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ORIGINAL ARTICLE

Metabolic stress-induced reciprocal loop of long noncoding RNA ZFAS1 and ZEB1 promotes epithelial–mesenchymal transition and metastasis of pancreatic cancer cells

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Abstract

Pancreatic cancer (PC) development faces significant metabolic stress due to metabolic reprogramming and a distinct hypovascular nature, often leading to glucose and glutamine depletion. However, the adaption mechanisms by which PC adapts to these metabolic challenges have not yet been completely explored. Here, we found that metabolic stress induced by glucose and glutamine deprivation led to an overexpression of ZNFX1 antisense RNA 1 (ZFAS1). This overexpression played a significant role in instigating PC cell epithelial–mesenchymal transition (EMT) and metastasis. Mechanistically, ZFAS1 enhanced the interaction between AMPK, a key kinase, and ZEB1, the primary regulator of EMT. This interaction resulted in the phosphorylation and subsequent stabilization of ZEB1. Interestingly, ZEB1 also reciprocally influenced the transcription of ZFAS1 by binding to its promoter. Furthermore, when ZFAS1 was depleted, the nutrient deprivation-induced EMT of PC cells and lung metastasis in nude mice were significantly inhibited. Our investigations also revealed that ZFAS1-rich exosomes released from cells suffering glucose and glutamine deprivation promoted the EMT and metastasis of recipient PC cells. Corroborating these findings, a correlated upregulation of ZFAS1 and ZEB1 expression was observed in PC tissues and was associated with a poor overall survival rate for patients. Our findings highlight the involvement of a long noncoding RNA-driven metabolic adaptation in promoting EMT and metastasis of PC, suggesting ZFAS1 as a promising novel therapeutic target for PC metabolic treatment.

KEYWORDS
EMT, exosome, lncRNA, metabolic stress, metastasis, pancreatic cancer

Abbreviations: AMPK, AMP-activated protein kinase; C.C, compound C; CM, condition medium; EMTs, epithelial–mesenchymal transition; GLS, glutaminase; GLS-AS: glutaminase-antisense; IHC, immunohistochemistry; lncRNA, long noncoding RNA; MUT, mutant type; PC, pancreatic cancer; RIP, RNA binding protein immunoprecipitation; RT-qPCR, quantitative RT-PCR; ZEB1, zinc finger E-box binding homeobox 1; ZFAS1, ZNFX1 antisense RNA 1; ZNFX1, zinc finger NFX1-Type containing 1.

Wenfeng Zhuo, Zhu Zeng, Yuhang Hu, and Ping Hu contributed equally to this work as first authors.

1 | INTRODUCTION

Glucose and glutamine are among the most plentiful nutrients found in circulation, serving as sources of energy, providers of reductive equivalents, and precursors for biosynthesis.[1,2] Research revealed that glucose and glutamine were significantly reduced in the tumor core compared to surrounding tissues.[3,4] Specifically, PC is a highly fibrotic tumor with a low degree of vascularization, and its nutrient level differs from that in plasma.[5] A metabolomics study with mass spectrometry found that poor tumor perfusion contributed to limited nutrients, including glucose and glutamine, in samples of PC patients compared to nearby benign tissues.[6] Cancer cells leverage alterations of oncogenes to participate in the metabolic stress adaptation response, thereby obtaining the energy and biosynthetic precursors needed for cell division to survive under such stress conditions.[7–9] Evidence indicated that metabolic stress induced by glucose and glutamine deficiency initiated EMT in various cancer cells. Glucose deprivation was reported to promote EMT and invasion of hepatic cancer cells under hypoxic conditions.[10] Research also showed that glutamine depletion promoted EMT and invasion of PC cells by upregulating the expression of EMT regulator Slug.[11] Therefore, further identifying the molecular basis of the stress-adaptation pathway under metabolic stress could provide insight into the specific vulnerability of PC cells.

Expanding evidence has revealed that lncRNAs are involved in remodeling tumor cells under nutritional stress. Our previous study discovered glutamine depletion inhibited GLS-AS expression, which consequently increased GLS expression and further promoted survival and migration of PC cells.[12] Results from Chen et al.[13] also revealed that lncRNA TP53TG1 promoted glioma cell proliferation and migration by regulating the expression of glucose metabolism-related genes under glucose deprivation. Accordingly, we sought to determine whether specific lncRNA contributes to PC's adaptive EMT and metastasis under glucose and glutamine deprivation.

In our study, we observed the morphological characteristics of EMT and the capacity of migration and invasion of PC cells during glucose- and glutamine-deficiency conditions. Furthermore, the lncRNA microarray was adopted to identify the coordinately upregulated lncRNA that was the key regulator for glucose and glutamine deficiency-induced EMT of PC cells. Moreover, we undertook RIP and RNA pull-down assays to determine the mechanism for the lncRNA-mediated EMT of PC cells during glucose and glutamine deprivation.

2 | MATERIALS AND METHODS

2.1 | Patients and tumor specimens

Pancreatic cancer and corresponding peritumor tissues of 60 surgical resections of patients without preoperative treatment were provided by the Institute of Pancreatic Diseases of Union Hospital from 2013 to 2017. All patients signed informed consent. The tissues were delivered to our laboratory within 20 min, then either formalin-fixed and paraffin-embedded or snap-frozen as described previously.[14]

2.2 | Statistical analysis

Each experiment was carried out a minimum of three times. The software tools SPSS 22.0 (SPSS Inc.) and GraphPad Prism version 8.0.0 (GraphPad Software) were used for statistical evaluation. Data are represented as mean ± SD and were analyzed using Student's *t*-test or one-way ANOVA. The $2^{-\Delta\Delta Ct}$ method was utilized for the analysis of relative gene expression. Survival curves were constructed using the Kaplan–Meier method and assessed by the log-rank test. All statistical examinations were two-sided. The data complied with the assumptions of the tests. An approximation of variation was carried out within each data group. Similar variances were observed across the groups. The depicted data represents the results from three independent experiments. Significance levels were set at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

2.3 | Supplementary methods

The methods of cell culture, cell proliferation assay, Transwell assay, wound healing assay, RT-qPCR, western Blot, Co-IP, RNA pulldown, transfection, ChIP, luciferase reporter assay, IHC, RNA-FISH, microarray analysis, RIP, exosome experiments, subcellular fractionation assay, xenograft assay, the sequence of PCR primers (Table S1), the sequence of siRNA, mimic, and inhibitor (Table S2), and the details of lentivirus are available in Appendix S1.

3 | RESULTS

3.1 | Both glucose and glutamine deprivation induced EMT of PC cells

To investigate malnutrition's effect on PC's metastatic ability, we cultured human PC cells under saturating nutrient medium or glucose-free/glutamine-free medium for up to 72 h. The results indicated that cells incubated in a medium lacking glucose and glutamine undergo dramatic EMT-like transformation, evidenced by alterations of morphology from an epithelial (a polygonal shape and cobblestone-like sheets) to a more mesenchymal (loose cell contacts, scattered from cell cluster and acquired an elongated, fusiform morphology with dendritic processes) phenotype (Figure S1A). Compared to cells grown in a nutrient-sufficient medium, PC cells cultured under glucose and glutamine-depleted medium displayed retarded proliferation and increased invasion and migration ability (Figure S1B–E). Moreover, nutrient depletion significantly elevated ZEB1 and vimentin protein

levels and decreased PC cells' E-cadherin protein levels (Figure S1F). The mRNA expression of E-cadherin and vimentin was consistent with the protein level. Of note, no obvious alteration of ZEB1 mRNA expression indicated a posttranscriptional regulation of ZEB1 expression during nutrient starvation (Figure S1G). Collectively, these data revealed that EMT would be an adaptive response of PC cells during glucose and glutamine deprivation.

