

## RESEARCH DESIGN AND METHODS

### Mice

GFRAL knockout (KO) mice were generated as previously described(1) and breeding pairs were kindly provided by Dr. Randy Seeley (University of Michigan). All mice used in the study were housed in accordance with the guidelines for Animal Use, and studies were approved by the Animal Ethics Research Board of McMaster University (AUP: 210104). All mice were males on a C57BL/6J background and housed on a 12-hour light/dark cycle with ad libitum access to food and water. All mice were housed in specific-pathogen free (SPF) microisolators in a room kept at 21-22°C, 40-60%RH. Before experiments, male mice were randomized and separated into different groups matched on body weight. Mice used in acute experiments were 8-16 weeks of age and placed on a chow diet. Male wild-type (WT) and GFRAL knockout (KO) mice were placed on a high-fat, high-fructose diet (HFD: 40 kcal% fat (mostly palm oil), 20 kcal% fructose with 0.02% cholesterol) (Research Diets, Inc., Code: D19101102) starting at 12-16 weeks of age for 12 weeks before being singly housed and treated with vehicle or linolenic acid via oral gavage for 7 days. Fatty acids were orally administrated at noon. Body weight and food weight were recorded daily during the treatment period. Food intake was calculated by subtraction of the amount of food content in food hoppers from the amount added the previous day. Spillage and grind of food in cages was carefully monitored every day. Blood from live animals was collected from a tail snip. Blood samples were centrifuged at 10,000 rpm for 10 min at 4°C after clotting at room temperature for 30 min, and the supernatant was collected. Serum was saved and stored at -80°C until use. Mice were anesthetized using ketamine/xylazine. Heart, liver, kidney, intestine, colon, stomach, cecum, lung, spleen, epididymal white adipose tissue (eWAT), inguinal WAT (iWAT), brown adipose tissue (BAT) and brain were snap-frozen and stored at -80°C until use.

### Biochemical analysis in serum

In acute treatment experiments with fatty acids, mice were placed on a chow diet. After oral gavage of vehicle control, palm oil, soybean oil, palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3) or glucose (2.25g/kg/day), tail blood was taken at 4, 8 or 13 h. Mouse GDF-15 (R&D Systems, Catalog#: DY6385)(2), insulin (Crystal Chem, Catalog#: 90080), ghrelin (Sigma, EZRGRT-91K) and GLP-1 (Sigma, EZGLP1T-36K) was measured by following the kit protocols.

### RNA isolation, cDNA synthesis and qPCR.

Frozen tissues were homogenized and lysed in TRIzol reagent. After centrifugation, the supernatant (aqueous phase) was applied to the RNeasy kit (Qiagen, ID: 74106) for subsequent total RNA extraction and purification according to its protocols. cDNA synthesis was using SuperScript® IV Reverse Transcriptase kit (Invitrogen, Catalog#: 18090010) according to the manufacturer's instructions. The detection of cDNA expression for specific genes was performed by qPCR using AmpliTaq Gold™ DNA Polymerase kit (Applied Biosystems, Catalog#: N8080241). Taqman® primers were purchased from ThermoFisher Scientific. Relative mRNA levels were quantified with the  $\Delta C_T$  method, using mouse *Actb* (Mm02619580\_g1) as an endogenous control. Gene specific primers were listed as following: *Gdf15* (Mm00442228\_m1), *Ddit3* (Mm01135937\_g1), *Atf4* (Mm00515325\_g1) and *Xbp1s* (Mm03464496\_m1).

## Immunoblotting

The kidney homogenates were prepared by using lysis buffer containing, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton, 2.5 mM, sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1  $\mu$ g/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and phosphatase inhibitor cocktail. Total lysate protein concentrations were determined using the BCA Protein Assay. Protein concentrations were adjusted and diluted in 5X LDS (lithium dodecyl sulfate gel sample buffer) containing 50 mM DTT. Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10%) Bis/Tris, MOPS running buffer. Separated proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. Nonspecific binding was blocked by 5% BSA for 1 h at room temperature, and membranes were probed with antibodies against ATF4 (1:1000, CST, Cat #: 11815) and  $\beta$ -actin (1:1500, CST, Cat #: 5125). After washing, membranes were incubated with an anti-rabbit secondary antibody (1:2000, CST, Cat #: 7074) at room temperature for 1 h. Protein bands were visualized with the MBI Fusion FX7 camera system and quantified using the ImageJ software (1.54f).

## RNAscope analysis

Mouse samples were fixed in 10% neutral buffered formalin for 36 hours and paraffin processed, embedded and stained at the Core Histology Facility at McMaster University. Slides for chromogenic *in situ* hybridization were cut and stained with RNAscope 2.5 LS -ISH probe Mm-GDF15 (318528) from Advanced Cell Diagnostics (ACD) and detected with RNAscope 2.5 LSX -Red Reagent kit (ACD, 322750). Staining was performed on the Leica Bond RX automated stainer using a program devised by ACD for the Leica Bond and included a mild pretreatment of slides with ER2 retrieval and Protease supplied in the reagent kit. The slides were covered by coverslip with VectaMount Permanent mounting medium from Vector Labs (H-5000). The quantification of GDF15 mRNA staining was measured by QuPath software (v0.2.3).

## Statistics

Statistical analyses were performed using GraphPad Prism (version 9.5.1). All values are reported as mean  $\pm$  SEM unless stated otherwise. Data were analyzed using one-way or two-way ANOVA with Tukey's or Šídák's post-hoc tests, or unpaired t test (two-sided) where appropriate. Differences were considered significant when  $P < 0.05$ .

## References

1. Frikke-Schmidt H, Hultman K, Galaske JW, Jorgensen SB, Myers MG, Jr., Seeley RJ: GDF15 acts synergistically with liraglutide but is not necessary for the weight loss induced by bariatric surgery in mice. *Mol Metab* 2019;21:13-21

2. Martinussen C, Svane MS, Bojsen-Møller KN, Jensen CZ, Kristiansen VB, Bookout AL, Jørgensen SB, Holst JJ, Albrechtsen NJW, Madsbad S, Kuhre RE: Plasma GDF15 levels are similar between subjects after bariatric surgery and matched controls and are unaffected by meals. *American Journal of Physiology-Endocrinology and Metabolism* 2021;321:E443-E452