2+/+) littermates to induce ESCC by treating them with chemical carcinogen 4-nitroquinoline N-oxide (4-NQO) (Fig. 3J). We observed esophageal atypical hyperplasia lesions or squamous cell carcinoma in Nsun2+/− mice after 4-NQO withdrawal for 4 or 8 weeks. IHC assays showed sequential upregulation of NSUN2 protein in normal esophageal epithelium, atypical hyperplasia lesions and ESCC tumor tissues (Fig. 3J). As expected, all (10/10) Nsun2+/− mice developed esophageal masses after 4-NQO withdrawal for 12 weeks, but not all (8/10) Nsun2+/− mice at this timepoint (Fig. 3K). Both tumor number and tumor size of Nsun2+/− mice were smaller than those of Nsun2+/+ mice (Fig. 3L, M). Histopathological analysis showed advanced esophageal tumor stages of Nsun2+/+ mice than those of Nsun2+/− mice (Fig. 3N; Supplementary Fig. 4D). Consistently, Nsun2+/− mice had worse prognosis than Nsun2+/− mice (Fig. 3O). These findings suggest that NSUN2 plays a critical role in the tumorigenesis and progression of ESCC.

NSUN2-mediated mC hypermethylation activates PI3K/AKT and ERK/MAPK signaling in ESCC

We next explore whether the oncogenic role of NSUN2 is mC-dependent in ESCC. We performed RNA bisulfite sequencing (RNA-BisSeq) [9] on poly(A)-enriched RNAs purified from seven paired ESCC samples (Supplementary Table 7) to elucidate the transcriptional mC profile of ESCC. Conversion rates (C to T) of the methylation conversion control Dhfr were all >99%. We identified 11,139 mC sites in 8263 transcripts. More than 90% of these mC sites occurred in mRNAs (Supplementary Fig. 5A) and were enriched in 5’UTR, CDS and 3’UTR (Supplementary Fig. 5B). Moreover, the identified mC sites were particularly accumulated in
regions immediately downstream of translation initiation sites (Supplementary Fig. 5C) and were enriched in the CG-rich environments (Supplementary Fig. 5D).

Among the 115,187 m5Cs, 4051 sites within 1362 transcripts were hypermethylated while 1627 sites within 626 transcripts were hypomethylated in tumors compared with those in normal tissues (Fig. 4A, B; Supplementary Data 1), indicating that m5C hypermethylation is a frequent event in ESCC. Furthermore, we found that RNA levels of NSUN2 but not NSUN6 were significantly upregulated in tumors from the seven paired ESCC samples (Supplementary Fig. 5E), suggesting that NSUN2 may be the main methyltransferase mediating the formation of aberrant mRNA m5C hypermethylation in ESCC. Then, pathway enrichment analysis using IPA software showed that the m5C-hypermethylated