3.2 | ZFAS1 is a critical regulator for metabolic stress-induced EMT of PC cells

Pioneering published works have elucidated that lncRNAs have critical roles in metabolic remodeling in tumor microenvironment.[15,16] An lncRNA microarray was used to explore the mechanism and action of lncRNAs in mediating EMT elicited by nutrient stress. Long noncoding RNA ZFAS1 is upregulated in both glucose- and glutamine-depleted BXPC-3 cells (Figure 1A). Both RT-qPCR and FISH assay suggested that ZFAS1 was significantly elevated in PC cells under glucose- and glutamine-depleted conditions (Figures 1B,C and S2A,B). It was also observed that ZFAS1 silencing significantly increased and decreased the mRNA and protein levels of E-cadherin and vimentin, respectively (Figures 1D,E and S2C–E). ZFAS1 inhibition decreased the protein level of ZEB1 with no change of the mRNA level, indicating the posttranscriptional regulation of ZEB1 expression during nutrient starvation might be attributed to the elevation of ZFAS1. Additionally, ZFAS1 knockdown significantly repressed invasion and migration in PC cells in response to glucose or glutamine starvation (Figures 1F–H and S2F–H). Thus, these results implied that upregulated ZFAS1 was decisive for the nutrient deprivation-induced EMT of PC cells.

3.3 | ZFAS1 promoted phosphorylation and stability of ZEB1

We hypothesized that ZFAS1 promotes EMT under nutrient deficiency by acting on posttranscriptional regulation of ZEB1, a key EMT regulator. Initially, the luciferase reporter assay results indicated that the promoter activity of ZEB1 had no visible change during glucose and glutamine depletion (Figure S3A). Exposure to glucose and glutamine starvation remarkably enhanced the half-life of ZEB1 protein in PANC-1/BxPC-3 cells (Figure 2A). ZFAS1 silencing significantly decreased the stability of ZEB1 protein in glucose and glutamine-depleted PANC-1/BxPC-3 cells (Figure 2B,C). Moreover, the nutrient depletion-induced ZEB1 expression could be abolished by the proteasome inhibitor MG132 (Figure 2D). These results implied that ZEB1 might be regulated at a posttranslational level under nutrient stress. Notably, the acetylation and ubiquitination of ZEB1 showed no significant change in PC cells with glucose and glutamine starvation, while phosphorylation of ZEB1 was profoundly increased (Figure S3B–D). Furthermore, the protein level of ZEB1 was elevated in both the cytoplasm and nucleus of PC cells cultured with limited glucose and glutamine, and ZFAS1 silencing could inhibit this effect (Figure S3E). More importantly, ZFAS1 inhibition significantly impeded the phosphorylation of ZEB1 induced by glucose and glutamine starvation (Figure 2E). Collectively, our results suggested that ZFAS1 regulated ZEB1 protein level at the posttranslational level through phosphorylation under glucose and glutamine starvation.

3.4 | AMPK promoted phosphorylation of ZEB1 at Thr220 during metabolic stress

Next, we sought to elucidate how ZFAS1 influences ZEB1 phosphorylation, thereby modulating ZEB1 protein stability. Previous studies have shown that glucose and glutamine deprivation, frequently encountered in PC, can activate AMPK, which subsequently phosphorylates several critical regulators involved in tumor or antitumor processes.[17,18] The AMPK substrates have a common phospho-AMPK substrate motif [LXRXX(pS/pT)].[19] Upon analysis, we identified a conserved phospho-AMPK substrate motif within the ZEB1 protein sequence at position 215-LERHMT-220 (Figure 3A). The Co-IP assay showed a direct interaction between ZEB1 and AMPK (Figure 3B). This assay further confirmed that ZEB1 could serve as a phosphorylation substrate for AMPK by employing an anti-phospho-AMPK (p-AMPK) substrate motif Ab (LXRXX (pS/pT), denoted as p-AMPK-Sub) (Figure 3C). Furthermore, depletion of AMPK via siRNA noticeably reduced ZEB1 protein levels without affecting mRNA levels (Figure 3D,E). Consistent with these findings, both AMPK knockdown and AMPK inhibitor (C.C) treatment[20] effectively diminished ZEB1 phosphorylation and expression, as well as the interaction between ZEB1 and p-AMPK, which were otherwise elevated under nutrient deprivation (Figure 3F,G). To further corroborate that AMPK stabilization of ZEB1 is contingent upon Thr220 phosphorylation, we transfected Flag-tagged WT and MUT (threonine-to-alanine mutation) ZEB1 plasmids into PANC-1 cells. The results from western blotting revealed that threonine-to-alanine replacement at Thr220 abolished the phosphorylation and expression of Flag-ZEB1, as well as the interaction between p-AMPK and Flag-ZEB1 during glucose and glutamine starvation (Figure 3H). Collectively, these findings suggest that ZEB1 protein upregulation is a consequence of AMPK-mediated phosphorylation and subsequent stabilization.

3.5 | ZFAS1 enhances the interaction between AMPK and ZEB1 during metabolic stress

Long noncoding RNA has been defined to serve as molecular scaffolds to connect different proteins that need to play a synergistic role.[21] Notably, the combination between p-AMPK and ZEB1 was substantially decreased when samples were pretreated with RNase (Figure 4A), indicating a possibility that lncRNA

