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ChREBP deficiency leads to diarrhea-predominant irritable bowel syndrome

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Abstract

Objective: Fructose malabsorption is a common digestive disorder in which absorption of fructose in the small intestine is impaired. An abnormality of the main intestinal fructose transporter proteins has been proposed as a cause for fructose malabsorption. However the underlying molecular mechanism for this remains unclear. In this study, we investigated whether carbohydrate response element-binding protein (ChREBP) plays a role in intestinal fructose absorption through the regulation of genes involved in fructose transport and metabolism and ion transport.

Methods: Wild type (WT) and *Chrebp* knockout (KO) mice (6 or 8 weeks old) were fed a control diet (55% starch, 15% maltodextrin 10) or high-fructose diet (HFrD, 60% fructose, 10% starch) for 3–12 days. Body weight and food intake were measured, signs of fructose malabsorption were monitored, and the expression of genes involved in fructose transport/metabolism and ion transport was evaluated. Furthermore, transient transfection and chromatin immunoprecipitation were performed to show the direct interaction between ChREBP and carbohydrate response elements in the promoter of *Slc2A5*, which encodes the fructose transporter GLUT5.

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Author contribution statement

A.-R.O. and J.-Y.C. designed this study. A.-R.O., S.S., J.L., J.-M.P, K.T.N., and J.-Y.C. performed the experiments and all authors participated in data analysis. A.-R.O., S.S., and J.-Y.C. wrote the manuscript, K.-B. H., Y.-B.K., and H.-J.L. made modifications. All authors approved the final version.

Disclosure statement

The authors declare no conflicts of interest.

Results: *Chrebp* KO mice fed the control diet maintained a constant body weight, whereas those fed a HFrD showed significant weight loss within 3–5 days. In addition, *Chrebp* KO mice fed the HFrD exhibited a markedly distended cecum and proximal colon containing both fluid and gas, suggesting incomplete fructose absorption. Fructose-induced increases of genes involved in fructose transport (GLUT5), fructose metabolism (*fructokinase*, *aldolase B*, *triokinase*, and *lactate dehydrogenase*), and gluconeogenesis (*glucose-6-phosphatase* and *fructose-1,6-bisphosphatase*) were observed in the intestine of WT but not of *Chrebp* KO mice. Moreover the Na⁺/H⁺ exchanger NHE3, which is involved in Na⁺ and water absorption in the intestine, was significantly decreased in HFrD-fed *Chrebp* KO mice. Consistent with this finding, the high-fructose diet-fed *Chrebp* KO mice developed severe diarrhea. Results of chromatin immunoprecipitation assays showed a direct interaction of ChREBP with the *Glut5* promoter, but not the *Nhe3* promoter, in the small intestine. Ectopic co-expression of ChREBP and its heterodimer partner Max-like protein X activated the *Glut5* promoter in Caco-2BBE cells.

Conclusions: ChREBP plays a key role in the dietary fructose transport as well as conversion into lactate and glucose through direct transcriptional control of genes involved in fructose transport, fructolysis, and gluconeogenesis. Moreover, ablation of *Chrebp* results in a severe diarrhea in mice fed a high-fructose diet, which is associated with the insufficient induction of GLUT5 in the intestine.

Keywords

ChREBP; Intestine; Fructose absorption; Diarrhea; GLUT5

1. Introduction

The increased consumption of sugar and high-fructose corn syrup, both of which consist of nearly equal amounts of glucose and fructose, is associated with obesity and related metabolic diseases including dyslipidemia, insulin resistance, and nonalcoholic fatty liver disease [1–3]. The metabolic effects of dietary fructose appear to be more harmful than those of glucose, with excessive fructose intake increasing *de novo* lipogenesis and serum TG levels [4]. Moreover, excess intake of fructose as a monosaccharide can easily overwhelm the absorptive capacity of the small intestine, leading to incomplete absorption, *i.e.*, fructose malabsorption [5, 6]. A high concentration of fructose within the gut lumen draws fluids into the intestine, and the fructose is fermented by anaerobic flora in colon, which can produce gas, bloating, abdominal pain, or diarrhea, and results in decreased food intake [7, 8].

Fructose is primarily absorbed in the small intestine through glucose transporter 5 (GLUT5, encoded by *Slc2A5*), a main fructose transporter in mammals [9, 10]. This is an energy-independent process, and its absorptive capacity is carrier-limited [11]. GLUT5 is normally expressed at low baseline levels throughout the suckling and weaning stages in neonatal rats [12]. Differentiated cells express GLUT5 on the apical border of enterocytes in the small intestine and marked increases in its mRNA and protein abundance occur within a few hours of fructose consumption [13, 14]. Although several regions of the *Glut5* promoter respond to developmental and dietary signals [15–18], little is known about transcription factors and *cis*-regulatory elements that respond to dietary fructose.

Carbohydrate response element-binding protein (ChREBP) is a basic helix-loop-helix/leucine zipper transcription factor that plays a key role in glycolytic and lipogenic gene expression in response to carbohydrate consumption [19–21]. ChREBP is expressed ubiquitously but is most highly expressed in liver and adipose tissues and more modestly expressed in the intestine, kidney, and pancreatic β -cells. Upon activation by glucose, ChREBP translocates from the cytosol into the nucleus, where it forms a heterodimer with Max-like protein X (MLX) and binds to target genes containing carbohydrate response elements [22, 23]. Although ChREBP was first identified as a glucose-responsive factor, a critical role of ChREBP in fructose metabolism was suggested by the observation that *Chrebp* knockout (KO) mice could not tolerate diets containing sucrose and fructose [19]. ChREBP is now known to be activated by fructose as much as glucose [24, 25]. Although it has been reported that ChREBP upregulates hepatic fructokinase (*Khk*) upon high fructose consumption [24], no published studies have reported the effect of ChREBP on intestinal fructose transport and metabolism. Here we aimed to identify the molecular mechanism underlying fructose intolerance in *Chrebp* KO mice, focusing on intestinal fructose malabsorption.

2. Materials and Methods

2.1. Mice and diets

C57BL/6J mice (wild type, WT) and *Chrebp* KO mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained on a normal diet containing 13% kcal fat, 25% protein, 55% starch, 5% sucrose, and 2% lactose (PicoLab Rodent Diet 20, Orient Bio, Gyeonggi Province, Korea) under a 12-h light/dark cycle. The 6-week-old male and 8-week-old female mice were fed a control diet (CD) containing 10% kcal fat, 20% protein, 55% starch, and 15% maltodextrin 10 (D12450K; Research Diets, Inc., New Brunswick, NJ) or high-fructose diet (HFrD) containing 10% kcal fat, 20% protein, 10% starch, and 60% fructose (D02022704; Research Diets, Inc., New Brunswick, NJ). Body weight, blood glucose, rectal temperature, and food intake were measured on days 0, 5, 8, and 12. For glucose measurements, blood was taken from the tail vein of the same animals sequentially on the indicated days. The 8-week-old male mice were fed a CD or HFrD for 3, 5, or 7 days. At the end of the experiment, the mice were fasted for 2 h, euthanized, and body weights were obtained. Blood samples (600–800 μ l) were collected immediately via the hepatic portal vein and plasma was obtained by centrifugation at $300 \times g$ for 15 min at 4°C. Plasma samples were stored at -80°C until biochemical analysis. Liver and intestine were removed, weighed, and either formalin-fixed, or snap-frozen in liquid nitrogen. The length of small intestine and contents of the colon and cecum were determined on days 3, 5, and 7.

In a pair-fed experiment, 8-week-old WT male mice were fed the same amount of food (CD or HFrD) as was consumed by the HFrD-fed *Chrebp* KO mice; body weight was measured daily for 7 days. In a recovery experiment, 8-week-old WT male mice were fed the HFrD for 15 days. *Chrebp* KO male mice of the same age were fed either the HFrD for 15 days or the HFrD for 7 days, and switched to the CD until day 15. All animal studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Lee Gil Ya Cancer and Diabetes Institute, Gachon University.

2.2. Oral administration of liquid fructose or glucose

Male mice were maintained on a normal diet for 12 weeks prior to the gavage study. To allow the mice to adapt to oral administration, Dulbecco's phosphate buffered saline (DPBS) was injected orally every day for 7 days. Mice were fasted for 4 h and then orally administered either liquid fructose or glucose (~250 μ l, 2 g/kg body weight of 20% D-fructose or 20% D-glucose). Blood samples (60 μ l) were taken from the tip of the tail at 0 min, 20 min, 4 h, and 8 h after administration.

