Clinical Cancer Research

LncRNA–FEZF1-AS1 Promotes Tumor Proliferation and Metastasis in Colorectal Cancer by Regulating PKM2 Signaling

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Abstract

Purpose: Long non-coding RNAs (lncRNAs) play key roles in human cancers. Here, FEZF1-AS1, a highly overexpressed lncRNA in colorectal cancer, was identified by lncRNA microarrays. We aimed to explore the roles and possible molecular mechanisms of FEZF1-AS1 in colorectal cancer.

Experimental Design: LncRNA expression in colorectal cancer tissues was measured by lncRNA microarray and qRT-PCR. The functional roles of FEZF1-AS1 in colorectal cancer were demonstrated by a series of *in vitro* and *in vivo* experiments. RNA pull-down, RNA immunoprecipitation and luciferase analyses were used to demonstrate the potential mechanisms of FEZF1-AS1.

Results: We identified a series of differentially expressed lncRNAs in colorectal cancer using lncRNA microarrays, and revealed that FEZF1-AS1 is one of the most overexpressed. Further validation in two expanded colorectal cancer cohorts confirmed the upregulation of FEZF1-AS1 in colorectal cancer, and revealed that increased FEZF1-AS1 expression is associated with poor survival. Functional assays revealed that FEZF1-AS1 promotes colorectal cancer cell proliferation and metastasis. Mechanistically, FEZF1-AS1 could bind and increase the stability of the pyruvate kinase 2 (PKM2) protein, resulting in increased cytoplasmic and nuclear PKM2 levels. Increased cytoplasmic PKM2 promoted pyruvate kinase activity and lactate production (aerobic glycolysis), whereas FEZF1-AS1–induced nuclear PKM2 upregulation further activated STAT3 signaling. In addition, PKM2 was upregulated in colorectal cancer tissues and correlated with FEZF1-AS1 expression and patient survival.

Conclusions: Together, these data provide mechanistic insights into the regulation of FEZF1-AS1 on both STAT3 signaling and glycolysis by binding PKM2 and increasing its stability. *Clin Cancer Res; 24(19); 4808–19. ©2018 AACR.*

Introduction

Colorectal cancer is the third most common cancer worldwide, and its incidence and mortality are increasing in China (1, 2). The development and progression of colorectal cancer involve a series of complex genetic and epigenetic changes (3). Recent findings have revealed that approximately 98% of human genome transcripts are non-coding RNAs (ncRNAs) with limited or no protein-coding capacity. Long ncRNAs (lncRNAs), greater than 200 nt in length, account for most of the ncRNA family. An increasing number of studies have demonstrated that lncRNAs play key roles in the development and progression of human cancers, including colorectal cancer (4–6).

Although several lncRNAs, such as UCA1, LINC00152, and PVT1, have been reported to be aberrantly expressed in colorectal cancer and shown to regulate colorectal cancer tumorigenesis and progression (7–10), the functions and mechanisms of most lncRNAs in colorectal cancer remain unknown. For example, CCAL could promote colorectal cancer progression by activating the Wnt/β-catenin pathway (10). Our previous studies revealed that UCA1 and LINC00152 function as oncogenes by promoting tumor growth and chemoresistance in colorectal cancer (7, 8).

In this study, we identified a series of lncRNAs with aberrant expression in colorectal cancer tissues using lncRNA expression microarrays. Subsequent validation in two expanded colorectal cancer cohorts confirmed that FEZF1 antisense RNA 1 (FEZF1-AS1; previously named AK057037 or LOC154860) is upregulated in more than 60% of colorectal cancer tissues and is associated with poor survival. Functional analyses revealed that FEZF1-AS1 enhances colorectal cancer cell proliferation and metastasis. Mechanistic studies revealed that FEZF1-AS1 promotes colorectal cancer tumorigenesis and progression by binding to pyruvate kinase M2 (PKM2) and enhancing the

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Translational Relevance

Long non-coding RNA (lncRNA) expression aberration has been observed in almost all human cancers, providing a group of potential diagnostic markers, prognostic factors and therapeutic targets in tumorigenesis. Our research revealed extensive aberrant expression of lncRNA in colorectal cancer and showed that FEZF1 antisense RNA 1 (FEZF1-AS1), one of the top upregulated lncRNAs in colorectal cancer, functions as an oncogene by promoting colorectal cancer cell growth and metastasis. FEZF1-AS1 could bind and increase the stability of pyruvate kinase 2 (PKM2) protein, resulting in the activating of STAT3 signaling and the increasing of aerobic glycolysis. High FEZF1-AS1 and PKM2 expression are associated with poor prognoses in patients with colorectal cancer, suggesting that they could serve as valuable prognostic factors. Targeting FEZF1-AS1/PKM2 signaling appears to be a promising treatment for colorectal cancer.

STAT3 signaling and aerobic glycolysis (Warburg effect). Our data reveal the novel regulatory roles of FEZF1-AS1 in STAT3 signaling and glycolysis via PKM2 in colorectal cancer, suggesting that FEZF1-AS1 could be a potential new therapeutic target for colorectal cancer.

Materials and Methods

Cell lines and clinical samples

The colorectal cancer cell lines LoVo, Caco2, HT29, HCT8, HCT116, and SW480 were purchased from the ATCC from 2008 to 2014. The cells were cultured following the instructions recommended by the ATCC. These cells were characterized by Genewiz Inc. (China) using short tandem repeat (STR) markers and were confirmed to be *Mycoplasma*-free (last tested in 2017).

Two colorectal cancer cohorts, comprising 108 and 138 human primary colorectal cancer tissues and their paired adjacent noncancerous tissues (NCTs) were obtained from Affiliated Hospital of Jiangnan University and Fudan University Shanghai Cancer Center, respectively (Supplementary Table S1). All patient materials were obtained with informed consent, and this project was approved by the Clinical Research Ethics Committees of the participating institutions. The study was conducted in accordance with the International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS).

Microarray analysis

Total RNA was extracted from five paired colorectal cancer tissues and NCTs using RNAiso reagent (Takara, Japan). RNA integrity was verified using an Agilent 2100 bioanalyzer (Agilent). LncRNA expression profiling was performed using ArrayStar Human LncRNA array 2.0. Data were analyzed using GeneSpring GX v11.5.1 software (Agilent). LncRNAs were deemed differentially expressed if their fold change between the colorectal cancer and NCT groups exceed 2.0, and their *P* values were less than 0.05.

Quantitative RT-PCR assay

Total RNA was reverse transcribed into cDNA using the PrimeScript II 1st Strand Synthesis Kit (TaKaRa). Quantitative RT-PCR (qRT-PCR) was performed on the ViiA7 real-time PCR system using the UltraSYBR Mixture (CWBIO, China). The relative gene expression levels were normalized to those of *β-actin* and calculated using the $2^{-\Delta\Delta Ct}$ method (11).

Vector constructs and siRNA

The FEZF1-AS1 sequence (NR_036484) was synthesized by GENEray Biotechnology (China) and cloned into the eukaryotic expression vector pcDNA3.1 and the lentiviral expression vector pWPXL. The siRNAs of FEZF1-AS1 and PKM2 were purchased from RiboBio (China). The validated shRNA sequence of FEZF1-AS1 was synthesized and cloned into the pSIH-H1 shRNA cloning and expression lentivector. The related sequences are listed in Supplementary Table S2.

Generation of colorectal cancer cell lines with stable FEZF1-AS1 overexpression or knockdown

The pWPXL, pWPXL-FEZF1-AS1, pSIH-H1, or pSIH-H1-shFEZF1-AS1 plasmid was co-transfected into HEK-293T cells along with the packaging plasmid ps-PAX2 and the envelope plasmid pMD2G using Lipofectamine 2000 (Invitrogen) as we previously described (12). The virus particles were harvested 48 hours after co-transfection and then individually used to infect colorectal cancer cells to generate corresponding stable cell lines. The efficiency of FEZF1-AS1 overexpression or knockdown was assessed using qRT-PCR.

