**Title:** GDF15 knockout does not impact maternal food intake or body composition but increases female offspring bodyweight in the first 14 days of life

**Authors:** Molly C. Mulcahy, Noura El Habbal, JeAnna R. Redd, Haijing Sun, Randy Seeley Brigid E. Gregg, Dave Bridges

**Affiliations:**

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**Author contribution:** MM, NEH, and DB conceived of experiments. Data were collected by MM, NEH, JRR, and HS. RS provided the experimental animals. MM carried out analysis and wrote the initial draft of the manuscript. All authors contributed to the editing of the manuscript and approved its final format before publication.

Corresponding Author:

Dave Bridges, PhD, Email address: [davebrid@umich.edu](mailto:davebrid@umich.edu), Postal address: 1863 SPH I 1415 Washington Heights, Ann Arbor, Michigan 48109-2029, Telephone: +1 (734) 764-1266

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# Abstract

# Introduction

# Materials and Methods:

## Animal Husbandry and Protocol

Animals were housed in a temperature and humidity-controlled facility with a 12-hour light:dark cycle. Male and female *Gdf15* null animals were generated by the Seeley lab as detailed in (Frikke-Schmidt et al., 2019). Briefly, C57BL6/J mice were obtained from Jackson laboratories (RRID IMSR\_JAX:000664). To generate the *Gdf15*-/-, gRNAs were designed to flank the second exon of *Gdf15*. Using CRISPR-Cas9, oligonucleotides corresponding to the exon 2 gRNAs were cloned into px330. These plasmids were then injected into embryos and transferred into female mice. Resultant pups were screened for loss exon 2 buy PCR and DNA sequencing.

## Genotyping

At 14 days of age, a small section of the tail was collected and digested in 100uL of lysis buffer (10 mM Tris (pH8.0), 150 mM NaCl, 10 mM EDTA, 0.1% SDS and 1 mg/ml proteinase K) at 55°C for 4 hours. Digested DNA sample was utilized with DreamTaq Green to generate PCR product (ThermoFisher Scientific, Catalog #K1081). Genotyping by PCR was conducted with 2 forward and one reverse primer (see table). Initiation of PCR was at 95 °C for 3 minutes, followed by 38 cycles of denaturation(95°C for 30 seconds), annealing (60°C for 40 seconds), and elongation (72°C for 1 minute), and a final amplification step at 72°C for 5 minutes. PCR product was put through gel electrophoresis on a 2% agarose gel at 130V and imaged on a gel documentation system using UV light. PCR product resulted in 2 visible bands, one at 200bp (KO) and another at 600bp (WT). Mice with both bands considered heterozygous.

## Mating

To generate study animals, heterozygous mating pairs (*Gdf15+/-*) were combined to generate the F1 experimental animals (dams and sires). This project used only mating pairs that were homozygous for *Gdf15* and are referred to as *Gdf15-/-*(KO), *Gdf15+/+* (WT) for the remainder of this work. Adult female mice (GDF15-/-n=8, GDF15+/+n=6), at least 70 days old, were singly housed with *ad libitum* access to water and a standard chow diet (CD, Picolab Laboratory Rodent diet, (5L0D; 5% of Calories from fat, 24% from protein, 71% from carbohydrates). Once single-housed, weekly food intake and body weight measurements began and continued throughout the experiment. After one week of food and body weight monitoring, homogenous genotype males were introduced for mating. Males were allowed to remain in the breeding cage until a copulatory plug was discovered, indicating pregnancy (E0.5). Body weight and food intake measurements continued weekly through gestation and postnatal day 14.5. Their resultant offspring (F2) were homozygous WT or KO and were studied until postnatal day 14 (PND14). All protocols were approved by the institutional animal care and use committee of the University of Michigan.

## PCR Primers

|  |  |  |
| --- | --- | --- |
| Gene | Forward 5’-3’ | Reverse |
| *Gdf15* primer set 1 | 5' GAT TCC CGC CCG AAT TAG C 3' | 5’ ATC CGT CCT ACT CTG GCT AAG 3' |
| *Gdf15* primer set 2 | 5' CCG AAT TAG CCT GGT CAC CC 3' | 5’ ATC CGT CCT ACT CTG GCT AAG 3' |

## Insulin tolerance test

On E16.5, dams underwent insulin tolerance testing. Dams were placed in clean cage without access to food but with ad libitum access to water at ZT 2. Dams were fasted for 6 hours (ZT2-ZT8). Baseline blood glucose was assessed using a tail clip and a handheld glucometer (OneTouch Ultra). After initial blood glucose measurement, an intraperitoneal injection of insulin was administered (Humulin, u-100; 0.75U/kg lean mass). Blood glucose was measured in 15-minute intervals for 2 hours. Area under the curve was calculated by taking the sum of all glucose values for each animal and averaging by genotype. 24 hours after ITT, we collected two fed blood samples: at ZT1 and ZT13. Dams were lightly anesthetized via inhaled isoflurane and whole blood was collected by retroorbital bleed in a heparinized capillary tube. Blood was allowed to clot on ice for 20 minutes then was spun down in a cold centrifuge (4°C, Eppendorf microcentrifuge, model 5415R) for 20 minutes at 2000 g. Serum was decanted off after centrifugation and stored at -80°C until used for analysis.