Fig. 2 Transcription factor E2F1 positively regulates NSUN2 expression in ESCC. A In silico analysis of potential transcription factors in NSUN2 promoter region. B, C Relative NSUN2 mRNA (B) and protein (C) levels in ESCC cells with or without knockdown of each of the four transcription factors indicated in (A). D Schema of the putative E2F1 binding site in NSUN2 promoter and the primers used for chromatin immunoprecipitation (ChIP) analysis. Highlighted are the consensus and mutant sequences for E2F1 binding. E ChIP-qPCR analysis of cells with anti-E2F1 antibody or IgG control. Left panel shows qPCR results and the right panel shows the images of agarose gel electrophoresis of the qPCR products. F Luciferase reporter assays in cells co-transfected with the indicated plasmids or siRNA for 48 h (upper panel). Knockdown efficiency of E2F1 was shown in the lower panel. pGL4-wt-promoter, pGL4-NSUN2-wt-promoter; pGL4-mut-promoter, pGL4-NSUN2-mut-promoter. G E2F1 RNA levels were significantly higher in ESCC tumors than in paired normal tissues (n = 215, SYSUCC cohort). H Spearman’s correlations between RNA levels of NSUN2 and E2F1 in ESCC tumors (n = 215). The center red line is the regression line. r, correlation coefficient. Data are shown as mean ± SEM in (B), (E), (F) and (G). All data are from at least three independent experiments. ACTIN was used as a control in (C) and (F). P values were calculated by two-sided Student’s t test (*P < 0.05, **P < 0.01 and ***P < 0.001) in (B), (E) and (F), and two-sided paired Wilcoxon signed-rank test in (G).
transcripts in tumors were mainly enriched in cancer-related pathways, such as PI3K/AKT, ERK/MAPK and cell-surface activated receptor-related pathways (Fig. 4C). Indeed, many oncogenes involved in these pathways exhibited m\(^5\)C-hypermethylated in tumors (Fig. 4D, E). We randomly selected several transcripts involved in these pathways and verified their upregulated m\(^5\)C levels in tumors (n = 215; Fig. 4F; Supplementary Table 1) using m\(^5\)C-RIP-qPCR, which was consistent with our RNA-BisSeq results.
Fig 3 NSUN2 promotes malignant phenotypes of ESCC cells and enhances the tumorigenesis and development of Nsun2 knockout mice. A–D Effects of NSUN2 overexpression or knockdown on abilities of ESCC cell proliferation (A, B), migration and invasion (C, D). E, F Wild-type but not mutant NSUN2 enhanced proliferation (E), migration and invasion abilities (F) of ESCC cells. F, H Wild-type but not mutant NSUN2 reversed the decreased abilities of proliferation (F), migration and invasion (H) in NSUN2-depleted ESCC cells. WT, wild-type NSUN2 plasmids; MUT1, NSUN2 plasmid with a point mutation at catalytic site (C321A); MUT2, NSUN2 plasmid with point mutations at both catalytic site (C321A) and releasing site (C271A). All three plasmids were insensitive to shNSUN2 plasmid. I Schematic diagram of the timeline of establishing the 4-NQO-induced ESCC cell model. Colored arrows indicate the time when different events occurred. J Pathological features (upper panel) or NSUN2 levels (lower panel) of normal esophagus or esophagus tissues after 4-NQO withdrawal 4 or 8 weeks from 4-NQO administration. K–M Morphological images (K), tumor number (L) or tumor size (M) of esophagus collected from Nsun2+/+ mice (n = 10) and their Nsun2−/− (n = 10) littermate after 4-NQO withdrawal for 12 weeks. N Pathological features (upper panel) or NSUN2 levels (lower panel) of esophagus collected from Nsun2+/+ mice and their Nsun2−/− littermates after 4-NOQ withdrawal for 12 weeks, as estimated by hematoxylin and eosi (H&E) staining or immunohistochemical staining (IHC). NSUN2-mediated m5C hypermethylation might promote ESCC malignancy. 

LIN28B recognizes m5C modification of GRB2 and stabilizes GRB2 mRNA

It is known that RNA methylation regulates its target RNA by its m5C catalytic activity (Fig. 4I; Supplementary Fig. 5F), indicating that NSUN2- mediated m5C hypermethylation may trigger PI3K/AKT and ERK/MAPK signaling. As expected, NSUN2 knockdown suppressed the activation of PI3K/AKT and ERK/MAPK pathways (Fig. 4J). The suppression could be reversed by overexpression of wild-type but not mutant NSUN2 (Fig. 4K). These results indicate that NSUN2-mediated m5C hypermethylation may trigger PI3K/AKT and ERK/MAPK pathways to promote ESCC malignancy.

GRB2 is a critical target via which NSUN2 stimulates PI3K/AKT and ERK/MAPK signaling

To identify downstream effectors involved in NSUN2-mediated activation of PI3K/AKT and ERK/MAPK signaling, we assessed potential targets with hypermethylated-m5Ccs in ESCC tumors. We focused on the adaptor protein GRB2 for the following reasons. Firstly, GRB2 plays a central role in signal transduction between cell-surface receptors and PI3K/AKT and ERK/MAPK signaling. As expected, NSUN2 knockdown suppressed the activation of PI3K/AKT and ERK/MAPK pathways (Fig. 4). This suppression could be reversed by overexpression of wild-type but not mutant NSUN2 (Fig. 4K). These results indicate that NSUN2-mediated m5C hypermethylation may trigger PI3K/AKT and ERK/MAPK pathways to promote ESCC malignancy.