Cell proliferation assay and colony formation assay

Cell viability was measured using the Cell Counting Kit 8 (CCK-8, Dojindo, Japan) according to the manufacturer's instructions. For the colony formation assay, 800 to 1,500 colorectal cancer cells were seeded into each well of a 6-well plate and maintained in a medium containing 10% FBS for 10 days. The colonies were fixed with methanol and stained with 0.1% crystal violet; the number of clones was counted using an inverted microscope.

Cell cycle and apoptosis analyses

Colorectal cancer cells were harvested and fixed in ice-cold 70% ethanol. The fixed cells were washed twice with PBS buffer and subjected to cell-cycle analysis using the Cell Cycle and Apoptosis Detection Kit (CWBIO). For cell apoptosis analysis, colorectal cancer cells were treated with 0.25 μg/mL 5-FU for 48 hours. The cells were then harvested and subjected to apoptosis analysis using the Annexin V-FITC/PI Apoptosis Detection Kit (CWBIO) or Annexin V-PE/7-ADD Apoptosis Detection Kit (CWBIO).

Cell migration and invasion assays

Cell migration and invasion assays were performed using Boyden Transwell chambers (8-mm pore size, BD Biosciences) as we previously described (13).

Tumor formation and metastasis assays in nude mouse models

LoVo (HCT116) cells stably expressing FEZF1-AS1 (shFEZF1-AS1) or the control vector were subcutaneously injected into either flank of the same athymic male BALB/c nude mouse at 5 weeks of age ($n = 6$ for each group). Four to 5 weeks after injection, the mice were sacrificed and examined for the growth of subcutaneous tumors. Two types of mouse models were used

to evaluate the effect of FEZF1-AS1 on colorectal cancer metastasis. For the *in vivo* lung metastasis assay, 2×10^6 LoVo cells stably expressing FEZF1-AS1 or the control vector were injected into the caudal vein of each nude mouse ($n = 10$ for each group). These mice were sacrificed after 5 weeks and examined for lung metastases. In addition, an orthotopic mouse model was used to evaluate the effect of FEZF1-AS1 on hepatic metastasis. Briefly, 2×10^6 HCT116 cells stably expressing FEZF1-AS1 were injected into the submucosal tissue of cecum of an athymic male BALB/c nude mouse at 8 weeks of age ($n = 5$ for each group). These mice were killed 6 weeks after injection and checked for hepatic metastasis. All animal care and handling procedures were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Clinical Research Ethics Committees of Affiliated Hospital of Jiangnan University

RNA-seq and computational analyses

RNA-seq was performed to detect the mRNA expression profiles of FEZF1-AS1–silenced colorectal cancer cells at RiboBio (China) using HiSeq3000 (Illumina). LifeScope v2.5.1 was used to align the reads to the genome, generate raw counts corresponding to each known gene (a total of 20, 345 genes), and calculate the RPKM (reads per kilobase per million) values. The differential genes were selected with fold change >1.5, and Gene Ontology (GO) analysis was used for pathway enrichment using Cytoscape (ClueGo) with $P < 0.01$.

RNA pull-down assays and mass spectrometry analyses

RNA pull-down assays were performed using the Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher) according to the manufacturer's instructions. Briefly, the FEZF1-AS1 sequence was *in vitro* transcribed with biotin RNA-labeling mix and T7 RNA polymerase (Invitrogen) according to the manufacturer's instructions. The biotinylated FEZF1-AS1 RNA was incubated with streptavidin-linked magnetic beads and total cell lysates at room temperature for 2 hours. The bead-RNA–protein complexes were washed with 1× binding washing buffer four times. The proteins were precipitated and diluted in protein lysis buffer. Finally, the retrieved proteins were measured on SDS-PAGE gels for one-shot mass spectrometry or Western blot analysis. Detailed information regarding the primers used for *in vitro* transcription is depicted in Supplementary Table S2.

Luciferase reporter assay

The reporter construct pGL4.47[Luc2P/SIE/Hygro] (SIE-pGL4.47, Promega), which contains five copies of the sis-inducible element (SIE) that drives expression of the luciferase reporter gene luc2P, was used to evaluate STAT3 transcriptional activity. HEK-293T cells were transiently transfected with the plasmids, as indicated, and the pRL-TK Renilla luciferase plasmid was used as a control to adjust for the differences among wells. Thirty-six hours after transfection, the cells were harvested and then assayed for luciferase activity with the Dual-Luciferase Reporter Assay System (Beyotime).

RNA Immunoprecipitation assay

RNA Immunoprecipitation (RIP) assays were performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) as we previously described (7). Briefly, 1×10^7 colorectal cancer cells were harvested and lysed with RIP lysis buffer. Cell extracts were co-immunoprecipitated with anti-PKM2 antibodies (CST), and the retrieved RNA was subjected to qRT-PCR analysis using FEZF1-AS1-specific primers. Total RNA (input controls) and normal mouse IgG controls were assayed simultaneously to confirm that the detected signals were from the RNA specifically binding to PKM2.

Western blotting

The separation of nuclear and cytoplasmic fractions was performed using the PARIS Kit (Life Technologies) according to the manufacturer's instructions. Extracted proteins were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% non-fat milk and incubated with primary antibodies for PKM2 (CST, 1:1,000), STAT3 (CST, 1:500), pSTAT3 (Tyr705; CST, 1:1,000), Ub (Santa Cruz Biotechnology, 1:500), HA (CST, 1:5,000), Histone H3.1 (Abmart, USA, 1:1,000), GAPDH (Thermo Fisher, 1:5,000), and β-actin (Beyotime, 1:1,000).

Pyruvate kinase activity and lactate detection

Pyruvate kinase activity and lactate production in colorectal cancer cells were measured using the Pyruvate Kinase Activity Assay Kit (Jiancheng, China) and the Lactic Acid Assay Kit (Jiancheng), respectively.

Immunohistochemistry

The expression levels of PKM2 protein were determined by immunohistochemistry (IHC) analysis using colorectal cancer tissue arrays constructed previously (12). IHC staining was performed on 4-mm sections of paraffin-embedded tissue samples. Briefly, the slides were incubated with an anti-PKM2 antibody (CST, 1:200) at 4°C overnight. The subsequent steps were performed using the GTVision III Detection System/Mo&Rb (Gene Tech, China).

Statistical analysis

All results are presented as the mean values ± SEM. Student *t* test, the Mann–Whitney *U* test, and the χ^2 test were used to compare the differences among different groups. The Kaplan–Meier method and log-rank test were applied to determine the differences in survival rates between two groups. Cox univariate and multivariate proportional hazards regression models were used to determine the independent factors that influence survival. All statistical analyses were carried out using SPSS20.0 (SPSS). *P* values <0.05 were considered statistically significant.

Results

FEZF1-AS1 is upregulated in colorectal cancer tissues and predicts poor prognosis

LncRNA expression profiles in five paired colorectal cancers and NCTs were analyzed to screen for differentially expressed lncRNAs in colorectal cancer. Of the approximately 33,000 human lncRNAs analyzed, 496 exhibited significantly differential expressions with 306 genes being upregulated and 190 genes being downregulated in colorectal cancer compared with NCT (fold change >2, $P < 0.05$, Supplementary Table S3). Of the 496 significantly dysregulated genes, unsupervised