2.3. Biochemical analysis

Plasma alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH), triglyceride (TG), and total cholesterol were determined by automated analysis (Model AU-480; Olympus, Tokyo, Japan). Liver TG was determined as previously described [26] using a Cleantech TG-S kit (Asan Pharmacy, Inc., Seoul, Korea). Liver cholesterol was determined using a Cholesterol/Cholesteryl Ester Quantitation Kit (BioVision, Inc., Milpitas, CA). Blood glucose was measured using the GlucoDr.TM Plus (AGM-3000; Allmedicus, Gyeonggi Province, Korea). Blood fructose concentration was measured using the EnzyChromTM Fructose Assay Kit (EFRU-100; BioAssay System, Hayward, CA). For lactate measurements, intestine and liver were homogenized in lactate assay buffer and centrifuged at 13,000 rpm 5 min at 4°C. Supernatant was transferred into new tube and enzyme was removed from supernatant using Deproteinizing Sample Preparation kit-TCA (ab204708; Abcam, Cambridge, MA). Lactate concentration was determined using a L-Lactate assay kit (ab65330; Abcam, Cambridge, MA).

2.4. RNA isolation and quantitative polymerase chain reaction

Total RNA was isolated from the small intestine using RNAiso Plus (Takara, Shiga, Japan). Purified total RNA was treated with RNase-free DNase (Roche, Penzberg, Germany) and reverse-transcribed using PrimeScriptTM RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan). Gene-specific primers were designed using Primer Express Software (PerkinElmer Life Sciences, Waltham, MA), and validated by analysis of template titration and dissociation curves. Quantitative polymerase chain reaction (qPCR) was performed on a CFX384 TouchTM Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) using SYBR[®] Premix Ex TaqTM II, ROX Plus (Takara, Shiga, Japan). Relative gene expression was determined using the 2⁻ Ct method [27], with the gene encoding ribosomal protein, large, P0 (*Rplp0*) serving as the invariant control. Primer sequences are shown in Supplementary Table 1.

2.5. Immunohistochemistry

Small intestine specimens were fixed in neutral-buffered formalin and embedded in paraffin blocks according to standard procedures. The paraffin-embedded tissue sections were incubated in the oven at 60°C for 1 h. After rehydration in graded ethanol, antigen retrieval was performed in 1× Tris-EDTA, pH 9.5 (Thermo Scientific, Boston, MA) by heating in a microwave for 10 min. Sections were incubated with 3% H₂O₂ (Dako, Denmark) at room temperature for 30 min, blocked for 5 min, and then incubated with the primary antibody overnight. The next day, sections were incubated with the secondary antibody for 30 min,

washed with DPBS containing Tween 20, and stained with 3,3'-diaminobenzidine. Primary antibodies against the following proteins were used: ChREBP (Novus Biologicals, Littleton, CO), GLUT5 (Santa Cruz Biotechnology, Santa Cruz CA), GLUT2 (Santa Cruz Biotechnology, Santa Cruz CA), and NHE3 (Santa Cruz Biotechnology, Santa Cruz CA).

2.6. Western blotting

Western blot analyses were performed according to standard protocols using antibodies against ChREBP (Novus Biologicals), GLUT5 (Santa Cruz Biotechnology), GLUT2 (Santa Cruz Biotechnology), NHE3 (Santa Cruz Biotechnology), and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Merck Millipore, Temecula, CA). GAPDH was used as a loading control.

2.7. Plasmid construction

ChoREs (CANNTGNNNNNNNNNTG [28]) in the *Glut5* and *Nhe3* promoters were identified by using Vector NTI software. Five ChoREs were located in the *Glut5* promoter between 0 and 2165 bp upstream from the transcription start site: -915 to -889 (ChoRE4 overlaps ChoRE5), -1620 to -1604, -1937 to -1853, and -2165 to -2149. One ChoRE was located in the *Nhe2* promoter, -1248 to -1264. The proximal region of the *Glut5* promoter (-2165/+0) was amplified from mouse genomic DNA by PCR and inserted into the SacI and EcoRV sites of the pGL4 basic vector (mGlut5-2165). Serial deletion constructs of the *Glut5* promoter were generated by PCR amplification and subcloned into the pGL4 basic vector. Mouse ChREBP and MLX expression vectors were kind gifts from Dr. Howard C. Towle [23].

2.8. Cell culture and luciferase assay

Well-differentiated mature monolayers of Caco-2BBE (human intestinal epithelial) cells were maintained in 25 mM glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Life Technologies, Seoul, Korea) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, Seoul, Korea), 1% penicillin-streptomycin (Gibco, Life Technologies, Seoul, Korea), and 0.01 mg/ml human transferrin (Gibco, Life Technologies, Seoul, Korea). Cell cultures were maintained in a 37°C incubator supplemented with 5% CO₂. Luciferase reporter plasmids containing the *Glut5* promoter constructs were transfected into Caco-2BBE cells with or without pcDNA expression vectors containing the *Chrebp* and *Mlx* genes. Luciferase activity was determined using the Luciferase Assay System (Promega Corporation, Fitchburg, WI) according to the manufacturer's instructions and expressed as arbitrary units normalized to β-galactosidase activity. All experiments were performed in triplicate and repeated at least three times.

2.9. Chromatin immunoprecipitation assay

Small intestine tissues were washed with formaldehyde (37°C, 1% final concentration) and then opened longitudinally on a glass plate. The mucosa was scraped and separated from the underlying muscle layers by a glass microscope slide. The scraped mucosal cells were then fixed in formaldehyde (37°C, 1% final concentration) for 10 min at room temperature. The chromatin immunoprecipitation (ChIP) assay was performed as described previously [28,

29] using the EpiQuik Tissue Chromatin Immunoprecipitation kit (P-2003, EpiGentek, Farmingdale, NY). Chromatin was immunoprecipitated with control IgG or anti-ChREBP antibody (Novus Biologicals), purified, and then analyzed by qPCR using a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). The following primers were used: ChoRE1 forward 5′GATTTC CTG CCG CAT TCA GA3′, reverse 5′TTTTTCAGAC CTC CCA GAT GGA3′; ChoRE2 forward 5′TCC ATC CAC ACA CTT TCA AAC C3′, reverse 5′CAA GCC ACG GCC AAC AG3′; ChoRE3 forward 5′TCC CCG GCT CAC CTC AA3′, reverse 5′TTC GGA GTG GGA GTC TGG TT3′; ChoRE4/5 forward 5′TGG TCA GTC CGG TAG CAG TTG3′, reverse 5′CCT TTG CAG GGC AGG CTA A3′; mNHE3 ChoRE1 forward 5′GGG AGG ATA TAG GGA ATT TG3′, reverse 5′CGA TAC TTG AAA CGT ATA TAT GT3′; mCyclophilin forward 5′GGT CTT TGG GAA GGT GAA AGA A3′, reverse 5′GCC ATT CCT GGA CCC AAA A3′.

2.10. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was carried out using SPSS (Version 17.0; SPSS Inc., Chicago, IL). Data were analyzed using the Mann–Whitney U test; $p < 0.05$ was considered significant.

3. Results

3.1. Fructose intolerance in *Chrebp* KO mice

To examine the role of ChREBP in fructose absorption in the intestine, 6-week-old male WT and *Chrebp* KO mice were fed either the HFrD (60% fructose, 10% starch) or CD (55% starch, 15% maltodextrin 10) for 3–12 days. WT mice maintained a constant body weight on both diets; however, *Chrebp* KO mice showed significant weight loss on the HFrD relative to the CD (Fig. 1 A). *Chrebp* KO mice fed the HFrD for 5 days showed approximately 20% reduction in body weight and became moribund in 2 weeks, necessitating termination of the study. Compared with WT mice, HFrD-fed *Chrebp* KO mice exhibited markedly lower blood glucose levels (Fig. 1B) and lower body temperature (Fig. 1C). Similar changes in body weight and temperature were observed in female mice (Fig. 1D & F). Blood glucose levels were slightly increased by HFrD in either group of mice (Fig. 1E). Of note, *Chrebp* KO mice younger than 5 weeks that were fed the HFrD became moribund within a few days, culminating in death in less than 1 week (>90% of the *Chrebp* KO mice; data not shown). To better understand the cause of weight loss in HFrD-fed *Chrebp* KO mice, we evaluated daily food consumption. We found that the average daily food intake for 3 days was similar between WT and *Chrebp* KO mice fed the CD but was significantly lower in *Chrebp* KO mice than in WT mice fed the HFrD (Fig. 1G). To determine whether reduced food intake was the sole cause of weight loss in *Chrebp* KO mice, we conducted a pair-feeding experiment. WT mice limited to the same amount of food (CD or HFrD) consumed by the HFrD-fed *Chrebp* KO mice showed no significant change in body weight over the duration of experiment (Fig. 1H). In addition, the weight loss of HFrD-fed *Chrebp* KO mice was reversible (Fig. 1I and Supplementary Fig. S1). Food intake in *Chrebp* KO mice was decreased by greater than 50% within 1 to 2 days after starting HFrD and increased right after switching back to the CD, and the symptom of diarrhea was significantly improved within 48 h. The body weight and food intake of HFrD-fed *Chrebp* KO mice fully recovered

within 6 days after switching back to the CD. Together, these results indicate that *Chrebp* KO mice show significant weight loss and food intake on the HFrD relative to the CD, however, reduced food intake was not the sole cause of weight loss in HFrD-fed *Chrebp* KO mice.