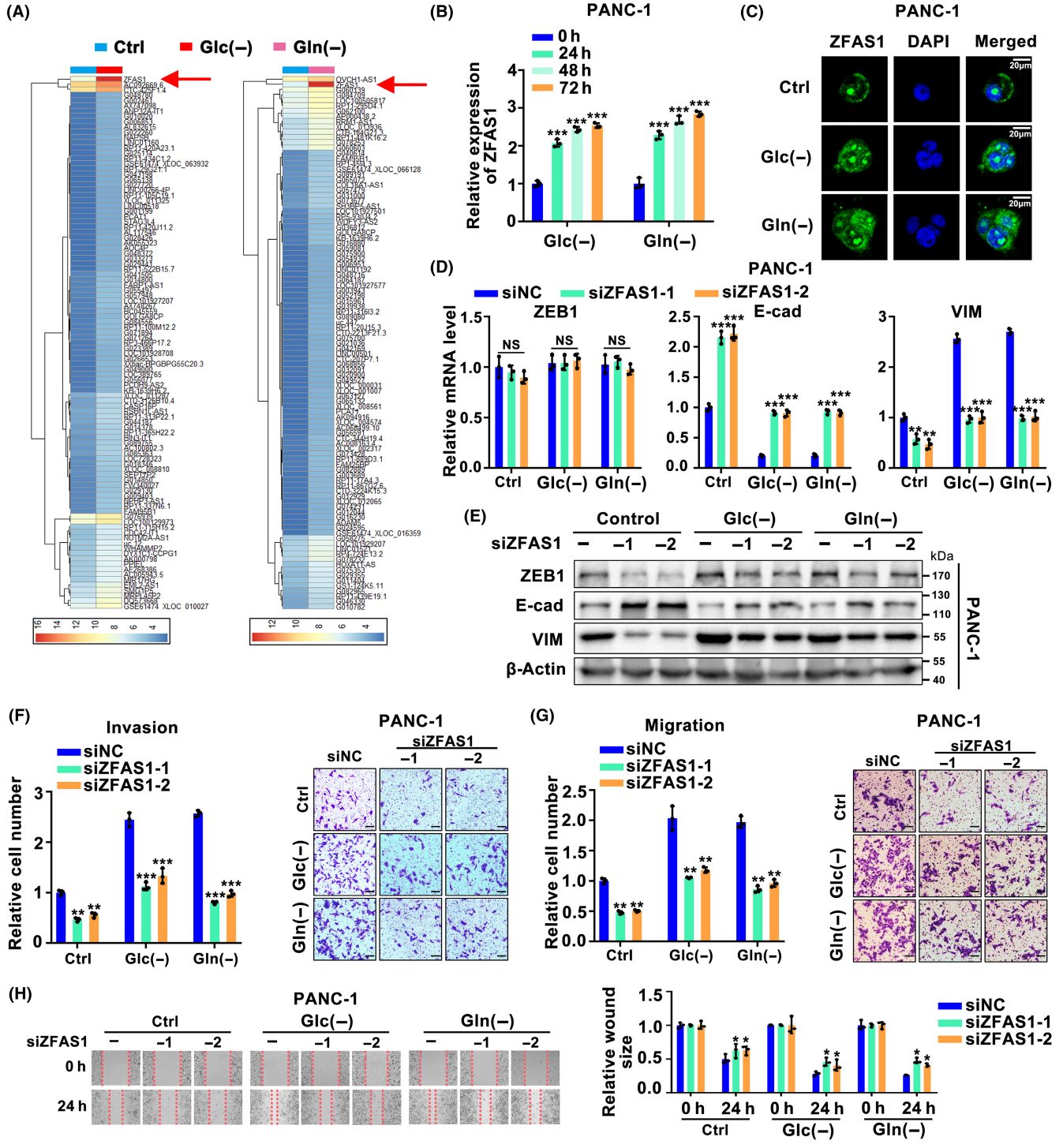

FIGURE 1 Upregulated ZFAS1 is a critical regulator for nutrient starvation-induced epithelial–mesenchymal transition of pancreatic cancer cells. (A) Microarray analysis for long noncoding RNAs (lncRNAs) was undertaken with BxPC-3 cells cultured with saturating nutrient medium (control, Ctrl), glucose (Glc)− medium, or glutamine (Gln)− medium for 48 h. The heatmap shows the 100 most upregulated lncRNAs under glucose and glutamine starvation (GSE233113). (B) The expression of ZFAS1 was detected with RT-qPCR in PANC-1/BxPC-3 cells cultured with saturating nutrient medium, Glc− medium, or Gln− medium for 0, 24, 48, and 72 h. (C) RNA-FISH assay shows the expression of ZFAS1 in PANC-1 cells cultured with saturating nutrient medium, Glc− medium, or Gln− medium for 48 h. Scale bar, 20 μm. (D, E) mRNA and protein levels of ZEB1, E-cadherin, and vimentin (VIM) were detected in ZFAS1 knockdown PANC-1 cells cultured with saturating nutrient medium, Glc− medium, or Gln− medium for 48 h. (F–H) Invasion and migration ability of ZFAS1 knockdown PANC-1 cells under glucose and glutamine starvation was assessed by Transwell assay and wound healing assay. Scale bar, 100 μm. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. NS, not significant; siNC, silence RNA negative control.

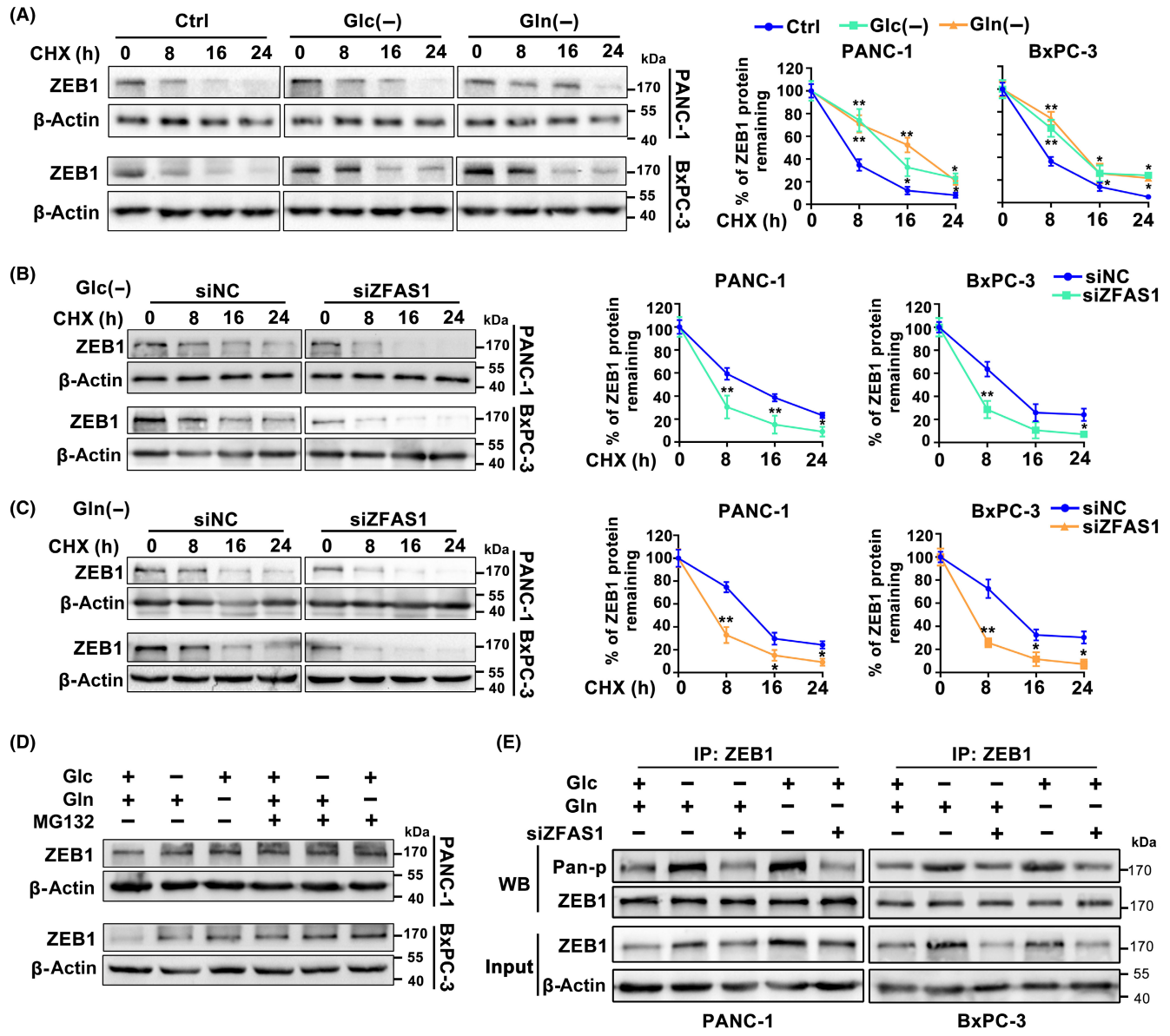

FIGURE 2 ZFAS1 regulated ZEB1 at the posttranslational level in response to nutrient depletion through phosphorylation modification. (A–C) After being cultured with saturating nutrient medium (control, Ctrl), glucose (Glc)− medium, or glutamine (Gln)− medium for 48 h, PANC-1/BxPC-3 cells with or without ZFAS1 knockdown were treated with cycloheximide (CHX, 50 μg/mL) for periods as indicated. Total protein extraction and western blot analysis of the protein level of ZEB1. The line chart quantifies the relative density of protein above. (D) Western blot (WB) assay analyzed the protein level of ZEB1 in PANC-1/BxPC-3 cells cultured with saturating nutrient medium, Glc− medium, or Gln− medium for 48 h with or without treatment with MG132 (20 μM) for 3 h. (E) Phosphorylation of ZEB1, detected in ZFAS1 knockdown PANC-1/BxPC-3 cells cultured with saturating nutrient medium, Glc− medium, or Gln− medium for 48 h, were analyzed in the cell lysates following anti-ZEB1 immunoprecipitation (IP). $^{*}p < 0.05$, $^{**}p < 0.01$. siNC, silence RNA negative control.