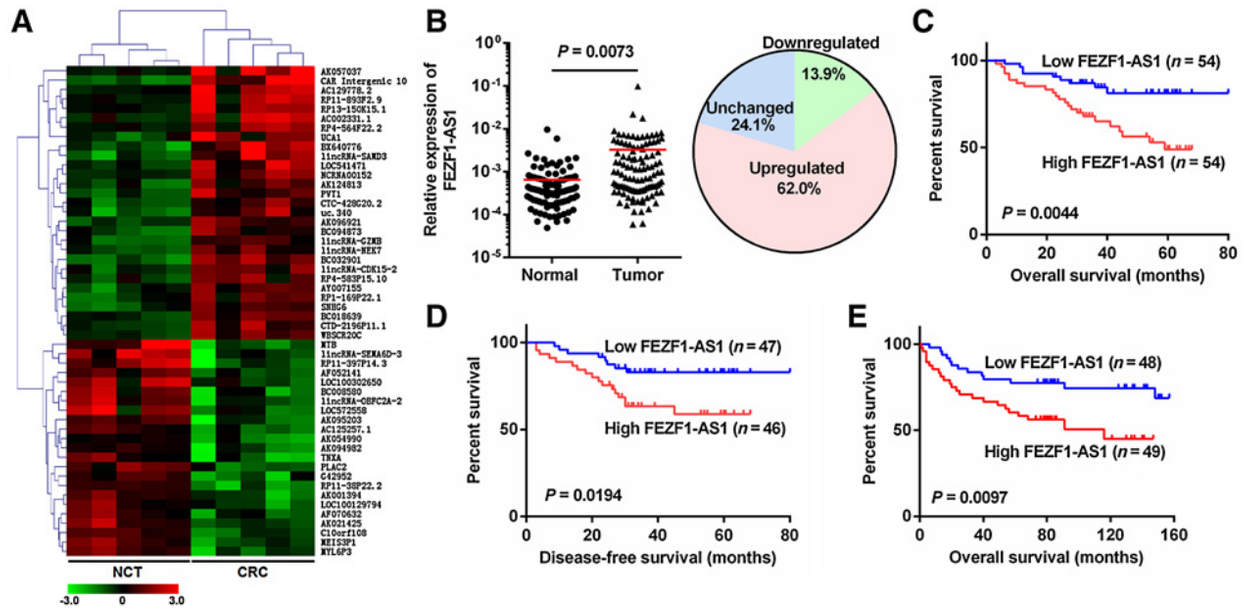

Figure 1.

FEZF1-AS1 is upregulated in colorectal cancer tissues. **A,** Heatmap of 52 dysregulated lncRNAs identified from microarray analysis by unsupervised hierarchical clustering. **B,** Relative expression levels of FEZF1-AS1 in 108 paired colorectal cancer and NCTs were quantified by qRT-PCR. FEZF1-AS1 was upregulated (>2-fold) in 62% (67 of 108) of the colorectal cancer tissues compared with that in the adjacent NCTs. **C** and **D,** Kaplan–Meier survival analysis of patient overall survival (**C**) and disease-free survival (**D**) according to FEZF1-AS1 levels in colorectal cancer tissues. **E,** Kaplan–Meier survival analysis of patient overall survival according to FEZF1-AS1 levels in an independent colorectal cancer cohort.

hierarchical clustering identified 52 lncRNAs that could distinguish colorectal cancers from corresponding NCTs (Fig. 1A, Supplementary Table S4). In addition to CAR Intergenic 10 (CAR10), an oncogenic lncRNA (14), AK057037 (now named as FEZF1-AS1) was the most overexpressed novel lncRNA of the 52 lncRNAs. Thus, we focused on FEZF1-AS1 for further studies. Expression validation in a small colorectal cancer cohort confirmed the upregulation of FEZF1-AS1 in colorectal cancer. Next, we examined FEZF1-AS1 expression in an expanded colorectal cancer cohort, revealing that it was significantly upregulated in colorectal cancer tissues compared with adjacent NCTs ($P < 0.01$) and that 62% (67 of 108) of the colorectal cancer tissues showed more than 2-fold upregulation of FEZF1-AS1 compared with the corresponding NCTs (Fig. 1B). Statistical analysis showed that FEZF1-AS1 expression in colorectal cancer was positively correlated with tumor stage ($P = 0.021$), and no significant association was found between FEZF1-AS1 expression and other parameters (Table 1). Survival analysis showed that high FEZF1-AS1 expression was significantly correlated with poor overall survival (log rank = 8.104, $P = 0.004$, Fig. 1C) and disease-free survival (log rank = 5.467, $P = 0.019$, Fig. 1D). To further evaluate the prognostic effect of FEZF1-AS1, both univariate and multivariate analyses were performed. The results indicated that FEZF1-AS1 expression is an independent prognostic factor for colorectal cancer [HR, 2.240; 95% confidence interval (CI), 1.028–4.878; $P = 0.042$]. In addition, an independent colorectal cancer cohort (Supplementary Table S1) was recruited as a test group, and the results confirmed overexpression of FEZF1-AS1 (Supplementary Fig. S1A–S1B) and its impact on patient survival (log rank = 6.690, $P = 0.0097$, Fig. 1E).

FEZF1-AS1 promotes colorectal cancer growth and metastasis *in vitro* and *in vivo*

The endogenous expression of FEZF1-AS1 was measured in colorectal cancer cell lines, and HCT116/LoVo cells with relatively higher/lower FEZF1-AS1 expression were selected for subsequent functional assays because of their relatively high

Table 1. Correlation of the expression of FEZF1-AS1 in colorectal cancer with clinicopathologic features

Characteristics	FEZF1-AS1 Low	FEZF1-AS1 High	*P*
Ages, y			
<60	34	26	0.120
≥60	20	28	
Gender			
Male	30	32	0.695
Female	24	22	
Tumor size (cm)			
<5	37	28	0.079
≥5	17	26	
Location			
Colon	27	31	0.441
Rectum	27	23	
Differentiation			
Well and moderately	48	42	0.128
Poorly	6	12	
Depth of tumor			
T1 + T2	12	8	0.461
T3	11	13	
T4	31	33	
Tumor stage			
I + II	28	16	0.021
III	22	30	
IV	4	8	

cell motility and transfection efficiencies (Supplementary Fig. S2A–S2C). CCK-8 and colony formation assays demonstrated that FEZF1-AS1 knockdown significantly inhibited colorectal cancer cell proliferation and colony formation, whereas ectopic FEZF1-AS1 expression promoted cell proliferation and colony formation (Fig. 2A and B). Moreover, silencing FEZF1-AS1 expression decreased the number of cells in S phase and increased cell apoptosis, whereas ectopic FEZF1-AS1 expression promoted G_1–S cell-cycle progression and inhibited colorectal cancer apoptosis levels compared with control cells (Fig. 2C–D and Supplementary Fig. S2D–S2E). Furthermore, silencing FEZF1-AS1 inhibited colorectal cancer tumorigenicity, whereas FEZF1-AS1 overexpression promoted tumorigenicity in mice (Fig. 2E). Collectively, these data show the growth-promoting functions of FEZF1-AS1 in colorectal cancer.

We further explored the effects of FEZF1-AS1 on colorectal cancer metastasis. Transwell assays showed that FEZF1-AS1 knockdown inhibited the migration and invasion of HCT116 and LoVo cells, whereas FEZF1-AS1 overexpression significantly promoted their migration and invasion (Fig. 2F and Supplementary Fig. S2F). In addition, we assessed the impact of FEZF1-AS1 on metastasis *in vivo* using two metastasis models, a lung metastasis mouse model and an orthotopic hepatic metastasis mouse model. These results revealed that FEZF1-AS1 overexpression significantly promote colorectal cancer pulmonary and hepatic metastasis (Fig. 2G and H).

FEZF1-AS1 is associated with PKM2

To elucidate the potential molecular mechanisms of FEZF1-AS1 in colorectal cancer, we measured gene expression profiling in FEZF1-AS1–silenced HCT116 cells. Hierarchical clustering revealed significantly altered expressions of 1,261 genes (fold change >1.5) in FEZF1-AS1–silenced HCT116 cells compared with the control cells (Supplementary Fig. S3A). Of the altered genes, enriched genes ($P < 0.01$) belonged to several key signal transduction pathways, including the JAK-STAT and HIF-1 pathways (Supplementary Fig. S3B) that have been proven to be closely related to tumorigenesis and progression (15, 16). Several key genes in these pathways were selected for further validation using qRT-PCR, and the results confirmed the expression profile analysis (Supplementary Fig. S3C). These data suggest that FEZF1-AS1 has extensive regulatory functions in colorectal cancer tumorigenesis and progression.

To further identify targets directly regulated by FEZF1-AS1, we pulled down FEZF1-AS1–binding proteins using an RNA pull-down assay. The retrieved proteins were subjected to SDS-PAGE electrophoresis analysis, and several additional differential bands were selected for a mass spectrum analysis (Fig. 3A). On the basis of the functional annotation of proteins predicted by mass spectrum analysis and the potential pathways regulated by FEZF1-AS1 (Supplementary Fig. S3B), PKM2, which could activate STAT3 signaling by acting as a protein kinase (17–20), was deemed an FEZF1-AS1–associated protein. Western blotting further confirmed the binding of FEZF1-AS1 to PKM2 using the retrieved proteins in the RNA pull-down assay (Fig. 3B). RIP assays performed with an anti-PKM2 antibody also demonstrated the association between PKM2 and FEZF1-AS1 (Fig. 3C). Taken together, these data suggest that FEZF1-AS1 physically associates with PKM2.

