

# Female mice exposed to low doses of dioxin during pregnancy and lactation have increased susceptibility to diet-induced obesity and diabetes



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## ABSTRACT

**Objective:** Exposure to persistent organic pollutants is consistently associated with increased diabetes risk in humans. We investigated the short- and long-term impact of transient low-dose dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD) exposure during pregnancy and lactation on glucose homeostasis and beta cell function in female mice, including their response to a metabolic stressor later in life.

**Methods:** Female mice were injected with either corn oil (CO; vehicle control) or 20 ng/kg/d TCDD 2x/week throughout mating, pregnancy and lactation, and then tracked for 6–10 weeks after chemical exposure stopped. A subset of CO- and TCDD-exposed dams was then transferred to a 45% high-fat diet (HFD) or remained on a standard chow diet for an additional 11 weeks to assess the long-term effects of TCDD on adaptability to a metabolic stressor. To summarize, female mice were transiently exposed to TCDD and then subsequently tracked beyond when TCDD had been excreted to identify lasting metabolic effects of TCDD exposure.

**Results:** TCDD-exposed dams were hypoglycemic at birth but otherwise had normal glucose homeostasis during and post-TCDD exposure. However, TCDD-exposed dams on a chow diet were modestly heavier than controls starting 5 weeks after the last TCDD injection, and their weight gain accelerated after transitioning to a HFD. TCDD-exposed dams also had an accelerated onset of hyperglycemia, impaired glucose-induced plasma insulin levels, reduced islet size, increased MAFA<sup>ve</sup> beta cells, and increased proinsulin accumulation following HFD feeding compared to controls. Overall, our study demonstrates that low-dose TCDD exposure during pregnancy has minimal effects on metabolism during the period of active exposure, but has detrimental long-term effects on metabolic adaptability to HFD feeding.

**Conclusions:** Our study suggests that transient low-dose TCDD exposure in female mice impairs metabolic adaptability to HFD feeding, demonstrating that dioxin exposure may be a contributing factor to obesity and diabetes pathogenesis in females.

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**Keywords** Beta cells; Diabetes; Dioxin; Metabolic plasticity; Obesity; Pregnancy

## 1. INTRODUCTION

Diabetes is a global health concern, with incidence rates tripling over the past 20 years [1]. This disease is characterized by chronic hyperglycemia, defects in insulin secretion from pancreatic beta cells, and impaired peripheral insulin action [2]. Type 2 diabetes accounts for 90–95% of all diabetes cases and is strongly associated with genetic and lifestyle determinants such as physical inactivity, poor diet, and obesity. Epidemiological studies consistently report significant associations between exposure to persistent organic pollutants (POPs) and increased diabetes incidence in humans [3,4], yet a causal association remains uncertain.

POPs are typically lipophilic chemicals that resist degradation, leading to widespread environmental dispersion and biomagnification [5]. Dioxins and dioxin-like compounds are a broad class of POPs that induce cellular toxicity through persistent activation of the aryl hydrocarbon receptor (AhR) and upregulation of AhR-target genes including cytochrome P450 (*Cyp1a1*) [6]. CYP enzymes are phase I xenobiotic metabolism enzymes that play an essential role in the oxidation of xenobiotic compounds for subsequent excretion from the body. However, this process generates highly reactive intermediate metabolites that could potentially cause oxidative stress and DNA/protein damage [7]. We previously showed that CYP1A1 is induced and functional in human and mouse pancreatic islets following exposure to

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**Abbreviations:** AhR aryl hydrocarbon receptor, CO corn oil; Cyp cytochrome P450, Cyp1a1 cytochrome P450 1A1; GDM gestational diabetes mellitus, GTT glucose tolerance test; HFD high-fat diet, ITT insulin tolerance test; POPs persistent organic pollutants, TCDD 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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the highly persistent dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, also referred to as dioxin) *in vitro* [8]. *Cyp1a1* was also upregulated in mouse islets after systemic TCDD administration *in vivo* [8,9], indicating that dioxins reach the endocrine pancreas, which may impact beta cell function and survival. Indeed, TCDD-exposed mouse and human islets had significantly reduced glucose-induced insulin secretion *in vitro* [8], and a single high-dose injection of TCDD (20 µg/kg) reduced plasma insulin levels for up to 6 weeks in male and female mice *in vivo* [9]. TCDD-exposed males also displayed modest fasting hypoglycemia for ~4 weeks post-injection, increased insulin sensitivity, and decreased beta cell area. In contrast, TCDD-exposed females became transiently glucose intolerant 4 weeks after the single high-dose TCDD injection [9]. Additionally, prolonged low-dose TCDD exposure (20 ng/kg/d, 2x/week) accelerated high-fat diet (HFD)-induced hyperglycemia and reduced glucose-induced plasma insulin levels in female but not male mice [10]. Our results suggest that dioxin-like compounds have long-lasting adverse effects on metabolism, beta cell function and islet composition, and that pollutant-exposed female mice may be more susceptible than males to maladaptive metabolic responses.

Pregnancy is a unique period of metabolic plasticity in females during which beta cells proliferate and increase insulin secretion to accommodate for changes in nutritional needs [11–14]. Failure to compensate for these changes can lead to hyperglycemia, gestational diabetes mellitus (GDM), and long-term metabolic complications in mothers and their offspring [15]. Most epidemiological studies investigating the association between POPs and diabetes have focused on adult populations subjected to acute dioxin exposure (e.g., war veterans, occupational workers, and victims of chemical disasters) [16–19] or populations with chronic high background exposure (e.g., populations with high fish consumption) [20–22]. The few studies that assessed the link between chronic low-dose POP exposure during pregnancy and GDM reported inconsistent findings of either a positive association between serum POP levels and GDM [23–26] or no association [26–28]. In addition, these were all cross-sectional studies that do not provide information about the timing of events and cannot rule out the possibility of reverse causality (i.e., whether POPs increase GDM risk or whether GDM increases serum POP accumulation). Lastly, long-term tracking is not possible with cross-sectional epidemiological studies.

The purpose of our study was first to determine whether low-dose TCDD exposure during pregnancy and lactation impacts pancreatic beta cell plasticity, glucose homeostasis, or body weight regulation during this critical period of metabolic stress in female mice ("TCDD injection" window). We then stopped TCDD administration when offspring were weaned and conducted metabolic assessments on the dams for an additional 10 weeks to determine whether transient TCDD exposure during pregnancy/lactation has lasting metabolic consequences ("Post-TCDD" window). Lastly, at the end of the "Post-TCDD" window, a subset of control and TCDD-exposed dams was transitioned to HFD feeding for another 11 weeks to assess whether transient TCDD exposure impairs adaptability to a metabolic stressor, thus increasing the long-term risk of developing diabetes ("Metabolic challenge" window).

## 2. MATERIALS AND METHODS

### 2.1. Animals

C57BL/6 mice, 5–8 weeks old (Charles River, Raleigh, NC, USA), were maintained on a 12-h light/dark cycle throughout the study and received *ad libitum* access to standard rodent chow (Harlan

Laboratories, Teklad Diet #2018, Madison, WI, USA). All experiments were approved by the Carleton University Animal Care Committee and conducted in accordance with the Canadian Council on Animal Care guidelines. Prior to beginning experimental protocols, animals were randomly assigned to treatment groups and matched for body weight and blood glucose to ensure that these variables were consistent between groups.

#### 2.1.1. Cohort 1 (Supplemental Figs. S1–S3)

As outlined in Figure 1A, female mice received subcutaneous (s.c.) injections of corn oil (CO) (25 ml/kg, vehicle control, n = 6) (#C8267-2.5L, Sigma–Aldrich, St. Louis, MO, USA) or a low dose of TCDD (20 ng/kg/d, n = 6) (Sigma–Aldrich, # 48,599) 2x/week starting one week prior to pairing with male mice and lasting throughout mating and pregnancy. All mice were euthanized on postnatal day 1 (P1). Whole pancreas was harvested and stored in 4% paraformaldehyde (PFA; #AAJ19943K2, Thermo Fisher Scientific, Waltham, MA, USA) for 24 h followed by long-term storage in 70% EtOH for histological analysis.