3.2. Distended bowel in HFrD-fed *Chrebp* KO mice

We next examined whether HFrD might induce pathological changes of the intestine. On day 3 of the diet, HFrD-fed *Chrebp* KO mice had enlarged ceca that contained fluid. By day 5, and through day 7, these mice exhibited enlarged, red intestines and extremely distended ceca that appeared to contain both fluid and gas (Fig. 2A). Such symptoms are indicative of bacterial fermentation and intestinal irritation, both of which might further aggravate fructose malabsorption in these HFrD-fed *Chrebp* KO mice. In contrast, the intestines of WT or *Chrebp* KO mice on the CD and HFrD-fed WT mice appeared normal on days 5 and 7. Colon length and cecum weight were significantly increased in HFrD-fed *Chrebp* KO mice relative to HFrD-fed WT mice (Fig. 2B). In addition, fecal pellets were significantly decreased in HFrD-fed *Chrebp* KO mice (Fig. 2C). These changes occurred within 3–5 days after starting the HFrD. Fructose levels in the feces were 2-fold higher in HFrD-fed *Chrebp* KO mice by day 3 (Fig. 2D), demonstrating that *Chrebp* KO mice developed overt fructose intolerance accompanied by clinical signs due to increased fructose in the colon lumen. Consistent with this finding, the HFrD-fed *Chrebp* KO mice developed diarrhea, which can result from incomplete intestinal absorption of fructose. These results indicate that without ChREBP, a high concentration of fructose in the gut cannot be absorbed, which can lead to water influx into the lumen due to osmotic pressure, and rapid propulsion of bowel contents into the colon. Bacterial fermentation of unabsorbed fructose causes gas, bloating, abdominal pain, or diarrhea and results in decreased food intake and weight loss [30, 31], such as is shown in Fig 1

3.3 Impaired fructose absorption and metabolism in HFrD-fed *Chrebp* KO mice

We next characterized the impact of HFrD feeding on metabolic parameters. Eight-week-old male WT and *Chrebp* KO mice were fed either the HFrD or CD for 3 days and plasma ALT, AST, LDH, TG, and cholesterol were examined. Plasma ALT, AST, and LDH as indicators of liver injury were significantly increased in HFrD-fed WT mice, but these increases were not observed in HFrD-fed *Chrebp* KO mice (Fig. 3A–C). Plasma and liver TG and cholesterol were not significantly changed with HFrD feeding in WT mice (Fig. 3D–G). *Chrebp* KO mice showed decreased plasma TG and cholesterol and liver TG compared with WT mice upon either diet (Fig. 3D–F). Together, these data indicate that *Chrebp* KO mice were resistant to liver damage by HFrD feeding, which could result from the reduced intestinal fructose absorption and transport into the liver. Based on the fact that a significant proportion of absorbed monosaccharides is converted to lactate in rats and humans [32–34], we measured lactate levels in the intestine and liver. Lactate levels in the intestine were significantly increased in HFrD-fed WT mice but this increase was not observed in HFrD-fed *Chrebp* KO mice (Fig. 3H). Lactate levels in the liver were higher than in the intestine regardless of genotype and they were not increased by the HFrD but, rather, were decreased in HFrD-fed *Chrebp* KO mice (Fig. 3I). These results indicate that HFrD feeding increases

fructose conversion into lactate in the intestine but not in the liver, a process which is impaired in *Chrebp* KO mice.

3.4. Changes in genes involved in intestinal fructose metabolism in HFrD-fed *Chrebp* KO mice

To explore possible mechanisms underlying fructose malabsorption, we first analyzed the expression of genes involved in fructose transport and metabolism in the intestine after mice consumed the HFrD for 3 days (Fig. 4A). We found that the HFrD had no significant effect on mRNA levels of *Chrebp* or *Mix* in WT mice. Compared with WT mice fed either diet, expression of *Glut2* (the high capacity glucose transporter) and *Slc5a1* (*Sglt1*, Na⁺/glucose transporter) was significantly lower in *Chrebp* KO mice fed either diet. However, in HFrD-fed WT mice, mRNA levels of *Glut5* (the main intestinal fructose transporter) were increased 8-fold compared to WT mice fed the CD. In HFrD-fed WT mice, mRNA levels of *Khk*, *AldoB*, and *Triok*, which encode fructokinase, aldolase B, and triokinase, were increased 2-fold, 4-fold, and 5.5-fold respectively. However, these fructose-induced increases were attenuated in *Chrebp* KO mice, and expression of these genes was also lower in *Chrebp* KO mice fed the CD. In accordance with the intestine lactate levels shown in Fig. 3H, *Ldh* expression was lower in *Chrebp* KO mice compared with WT mice and a fructose-induced increase was observed only in WT mice. Previously, it was reported that mRNA levels of key gluconeogenic enzymes, glucose-6-phosphatase (*G6pc*) and fructose-1,6-bisphosphatase (*Fbp1*) increased significantly in fructose-perfused intestines [35]. Consistent with this result, the expression of these two gluconeogenic genes was dramatically increased by fructose in WT mice but the induction was completely blunted in *Chrebp* KO mice.

We next evaluated the expression of genes involved in electrolyte transport, because decreased Na⁺ absorption and increased electrogenic Cl⁻ secretion can cause luminal fluid accumulation and diarrhea [36]. The mRNA levels of *Slc9a3* (*Nhe3*), which encodes the Na⁺/H⁺ exchanger NHE3, were significantly decreased by the HFrD in *Chrebp* KO mice but not in WT mice. In contrast, the mRNA levels of *Slc26a3* (*Dra*), which encodes the Cl⁻/HCO₃⁺ exchanger DRA, were not significantly changed by HFrD in either group of mice. Changes in protein levels of ChREBP, GLUT5, GLUT2, and NHE3 correlated with the corresponding mRNA levels (Fig. 4B & C). These results suggest that intestinal ChREBP plays a key role in the dietary fructose transport as well as conversion into lactate and glucose through the fructose-induced increase of genes involved in fructose transport (*Glut5*), fructolysis (*Khk*, *AldoB*, *Triok*, *Ldh*), and gluconeogenesis (*G6pc* and *Fbp1*).

3.5. ChREBP is necessary for fructose-mediated *Glut5* induction

To identify direct targets of ChREBP that mediate the effects of fructose on fructose transport and metabolism, liquid fructose or glucose was administered by oral gavage to WT and *Chrebp* KO mice after a 4-h fast. Previous studies have shown that *Glut5* mRNA levels and fructose transport rates in rodents increase within 2–4 h of fructose consumption or after intestinal perfusion of fructose during weaning [12, 37]. Therefore, we evaluated intestinal gene expression 4 h after oral administration of liquid fructose or glucose. Blood glucose or plasma fructose concentrations were increased in both WT and *Chrebp* KO mice within 20

min after administration (Fig. 5A). It is noteworthy that plasma fructose concentration in *Chrebp* KO mice was comparable with WT mice at any time point after oral fructose gavage, partially due to the impaired fructose transport and fructolysis in the peripheral tissues [38].

Acute fructose administration produced a 3-fold increase in *Glut5* mRNA level in WT mice but did not significantly change *Glut5* expression in *Chrebp* KO mice (Fig. 5B). Compared with WT mice, *Chrebp* KO mice showed lower basal mRNA levels of *Glut2* and *Khk*, which were not changed by acute glucose or fructose administration. The basal level of *AldoB* mRNA was lower in *Chrebp* KO mice compared to WT mice. *AldoB* expression was increased slightly with fructose administration in WT mice but not in *Chrebp* KO mice. In contrast, the mRNA levels of *AldoA* and *AldoC* were not significantly changed with administration of glucose or fructose in either group of mice. The *Nhe3* mRNA level was not significantly changed with glucose or fructose administration in WT mice but decreased slightly with fructose administration in *Chrebp* KO mice. The mRNA levels of *Sglt1* and *Dra* were not changed by fructose administration in either group of mice. These data suggest that ChREBP functions as a transcriptional activator for fructose-mediated *Glut5* induction and is also required for appropriate expression of *Nhe3* in the intestine upon high fructose ingestion.

