IGF2BP3 is an essential N6-methyladenosine biotarget for suppressing ferroptosis in lung adenocarcinoma cells

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\section*{1. Introduction}

Lung cancer is one of the leading causes of cancer mortality worldwide [1]. Although modern technology has greatly improved the efficacy of lung cancer treatment over the last decade, five-year survival rates are still only approximately 10\%–20\% [2]. Patients with the more
severe metastatic stage IV lung cancer have a five-year survival rate of less than 5% [3]. One of the key reasons for this is a lack of effective tumor targets for treating this disease. Lung adenocarcinoma (LUAD) is the most common pathological type of lung cancer [4]; however, knowledge of potential targets for treatment of LUAD is equally limited.

As a class of potential targets in tumors, N6-methyladenosine (m6A)-related proteins include methyltransferases, demethylases, and methyl-ation recognition proteins – called “writers,” “erasers,” and “readers,” based on their functions. m6A-related proteins regulate the biological effects of m6A, the most common RNA methylation modification [5]. Important roles for m6A have been demonstrated in various types of tumors, including those of liver and ovarian cancer [6–8]. Abnormalities of m6A-related proteins are crucial for the occurrence, progression, metastasis, drug resistance, and recurrence of tumors [9]. In LUAD, m6A/A and m6A-related proteins are also essential for tumorigenesis [10–12]. To develop a cost-effective biomaterial-associated anti-tumor strategy, evaluation of the therapeutic value of individual m6A-related proteins is particularly important. However, to the best of our knowledge, no such work has yet been reported.

m6A readers are particularly important because they are terminal effectors that can recognize target m6A-methylated RNA. Insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3), an m6A reader that belongs to the IGF2BP family, is quite well conserved between species [13]. Prior studies confirmed that IGF2BP3 is an RNA-binding protein modulated by m6A methylation, and its function is to promote mRNA translation and stability [14]. IGF2BP3 is highly expressed in at least 15 kinds of tumors, indicating a remarkable effect of IGF2BP3 on tumor occurrence and development [15]. In LUAD, IGF2BP3 accelerates cell proliferation by regulating alternative splicing of pyruvate kinase M1/2 (PKM) [16], non-coding RNA signaling [17], and translational activation [18]. Therefore, IGF2BP3 may play an oncogenic role in LUAD. However, comprehensive study to further define its necessity in the tumorigenesis of LUAD is still lacking.

Ferroptosis is a new type of regulated cell death that is iron-dependent and distinct from other types of cell death, such as apoptosis, necrosis, and autophagy [19]. The hallmarks of ferroptosis include continuous accumulation of intracellular lipid reactive oxygen species (ROS), which eventually destroy the continuity of the plasma membrane [19,20]. Ferroptosis has been implicated in cancers [21], and the connection between m6A and ferroptosis has raised enormous interest in cancer communities. Several studies have proved that m6A-related proteins, such as methyltransferase-like 3 (METTL3), fat mass and obesity-associated gene (FTO), and YTH domain family 2 (YTHDF2), regulate ferroptosis in lung and many other cancers [22–24]. Nevertheless, the importance of m6A-related proteins has attracted attention and it is unknown which of these proteins are suitable targets for anti-tumor biomaterials.

In this study, m6A-related proteins were comprehensively evaluated in LUAD through multi-omics studies. IGF2BP3 was identified to have clinical significance and be associated with ferroptosis in LUAD cells. The mechanism underlying the suppression of ferroptosis by IGF2BP3 and potential therapeutic small molecules targeting IGF2BP3 were subsequently investigated. Because of the critical pro-tumorigenic role of IGF2BP3 in LUAD, developing more therapeutic biomaterials may be helpful to treat LUAD in future.

2. Materials and methods

2.1. Clinical specimens

Fifty clinical samples of paired adjacent and LUAD tissues were obtained from the Department of Biobank, Shanghai Chest Hospital, between January 2019 and December 2020. Patient information is listed in Supplementary Table S1. Written informed consent was obtained from each patient, following the guidelines of the Declaration of Helsinki. This study was approved by the institutional ethics committee of Shanghai Chest Hospital.

2.2. Cell culture

The human bronchial epithelial cell line BEAS-2B, human LUAD cell line NCI-H1299, human pancreatic cancer cell line SW1990, and human colon adenocarcinoma cell line HCT-116 were purchased from FuHeng Biotechnology (Shanghai, China). Cells were validated by short tandem repeat analysis, and cultured in Dulbecco's Modified Eagle's Medium (DMEM, HyClone, Logan, UT, USA) with 10% fetal bovine serum (FBS, HyClone) and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA) in an incubator with 5% CO2 at 37 °C.

2.3. Animal experiments

IGF2BP3−/− mice were generated by Cyagen Biosciences (Guangzhou, China). Met3−/− mice were obtained as described in our previous study [25]. H1299 cells with or without IGF2BP3 overexpression were digested and adjusted to a density of 5 × 106 cells/200 μL. Next, 200 μL of cells were injected into the right armpit of each 4–6-week-old athymic nude mouse (Nudeijie, Shanghai, China). The weight and tumor size of nude mice were measured. Each group contained five mice. After 2 weeks, mice were injected daily with dimethyl sulfoxide (DMSO, Beyotime Biotechnology, Shanghai, China) with or without imidazole ketone erasto (IKE, 50 mg/kg, MedChemExpress, Monmouth, NJ, USA) or rigosertib (RIG, 250 mg/kg, Selleck, Houston, TX, USA). Mice were euthanized 28 d after implantation. Tumor growth was monitored and sizes were calculated as follows: 0.5 × L × W2, where L indicates length and W indicates width. All animal experiments were approved by the institutional ethics committee of Shanghai Chest Hospital.