*Serum Gdf15 Quantification*

Serum *Gdf15* analysis was completed using maternal serum collected 24 hours after ITT. Gdf15 levels were determined via ELISA according to manufacturer guidelines (R&D system, catalog # MGD150).

## Offspring

Pups were counted and body weights were recorded within 24 hours of birth, postnatal day (PND 0.5). Gestational age was determined as difference between birth dates and dates of appearance of copulatory plug. At PND 3.5, litter sizes were culled to 2 male and 2 female pups, to standardize amount of nutrition provided to each pup. Survival of pups to PND 3.5 was assessed by comparing the number of pups present at PND 3.5 to the number present on PND 0.5 and is expressed as a percentage. Body weight was assessed for each pup on PND 0.5, 3.5, 7.5, 10.5, and 14.5. Pups were euthanized by decapitation on the 2 hours before milk collection began (PND 14.5-17.5).

## Weigh-suckle-weigh, milk volume production

On postnatal day 10.5, we assessed milk volume production by the weigh-suckle-weigh method (ref). Dams were weighed using an analytical scale to the nearest 0.01 gram and placed in a clean cage with free access to food and water. Pups were then weighed in aggregate and placed in a clean cage on top of a heating pad without access to food or water. Dam and pups remained separated for 2 hours. After 2 hours, weight measurements were repeated, and pups were reintroduced to the dam’s cage where they remained for 1 hour. After one hour, the final weights were taken for both dams and pups in aggregate. Volume of milk produced is expressed the average weight lost by each dam after 1 hour of nursing divided by the number of pups in the litter.

## Milk collection

Milk collection took place on PND 14.5-17.5. Pups were separated from dams and sacrificed 2 hours before milk collection began. Dams were allowed to *ad libitum* access to food and water in a clean cage during that time. Dams were anesthetized with intramuscular injection of Ketamine/Xylazine (0.1275g/kg body weight) into forelimb muscle. Once the dam was fully anesthetized, an oxytocin injection (2U per dam) was given in the forelimb muscle to begin let-down. Milk was collected with a pipette after manually expressing milk from nipples and stored in a 1.5 mL Eppendorf tube. Following milk collection, dams were immediately euthanized via isoflurane inhalation and cervical dislocation. Mammary glands were dissected.

## Milk fat percentage determination

Whole milk was collected from dams at Postnatal day 14.5-17.5 and was stored at -80° C until analyzed. Whole milk was thawed on wet ice then homogenized by pipetting up and down. Milk was then diluted in PBS+EDTA in a 1:3 ratio and mixed thoroughly by pipetting up and down.

Capillary tubes were filled with the diluted milk solution and one end was double-sealed with crit-o-seal. Sample tubes were spun in 8 consecutive 120-second cycles in a mini hematocrit spinner (Iris Sample Processing, StatSpin CritSpin M961-122). In the capillary after 16 total minutes of spinning, total fat and aqueous layers were visible. These layers were measured using a 150mm dial caliper (General Tools, 6” Dial Caliper). Percentage of milk fat was determined with based on total volume of diluted milk sample. Milk samples were analyzed in duplicate, or triplicate if milk fat percentage differed by more than 25% in the first two samples.

## Statistical Analyses

Data were analyzed in R Studio version4.2.0 (R Core Team, 2021) and are represented as mean ± standard error. Longitudinal analyses, such as food intake, body composition, and insulin tolerance testing were assessed using linear mixed effects modeling using R package Lme4 (Bates et al., 2015) with random effect of mouse ID and dam and fixed effects of genotype, age, and sex. Models of offspring body weight were assessed using a two-way ANOVA for sex and genotype, with an interaction between the two. If a significant interaction was observed, sex-stratified models were then used and the p-value for the interaction was reported. Otherwise, sex was used as a covariate in a non-interacting model. Pairwise values were assessed for normality by the Shapiro-Wilk test and equivalence of variance by Levene’s test. Variables that were not normally distributed or of equivalent variance underwent non-parametric testing via Mann-Whitney U test. Those that were normally distributed and of equivalent variance were assessed via Student’s t-test. P-values <0.05 were considered statistically significant.

# Results

# Conclusion

# Discussion

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# References

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