To identify which region of FEZF1-AS1 binds to PKM2, we constructed a series of FEZF1-AS1 deletion mutants based on the predicted secondary structure of FEZF1-AS1 in the LNCipedia database (http://www.lncipedia.org/; Fig. 3D). RNA fragments were *in vitro* transcribed from these deletion mutant constructs and used for RNA pull-down assays. Immunoblotting analysis of PKM2 in protein samples pulled down by these different FEZF1-AS1 constructs showed that RNA fragments with the 1,200 to 1,800 deletion nearly completely lost their ability to bind PKM2, suggesting that the region is essential for FEZF1-AS1 binding to PKM2. A potential PKM2-binding region of FEZF1-AS1 (1,400–1,550) was also predicted in our identified location using catRAPI omics (http://s.tartaglialab.com/page/catrapid_omics_group; Supplementary Fig. S4). To further investigate which domain of PKM2 accounted for its interaction with FEZF1-AS1, we performed RIP assays using a series of HA-tagged PKM2 deletion mutants (21). As Fig. 3E shows, the A2 domain (219–390) of PKM2 is essential for the binding of PKM2 to FEZF1-AS1.

FEZF1-AS1 increases the stability of PKM2 protein

To determine whether FEZF1-AS1 regulates PKM2 activity, we first investigated the subcellular localization of FEZF1-AS1 by qRT-PCR as previously described (7). FEZF1-AS1 is localized in both the cytoplasm and nucleus of HCT116 cells (Supplementary Fig. S5A), suggesting its complicated regulatory mechanisms in colorectal cancer. Considering this, we measured the expression and subcellular localization of PKM2 in FEZF1-AS1–depleted or FEZF1-AS1–overexpressing colorectal cancer cells. No significant changes in the mRNA levels of PKM2 were observed in FEZF1-AS1–depleted HCT116 cells or FEZF1-AS1–overexpressing LoVo cells compared with those in their corresponding controls (Supplementary Fig. S5B). However, both the total and nuclear PKM2 levels were significantly decreased in FEZF1-AS1–silenced HCT116 cells and increased in FEZF1-AS1–overexpressing LoVo cells (Fig. 3F and Supplementary Fig. S5C), suggesting that FEZF1-AS1 can increase the levels of PKM2 protein at the post-transcriptional level. To further validate this observation, we used the protein synthesis inhibitor cycloheximide (CHX) to evaluate the effect of FEZF1-AS1 on the degradation of PKM2. FEZF1-AS1 overexpression in LoVo cells prolonged the half-life of PKM2 (Fig. 3G). Moreover, we observed that inhibiting proteasome activity prevented si-FEZF1-AS1–induced endogenous PKM2 downregulation in HCT116 cells, suggesting that PKM2 degradation via the ubiquitin-proteasome pathway is inhibited by FEZF1-AS1 (Fig. 3H). To confirm these data, we evaluated the effect of FEZF1-AS1 on PKM2 ubiquitination in colorectal cancer cells. Ectopic FEZF1-AS1 expression could increase the stability of PKM2 via reducing its ubiquitous modification (Fig. 3I), whereas the deletion mutant (1,200-1,800) of FEZF1-AS1 (FEZF1-AS1Δ600) failed to inhibit the ubiquitination of PKM2 (Supplementary Fig. S6A). Together, these results demonstrate that FEZF1-AS1 increases the levels of PKM2 by binding and increasing its stability.

FEZF1-AS1 exerts tumor-promoting functions in colorectal cancer by regulating PKM2

To investigate whether FEZF1-AS1 exerts tumor-promoting functions in colorectal cancer by modulating PKM2, we first examined the effect of PKM2 on FEZF1-AS1–induced cell proliferation, and observed that PKM2 overexpression could mimic the tumor-promoting effects of FEZF1-AS1, whereas

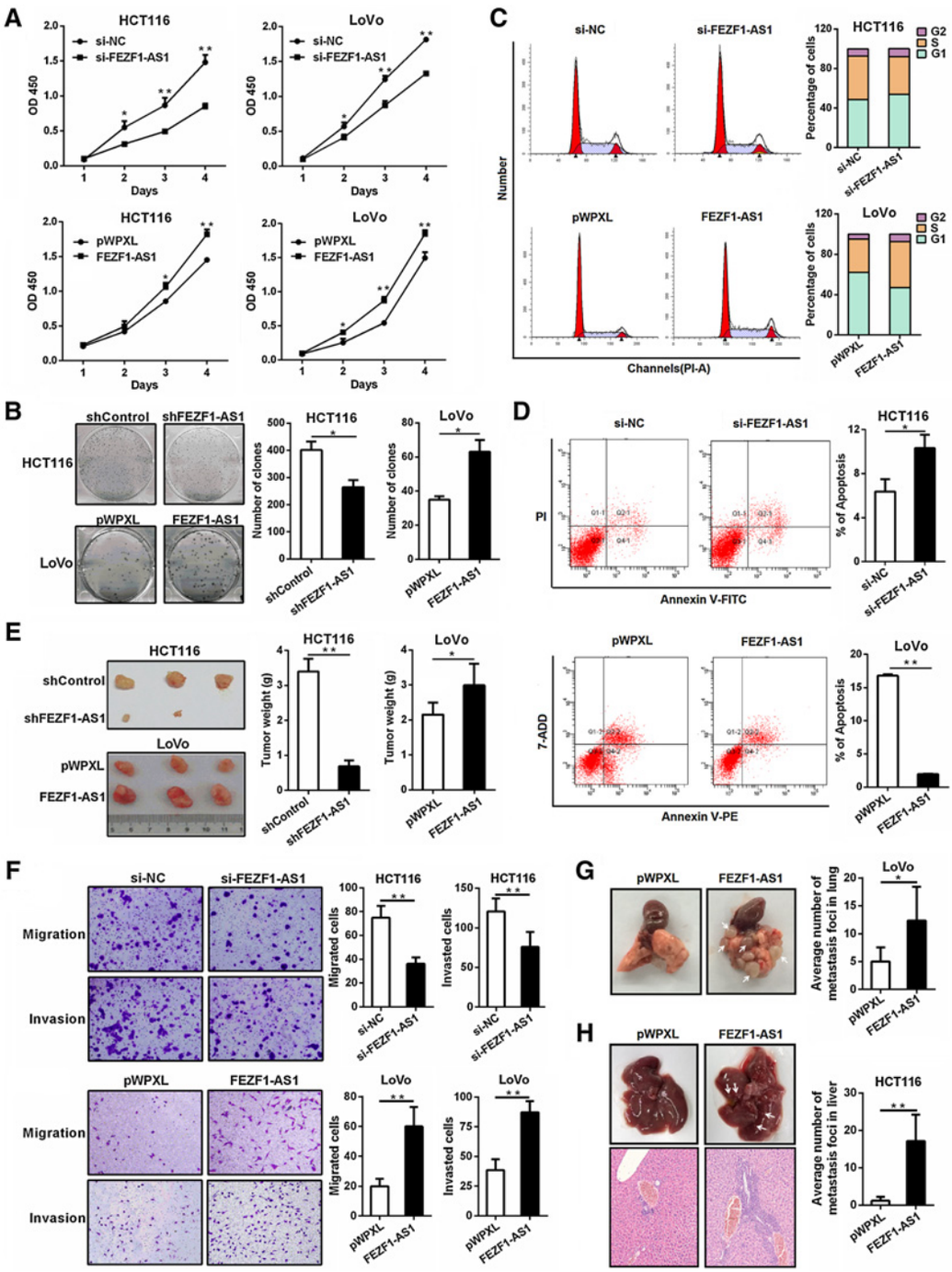

Figure 2.

