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Intestinal Reg4 deficiency confers susceptibility to high-fat diet-induced liver steatosis by increasing intestinal fat absorption in mice

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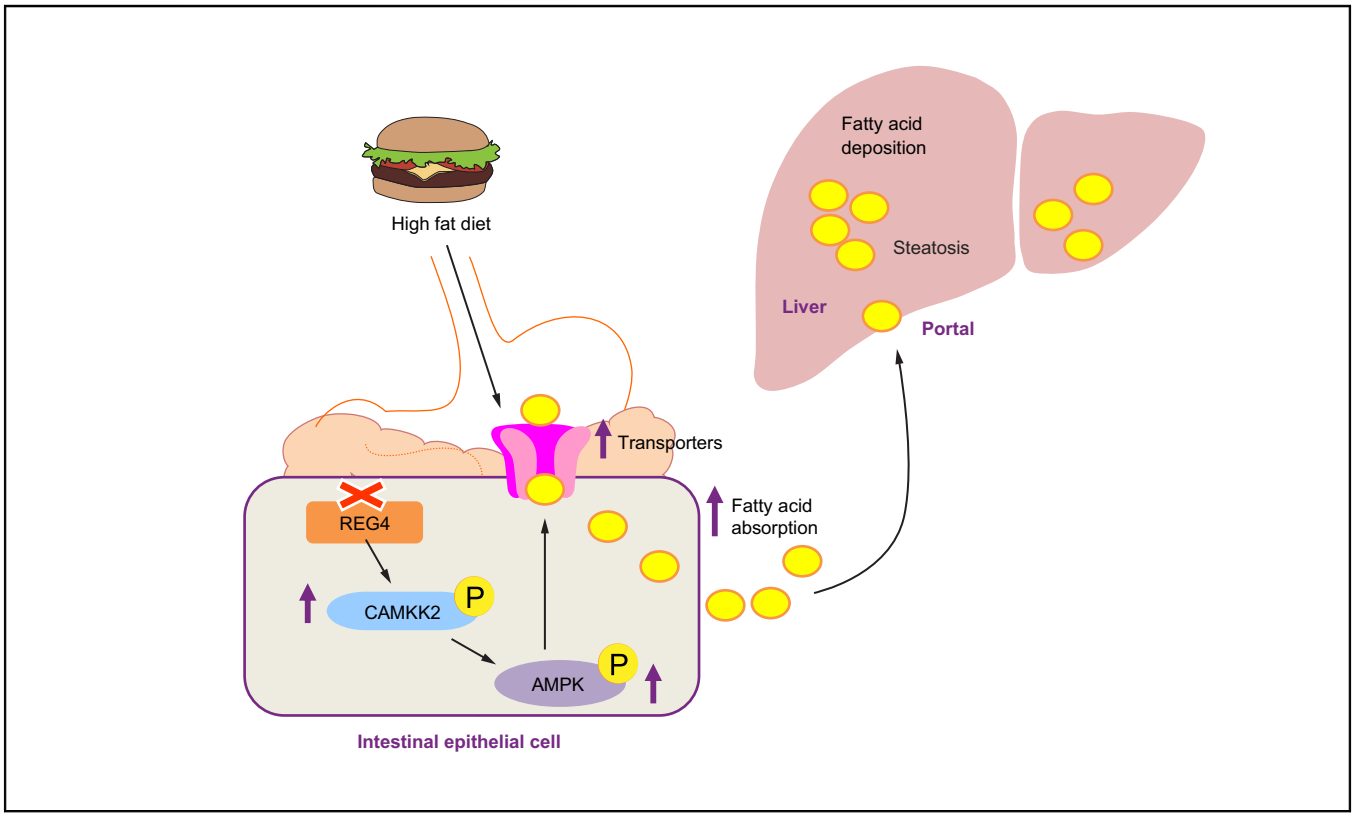
Graphical abstract



Highlights

- Intestinal *Reg4* deletion is prone to high-fat-diet-induced liver steatosis in mice.
- Intestinal *Reg4* deficiency increases intestinal fat absorption with AMPK signalling activation in mice.
- REG4 inhibits fat uptake in intestinal epithelial cells via altering AMPK activation.
- Serum REG4 levels are reduced with liver steatosis progression in children with obesity.

Impact and Implications

Hepatic steatosis is a key histological feature of non-alcoholic fatty liver disease, which is the leading chronic liver disease in children leading to the development of metabolic diseases; however, little is known about mechanisms induced by dietary fat. Intestinal REG4 acts as a novel enteroendocrine hormone reducing high-fat-diet-induced liver steatosis with decreasing intestinal fat absorption. REG4 may be a novel target for treatment of paediatric liver steatosis from the perspective of crosstalk between intestine and liver.

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Intestinal Reg4 deficiency confers susceptibility to high-fat diet-induced liver steatosis by increasing intestinal fat absorption in mice

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Background & Aims: Regenerating gene family member 4 (REG4) is a novel marker for enteroendocrine cells and is selectively expressed in specialised enteroendocrine cells of the small intestine. However, the exact roles of REG4 are largely unknown. In this study we investigate the effects of REG4 on the development of dietary fat-dependent liver steatosis and the mechanisms involved.

Methods: Mice with intestinal-specific *Reg4* deficiency (*Reg4*$^{\Delta IEC}$) and *Reg4*-floxed alleles (*Reg4*$^{fl/fl}$) were generated to investigate the effects of Reg4 on diet-induced obesity and liver steatosis. Serum levels of REG4 were also measured in children with obesity using ELISA.

Results: *Reg4*$^{\Delta IEC}$ mice fed a high-fat diet demonstrated significantly increased intestinal fat absorption and were prone to obesity and hepatic steatosis. Importantly, *Reg4*$^{\Delta IEC}$ mice exhibit enhanced activation of adenosine monophosphate-activated protein kinase (AMPK) signalling and increased protein abundance of the intestinal fat transporters, as well as enzymes involved in triglyceride synthesis and packaging at the proximal small intestine. Moreover, REG4 administration reduced fat absorption, and decreased the expression of intestinal fat absorption-related proteins in cultured intestinal cells possibly via the CaMKK2-AMPK pathway. Serum REG4 levels were markedly lower in children with obesity with advanced liver steatosis (p <0.05). Serum REG4 levels were inversely correlated with levels of liver enzymes, homeostasis model assessment of insulin resistance, low-density lipoprotein cholesterol, and triglycerides.

