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# IGFBP-3 promotes cachexia-associated lipid loss by suppressing insulin-like growth factor/insulin signaling

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**Abstract**

**Background:** Progressive lipid loss of adipose tissue is a major feature of cancer-associated cachexia. In addition to systemic immune/inflammatory effects in response to tumor progression, tumor-secreted cachectic ligands also play essential roles in tumor-induced lipid loss. However, the mechanisms of tumor-adipose tissue interaction in lipid homeostasis are not fully understood.

**Methods:** The *yki*-gut tumors were induced in fruit flies. Lipid metabolic assays were performed to investigate the lipolysis level of different types of insulin-like growth factor binding protein-3 (IGFBP-3) treated cells. Immunoblotting was used to display phenotypes of tumor cells and adipocytes. Quantitative polymerase chain reaction (qPCR) analysis was carried out to examine the gene expression levels such as *Acc1*, *Acly*, and *Fasn* et al.

**Results:** In this study, it was revealed that tumor-derived IGFBP-3 was an important ligand directly causing lipid loss in matured adipocytes. IGFBP-3, which is highly expressed in cachectic tumor cells, antagonized insulin/IGF-like signaling (IIS) and impaired the balance between lipolysis and lipogenesis in 3T3-L1 adipocytes. Conditioned medium from cachectic tumor cells, such as Capan-1 and C26 cells, contained excessive IGFBP-3 that potently induced lipolysis in adipocytes. Notably, neutralization of IGFBP-3 by neutralizing antibody in the conditioned medium of cachectic tumor cells significantly alleviated the lipolytic effect and restored lipid storage in adipocytes. Furthermore, cachectic tumor cells were resistant to IGFBP-3 inhibition of IIS, ensuring their escape from IGFBP-3-associated growth suppression. Finally, cachectic tumor-derived ImpL2, the IGFBP-3 homolog, also impaired lipid homeostasis of host cells in an established cancer-cachexia model in *Drosophila*. Most importantly, IGFBP-3 was highly expressed in cancer tissues in pancreatic and colorectal cancer patients, especially higher in the sera of cachectic cancer patients than non-cachexia cancer patients.

**Conclusion:** Our study demonstrates that tumor-derived IGFBP-3 plays a critical role in cachexia-associated lipid loss and could be a biomarker for diagnosis of cachexia in cancer patients.

**Keywords:** Biomarker; Cancer; Cachexia; Insulin-like growth factor binding protein-3; Lipid loss

## Introduction

Cancer-associated cachexia is a systemic syndrome with metabolic dysfunction and is characterized by excessive weight loss, skeletal muscle wasting, and atrophy of adipose tissues.[1-2] It is estimated that 60–80% of cancer patients are affected by cachexia and about 20% of them died of cachexia.[3] Cachexia not only is associated with increased morbidity and mortality but also accounts for the poor response to chemotherapy and increased susceptibility to chemotherapeutic toxicity.[4-5] Although advances have been made in exploring the mechanisms of cachexia, how to make use of them in clinical practice remains challenging.

Increasing evidence has revealed that multiple tumor-derived cytokines and pathways are involved in skeletal muscle degradation and fat mass loss to cause cancer cachexia.[6-7] Muscle wasting is orchestrated by extracellular ligands

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including pro-inflammatory cytokines derived from immune or tumor cells. Tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) have been shown to trigger muscle wasting through activating the nuclear factor kappa B (NF-κB) and Janus Kinase–Signal Transducer and Activator of Transcription (JAK-STAT) signaling pathways.[8] Myostatin and activin are transforming growth factor-beta (TGF-β) superfamily ligands that serve as negative regulators of insulin-like growth factor 1 (IGF1) signaling to promote skeletal muscle degradation.[9] The ubiquitin-proteasome system (UPS) is also involved in the muscle proteolytic pathways, and muscle ring-finger protein 1 (MuRF-1) and atrophy gene-1 (Atrogin-1) act as two main regulators of muscle protein breakdown.[10] However, the involvement of adipose tissue in cachexia is largely overlooked. Recently, accumulating evidence has demonstrated its unveiled roles in the development of cachexia through remodeling of adipose tissue which is associated with adipocyte atrophy, impairment of lipid turnover, modification of extracellular matrix, enhanced inflammatory signaling, and dysfunction of energy metabolism.[11] Adipogenesis represents the deposition of triglycerides (TGs) in cytoplasmic lipid droplets, while adipose triglyceride lipase (ATGL) as well as the hormone-sensitive lipase (HSL) mediate intracellular lipolysis.[12] Noticeably, unbalanced lipid turnover including increased lipolysis and impaired adipogenesis plays an important role in cachexia. In human cancer-related cachexia, it is associated with normal lipogenesis by elevated lipolysis in white adipose tissue, indicating lipid catabolism is more relevant with lipid loss in patients with cachexia.[13] It has been revealed that tumor or host cell-derived cytokines like TNF-α, IL-1β, and IL-6 contribute to cancer-associated cachexia by promoting lipolysis.[14] However, the underlying mechanism of tumor-derived cytokines in lipid loss is poorly understood.

In addition to the above-mentioned pro-inflammatory cytokines, IGF-binding protein-3 (IGFBP3) was recently discovered to be associated with cancer-associated cachexia. Adult *Drosophila* could be used as a model for organ wasting in virtue of the over-proliferation of its intestinal stem cells induced by activation of Yorkie, the *Yap1* oncogene ortholog.[15] It has been demonstrated that the organ-wasting phenotypes are associated with the increased secretion of ImpL2, the IGFBP-3 homolog that is the IGF-antagonizing peptide.[16] It was further uncovered that $yki^{3SA}$ tumors produced the platelet-drived growth factor (PDGF)- and vascular endothelial growth factor (VEGF)-related factor 1 ligand to activate host methyl ethyl ketone (MEK) signaling pathway, leading to muscle wasting.[17] In pancreatic cancer, IGFBP3 has been found to be dramatically up-regulated in tumor samples and induce muscle wasting in C2C12 muscle cells.[18] However, how cachectic tumor-derived IGFBP3 causes lipid loss and the mechanisms of tumor-adipose tissue interaction in lipid homeostasis have not been fully elucidated. Cancer-related cachexia is frequently accompanied by a loss of body fat. The neurohumoral factors that lead to body fat destruction were investigated in this study by inducing malignant tumorigenesis and ascites in *Drosophila* to mimic the phenotype of cancer-associated cachexia.

## Methods

### Cells and reagents

We purchased 3T3-L1 (CL-173) and Capan-1 (HTB79) cells from American Type Culture Collection (ATCC; Manassas, Virginia, USA). Synthetic IGFBP-3 (R&D, Minnesota, USA, 675-B3-025) and IGF-1 (R&D, 291-G1-200), IGFBP3 antibody (Santa Cruz, Dallas, Texas, USA, sc-9028), phosphorylated Akt (pAkt)-S473 antibody (Cell Signaling, Danvers, Massachusetts, USA, 9271), pAkt-T308 antibody (Cell Signaling, 13038), Akt antibody (Cell Signaling, 9272), phospho-protein kinases A (pPKA)-substrate (RRXS*/T*; Cell Signaling, 9624), α-Tubulin (Sigma, Merck KGaA, Darmstadt, Germany, T9026), and β-actin (Cell Signaling, 4970) were used in this study. Free glycerol reagent (Sigma, F6428), TG reagent (Sigma, T2449), D-glucose assay kit (K-GLUC, Megazyme, Bray, Ireland), Glycerol standard (Sigma, G7793), [U-$^{14}$C]-Glucose (PerkinElmer, Waltham, Massachusetts, USA, NEC042X050UC), and Bodipy493 (Thermo Fisher, Waltham, Massachusetts, USA, D3922) were used in this study. C26 colon cancer cells were a kind of gift from Robert Jackman.