FEZF1-AS1 promotes colorectal cancer cell proliferation and metastasis *in vitro* and *in vivo*. **A,** Effects of FEZF1-AS1 on colorectal cancer cell proliferation were measured by CCK-8 assays. **B,** Effects of FEZF1-AS1 on colony formation in HCT116 and LoVo cells. **C** and **D,** FACS analysis of the effects of FEZF1-AS1 on cell cycle and apoptosis in HCT116 and LoVo cells. **E,** Effect of FEZF1-AS1 on tumor formation in a nude mouse xenograft model. Representative images of tumors from the FEZF1-AS1 (shFEZF1-AS1) and control groups ($n = 6$ for each group). **F,** Representative images and bar graphs depicting the migration and invasion abilities of FEZF1-AS1–silenced HCT116 cells or FEZF1-AS1 overexpressing LoVo cells. **G,** Effect of FEZF1-AS1 on tumor metastasis in a lung metastasis mouse model. LoVo cells stably expressing FEZF1-AS1 or the control (2×10^6) were injected into the caudal vein of nude mice ($n = 10$ for each group). The white arrows show the metastases. Overexpression of FEZF1-AS1 significantly increased the number of lung metastases. **H,** Effect of FEZF1-AS1 on colorectal cancer hepatic metastasis was evaluated using an orthotopic mouse model. A total of 2×10^6 HCT116 cells stably expressing FEZF1-AS1 were injected into the submucosal tissue of cecum of nude mice ($n = 5$ for each group). Representative liver tissues and their H&E-stained sections (×100 magnification) are shown in the left. The number of metastatic foci in liver tissues is quantified in the right. The white arrows show the metastases; *, $P < 0.05$; **, $P < 0.01$.

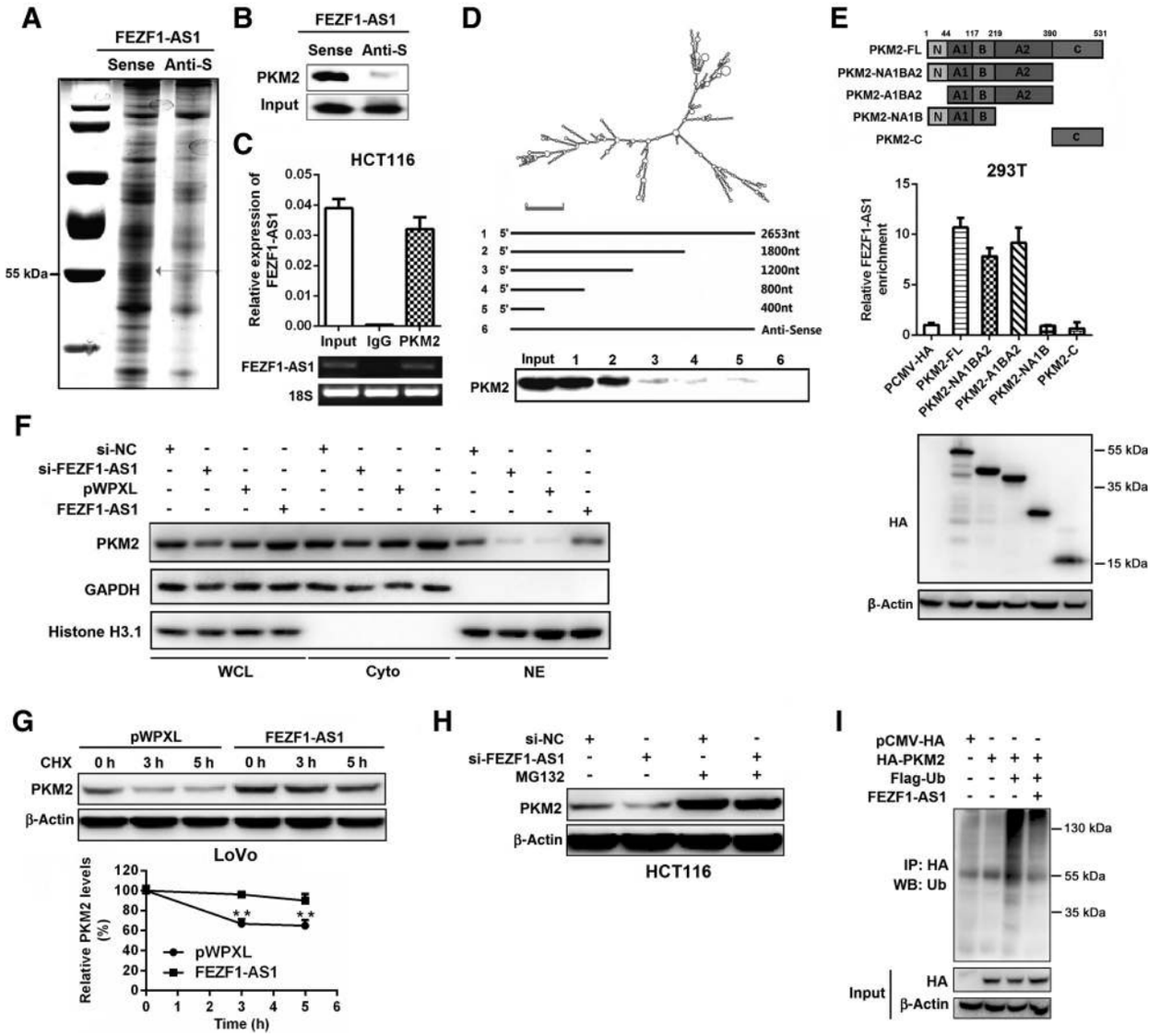

Figure 3.

FEZF1-AS1 increases PKM2 protein levels by binding to the PKM2 protein and enhancing its stability. **A**, Proteins retrieved from the FEZF1-AS1 pull-down assay were analyzed by SDS-PAGE. **B**, Western blot analysis of the proteins retrieved from the FEZF1-AS1 pull-down assay using an anti-PKM2 antibody. **C,** RIP assays using an anti-PKM2 antibody showed that PKM2 interacts with FEZF1-AS1 in HCT116 cells. The qRT-PCR results of RIP assays are shown in the top. The results of agarose electrophoresis of the PCR products are shown in the bottom. **D,** Immunoblot detection of the PKM2 protein in HCT116 cells as retrieved by *in vitro* transcribed biotinylated RNAs of different constructs of FEZF1-AS1 or its antisense sequence (negative control). **E,** RIP assays were performed using an anti-HA antibody in 293T cells transfected with HA-tagged pCMV-PKM2 or its deletion mutants. QRT-PCR was used to measure the enrichment of FEZF1-AS1. Western blot was used to evaluate the expression of HA-tagged pCMV-PKM2 or its deletion mutants. **F,** The subcellular localization of PKM2 in colorectal cancer cells. PKM2 protein levels were analyzed by immunoblot of the whole-cell lysates (WCL), cytoplasm extracts (Cyto), and nuclear extracts (NE) of HCT116 (LoVo) cells transfected with si-FEZF1-AS1 (FEZF1-AS1) or its control. GAPDH was used as a loading control for WCL/Cyto and a quality control for NE. **G,** The protein levels of PKM2 were measured in FEZF1-AS1–overexpressing LoVo cells by Western blot. Cells were treated with CHX (50 μg/mL) for 3 or 5 hours before harvest. **H,** The protein levels of PKM2 were checked in FEZF1-AS1–depleted HCT116 cells by Western blot. Cells were treated with MG132 (20 μmol/L) for 3 hours before harvest. **I,** Ectopic FEZF1-AS1 expression inhibited the ubiquitous modification of PKM2. 293T cells were transfected with HA-PKM2, Flag-Ub, or FEZF1-AS1 plasmid and treated with MG132 (20 μmol/L) for 3 hours. The ubiquitinylated PKM2 was measured by Western blot using an anti-Ub antibody following the immunoprecipitation of HA-PKM2 with an anti-HA antibody. **, $P < 0.01$.