affected the binding between p-AMPK and ZEB1. Therefore, we hypothesized that ZFAS1 interacted with p-AMPK and ZEB1 during nutrient deprivation. The results showed that ZFAS1, but not antisense of ZFAS1 and other EMT-related transcription factors such as Twist and Snail, bound to p-AMPK and ZEB1 (Figure 4B,C). Moreover, domain truncation constructs of ZFAS1 were used to clarify the structural basis for these interactions. It is interesting to note that both the full length and 1–250 nt of ZFAS1 were observed to bind to both ZEB1 and p-AMPK (Figure 4D). In further support of the assembly of ZEB1 and AMPK by ZFAS1, we carried out two-step RIP using Flag-ZEB1 and HA-AMPK transfected cells. The results showed that AMPK and ZFAS1 were precipitated simultaneously during the first precipitate using the anti-Flag Ab against Flag-ZEB1 (Figure 4E). ZEB1 and ZFAS1 were also precipitated in the second immunoprecipitation with anti-HA Ab against HA-AMPK, indicating a trimer formed between ZFAS1, ZEB1, and AMPK. In addition, ZFAS1 overexpression significantly enhanced the combination between p-AMPK and ZEB1, and the protein level of ZEB1 (Figure S4A). Consistently, the amount of coassociated AMPK within ZEB1 immunoprecipitation and ZEB1

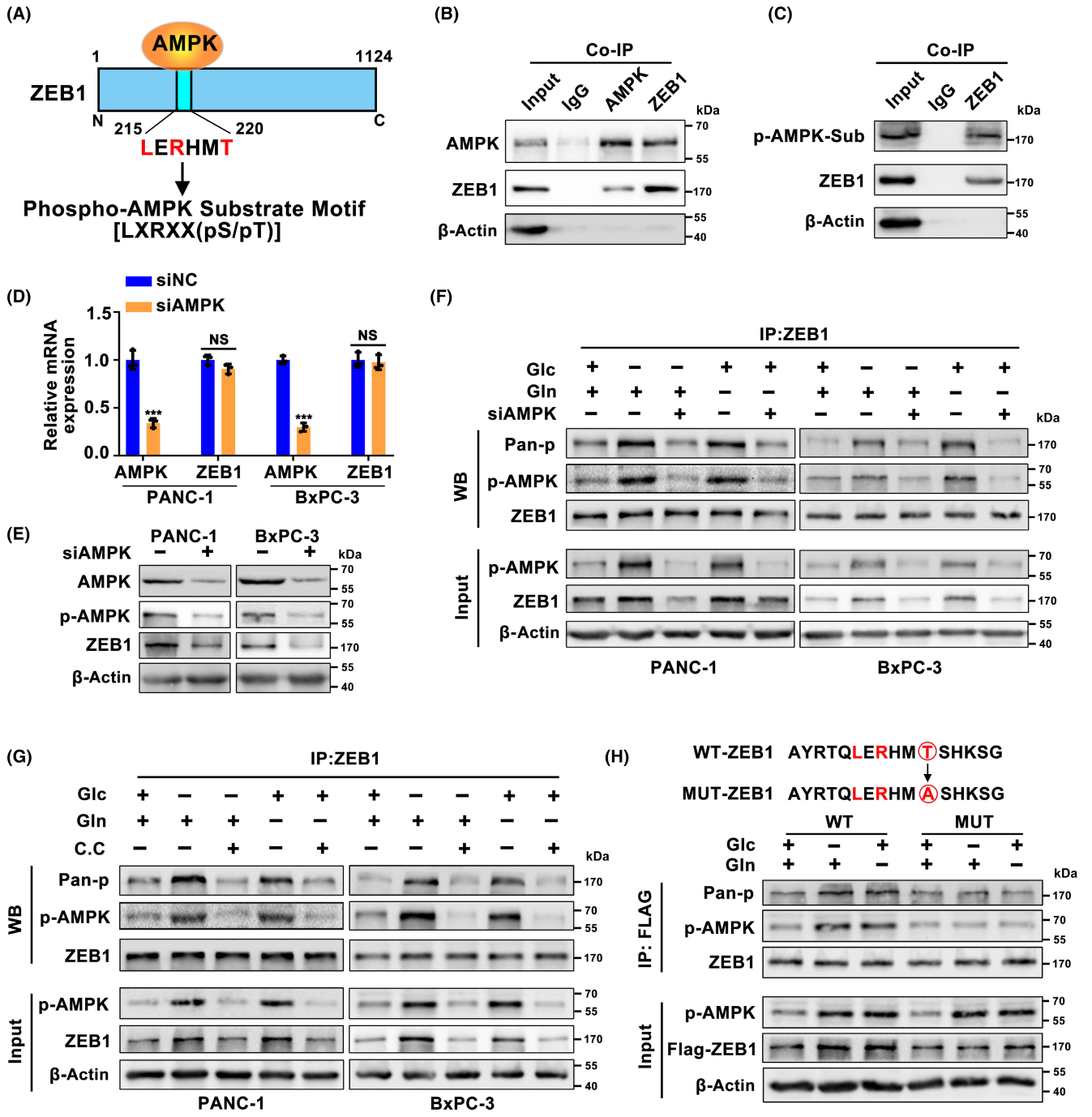

FIGURE 3 AMP-activated protein kinase (AMPK)-dependent phosphorylation at Thr220 mediates the stabilization of ZEB1 under nutrient starvation. (A) Schematic model presenting the potential phosphorylation sites of AMPK on ZEB1 protein sequence. (B) AMPK, ZEB1, and β-actin protein levels were detected in PANC-1 cell lysates following anti-IgG, anti-AMPK, or anti-ZEB1 immunoprecipitation (IP). (C) Proteins, detected with anti-phospho-AMPK substrate motif Ab (pS/pT), anti-ZEB1, and anti-β-actin Ab in PANC-1 cells, were analyzed in the cell lysates following anti-ZEB1 immunoprecipitation. (D, E) mRNA and protein levels of AMPK and ZEB1 were detected by RT-qPCR and western blotting (WB) in PANC-1/BxPC-3 cells transfected with siAMPK (a mixture of AMPKα1 and AMPKα2 siRNA) or correlated control. (F, G) PANC-1/BxPC-3 cells, cultured with saturating nutrient medium, glucose (Glc)− medium, or glutamine (Gln)− medium, were transfected with siNC/siAMPK or treated with or without compound C (C.C, 5 μM) for 48 h. Phosphorylated ZEB1, phosphorylated AMPK, and ZEB1 protein levels were analyzed in the cell lysates following anti-ZEB1 immunoprecipitation. (H) PANC-1 cells, cultured with saturating nutrient medium, Glc− medium, or Gln− medium, were transfected with the WT and mutant type (MUT; threonine to alanine) of ZEB1 plasmids for 48 h. Phosphorylated ZEB1, phosphorylated AMPK, and ZEB1 protein levels were analyzed in the cell lysates following anti-Flag immunoprecipitation. ***$p < 0.001$. NS, not significant.