### Induction of yki-gut tumors in fruit flies

$w^{1118}$, upstream activating sequence (*UAS*)-*ILP2-FLAG*, and $esg^{TS}$ (tub-GAL80$^{TS}$, esg-GAL4, and UAS-green fluorescent protein (GFP)) were kind gifts from Norbet Perrimon. UAS-yki (BLM 228817) was obtained from the Bloomington Drosophila Stock Center (Bloomington, India). RNA interference line 15009R-3 against ImpL2 was obtained from the National Institute of Genetics (Mishima City, Japan). To induce *yki* expression in intestinal stem cells, the crosses were set up with esg$^{TS}$ at room temperature and transferred to 18°C after 24 h to activate *GAL80*$^{TS}$, thus restricting the expression of *GAL4*-induced transgenes. Four-day-old adult progenies were placed at 29°C to induce the transgenes for 8 days. Flies were transferred onto fresh food every two days. All animal experiments in this study strictly complied with the regulations and requirements of animal experiments.

### Cell culture and preparation of conditioned medium (CM)

3T3-L1 pre-adipocytes were cultured in growth medium, high-glucose dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, and 100 U/mL penicillin, in an atmosphere of 5% $CO_2$ and 95% air at 37°C. For differentiation, 3T3-L1 pre-adipocytes were switched into the DMEM containing 10% FBS, antibiotics, 0.25 mmol/L dexamethasone, 0.5 mmol/L isobutylmethylxanthine (IBMX), 2 mmol/L rosiglitazone, and 0.1 mg/mL insulin for two days; and switched to the DMEM with 10% FBS, antibiotics, 1 mg/mL insulin, and 2 mmol/L rosiglitazone for another two days. Differentiated 3T3-L1 adipocytes were then maintained in growth medium for at least 4 days. Capan1 and C26 adenocarcinoma cells were cultured in the DMEM with 10% FBS plus antibiotics. For the CM preparation, Capan-1 cells and Colon-26 cells were

plated at a density of 50,000 cells/cm$^2$, and after 12 h, cells were washed twice with phosphate buffer saline (PBS) and then cultured for another 24 h. CMs were centrifuged at 1200 × *g* for 10 min and filtered with a 0.2 mm syringe filter, and they were used immediately or stored at −80°C.

### Lipid and glucose metabolic assays

For the lipolysis assay, 3T3-L1 adipocytes in 24-well plates were treated with control medium plus synthetic IGFBP-3 or CM from cancer cells plus anti-IGFBP-3 antibodies, washed with PBS, and incubated with 200 mL serum- and phenol-free DMEM containing 4% fatty-acid-free bovine serum albumin (BSA) for 2 h. The amount of glycerol derived from TGs through lipolysis in the medium was measured using the Free Glycerol Reagent (F6428, Sigma) to calculate lipolytic rates. We followed the procedures described previously.[19]

After treatment with IGFBP-3 or CM containing anti-IGFBP-3 antibody, 3T3-L1 adipocytes was incubated with 500 mL Krebs–Ringer buffer containing 0.5% BSA, 25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 mmol/L glucose, 0.5 mCi/mL [U-$^{14}$C]-Glucose (NEC042V250UC) for 2 h. The metabolism was stopped by trichloroacetic acid (TCA). Adipocytes were washed twice and were scraped in 1 mL of 0.6% (w/v) NaCl on ice. The lipids were extracted using chloroform/methanol mixture, and $^{14}$C-labeled lipids in the chloroform phase were measured by liquid scintillation counting to reflect lipogenic rates. Lipogenic rates were calculated and normalized with control essentially as described previously.[20]

3T3-L1 adipocytes were treated with IGFBP-3 or CM containing anti-IGFBP-3 antibody, washed, and harvested in 100 mL TNET buffer: 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 2 mmol/L ethylene diamine tetraacetic acid (EDTA), and 1% Triton X-100 with 0.5% cholate. Dissolved triacylglycerol (TAG) was measured using a Serum Triglyceride Determination kit (TR0100, Sigma) and normalized to protein level.

Glucose levels in the media before and after the treatments were measured using K-GLUC (Megazyme). Adipocytes were then lysed using radio immunoprecipitation assay lysis buffer (RIPA; 50 mmol/L) Tris-HCl [pH 7.4], 150 mmol/L NaCl, 1% sodium deoxycholate, 1 mmol/L EDTA, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40 (NP-40) containing protease and phosphatase inhibitor cocktails (Sigma) and the total protein amounts were determined by Bradford kit (Sigma). The glucose uptaken by adipocytes was calculated by normalizing the substation of glucose levels to protein amount.

### Immunoblotting and quantitative polymerase chain reaction (qPCR) analysis

Adipocytes and adult fruit flies were lysed using RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma). The total protein was quantified using a Pierce bicinchoninic acid assay (BCA) protein assay kit (Thermo Scientific, Waltham, Massachusetts, USA). Equal amounts of protein lysate were loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Merck KGaA, Darmstadt, Germany). The membranes were incubated overnight at 4°C with primary antibodies against IGFBP-3, Akt, pAkt-S473, pAkt-T208, pPKA substrate, α-Tubulin, and β-actin followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoblotting was visualized using Fluorchem FC3 system (ProteinSimple, McKinley, Minneapolis, USA) by chemiluminescence (Millipore) and quantified by densitometry using AlphaView software (Version 3.4.0.729, ProteinSimple).

### BODIPY staining

Cells were cultured on coverslips and fixed with 4% paraformaldehyde (433689M, AlfaAesar, Heysham, Lancashire, United Kingdom) in PBS at room temperature for 10 min. Cells were then stained with Dipyrromethene Boron Difluoride (BODIPY) 493/503 (ThermoFisher, D-3922, 20 μmol/L) and 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) in PBS at room temperature for 30 min. Subsequently, coverslips were mounted with mounting media onto glass slides followed by image acquisition.

### Real-time (RT) quantitative polymerase chain reaction analysis

To analyze gene expression patterns, the RNA of adipocytes and adult flies were extracted using Trizol (Invitrogen) and complementary DNA (cDNA) was transcribed using the iScript cDNA Synthesis Kit (Biorad, Hercules, California, USA) on 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, Massachusetts, USA). The relative gene expression was calculated using $2^{-\Delta\Delta Ct}$. The sequences of primers are listed in Supplementary Table 1, http://links.lww.com/CM9/B482.

### Clinical specimens

Human serum samples were collected from CRC patients with ($n = 14$) and without cachexia ($n = 31$) from Xuanwu Hospital of Capital Medical University (Beijing, China). According to the diagnosis standard of cachexia, we defined cachexia as weight loss >5% within the past six months with clinical phenotype or body mass index (BMI) <20 kg/m$^2$. The blood samples were centrifuged at 3000 × *g* for 10 min to extract the serum. The level of serum IGFBP-3 was measured using Human IGFBP-3 enzyme linked immunosorbent assay (ELISA) kit (Raybiotech, Gwinnett county, Georgia USA, ELH-IGFBP3-1). All samples were stored at −80°C until needed. All procedures were conducted with the approval of the Institutional Review Board of Xuanwu Hospital of Capital Medical University (No. [2022]073). The informed consents were obtained from all patients. The clinicopathological features are shown in Supplementary Table 2, http://links.lww.com/CM9/B482.

### Gene Expression Profiling Interactive Analysis (GEPIA) database

The GEPIA (http://GEPIA.cancer-pku.cn/index.html) online database was used to analyze gene sequencing data from the cancer genome atlas (TCGA) and Genotype-Tissue Expression (GTEx). GEPIA database analysis was used to assess the expression levels of IGFBP-3 in the patients with pancreatic adenocarcinoma, rectal adenocarcinoma, and colonic adenocarcinoma.

### Statistical analysis

Unpaired Student's *t* test and one-way analysis of variance (ANOVA) followed by *post hoc* test were performed to assess the differences. Data were presented as the mean ± standard error of mean. A *P*-value <0.05 was considered statistically significant. We performed statistical analysis by using IBM SPSS Statistics software (version 26.0, IBM Inc, Armonk, NY, United States).