mouse model of ESCC (Fig. 5G). Higher levels of GRB2 and the activation levels of both pathways were also observed in esophageal tissues of Nsun2+/+ mice than in those of Nsun2−/− mice (Fig. 5H). Moreover, positive correlations were found between GRB2 RNA levels and NSUN2 RNA levels (Fig. 5I) or GRB2 mRNA levels (Fig. 5J) in ESCCs (n = 215; Supplementary Table 1). Since the m5C site of GRB2 is in its 3′UTR, it is possible that NSUN2-mediated m5C modification maintains GRB2 expression by enhancing its mRNA stability. We treated ESCC cells with actinomycin D and found that NSUN2 silencing significantly reduced half-life of GRB2 RNA (Fig. 5K). This reduction could be restored by overexpressing wild-type but not mutant NSUN2 (Fig. 5L). Luciferase reporter assays showed substantial decreased luciferase expression of vector with wild-type GRB2 3′UTR (GRB2-WT), but not of that with m5C site mutant GRB2 3′UTR (GRB2-MUT) in NSUN2-depleted cells (Fig. 5M). These results suggest a central role of GRB2 in the NSUN2-enhanced activation of PI3K/AKT and ERK/MAPK signaling.

LIN28B recognizes m5C modification of GRB2 and stabilizes GRB2 mRNA

It is known that RNA methylation regulates its target RNA by reader proteins [28]. Since m5C modification could stabilize GRB2 mRNA, we first examined the effect of the known reader YBX1 on GRB2 and found that silencing YBX1 had no influence on GRB2 levels (Supplementary Fig. 6A). To identify the m5C mediators involved in GRB2 regulation, we performed mass spectrometry analysis of proteins obtained by RNA pulldown using biotin-labelled 50-bp GRB2 or m5C-GRB2 RNA probes (Supplementary Data 2). We identified seven proteins preferentially binding to m5C-GRB2 probes (Fig. 6A; Supplementary Fig. 6B), and among which, only LIN28B was verified by western blotting (Fig. 6B; Supplementary Fig. 6C) and REMSA assays (Fig. 6C). Since LIN28B has a conserved cold shock domain (CSD) similar to YBX1 [29], an m5C reader targeting m5C-modified RNAs through its CSD domain [12, 13], we hypothesized that LIN28B binds m5C-modified GRB2 through CSD domain. REMSA experiment confirmed this notion that truncation of LIN28B CSD domain (LIN28B-ΔCSD) led to reduction in binding affinity of LIN28B toward the m5C-containing GRB2 oligo compared with full-length LIN28B (LIN28B-WT) (Fig. 6D; Supplementary Fig. 6F). To further screen key residues for LIN28B to bind to m5C site of GRB2, we performed structural modeling for LIN28B in complex with the m5C containing GRB2 RNA hexamer oligo using YBX1-m5C RNA complex as a reference model [12]. According the modeled LIN28B-m5C RNA complex structure, W36, M41, F43, D61, H65, and K92 might be the key interacting residues (<3 angstrom), and N38 and D61 had polar contacts with m5C RNA fragment. Furthermore, the residue interaction network generated by RING software [30] showed that there was Van der Waals force
between the W36 residue and m\textsuperscript{5}C, which indicated that W36 was critical for the LIN28B-m\textsuperscript{5}C RNA interaction (Supplementary Fig. 6E–G). REMSA assays further confirmed W36 as the key residue for LIN28B to distinguish and bind to m\textsuperscript{5}C-modified GRB2 RNA (Supplementary Fig. 6D; Supplementary Fig. 6H). LIN28B-PAR-CLIP further showed direct binding of LIN28B to GRB2 m\textsuperscript{5}C site (Fig. 6E, F). This interaction was reduced in NSUN2-silenced cells (Fig. 6G) where LIN28B protein level was not altered (Fig. 6J).
6H) and overexpressing wild-type but not mutant NSUN2 could reverse this reduced interaction (Fig. 6I). In addition, we found that LIN28B RNA levels were upregulated in ESCC tumors and were positively associated with GRB2 RNA levels (n = 215; Fig. 6J, K; Supplementary Table 1). Since LIN28B could bind and stabilize its target RNAs [31], we assumed that NSUN2-enhanced GRB2 stability depends on LIN28B. As expected, LIN28B silencing markedly decreased mRNA (Fig. 6L) and protein (Fig. 6M) levels of GRB2, and also reduced GRB2 mRNA stability (Fig. 6N). In addition, luciferase expression of GRB2-WT was strongly decreased in LIN28B-knockdown cells, whereas GRB2-MUT had no such effects (Fig. 6O). These findings suggest that LIN28B is a novel m^5C mediator recognizing the m^5C-modified GRB2 and stabilizing GRB2 mRNA. We then evaluated whether LIN28B binding to other m^5C-modified RNAs. A substantial decrease of LIN28B RNA-binding affinity was observed in ESCC cells when NSUN2 was silenced (Supplementary Fig. 6I). By further analyzing RNA-BisSeq and LIN28B-PARC-CLIP-Seq data, we observed a substantial overlap of m^5C-modified RNAs and LIN28B-bonding target RNAs (Supplementary Fig. 6J). Moreover, ~29% of the m^5C sites were localized within the LIN28B peaks (Supplementary Fig. 6K). In addition, a significant enrichment of m^5C modifications in LIN28B-bound RNAs was observed (Supplementary Fig. 6L). These findings indicate that LIN28B may also recognize m^5C-modified RNAs other than GRB2.