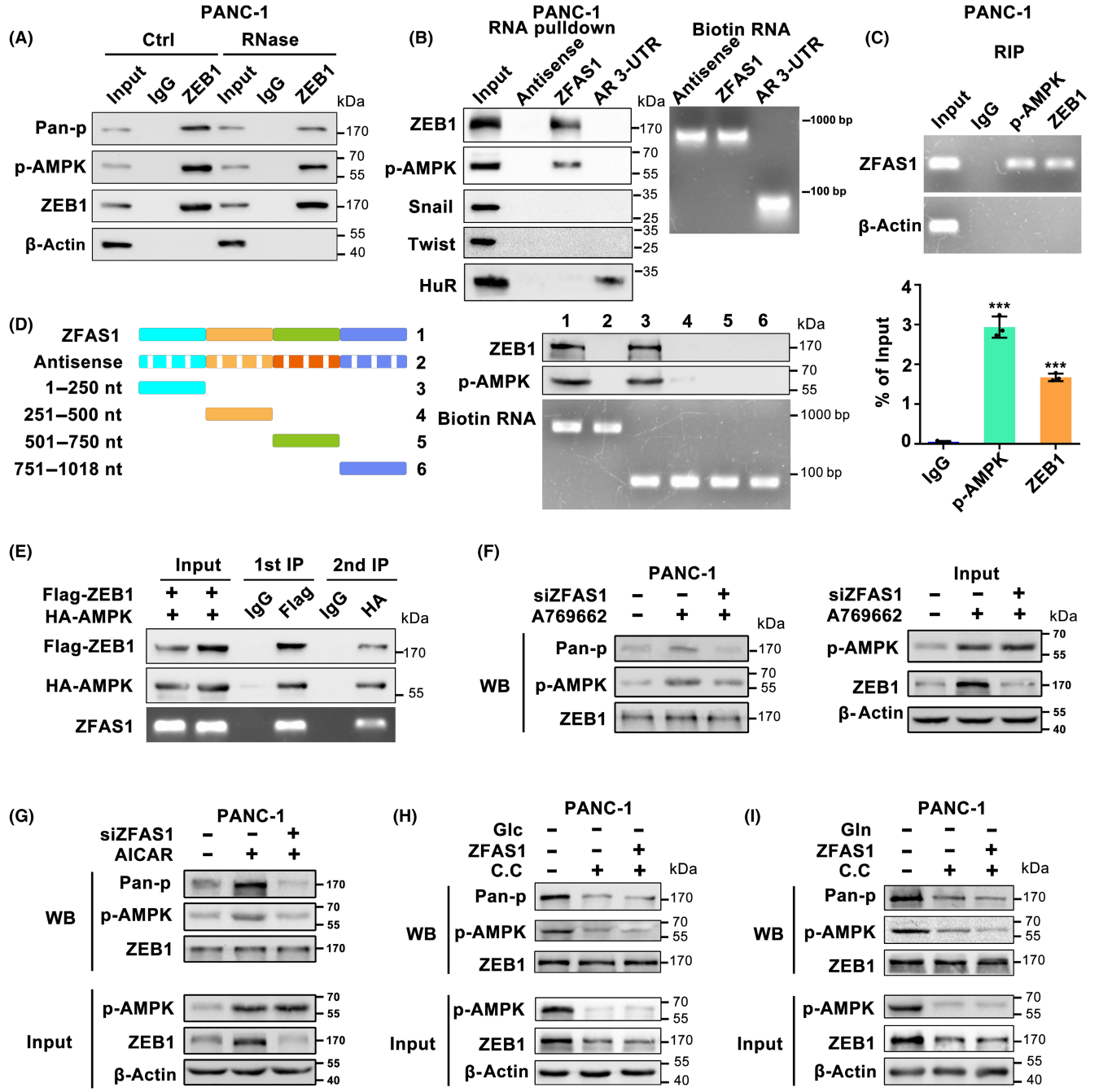

FIGURE 4 ZFAS1 enhances ZEB1 protein stability by facilitating AMP-activated protein kinase (AMPK)-mediated phosphorylation. (A) Protein level of phosphorylated ZEB1, p-AMPK, ZEB1, and β-actin was analyzed by western blotting (WB) in PANC-1 cell lysates treated with or without RNase following anti-ZEB1 immunoprecipitation (IP). (B) RNA pulldown assay was undertaken to verify the binding between ZFAS1 and ZEB1/p-AMPK with a biotinylated ZFAS1 probe in PANC-1 cells. Antisense of ZFAS1 and AR 3′-UTR served as a negative and positive control, respectively. (C) RNA binding protein immunoprecipitation assay was used to verify the binding between ZEB1/p-AMPK and ZFAS1 with anti-ZEB1 and anti-p-AMPK Abs in PANC-1 cells. RT-qPCR and 2% agarose gel electrophoresis were carried out to detect the ZFAS1 and β-actin. (D) Schematic representation of the ZFAS1-depleted mutants used in RNA pulldown. Phosphorylated AMPK (p-AMPK) and ZEB1 protein levels were analyzed in ZFAS1 overexpression cell lysates following anti-ZEB1 and anti-p-AMPK immunoprecipitation. (E) PANC-1 cells, cotransfected with Flag-tagged ZEB1 and HA-tagged AMPK plasmid for 48 h, were used to carry out sequential immunoprecipitations with anti-Flag and anti-HA Abs. (F) PANC-1 cells were transfected with siNC/siZFAS1 for 48 h and treated with or without A769662 (100 mM) for 4 h. Phosphorylated ZEB1, p-AMPK, and ZEB1 protein levels were analyzed in the cell lysates following anti-ZEB1 immunoprecipitation. (G) PANC-1 cells were transfected with siNC/siZFAS1 for 48 h and treated with or without AICAR (0.5 mM) for 5 h. Phosphorylated ZEB1, p-AMPK, and ZEB1 protein levels were analyzed in cell lysates following anti-ZEB1 immunoprecipitation. (H, I) PANC-1 cells, cultured under glucose-free (Glc−) or glutamine-free (Gln−) conditions, were transfected with ZFAS1 overexpression plasmid or treated with or without compound C (C.C, 5 μM) for 48 h. Phosphorylated ZEB1, p-AMPK, and ZEB1 protein levels were analyzed in the cell lysates following anti-ZEB1 immunoprecipitation. ***$p < 0.001$.

protein level was elevated in PANC-1 cells transfected with ZFAS1-1 overexpression plasmid, which encodes the 1–250 nt of ZFAS1, but not in that of ZFAS1-dt1 plasmid with the 1–250 nt of ZFAS1 deletion. These findings suggest that ZFAS1 drives the association between p-AMPK and ZEB1.

Moreover, the Co-IP assay results indicated that the AMPK activators, A769662 and AICAR, significantly enhanced ZEB1 phosphorylation, an effect subsequently nullified by ZFAS1 inhibition (Figures 4F,G and S4B–D). Additionally, glucose starvation-induced coassociation of p-AMPK with ZEB1 and ZEB1 phosphorylation was notably hindered by the AMPK inhibitor, C.C. This inhibition could not be counteracted by ZFAS1 overexpression (Figures 4H and S4E). A similar pattern was noted in glutamine-deprived PC cells (Figures 4I and S4F). Collectively, these data propose that ZFAS1 is essential for AMPK-mediated ZEB1 phosphorylation and stabilization under glucose and glutamine depletion.