## Results

### IGFBP-3 promoting lipolysis and inhibiting lipogenesis in adipocytes

Lipolysis and lipogenesis are two major regulators of lipid homeostasis in adipocytes. In order to investigate the role of IGFBP-3 on lipid metabolism, we first examined whether IGFBP-3 affected lipolytic rates in adipocytes. We added different doses of synthetic IGFBP-3 into mature 3T3-L1 adipocytes cultured with FBS-containing complete medium and measured lipolytic rates. Interestingly, we found that IGFBP-3 treatment significantly increased lipolysis in 3T3-L1 adipocytes, indicated by glycerol release into the medium, in a dose-dependent manner [Figure 1A]. To test the effect of IGFBP-3 on lipogenesis, we treated mature 3T3-L1 adipocytes with $^{14}$C-labeled glucose with or without IGFBP-3 and measured lipids derived from $^{14}$C-carbon backbones. We observed that IGFBP-3 treatment dose-dependently decreased $^{14}$C-carbon-derived lipids [Figure 1B], suggesting a suppression of lipogenesis. Consistent with lipolytic and lipogenic changes, IGFBP-3 treatment statistically significantly decreased TAG levels in 3T3-L1 adipocytes, as indicated by both biochemical assays and staining of BODIPY, a specific dye of neutral lipids [Figures 1C,D]. Collectively, these results demonstrated that IGFBP-3 diminished lipid storage by disrupting the balance between lipolysis and lipogenesis in mature adipocytes.

### IGFBP-3 modulating insulin/IGF signaling to affect expression of lipid metabolic genes

To understand whether and how IGFBP-3 modulates insulin/IGF signaling to cause lipid dysregulation in adipocytes, we treated the differentiated 3T3-L1 adipocytes with IGFBP-3 at different doses. It was found that IGFBP-3 significantly decreased Akt phosphorylation at Ser473, an important indicator of IGF/insulin signaling, in adipocytes in a dose-dependent manner. On the other hand, phosphorylation of PKA substrate was not changed [Figure 2A], indicating that IGFBP-3 did not activate PKA cascade. Many genes important in lipid metabolism are regulated by IGF/insulin signaling. For instance, lipogenic genes *Acc1*, *Acly*, and *Fasn* are regulated by sterol regulatory element-binding transcription factor 1 (SREBP1c), whereas lipolytic gene *Atgl* is regulated by Forkhead box protein O (FoxO).[21] We measured their expression pattern after IGFBP-3 treatment and found that IGFBP-3 potently suppressed the expressions of *Acc1*, *Acly*, and *Fasn* [Figure 2B], while promoted *Atgl* levels. Of note, *Hsl*, another important lipase gene that is independent of IGF/insulin signaling, was not affected statistically significantly [Figure 2C]. Thus, our results suggest that exogenous IGFBP-3 modulates the expression of lipolytic and lipogenic genes through impairing insulin/IGF signaling.

Classically, IGFBP-3 binds to IGFs to form the ternary and binary complex and by which inactivates IGFs, leading to failure of binding to type I IGF-receptor (IGF-1R) to regulate cellular proliferation, growth, and survival.[22] FBS-containing medium includes plenty of IGF-1 and insulin, we thus hypothesized that IGFBP-3 might block their bioavailabilities to suppress insulin/IGF signaling via binding to IGFs. We treated the matured adipocytes with IGFBP-3 and IGF-1 in a serum-free medium. As expected, IGF-1 alone robustly activated insulin/IGF signaling, as indicated by the pAkt at Ser473 as well as T308 in adipocytes. However, IGFBP-3 completely abolished IGF-1-dependent phosphorylation of Akt at both sites [Figure 2D]. Consistently, IGFBP-3 also impaired IGF-1-associated suppression of *Atgl* expression and lipolysis in 3T3-L1 adipocytes [Figure 2E]. It is noteworthy that, IGFBP-3 alone failed to change either lipid metabolic expression or lipolysis, suggesting that metabolic regulation of IGFBP-3 impinges on IGF-1 function.

### Cachectic cancer cells producing IGFBP-3 to suppress IGF signaling in adipocytes

Since cachectic tumors have been shown to impair IGF signaling and anabolism, we hypothesized that tumor cells produced IGFBP-3 to affect host tissues. We examined the IGFBP-3 expression in different tumor samples such as pancreatic tumors that are most likely to develop severe wasting syndrome, and analyzed the gene expression in two independent published datasets from both pancreatic tumor and control samples. Interestingly, the mRNA levels of IGFBP-3 were significantly increased in pancreatic tumors compared with control samples, as indicated by different probes in the two datasets [Figures 3A,B]. In addition, we analyzed IGFBP-3 levels in both C26 and Capan-1 cancer cells that have robust lipid loss in adipocytes,[23-24] and observed an increase in IGFBP-3 protein levels [Figure 3C].

We next investigated whether cancer cells could secret IGFBP-3 to target adipocytes and suppress their IGF signaling. We treated differentiated adipocytes with CM from the cachectic cancer cells that contain plenty of IGFBP-3 and, interestingly, we found that the IGF signaling as indicated by pAkt was dramatically decreased