Conclusions: Our findings directly link *Reg4* deficiency with increased fat absorption and obesity-related liver steatosis, and suggest that REG4 may provide a potential target for prevention and treatment of liver steatosis in children.

Impact and Implications: Hepatic steatosis is a key histological feature of non-alcoholic fatty liver disease, which is the leading chronic liver disease in children leading to the development of metabolic diseases; however, little is known about mechanisms induced by dietary fat. Intestinal REG4 acts as a novel enteroendocrine hormone reducing high-fat-diet-induced liver steatosis with decreasing intestinal fat absorption. REG4 may be a novel target for treatment of paediatric liver steatosis from the perspective of crosstalk between intestine and liver.

Introduction

With the growing epidemic of childhood obesity, 55–80% of obese children present with fatty liver, which has been the most common cause of chronic liver diseases in the paediatric population worldwide.[1–3] Fatty liver (hepatic steatosis) is referred to as excess fat accumulation in hepatocytes and comprises a continuum of liver conditions, from simple non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH).[4] However, the pathogenesis of fatty liver in paediatrics has not been fully understood. It is reported that *de novo* lipogenesis in the liver, flow of the plasma fatty acid pool with adipose tissue, and excessive dietary fatty acids from the intestine are three main ways to produce fat in the liver.[5] Indeed, studies recently showed that intestinal-derived or absorbed fatty acid flux to hepatocytes was an important issue as about 15% of fatty acids incorporated into triglycerides in the liver originated from intestinal absorption or were found in individuals with non-alcoholic fatty liver disease (NAFLD).[6–8] It thus hypothesised that the absorption of dietary fatty acid in the intestine might be involved in the onset or pathogenesis of paediatric fatty liver.

Keywords: Liver steatosis; Fat absorption; Reg4; AMPK; Childhood obesity.
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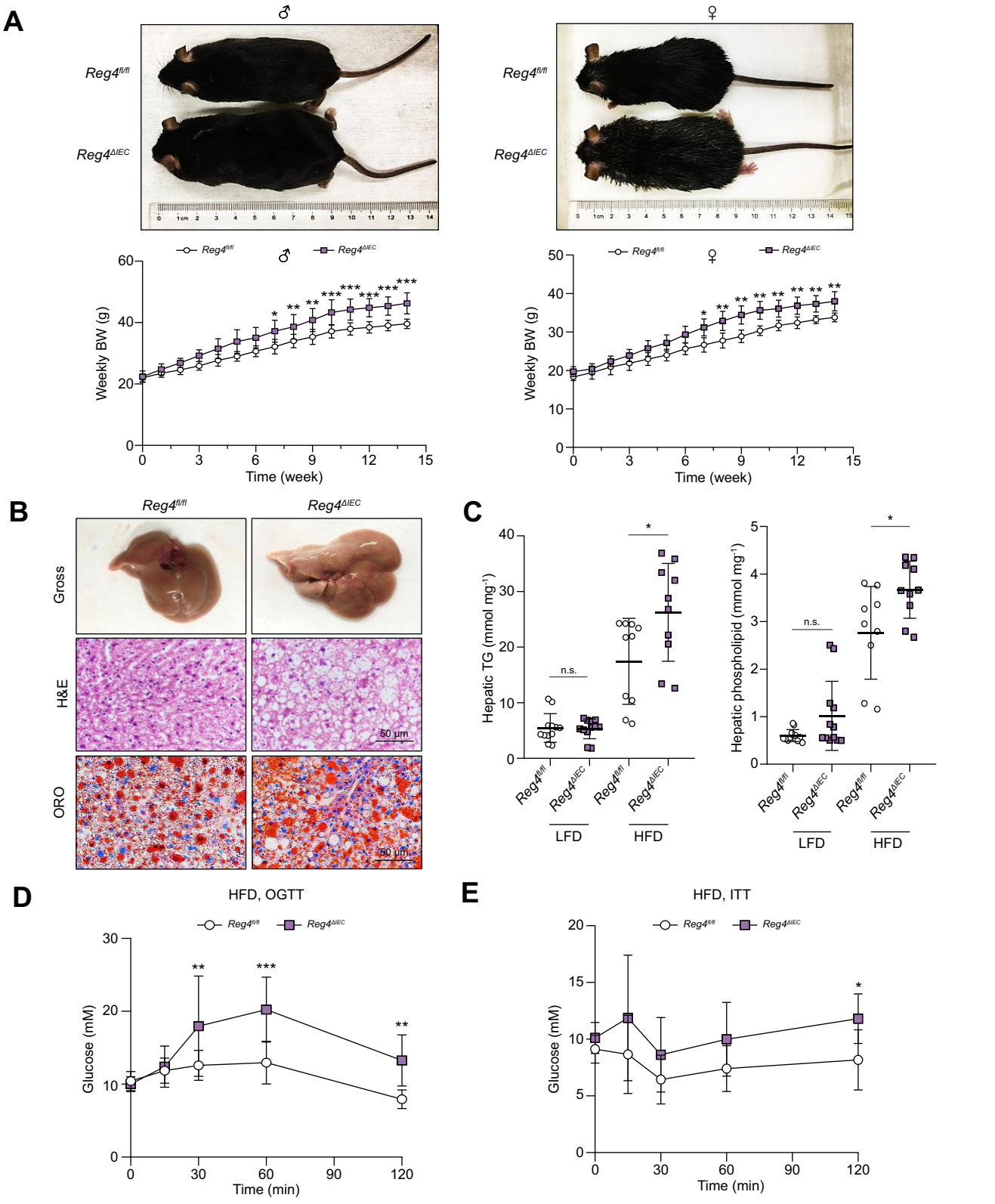