#### 2.1.2. Cohort 2 (Figures 1–6; Supplemental Figs. S3–S5)

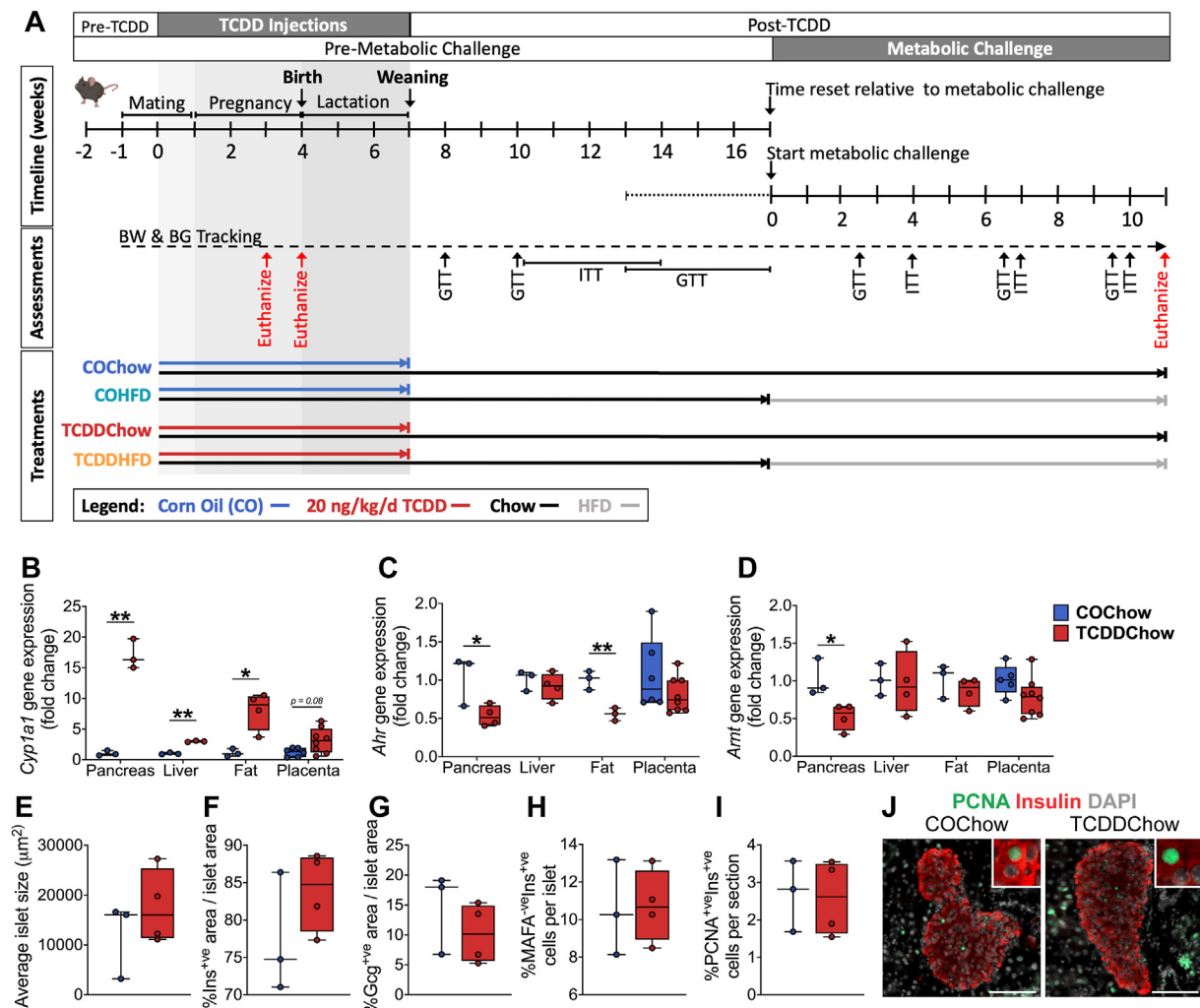
As outlined in Figure 1A, female mice received s.c. injections of CO (25 ml/kg, n = 12) or a low dose of TCDD (20 ng/kg/d; n = 14) 2x/week starting one week prior to pairing with male mice and lasting throughout mating, pregnancy, and lactation ("TCDD injection" window: study weeks 0–7). Litters were culled to 6 pups/litter at P1 to ensure that litter size did not alter the metabolic outcomes in our study. Metabolic assessments were conducted on chow-fed dams for 6–10 weeks following the last TCDD exposure at weaning ("Post-TCDD" window: study weeks 7–17). This range reflects differences in the duration of mating, with a 4-week difference between the first and last pregnancies. As outlined in Figure 1A, a subset of CO- and TCDD-exposed dams was then transferred to a 45% HFD (Research Diets D12451, New Brunswick, NJ, USA) or remained on a standard chow diet for an additional 11 weeks ("Metabolic challenge" window), generating the following experimental groups (n = 4–5 per group): COChow, COHFD, TCDDChow, and TCDDHFD. Whole pancreas, liver, perirenal fat, and placenta were harvested from a subset of mice at approximately day 15.5 of pregnancy (GD15.5) (n = 3–4/group). Pancreas and hypothalamic brain tissue were collected at week 11 of the metabolic challenge (n = 4–5/group). Tissues were stored in RNAlater or flash frozen for qPCR analysis, or stored in 4% PFA.

#### 2.1.3. Cohort 3 (Figure 7)

As outlined in Figure 7A, non-pregnant female mice received intraperitoneal (i.p.) injections of CO (25 ml/kg, vehicle control) or a low dose of TCDD (20 ng/kg/d) 2x/week for 12 weeks. During this period of active chemical exposure, a subset of CO- and TCDD-exposed females were fed either a standard chow diet or 45% HFD, generating the following experimental groups (n = 3–4/group): COChow, COHFD, TCDDChow, and TCDDHFD. After 12 weeks of exposure, pancreatic islets were isolated from all mice and stored in RNAlater for subsequent analysis by RNAseq and qPCR. A detailed description of this cohort can be found in [10]. RNAseq analysis of this cohort was used to support our findings from cohorts 1 and 2.

### 2.2. Metabolic assessment

All metabolic analyses were conducted in conscious, restrained mice, and blood samples were collected via the saphenous vein using heparinized microhematocrit tubes. Blood glucose levels were measured using a handheld glucometer (LifeScan, Burnaby, BC, Canada).



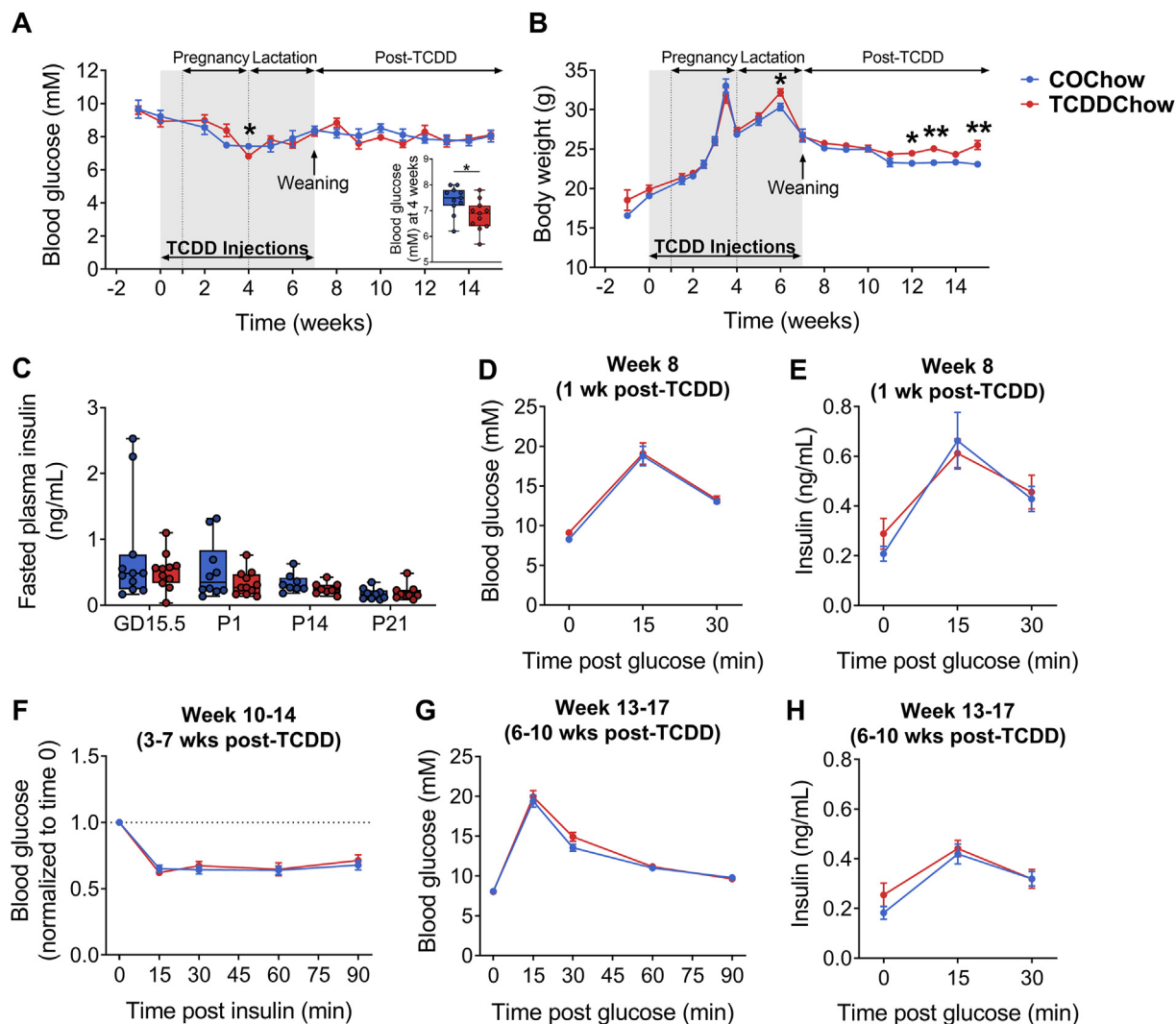
**Figure 1:** Transient low-dose TCDD exposure induced *Cyp1a1* expression more strongly in the pancreas than other tissues at mid-gestation. (A) Schematic summary timeline of the study. Female mice were injected with either corn oil (CO) or 20 ng/kg/d TCDD 2x/week during mating, pregnancy, and lactation ("TCDD injection" window), and then tracked for 6–10 weeks after chemical exposure stopped ("Post-TCDD" window) (i.e., study weeks 13–17). This range reflects differences in the duration of mating, with a 4-week difference between the first and last pregnancies. At study weeks 13–17, a subset of CO- and TCDD-exposed dams were transitioned to a 45% HFD or remained on standard chow. All mice were then assessed for another 11 weeks. BW = body weight, BG = blood glucose, GTT = glucose tolerance test, and ITT = insulin tolerance test. (B–D) Gene expression was measured in whole pancreas, liver, perirenal fat, and placenta on day 15.5 of pregnancy, including (B) *Cyp1a1*, (C) *Ahr*, and (D) *Amt*. Gene levels are expressed as fold change relative to control. (E–J) Histological analysis of pancreas collected from the dams mid-gestation, including quantification of (E) average islet size, (F) % insulin (Ins)<sup>+</sup>ve area/islet area, (G) % glucagon (Gcg)<sup>+</sup>ve area/islet area, (H) % MAFA<sup>+</sup>ve Ins<sup>+</sup>ve cells per islet, and (I) % PCNA<sup>+</sup>ve Ins<sup>+</sup>ve cells per pancreatic section. (J) Representative images of pancreas sections showing immunofluorescent staining for insulin and PCNA. Inset regions show PCNA<sup>+</sup>ve Ins<sup>+</sup>ve cells. Scale bar = 100 μm. All data are presented as median with min/max values. Individual data points on box and whisker plots represent biological replicates (different mice). \*p < 0.05 and \*\*p < 0.01 vs control. The following statistical tests were used: (B) two-tailed unpaired t test for pancreas, liver, and fat; Mann–Whitney test for placenta; and (C–D and E–I) two-tailed unpaired t test.