2.4. Plasmids and drugs

IGF2BP3 single-guide RNAs (sgRNAs) were subcloned into the lentiviral vector LentiCrisprV2 system. IGF2BP3 WT and IGF2BP3 K384A were generated by ZuoRun Biotech (Shanghai, China). Mex-3 RNA binding family member A (MEX3A)-overexpressing plasmids were generated by BioVision Technology (Shanghai, China). METTL3-overexpressing and METTL3-knockout plasmids were obtained from our previous studies [26,27]. Glutathione peroxidase 4 (GPX4) and solute carrier family 3 member 2 (SLC3A2) overexpressing plasmids were generated by ZuoRun Biotech (Shanghai, China). The sequences of sgRNAs are listed in Supplementary Table S2. RS3L (2 μM, MedChemExpress), erastatin (40 μM, MedChemExpress), ferrostatin-1 (2 μM, MedChemExpress), and RIG (1 μM, Selleck) were used as treatments in cellular experiments. United Stated Food and Drug Administration (FDA)-approved drugs were obtained from Selleck.

2.5. Quantitative reverse transcription PCR (qRT-PCR), immunoblotting (IB), immunohistochemistry (IHC), and enzyme-linked immunosorbent assay (ELISA)

For qRT-PCR, TRizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA, and cDNA was synthesized using HiScript III RT SuperMix (Vazyme, Nanjing, China). qRT-PCR was performed using a HiScript II One Step qRT-PCR SYBR Green Kit (Vazyme). Primer sequences are listed in Supplementary Table S2. IB, IHC, and ELISA were performed using conventional protocols. Primary antibodies included: anti-METTL3 (Abcam, Cambridge, UK), anti-MEX3A (Sigma, St. Louis, MO, USA), anti-IGF2BP3 (Abcam), anti-IGF2BP3 (Abcam), anti-HA (Abcam), anti-GPX4 (Abcam), anti-SLC3A2 (Abcam), Wuhan, China), anti-acyl-CoA synthetase long chain family member 3 (ACSL3, Abcam), anti-ferroptotic heavy chain 1 (FTH1, Abcam), anti-insulin like growth factor 2 mRNA binding protein 1 (IGF2BP1, Abcam), anti-YTHDF2 (Abcam), and anti-glyceroldehyde-3-phosphate dehydrogenase (GAPDH, Cell Signaling Technology, Danvers, MA, USA). Tissue microarray assay (TMA) slides were obtained and measurement of IHC scores was performed as described in our previous study [26]. IGF2BP3, SLC3A2, and ACSL3
proteins were determined in tissues using ELISA kits (Yingxin Biotech, Shanghai, China) in accordance with the manufacturer's instructions.

### 2.6. Measurements of cell death, lipid ROS, malondialdehyde (MDA), and propidium iodide (PI) staining

Cells were stained with SYTOX Green (1 μM, Invitrogen) for 20 min and then analyzed by flow cytometry to measure cell death. Lipid ROS was measured by adding C11-BODIPY (1.5 μM, Invitrogen) for 20 min before cell harvest, and then assessed by flow cytometry. For intracellular MDA measurement, a Lipid Peroxidation MDA Assay Kit (Beyotime) was used according to the manufacturer's instructions. For PI staining, cells were stained with PI (Servicebio, Wuhan, China) for 10 min and photographed under a microscope (Leica, Wetzlar, Germany).

### 2.7. m^6^A dot blot

Total RNA was extracted with Trizol reagent and denatured by heating at 100 °C for 10 min, followed by chilling on ice immediately. Next, RNA was spotted on Biodyne Nylon Transfer Membranes ( Pall, New York, NY, USA) and cross-linked by 365 nm UV for 10 min m^6^A levels were measured using an anti-m^6^A antibody (Synaptic Systems, Goettingen, Germany) through IB.

### 2.8. RNA immunoprecipitation (RIP)

A Magna RIP Kit (Millipore, Billerica, MA, USA) was used. Briefly, cells were lysed by lysis buffer and incubated with magnetic beads loaded with 5 μg of the following primary antibodies: anti-HA (Abcam), anti-IGF2BP3 (Abcam), anti-m^6^A antibody (Synaptic Systems), or IgG (Beyotime Biotechnology) at 4 °C overnight. Next, cells were digested at 55 °C for 30 min using proteinase K. Finally, the remaining RNA was extracted using TRIzol reagent and measured by qRT-PCR.

### 2.9. Measurement of mRNA decay

Cells were treated with actinomycin D (ActD, 5 μg/mL, MedChemExpress) before detection of mRNA expression using qRT-PCR at the indicated time.

### 2.10. RNA pull-down experiments

Probes containing partial SLC3A2 mRNA with or without m^6^A methylation were obtained from Generay (Shanghai, China). Probes were incubated with cell lysates at 4 °C overnight. Subsequently, streptavidin magnetic beads (MedChemExpress) were added to the reaction for 3 h. After washing, the enriched proteins were subjected to IB.