**GRB2 serves as an oncogene in ESCC, and the NSUN2-GRB2 axis is clinically relevant to ESCC**

We further investigate the role of GRB2 in ESCC. Functionally, GRB2 knockdown dramatically suppressed cell proliferation, migration, and invasion (Supplementary Fig. 7A, B). Clinically, we found higher m^5C and RNA levels of GRB2 in tumors than in adjacent normal tissues from our 215 paired ESCC cohort (Fig. 4F; Fig. 7A; Supplementary Table 1–2) and from the seven paired sequencing ESCC samples (Fig. 4G; Fig. 7B; Supplementary Table 7). Furthermore, patients with high GRB2 mRNA or m^5C levels had shorter OS than those with low levels (Fig. 7C, D; Supplementary Table 3). Consistently, GRB2 protein levels were significantly higher in ESCC tumors than in paired normal tissues detecting by western blotting (n = 10; Fig. 7E) and IHC assays (n = 59; Fig. 7F; Supplementary Table 4–5). Patients with higher GRB2 protein level also had worse prognosis (Fig. 7H; Supplementary Table 6). These findings indicate that GRB2 was an independent prognostic factor in ESCC.

Finally, we explored the clinical importance of the NSUN2-GRB2 axis in ESCC. As expected, overexpressing GRB2 rescued the inhibition of malignant phenotypes in NSUN2-depleted cells (Supplementary Fig. 7C, D). Clinically, approximately 89% of the specimens with lower NSUN2 protein level presented weaker IHC staining of GRB2, while nearly 72% of the specimens with higher NSUN2 showed stronger IHC staining of GRB2 (Fig. 7J). Spearman’s correlation analysis also revealed a positive correlation between protein level of NSUN2 and GRB2 (Fig. 7K). These results demonstrate the clinical correlation between NSUN2 and GRB2, and reveal an oncogenic role of the NSUN2-GRB2 axis in ESCC.