3.6 | ZEB1 reciprocally upregulates ZFAS1 under glucose and glutamine deprivation

Next, we sought to elucidate the mechanism of ZFAS1 upregulation in PC cells under glucose and glutamine starvation. Luciferase reporter was designed to test the transcriptional activity of ZFAS1. The result indicated that the transcriptional activity of ZFAS1 was significantly enhanced in glucose and glutamine-depleted PC cells (Figure 5A). ZEB1 is validated as a multifunctional transcription factor that activates or inhibits transcription.[22,23] Thus, we wondered whether upregulated ZEB1 might mediate the elevation of ZFAS1 under glucose and glutamine-depleted conditions. Analysis of transcription factor binding profiles by Jaspar indicated four ZEB1 potential binding sites located on the promoter of the *ZFAS1* gene (Figure 5B). In support, the ChIP assay showed that the ZFAS1 promoter sequence containing −152 nt -CTCACCCGGCT- −162 nt sequence was captured by ZEB1 immunoprecipitation, which was enhanced in glucose and glutamine starved PC cells (Figure 5C). To prove that ZEB1 binds explicitly to the ZFAS1 promoter, ZFAS1 promoter sequences carrying −152 nt -CTCACCCGGCT- −162 nt sequence (WT) or mutant −152 nt -CTAAAAAAAAA- −162 nt sequence (MUT) were inserted into a luciferase reporter vector and transfected into PC cells (Figure 5D). The results showed that the promoter activity of ZFAS1 in WT cells, but not MUT cells, was notably enhanced during glucose and glutamine depletion, which could be reversed by ZEB1 knockdown (Figure 5E). Coincidently, RT-qPCR assays further validated that ZEB1 knockdown remarkably inhibited the increase of ZFAS1 upon glucose and glutamine depletion (Figure 5F). Accordingly, the AMPK activator, A769662 and AICAR, enhanced both promoter activity and expression of ZFAS1, which was abolished by ZEB1 knockdown (Figure 5G,H). Collectively, these results verified a requirement of ZEB1 for the upregulation of ZFAS1 in PC cells under nutrient depletion, forming reciprocal feedback between ZEB1 and ZFAS1.

3.7 | Targeting ZFAS1 hampers metabolic stress-induced EMT and metastasis of PC cells

Next, we characterize the role of the ZFAS1/ZEB1 axis in PC cells under glucose and glutamine starvation. The data showed that ZFAS1 knockdown impaired and enhanced the mRNA and protein levels of vimentin and E-cadherin under glucose and glutamine depleted conditions, respectively. ZEB1 overexpression completely abrogated the effect of ZFAS1 knockdown (Figure 6A). Meanwhile, ZFAS1 knockdown distinctively inhibited the enhanced invasion and migration of PC cells in response to glucose and glutamine starvation, which was entirely neutralized by ZEB1 overexpression (Figure 6B). In a further study, we found that athymic nude mice injected with PANC-1 cells with stable ZFAS1 depletion showed decreased lung metastatic colonies and longer survival, which was remarkably reversed by ZEB1 overexpression (Figure 6C–F), indicating that the ZFAS1/ZEB1 loop is critical for the metastasis of PC cells. Overall, these findings indicated that ZFAS1 was involved in the role of ZEB1 in promoting EMT and metastasis of PC cells under glucose and glutamine depletion.

3.8 | ZFAS1 is essential for nutrient stressed exosome-promoted EMT and metastasis in cancer cells with saturating nutrient

Our prior investigation revealed that hypoxic PC cells could transfer chemoresistance to normoxic cells.[14] This prompted us to question whether cells cultured under nutrient-limited conditions might similarly confer EMT and metastasis traits to cells in nutrient-rich conditions. To examine this possibility, we cocultured these two cell types in a chamber. Intriguingly, we found that PC cells cocultured with nutrient-depleted PC cells displayed enhanced invasion and migration capabilities (Figure S5A), suggesting that cells in nutrient-deficient environments could impart invasiveness and migratory capacity to cells in nutrient-adequate conditions through noncontact mechanisms.

We thus hypothesized that exosomes could facilitate cell communication under nutrient-limited and -sufficient conditions. We harvested exosomes from cells cultured under glucose and glutamine depletion and discovered that cells cultured under metabolic stress secrete more exosomes than cells cultured under normal conditions (Figure S5B,C). Meanwhile, ZFAS1 levels were elevated in exosomes released by cells cultured under these nutrient-deprived conditions (Figure S5D). Furthermore, ZFAS1 expression remained unchanged in glucose and glutamine-depleted CM treated with RNase R. However, it significantly decreased when cotreated with RNase R and Triton X-100 (Figure S5E), implying that extracellular ZFAS1 was primarily encapsulated within exosomes rather than freely circulating in the CM.

We then sought to determine the role of ZFAS1 in the interaction between cells under nutrient-poor and -rich conditions.