### 2.11. Luciferase reporter assay

Regions encoding GPX4, SLC3A2, ACSL3, and FTH1 were inserted into the pmir-GLO luciferase reporter plasmid, which is often used to confirm putative m^6^A sites [25,26,28], and generated by ZuoRun Biotech. Luciferase activities were detected by a dual luciferase reporter gene assay kit (Promega, Madison, WI, USA). Firefly luciferase activities were normalized to Renilla luciferase activity.

### 2.12. Cell proliferation assay

Cell proliferation was analyzed with a Cell Counting Kit 8 (CCK-8) (Servicebio). Briefly, 6 × 10^3 cells were added into 96-well plates for culture. After cells adhered to the well, the required reagents or drugs were added. The absorbance value of each well at 450 nm was measured with a microplate reader (BioTek, Winooski, VT, USA).

### 2.13. Bioinformatics

Expression levels of m^6^A-related proteins were analyzed using GEO DataSets (http://www.ncbi.nlm.nih.gov/geo/) [29]. Expression levels of genes in normal and tumor tissues were analyzed using GEO DataSets and the starBase database (http://starbase.sysu.edu.cn/) [30], GEPIA database (http://geopia.cancer-pku.cn/) [31], and Ualcan database (http://ualcan.path.uab.edu) [32]. Interconnections among proteins were determined using the STRING database (https://string-db.org) [33]. The GEPIA database, Kaplan–Meier plotter database (https://kmplot.com) [34], and OncoLnc database (https://www.oncolnc.org/) [35] were used to analyze survival information. To identify targets of IGF2BP3, data were analyzed using enhanced crosslinking immunoprecipitation and high-throughput sequencing (eCLIP-seq), transcriptomic RNA sequencing (RNA-seq), and methylated RNA immunoprecipitation sequencing (MeRIP-seq/m^6^A-seq) data, which were extracted from GEO DataSets and the m6A2target database (http://m6a2target.canceromics.org) [36]. Potential m^6^A sites were predicted using the online tool SRAMP (http://www.cuilab.cn/sramp) [37]. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed by DAVID (https://david.ncifcrf.gov) [38,39].

### 3. Results

#### 3.1. High expression of IGF2BP3 indicates poor prognosis in LUAD

A schematic presentation of the experimental workflow to identify and verify promising therapeutic targets among m^6^A-related proteins in LUAD is shown in Fig. 1. First, we explored the expression profiles of 20 well-established m^6^A-related proteins in LUAD from the GEO database, including METTL3, methyltransferase-like 14 (METTL14), methyltransferase-like 16 (METTL16), Wilms' tumor 1-associated protein (WTAP), FTO, alkB homolog 5 (ALKBHS5), YTH domain containing 1/2 (YTHDC1/2), YTH domain family 1/2/3 (YTHDF1/2/3), insulin-like growth factor 2 mRNA binding protein 1/2 (IGF2BP1/2), KIAA1429 (also called vir-like m^6^A methyltransferase associated, VIRMA), heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPAB1), heterogeneous nuclear ribonucleoprotein C (HNRNPC), zinc finger CCCH-type containing 13 (ZC3H13), RNA binding motif protein 15 (RBM15), and RNA binding motif protein 15B (RBM15B). We noticed that only IGF2BP3 was highly expressed in LUAD compared with normal or adjacent lung tissues by analyzing GSE40275, GSE7670, and GSE1037 datasets (Fig. 2A). To confirm the importance of IGF2BP3 in LUAD, IGF2BP3 expression levels were evaluated in the GSE32863 dataset (Fig. 2B) and using the Ualcan database (Fig. 2C). As expected, IGF2BP3 was elevated in LUAD compared with adjacent and normal lung tissues (Fig. 2B and C). Because blocking tumor progression is an ideal anti-tumor approach, we examined IGF2BP3 expression profiles in LUAD patients at distinct stages and found that expression of IGF2BP3 was higher in the late stages (II to IV) than the early stage (I, Fig. 2D), suggesting that IGF2BP3 is critical for tumor progression. Using our own LUAD cohort (n = 192), IGF2BP3 expression levels in tumors were much higher than that of adjacent lung tissues (Fig. 2E-G). To link IGF2BP3 expression with clinical outcomes in LUAD, we investigated the role of IGF2BP3 in patient survival and found that higher IGF2BP3 expression indicated poorer overall survival (Fig. 2H and I). In addition to IGF2BP3, we upregulated in LUAD, its expression levels were associated with smoking history in patients with LUAD (Supplementary Fig. S1).
Collectively, the above results reveal that IGF2BP3 is commonly upregulated in LUAD and higher IGF2BP3 correlates with poorer overall survival.

3.2. IGF2BP3 is associated with ferroptosis

We next investigated the function of IGF2BP3. Because IGF2BP3 is an RNA-binding protein and its function relies on m^6^A modification, we wanted to determine which mRNAs can be bound and regulated by IGF2BP3. Expression levels of these RNAs should be manipulated by m^6^A writers, such as METTL3, a key factor of the m^6^A methyltransferase complex. To address this, we comprehensively analyzed eCLIP-seq data using anti-IGF2BP3 antibodies (GSE92220), RNA-seq comparison of mRNA expression profiles before and after IGF2BP3 knockdown (KD, GSE90684), and m^6^A-seq to reveal which mRNAs were m^6^A methylated after METTL3 knockdown (KD, GSE102493). Through the above strategies, we identified 1925 potential target mRNAs of IGF2BP3 (Fig. 3A).