**DISCUSSION**

RNA m^5C modification is a common posttranscriptional RNA modification participating in many cellular and physiological processes [32]. Abnormal m^5C modification is associated with various diseases, including cancer [12, 33], inflammation [14], intellectual disabilities [22], neurodevelopmental disorders [34], infertility [35], and mitochondrial dysfunction [36]. However, the precise correlations between RNA m^5C modification and tumor development remains largely unclear. In this study, we described a transcriptome-wide m^5C profile in ESCC for the first time, which showed aberrantly increased levels of RNA m^5C modification in ESCC tumors due to the overexpression of NSUN2. NSUN2 plays a critical role in ESCC by positively regulating GRB2 through the m^5C-LIN28B-based posttranscriptional regulation, while its own transcriptome-wide m^5C hypermethylation in ESCC and activates PI3K/AKT and ERK/MAPK signaling. A, B Heatmap (A, left) and distribution (B) of the differential methylation levels of m^5C sites between ESCC tumors and paired normal tissues (n = 7). C Canonical pathway analysis of genes with m^5C hypermethylated transcripts (n = 1362) in ESCC tumors than in paired normal tissues (n = 7) using IPA software. D Heatmap (upper) showing m^5C hypermethylation of 45 representative genes involved in ten canonical cancer-related pathways in ESCC tumors. E Schematic diagram of genes involved in these cancer-related pathways. Genes with m^5C hypermethylated transcripts in tumors are highlighted in red. F, G Substantially hypermethylated m^5C of representative transcripts involved in these cancer-related pathways in ESCC tumors than in paired normal tissues by m^5C-RIP-qPCR (F, n = 215) and RNA-BisSeq (G; n = 7). H Spearman’s correlation analysis between NSUN2 RNA levels and m^5C levels of transcripts mentioned in (F) in ESCC tumors (n = 215). I Relative m^5C levels of transcripts mentioned in (F) were increased or decreased in ESCC cells with NSUN2 overexpression or knockdown. J, K Western blotting analysis showing that NSUN2 depletion substantially suppressed AKT, MEK and ERK phosphorylation and activation in ESCC cells (J), while wild-type but not mutant NSUN2 reversed the decrease of AKT, MEK and ERK phosphorylation and activation caused by NSUN2 knockdown (K). Heatmaps in (A, right) and (D, lower) showing the z-score of m^5C levels. Colors from blue to red indicate low to high. Data in (F, G) are displayed in boxplots. Data in (J) are shown as mean ± SEM from three independent experiments. The relative m^5C levels in target transcripts in (F) and (I) were evaluated with input normalization. ACTIN was used as a control in (J, K). P values were calculated by two-sided paired Wilcoxon signed-rank test in (F), two-sided Mann–Whitney test in (G) and two-sided Student’s t test in (I) (⁎P < 0.05, ⁎⁎P < 0.01 and ⁎⁎⁎P < 0.001).
Another interesting finding is the identification of E2F1 as an NSUN2 transcriptional activator. In this study, we found that overexpression of NSUN2 in ESCC was not caused by genomic changes as indicated by no significant alterations of mutations, CNV and DNA methylation status at NSUN2. Integrated analysis suggests that E2F1 was a potential regulator of NSUN2. E2F1 is a well-known TF that has been shown to be aberrant expressed in ESCC tumors [40]. Consistently, in our study, we demonstrated that E2F1 was upregulated in ESCC tumors and was positively correlated to NSUN2 expression, supporting a positive regulation.
of NSUN2 expression by E2F1. Moreover, we demonstrated that E2F1 could bind to the promoter region of NSUN2, stimulating NSUN2 transcription. These findings reveal for the first time that overexpression of NSUN2 in ESCC may be partially mediated by trans-element(s).

To further address the oncogenic function of NSUN2-mediated m^5^C methylation in ESCC, we provided an RNA m^5^C landscape in ESCC and demonstrated an oncogenic role of RNA m^5^C-hypermethylation in ESCC. In ESCC, a number of genes with landscape in ESCC and demonstrated an oncogenic role of RNA methylation rely on the binding of LIN28B to m^5^C-RNA through the indole ring of W65 in its CSD [12]. Another interesting finding is a new RNA m^5^C mediator. The biological importance of RNA methylation relies on reader proteins [28]. Previous studies have reported ALYREF and YBX1 as m^5^C readers [9, 12, 13]. In our study, we showed a m^5^C-mediated RNA stabilization of GRB2, which was YBX1-independent. Then, by mass spectrometry analysis, we have shown that LIN28B preferentially interacts with m^5^C-modified GRB2. This notion is further confirmed by several experiments showing that the binding of LIN28B to GRB2 is m^5^C-dependent. LIN28B is a known oncoprotein aberrantly expressed in a subset of human cancers [51], including ESCC [52], which is consistent with our results. It contains a conserved CSD domain [29], which is necessary for YBX1 binding to m^5^C-modified RNAs [12]. In our study, we have identified a critical role of CSD domain for LIN28B binding to m^5^C-carrying GRB2. By performing structure modeling analysis and in-vitro RNA-protein interaction assays, we have identified the LIN28B as an m^5^C mediator preferentially recognizing m^5^C-carrying GRB2 RNA through the W36 residue in its CSD. The mode of LIN28B binding to m^5^C-modified GRB2 RNA is extremely similar to YBX1, which binds to m^5^C-modified RNAs through the indole ring of W65 in its CSD [12]. However, this binding mechanism is different from that of ALYREF, which recognizes and interacts with m^5^C-modified RNAs.
mainly through a conserved positively charged residue, K171 [9]. It has been shown that LIN28B regulates RNA stability mainly through inhibiting let-7 microRNAs biogenesis [53] or through directly binding to its target RNAs [31, 54]. As our observations suggest, LIN28B could indeed stabilize GRB2 transcripts. However, this stabilization is m^C-dependent, which might be a novel mechanism of LIN28B-dependent regulation to its target RNAs. Our results strongly indicate that LIN28B is likely an m^C
Fig. 6 LIN28B stabilizes GRB2 mRNA by recognizing its m5C site. A Scatter plot of proteins binding to 50 bp GRB2 probe with or without m5C modification in KYSE30 cells. The filled red circles indicate proteins preferentially binding to GRB2(m5C) probes. B Western blotting analysis of potential GRB2(m5C) binding proteins obtained from RNA pull-down assays with 50 bp GRB2 probe with or without m5C modification shows specific association of LIN28B with GRB2(m5C) probes. C RNA Electrophoretic mobility shift (REMSA) assays of purified FLAG-tagged LIN28B with unmethylated or methylated GRB2 probes. The probes were maintained constantly while a gradient of 0–8 μM purified LIN28B was added to the reactions. D REMSA assays of GRB2(m5C) probes with purified FLAG-tagged LIN28B (wild-type or CSD domain truncation mutants). E Integrative-genomics-viewer (IGV) profiles showing the m5C levels of GRB2 in tumors and adjacent normal samples as well as the LIN28B-binding groups in PAR-CLIP-Seq data. The filled red circle represents the m5C site (chr17:75318971) in GRB2.