3.6. ChREBP directly regulates *Glut5* gene expression through functional ChoREs

To establish the mechanism by which ChREBP regulates *Glut5* gene expression, we performed promoter analysis. *In silico* analysis of the mouse *Glut5* promoter revealed five putative ChoREs in the 2-kb upstream region of the gene. Therefore, a nearly 2-kb fragment of the 5'-flanking region of the gene was cloned and ligated into a luciferase reporter plasmid (mGLUT5-2165, Fig. 6A) and transfected into Caco-2BBE human intestinal cells. The full-length native promoter increased luciferase activity by 150-fold compared with the empty vector, confirming *Glut5* promoter activity in intestinal cells. We then tested the effect of fructose on promoter activity in the presence or absence of the transcription factors ChREBP and MLX. Our results showed increased luciferase activity of mGLUT5-2165 upon fructose treatment only when the reporter construct was transfected along with expression vectors for ChREBP and its heterodimer partner MLX (Fig. 6B). To evaluate putative ChoRE sequences in the *Glut5* promoter, serially deleted promoter constructs were transfected along with expression vectors for ChREBP and MLX (Fig. 6C). Transcriptional activation by ChREBP/MLX was completely abolished with the deletion of ChoRE1, indicating that ChREBP activates the *Glut5* promoter through ChoRE1 in Caco-2BBE cells. To confirm the *in vivo* association of ChREBP with the *Glut5* promoter, ChIP-qPCR was performed using primers specifically designed for the ChoREs (Fig. 6D). Our results showed binding of ChREBP to ChoRE1 in the intestinal tissue of HFrD-fed WT mice but not in WT mice fed the CD or *Chrebp* KO mice fed either diet. We also evaluated whether ChREBP binds to the putative ChoRE on the *Nhe3* promoter using ChIP-qPCR. Our results show that ChREBP did not bind to the *Nhe3* promoter, suggesting that *Nhe3* is an indirect target of ChREBP (Supplementary Fig. S2). Collectively, these results demonstrate that ChREBP directly binds to ChoRE1 of the *Glut5* promoter in the intestine, and this protein-DNA interaction is dependent on diet.

4. Discussion

Excessive fructose consumption leads to fructose malabsorption, which is linked to irritable bowel syndrome (IBS). Although impaired fructose transport is considered a main cause of fructose malabsorption, the molecular mechanism underlying fructose-induced upregulation of fructose transporters is poorly understood. Here we demonstrate that ChREBP deficiency results in severe fructose malabsorption mainly due to a decreased capacity for fructose absorption resulting from insufficient induction of GLUT5 upon high fructose ingestion (Fig. 7). As a result, a high concentration of fructose in the gut cannot be absorbed, which can lead to water influx into the lumen due to osmotic pressure, and rapid propulsion of bowel contents into the colon. Fermentation of unabsorbed fructose in the colon might cause gas, bloating, abdominal pain, or diarrhea and results in decreased food intake. As shown in IBS patients [31, 39], avoiding fructose in the diet was found to improve all of the IBS symptoms that we observed in HFrD-fed *Chrebp* KO mice. This recovery in human patients is consistent with our findings that HFrD-induced weight loss and diarrhea are reversible in *Chrebp* KO mice, indicating that the clinical effects of fructose malabsorption and metabolism are reversible.

Chrebp KO mice exhibited normal food intake and growth when fed low-fructose or fructose-free diets but rapidly lost weight when fed the HFrD, as the unabsorbed fructose caused distention, localized fluid retention, and nutrient malabsorption in the gastrointestinal tract. Another contributing, though not exclusive, factor to the weight loss in HFrD-fed *Chrebp* KO mice is decreased food intake. The reason for the decreased appetite in HFrD-fed *Chrebp* KO mice is unclear, but pain, flatulence, and diarrhea resulting from incomplete intestinal absorption of fructose could all be factors. Although not investigated here, we speculate that the disturbed secretion of gut hormones, known to influence appetite and metabolism, may also be a factor in HFrD-fed *Chrebp* KO mice. Therefore, the reasons for the decreased appetite in HFrD-fed *Chrebp* KO mice warrant further investigation.

In our study *Chrebp* KO mice showed severe diarrhea as early as 3–5 days after the start of the HFrD, whereas *Glut5* KO mice have been reported to exhibit relatively well-formed stools when eating a similar diet [13]. These different phenotypes could be explained by differential expression of NHE3. Intestinal NHE3 is the major Na^+/H^+ exchanger at the apical membrane of intestinal epithelial cells [40] and its transport function is essential for the regulation of salt and water homeostasis. In mice, NHE3 deficiency leads to mild diarrhea and distention in all intestinal segments [41]. Consistent with these results, HFrD-fed *Chrebp* KO mice showed significantly decreased NHE3 expression, whereas HFrD-fed *Gluts* KO mice showed increased NHE3 expression [13]. However, we did not observe the direct binding of ChREBP to the putative ChoRE in the *Nhe3* promoter in HFrD-fed mice (Supplementary Fig. S2), suggesting that *Nhe3* is an indirect target of ChREBP. Further studies are needed to identify the mechanism by which ChREBP regulates NHE3 protein levels in response to fructose consumption.

In this study, we observed acute liver damage in HFrD-fed WT mice but not in *Chrebp* KO mice. The HFrD feeding for 3 days fails to increase plasma and liver TG in WT mice. Therefore a possible mechanism by which fructose may induce liver toxicity involves the

influx of fructose itself into the liver [42, 43]. Fructose is metabolized by KHK, which phosphorylates fructose to fructose-1-phosphate rapidly, resulting in marked ATP depletion coupled with the absence of a feedback inhibition. In WT mice, excessive fructose consumption increases intestinal fructose uptake and delivery into the liver, resulting in increased production of fructose-1-phosphate and ATP depletion. Whereas, in *Chrebp* KO mice, low fructose delivery to the liver due to insufficient fructose uptake in the intestine may prevent such ATP depletion and liver damage.

Recently Jang *et al.* [34] reported that most dietary fructose is cleared by the intestine and that clearance requires KHK. They found that the intestine converts fructose to glucose and various organic acids (lactate, glycerate, TCA intermediates, and amino acids). In agreement with their results, we also observed the significantly increased expression of genes involved in fructose transport (*Glut5*), fructolysis (*Khk*, *AldoB*, *Triok*, and *Ldh*), and gluconeogenesis (*G6pc* and *Fbp1*) in HFrD-fed WT mice. In *Chrebp* KO mice, expression of these genes is significantly decreased and fructose-induced increased expression of these genes is blunted. It is known that the fructose-induced expression of these genes requires increased GLUT5 activity. Therefore, further studies are needed to clarify whether these genes are directly regulated by ChREBP or indirectly by increased fructose and/or its metabolites downstream of GLUT5 activity. Intestine-specific induction of GLUT5 in *Chrebp* KO mice will be a valuable tool to test this question.

Fructose absorption in humans appears to be limited by high fructose consumption [7], and this is consistent with the restricted absorption capacity of a facilitative transport system. The inability to upregulate GLUT5 protein levels to match fructose levels in the diet may also be a cause of fructose malabsorption and diarrhea-predominant irritable bowel syndrome in humans. Several studies showed no correlation between fructose malabsorption and *Glut5* gene expression or variants in humans [44, 45]. These results do not necessarily preclude a role for GLUT5 in fructose malabsorption, because those studies were performed on fasted patients and the cohort size was small. It is possible that reduced transport could result from impaired upregulation of gene expression or activity of GLUT5 in response to dietary fructose. Our findings underscore the need to evaluate genetic defects in ChREBP and the *Glut5* promoter region in adult patients with fructose malabsorption and diarrhea-predominant irritable bowel syndrome.

In conclusion, our study demonstrated the role of ChREBP in intestinal fructose absorption in mice challenged with a high-fructose diet and supports the use of *Chrebp* KO mice as a model for irritable bowel syndrome with diarrhea. Further studies are needed to better understand tissue-specific actions of the ChREBP pathway in response to a high-fructose diet. Targeting ChREBP may be a potential therapy for fructose-induced obesity, hepatic steatosis, and hypertriglyceridemia, but such therapy could be accompanied by intestinal fructose malabsorption as shown in this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CD	control diet
ChREBP	carbohydrate response element-binding protein
FBP1	fructose-1,6-bisphosphatase
GLUT2	glucose transporter 2
GLUT5	glucose transporter 5
G6PC	glucose-6-phosphatase
IBS	irritable bowel syndrome
HFrD	high-fructose diet
KHK	fructokinase
KO	knockout
LDH	lactate dehydrogenase
MLX	Max-like protein X
NHE3	Na ⁺ /H ⁺ exchanger type3
TG	triglyceride
TrioK	triokinase
WT	wild type