PKM2 knockdown blocked the FEZF1-AS1–induced cell growth in colorectal cancer cells (Fig. 4A and B). In addition, the increased cell mobility in FEZF1-AS1–overexpressing colorectal cancer cells was reversed by PKM2 knockdown, and the decreased cell mobility in FEZF1-AS1–silenced colorectal cancer cells was partially restored by PKM2 overexpression (Fig. 4C and D). We then evaluate the functional importance of potential regions accounting for the binding between

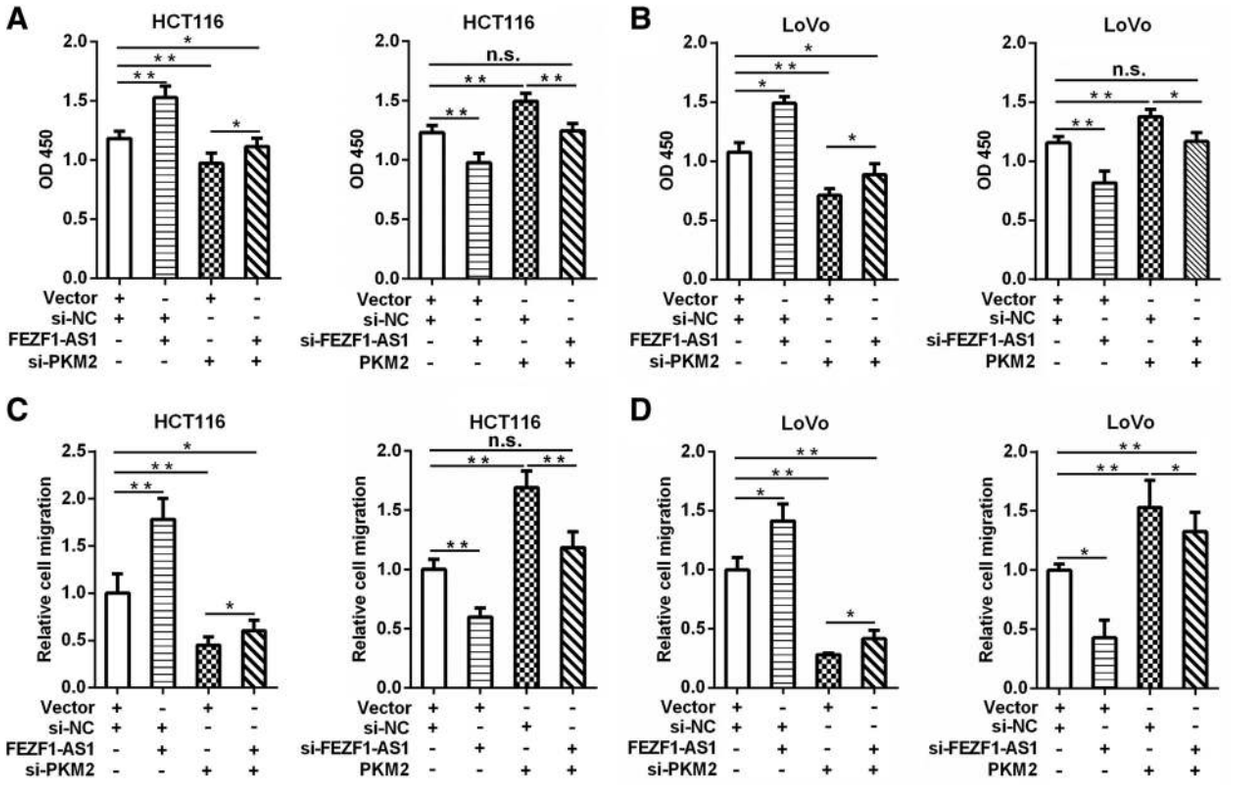

Figure 4.

PKM2 mediates the tumor-promoting functions of FEZF1-AS1 in colorectal cancer cells. **A** and **B,** Increased cell viability in FEZF1-AS1–overexpressing colorectal cancer cells was abolished by PKM2 knockdown, whereas the decrease in cell viability in FEZF1-AS1–silenced colorectal cancer cells was restored by PKM2 overexpression. The cell viability was measured by CCK-8 assays. **C** and **D,** PKM2 knockdown partially blocked FEZF1-AS1–induced cell migration, whereas ectopic PKM2 expression rescued the decreased cell motility in FEZF1-AS1–silenced colorectal cancer cells. *, $P < 0.05$; **, $P < 0.01$; n.s., not significant.

FEZF1-AS1 and PKM2 in colorectal cancer cells using the FEZF1-AS1Δ600 mutant and the PKM2 mutant lacking A2 domain (PKM2ΔA2). The FEZF1-AS1 mutant did not restore the cell growth inhibited by PKM2 knockdown in colorectal cancer cells (Supplementary Fig. S6B), and the PKM2 mutant did not rescue cell growth in FEZF1-AS1–depleted colorectal cancer cells (Supplementary Fig. S6C–S6D), suggesting that the 1,200 to 1,800 region of FEZF1-AS1 and the A2 domain of PKM2 are essential for their oncogenic roles. Taken together, these data demonstrate that FEZF1-AS1 exerts tumor-promoting functions in colorectal cancer, at least partially, by binding and promoting PKM2 activity.

PKM2 is upregulated in colorectal cancer and positively correlates with FEZF1-AS1 expression

To further establish the correlation between FEZF1-AS1 and PKM2 in colorectal cancer tissues, we first evaluated the protein expression of PKM2 in 138 paired colorectal cancer and NCT samples using IHC (Fig. 5A). The IHC staining results revealed that more than half of colorectal cancers (55.1%, 76/138) showed increased PKM2 expression compared with their paired NCTs (Fig. 5B). The PKM2 protein levels in colorectal cancer tissues were positively correlated with the FEZF1-AS1 levels ($r = 0.382$, $P < 0.001$, Fig. 5C), confirming the positive regulation of PKM2 by FEZF1-AS1 in clinical colorectal cancer tissues. Meanwhile, the positive relationship between FEZF1-AS1 and PKM2 levels was also observed in colorectal cancer xenografts in nude mice (Supplementary Fig. S7).

Higher PKM2 expression was significantly associated with lymphatic invasion ($P = 0.014$) and advanced tumor stage ($P = 0.031$). Survival analysis further revealed that PKM2 expression was associated with poor prognosis in colorectal cancer (log rank = 13.9, $P = 0.0002$, Fig. 5D). After adjusting for age, gender, tumor size, location, differentiation, and TNM stage, multivariate analysis showed that PKM2 expression was an independent risk factor for the survival of patients with colorectal cancer (HR, 2.236; 95% CI, 1.190–4.201; $P = 0.012$). Given these results, we conclude that FEZF1-AS1 can regulate the *in vivo* activity of PKM2 in colorectal cancer.

FEZF1-AS1 activates the PKM2/STAT3 pathway

Nuclear PKM2 was increased significantly in FEZF1-AS1–overexpressing colorectal cancer cells (Fig. 3F), and expression profile analysis showed that the STAT3 pathway was potentially modulated by FEZF1-AS1 (Supplementary Fig. S3B), suggesting that the tumor-promoting functions of FEZF1-AS1 are mainly mediated by the protein kinase activity of PKM2. To determine whether FEZF1-AS1 could activate STAT3 signaling by