Subsequently, these targets were used as inputs for GO and KEGG pathway enrichment analysis (Fig. 3B–E). GO contains a wide variety of biological ontologies, providing a three-layer system to describe the function of gene products, namely: cellular component (CC), molecular function (MF), and biological process (BP). Cell components are used to describe the location of gene products in cells, molecular functions mostly refer to the function of a single gene product, and biological processes refer to an orderly biological process with multiple steps. GO is a pure gene set, which does not define the relationship between genes. In comparison, KEGG has a gene set, but also defines the complex relationship between genes. Using GO, we found that IGF2BP3 is multifunctional and may be critical for sustaining transformative phenotypes in tumor cells. As ferroptosis is a newly identified form of cell death, its induction is considered a new and alternative method to treat tumors [21]. Thus, the involvement of ferroptosis, as predicted by KEGG, raised our particular interest (Fig. 3E). According to the results shown in Fig. 3A and E, 12 potential ferroptosis-associated targets of IGF2BP3 were picked up and further analyzed using STRING, a useful tool to predict protein-protein interactions. We found that the products of these 12 genes are close interactors (Fig. 3F). Because nuclear receptor coactivator 4 (NCOA4), SLC3A2, GPX4, ACSL3, and FTH1 were previously established to control ferroptosis in tumor cells [40–43], we focused on the role of IGF2BP3 in ferroptosis in the current study.

3.3. IGF2BP3 desensitizes ferroptosis by sustaining anti-ferroptosis factors

Next, we examined whether IGF2BP3 modulates ferroptosis and...
regulates its targets. Because IGF2BP3 is an m6A reader, we wondered how IGF2BP3 exerts its function in an m6A-dependent manner. To address this, we generated LUAD H1299 cells with IGF2BP3 either overexpressed or knocked out (KO, Fig. 4A). To avoid competitive interference in H1299 cells by endogenous IGF2BP3, we also reconstructed IGF2BP3 expression in IGF2BP3–KO H1299 cells with wild-type (WT) or mutant IGF2BP3 without the KH3–4 m6A-reading domain (Fig. 4A). We found that overexpressing IGF2BP3 desensitized ferroptosis induced by RSL3 and erastin, two pharmaceutical ferroptosis inducers. However, ferroptosis was sensitized when IGF2BP3 was knocked out (Fig. 4B and C; Supplementary Fig. S2A). Notably, compared with WT IGF2BP3, mutant IGF2BP3 without the KH3–4 m6A-reading domain failed to rescue IGF2BP3–KO-induced sensitization of ferroptosis (Fig. 4B and C), suggesting that the m6A-reading domain of IGF2BP3 is essential for IGF2BP3 to suppress ferroptotic cell death. Excessive lipid peroxidation is the hallmark of ferroptosis. Our evaluation of lipid ROS revealed that, as observed for cell death, IGF2BP3 also suppressed lipid peroxidation in a manner dependent on the m6A-reading domain (Fig. 4D and E). To exclude non-ferroptotic effects, ferrostatin-1, a ferroptosis inhibitor, was used in parallel. Regardless of the IGF2BP3 status, ferroptotic
Fig. 3. Prediction of IGF2BP3 targets and their functions.
(A) The 1925 IGF2BP3 mRNA targets, as revealed by comparing eCLIP-seq, RNA-seq, and m^A^-seq data from GEO and m6a2target databases.
(B–E) Possible biological process (B), cellular component enrichment (C), molecular function (D), and involved pathways (E) were predicted by GO and KEGG enrichment analysis.
(F) Associations between the 12 IGF2BP3 targets and KEGG-predicted ferroptosis-associated genes were reanalyzed using STRING.
changes induced by RSL3 and erastin were successfully prevented by pretreatment with ferrostatin-1 (Fig. 4B–E; Supplementary Fig. S2A).

We next assessed whether IGF2BP3 regulates the proteins identified as potential targets by comparing mRNA expression profiles before and after IGF2BP3 KO in H1299 cells. We found that seven of the 12 potential targets illustrated in Fig. 3F were altered (Fig. 4F). Among these seven candidates, ACSL3, SLC3A2, GPX4, and FTH1 are known anti-ferroptotic proteins. Consistent with mRNA results, these proteins could also be positively regulated by IGF2BP3 (Fig. 4G), suggesting that protein alterations might result from changes in the mRNA. Interestingly, treatment of H1299 cells with RSL3 and erastin led to the suppression of both IGF2BP3 and these anti-ferroptotic factors (Fig. 4H), indicating that the

Fig. 4. IGF2BP3 desensitizes ferroptosis and sustains anti-ferroptosis factors.

(A) Verification of IGF2BP3 expression levels in control cells and H1299 cells with IGF2BP3 either overexpressed or knocked out (KO, upper panel). IGF2BP3 expression was also evaluated in IGF2BP3–KO H1299 cells reconstituted with either WT or IGF2BP3 without the KH3–4 domain (IGF2BP3ΔKH3–4, lower panel).

(B–E) Measurements of cell death (B–C) and lipid peroxidation (D–E) by SYTOX Green and C11-BODIPY in control cells and H1299 cells under the indicated conditions through flow cytometry, with treatment of RSL3 (2 μM, 12 h; B and D) or erastin (40 μM, 12 h; C and E). H1299 cells were also pre-treated with or without ferrostatin-1 (2 μM, 12 h) before RSL3 or erastin treatment. At least three replicates were carried out.

(F) mRNA expression levels of ferroptosis-associated genes in control cells and H1299 cells with IGF2BP3 knockout (KO), as measured by qPCR.