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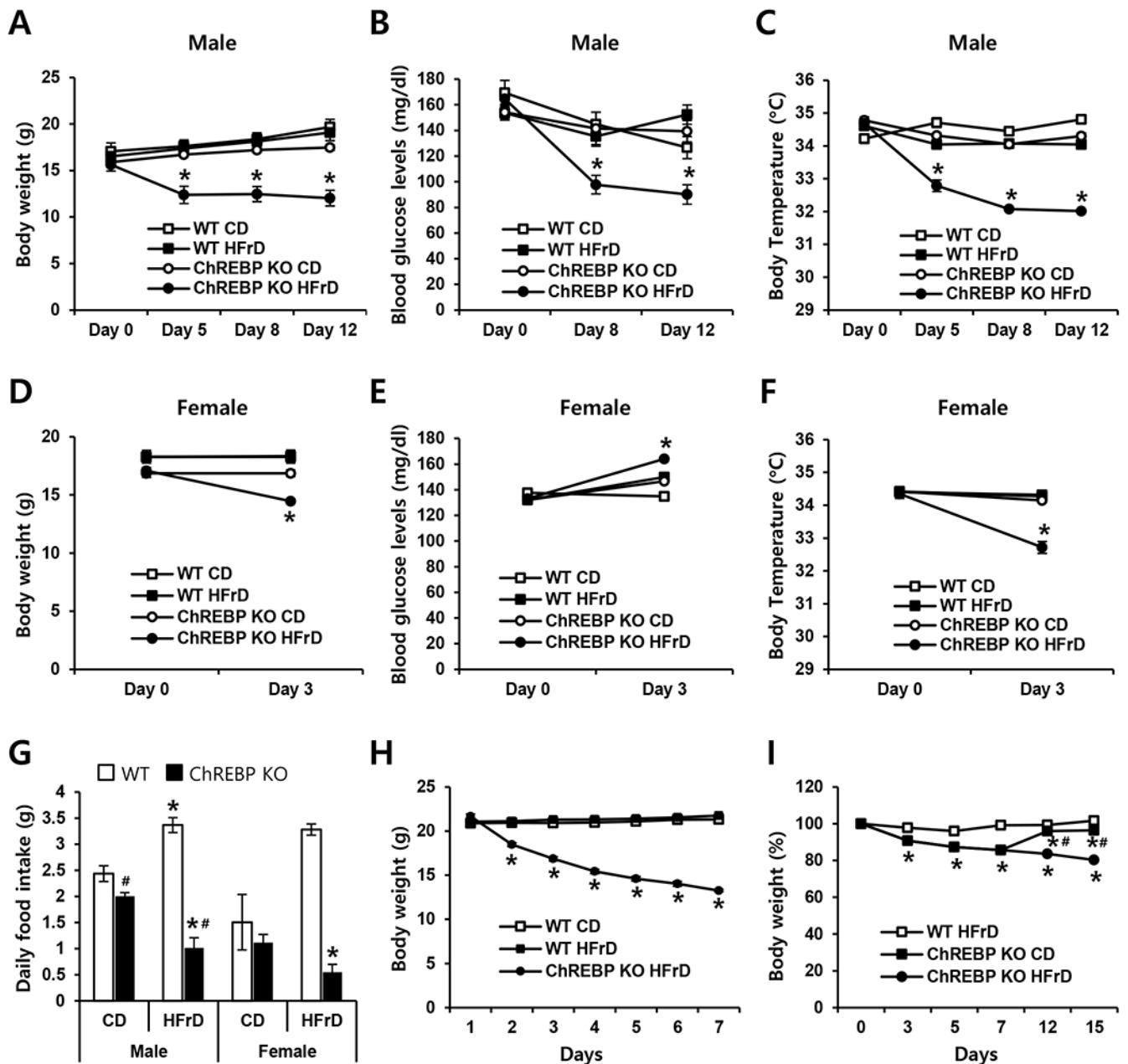


Fig. 1. Mice lacking ChREBP lost body weight and appetite with high-fructose feeding
 WT (n=9) and *Chrebp* KO (n=8) mice were fed with either the control diet (CD) or high-fructose diet (HFrD) for 3 to 12 days starting at 6 weeks of age. (A) Body weight, (B) peripheral blood glucose, and (C) body temperature of male mice were measured sequentially on days 0, 5, 8, and 12. (D) Body weight, (E) blood glucose, and (F) body temperature of female mice were measured on days 0 and 3. (G) Daily food intake (average for 3 days) was measured in male and female mice. (H) A pair-feeding experiment was conducted for 7 days, with WT mice fed the same amount of food (CD or HFrD) as was consumed by HFrD-fed *Chrebp* KO mice. (I) Body weight of WT mice fed the HFrD (n=9) and *Chrebp* KO (n=8) mice that were fed either the HFrD for 15 days or the HFrD for 7 days

and then switched to the CD until day 15. Results are expressed as mean \pm SEM. * $p < 0.05$ vs. the WT HFrD group and *Chrebp* KO CD group (A-F), * $p < 0.05$ vs. genotype-matched, CD-fed group; # $p < 0.05$ vs. diet-matched, genotype control (G). * $p < 0.05$ vs. the WT CD group (H), * $p < 0.05$ vs. the WT HFrD group; # $p < 0.05$ vs. genotype-matched, HFrD group (I).

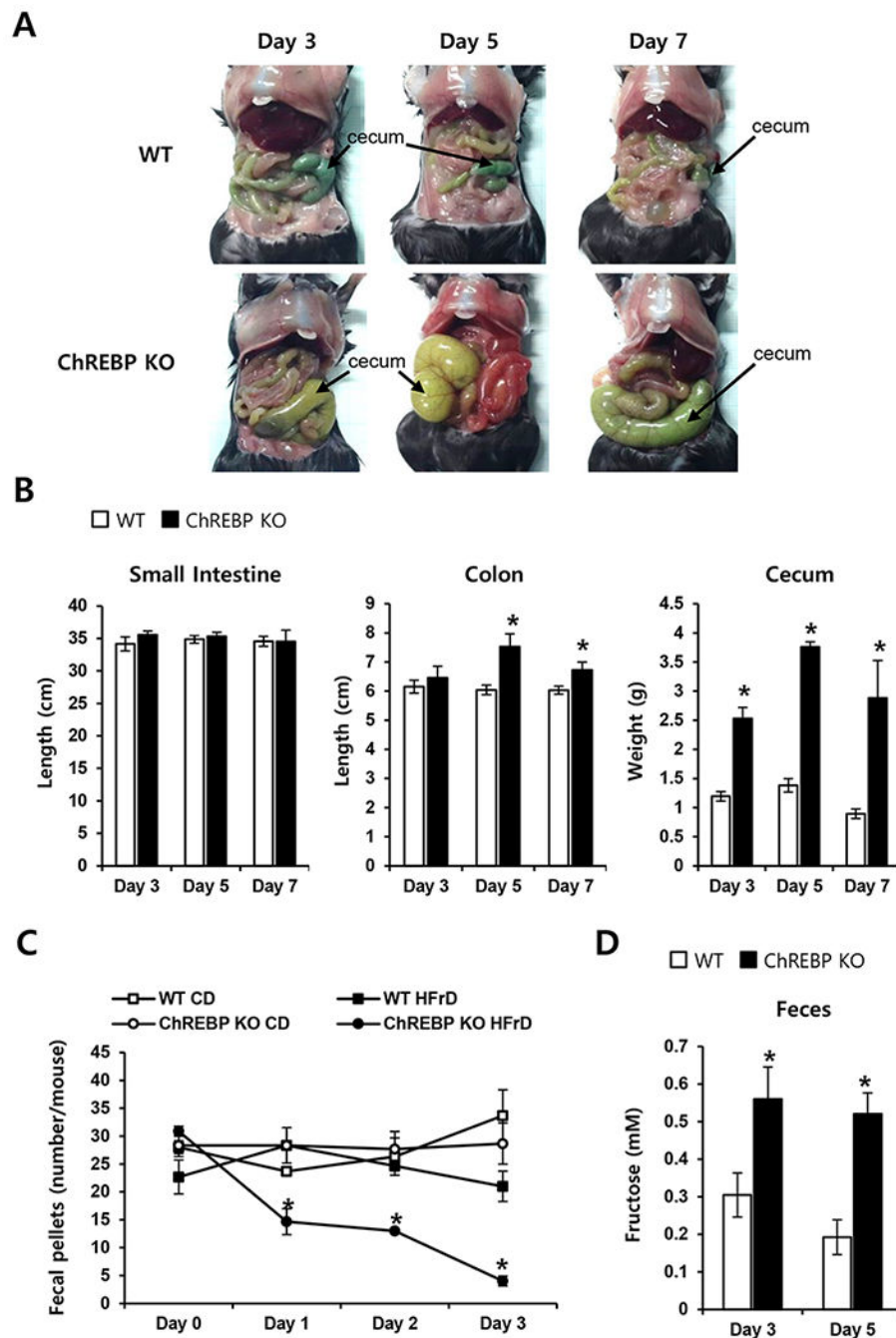


Fig. 2. *Chrebp* deletion caused fructose malabsorption upon high-fructose feeding
 WT (n 5) and *Chrebp* KO (n 5) mice were fed either the control diet (CD) or high-fructose diet (HFrD) for 7 days starting at 8 weeks of age. On days 3, 5, and 7, the small intestine, colon, and cecum of HFrD-fed *Chrebp* KO mice were distended, whereas the intestines of HFrD-fed WT mice appeared normal. (A) The cecum and colon of HFrD-fed *Chrebp* KO mice were distended, whereas the intestines of HFrD-fed WT mice appeared normal. (B) Intestine length and cecum weight of HFrD-fed WT and *Chrebp* KO mice. (C) Formed stools (fecal pellets) were measured on days 0–3, and (D) the fructose concentration of feces was

determined on days 3 and 5 in HFrD-fed WT and *Chrebp* KO mice. Results are expressed as mean \pm SEM. * $p < 0.05$ vs. the WT HFrD group.