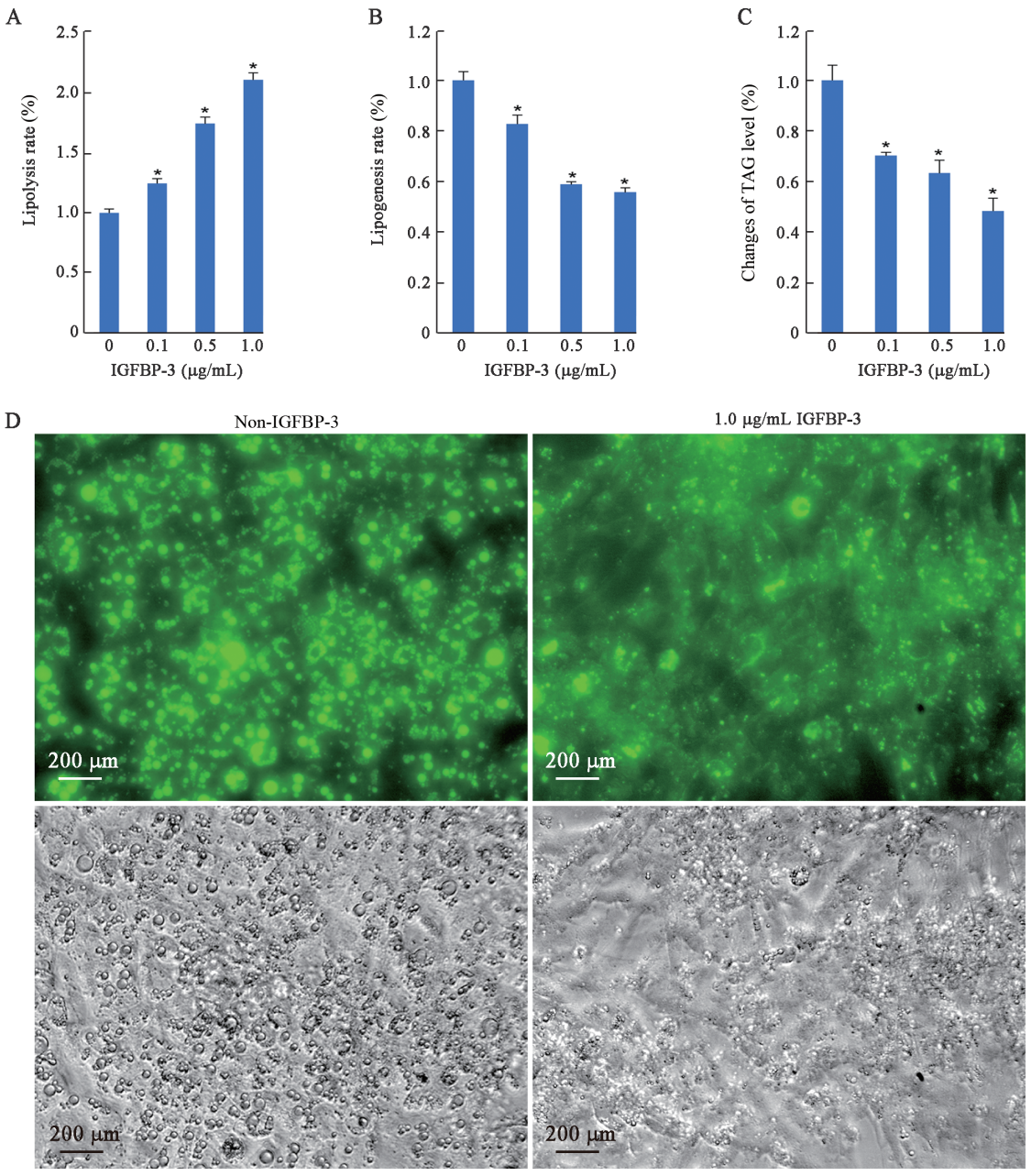


**Figure 1:** IGFBP-3 decreased lipogenesis and increased lipolysis. (A) 3T3-L1 adipocytes were treated with different doses of synthetic IGFBP-3 as indicated for 12 h. Lipolysis rate was determined using the Free Glycerol Reagent (Sigma). (B) 3T3-L1 adipocytes were treated with $^{14}$C-labeled glucose together with different doses of synthetic IGFBP-3 for 12 h as indicated. Lipogenesis rate was measured by liquid scintillation counting. (C, D) 3T3-L1 adipocytes were treated with synthetic IGFBP-3 for 12 h, and TAG levels were determined by biochemical assay. Representative photos indicate the neutral lipids stained by BODIPY. $*P < 0.05$ by two-tailed unpaired Student's *t* test. BODIPY: Dipyrromethene Boron Difluoride; IGFBP-3: Insulin-like growth factor (IGF) binding protein-3; TAG: Triacylglycerol.

[Figures 3D,E]. The expression levels of IGF-downstream lipid metabolic genes, like *Atgl* and *Fasn*, were also affected by CM of either Capan-1 and C26 cells [Figures 3F,G]. To examine whether the suppression of IGF signaling was caused by cancer cell-derived IGFBP-3 in CM, we neutralized the bioactive IGFBP-3 in the CM with a specific IGFBP-3 neutralizing antibody. Strikingly, the IGF signaling activity, as well as lipid metabolic gene expression, were changed by IGFBP-3 neutralizing antibody in the CM of either Capan-1 and C26 cells [Figures 3F,G]. These results demonstrated that either C26 or Capan-1 cancer cells produced IGFBP-3 to suppress IGF signaling in adipocytes.

### *Cachectic cancer cells producing IGFBP-3 to impair lipid homeostasis in adipocytes*

Furthermore, we investigated whether cancer cell-derived IGFBP-3 could impair lipid metabolism in differentiated 3T3-L1 adipocytes. The activities of lipolysis and lipogenesis were examined. Consistent with changes in IGF signaling and the expression of genes related to

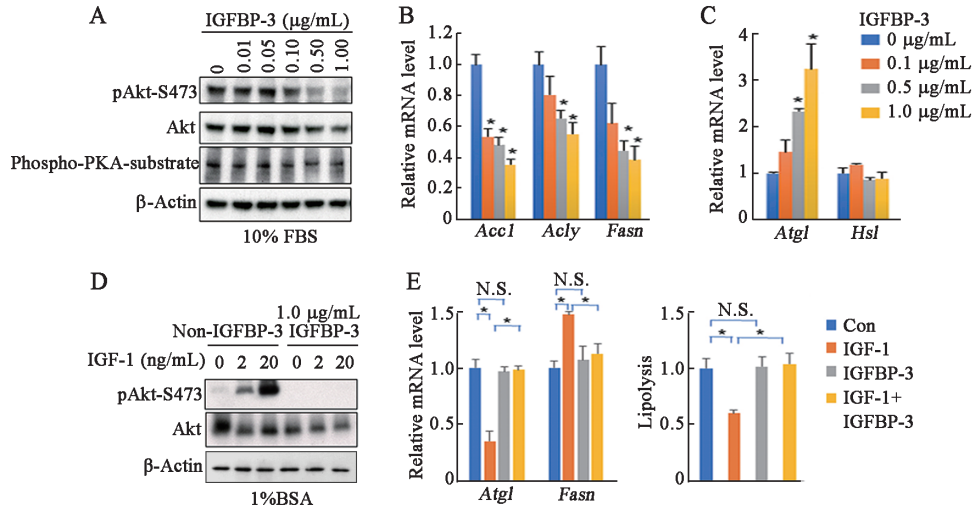


**Figure 2:** IGFBP-3 decreased IGF/insulin signaling and dysregulated the expression of lipid metabolic genes. (A) 3T3-L1 adipocytes were treated with different doses of synthetic IGFBP-3 as indicated for 12 h. The expression of phosphorylation of Akt at Ser473 and Akt protein as well as phosphorylation of PKA substrate was determined using Western blot analysis. (B, C) RT-qPCR was performed to examine the mRNA level of lipogenic genes (*Acc1*, *Acly*, and *Fasn*) and lipolytic genes (*Atgl* and *Hsl*). *$P$ <0.05 *vs.* control. (D) 3T3-L1 adipocytes were treated with different doses of IGF-1 (0 ng/mL, 2 ng/mL, and 20 ng/mL) plus 1 μg/mL IGFBP-3 for 1 h. Western blot analysis was performed to determine the expression of p-Akt (Ser473) and Akt. (E) RT-qPCR analysis of the expression of *Atgl* and *Fasn* in 3T3-L1 adipocytes treated with IGF-1, IGFBP-3, or both for 12 h. Lipolysis was determined using the Free Glycerol Reagent (Sigma). BSA: Bovine serum albumin; Con: Control; FBS: Fetal bovine serum; IGFBP-3: Insulin-like growth factor (IGF) binding protein-3; mRNA: Messenger ribonucleic acid; N.S.: No significance; pAkt: Phosphorylated Akt; PKA: Protein kinase A; RT-qPCR: Real-time quantitative polymerase chain reaction.