Fig. 1. Intestinal-specific *Reg4* deficiency aggravates obesity and liver steatosis in mice. (A) Representative male and female *Reg4*$^{fl/fl}$ mice and *Reg4*$^{\Delta IEC}$ mice fed a high-fat diet (HFD) for 14 weeks (top). Body weight (BW) was measured weekly (bottom) (male *Reg4*$^{fl/fl}$ mice n = 11 mice and *Reg4*$^{\Delta IEC}$ mice n = 10; female *Reg4*$^{fl/fl}$ mice n = 11 and *Reg4*$^{\Delta IEC}$ mice n = 10). (B) Gross, haematoxylin and eosin (H&E) and Oil Red O (ORO) imaging of livers of male *Reg4*$^{\Delta IEC}$ and *Reg4*$^{fl/fl}$ mice fed a HFD for 14 weeks (male mice, each group n = 11). (C) The contents of hepatic triglyceride (TG) and phospholipid were determined in male *Reg4*$^{\Delta IEC}$ and *Reg4*$^{fl/fl}$ mice fed a low-fat diet (LFD) or HFD for 14 weeks (LFD male *Reg4*$^{fl/fl}$ mice n = 12, *Reg4*$^{\Delta IEC}$ mice n = 12; HFD male *Reg4*$^{fl/fl}$ mice n = 9 – 10, *Reg4*$^{\Delta IEC}$ mice n = 10). (D, E) Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) of *Reg4*$^{\Delta IEC}$ and *Reg4*$^{fl/fl}$ mice fed a HFD for 8 weeks (male mice, *Reg4*$^{fl/fl}$ mice n = 10 and *Reg4*$^{\Delta IEC}$ mice n = 10). All data are mean ± standard deviation (SD). Linear mixed model for A, two-way variance (ANOVA, or mixed model, multiple comparisons) analysis for A; unpaired two-tailed Student *t* test with Welch's correction analysis for C; Two-way ANOVA analysis for D and E. n.s., not significant, *p <0.05, **p <0.01, ***p <0.001.

Regenerating gene family member 4 (REG4), a member of the calcium-dependent (C-type) lectin superfamily, is predominantly expressed in gastrointestinal tract tissues including the colon, small intestine, stomach, and pancreas.[9,10] It has been reported that aberrant expression of REG4 is associated with several diseases including gallbladder carcinoma, gastric cancer, colon cancer, pancreatic cancer, prostate cancer, and inflammatory bowel disease.[11–16] Single-cell messenger RNA sequencing recently revealed REG4 is a novel marker for enteroendocrine cells,[17,18] a rare population of hormone-producing intestinal cells. However, the exact role of Reg4 in the enteroendocrine cell has not been reported to date. In this study, we conditionally knocked out intestinal *Reg4* in mice and investigated the roles of Reg4 in the high-fat diet (HFD)-induced fatty liver. We found that intestinal-specific *Reg4* deletion aggravated HFD-induced liver steatosis by increasing the fatty acid uptake.

Materials and methods

High-fat diet-induced obesity in mice

For diet-induced obesity studies, 4–6-week-old male and female $Reg4^{\Delta IEC}$ and $Reg4^{fl/fl}$ mice were placed on a 60% HFD or 10% LFD (Bofan Biological Technology, Shanghai, China) for 8–14 weeks. Body weight and food intake were measured weekly. All animals used in this study received human care and the study protocols comply with the institution's guidelines. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the Xin Hua Hospital, School of Medicine, Shanghai Jiao Tong University (XHEC-F-2022-009).

Children with obesity

A total of 52 overweight/obese children (46 boys and six girls) and 21 age-matched controls (12 boys and nine girls) were from Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China. The children with obesity were identified according to the BMI cut-off points for overweight and obesity in Chinese children and adolescents aged 7–17 years. Written consent forms were obtained from the parents of all participants. The study protocol was reviewed and approved by the Ethics Committees of Xin Hua Hospital, School of Medicine, Shanghai Jiao Tong University (XHEC-D-2022-010).

Statistical analysis

The statistics are presented as mean ± standard deviation (SD). ANOVA or the Student *t* test was used to compare differences between groups. Correlations between serum REG4 and serum biochemical indexes were tested using the Spearman rank correlation test. The level of statistical significance was set at 0.05.

Detailed protocols are provided in the Supplementary material.

Results

Intestinal-specific *Reg4* deletion is prone to HFD-induced liver steatosis

As shown in Fig. S1A, the quantitative real-time PCR (qRT-PCR) showed that *Reg4* mRNA was exclusively expressed in the gastrointestinal tract, including stomach, intestinal mucosa, but not in the liver (Fig. S1A). In proximal intestine of mice, the colorimetric *in situ* hybridisation (CISH) assay indicated that *Reg4* mRNA was selectively expressed at crypts and villus (Fig. S1B). Immunofluorescence staining showed that Reg4 protein was located mainly in the epithelial cells of the bottom or middle of the villus (Fig. S1C). The REG4 protein was also observed in the human small intestine (Fig. S1D).

To investigate the entero-endocrinal roles of Reg4, we initially depleted the intestinal-specific *Reg4* ($Reg4^{\Delta IEC}$). As shown in Fig. S2, small intestine length, villus height, and crypt number in $Reg4^{\Delta IEC}$ mice did not differ from the $Reg4^{fl/fl}$ littermates that were fed a standard diet (10% kcal from fat) (Fig. S2A and B). When challenged with a HFD (60% kcal from fat), body weight was higher in both male and female $Reg4^{\Delta IEC}$ mice compared with that of $Reg4^{fl/fl}$ mice (Fig. 1A). After 14 weeks of HFD feeding, almost all the fat pads from $Reg4^{\Delta IEC}$ mice were significantly larger and heavier than those in $Reg4^{fl/fl}$ mice (Fig. S3A and C). H&E staining revealed that the adipocytes of the inguinal fat, gonadal fat, mesenteric fat, and retroperitoneal fat depots in $Reg4^{\Delta IEC}$ mice were significantly larger than those in $Reg4^{fl/fl}$ mice (Fig. S3B and D). As indicated in Fig. 1B, more severe liver steatosis was observed in $Reg4^{\Delta IEC}$ mice than in $Reg4^{fl/fl}$ mice as illustrated by H&E and Oil Red O staining (Fig. 1B and Fig. S4A). The higher levels of hepatic triglycerides and phospholipids were detected in livers of $Reg4^{\Delta IEC}$ mice than those in $Reg4^{fl/fl}$ mice (Fig. 1C). Moreover, the $Reg4^{\Delta IEC}$ mice had slightly higher levels of liver enzymes alanine transaminase (ALT) and aspartate aminotransferase (AST) in serum (Fig. S4B). The inflammatory genes including the interleukin-6 (*Il6*) and interleukin-1-beta (*Il1b*) increased in the livers of $Reg4^{\Delta IEC}$ mice than those in $Reg4^{fl/fl}$ mice, but it did not reach significance (Fig. S4C). Moreover, $Reg4^{\Delta IEC}$ mice showed a decrease in glucose tolerance and insulin sensitivity compared with $Reg4^{fl/fl}$ mice when subjected to an oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) (Fig. 1D and E).