F–G PAR-CLIP-qPCR assays showing direct in-vivo binding of LIN28B to GRB2 in ESCC cells (F), and significant reduction of LIN28B binding to GRB2 when NSUN2 was silenced (G). H NSUN2 depletion had no influence on LIN28B protein levels. I Wild-type but not mutant NSUN2 reversed the reduction of LIN28B binding to GRB2 caused by NSUN2 depletion. J LIN28B RNA levels were significantly higher in ESCC tumors than in paired normal tissues (n = 215, SYSUCC cohort). K Spearman’s correlation analysis between LIN28B and GRB2 RNA levels in ESCC tumors (n = 215). L–M LIN28B knockdown substantially decreased the RNA (L) and protein (M) levels of GRB2. N Effects of LIN28B knockdown on mRNA half-life of GRB2 by RNA stability assays. O Luciferase reporter assays of luciferase reporter gene with wild-type GRB2-m5C site (GRB2-WT) or mutant m5C site (GRB2-MUT) in the control or stable LIN28B knockdown ESCC cells. Data in (F), (G), (I), (J), (L), (N) and (O) are displayed as mean ± SEM. All data are from at least three independent experiments. P values are calculated by two-sided Student’s t test (*P < 0.05, **P < 0.01 and ***P < 0.001. ns not significant) in (F), (G), (I), (J), (L), (N) and (O), and by two-sided paired Wilcoxon signed-rank test in (J). IgG served as an isotype control in (F), (G) and (I). ACTIN was served as a control in (B), (H) and (M).

mediator stabilizing m5C-modified GRB2. This function is similar to YBX1 that plays an essential role in maintaining m5C-carrying RNA stability [12] but not ALYREF that exerts an RNA-export-promoting function through recognizing m5C-modified RNAs [9]. Since the m5C modification was located in 3'UTR of GRB2 RNA, which was previously shown to be bound by miR-333-3p in ESCC [46], it would be worth exploring the association between m5C modification and miRNA binding in GRB2 3'UTR. Previous study has suggested that presence of RNA m5Amethylated on some transcripts could affect the RBP-RNA interactions and the miRNA targeting in 3'UTR region of RNAs [55], whether m5C modification exerts similar regulatory function remains to be further clarified. Although we have also shown the overlap of LIN28B-binding targets and m5C-modified RNAs, whether LIN28B is a common reader and the exact molecular mechanism for the association of LIN28B and other m5C-modified RNAs are warranted to further investigation.

In conclusion, our current work elucidates a vicious role of the NSUN2-m5C-GRB2-P13K/AKT and ERK/MAPK signaling axes in the initiation and the progression of ESCC. These findings illustrate an m5C-mediated epigenetic regulation mechanism of ESCC and highlight the opportunity for epitranscriptomic-targeted therapy for ESCC.

MATERIALS AND METHODS
Patient sample collection
Written informed consent was obtained from each patient, and this study was approved by the Institutional Review Board of the SYSUCC.

Cell lines and cell culture
Human ESCC cell lines KYSE30 and EC109 were kind gifts from Dr. Xinyuan Guan at SYSUCC.