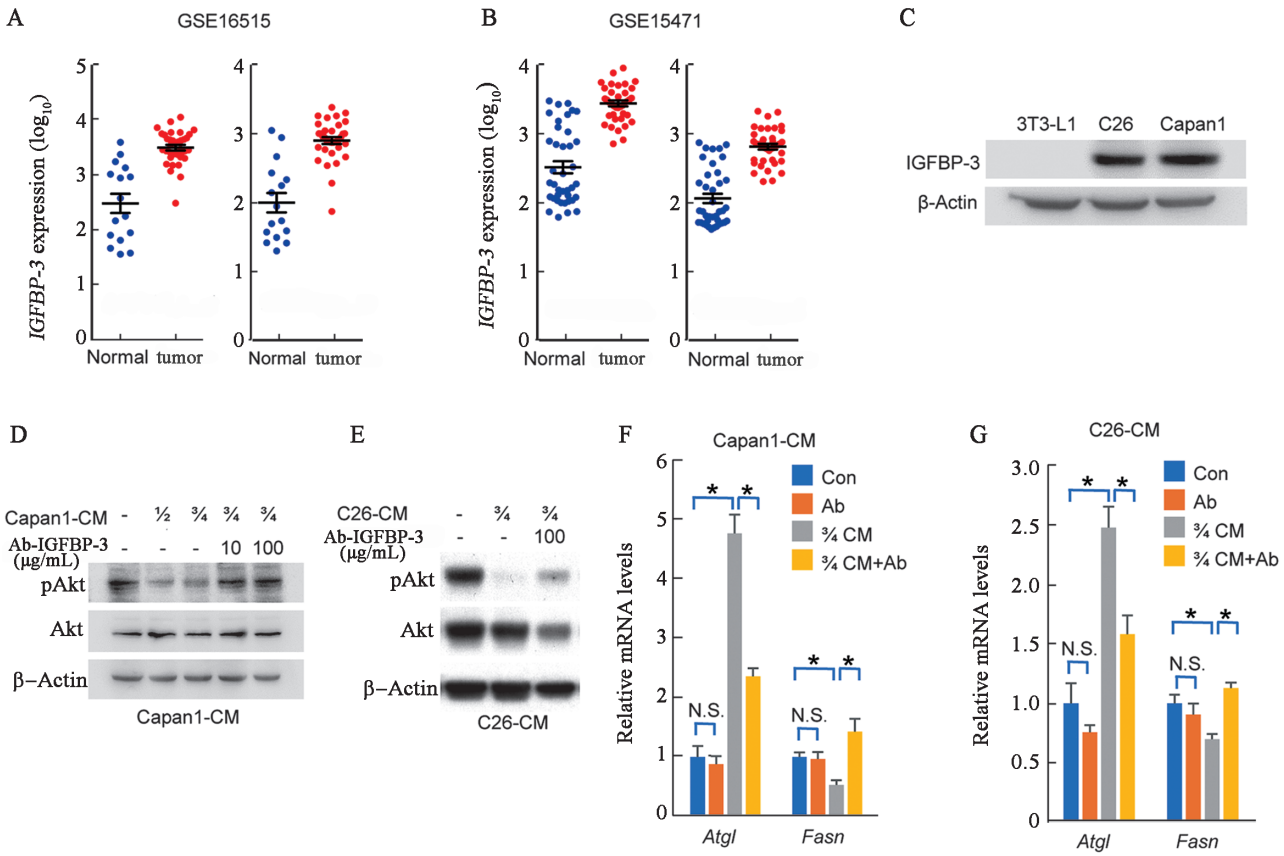


**Figure 3:** Cachectic tumor cells produced IGFBP-3 to suppress IGF signaling in adipocytes. (A, B) The expression of IGFBP-3 mRNA was analyzed in two independent published datasets (GSE16515 and GSE15471). (C) Western blot analysis on the expression of IGFBP-3 was performed in 3T3-L1 adipocytes as well as C26 and Capan-1 cells. (D, E) 3T3-L1 adipocytes were treated with conditioned medium (CM) from Capan1 or C26 cells with or without IGFBP-3 neutralizing antibody for 1 h. Western blot analysis of the expression of p-Akt (Ser473) and Akt was shown. (F, G) RT-qPCR was performed to determine the mRNA level of *Atgl* and *Fasn* in 3T3-L1 adipocytes treated as indicated for 12 h. *$P$ <0.05 *vs.* control. Ab: Antibody; Ab-IGFBP-3: Antibody of IGFBP-3; Con: Control; IGFBP-3: Insulin-like growth factor (IGF) binding protein-3; mRNA: Messenger ribonucleic acid; N.S.: No significance; pAkt: Phosphorylated Akt; RT-qPCR: Real-time quantitative polymerase chain reaction.

lipid metabolism, the lipolytic rates were significantly enhanced, while the lipogenic rates were reduced in adipocytes treated with CM from Capan-1 cells [Figures 4A,B]. The TAG storage in adipocytes as measured by both biochemical assays and after staining with BODIPY, were also significantly decreased [Figures 4C,D]. The same with C26 cells [Figures 4E,F,G,H]. Importantly, the antibody added to neutralize IGFBP-3 in the CM significantly improved lipid metabolic imbalance and increased TAG storage in the adipocytes [Figures 4A–H]. It should be noted that, in comparison with Capan-1 cells, the effects of IGFBP-3 antibody neutralization in the CM from C26 cells were comparatively insignificant in terms of improving lipid metabolism [Figures 4A–H], suggesting that C26 cells may also secrete additional proteins that can alter IGF signaling and lipid metabolism in adipocytes.

### *IGFBP-3 failing to affect the growth of cachectic cancer cells*

As demonstrated above, cancer cells produced IGFBP-3 to impair IGF signaling and lipid metabolism in adipocytes. We next investigated whether IGFBP-3 could affect tumor growth. Previous studies have indicated that IGFBP-3 fails to suppress Capan-1 cell growth unless at a very high concentration,[18] suggesting an unknown resistance to IGFBP-3 treatment in Capan-1 cells. We therefore treated C26 cells with different doses of IGFBP-3 to examine whether IGFBP-3 affected C26 cell growth. Similar to Capan-1 cells, we found that IGFBP-3 failed to affect either IGF signaling [Figure 5A] or proliferation rates [Figures 5B–C] of C26 cells even at the high concentration of 5 μg/mL. We also investigated whether endogenous IGFBP-3, in addition to exogenous one, was required for C26 cell growth, and neutralized IGFBP-3 via adding specific antibodies in the medium. Again, we did not observe any changes in IGF signaling or proliferation rates of C26 cells [Figures 5D–F]. These data collectively demonstrated that cachectic cancer cells that produced a large amount of IGFBP-3 were resistant to IGFBP-3 signaling.

### *Gut tumors producing IGFBP-3 homolog to cause lipid loss in Drosophila*

A conserved *Drosophila* model mimicking tumor-induced organ wasting, including lipid loss was established in previous studies.[15,17] Researchers found that malignant gut tumors, which were induced by oncogene *yki*, produced ImpL2, a fly IGFBP homolog, to block systemic IGF/insulin signaling and caused wasting.[14] However, whether tumor-derived ImpL2 impairs lipid metabolism in a manner similar to mammalian adipocytes is not well understood. We thus investigated the molecular regulation of lipid metabolic genes in this gut tumor model. As expected, induction of *yki* in the gut caused tumor formation and subsequent abdomen bloating, lipid loss, and hyperglycemia [Figures 6A–E]. Consistent with the findings in cultured adipocytes, we found that the expression levels of lipogenic gene *Fas*, the homolog of mouse *Fasn*, was significantly suppressed, while the lipolytic gene *bmm*, the homolog of mouse *Atgl*, was dramatically induced, in yki-tumor-bearing