Intestinal *Reg4* deficiency increases intestinal fat absorption with AMPK signalling activation

When mice were given a HFD for 14 weeks, fasted overnight, and re-fed with HFD for 1 h, faecal triglyceride content was decreased by about 40% in $Reg4^{\Delta IEC}$ compared with that of $Reg4^{fl/fl}$ mice (Fig. 2A, left), whereas proximal intestinal triglyceride content was increased significantly in $Reg4^{\Delta IEC}$ mice (Fig. 2A, right). Consistently, after the mice were gavaged with olive oil, Oil Red O staining and transmission electron microscope (TEM) analysis showed increased number and larger fat droplets in the mucosa of the proximal intestine of $Reg4^{\Delta IEC}$ mice (Fig. 2B and Fig. S5). We further analysed the expression of the main components that are responsible for fat uptake in the proximal small intestine. The triglyderide synthesis enzymes and proteins, including acyl CoA: monoacylglycerol acyltransferase-2 (MOGAT2), diacylglycerol O-acyltransferase 2 (DGAT2), and fatty-acid-binding protein 2, intestinal (FABP2), were expressed at higher levels at the proximal small intestine of $Reg4^{\Delta IEC}$ mice compared with that of $Reg4^{fl/fl}$ mice (Fig. 2C and D). The triglyceride chylomicron packaging and formation proteins microsomal triglyceride transfer protein (MTTP) and tail-interacting protein 47 (TIP47) increasingly expressed at the proximal small intestine of $Reg4^{\Delta IEC}$ mice (Fig. 2C and D).

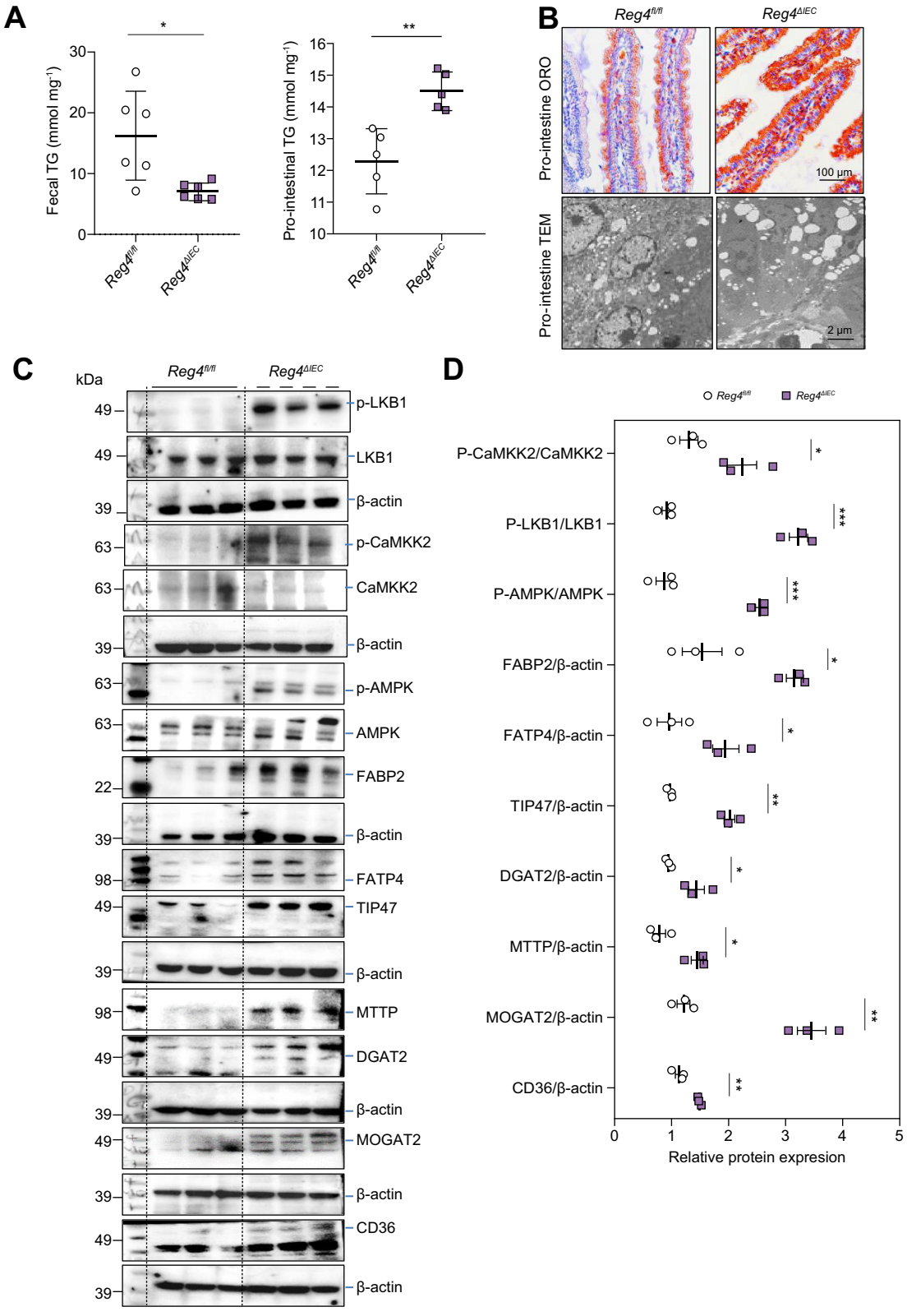

Fig. 2. Intestinal-specific *Reg4* deficiency increases intestinal fat absorption. (A) Triglyceride (TG) contents were quantified in proximal intestines and faeces from *Reg4*$^{\Delta IEC}$ and *Reg4*$^{fl/fl}$ mice following overnight fast and high-fat diet (HFD) re-fed (male mice, n = 6). (B) Representative images of Oil Red O (ORO) staining (male mice, n = 5) and transmission electron microscope (TEM) analysis (male mice, n = 3) of proximal intestines from *Reg4*$^{\Delta IEC}$ and *Reg4*$^{fl/fl}$ mice with fed a 4-day HFD, fasted overnight, and followed by HFD re-fed for another 1 h. (C) Western blotting of the extracts of proximal small intestine isolated from male and female *Reg4*$^{\Delta IEC}$ and *Reg4*$^{fl/fl}$ mice after 4-day HFD feeding. Mice were fasted overnight and re-fed with HFD for 1 h. The expression levels of liver kinase B1 (LKB1), phosphorylated-LKB1 (P-LKB1), Calcium/calmodulin-dependent protein kinase 2 (CaMKK2), phosphorylated- CaMKK2 (P-CaMKK2), adenosine monophosphate-

Moreover, the fatty acid transporting proteins cluster of differentiation 36 (CD36) and fatty acid transport protein 4 (FATP4) were also upregulated in the proximal small intestinal mucosa of *Reg4*$^{\Delta IEC}$ mice (Fig. 2C and D). Adenosine monophosphate-activated protein kinase (AMPK) is a critical regulator of cellular and whole-body energy homeostasis mediating the function of a variety of hormones and controlling fat absorption.[19–21] AMPK can be directly phosphorylated on Thr172 by its major upstream AMPK kinases, liver kinase B1 (LKB1) and calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2).[22,23] In the proximal small intestinal mucosa of mice, the phosphorylated-AMPK, phosphorylated-LKB1 as well as phosphorylated-CaMKK2 were enhanced in *Reg4*$^{\Delta IEC}$ mice compared with *Reg4*$^{fl/fl}$ mice fed on a HFD (Fig. 2C and D).