(G–K) Representative IB images of IGF2BP3, ACSL3, SLC3A2, GPX4, and FTH1 in H1299 cells (G–H and J) with indicated treatments or in tails from WT and Igf2bp3−/− mice (I and K).

Statistical analysis was performed using one-way ANOVA (B–E), or t-tests (F). Data are presented as mean ± SD from three independent experiments. *p < 0.05, **p < 0.01, and N.S. indicates non-significance.
alteration of IGF2BP3 might precede the alterations of its targets. In vivo data from mouse tails also confirmed that Igf2bp3 stimulates expression of anti-ferroptotic factors (Fig. 4I). Because m^A^-methylated is a prerequisite for the function of IGF2BP3, we next determined whether anti-ferroptotic factors are also regulated by m^A-. Indeed, these factors can be positively regulated by METTL3 in human LUAD cells and mice (Fig. 4J and K). Collectively, IGF2BP3 desensitizes ferroptosis by sustaining the expression of anti-ferroptotic factors through its m^A^-reading function.

To assess whether these anti-ferroptotic factors are downstream effectors of IGF2BP3, we overexpressed GPX4 and SLC3A2 in IGF2BP3 KO H1299 cells and evaluated whether such treatments affected ferroptosis sensitivity. We found that IGF2BP3 KO-sensitized RSL3 and erastin-induced cell death and lipid ROS accumulation could be partially reversed by overexpression of either GPX4 or SLC3A2 (Supplementary Figs. S2B and C). Moreover, colocalization of IGF2BP3 with GPX4, SLC3A2, ACSL3, and FTH1 were observed in H1299 cells (Supplementary Fig. S2D), indicating that IGF2BP3 and its downstream effectors might closely interact.

To further verify whether IGF2BP3 inhibits ferroptosis in vivo, xenograft mice were generated using control H1299 cells and H1299 cells overexpressing IGF2BP3. Following administration of IKE to mice, we found that IGF2BP3 prevented xenograft tissues from IKE-induced lift of m^A- and K). Collectively, IGF2BP3 desensitizes ferroptosis by sustaining the expression of anti-ferroptotic factors through its m^A^-reading function. To further verify whether IGF2BP3 inhibits ferroptosis in vivo, xenograft mice were generated using control H1299 cells and H1299 cells overexpressing IGF2BP3. Following administration of IKE to mice, we found that IGF2BP3 prevented xenograft tissues from IKE-induced lift of m^A- and K). Collectively, IGF2BP3 desensitizes ferroptosis by sustaining the expression of anti-ferroptotic factors through its m^A^-reading function.

3.4. IGF2BP3 recognizes m^A^-methylated mRNAs of anti-ferroptosis factors

The fundamental role of IGF2BP3 is to recognize m^A^-methylated target mRNAs. However, whether IGF2BP3 recognizes and binds m^A^-methylated mRNAs encoding anti-ferroptotic factors is unknown. Thus, we first predicted by SRAMP online software (Fig. 5A–D). Next, sites A193 of GPX4 mRNA, A1811 of SLC3A2 mRNA, A2564 of ACSL3 mRNA, and A361 of FTH1 mRNA, as well as their flanking regions, were selected for further analysis to investigate interactions between IGF2BP3 and m^A^-methylated anti-ferroptotic mRNAs because of their highest combined scores (Fig. 5A–D). Notably, these m^A^-sites are close to either the N- or C-terminus of the coding sequence (CDS, Fig. 5A–D), suggesting that these are regulatory elements. In addition, the common m^A^- motif among these anti-ferroptotic mRNAs was “GGACU,” which is also regarded as the consensus m^A^- motif among species [44], suggesting that our prediction results were reliable.

We next performed experimental verification. Regions encoding GPX4, SLC3A2, ACSL3, and FTH1 mRNA with (WT) or without (Mut) the putative m^A^-site were cloned downstream of the Firefly luciferase gene of the pmir-GLO luciferase reporter plasmid (Supplementary Fig. 3A). We found that luciferase activities from pmir-GLO plasmids containing Mut mRNAs were much weaker compared plasmids containing WT mRNAs (Supplementary Figs. S3B–E), confirming that the m^A^-sites within these anti-ferroptosis factors are genuine. RIP experiments were performed in H1299 cells expressing either exogenous IGF2BP3-HA or an m^A^-unrelated negative control MEX3A-HA (Supplementary Fig. 3F). Enrichment of these anti-ferroptotic mRNAs in RIP products pulled down by anti-HA antibodies were notably higher in H1299 cells expressing IGF2BP3-HA compared with those expressing MEX3A-HA (Fig. 5G–J), demonstrating that IGF2BP3 specifically binds anti-ferroptotic mRNAs. We also performed RIP experiments in IGF2BP3-KO H1299 cells reconstituted with either WT or IGF2BP3 without the KH3–4 domain, which confirmed that IGF2BP3 binding to anti-ferroptotic mRNAs relied on its m^A^-reading domain (Fig. 5K–N). In addition to RIP experiments, RNA pull-down experiments using SLC3A2 probes with or without m^A^-methylated SLC3A2 mRNA (Supplementary Fig. S3G). However, to the best of our knowledge, four other non-ferroptosis associated mRNAs did not bind with IGF2BP3 (Supplementary Fig. S3H), suggesting that IGF2BP3 may interact with anti-ferroptosis factors by a specific mechanism. By stimulating global m^A^-methylated mRNAs expression via overexpressing METTL3 (Supplementary Fig. S3I), we found that sites of interactions between IGF2BP3 and anti-ferroptotic mRNAs were m^A^-dose-dependent (Fig. 5O–R). We also evaluated whether lifting global m^A^-mRNA expression could affect ferroptosis sensitivity, and found that overexpressing METTL3 only slightly suppressed RSL3 and erastin-induced cell death (Supplementary Fig. S3J). This may result from the cystine–glutamate antiporter (Xc^-) system, which is essential for synthesis of the glutathione (GSH), an antioxidant that can be reduced following stimulation of global m^A^- (27,45). Such actions may offset the effects of IGF2BP3. Unfortunately, overexpression of IGF2BP3 did not alter global m^A^- (Supplementary Fig. S3K), demonstrating that IGF2BP3 barely acts downstream of m^A^-mRNA. Altogether, these results suggest that IGF2BP3 may interact with m^A^-methylated mRNAs to modulate ferroptosis.