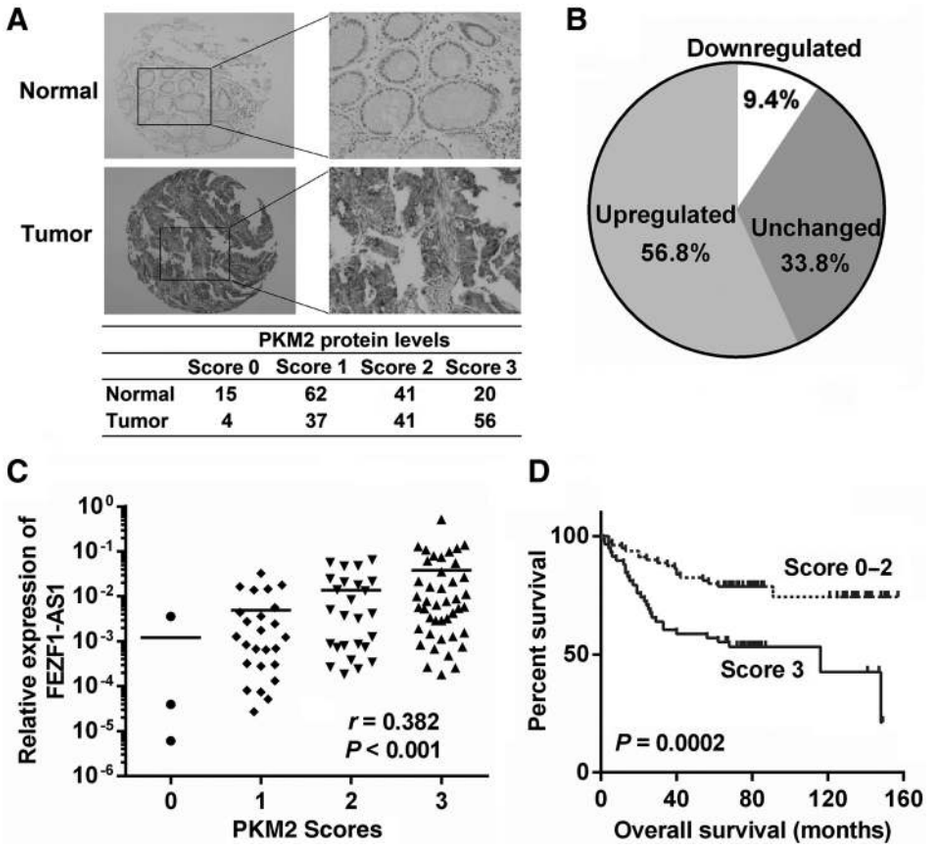

Figure 5.
PKM2 protein levels are overexpressed in colorectal cancer and positively correlate with FEZF1-AS1 levels. **A,** Immunohistochemical staining of PKM2 in 138 paired colorectal cancer tissues and adjacent NCTs. **B,** PKM2 protein expression was frequently increased in colorectal cancer tissues compared with that in matched NCTs. **C,** Expression levels of PKM2 were positively correlated with FEZF1-AS1 expression levels in colorectal cancer tissues ($n = 97$). **D,** Kaplan–Meier survival analysis according to PKM2 expression levels in colorectal cancer tissues.

regulating nuclear PKM2 activity, we evaluated the status of the STAT3 pathway in colorectal cancer cells with different FEZF1-AS1 and PKM2 expression levels using luciferase assays. STAT3 signaling was activated in FEZF1-AS1 or PKM2-overexpressing colorectal cancer cells, and silencing PKM2 expression blocked FEZF1-AS1–induced STAT3 activation. In contrast, FEZF1-AS1 knockdown inhibited STAT3 activation, which was rescued by PKM2 overexpression (Fig. 6A). Western blot results further showed that ectopic FEZF1-AS1 expression increased the phosphorylation of STAT3 (Tyr705), which was blocked by PKM2 knockdown (Fig. 6B). On the contrary, FEZF1-AS1 knockdown inhibited STAT3 phosphorylation, which was rescued by the overexpression of PKM2 (Fig. 6B). Consistent with these results, downstream targets of the STAT3 pathway (*MCL1*, *BIRC5*, *CCND1*, *BCL2L1*, *CDH1*, *MMP2*, *and MMP9*) were also significantly upregulated in FEZF1-AS1–overexpressing colorectal cancer cells, and their expression was partially inhibited by PKM2 knockdown (Fig. 6C). Collectively, these data demonstrate that FEZF1-AS1 activates the STAT3 pathway via PKM2.

FEZF1-AS1 prompts aerobic glycolysis via PKM2

PKM2 tetramer, the active form of PKM2 in cytoplasm, has high pyruvate kinase activity that is a rate-limiting factor for aerobic glycolysis. Cytoplasmic PKM2 was also regulated by FEZF1-AS1 (Fig. 3F and Supplementary Fig. S5C), suggesting that FEZF1-AS1 may also regulate aerobic glycolysis. To prove this, we analyzed whether FEZF1-AS1 influence the pyruvate kinase activity of PKM2. FEZF1-AS1 overexpression increased the pyruvate kinase activity and lactate production, which was significantly impaired by PKM2 knockdown in colorectal cancer cells (Fig. 6D and E and Supplementary Fig. S8A). In comparison, FEZF1-AS1 knockdown inhibited the pyruvate kinase activity and lactate production in colorectal cancer cells, which was rescued by PKM2 overexpression (Fig. 6D and E and Supplementary Fig. S8B). The FEZF1-AS1Δ600 mutant failed to increase the pyruvate kinase activity and lactate production like the full-length FEZF1-AS1 (Supplementary Fig. S9A–S9B). The PKM2ΔA2 mutant also could not mediate the effects of FEZF1-AS1 on the pyruvate kinase activity and lactate production (Supplementary Fig. S9C–S9D). Together, these data suggest that, in addition to its main regulatory role on the protein kinase activity of PKM2, FEZF1-AS1 may also promote aerobic glycolysis by increasing the pyruvate kinase activity of PKM2.

Discussion

LncRNAs can regulate gene expression via various aspects, including chromatin modification, transcriptional and post-transcriptional processing. Many studies have revealed the extensive regulatory functions of lncRNA in tumorigenesis and progression. In this study, we identified a series of differentially expressed lncRNAs in colorectal cancer, including CAR10, UCA1, LINC00152, and PVT1, that have been identified as potential oncogenes by us (7, 8, 22) and other groups (9, 14, 23, 24). Of these lncRNAs, FEZF1-AS1, a top overexpressed lncRNA in colorectal cancer, is a newly identified oncogenic lncRNA in human digest tract cancers (25–27). However, the

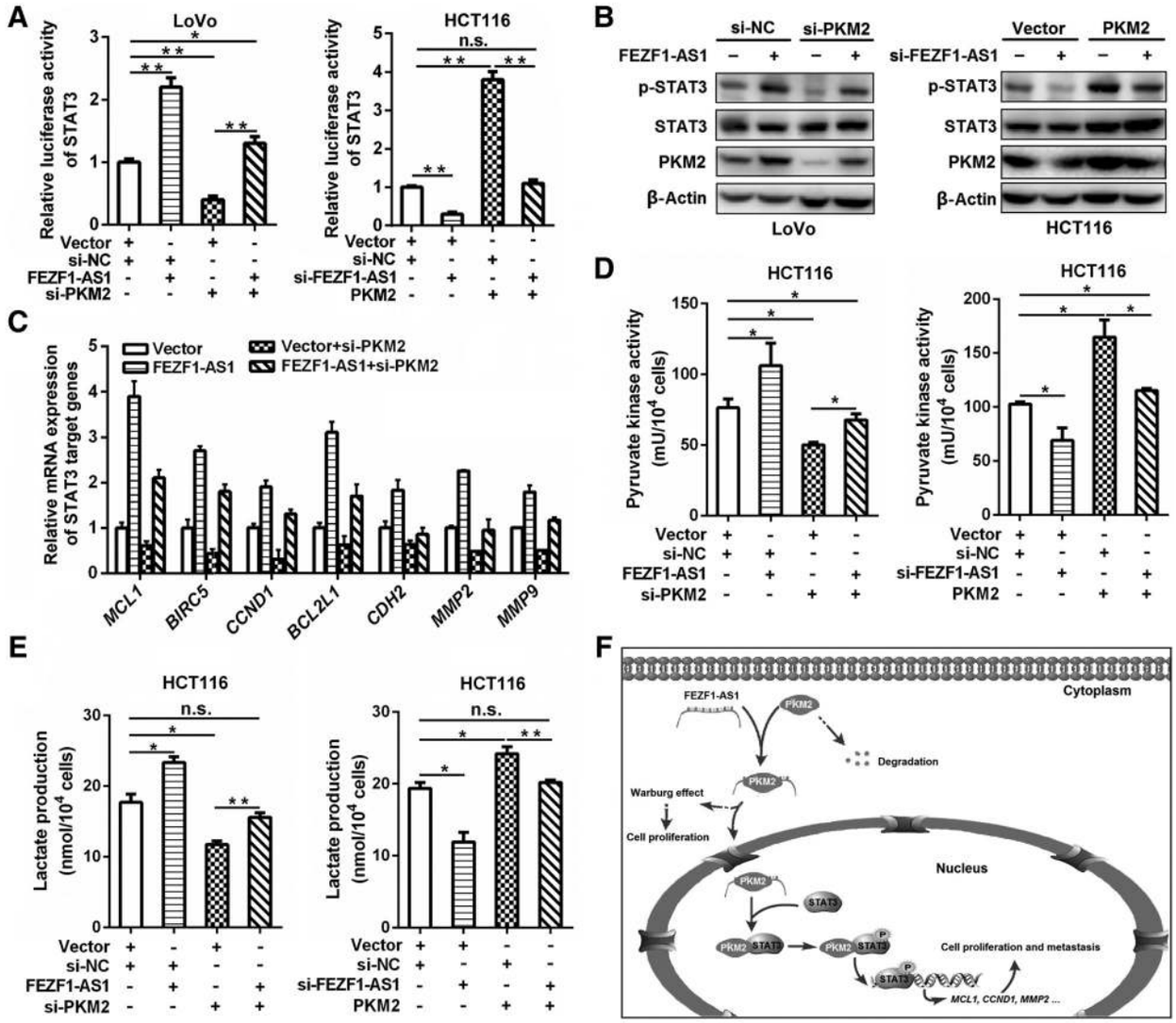

Figure 6.