REG4 inhibits fat uptake in intestinal epithelial cells by altering AMPK activation

Treatment of human intestinal epithelial cells (FHs 74 Int) with oleate led to an increase in fatty acid uptake, which was inhibited by human recombinant REG4 protein (Fig. S6A). The REG4 treatment also reduced oleate-induced activation of AMPK signalling (AMPK, LBK1, and CaMKK2) and the expression of the main mechanism components of fatty acid uptake (*e.g.* CD36, FATP4, FABP2) (Fig. S6B and C). We next showed that knockdown of the CaMKK2 reduced the oleate-mediated phosphorylation of AMPK (Fig. S7), which is consistent with previous findings.[24] Pharmacological inhibition of AMPK with AMPK-specific inhibitor compound C significantly reduced the oleate uptake and decreased the expression of fatty acid absorption proteins (Fig. 3A–D). Similarly, REG4 treatment abolished the AMPK-pharmacological activator 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR)-induced oleate uptake and fat-absorption-related proteins (Fig. 3A–D).

Serum levels of REG4 are reduced with liver steatosis progression in children with obesity

Serum REG4 levels were markedly lower in children with obesity, and continually decreased with liver steatosis progression (Fig. 4A and B). Serum REG4 levels were inversely correlated not only with concentrations of ALT (r = -0.4583, p = 0.0018), AST (r = -0.5223, p = 0.0003), and alkaline phosphatase (AKP; r = -0.2948, p = 0.0521), but also with homeostasis model assessment of insulin resistance (HOMA-IR, r = -0.4711, p = 0.0012), LDL cholesterol (r = -0.5296, p =0.0002) and triglycerides (r = -0.3528, p = 0.0118) levels (Fig. 4C).

Discussion

In this study, we demonstrate that intestinal Reg4 acts as an enteroendocrine hormone protecting mice from high-fat diet-induced liver steatosis via reducing intestinal fat absorption. First of all, overall body weights and fat contents were significantly increased in *Reg4*$^{\Delta IEC}$ mice given a HFD. Second, the small intestine of *Reg4*$^{\Delta IEC}$ mice dramatically increased fatty acid absorption and related protein levels as well as activation of AMPK signalling. Importantly, we indicated that REG4 reduced intestinal fat absorption at least in part via inhibiting the activation of the CaMKK2-AMPK pathway.

REG4 recently has been identified as a novel marker for enteroendocrine cells.[17,18] but its role in digestion and energy homeostasis is unknown. Here we reported that intestinal *Reg4* deficient (*Reg4*$^{\Delta IEC}$) mice were susceptible to HFD-induced liver steatosis characterised by increased hepatic fat accumulation and insulin resistance. Because *Reg4* has a highly restricted tissue expression pattern, we indicated it is selectively expressed in the intestinal tract, but it is hardly detected in the liver. Intestinal-absorbed fatty acid from dietary has been identified as an important source of liver fat accumulation.[7,8] We thus hypothesise that the intestinal Reg4 may protect against HFD-induced hepatic steatosis via reducing intestinal fatty acid uptake. Indeed, *Reg4*$^{\Delta IEC}$ mice fed a HFD had an increase in proximal intestinal fatty acid droplets. In *in vitro* experiments, REG4 treatment attenuated oleate absorption in human small intestinal epithelial cells (FHs 74 Int). Taken together, our findings suggested that intestinal REG4 acted as an important enteroendocrine hormone to reduce fatty acid uptake at the proximal intestine.

There are several main components responsible for intestinal fat absorption. The transporters including CD36 and FATP4 are important mediators of long-chain fatty acids and their acyl-CoA esters transportation.[25,26] The triglyceride synthesis enzyme levels of MOGAT2, FABP2, and DGAT2 determine the rate of MAG uptake from the lumen into enterocytes in a cell-autonomous manner.[27,28] The proteins MTTP and TIP47 are responsible for triglyceride chylomicron packaging and formation.[29,30] *Reg4*$^{\Delta IEC}$ mice here had an increase in the protein level of the main components responsible for intestinal fat absorption during feeding with a HFD. Although the human intestinal epithelial cells FHs 74 Int had reduced expression of these proteins after treated with REG4 protein. Recent studies showed that activation of AMPK could stimulate fatty acid uptake in intestinal epithelial cells.[21,24,31] We here showed that activation of AMPK and its two upstream activators LBK1 and CaMMK2 increased in the proximal intestinal mucosa of *Reg4*$^{\Delta IEC}$ mice. *In vitro*, REG4 addition inhibited oleate or AICAR-induced AMPK activation. AMPK has been reported to facilitate fatty acid uptake by manipulating CD36 expression and translocation.[31,32] Knockdown of the CaMKK2 but not LBK1 decreased oleate-stimulated AMPK activation, which is consistent with a previous study.[24] Together, these findings suggest REG4 reduces the fatty acid absorption possibly by inhibition of CaMKK2-AMPK signalling.

With this population-based cross-sectional study, we showed that decreased serum REG4 levels reflected the presence and the degree of liver steatosis in children with obesity. Children with more advanced steatosis had lower serum REG4 levels compared with those with milder steatosis. Moreover, serum REG4 levels

activated protein kinase (AMPK), phosphorylated-AMPK (P-AMPK), cluster of differentiation 36 (CD36), acyl CoA: monoacylglycerol acyltransferase-2 (MOGAT2), fatty acid transport protein 4 (FATP4), diacylglycerol O-acyltransferase 2 (DGAT2), fatty-acid-binding protein 2, intestinal (FABP2), microsomal triglyceride (TG) transfer protein (MTTP), and tail-interacting protein 47 (TIP47) were measured. (D) Quantification of proteins in (C). β-Actin was used as an internal reference. Each group, n = 3. Unpaired two-tailed Student t test with Welch's correction analysis for A and D; *p <0.05, **p <0.01, ***p <0.001.