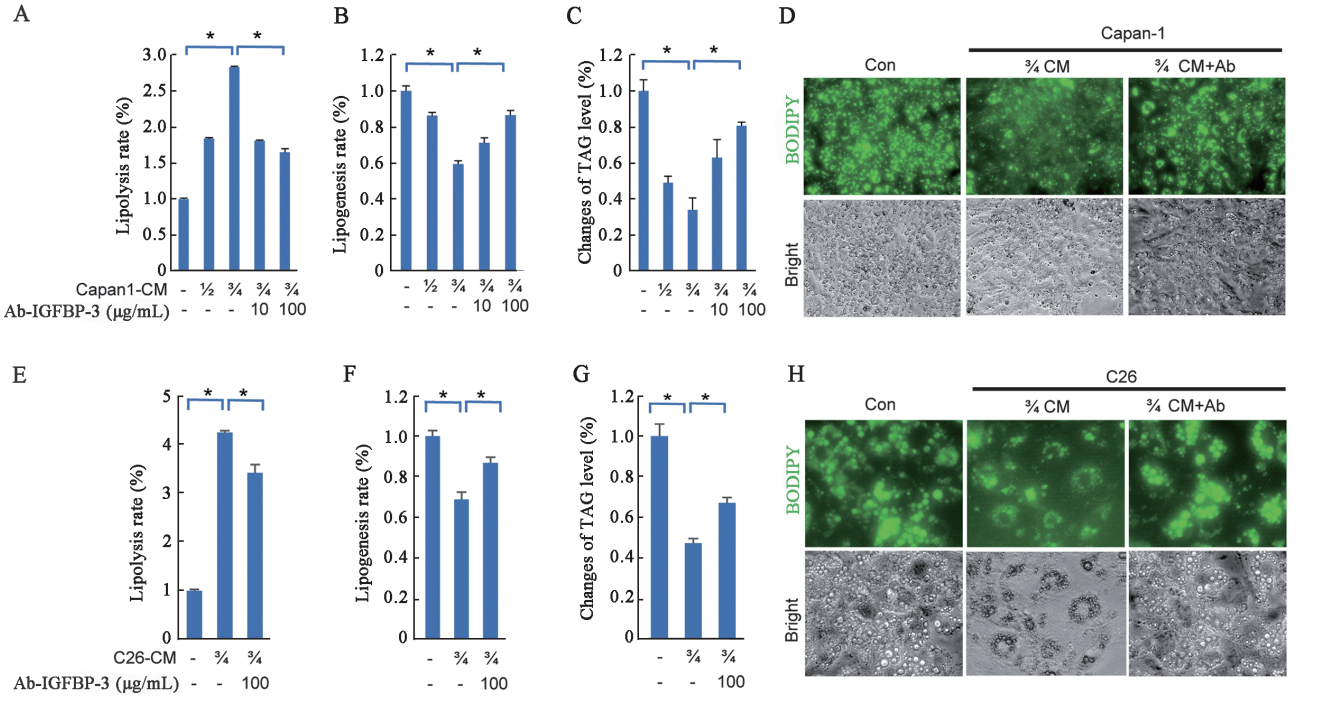


**Figure 4:** Cachectic tumor cells secreted IGFBP-3 to cause lipid loss in adipocytes. 3T3-L1 adipocytes were treated with CM from Capan1 or C26 cells without or with different doses of IGFBP-3 neutralizing antibody for 12 h. Lipolysis rate (A, E), lipogenesis rate (B, F), and TAG level (C, G) were examined. (D original magnification × 200, H original magnification × 200) The neutral lipids were stained with BODIPY and representative photos were indicated. Ab: Antibody; CM: Conditioned medium; Con: Control; IGFBP-3: Insulin-like growth factor (IGF) binding protein-3; TAG: Triacylglycerol.

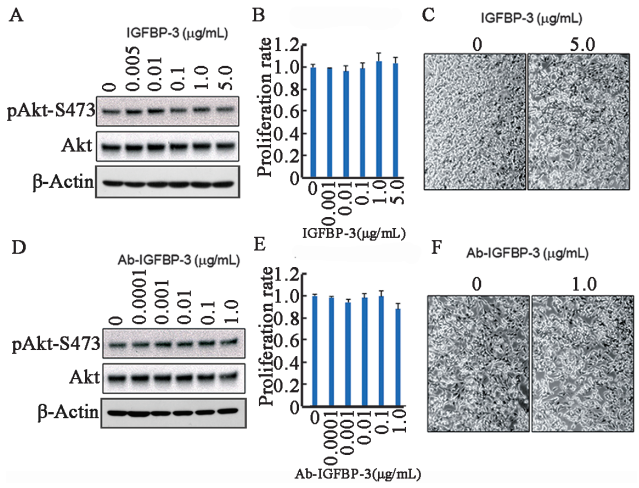


**Figure 5:** IGFBP-3 failed to affect the growth of cachectic cancer cells. (A, D) C26 cells were treated with different doses of IGFBP-3 or IGFBP-3 neutralizing antibody as indicated for 12 h. The expression of pAkt (Ser473) and Akt was shown using Western blot analysis. (B, E) Cell proliferation after IGFBP-3 treatment for three days was examined by MTT assay. (C, F) Cell morphology was observed by optical microscopy (C and F original magnification × 200). Ab: Antibody; IGFBP-3: Insulin-like growth factor (IGF) binding protein-3; MTT: Methyl thiazolyl tetrazolium assay; pAkt: Phosphorylated Akt.

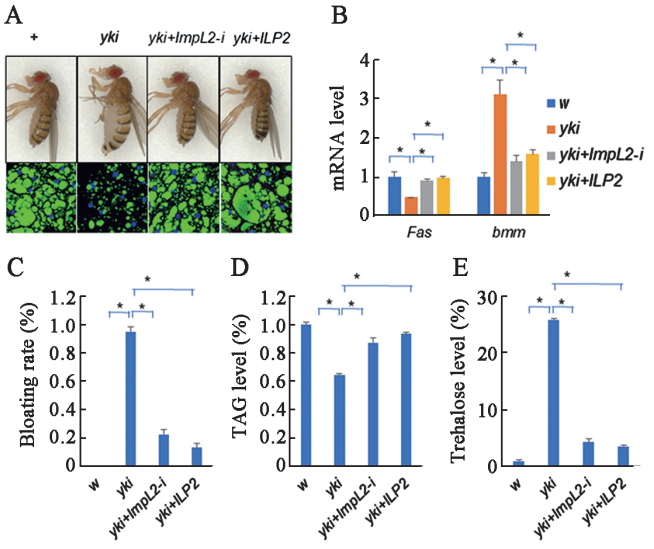


**Figure 6:** Gut tumors produced IGFBP-3 homolog to cause lipid loss in *Drosophila*. (A, B) Knockdown of *ImpL2* or overexpression of ILP2 in *yki*-tumors at day 8 improved host abdominal fat in *yki*-gut *Drosophila*, and statistical analysis of abdomen bloating was performed ($n = 3$, 5 flies/replicate) (A, [original magnification × 400]). Fat-body-specific reporter indicates depletion of this tissue in the abdomen of hosts in the presence of the tumor. Green indicates lipid droplets, which are the storage vesicles of the adipose tissue. (B) RT-qPCR was performed to determine the mRNA level of *Fas* and *Bmm* in gut tumors of *Drosophila*. (C) The bloating rates of wild-type *yki*, knock-down *ImpL2* in *yki*-tumors and overexpression of ILP2 in *yki*-tumors, respectively. (D, E) The levels of TAG and trehalose were determined ($n = 3$, 30 flies/group). *$P$ <0.05 by two-tailed unpaired Student's $t$-test. IGFBP-3: Insulin-like growth factor binding protein-3; ILP2: Insulin-like peptide 2; *ImpL2*-i: *ImpL2*-RNAi; mRNA: Messenger ribonucleic acid; RT-qPCR: Real-time quantitative polymerase chain reaction; TAG: Triacylglycerol; w: Wild type.

flies [Figure 6B]. To investigate whether tumor-derived ImpL2 promoted lipid imbalance, *ImpL2* was knocked down specifically in the *yki*-gut tumors. Interestingly, the expression levels of *Fas* and *bmm* were both restored to normal [Figure 6B]. So were the TAG storages, glycemic levels, as well as abdomen bloating phenotypes [Figures 6A–E]. Our results indicate that malignant gut tumors produce IGFBP-3 homolog to impair systemic IGF signaling and lipid homeostasis.

Similar to mammalian IGF1/2 restricted by excessive IGFBP-3, fly insulin-like peptide 2 (ILP2) is the major target restricted by ImpL2. We wondered whether enhancing ILP2 production could conquer the suppression of systemic IGF/insulin signaling by tumor-derived ImpL2 and improve wasting by overexpressing ILP2 in the *yki*-gut tumors. Strikingly, we found that lipid metabolic genes like *Fas* and *bmm* were both restored by ILP2 overexpression in the gut tumors [Figure 6B]. Moreover, the TAG storage, glycemic levels, as well as abdomen bloating phenotypes were all restored to normal levels [Figures 6C–E]. These results indicate that increasing bioactive ILP2 is sufficient to break ImpL2 restriction on systemic IGF/insulin signaling and lipid homeostasis.