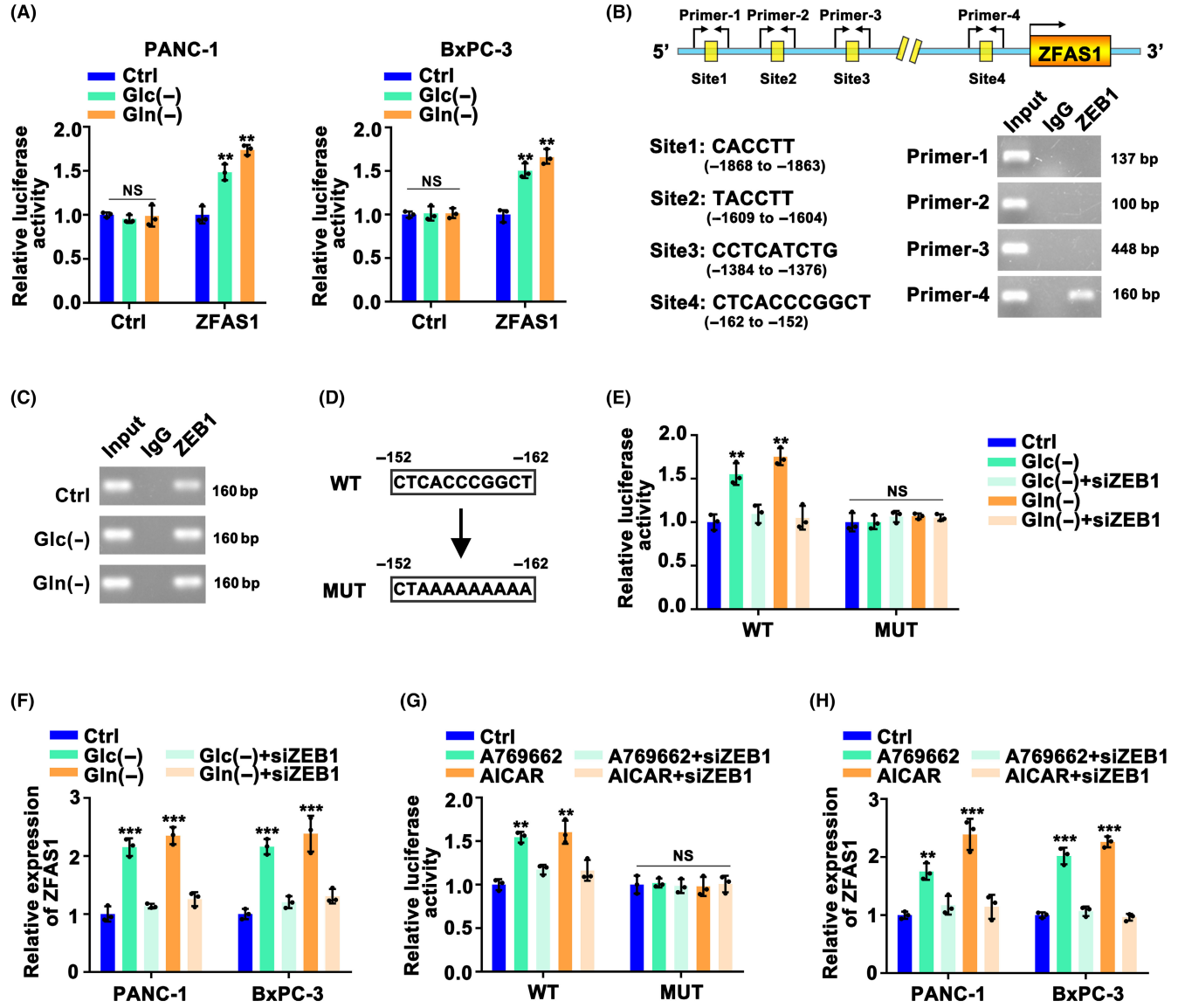

FIGURE 5 ZEB1 transcriptionally regulates ZFAS1 under glucose (Glc) and glutamine (Gln) deprivation. (A) PANC-1/BxPC-3 cells, cultured under glucose-free (Glc−) or glutamine-free (Gln−) conditions, transfected with pGL3 vector or ZFAS1 promoter with firefly luciferase plasmid and pRL-TK plasmid encoding *Renilla* luciferase for 48 h. The luciferase activity of cells was assessed by luciferase reporter assay. (B) Schematic model presenting four potential binding sites of ZEB1 on ZFAS1 promoter sequence (Up). ChIP assay was carried out using anti-IgG or anti-ZEB1 Ab to verify the binding of ZEB1 protein on the promoter of ZFAS1 (Down). (C) PANC-1 cells were cultured with saturating nutrient medium (Ctrl), Glc− medium, or Gln− medium for 48 h. The cells were lysed for the ChIP assay with anti-IgG or anti-ZEB1 Ab to verify the binding of ZEB1 protein on the promoter of ZFAS1. (D) Schematic model presenting the WT and mutant type (MUT) of ZFAS1 promoter sequence. (E) PANC-1 cells, cultured with saturating nutrient medium, Glc− medium, or Gln− medium, were cotransfected with pGL3 ZFAS1 promoter (WT/MUT) with firefly luciferase plasmid, pRL-TK plasmid encoding *Renilla* luciferase, and siNC/siZEB1 for 48 h. The luciferase activity of cells was assessed by luciferase reporter assay (right). (F) Expression of ZFAS1 was detected with RT-qPCR in PANC-1/BxPC-3 cells transfected with siNC/siZEB1 and cultured with saturating nutrient medium, Glc− medium, or Gln− medium. (G) PANC-1 cells were cotransfected with pGL3 ZFAS1 promoter (WT/MUT) with firefly luciferase plasmid, pRL-TK plasmid encoding *Renilla* luciferase, and siNC/siZEB1 for 48 h and treated with or without A769662/AICAR. The luciferase activity of cells was assessed by luciferase reporter assay. (H) Expression of ZFAS1 was detected with RT-qPCR in PANC-1/BxPC-3 cells transfected with siNC/siZEB1 for 48 h and treated with or without A769662/AICAR. $^{**}p < 0.01$, $^{***}p < 0.001$. NS, not significant.

We found that PHK26-labeled exosomes could fuse with recipient cells (Figure S5F) and that ZFAS1 was notably upregulated in exosomes derived from cells cultured in glucose and glutamine-depleted medium (Figure S5G). Moreover, exosomes from cells cultured under glucose and glutamine-limited conditions significantly stimulated EMT, invasion, and migration of BxPC-3/PANC-1 cells under nutrient-sufficient conditions (Figure S5H–J). Interestingly, the RNA synthesis inhibitor ActD marginally reduced the increase in ZFAS1 in exosomes sourced from glucose and glutamine-depleted cells (Figure S5K), suggesting that ZFAS1 augmentation in recipient

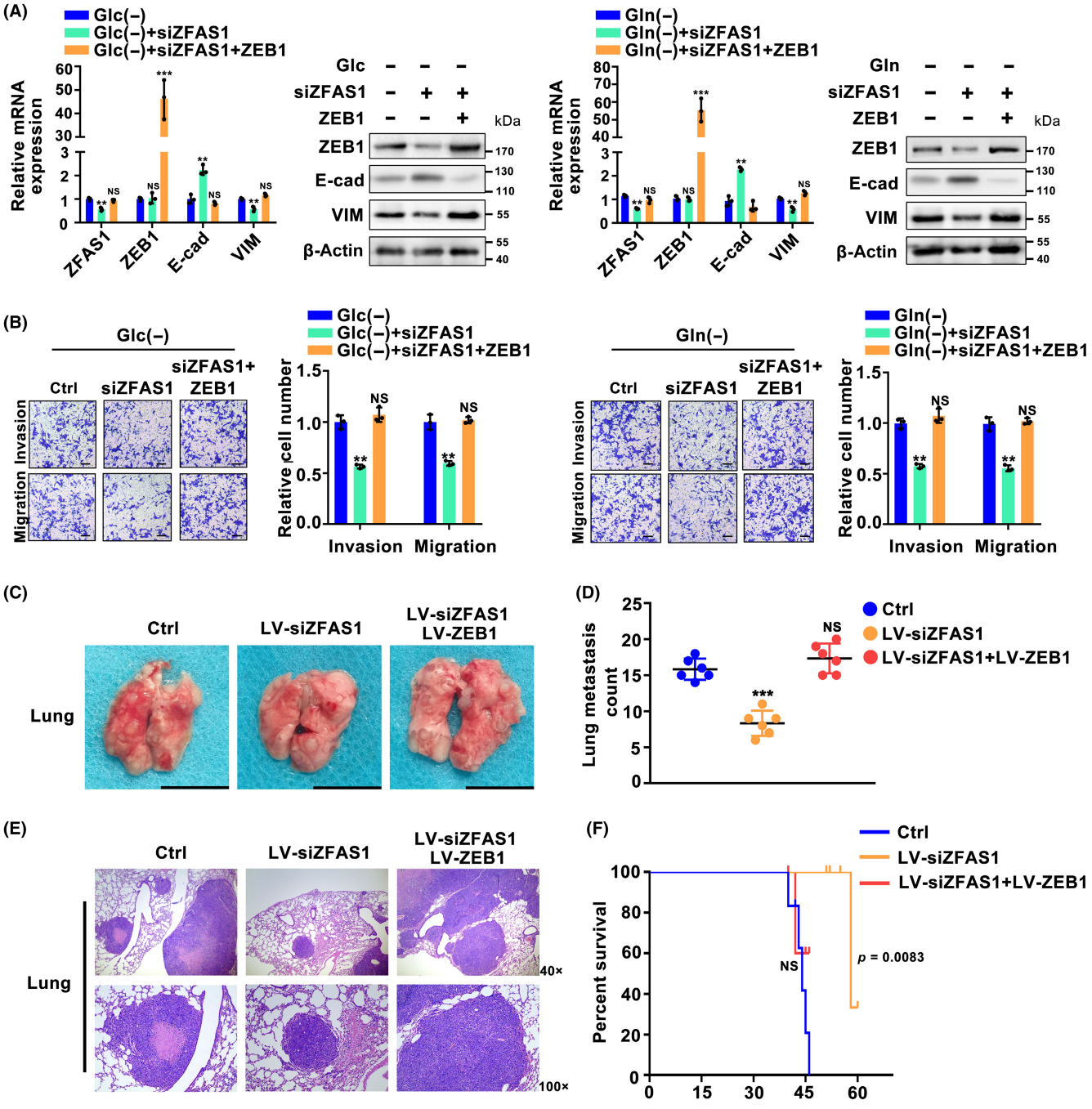

FIGURE 6 Targeting the ZFAS1/ZEB1 axis hampers the nutrient stress-induced epithelial–mesenchymal transition and metastasis of pancreatic cancer cells. (A, B) PANC-1 cells, cultured with saturating nutrient medium, glucose (Glc)− medium, or glutamine (Gln)− medium, were transfected with siZFAS1, ZEB1 overexpression plasmid, or correlated control for 48 h. (A) ZFAS1 expression and ZEB1, E-cadherin (E-cad), and vimentin (VIM) mRNA and protein levels were detected by RT-qPCR and western blotting. (B) Invasion and migration of PANC-1 cells was assessed by Transwell assay. Scale bar, 100 μm. (C–F) Nude mice were treated with tail vein injection of PANC-1 cells stably transfected with LV-NC, LV-siZFAS1, or/and LV-ZEB1. (C) Representative images of the lungs of nude mice. Scale bar, 1 cm. (D) Quantification of metastatic lung colonization of nude mice. (E) Representative images of H&E staining of lungs of nude mice. (F) Kaplan–Meier curves showing overall survival of nude mice. $^{**}p < 0.01$, $^{***}p < 0.001$. NS, not significant.