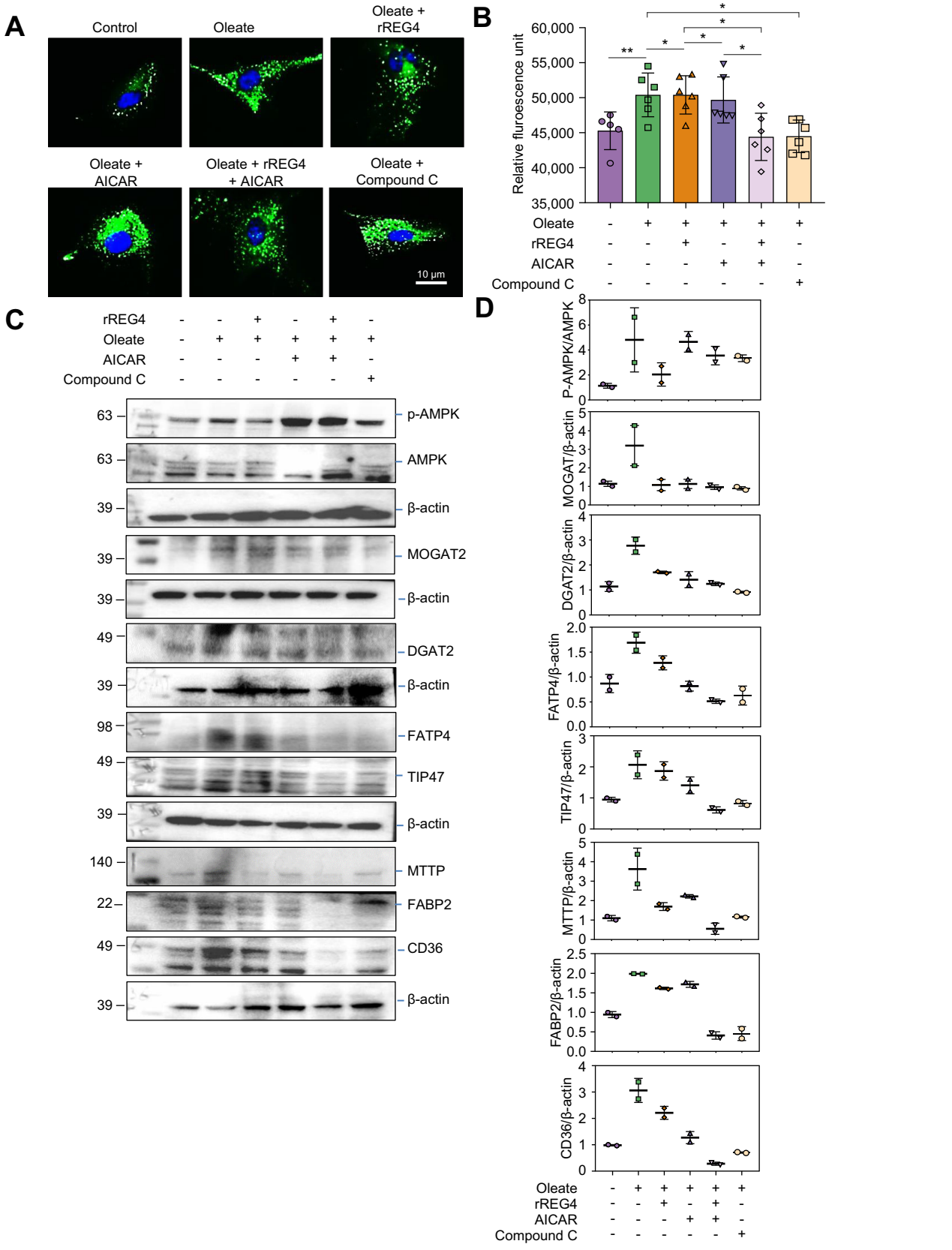

Fig. 3. REG4 represses the activation of AMPK and fat uptake in cultured intestinal epithelial cells. (A) FHs 74 Int cells were pre-treated with or without REG4 (10 μg/ml) and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR; 1 mM) for 30 min which followed by oleate (0.1 mM) or bovine serum albumin (BSA) for 1 h. FHs 74 Int cells were pre-treated with Compound C (5 μM) for 30 min and followed by oleate (0.1 mM) or BSA for 1 h. Representative images of neutral lipid staining on FHs 74 Int cells. (B) Fatty acid uptake was measured in the cells with above treatments (repeats n = 5–6). (C) Western blotting of the extracts of cells with above treatments for the adenosine monophosphate-activated protein kinase (AMPK), phosphorylated-AMPK (P-AMPK), cluster of differentiation 36 (CD36), acyl CoA: monoacylglycerol acyltransferase-2 (MOGAT2), fatty acid transport protein 4 (FATP4), diacylglycerol O-acyltransferase 2