### *IGFBP-3 highly expressed in cancer tissues and served as a biomarker for cachexia in cancer patients*

In order to explore the expression of IGFBP-3 in tissues of cancer patients, we used online GEPIA database and found that the expression of IGFBP-3 was statistically significantly increased in cancer tissues of pancreatic cancer compared with normal tissues ($P$ <0.05). The expression of IGFBP-3 was also increased in colorectal cancer tissues although there was no statistical significance [Figure 7A]. Further Kaplan–Meier (KM) plotter (https://kmplot.com/analysis) analysis indicated that the expression of IGFBP-3 was significantly associated with poor overall survival in both pancreatic and colorectal cancer patients [Figures 7B, C]. Moreover, we found that the serum level of IGFBP-3 was significantly increased in cachectic colorectal cancer compared with non-cachectic controls [Figure 7D]. In addition, there was more liver metastasis in cachectic patients than that of non-cachectic counterparts [Supplementary Table 2, http://links.lww.com/CM9/B482], indicating that serum IGFBP-3 could serve as a potential biomarker for diagnosis of cachexia and predicting metastasis in cancer patients. Overall, our study demonstrates cachectic tumor cells-derived IGFBP-3 stimulates lipid loss by antagonizing IGF/insulin signaling [Figure 8].

## Discussion

Cancer-associated cachexia is a metabolic syndrome that is characterized by the loss of skeletal muscle mass and fat mass.[25-26] In the cachectic stage of cancer, proteins lost due to muscle atrophy are recycled by the liver through making amino acids. The associative alanine and glutamine provide energy for gluconeogenesis and tumor growth, thus promoting cancer cachexia. At the cachectic stage, as hepatic gluconeogenesis progresses, there is significant insulin resistance and a significant decrease of IGF-1 in the blood, which significantly reduce glucose utilization in the muscle, inhibit muscle growth, and lead to muscle wasting.[27] Although deple-

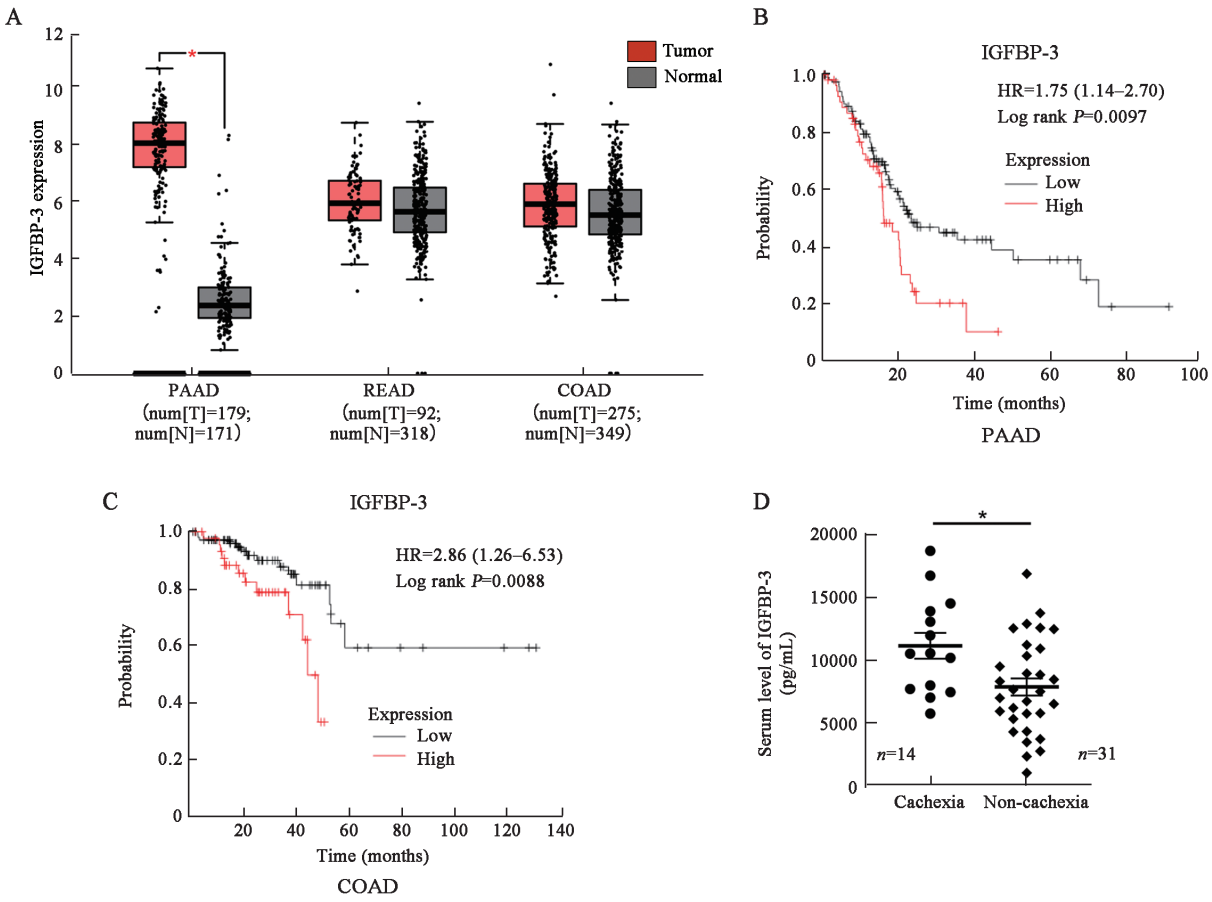


**Figure 7:** IGFBP-3 was highly expressed in cancer tissues and serum of cachectic cancer. (A) Gene Expression Profiling Interactive Analysis (GEPIA) indicated IGFBP-3 was highly expressed in pancreatic cancer tissues ($P$ <0.05) and colorectal cancer tissues ($P$ >0.05) compared with normal tissues. (B, C) The association of the expression of IGFBP-3 with overall survival was analyzed using Kaplan–Meier (KM) plotter (https://kmplot.com/analysis) in both PAAD and COAD. The patients were divided by high and low IGFBP-3 expression levels using the auto-select best cutoff, and log-rank $P$-value was shown. (D) The serum level of IGFBP-3 in cachectic and non-cachectic colorectal cancer patients was determined by ELISA assay. This graph was generated using Graphpad prism 9. *$P$ <0.05. COAD: Colonic adenocarcinoma; ELISA: Enzyme linked immunosorbent assay; HR: Hazard ratio; IGFBP-3: Insulin-like growth factor (IGF) binding protein-3; PAAD: Pancreatic adenocarcinoma; READ: Rectal adenocarcinoma.

tion of body mass is proposed to be the critical phenotype observed in cancer cachexia, increasing evidence showed that lipolysis and lipid wasting may occur before muscle loss.[1] Fat loss in cachectic cancer patients regularly occurs via breakdown of white adipose tissue in response to anorexia and other negative energy balance.[28-29] This study found that in cancer-associated cachexia mice and patients, white fat was converted significantly to calorie-burning brown fat, leading to increased energy expenditure and organ failure. It was also noted that inflammation had an important role in the conversion of white fat to brown fat during cancer-related cachexia, implying a potential therapeutic target.[30] In addition to the well-known pro-inflammatory/immune cytokines derived from cachectic tumors, IGFBP-3 was recently proposed to play an essential role in tumor-induced muscle wasting.[18] In this study, we revealed that tumor-derived IGFBP-3 played an evolutionarily conserved role in cachexia-associated lipid loss and could be used as a biomarker for the diagnosis of cachexia in cancer patients. These results shed light on the mechanisms of tumor-adipose interaction in regulating lipid homeostasis in cancer cachexia.

Excessive IGFBP-3 binds to insulin-like growth factors (IGFs) to form complexes and prevent IGFs from targeting IGF receptors to regulate cellular proliferation, growth, survival, migration, and development. In the present study, we revealed the direct role of IGFBP-3 in lipid loss in mature adipocytes by promoting lipolysis and inhibiting lipogenesis in an insulin/IGF signaling-dependent manner. Mechanistically, IGFBP-3 decreased IGF/insulin signaling, as indicated by Akt phosphorylation at S473, to modulate both sterol regulatory element binding protein (SREBP)- and forkhead box, sub-group O (FoxO)-mediated gene expression involved in lipid metabolism,[31] tilting the balance toward lipolysis in adipocytes. As IGF/insulin signaling regulated glucose uptake in adipocytes, our results suggest that IGFBP-3 may impair IGF/insulin-mediated glucose uptake. Of note, IGFBP-3 can also function in an IGF-independent manner.[32] Whether and how IGF-independent actions of IGFBP-3 participate in the adipocyte functional modulation would be another interesting question in future studies.