Body weight and blood glucose were measured weekly or 2x/week following a 4-h morning fast. Saphenous blood was collected at GD15.5, P1, lactation (P14), and weaning (P21) following a 4-h morning fast to measure plasma insulin levels by ELISA (#80-INSMSU-E01, ALPCO, Salem, NH, USA). For all metabolic tests, time 0 indicates the blood samples collected prior to the administration of glucose or insulin. For glucose tolerance tests (GTTs), the mice received an i.p. bolus of glucose (2 g/kg) following a 4-h morning fast. Blood samples were collected at 0, 15, and 30 min to measure plasma insulin levels by ELISA. For insulin tolerance tests (ITTs), the mice received an i.p. bolus of insulin (0.7 IU/kg: Figures 2F and 4D, and Supplemental Fig. S4B; 1.0 IU/kg: Figure 4B) (#02024233, Novolin ge Toronto, Novo Nordisk Canada) following a 4-h morning fast. Cardiac blood was collected at week 11 of the metabolic challenge to measure

non-fasted plasma leptin levels (#90030, Crystal Chem, Elk Grove Village, IL, USA), proinsulin levels (ALPCO, #80-PINMS-E01), and insulin levels (ALPCO, #80-INSMSU-E01) by ELISA.

### 2.3. Food intake analysis

Food intake was assessed at mid-pregnancy, lactation, and week 7 of the metabolic challenge. All dams were singly housed for this experiment. The dams were transferred to a clean cage and the amount of food in their feeding trays was weighed (day 0). Food weight was measured again 2 days later (day 2), ensuring that any large pieces of food that had fallen into the cage were included in the measurement. Food intake in kcal was determined as follows: [(weight<sub>day 0</sub> – weight<sub>day 2</sub>) x food energy density], where standard chow had an energy density of 13.0 kJ/g and 45% HFD had a density of 19.7 kJ/g.



**Figure 2:** Transient TCDD exposure during pregnancy and lactation causes hypoglycemia at P1 and promotes weight gain post-exposure. Female mice were injected with either corn oil (CO) or 20 ng/kg/d TCDD 2x/week during mating, pregnancy, and lactation, and then tracked for 6–10 weeks after exposure (see Figure 1A for the study timeline). GTT = glucose tolerance test and ITT = insulin tolerance test. (A) Blood glucose and (B) body weight were measured weekly following a 4-h morning fast. Blood glucose at postnatal day 1 (P1) is shown in the inset graph in (A). (C) Fasted plasma insulin levels were measured at day 15.5 of pregnancy, P1, mid-lactation (P14), and weaning (P21) following a 4-h morning fast. (D and G) Blood glucose and (E and H) plasma insulin levels during a GTT at (D and E) 1-week post-TCDD (study week 8) and (G and H) 6–10 weeks post-TCDD (study weeks 13–17). (F) Blood glucose levels during ITTs at 3–7 weeks post-TCDD (study weeks 10–14). Values are normalized relative to time zero for each mouse. All data are presented as mean  $\pm$  SEM in line graphs or median with min/max values in box and whisker plots. Individual data points in box and whisker plots represent biological replicates (different mice). \* $p < 0.05$  and \*\* $p < 0.01$  vs controls. The following statistical tests were used: (A and B) line graphs, two-way REML-ANOVA with uncorrected Fisher's LSD test; box and whisker plots, two-tailed unpaired t test; (C) two-tailed unpaired t test at P14; two-tailed Mann–Whitney test at GD15.5, P1, and P21; and (D–H) two-way RM ANOVA with Sidak's test.

#### 2.4. EchoMRI analysis

Fat and lean mass were measured by the University of Ottawa Animal Behavior Core (Ottawa, ON, Canada) at week 10 of the metabolic challenge using an EchoMRI-700 instrument (EchoMRI LLC, Houston, TX, USA). Percent fat and lean mass were determined relative to total body weight measured immediately prior to the MRI. The instrument was calibrated per the manufacturer's instructions prior to measurements.

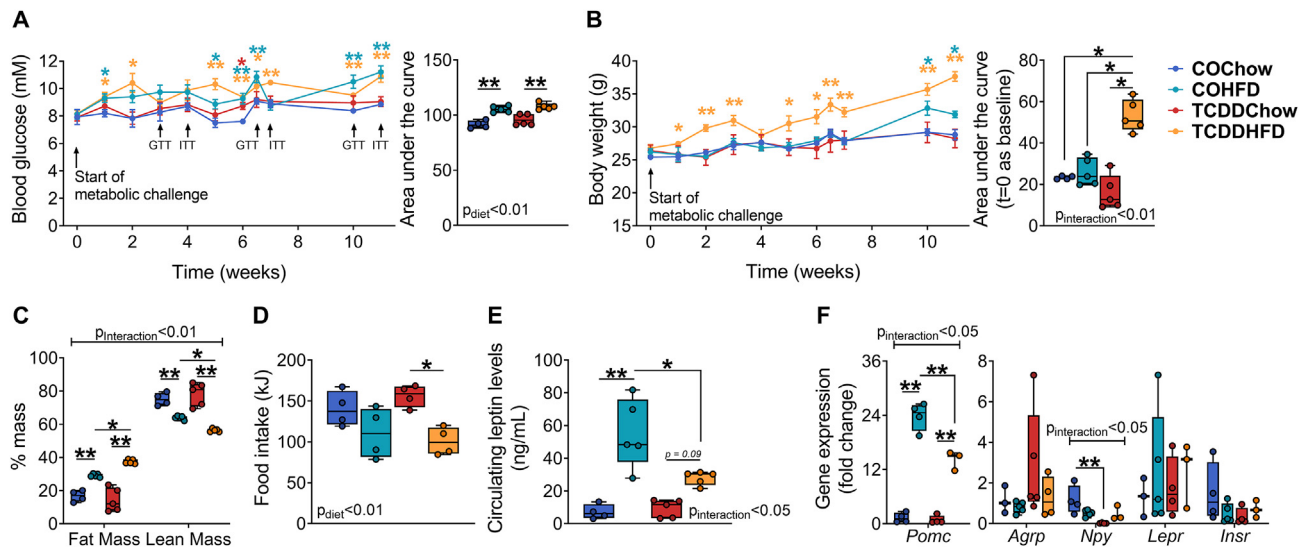
#### 2.5. Islet isolation and culture

Islets were isolated by pancreatic duct injection with collagenase (#C7657, 1000 U/ml; Sigma–Aldrich) as previously described [8]. In brief, inflated pancreatic tissues were excised and incubated at 37 °C

for 12 min, vigorously agitated, and collagenase reactions quenched. The pancreatic tissue was washed three times in HBSS + CaCl<sub>2</sub> and resuspended in Ham's F-10 (#SH30025.01, HyClone, GE Healthcare Life Sciences, Pittsburgh, PA, USA). Pancreatic tissue was filtered through a 70  $\mu$ m cell strainer and islets were handpicked under a dissecting scope to >95% purity.