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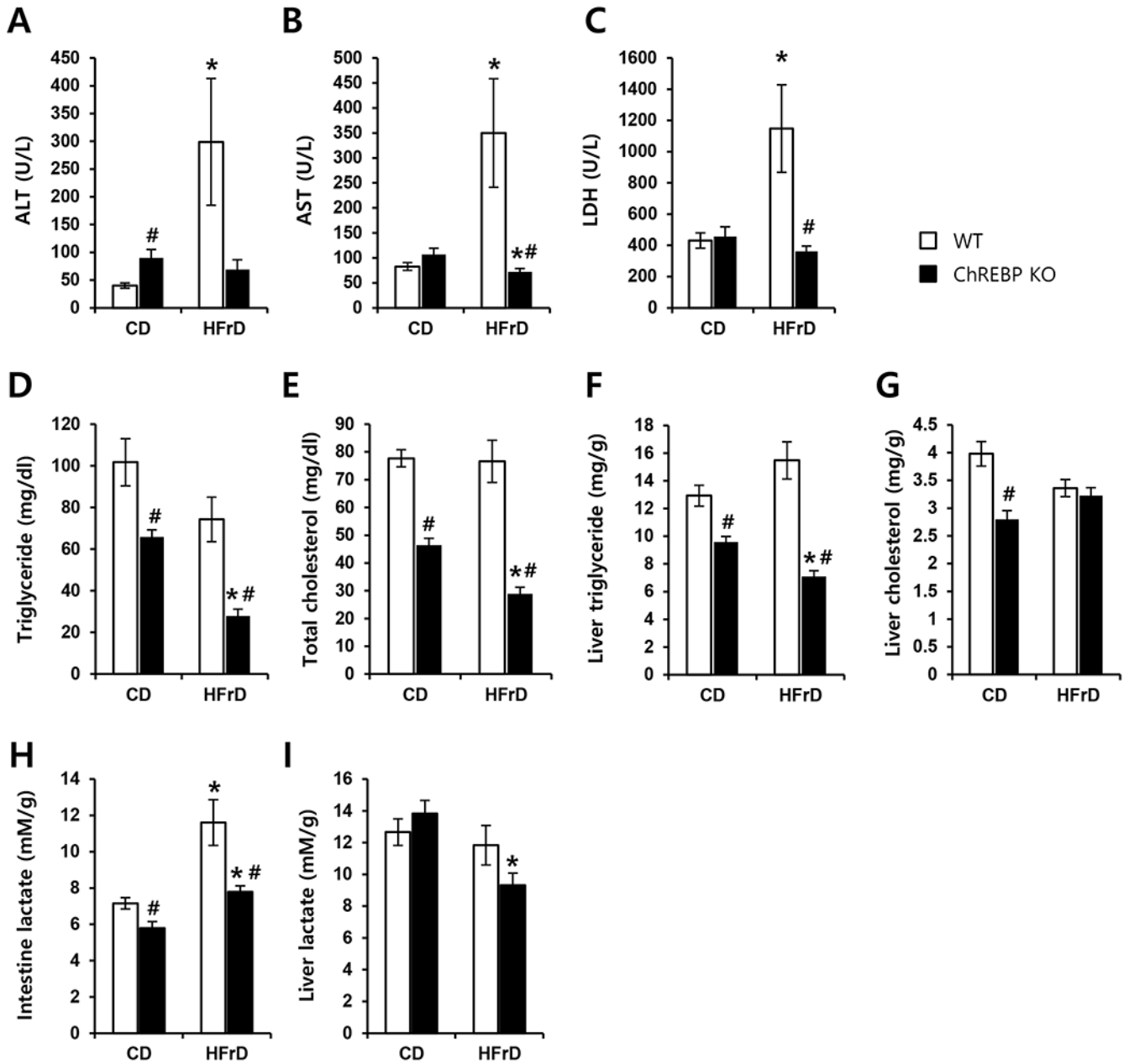


Fig. 3. *Chrebp* deletion resulted in impaired fructose transport into the liver and impaired conversion to lactate in the intestine

WT (n=8) and *Chrebp* KO (n=8) mice were fed either the control diet (CD) or high-fructose diet (HFrD) for 3 days starting at 8 weeks of age; they were then fasted for 2 h to achieve synchronization. Plasma levels of (A) ALT, (B) AST, (C) LDH, (D) triglyceride, and (E) total cholesterol, (F) liver triglyceride, and (G) liver cholesterol were measured from portal samples. Lactate levels in (H) the intestine and (I) liver were measured. Results are expressed as mean \pm SEM. * $p < 0.05$ vs. genotype-matched, CD-fed group; # $p < 0.05$ vs. diet-matched, genotype control.