3.5. IGF2BP3 sustains the stability of anti-ferroptotic mRNAs, the relationships of which correlate with clinical outcome

Although IGF2BP3 recognizes and binds m^A^-methylated mRNAs encoding anti-ferroptotic factors, how IGF2BP3 regulates these mRNAs is unknown. By treating cells with ActD, which inhibits nascent mRNA transcription [46], we evaluated the remaining intracellular mRNA in H1299 cells with or without IGF2BP3 overexpression. We found that the remaining mRNA levels of these anti-ferroptotic factors decreased much slower compared with control cells (Fig. 6A–D), suggesting that IGF2BP3 sustains the mRNA stability of anti-ferroptotic factors. Similarly, overexpressing METTL3 prolonged the life-halves of these mRNAs (Fig. 6E–H), indicating that m^A^- is indispensable for the regulation of mRNA stability by IGF2BP3.

We next investigated the close interactions between IGF2BP3 and anti-ferroptotic factors in clinical samples. Our previous study [27] demonstrated that YTHDC2 suppresses LUAD cells by indirectly inhibiting SLC3A2, leading to sensitization of ferroptosis. Therefore, we evaluated whether SLC3A2 is associated with other m^A^-readers, such as IGF2BP3, in regulating ferroptosis. In addition, because few previous studies focused on ACSL3 and m^A^-readers, we selected ACSL3 for further study. We evaluated expression of SLC3A2 and ACSL3 in The Cancer Genome Atlas (TCGA) database. IGF2BP3 and ACSL3 were more highly expressed in LUAD compared with normal lung (Fig. 6I and J). We also examined SLC3A2, ACSL3, and IGF2BP3 in our LUAD cohort (n = 50) and found significant positive correlations between IGF2BP3 and SLC3A2, and between IGF2BP3 and ACSL3 (Fig. 6K and L). Moreover, higher levels of SLC3A2 and ACSL3 indicated shorter overall survival (Fig. 6M and N). These results demonstrate close relationships between IGF2BP3 and anti-ferroptotic factors that are indicative of poor clinical outcomes in LUAD.

3.6. Potential strategy to target IGF2BP3 and suppress tumorigenesis

The aim of the present study was to identify therapeutic drugs that can target m^A^-related proteins. To achieve this, a library containing ~1800 FDA-approved small molecules were screened to assess whether IGF2BP3 can be downregulated (Fig. 7A). RIG was found to have the capacity to reduce IGF2BP3, but not other m^A^-related proteins (Fig. 7B), suggesting that it might specifically inhibit IGF2BP3. Also, cotreatment with RIG sensitized H1299 cells to RSL3 and erastin-induced ferroptotic cell death (Fig. 7C) and accumulation of lipid ROS (Fig. 7D). We also evaluated whether administration of RIG was harmful to non-tumor cells by testing RIG in parallel in H1299 and BEAS-2B cells. As expected, RIG-impaired cell proliferation in BEAS-2B cells was much less obvious compared with H1299 cells (Fig. 7E), suggesting that RIG administration is a safe strategy to treat tumors. In vivo mouse experiments also demonstrated that RIG has a strong capacity to reduce IGF2BP3 expression and tumor growth (Fig. 7F). Taken together, we found that RIG is a
(caption on next page)
safe drug to suppress LUAD, possibly by targeting IGF2BP3.

Finally, we evaluated whether IGF2BP3 plays a similar role to desensitize ferroptosis in tumor cells other than LUAD by knocking out or overexpressing IGF2BP3 in SW1990 cells (a pancreatic cancer cell line) and HCT116 cells (a colon adenocarcinoma cell line). Our results show that IGF2BP3 still desensitized SW1990 and HCT116 cells to ferroptotic cell death (Supplementary Figs. S4A and B). In comparison, the anti-ferroptotic role of IGF2BP3 was very weak in BEAS-2B cells (Supplementary Fig. S4C). These results demonstrate that the anti-ferroptosis function of IGF2BP3 is strong and common among tumor cells but may not be critical to suppress ferroptosis in non-tumor cells. Thus, once again our results emphasize that targeting IGF2BP3 might be useful to induce ferroptosis in tumor cells.

4. Discussion

Although some potential targets have been revealed during studies of LUAD, therapeutic strategies for LUAD are still far from satisfactory. This ineffectiveness may largely occur because most strategies involve one-point to one-point targets. To enhance efficacy, it is urgent to develop multifunctional targets.