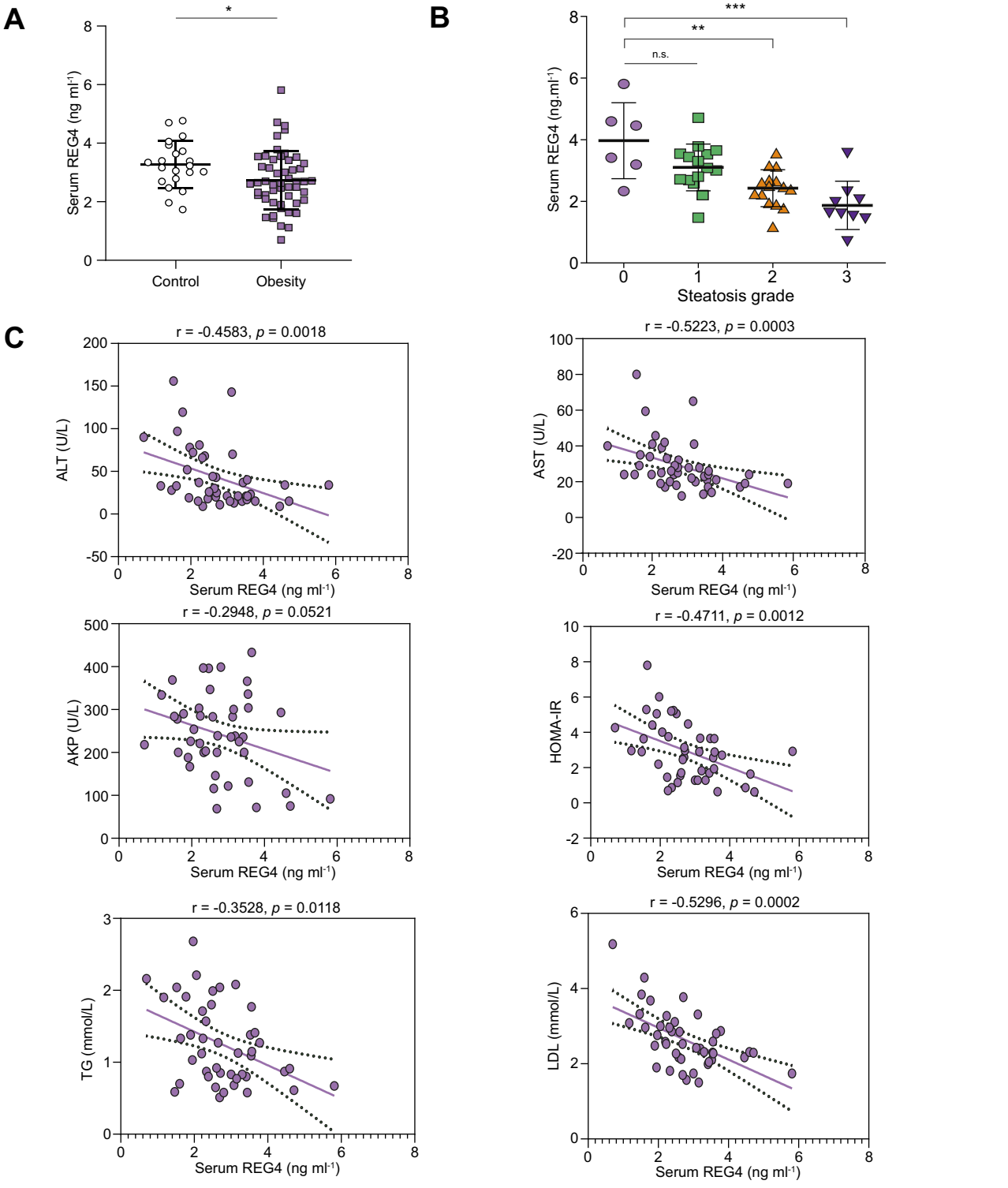

Fig. 4. Serum REG4 levels were measured in children with obesity. (A) The serum REG4 levels in children with obesity and controls using ELISA. (B) The serum REG4 levels in the children with obesity with different grades of liver steatosis (0–3) using ELISA. (C) The correlation of serum REG4 levels with levels of serum liver function markers, homeostasis model assessment of insulin resistance (HOMA-IR), low-density lipoprotein cholesterol (LDL) and serum triglycerides (TG). Unpaired two-tailed Student *t* test with Welch's correction analysis for A; ordinary one-way ANOVA analysis for B; two-tailed Spearman's correlation (non-parametric) analysis for C. n.s., not significant, $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001.

(DGAT2), fatty-acid-binding protein 2, intestinal (FABP2), microsomal triglyceride (TG) transfer protein (MTTP), and tail-interacting protein 47 (TIP47). (D) Quantification of proteins in (C). β-Actin was used as an internal reference. Independent experiment at least two times. Ordinary one-way ANOVA analysis for B; $^{*}p$ <0.05, $^{**}p$ <0.01.

were negatively correlated with levels of serum triglycerides, liver function enzymes, and insulin resistance. Although there were some limitations to our study, including a small sample size and wide age range of the children, this study provides novel data on obesity-associated liver steatosis and its association with REG4 levels in serum.

In conclusion, this study found that ablation of intestinal *Reg4* contributed to hepatic steatosis possibly via AMPK mediating increased intestinal fatty acid uptake. Serum REG4 levels were reduced with liver steatosis progression in children with children. REG4 may provide a potential target for prevention and treatment for liver steatosis in children.

Abbreviations

AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AKP, alkaline phosphatase; ALT, alanine transaminase; AMPK, adenosine monophosphate-activated protein kinase; AST, aspartate aminotransferase; CaMKK2, calcium/calmodulin-dependent protein kinase kinase 2; CD36, cluster of differentiation 36; CISH, colorimetric *in situ* hybridisation; FATP4, fatty acid transport protein 4; HFD, high-fat diet; HOMA-IR, homeostasis model assessment of insulin resistance; ITT, insulin tolerance test; LKB1, liver kinase B1; MTTP, microsomal triglyceride transfer protein; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OGTT, oral glucose tolerance test; ORO, Oil Red O; qRT-PCR, quantitative real-time PCR; REG4, regenerating gene family member 4; TIP47, tail-interacting protein 47.

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Conflicts of interest

The authors have no conflicts of interest to disclose.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conceptualisation: YW, WY, WC, YX, YL, JD, XT, YX. Formal analysis: YW, WY, WC, YX. Investigation: YW, WY, WC, YX, YL, JD, XT, BW, SP, BG. Methodology: YW, WY, WC, YX, YL, JD, XT, BW, SP, BG. Visualisation: YW, WY, WC, YX, YL, JD, XT, BW, SP, BG. Writing – original draft: YW, WY, WC, YX, YL, JD, XT. Writing – review and editing: YW, WY, WC, YX, YL, JD, XT. Data curation: YW, WY, YL, JD, XT, YX. Funding acquisition: YW, WY, YL, JD, XT, YX. Project administration: YW, WY, YL, JD, XT, YX. Resources: YW, WY, YL, JD, XT, YX. Supervision: YW, WY, YL, JD, XT, YX. Software: YL, JD, XT, BW, SP, BG. Validation: YL, JD, XT, BW, SP, BG. All the authors approved this version of the manuscript to be published.

Data availability statement

The data that support the findings of this study are available from the corresponding authors, upon reasonable request.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2023.100700.

References

Author names in bold designate shared co-first authorship

[1] Schwimmer JB, Deutsch R, Kahen T, Lavine JE, Stanley C, Behling C. Prevalence of fatty liver in children and adolescents. Pediatrics 2006;118:1388–1393.

[2] Schwimmer JB, Newton KP, Awai HI, Choi LJ, Garcia MA, Ellis LL, et al. Paediatric gastroenterology evaluation of overweight and obese children referred from primary care for suspected non-alcoholic fatty liver disease. Aliment Pharmacol Ther 2013;38:1267–1277.

[3] Friedman SL, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. Nat Med 2018;24:908–922.