#### 2.6. Quantitative real-time PCR

RNA was isolated from whole pancreas, liver, perirenal fat, placenta, and hypothalamic brain using TRIzol (#15596018, Invitrogen, Carlsbad, CA, USA) per the manufacturer's instructions. RNA was isolated from islets using a RNeasy Micro Kit (#74004, Qiagen) per the manufacturer's instructions, with the following amendment: 7 volumes of



**Figure 3:** Transient TCDD exposure promotes diet-induced obesity and alters energy homeostasis. Following 6–10 weeks of post-TCDD tracking in chow-fed mice (study weeks 13–17), a subset of the CO- and TCDD-exposed dams were transitioned to a 45% HFD or remained on standard chow and assessed for another 11 weeks ("Metabolic challenge" window) (see Figure 1A for the study timeline). (A) Blood glucose and (B) body weight were measured weekly following a 4-h morning fast. (C) Fat mass and lean mass were measured by EchoMRI 10 weeks into the metabolic challenge. (D) Caloric intake was measured at week 7 of the challenge. (E) Non-fasted circulating leptin levels were measured in cardiac blood at week 11 of the challenge. (F) Gene expression for markers of energy homeostasis were measured in the hypothalamus at week 11 of the challenge. All data are presented as mean  $\pm$  SEM in line graphs or median with min/max values in box and whisker plots. Individual data points in box and whisker plots represent biological replicates (different mice). \* $p < 0.05$  and \*\* $p < 0.01$ , colored stars are vs COChow. The following statistical tests were used: (A and B) line graphs, two-way RM ANOVA with uncorrected Fisher's LSD test; box and whisker plots, two-way ANOVA with Tukey's multiple comparison test; and (C–F) two-way ANOVA with Tukey's multiple comparison test.

buffer RLT + DTT were added to the samples prior to lysing with 70% EtOH. DNase treatment was conducted prior to cDNA synthesis with an iScript gDNA Clear cDNA Synthesis Kit (#1725035, Bio-Rad, Mississauga, ON, Canada). qPCR was performed using SsoAdvanced Universal SYBR Green Supermix (#1725271, Bio-Rad) or SensiFAST SYBR No-ROX Supermix (#BIO-980050, Biorline, London, UK) and run on a CFX96 or CFX384 (Bio-Rad). *Hprt* or *Ppia* were used as the reference genes since these genes displayed stable expression under control and treatment conditions. Data were analyzed using the  $2^{\Delta\Delta CT}$  relative quantitation method. Primer sequences are listed in Table S1

### 2.7. RNA sequencing

Gene expression in whole islets isolated from females in cohort 3 ( $n = 3\text{--}4/\text{group}$ ) was measured using a TempO-Seq Mouse Whole Transcriptome panel (BioSpyder Technologies Inc., Carlsbad, CA, USA) as described in [10]. Raw counts per million were extracted for genes of interest and analyzed by 2-way ANOVA. A larger-scale pathway analysis of this dataset is provided elsewhere [10].

### 2.8. Immunofluorescent staining and image quantification

Whole pancreas tissues were processed and paraffin-embedded by the University of Ottawa Heart Institute Histology Core Facility (Ottawa, ON, Canada). Tissues were sectioned (5  $\mu\text{m}$  thick) using a Thermo Fisher Scientific Microm HM 355S. Immunofluorescent staining was conducted as previously described [8]. In brief, slides were deparaffinized with sequential incubations in xylene and ethanol. Heat-induced epitope retrieval was performed in 10 mM citrate buffer or 10 mM Tris base at 95  $^{\circ}\text{C}$  for 10–15 min, and slides were incubated with Dako Serum Free Protein Block (#X090930-2, Agilent, Santa Clara, CA, USA) for 30 min. Slides were incubated overnight at 4  $^{\circ}\text{C}$  with primary antibodies and then incubated with secondary antibodies for 1-h at room temperature. Coverslips were mounted using Vectashield HardSet mounting medium with DAPI (#H-1500, Vector

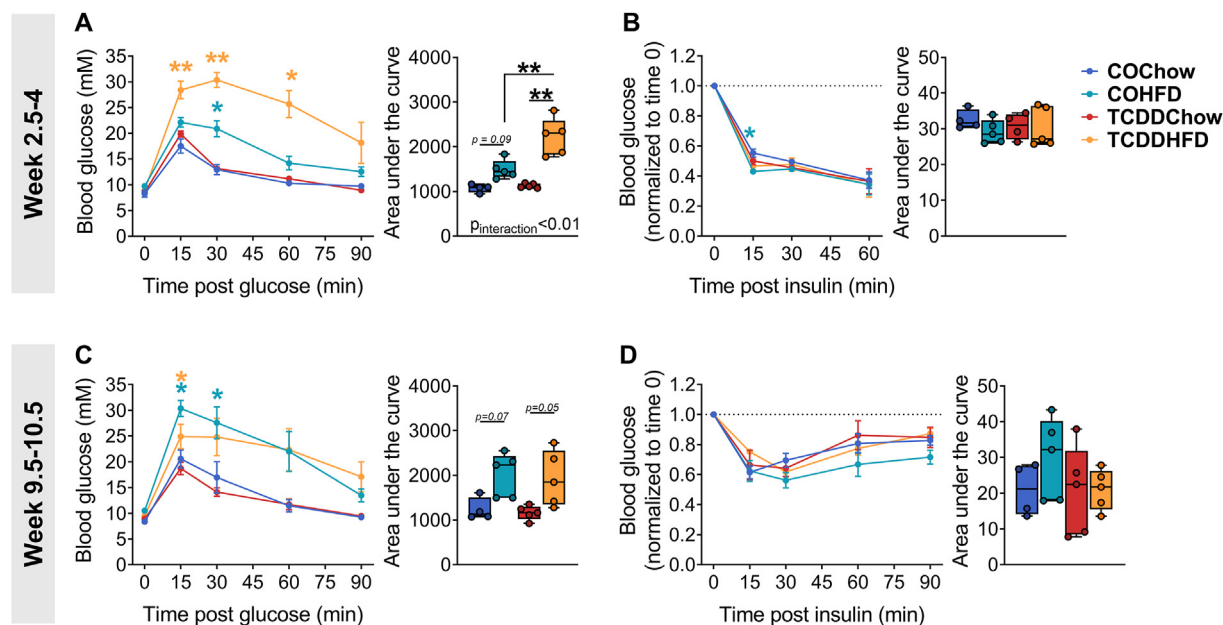
Laboratories, Burlingame, CA, USA) or ProLong Gold antifade mountant (#P36935, Thermo Fisher Scientific) to counterstain nuclei.

The following primary antibodies were used: rabbit anti-insulin (#3014, 1:200, C27C9, Cell Signaling Technology, Danvers, MA, USA), mouse anti-insulin (#8138S, 1:250, L6B10, Cell Signaling), mouse anti-glucagon (#G2654, 1:250, Sigma–Aldrich), mouse anti-PCNA (#610665, 1:100, BD Transduction Laboratories, San Jose, CA, USA), rabbit anti-MAFA (1:1000, shared by Dr. Timothy Kieffer), and mouse anti-proinsulin (GS-9A8-s, 1:50, DSHB, Iowa City, IA, USA). The following secondary antibodies were used: goat anti-rabbit IgG (H + L) secondary antibody, Alexa Fluor 594 (#A11037, 1:1000, Invitrogen), and goat anti-mouse IgG (H+L) secondary antibody, Alexa Fluor 488 (#A11029, 1:1000, Invitrogen).

For islet morphology quantification, a range of 5–13 islets were imaged per mouse with an Axio Observer 7 microscope and the average of all islet measurements was reported for each biological replicate. Immunofluorescence was manually quantified using Zen Blue 2.6 software (Carl Zeiss, Oberkochen, Germany). The % of hormone<sup>+</sup> area per islet was calculated as [(hormone<sup>+</sup> area/islet area)  $\times$  100]. The % of PCNA<sup>+</sup> insulin<sup>+</sup> cells per islet was calculated as [(# of PCNA<sup>+</sup> insulin<sup>+</sup> cells)/(total # of insulin<sup>+</sup> cells per islet)  $\times$  100], with an average of 590 cells counted per mouse. The % of MAFA<sup>+</sup> insulin<sup>+</sup> cells per islet was calculated as [(# of MAFA<sup>+</sup> insulin<sup>+</sup> cells per islet)/(total # of insulin<sup>+</sup> cells per islet)  $\times$  100], with an average of 750 cells counted per mouse. The % of beta cells with an accumulation of proinsulin was calculated as [(# of insulin<sup>+</sup> cells with accumulated cytoplasmic proinsulin)/(total # of insulin<sup>+</sup> cells per islet)  $\times$  100], with an average of 400 cells counted per mouse.