To reveal such powerful targets, we focused on m6A-related proteins. Through comprehensive multi-omics studies, IGF2BP3 was identified as being highly expressed in LUAD and positively controlling multiple essential regulators in the process of ferroptosis. As an m6A reader, the biological effects of IGF2BP3 are ultimately executed through its specific recognition of m6A-methylated RNAs [47]. IGF2BP3 modulates cancer progression by regulating cell proliferation, cell cycle, angiogenesis, glycolysis, metastasis, and immune escape [48–50]; however, whether IGF2BP3 can be regarded as a potent therapeutic target in LUAD is still unknown. The concept of IGF2BP3 as a critical LUAD regulator has gradually been accepted because a series of studies have reported specific effectors of IGF2BP3 in other tumorigenic processes in addition to ferroptosis [51–53]. In recent years, interactions between m6A methylation and ferroptosis have garnered enormous interest in cancer research communities. Emerging studies have proved that m6A-related proteins are essential for modulating ferroptosis in tumors [22–24,27]. However, whether and how IGF2BP3 affects ferroptosis remains unknown until the relationship between IGF2BP3 and anti-ferroptosis factors is elucidated. Our study also demonstrates that IGF2BP3 can suppress ferroptosis by sustaining expression of anti-ferroptosis factors (Fig. 7G). Herein, we provide further evidence suggesting that IGF2BP3 is a promising therapeutic target for LUAD treatment.

Ferroptosis is related to iron-dependent accumulation of lipid hydroperoxides at lethal levels [54]. Emerging evidence suggests that ferroptosis plays an important role in tumor development. m6A-associated mechanisms have been linked to ferroptosis [55–57]. METTL3, METTL14, and FTO can reportedly modulate ferroptosis in tumors [22–24]. Notably, besides the currently reported m6A reader IGF2BP3, the m6A reader YTHDC2 was previously reported to be closely associated with ferroptosis [27], thus hinting at critical roles for m6A readers in modulating ferroptosis. GPX4, SLC3A2, ACSL3, and FTH1 are also pivotal anti-ferroptosis factors. GPX4 prevents ferroptosis by eliminating intracellular lipid ROS, and inhibition of GPX4 triggers ferroptosis [58]. Increasing evidence suggests that GPX4 is related to the pathogenesis of tumors. GPX4 is highly expressed in tumors compared with normal tissues in nine types of tumors in TCGA, including LUAD [59]. As the primary regulator preventing ferroptosis, GPX4 downregulation is a critical indicator of ferroptosis [60]. SLC3A2 and solute carrier family 7 member 11 (SLC7A11) are two subunits of the cysteine–glutamate antiporter Xc system, and cystine is one of the major raw materials for the synthesis of the antioxidant GSH [61]. Inhibition of SLC3A2 was previously reported to sensitize ferroptosis [62]. Notably, our previous study demonstrated that SLC3A2 is regulated by m6A by an indirect mechanism [27]. SLC3A2 deficiency leads to impaired tumorigenesis in nude mice [63]. ACSL3, a member of the acyl-CoA synthetase long chain family that activates free fatty acids to produce fatty acyl-CoAs, inhibits ferroptosis by activating exogenous monounsaturated fatty acids, which suppress ferroptosis [64]. ACSL3 can also promote oleic acid secretion from adipocytes to inhibit lipid peroxidation and the ensuing ferroptotic cell death [65]. FTH1 is the heavy subunit of ferritin, which plays an essential role in the maintenance of intracellular iron homeostasis to prevent harmful effects caused by iron overload [66,67]. Accordingly, inhibition of FTH1 plays a role in sensitizing ferroptosis. Excessive lipid peroxidation, unbalanced redox hemostasis, and liable iron overload are three major hallmarks of ferroptosis. Notably, GPX4, SLC3A2, ACSL3, and FTH1 cover all three of these aspects. The ideal cost-effective strategy to induce ferroptosis is to simultaneously inhibit these four anti-ferroptosis factors. Interestingly, this goal can be achieved by suppressing IGF2BP3 because of its common regulatory mechanism. Interestingly, the m6A sites within GPX4, SLC3A2, ACSL3, and FTH1 mRNAs are close to either the N- or C-terminus of the CDS, suggesting that such localization may prolong the half-lives of these mRNAs. Recently, several studies [68–70] pointed out that m6A methylation of mRNA might be essential to maintain RNA stability, consistent with our conclusion.

Because tumor cells are more sensitive to ferroptosis compared with normal cells, ferroptosis is a new and alternative method to treat cases of LUAD with resistance to conventional drugs. Therapeutic drugs inducing ferroptosis have been developed, some of which are undergoing experimental verification. For example, RSL3, ML162, diphenylethionidom chloride (DPI) compounds, FIN56, and FINO2 are known to target GPX4 [54]. In addition, sorafenib, erastin and its derivatives are capable of inhibiting the Xc system and preventing cystine uptake [71], while buthionine sulfoximine and DPI2 have the capacity to deplete GSH [72]. Although small molecules have been gradually discovered and developed, challenges still exist. First, most inhibitors have single targets, and those targets involve GPX4 or the Xc system. Second, many inhibitors show poor (or unclear) pharmacological properties in animal models, limiting their clinical application potential [73]. Last, but not least, whether these drugs are specific and leave normal tissue unharmed is unclear; accordingly, how to improve their specificity requires further exploration.