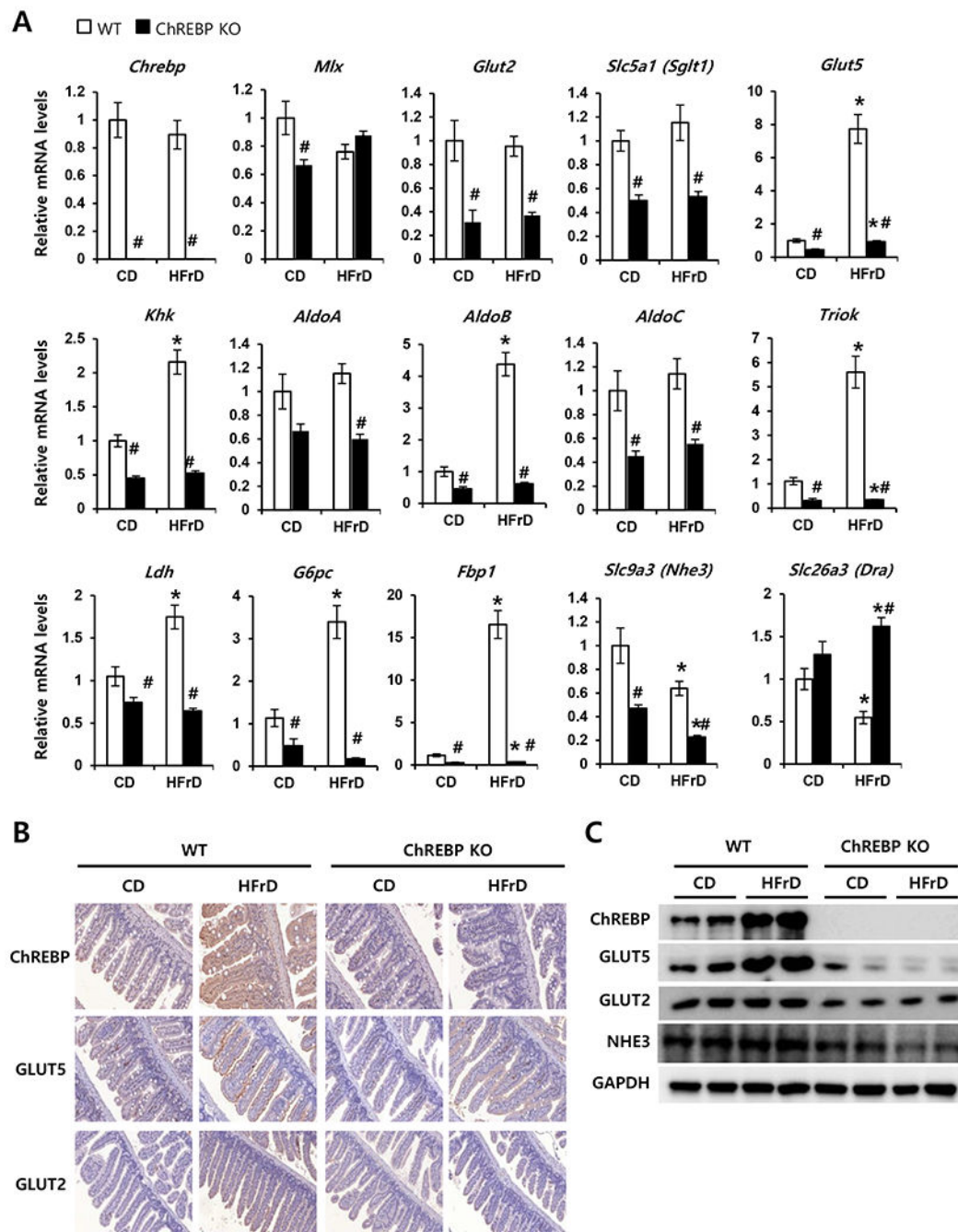


Fig. 4. *Chrebp* deletion decreased fructose-induced transporter and fructolytic gene expression
 WT (n=8) and *Chrebp* KO (n=8) mice were fed either the control diet (CD) or high-fructose diet (HFrD) for 3 days starting at 8 weeks of age. (A) Relative expression of genes encoding the transcription factors *Chrebp* and *Mix*, glucose and/or fructose transporter genes (*Glut2*, *Sgt1*, and *Glut5*), fructolytic genes (*Khk*, *AldoA*, *AldoB*, *AldoC*, *TrioK*, and *Ldh*), gluconeogenic genes (*G6pc* and *Fbp1*), and ion transporter genes (*Nhe3*, and *Dra*) in the intestine were analyzed by qPCR. Target gene expression was normalized to the expression of *Rplp0* and expressed as mean \pm SEM. * p <0.05 vs. genotype-matched, CD-fed group;

[#] $p < 0.05$ vs. diet-matched, genotype control. (B) Representative immunohistochemistry images of ChREBP, GLUT5, and GLUT2 in the small intestine of WT (C57BL/6J) and *Chrebp* KO mice. (C) Western blot analysis of ChREBP, GLUT5, GLUT2, and NHE3 in the small intestine of WT and *Chrebp* KO mice fed the CD or HFrD.

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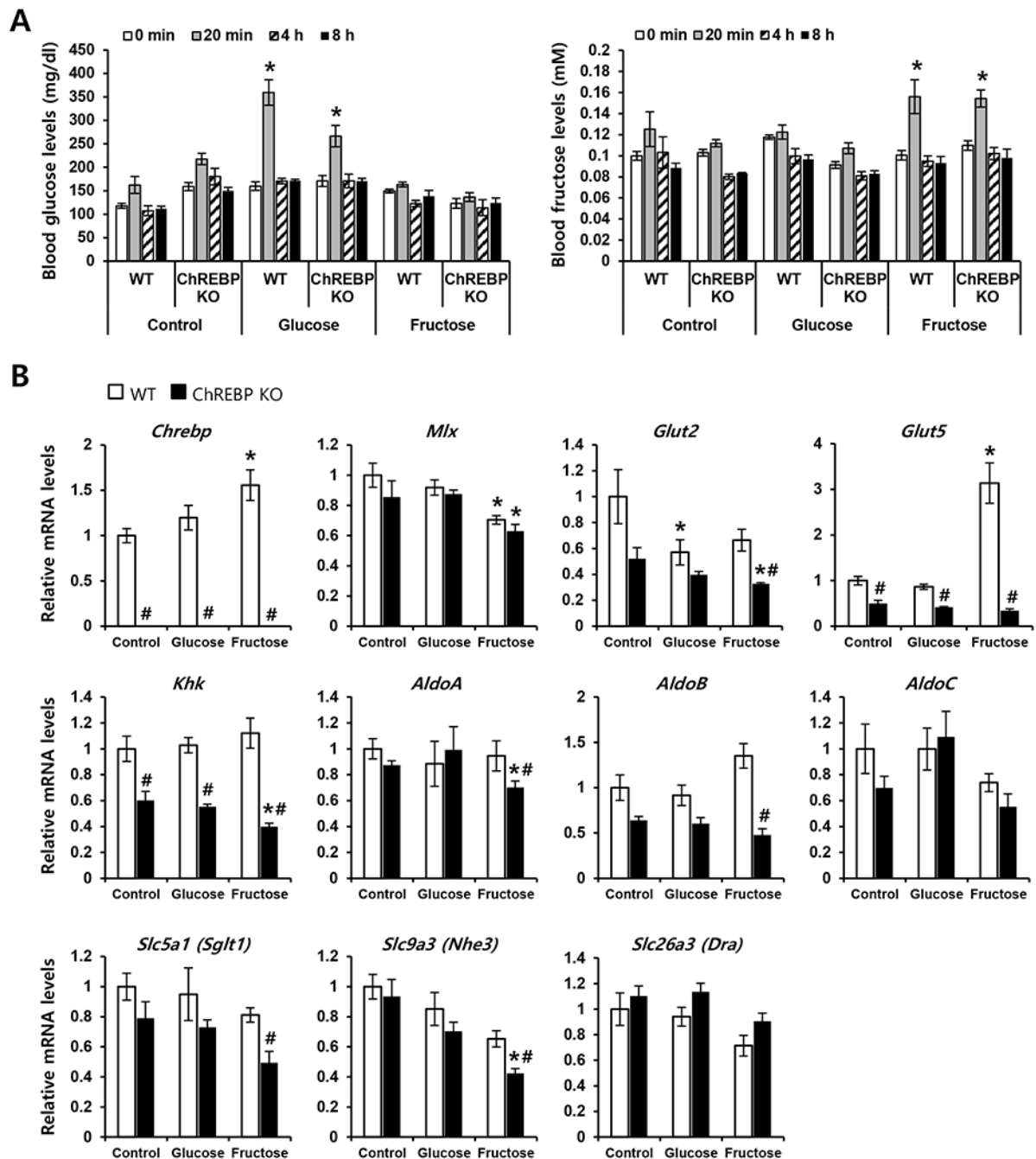


Fig. 5. ChREBP directly increases fructose-mediated *Glut5* gene expression

WT and *Chrebp* KO mice were given Dulbecco's phosphate buffered saline (control), 20% fructose, or 20% glucose by oral gavage (n=4 each group). (A) Plasma fructose and glucose concentrations were measured at 0 min, 20 min, 4 h, and 8 h. (B) After 4 h, the expression of genes encoding the transcription factors *Chrebp* and *Mlx*, fructose transporter genes *Glut2* and *Glut5*, fructolytic genes *Khk*, *AldoA*, *AldoB*, and *AldoC*, and ion transporter genes *Sgt1*, *Nhe3*, and *Dra* were analyzed by qPCR. The expression of target genes was

normalized to *Rplp0* expression and expressed as mean \pm SEM. * $p < 0.05$ vs. genotype-matched, control group; # $p < 0.05$ vs. administration-matched, genotype control.

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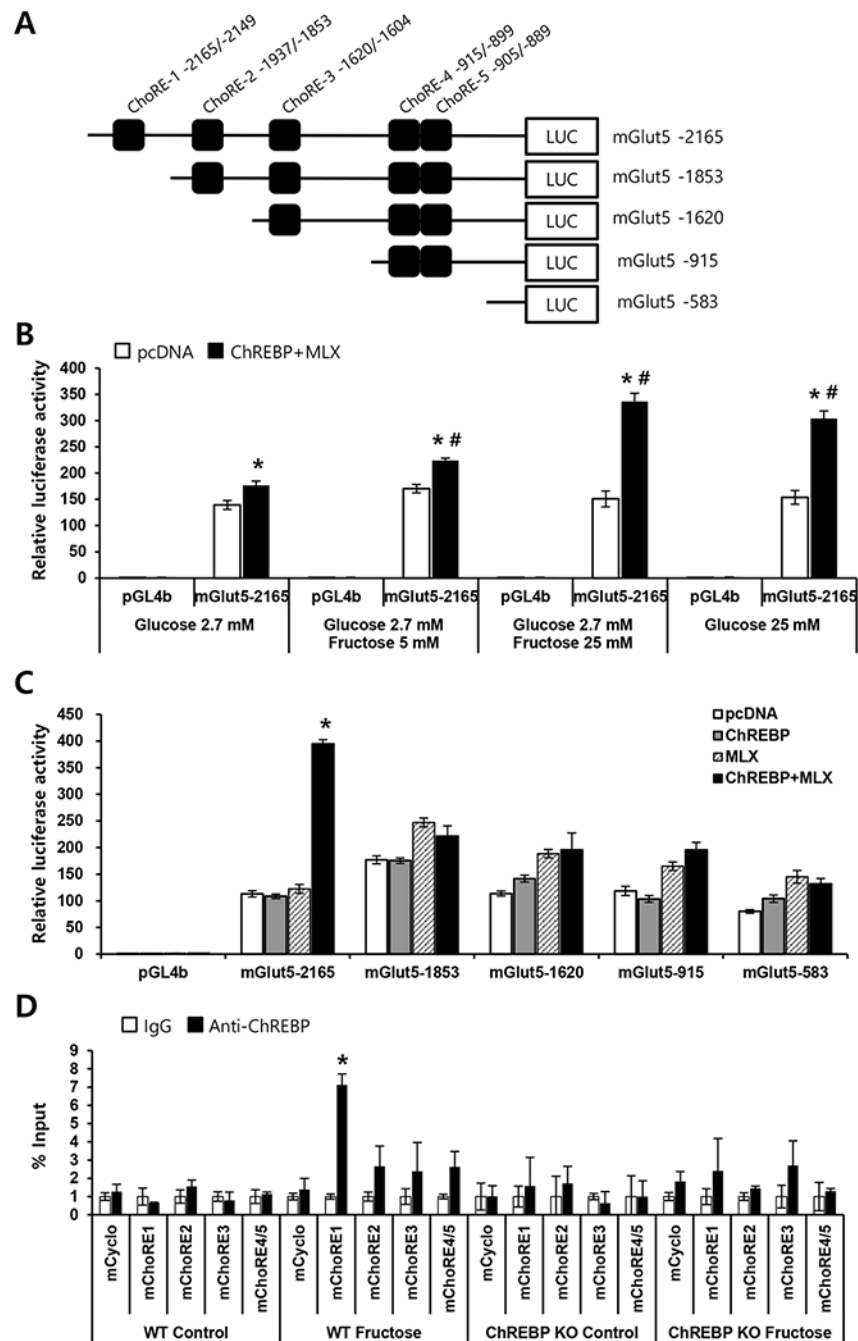