### 2.9. Quantification and statistical analysis

All statistical analyses were conducted using GraphPad Prism 8.4.2 (GraphPad Software Inc., La Jolla, CA, USA). Specific statistical tests are indicated in the figure legends and summarized in Table S2. Verifications of



**Figure 4:** TCDD exposure accelerates the onset of glucose intolerance in HFD-fed dams without altering insulin sensitivity. Glucose and insulin tolerance were assessed *in vivo* at weeks 2.5–4 and 9.5–10.5 of the metabolic challenge (see Figure 1A for the study timeline). (A and C) Blood glucose levels during glucose tolerance tests at (A) week 2.5 and (C) week 9.5 of the metabolic challenge. (B and D) Blood glucose levels during insulin tolerance tests at (B) week 4 and (D) week 10.5 of the metabolic challenge. Values are normalized relative to time 0 for each mouse. All data are presented as mean  $\pm$  SEM in line graphs or median with min/max values in box and whisker plots. Individual data points in box and whisker plots represent biological replicates (different mice). \* $p < 0.05$  and \*\* $p < 0.01$ , colored stars are vs COChow. The following statistical tests were used: (A–D) line graphs, two-way RM ANOVA with Tukey's multiple comparison test; box and whisker plots, two-way ANOVA with Tukey's multiple comparison test.

parametric assumptions are shown in Table S2. Sample sizes are described in Section 2.1 Animals. For all analyses,  $p < 0.05$  was considered statistically significant. Statistically significant outliers were detected by Grubbs' test with  $\alpha = 0.05$ . All data were tested for normality using the Shapiro–Wilk test and for equal variance using either the Brown–Forsythe test for one-way ANOVAs or the F test for unpaired t tests. Non-parametric statistics were used in cases where the data failed normality or equal variance tests. Parametric tests were used for all two-way ANOVAs, but normality and equal variance were tested on area under the curve values and by one-way ANOVAs (Table S2). A two-way mixed-effect model (REML) ANOVA was used in cases where data points were missing due to random reasons. Box and whisker plots display the median, with whiskers representing the minimum to maximum values. Bar plots display mean  $\pm$  SEM. Individual data points on box and whisker or bar plots are always biological replicates (i.e., different mice).

### 2.10. Data and resource availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. RNAseq data were submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) and are accessible under accession number GSE144765.

## 3. RESULTS

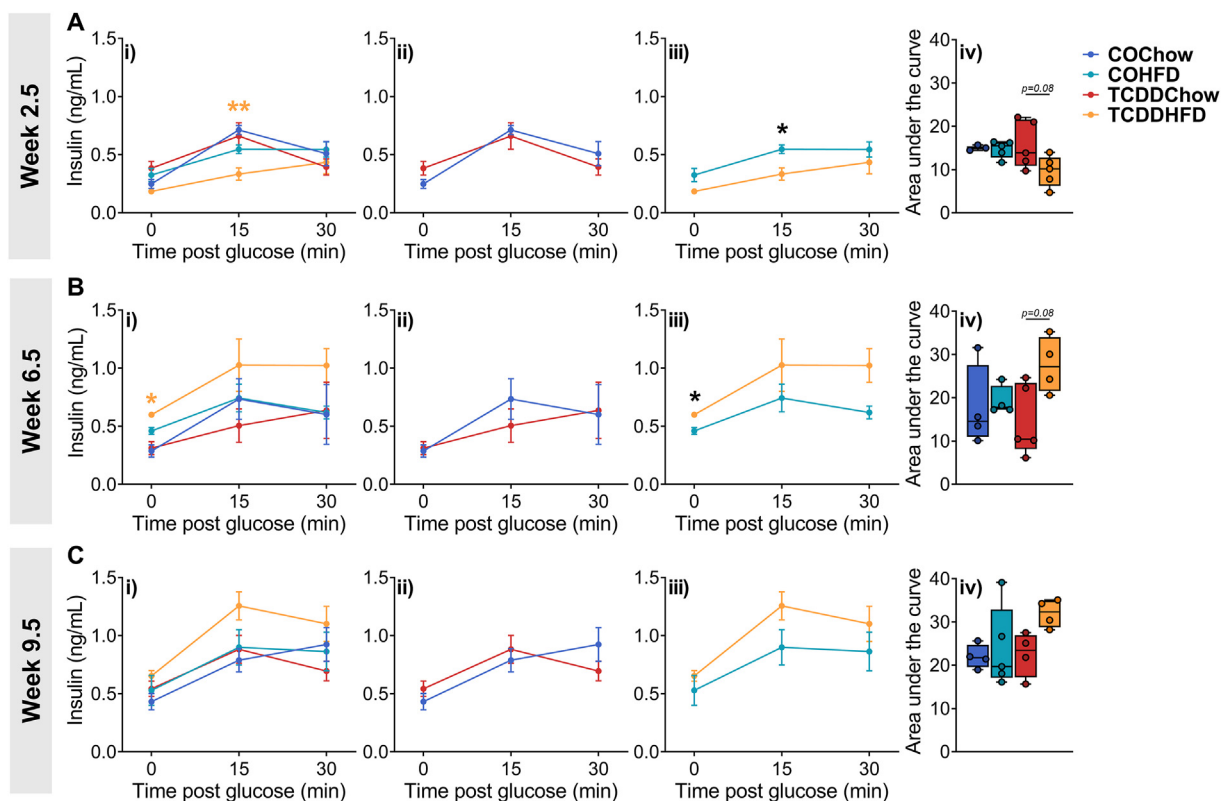
### 3.1. Transient low-dose TCDD exposure induces *Cyp1a1* gene expression more strongly in the pancreas than other tissues at mid-gestation

Briefly, female mice were injected with either corn oil (CO, vehicle control) or 20 ng/kg/d TCDD 2x/week during mating, pregnancy, and lactation ("TCDD injection" window), and then tracked for 6–10 weeks

after chemical exposure stopped ("Post-TCDD" window) (Figure 1A). A subset of CO- and TCDD-exposed dams was then transferred to a 45% HFD or remained on a standard chow diet for an additional 11 weeks to assess the long-term effects of TCDD on adaptability to a metabolic stressor ("Metabolic challenge" window) (Figure 1A).

Before examining the effects of low-dose TCDD exposure on glucose homeostasis and beta cell adaptation during pregnancy, we first validated our model by ensuring that TCDD reached the dam pancreas mid-gestation using *Cyp1a1* as an indicator of direct pollutant exposure [8]. TCDD induced *Cyp1a1* expression  $\sim$ 17-fold in the pancreas compared to  $\sim$ 3-fold,  $\sim$ 8-fold, and  $\sim$ 3.5-fold in the liver, perirenal fat, and placenta, respectively, on day 15.5 of pregnancy relative to controls (Figure 1B). *Ahr* expression was reduced  $\sim$ 2-fold in the pancreas and perirenal fat of TCDD-exposed dams compared to controls, and not changed in the liver or placenta (Figure 1C). *Arnt* was also reduced  $\sim$ 2-fold in the pancreas but not changed in the other tissues compared to controls (Figure 1D). These results indicate that TCDD reaches the pancreas of pregnant dams and strongly induces *Cyp1a1* expression, which leads to negative feedback regulation of the AhR pathway.

We next assessed whether maternal TCDD exposure altered islet morphology or beta cell characteristics mid-pregnancy, a period known to be associated with increased beta cell proliferation [11–14]. TCDD exposure did not alter average islet size (Figure 1E,J), % insulin<sup>+</sup> area per islet (Figure 1F), % glucagon<sup>+</sup> area per islet (Figure 1G), % MAFA<sup>-ve</sup> beta cells (Figure 1H), or % PCNA<sup>+</sup> beta cells (Figure 1I–J). These results suggest that low-dose TCDD exposure during pregnancy in mice does not influence beta cell proliferation, beta cell maturity, or islet composition on gestation day 15.5. Of note, a small sample size was used to measure these parameters.



**Figure 5:** HFD feeding impairs glucose-induced plasma insulin levels in TCDD-exposed dams. Glucose-induced plasma insulin levels were assessed *in vivo* at (A) week 2.5, (B) week 6.5, and (C) week 9.5 of the metabolic challenge (see Figure 1A for the study timeline). Insulin data are presented as (i) all groups compared to COChow, (ii) TCDDChow vs COChow, (iii) TCDDHFD vs COHFD, and (iv) area under the curve for all groups. All data are presented as mean  $\pm$  SEM in line graphs or median with min/max values in box and whisker plots. Individual data points in box and whisker plots represent biological replicates (different mice). \* $p < 0.05$ , colored stars are vs COChow. The following statistical tests were used: (A-C i-iii) two-way RM ANOVA with Tukey's multiple comparison test; (A-C iv) two-way ANOVA with Tukey's multiple comparison test.