Cell proliferation, migration and invasion assays
Details of in-vitro functional experimental procedures could see in Supplementary Materials and methods.

RNA extraction and quantitative real-time PCR (qRT-PCR)
The primer sequences are shown in Supplementary Table 8.

Western blotting analysis
Antibodies against the interest proteins are shown in Supplementary Table 9.

Chromatin immunoprecipitation (ChIP) assays
Specific primers used are listed in Supplementary Table 8. qPCR products were used for agarose gel electrophoresis.

4-NQO-induced ESCC model in Nsun2 knockout transgenic mice
Nsun2 knockout (Nsun2+/−) or Nsun2 wild-type (Nsun2+/+) C57BL/6J mice were donated from Nanjing Medical University. Animal experiments were carried out with protocols and guidelines approved by the Institutional Animal Care and Use Committee of SYSUCC.

Construction of RNA-BisSeq and RNA-Seq libraries
The procedure was performed according to a previous study with some modifications [9]. Analysis of the Dhfr spike-in showed C to T conversion rates >99%.

Differential m5C methylation analysis
m5C sites with P ≤ 0.05 and a mean m5C level difference ≥0.05 (|mean m5C level tumor − mean m5C level normal|) were considered to contain statistically significantly different m5C methylation.

Pathway analysis via Ingenuity Pathway Analysis (IPA)
Genes with m5C-hypermethylated transcripts were uploaded into IPA software for core analysis to identify canonical pathways (FDR ≤ 0.1) [56].

m5C RNA immunoprecipitation followed by qRT-PCR (m5C-RIP-qPCR)
The m5C-RIP-qPCR procedure was performed according to a previous study with some modifications [13]. Gene-specific primers are shown in Supplementary Table 8. Relative m5C levels of the indicated transcripts were evaluated with input normalization.

Photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP)
Cells were cultured in medium supplemented with 4-thiouridine for 14 h, followed by UV light crosslinking. The relative enrichment of the interest transcripts was calculated with input normalization.

RNA interference
Small interfering RNA (siRNA) targeting TFAP2C, SP1, NRF1, EZF1 or YBX1 genes are listed in Supplementary Table 10.

Plasmids, lentivirus production and transduction
Short hairpin RNA specifically targeting NSUN2, LIN28B or GRB2 are listed in Supplementary Table 10.

RNA stability assay
Cells were treated with actinomycin D and the mRNA half-life time was calculated as previously described [57].
**Luciferase reporter gene assays**

The luciferase activity or RNA level was examined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) or qRT-PCR, respectively.

**RNA pulldown and mass spectrometry analysis**

Biotin-labeled RNA fragment containing 50 bp GRB2 RNA sequences with (GRB2[m^5C]) or without (GRB2[\text{C}]) m^5C modification at m^5C site (chr17:75318971) were listed in Supplementary Table 10.
RNA electrophoretic mobility shift assays (REMSA)
Assays were performed using the LightShift Chemiluminescent RNA EMSA Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Public data processing
Details are described in Supplementary Materials and methods.

Statistical analysis
All the statistical analyses were performed using SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA) or GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA, USA). P < 0.05 was considered statistically significant.

Supplementary materials and methods
For the details of other experimental methods, see the Supplementary Materials and methods.

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ACKNOWLEDGEMENTS
We thank Dr. Bin Shen (Department of Histology and Embryology, Nanjing Medical University, Nanjing, China) for his generous donation of Nsun2 −/−/C57BL/6J mice. This study was supported by the Program for Guangdong Introducing Innovative and Entrepreneurial Teams (2017ZT075096 to DL), Natural Science Foundation of China (U1601229 to DL), National Young Top-notch Talent Support Program (to JZ) and Sun Yat-sen University Intramural Funds (to DL and to JZ).

AUTHOR CONTRIBUTIONS
JZheng and DL conceptualized and supervised the research. JS, XH, YZ, and RB designed and performed most experiments. GW, JZheng and LZhuang performed animal experiments. YY, RL, SD, ZL and ZZ were engaged in biostatistics and bioinformatics analysis. JL, ML, LP, JD, LZeng and SZ were responsible for patient recruitment, biospecimen and clinical data collection. JZheng, DL and JS drafted the paper.

COMPETING INTERESTS
The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41388-021-01978-0.

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