cells could be a result of both exosomal delivery and induction of endogenous transcription. These findings suggest a potential role for ZFAS1 in mediating the transmission of EMT and metastasis traits through exosomes between cells under nutrient-depleted and -saturated conditions.

3.9 | ZFAS1 is highly expressed in PC and correlated with poor prognosis

To determine whether the findings above are clinically relevant, we compared ZFAS1 expression in various cancer tissues. Analysis from

the database GEPIA showed that ZFAS1 expression was upregulated in multiple tumor tissues (Figure S6A,B). Moreover, the data from GEO datasets (GSE15471 and GSE165115) indicated that ZFAS1 expression was augmented in PC tissues compared to normal tissues (Figure S6C,D). Consistently, RT-qPCR assay indicated a stronger expression of ZFAS1 in 60 PC tissues, compared with adjacent normal tissues (Figure S6E). Kaplan–Meier survival curve analysis indicated that higher ZFAS1 expression in PC patients was associated with shorter survival (Figure S6F). We also found that ZFAS1 was negatively correlated with mRNA level of E-cadherin, but positively correlated with that of vimentin in PC tissues (Figure S6G,H). Additionally, the IHC assay showed that ZFAS1 was positively correlated with the protein level of ZEB1 and vimentin, but negatively associated with protein expression of E-cadherin (Figure S6I). Therefore, these results further support that ZFAS1 is an oncogenic lncRNA for PC.

Our results indicate that ZFAS1 is a nutrient stress-adapted oncogenic lncRNA and functions as a molecular scaffold facilitating AMPK-mediated phosphorylation and stabilization of ZEB1 to promote EMT and metastasis of PC cells (Figure 7).

4 | DISCUSSION

Cancer cells are believed to deal with fluctuations and limit nutrient levels in an unstable microenvironment to ensure vitality and reproduction. Glucose and glutamine are the crucial metabolites of catabolism and anabolism, which are the focus of many diagnostic methods and therapeutic targets.[24] Understanding the adaption of tumor cells during nutrient stress, including glucose and glutamine depletion, is of great significance to tumor treatment. Here, we revealed that PC cells exhibited the EMT process with increased invasion and migration abilities caused by glucose or glutamine starvation. Our work elucidated a new mechanism that the feedback between lncRNA-ZFAS1 and ZEB1 was the hub regulator for the adaption of PC cells during nutrient deprivation.

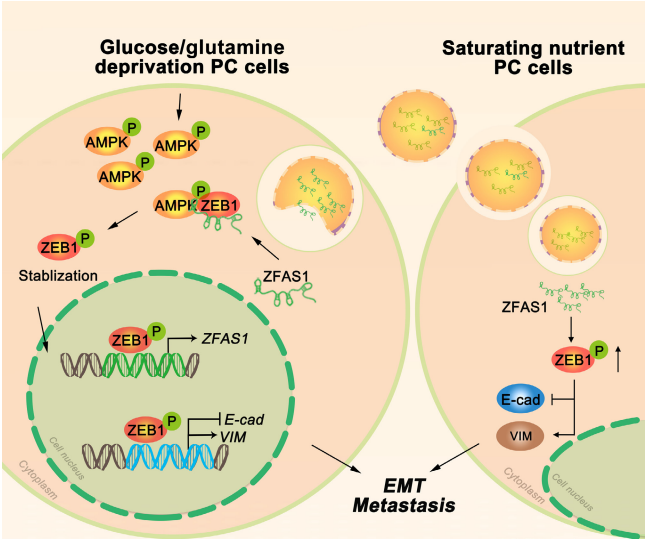

FIGURE 7 Schematic illustration depicting ZFAS1 as a nutrient stress-adapted oncogenic long noncoding RNA that promotes epithelial–mesenchymal transition (EMT) and metastasis of pancreatic cancer (PC) cells. AMPK, AMP-activated protein kinase; E-cad, E-cadherin; VIM, vimentin.

The accelerated growth rate of tumor cells generates solid and fluid pressure within the tumor, causing the surrounding abnormal blood vessels to compress and create a densely packed tumor mass. This situation leads to a highly hypoxic, acidic, and nutrient-poor microenvironment.[24–26] Previous research suggests that PC cells can endure these harsh conditions, such as low oxygen levels and an acidic external environment, and sustain their survival by enhancing EMT and the metastasis of PC cells.[27,28] In this study, we observed that glucose and glutamine deprivation could instigate EMT and metastasis in PC cells. These findings propose that alterations in the microenvironment leading to metabolic stress prompt cancer cells to increase metastasis to procure the necessary energy and nutrients for survival.

Notably, we revealed that ZFAS1 regulated ZEB1 expression by promoting the stability of ZEB1 protein. The posttranslational modifications of proteins include phosphorylation, acetylation, and ubiquitination.[29] The study elucidated that ZEB1 was phosphorylated and stabilized by the kinase ATM in response to DNA damage, which promoted tumor cell radioresistance in vitro and in vivo.[30] Similarly, our results showed that only phosphorylation but not ubiquitination and acetylation of ZEB1 was increased under nutrient deprivation, which was abrogated by ZFAS1 depletion. Moreover, numerous pieces of evidence indicated that lncRNA plays the role of "recruiting" proteins in cells and acts as the "skeleton" of the protein complex.[31,32] Additionally, several published reports have elucidated that ZFAS1 activated ZEB1 expression by adsorption of miRNA.[33–35] In our exploration, we found that ZFAS1 plays a transacting role in promoting the ZEB1 expression by promoting AMPK phosphorylation of ZEB1 as a scaffold.

Multiple mechanisms have been identified in reports that tumor-derived exosomes promote tumor progression and malignance.[36] Of note, exosomes have been found to carry miRNAs to promote recipient cells to a prometastatic phenotype.[37] Nevertheless, the function of lncRNA embedded in exosomes in EMT and metastasis remains poorly reported. Our study found that exosomal lncRNA ZFAS1, secreted from nutrient-depleted cells, conferred enhanced EMT and metastasis phenotype to recipient PC cells.

Our findings imply that ZFAS1 is a nutrient stress-adapted oncogenic lncRNA and functions as a molecular scaffold facilitating AMPK-mediated phosphorylation and stabilization of ZEB1 to promote EMT and metastasis of PC cells.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

Approval of the research protocol by an institutional review board: The Ethics Committee of the Academic Medical Center of Huazhong University of Science and Technology approved the procedure of human specimen collection (Permission No: 2013, S199).

Informed consent: All patients provided signed informed consent.

Registry and the registration no. of the study/trial: N/A.

Animal studies: The Ethics Committee of the Academic Medical Center of Huazhong University of Science and Technology approved the procedure of animal studies.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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