### 3.2. TCDD exposure during pregnancy and lactation causes hypoglycemia at P1 and promotes weight gain post-exposure

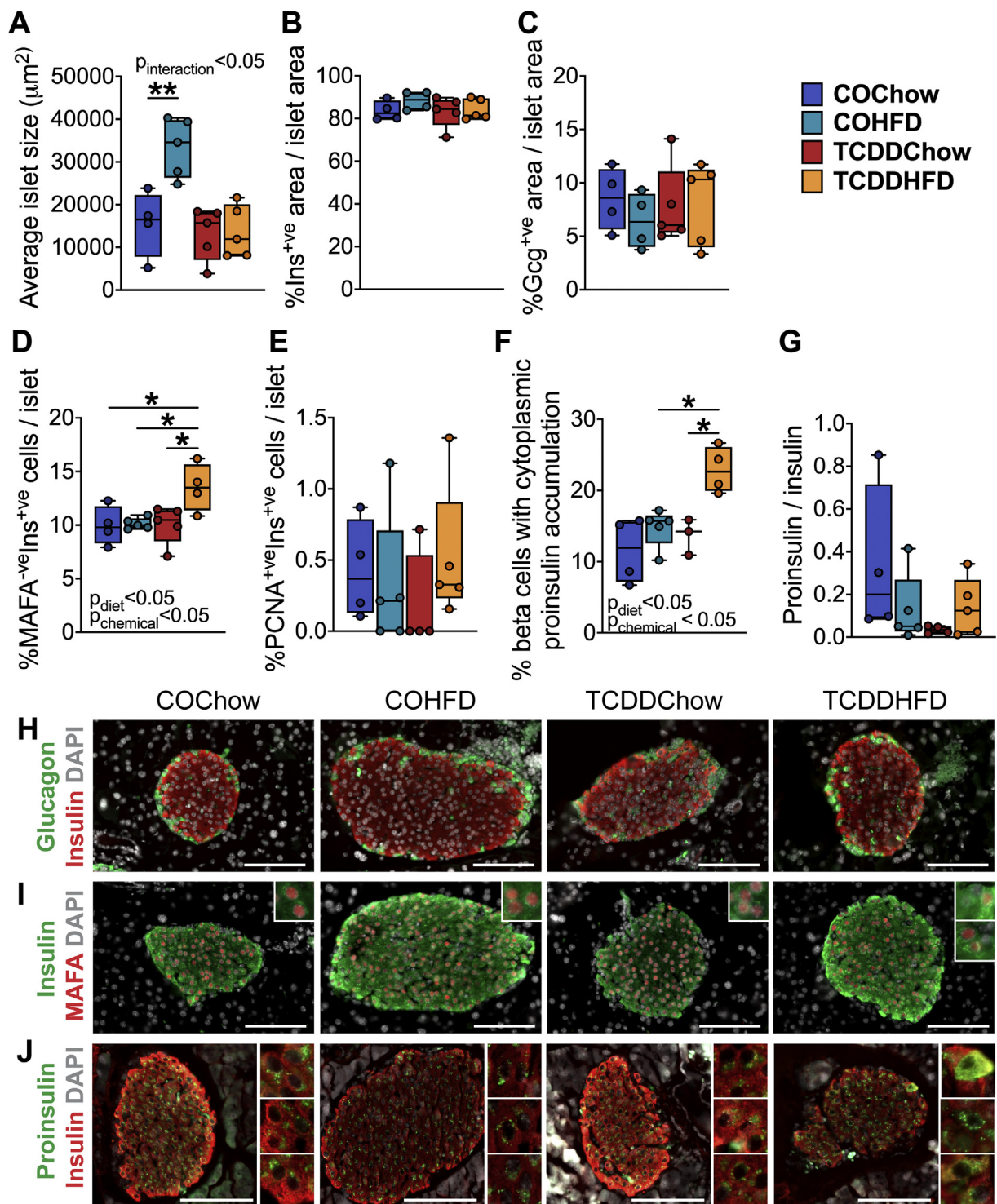
We next investigated whether transient TCDD exposure during pregnancy and lactation had short- or long-term effects on dam metabolism (i.e., while TCDD was still present or after TCDD had been excreted, respectively). TCDD exposure during pregnancy did not alter litter size (Supplemental Fig. S3A), so this was not a confounding variable in our study. TCDD-exposed dams were transiently hypoglycemic on postnatal day 1 (P1) compared to control dams (Figure 2A-inset), but otherwise had normal fasting glycemia during TCDD exposure and for 10 weeks after exposure ended (i.e., "Post-TCDD" window; Figure 2A). Importantly, TCDD-induced hypoglycemia at P1 was replicated in a second independent cohort of mice (Supplemental Fig. S1A). There were no changes in fasting plasma insulin levels during pregnancy or lactation with either cohort (Figure 2C and Supplemental Fig. S1C), or in islet morphology within the dam pancreas at P1 (Supplemental Figs. S2A–G). Since we did not observe any signs of GDM development in TCDD-exposed dams, we did not conduct glucose or insulin tolerance tests during pregnancy to avoid unnecessary stress. However, we did conduct extensive metabolic testing on the dams for 10 weeks after TCDD exposure stopped at the end of lactation to assess long-term metabolic effects following excretion of TCDD. TCDD exposure did not cause any long-term changes to glucose tolerance (Figure 2D, 2G, and Supplemental Fig. S3C), glucose-induced plasma insulin levels (Figure 2E, 2H, and

Supplemental Fig. S3D), or insulin sensitivity (Figure 2F) in the 10 weeks post-TCDD (i.e., study weeks 7–17).

We also assessed the impact of TCDD on body weight during and after exposure. Throughout pregnancy, TCDD-exposed dams had normal body weight (Figure 2B and Supplemental Fig. S1B) and food intake (Supplemental Fig. S3B). However, TCDD-exposed dams were transiently heavier than controls mid-lactation (Figure 2B), with no change in food intake (Supplemental Fig. S3B). We also observed a modest but statistically significant increase in body weight in TCDD-exposed dams starting 5 weeks after their last TCDD exposure (Figure 2B and Supplemental Figs. S5A–E). Based on these results, we speculated that female mice exposed to TCDD during pregnancy and lactation might be more susceptible to weight gain when challenged with an obesogenic diet (45% HFD).

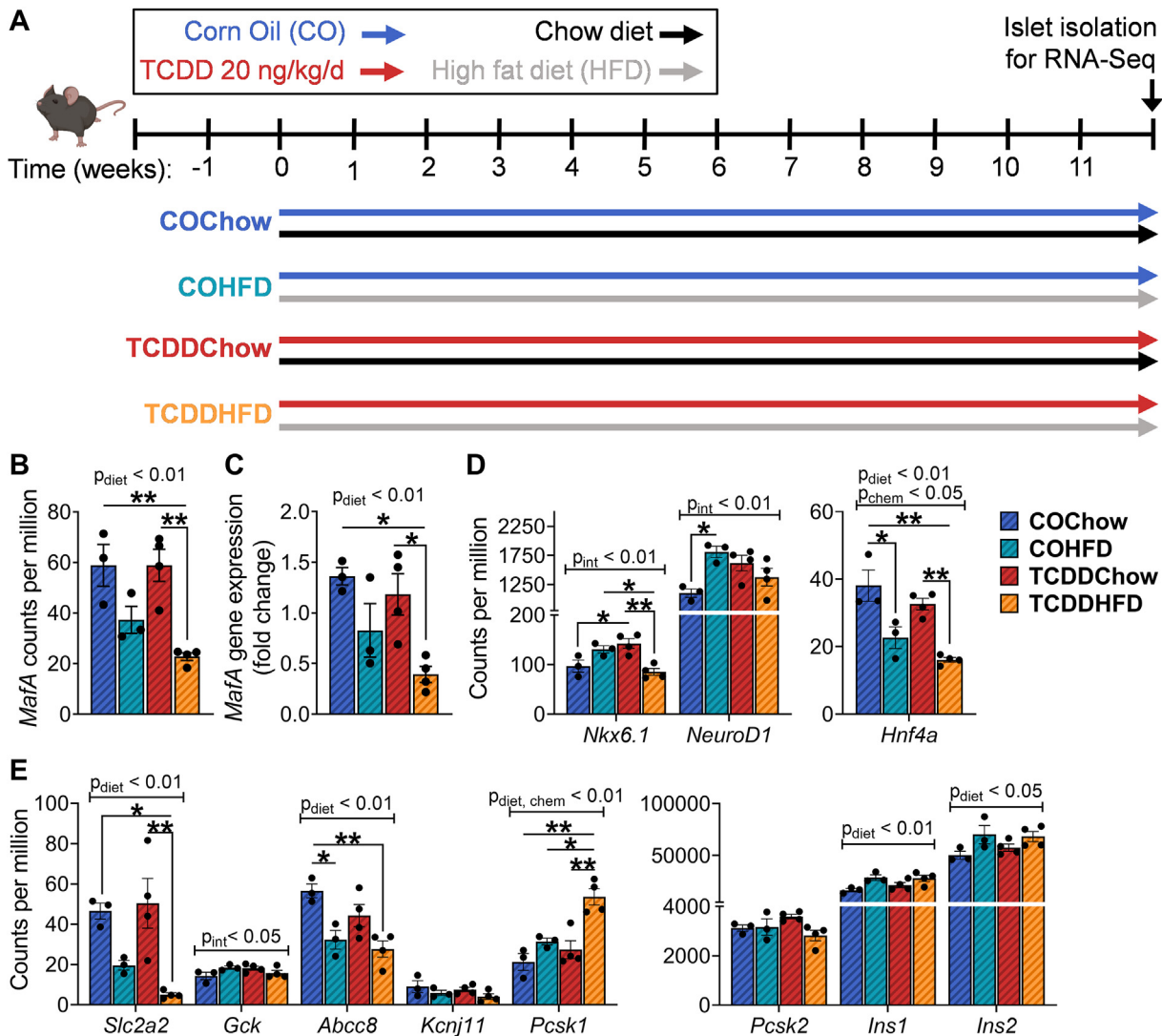
### 3.3. Transient TCDD exposure promotes diet-induced obesity and alters energy homeostasis