[4] Brunt EM, Wong VW, Nobili V, Day CP, Sookoian S, Maher JJ, et al. Nonalcoholic fatty liver disease. Nat Rev Dis Primers 2015;1:15080.

[5] Postic C, Girard J. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. J Clin Invest 2008;118:829–838.

[6] Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. J Clin Invest 2005;115:1343–1351.

[7] Cabre E, Hernandez-Perez JM, Fluvia L, Pastor C, Corominas A, Gassull MA. Absorption and transport of dietary long-chain fatty acids in cirrhosis: a stable-isotope-tracing study. Am J Clin Nutr 2005;81:692–701.

[8] Yamamoto Y, Hiasa Y, Murakami H, Ikeda Y, Yamanishi H, Abe M, et al. Rapid alternative absorption of dietary long-chain fatty acids with upregulation of intestinal glycosylated CD36 in liver cirrhosis. Am J Clin Nutr 2012;96:90–101.

[9] Hartupee JC, Zhang H, Bonaldo MF, Soares MB, Dieckgraefe BK. Isolation and characterization of a cDNA encoding a novel member of the human regenerating protein family: Reg IV. Biochim Biophys Acta 2001;1518:287–293.

[10] Kamarainen M, Heiskala K, Knuutila S, Heiskala M, Winqvist O, Andersson LC. RELP, a novel human REG-like protein with up-regulated expression in inflammatory and metaplastic gastrointestinal mucosa. Am J Pathol 2003;163:11–20.

[11] Violette S, Festor E, Pandrea-Vasile I, Mitchell V, Adida C, Dussaulx E, et al. Reg IV, a new member of the regenerating gene family, is overexpressed in colorectal carcinomas. Int J Cancer 2003;103:185–193.

[12] Oue N, Mitani Y, Aung PP, Sakakura C, Takeshima Y, Kaneko M, et al. Expression and localization of Reg IV in human neoplastic and non-neoplastic tissues: Reg IV expression is associated with intestinal and neuroendocrine differentiation in gastric adenocarcinoma. J Pathol 2005;207:185–198.

[13] Zheng HC, Xu XY, Yu M, Takahashi H, Masuda S, Takano Y. The role of Reg IV gene and its encoding product in gastric carcinogenesis. Hum Pathol 2010;41:59–69.

[14] Tamura H, Ohtsuka M, Washiro M, Kimura F, Shimizu H, Yoshidome H, et al. Reg IV expression and clinicopathologic features of gallbladder carcinoma. Hum Pathol 2009;40:1686–1692.

[15] Takayama R, Nakagawa H, Sawaki A, Mizuno N, Kawai H, Tajika M, et al. Serum tumor antigen REG4 as a diagnostic biomarker in pancreatic ductal adenocarcinoma. J Gastroenterol 2010;45:52–59.

[16] Nanakin A, Fukui H, Fujii S, Sekikawa A, Kanda N, Hisatsune H, et al. Expression of the REG IV gene in ulcerative colitis. Lab Invest 2007;87:304–314.

[17] Grun D, Lyubimova A, Kester L, Wiebrands K, Basak O, Sasaki N, et al. Single-cell messenger RNA sequencing reveals rare intestinal cell types. Nature 2015;525:251–255.

[18] **Haber AL**, **Biton M**, **Rogel N**, Herbst RH, Shekhar K, Smillie C, et al. A single-cell survey of the small intestinal epithelium. Nature 2017;551:333–339.

[19] Xue B, Kahn BB. AMPK integrates nutrient and hormonal signals to regulate food intake and energy balance through effects in the hypothalamus and peripheral tissues. J Physiol 2006;574:73–83.

[20] Lim CT, Kola B, Korbonits M. AMPK as a mediator of hormonal signalling. J Mol Endocrinol 2010;44:87–97.

[21] Chopra AR, Kommagani R, Saha P, Louet JF, Salazar C, Song J, et al. Cellular energy depletion resets whole-body energy by promoting coactivator-mediated dietary fuel absorption. Cell Metab 2011;13:35–43.
[22] Hardie DG. AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. Genes Dev 2011;25:1895–1908.
[23] Carling D, Mayer FV, Sanders MJ, Gamblin SJ. AMP-activated protein kinase: nature's energy sensor. Nat Chem Biol 2011;7:512–518.
[24] Li J, Song J, Zaytseva YY, Liu Y, Rychahou P, Jiang K, et al. An obligatory role for neurotensin in high-fat-diet-induced obesity. Nature 2016;533:411–415.
[25] Wang TY, Liu M, Portincasa P, Wang DQ. New insights into the molecular mechanism of intestinal fatty acid absorption. Eur J Clin Invest 2013;43:1203–1223.
[26] Stahl A, Hirsch DJ, Gimeno RE, Punreddy S, Ge P, Watson N, et al. Identification of the major intestinal fatty acid transport protein. Mol Cel 1999;4:299–308.
[27] **Nelson DW**, **Gao Y**, Yen MI, Yen CL. Intestine-specific deletion of acyl-CoA: monoacylglycerol acyltransferase (MGAT) 2 protects mice from diet-induced obesity and glucose intolerance. J Biol Chem 2014;289:17338–17349.
[28] Luo H, Jiang M, Lian G, Liu Q, Shi M, Li TY, et al. AIDA selectively mediates downregulation of fat synthesis enzymes by ERAD to retard intestinal fat absorption and prevent obesity. Cel Metab 2018;27:843–853.e846.
[29] Lee B, Zhu J, Wolins NE, Cheng JX, Buhman KK. Differential association of adipophilin and TIP47 proteins with cytoplasmic lipid droplets in mouse enterocytes during dietary fat absorption. Biochim Biophys Acta 2009;1791:1173–1180.
[30] Rajan S, Hofer P, Christiano A, Stevenson M, Ragolia L, Villa-Cuesta E, et al. Microsomal triglyceride transfer protein regulates intracellular lipolysis in adipocytes independent of its lipid transfer activity. Metabolism 2022;137:155331.
[31] Wu W, Wang S, Liu Q, Shan T, Wang X, Feng J, et al. AMPK facilitates intestinal long-chain fatty acid uptake by manipulating CD36 expression and translocation. FASEB J 2020;34:4852–4869.
[32] Habets DD, Coumans WA, Voshol PJ, den Boer MA, Febbraio M, Bonen A, et al. AMPK-mediated increase in myocardial long-chain fatty acid uptake critically depends on sarcolemmal CD36. Biochem Biophys Res Commun 2007;355:204–210.