FEZF1-AS1 activates STAT3 signaling and promotes glycolysis via PKM2. **A,** FEZF1-AS1 activated the STAT3 pathway via PKM2. A luciferase assay was performed to assess the effects of FEZF1-AS1 and PKM2 on the STAT3 pathway. **B,** FEZF1-AS1 induced STAT3 phosphorylation (Tyr 705) in colorectal cancer cells via PKM2. **C,** FEZF1-AS1 activated the expression of downstream targets of the STAT3 pathway via PKM2. The mRNA levels of these genes were measured by qRT-PCR. **D** and **E,** FEZF1-AS1 enhanced the pyruvate kinase activity (**D**) and lactate production (**E**) by regulating PKM2 in HCT116 cells. **F,** Schema illustrating the mechanism by which FEZF1-AS1 regulates STAT3 signaling and the Warburg effect (aerobic glycolysis) by increasing PKM2 activity in colorectal cancer cells. *, $P < 0.05$; **, $P < 0.01$; n.s., not significant.

detailed role and molecular mechanisms of FEZF1-AS1 in colorectal cancer remain to be clarified.

We showed that FEZF1-AS1 was upregulated in colorectal cancer tissues, and predicted poor prognosis, which was consistent with the previous reports (25, 26). Functional experiments showed that FEZF1-AS1 significantly promoted colorectal cancer tumorigenesis and metastasis *in vitro* and *in vivo*. Chen and colleagues (25) also observed similar effects of FEZF1-AS1 on cell proliferation and migration in colorectal cancer. Interestingly, they reported that FEZF1-AS1 does not regulate apoptosis in colorectal cancer cells. However, we observed that FEZF1-AS1 inhibited the apoptosis of colorectal cancer cells, which was consistent with the observations in other tumors (26, 28). The discrepant results may have been due to the different cell lines and methodologies used in the analyses.

FEZF1-AS1 is located on the opposite strand of FEZF1 on chromosome 7. FEZF1-AS1 acts as an oncogene in gastric cancer by suppressing p21 expression or activating Wnt signaling pathway (26, 29). The oncogenic roles of FEZF1-AS1 were also observed in lung cancer (30) and colorectal cancer (25). Although Chen and colleagues (25) suggested that FEZF1-AS1 may exert tumor-promoting effects by increasing the expression of FEZF1 in colorectal cancer, they did not uncover how FEZF1-AS1 increases FEZF1 activity. FEZF1 is a highly conserved transcriptional repressor that regulates nervous system development (31). The role of FEZF1 in human cancers is largely unclear (32). We also observed

the potential positive regulation of FEZF1-AS1 on FEZF1 and the upregulation of FEZF1 in colorectal cancer (Supplementary Fig. S10A–S10B); however, we noticed that the expression levels of FEZF1 in colorectal cancer are relative low (Supplementary Fig. S10C–S10D). Consequently, we speculated that other mechanisms mediate the functions of FEZF1-AS1 in colorectal cancer.

Gene expression profiling analysis showed that FEZF1-AS1 potentially regulates the JAK-STAT pathway. STATs, especially STAT3, are well known for their regulatory effects on cell proliferation, apoptosis, and metastasis in human cancers (33). By experimental screening and validation, PKM2, a regulator of STAT3 signaling in colorectal cancer (18–20), was identified as a key downstream target of FEZF1-AS1. PKM2 could function as a protein kinase in the nucleus and regulate many tumor-associated genes and pathways, including HIF-1, Oct-4, cyclin D1 and beta-catenin (17, 34, 35). Further assays revealed that FEZF1-AS1 promotes colorectal cancer development and progression via binding PKM2 and increasing its stability, identifying PKM2 as a novel executor of FEZF1-AS1 in colorectal cancer. Although the 1,200-1,800 bp region of FEZF1-AS1 was determined to bind with the A2 domain of PKM2, the precise PKM2-binding site of FEZF1-AS1 requires further research.

In addition to its central role in metabolic reprogramming, PKM2 could also function as a multifunctional signaling molecule to promote cancer proliferation and progression (35). PKM2 exists in both a dimeric and a tetrameric form. Unlike the PKM2 tetramer (the active form of PKM2 in the cytoplasm), which has high glycolytic activity, the PKM2 dimer (the active form of PKM2 in the nucleus) functions as a protein kinase and promotes tumorigenesis and progression by regulating gene expression (36). Our results revealed that FEZF1-AS1 increased both total and nuclear PKM2 activity and consequently activate STAT3, which is consistent with previous reports on the tumor-promoting role of nuclear PKM2 (18, 19). In addition, cytoplasmic PKM2 was also moderately increased in FEZF1-AS1–overexpressing colorectal cancer cells and led to enhanced aerobic glycolysis, suggesting that FEZF1-AS1 promotes colorectal cancer tumorigenesis and progression by regulating both the protein kinase and pyruvate kinase activities of PKM2 (Fig. 6F). PKM2 expression is transcriptionally or post-transcriptionally controlled by many factors, including Sp1, Sp3, HIF-1, MYC, miR-326, and miR-130 (34, 37–39). PKM2 activity is also modulated by post-translational mechanisms, including phosphorylation, sumoylation, acetylation, and prolyl hydroxylation (35). In this study, we revealed a novel lncRNA-mediated regulatory mechanism of PKM2 activity, indicating that regulating PKM2 activity is elaborate and complicated. Whether or how FEZF1-AS1 regulates the sub-cellular location of PKM2 requires further investigation, and additional efforts should be made to identify whether "cross-talk" exist among these regulatory mechanisms.

Microsatellite instability (MSI) and chromosome instability (CIN) are key genetic mechanisms mediating colorectal cancer tumorigenesis and have important application in molecular sub-typing and immunotherapy in colorectal cancer. We analyzed the relationship between FEZF1-AS1 and the MSI status in colorectal cancer using the data from The Cancer Genome Atlas (TCGA) database, and no significant association was observed (Supplementary Fig. S11). However, whether FEZF1-AS1 is associated with CIN remains unknown, which requires further investigation.

In summary, the work presented here shows that FEZF1-AS1 is upregulated in colorectal cancer and associated with poor survival. FEZF1-AS1 increases colorectal cancer cell proliferation and metastasis by regulating PKM2/STAT3 signaling and glycolysis, providing the first evidence of a FEZF1-AS1/PKM2 network in colorectal cancer (Fig. 6F). These data suggest that FEZF1-AS1/PKM2 signaling may be a promising therapeutic target in colorectal cancer treatment. However, further studies should be performed to identify the precise sites mediating the association between FEZF1-AS1 and PKM2, which is the key to develop specific inhibitors targeting FEZF1-AS1/PKM2 signaling.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Z. Bian, S. Huang, J. Zou, Z. Huang
Development of methodology: Z. Bian, J. Zhang, M. Li, Y. Feng
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Bian, M. Li, Y. Feng, J. Zhang, S. Yao, Y. Yin, B. Fei, J. Zou, Z. Huang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Bian, X. Wang, Z. Huang
Writing, review, and/or revision of the manuscript: Z. Bian, J. Zou, Z. Huang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Bian, X. Wang, G. Jin, J. Du, W. Han, B. Fei, J. Zou, Z. Huang
Study supervision: J. Zou, Z. Huang

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