Between 6 and 10 weeks after the last TCDD exposure (study weeks 13–17) and following complete excretion of TCDD, a subset of CO- and TCDD-exposed dams was transitioned to a 45% HFD (COHFD and TCDDHFD) while the other subset remained on standard chow (COChow and TCDDChow) for 11 weeks (see Figure 1A for the study timeline). Consistent with our findings shown in Figure 2A, TCDDChow dams continued to maintain normal fasting glycemia compared to COChow dams throughout the study (Figure 3A). HFD-fed dams



**Figure 6:** TCDDHFD dams had decreased average islet size, a higher proportion of MAFA<sup>-ve</sup> beta cells, and increased proinsulin accumulation compared to COHFD dams. Whole pancreas and cardiac blood were harvested at week 11 of the metabolic challenge for analysis (see Figure 1A for the study timeline). (A) Average islet size, (B) % insulin (Ins)<sup>+</sup> area/islet area, (C) % glucagon (Gcg)<sup>+</sup> area/islet area, (D) % MAFA<sup>-ve</sup> Ins<sup>+</sup> cells per islet, (E) % PCNA<sup>+</sup> Ins<sup>+</sup> cells per islet, and (F) % beta cells with cytoplasmic proinsulin accumulation as determined by immunofluorescent staining. (G) Non-fasted circulating proinsulin/insulin levels in cardiac blood. (H–J) Representative images of pancreas sections showing immunofluorescence staining for (H) insulin and glucagon, (I) insulin and MAFA, or (J) insulin and proinsulin. Inset regions in (I) show MAFA<sup>+</sup>Ins<sup>+</sup> cells. Inset regions in (J) show perinuclear proinsulin immunoreactivity vs proinsulin accumulation in the cytoplasm. Scale bar = 100  $\mu\text{m}$ . All data are presented as median with min/max values. Individual data points in box and whisker plots represent biological replicates (different mice). \* $p < 0.05$  and \*\* $p < 0.01$ . The following statistical tests were used: (A–G) two-way ANOVA with Tukey's multiple comparison test.





**Figure 7:** TCDDHFD females have reduced *MafA*, *Nkx6.1*, and *Slc2a2* gene expression and increased *Pcsk1* expression compared to TCDDChow females in a non-pregnancy model. (A) Schematic summary of the study timeline. Female mice were injected with corn oil (CO) or 20 ng/kg/d TCDD 2x/week for 12 weeks and either fed standard chow or 45% HFD. Islets were isolated at 12 weeks for subsequent gene expression analysis by RNAseq and qPCR. (B and D–E) RNAseq data showing gene counts per million for (B) *MafA*, (D) beta cell transcription factors, and (E) genes associated with insulin processing and secretion. (C) qPCR analysis of *MafA* expression. All data are presented as mean  $\pm$  SEM. Individual data points on bar graphs represent biological replicates (different mice). \* $p < 0.05$  and \*\* $p < 0.01$ . The following statistical tests were used: (B–E) two-way ANOVA with Tukey's multiple comparison test.

developed moderate fasting hyperglycemia irrespective of chemical exposure (Figure 3A). During this "Metabolic challenge" window, we were particularly interested in whether TCDD-induced weight gain would persist long term (i.e., TCDDChow vs COChow) and whether HFD feeding would exacerbate these effects (i.e., TCDDHFD vs TCDDChow). The increased body weight in TCDDChow dams (Figure 2B and Supplemental Figs. S5A–E) returned to control levels after longer tracking (Figure 3B and Supplemental Fig. S5E). However, TCDDHFD dams showed a rapid and sustained increase in body weight starting 1 week after being transferred to HFD, whereas COHFD dams only had a modest increase in body weight after  $\sim 10$  weeks of HFD feeding (Figure 3B and Supplemental Fig. S5E). Both HFD-fed groups had significantly increased % fat mass and decreased % lean mass compared to chow-fed dams after 10 weeks of HFD feeding, but these changes in body composition were exacerbated in TCDDHFD dams compared to COHFD dams (Figure 3C).

To better understand why TCDD-exposed mice gained more fat mass than control mice after HFD feeding, we investigated other aspects of energy homeostasis. We observed a significant decrease in caloric intake in TCDDHFD dams after 7 weeks of HFD feeding compared to TCDDChow dams, but this was not different than COHFD dams so unlikely to explain the dramatic differences in weight gain between HFD groups (Figure 3D). As expected, circulating leptin levels were significantly increased  $\sim 7.7$ -fold in COHFD dams compared to COChow dams after 11 weeks on HFD (Figure 3E). In contrast, TCDDHFD dams only showed a non-significant trend toward having  $\sim 3$ -fold higher circulating leptin compared to TCDDChow dams, and leptin levels in TCDDHFD dams were significantly lower than in COHFD dams (Figure 3E). To assess the contribution of central energy balance in TCDD-mediated obesity, we measured gene expression of key regulators within the brain. Proopiomelanocortin (*Pomc*) expression in the hypothalamus was dramatically induced by HFD feeding in both

CO- and TCDD-exposed dams, but similar to leptin, *Pomc* levels were significantly lower in TCDDHFD dams than in COHFD dams (Figure 3F). There was no effect of diet or chemical exposure on gene expression of agouti-related peptide (*Agrp*), leptin receptor (*LepR*), or insulin receptor (*Insr*) in the hypothalamus after 11 weeks of HFD feeding (Figure 3F). Neuropeptide Y (*Npy*) expression was decreased in TCDDChow dams compared to COChow dams but unchanged in TCDDHFD dams (Figure 3F). Taken together, these data suggest that diet-induced weight gain in TCDD-exposed dams (relative to CO) involves insufficient leptin levels and abnormal energy homeostasis.

### 3.4. TCDD exposure accelerates the onset of glucose intolerance in HFD-fed dams without altering insulin sensitivity

Obesity is a major risk factor for developing diabetes, so although there were no long-term changes in fasting glycemia following TCDD exposure (Figure 3A), we investigated whether the diet-induced weight gain observed in TCDDHFD dams was associated with changes in glucose or insulin tolerance. As expected, COHFD dams displayed modest hyperglycemia during a GTT after 2.5 weeks of HFD feeding (Figure 4A). In contrast, TCDDHFD dams were severely hyperglycemic throughout the GTT, with a peak glycemia of ~30 mM compared to ~22 mM in COHFD dams (Figure 4A), and a significant increase in overall glucose excursion compared to COHFD dams (Figure 4A-AUC). Following 6.5 (Supplemental Fig. S4A) and 9.5 weeks (Figure 4C) of HFD feeding, both COHFD and TCDDHFD dams had similar levels of hyperglycemia during the GTT. There were no differences in overall insulin tolerance between any groups at weeks 4, 7, and 10.5 of the metabolic challenge (Figure 4B, D, and Supplemental Fig. S4B).

### 3.5. HFD feeding impairs *in vivo* glucose-induced plasma insulin levels in TCDD-exposed dams

Since defective glycemic control is often triggered by improper insulin secretion, we assessed changes in glucose-stimulated plasma insulin levels *in vivo* following HFD feeding. Corresponding with the profound hyperglycemia observed after 2.5 weeks of HFD feeding (Figure 4A), TCDDHFD dams had significantly lower plasma insulin levels 15 min after an i.p. glucose bolus compared to COHFD dams (Figure 5Aiii) and trended toward having reduced overall plasma insulin levels during the GTT compared to TCDDChow dams (Figure 5Aiv). Interestingly, after 6.5 weeks of HFD feeding, TCDDHFD dams had significantly higher basal insulin levels than COHFD dams (Figure 5Biii) and trended toward having higher plasma insulin levels overall during the GTT compared to TCDDChow dams (Figure 5Biv). After 9.5 weeks of HFD feeding, plasma insulin levels in TCDDHFD dams had normalized back to COHFD levels (Figure 5C iii and iv). Prior TCDD exposure had no impact on plasma insulin levels in chow-fed dams at any time point (i.e., TCDDChow vs COChow; Figure 5Aii-5Cii).