Our results indicated that both Capan-1 pancreatic cancer cells and C26 colon cancer cells produced IGFBP-

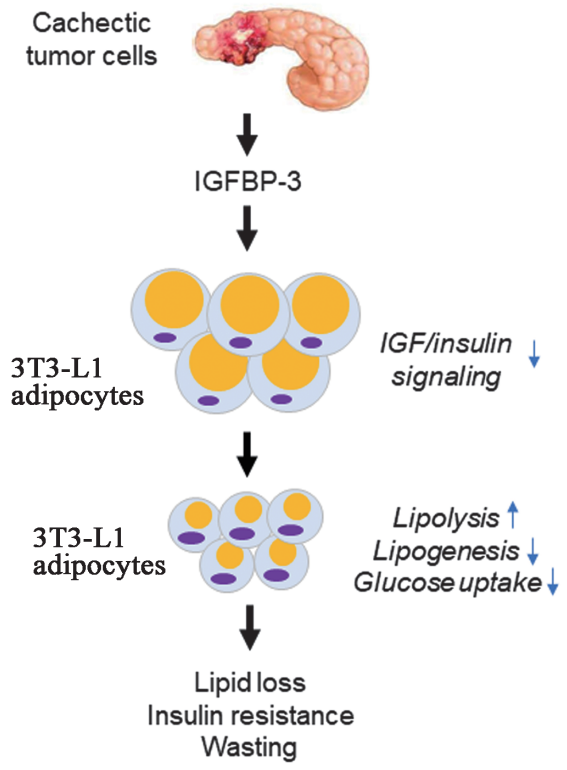


**Figure 8:** Schematic depiction of cachectic tumor cells-derived IGFBP-3 stimulates lipid loss by antagonizing IGF/insulin signaling. IGFBP-3: Insulin-like growth factor (IGF) binding protein-3.

3 to restrain IGF response and impact adipocyte wasting, including lipid loss and insulin resistance, indicating a general mechanism of tumor-adipocyte interaction. However, we noticed the different rescue effects of IGFBP-3 neutralizing antibody between these two cell lines. As compared to almost full restoration of IGF signaling, lipid metabolic gene expression, as well as TAG storage, in adipocytes by IGFBP-3 antibody neutralization in Capan-1 CM, the effects in C26 CM were relatively mild. The possible mechanism could be that C26 produces other secreted IGFBP family members,[33] in addition to IGFBP-3, to block IGF response. A few inflammatory cytokines such as IL-6 and IL-6 family cytokine (LIF) have been reported to impair IGF/insulin signaling in adipocytes and are found to be produced by C26 cells,[34-35] and these cytokines might also contribute to C26-disrupted IGF response and lipid homeostasis in adipocytes.

Cultured 3T3-L1 adipocytes only partially mimic the functions of white adipose tissues, like lipogenesis, lipolysis, as well as glucose uptake.[36] We found that tumor-derived IGFBP-3 contributed to the development of cachexia due to the increased lipolysis and reduced lipogenesis. These results were consistent with the findings that *Atgl* and *Hsl* knockout mice exhibited resistance to the development of cancer-associated cachexia.[12] Lipolysis is a sequential process orchestrated mainly by *Atgl* and *Hsl*. It can also be activated by inflammatory cytokines like IL-1, IL-6, and TNF-α. Elevated lipolysis is a characteristic of cachexia in both patients and rodent models.[12,37] In addition to increased lipolysis, reduction in lipogenesis and impaired adipocyte turnover are also responsible for the loss of adipose tissue.[4,38] Our study highlights the pro-cachectic role of IGFBP-3 by enhancing *Atgl* activity and suppressing lipogenic enzymes to produce lipid loss. However, the beige adipocytes and their transformed brown adipocytes are also very essential for systemic lipid metabolism.[39] Particularly, cachectic tumors cause lipid loss via the regulation of thermogenesis and browning of beige fat.[40-41] Since IGF/insulin signaling globally exists in each single cell and modulates their physiological process, whether IGFBP-3 directly controls beige/brown adipocyte functions and contributes to tumor-induced lipid loss warrant future investigation.

IGFBP-3, one of IGFBP family members, is mostly abundant in blood circulation. Accumulated studies have reported that IGFBP-3 affects multiple signaling pathways, such as mechanistic target of rapamycin complex 1 (mTORC1) and p53, central regulators of cell growth and metabolisms.[42-44] As a result, IGFBP-3 has been used as a promising diagnostic and prognostic biomarker in gastrointestinal tumors. For instance, combination analysis revealed that the gene variants of IGF2 and IGFBP-3 had great effects on the risk factors of gastric cancer.[45] Murphy *et al*[46] evaluated IGF-1 and IGFBP-3 circulating levels in colorectal cancer patients and showed that, based on the genetic factors, IGFBP-3 was associated with colorectal cancer risk. IGF3 and IGFBP-3 also have profound effects on therapeutic values. Radiotherapy is an important treatment method for oral squamous cell carcinoma. After receiving radiotherapy, IGFBP-3 induced the activation of NF-κB, the secretion of cytokines, and the production of reactive oxygen species, providing the evidence that IGFBP-3 can be a biomarker for radiotherapy.[47] Finally, IGFBP-3 was proposed to be involved in the skeletal muscle wasting induced by pancreatic ductal adenocarcinoma. This model was associated with systemic inflammatory reaction which has great value in digestive system cancer treatment.[48]

Notably, the effects of IGFBP-3 in adipose tissue were recapitulated in an evolutionarily conserved *Drosophila* model, in which malignant *yki*-gut tumors caused abdomen bloating, lipid loss, and hyperglycemia in the fly via production of ImpL2, an established IGFBP-3 homolog. ImpL2 blocks the dissemination of IGF-like peptides in the blood, blocks the IGF/insulin pathway in adipose and muscle, and decreases protein synthesis in adipose and muscle, thus leading to fat loss and muscle dysfunction. Since activated *yki* in tumors can autonomously activate IGF/insulin pathway, the activation of intracellular IGF/insulin pathway can still be maintained in the presence of large amounts of ImpL2 blocking extracellular IGF-like peptides, maintaining tumor proliferation.[15] Strikingly, we revealed that ILP2 overexpression, in addition to ImpL2 removal, in the *yki*-gut tumors remarkably conquered ImpL2-impaired systemic IGF/insulin signaling and lipid metabolism, indicating that an increase in IGF/insulin response in the host

organs might be sufficient to alleviate lipid loss in tumor-bearing flies. The *Drosophila* intestinal stem cell tumor model is a reliable platform for screening therapeutic peptides and also allows for convenient studies of related mechanisms.[15,17,49] Although similar effects have not yet been characterized in mammals, our findings imply a therapeutically novel concept for prevention or treatment of cancer-associated cachexia in mammals. Importantly, IGFBP-3 was highly expressed in cancer tissues compared with normal tissues and was associated with poor overall survival in both pancreatic and colorectal cancer patients. More importantly, the serum level of IGFBP-3 was higher in cachectic cancer than that of non-cachectic controls, indicating serum IGFBP-3 could be a potential biomarker for cachectic patients. While we proposed that tumor-derived IGFBP-3 participated in modulating lipolysis in matured adipocytes, further studies are still required to unveil the intracellular signaling pathway. In addition, we used *Drosophila* to investigate the functional mechanisms of *lmpL2* in *yki*-gut tumors, but the difference of species such as *Drosophila*, mice and people still could not be excluded. Thus, further functional studies for concrete regulatory mechanisms are of great interest.

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### Conflicts of interest

None.

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