Herein, we showed that a series of anti-ferroptosis factors are commonly regulated by IGF2BP3, indicating that IGF2BP3 exerts a wide...
regulatory mechanism to modulate ferroptosis. With the continuous progress of modern technology, targeting IGF2BP3 is not difficult. One option is RNAi agents, which are widely used with good tolerance and less damage to normal tissues. In addition, readily available FDA-approved small molecule libraries also exist, which can be used to screen possible drugs targeting IGF2BP3. Using such screening methods, we identified RIG as a potent small molecule that can suppress tumorigenesis by downregulating IGF2BP3. In addition, we experimentally confirmed that RIG is a safe drug that does not harm non-tumor cells. Thereby, we established a successful example of screening for drug targets against m^6^A-related proteins to reduce tumorigenesis, and we may take advantage of IGF2BP3 as a promising target.

Notably, IGF2BP3 is reported as being highly expressed in at least 15 kinds of tumors [15], thus targeting IGF2BP3 might be helpful for tumors other than LUAD. In summary, the efficacy of additional anti-IGF2BP3 drugs should be urgently verified to accelerate future clinical applications.

Statistical analysis was performed using two-way ANOVA (A–H), or Pearson correlation analysis (K–L). Data are presented as mean ± SD from three independent experiments. **p < 0.01.

Fig. 6. IGF2BP3 correlates with anti-ferroptosis factors and their clinical significance.

(A–D) ActD chase experiments to analyze mRNA levels of GPX4 (A), SLC3A2 (B), ACSL3 (C), and FTH1 (D) in control cells and H1299 cells overexpressing IGF2BP3, as measured by qPCR.

(E–H) ActD chase experiments for analyzing mRNA levels of GPX4 (E), SLC3A2 (F), ACSL3 (G), and FTH1 (H) in control cells and H1299 cells overexpressing METTL3, as measured by qPCR.

(I, J) Expression of SLC3A2 (I) and ACSL3 (J) in normal (n = 59) and LUAD (n = 515) using TCGA data and analysis with the Ualcan database.

(K, L) Correlation between SLC3A2 and IGF2BP3 (K), and between ACSL3 and IGF2BP3 (L) in LUAD (n = 50).

(M, N) Overall survival curves based on expression of SLC3A2 (M) and ACSL3 (N), as analyzed by the OncoLnc database.

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(K, L) Correlation between SLC3A2 and IGF2BP3 (K), and between ACSL3 and IGF2BP3 (L) in LUAD (n = 50).

(M, N) Overall survival curves based on expression of SLC3A2 (M) and ACSL3 (N), as analyzed by the OncoLnc database.

Statistical analysis was performed using two-way ANOVA (A–H), or Pearson correlation analysis (K–L). Data are presented as mean ± SD from three independent experiments. **p < 0.01.
Fig. 7. Rigosertib is a potent anti-tumor small molecule targeting IGF2BP3.

(A) Workflow for screening small molecules that targeting against IGF2BP3.

(B) Representative IB images of IGF2BP3, IGF2BP1 and YTHDF2 in H1299 cells treated with DMSO or rigosertib (1 μM, 24h).

(C, D) Measurements of cell death (C) and lipid peroxidation (D) by SYTOX Green and C11-BODIPY in H1299 cells pretreated with DMSO or rigosertib (1 μM, 24 h) followed by treatment with or without DMSO, RSL3 (2 μM, 12 h), or erastin (40 μM, 12 h). H1299 cells were also pretreated with or without ferrostatin-1 (2 μM, 12 h). At least three replicates were carried out.

(E) Cell proliferation was measured by CCK-8 assay in BEAS-2B and H1299 cells treated with DMSO or rigosertib (1 μM, 24h) for indicated time.

(F) Xenograft mice were administrated DMSO or rigosertib (250 mg/kg) for the indicated time when tumors were obvious in mice. Tumor volumes were then graphed. IGF2BP3 was also measured by IB in tumors. N = 5 mice/group.

(G) Schematic of the proposed model in the present study. Briefly, the m6A reader IGF2BP3 recognizes and binds target mRNAs that can be m6A-methylated by m6A writers, such as METTL3 in LUAD cells. Subsequently, the stability of IGF2BP3 target mRNAs encoding anti-ferroptosis factors are sustained. Ferroptosis is thus suppressed and tumorigenesis is stimulated. Targeting IGF2BP3 using RIG or other potential biomaterial-associated therapeutic drugs may be a possible treatment for LUAD in the future.

Statistical analysis was performed using one-way ANOVA (C, D), or two-way ANOVA (E, F). Data are presented as means ± SD from indicated samples or experiments (n = 3 for C, D, E, and n = 5 for F). **p < 0.01, and N.S. indicates non-significance.
IGFBP3 is a key m6A-regulated protein that is highly expressed in LUAD and associated with poor prognosis. IGFBP3 desensitizes ferroptosis by sustaining m6A-methylated mRNAs encoding anti-ferroptosis factors. Our discovery reveals a new potential strategy of targeting IGFBP3 to inhibit LUAD by sensitizing ferroptosis.

Credit author statement

Xin Xu: Conceptualization, Methodology, Formal analysis, Investigation, Data curation. Hong Wang: Formal analysis, Investigation. Lifa Ma: Data curation. Xiaozhao: Data curation. Wanzuo Xin: Investigation. Xiange Xu: Investigation. Yikun Wang: Investigation. Shiyu Qiu: Investigation. Xiaotian Tang: Methodology. Yatou Miao: Methodology. Mengyi Wu: Methodology. Yongchun Yu: Supervision. Yunhua Xu, Jiayi Wang, and Yongxia Qiao: Conceptualization, Methodology, Writing—review and editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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