Fig. 6. ChREBP directly binds to carbohydrate response elements in the *Glut5* promoter (A) Locations and sequences of carbohydrate response elements (ChoREs) in the mouse *Glut5* promoter and schematic diagram of the serial deletion *Glut5* promoter-luciferase reporter constructs. (B) The full-length *Glut5* promoter-luciferase reporter construct (mGlut5–2165) was transfected into Caco-2BBE cells along with vectors expressing *Chrebp* and *Mix* or empty pcDNA vector (control). Transfected cells were maintained in DMEM containing the indicated concentration of glucose and/or fructose for 48 h. Results are expressed as fold increase in luciferase activity relative to that of the control vector (pGL4b)

in 2.7 mM glucose. * $p < 0.05$ vs. pcDNA, # $p < 0.05$ vs. 2.7 mM glucose-treated group. (C) Serially deleted *Glut5* promoter-luciferase reporter constructs were transfected into Caco-2BBE cells, with or without vectors expressing *Chrebp* and/or *Mix*. After 48 h, luciferase activity was determined. Results are expressed as fold increase in luciferase activity (mean \pm SEM) relative to that of the control vector pGL4b. * $p < 0.05$ vs. pcDNA. (D) ChIP assay for ChREBP binding to ChoREs in the *Glut5* gene promoter. Chromatin was extracted from intestinal tissue of WT or *Chrebp* KO mice that received DPBS (control) or 20% fructose (Fructose) for 4 h (n=3 per group) by oral gavage. Chromatin was immunoprecipitated with control IgG or anti-ChREBP antibody and amplified by qPCR. Target gene enrichment was normalized to the enrichment of cyclophilin. Results are expressed as fold increase in the signal (mean \pm SEM) compared to control IgG. * $p < 0.05$ vs. IgG.

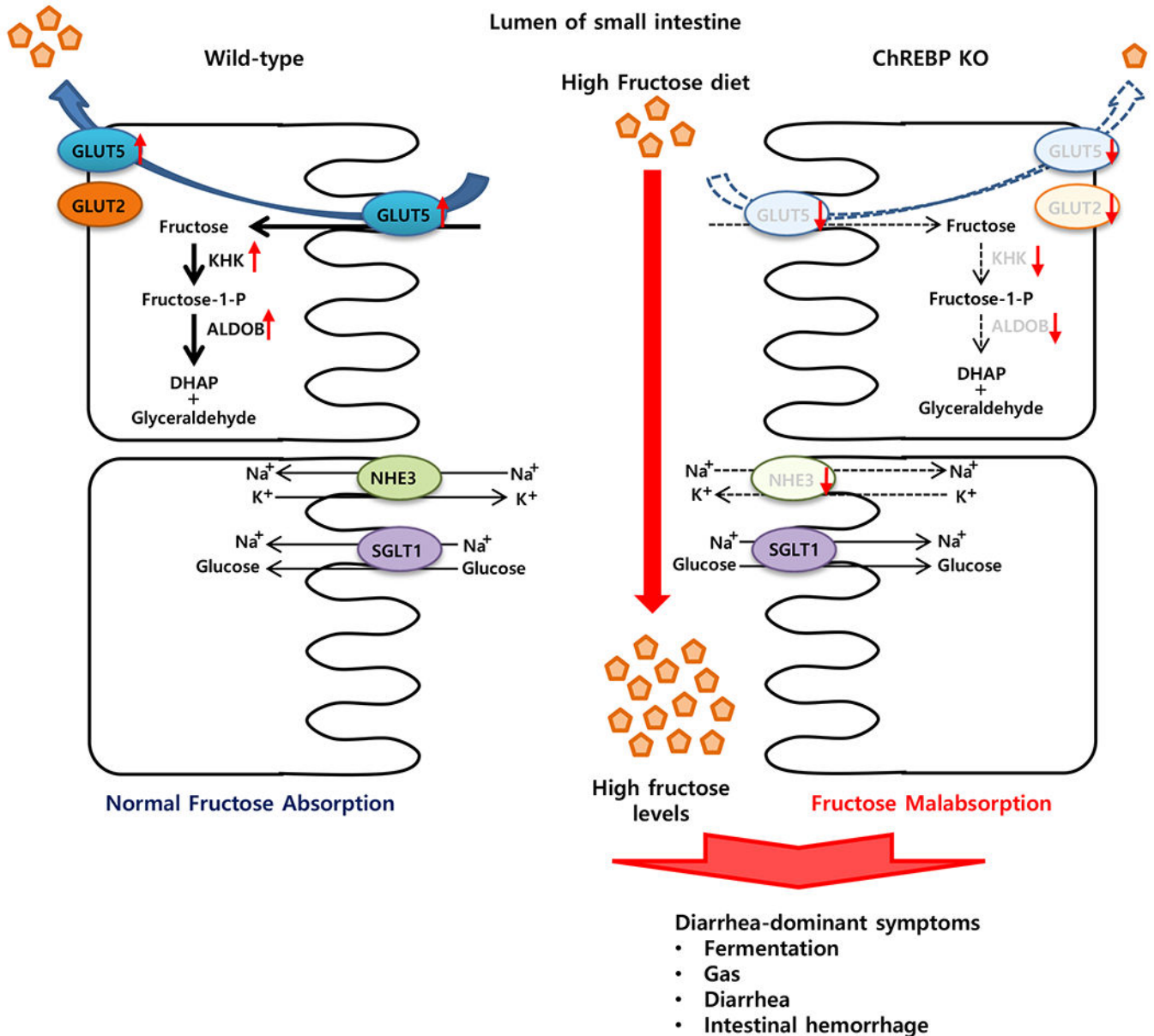


Fig. 7. Model showing the effects of ChREBP deletion on fructose transport and metabolism upon high-fructose diet feeding

Upon high fructose ingestion, genes involved in fructose transport (*Glut5*), fructolysis (*Khk*, *AldoB*, *Triok*, and *Ldh*), and gluconeogenesis (*G6pc* and *Fbp1*) are significantly increased in the intestinal cells. However, *Chrebp* KO mice show impaired fructose absorption and diarrhea, due to the inability to upregulate GLUT5 as well as fructolytic and glucogenic enzymes to match fructose levels in the diet, and the decreased expression of NHE3, a contributor to Na^+ and water absorption in the intestine. As a result, HFrD-fed *Chrebp* KO mice develop diarrhea-dominant irritable bowel symptoms such as gas, bloating, abdominal pain, or diarrhea. Significantly increased genes by high fructose in WT mice but not in *Chrebp* KO mice are indicated in red color. ALDOB, aldolase B; ChREBP, carbohydrate response element-binding protein; DHAP, dihydroxyacetone phosphate; F-1-P, fructose-1-

phosphate; F-1,6-BP, fructose-1,6-bisphosphate; FBP1, fructose-1,6-bisphosphatase; GA, glyceraldehyde; GAP, glyceraldehyde-3-phosphate; GLUT2, glucose transporter 2; GLUT5, glucose transporter 5; G-6-P, glucose-6-phosphate; G6PC, glucose-6-phosphatase; KHK, fructokinase; LDH, lactate dehydrogenase; NHE3, Na⁺/H⁺ exchanger type3; SGLT1, Na⁺/glucose transporter; TrioK, triokinase.

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