### 3.6. TCDDHFD dams have decreased islet size, a higher proportion of MAFA<sup>ve</sup> beta cells, and increased proinsulin accumulation compared to COHFD dams

We next assessed whether the observed changes in glucose-induced plasma insulin levels were associated with defects in islet morphology and other beta cell characteristics at week 11 of the metabolic challenge. As expected, HFD feeding caused a significant ~2-fold increase in average islet size in control mice (Figure 6A and 6H-J). However, TCDDHFD dams did not show the same adaptation to HFD feeding, but rather had an average islet size that was comparable to chow-fed dams (Figure 6A and 6H-J). TCDDHFD dams also had a significantly higher proportion of MAFA<sup>ve</sup> beta cells compared

to all other groups (Figure 6D,I), suggesting a loss of beta cell identity. We also observed a significant increase in the proportion of beta cells with proinsulin accumulation throughout the cytoplasm as opposed to the expected perinuclear pattern of immunoreactivity in TCDDHFD mice compared to COHFD and TCDDChow dams (Figure 6F,J), although this was not associated with changes in the non-fasted circulating proinsulin:insulin ratio in cardiac blood at week 11 of the metabolic challenge (Figure 6G). There was no change in % insulin<sup>+</sup> area per islet (Figure 6B,H), % glucagon<sup>+</sup> area per islet (Figure 6C,H), or % PCNA<sup>+</sup> beta cells (Figure 6E) in TCDDHFD dams compared to the other groups. TCDDChow mice did not display any changes in islet composition compared to COChow dams (Figure 6A–F and H–J).

### 3.7. TCDDHFD females have reduced *MafA*, *Nkx6.1*, and *Slc2a2* gene expression and increased *Pcsk1* expression compared to TCDDChow females in a non-pregnancy model

Due to our experimental design, we did not have sufficient animal numbers to isolate islets for RNA analysis from the dams in this study. Instead, we used RNAseq analysis on islets from a separate but related cohort of female mice to support our histological findings (Figure 6). Briefly, non-pregnant female mice were injected with corn oil (CO; vehicle control) or 20 ng/kg/d TCDD 2x/week for 12 weeks and simultaneously fed either standard chow or a 45% HFD (Figure 7A). Islets were isolated after 12 weeks of exposure and gene expression was measured by RNAseq (Figure 7B and 7D-E) and qPCR (Figure 7C). To reiterate the main differences, the second model involved simultaneous exposure to TCDD and/or HFD in non-pregnant female mice (Figure 7) compared to our primary model of sequential exposure to TCDD transiently during pregnancy/lactation followed by a 10-week period of chow feeding and then 11 weeks of chow or HFD feeding (Figures 1-6 and Supplemental Figs. S1-5). Despite the differences in experimental designs, we saw similar phenotypes in TCDDHFD females in both models. For example, both models showed accelerated glucose intolerance and abnormal glucose-induced plasma insulin levels, with no change in insulin sensitivity [10]. As such, this second model provides a useful dataset to follow up on our histological observations to determine whether low-dose TCDD consistently alters islet composition in females (Figure 6). A detailed RNAseq analysis of this dataset is published elsewhere [10].

Consistent with our histological finding of increased % MAFA<sup>ve</sup> beta cells in TCDDHFD dams (Figure 6D, I), RNAseq analysis showed that TCDDHFD females from the simultaneous exposure model had significantly lower *MafA* gene expression compared to COChow and TCDDChow females (Figure 7B). This finding was also validated by qPCR (Figure 7C). We next examined whether TCDD combined with HFD feeding altered the expression of other beta cell transcription factors. TCDDHFD females had significantly lower *Nkx6.1* expression than TCDDChow and COHFD females (Figure 7D), supporting our hypothesis of loss of beta cell identity. Furthermore, we observed a diet-induced decrease in *Hnf4a* irrespective of chemical exposure (Figure 7D) and significant upregulation of *NeuroD1* in COHFD females compared to COChow only (Figure 7D). Next, we examined the expression of genes involved in glucose-stimulated insulin secretion. Interestingly, TCDDHFD females had lower expression of the glucose transporter gene *Slc2a2* compared to TCDDChow females, suggesting a possible defect in glucose sensing (Figure 7E). The K<sub>ATP</sub> channel regulatory subunit *Abcc8* was also downregulated in TCDDHFD females, but a similar decrease was observed in COHFD females and is therefore unlikely to contribute to a chemical-induced phenotype.

Neither chemical or diet exposure affected the glucokinase gene *Gck* or  $K_{ATP}$  channel pore-forming subunit *Kcnj11* (Figure 7E). Finally, we assessed whether combined TCDD and HFD exposure affected insulin genes and/or prohormone convertase enzymes PC1 and PC2 since we had observed a significant increase in proinsulin accumulation in beta cells from TCDDHFD dams in our study (Figure 6F,J). In the simultaneous exposure model, TCDDHFD females had a significant increase in *Pcsk1* expression compared to all other experimental groups, supporting the hypothesis of abnormal insulin processing in TCDDHFD conditions. There was no change in *Pcsk2*, and the expression of *Ins1* and *Ins2* was affected by diet only (Figure 7E). Of note, RNAseq analysis was conducted on a small sample size ( $n = 3-4$ ) and therefore non-significant changes in gene expression should be further validated with a larger sample size.

#### 4. DISCUSSION

Our study demonstrates that low-dose exposure to TCDD throughout pregnancy and lactation in mice has modest metabolic consequences during pregnancy but profoundly increases susceptibility to weight gain and diabetes later in life. TCDD-exposed dams had normal body weight, basal blood glucose levels, fasted plasma insulin levels, and beta cell plasticity during pregnancy, but were transiently hypoglycemic at P1 (1 day after birth) and heavier at P14 (mid-lactation) compared to CO-exposed dams. After chemical exposure stopped at the end of lactation, TCDD-exposed dams maintained normal glucose homeostasis but showed a modest increase in body weight compared to CO-exposed dams within 5 weeks post-TCDD. Once challenged with HFD feeding, TCDD-exposed dams rapidly became obese, severely glucose intolerant, and showed beta cell dysfunction. Although the TCDD-induced exacerbation of glucose intolerance and dysregulation of glucose-induced plasma insulin levels did not persist long term, TCDDHFD mice remained significantly heavier than COHFD mice throughout the study. TCDDHFD dams also displayed defects in proinsulin processing, reduced islet size, and had a larger proportion of MAFA<sup>ve</sup> beta cells compared to COHFD dams. Taken together, these data suggest that low-dose dioxin exposure in female mice impairs metabolic adaptability to HFD feeding long after exposure ceases, leading to a sustained susceptibility to developing diet-induced obesity and glucose intolerance.

To our knowledge, this is the first study assessing the association between low-dose dioxin exposure during pregnancy/lactation and GDM or long-term diabetes risk in a mouse model. Our study shows that dioxin dramatically induces *Cyp1a1* expression in the pancreas during pregnancy, which is indicative of direct chemical exposure to pancreatic tissue. Interestingly, unlike our previous studies in non-pregnant mice [8,9], the degree of *Cyp1a1* induction in the pancreas was much higher than in the liver mid-pregnancy. It is unclear whether pregnancy promotes greater dioxin accumulation in the pancreas compared to other tissues, potentially due to an increase in blood flow to the pancreas in response to an increase in metabolic demand, or if the pancreas may be more sensitive to AhR-mediated dioxin signaling mid-pregnancy. We also observed a decrease in *AhR* and *Arnt* expression in the pancreas but not liver mid-pregnancy, indicating negative feedback regulation of the AhR pathway. Interestingly, we previously reported upregulation of CYP1A1 enzyme activity for at least 2 weeks in islets following acute high-dose TCDD exposure [8], suggesting that CYP1A1 activation may persist after AhR suppression. Whether induction of *Cyp1a1* or suppression of *AhR* in the pancreas

following low-dose dioxin exposure has protective or deleterious effects remains an important area of investigation.

Based on our previous work with acute high-dose TCDD [8–10], we predicted that low-dose TCDD exposure to pancreatic tissue during pregnancy could have both immediate and long-term implications on islet physiology and diabetes risk. Interestingly, low-dose TCDD exposure alone did not cause overt GDM in this study. In fact, rather than developing hyperglycemia, TCDD-exposed dams were transiently hypoglycemic at P1. Labor is an acute form of metabolic stress characterized by elevated blood glucose levels to compensate for increases in energy demand [29]. TCDD-induced hypoglycemia at P1 suggests that dioxin exposure during pregnancy may impair adaptability to a metabolic stressor; however, in the absence of a prolonged stressor, the dams returned to euglycemia. Future studies should investigate whether combining TCDD exposure during pregnancy with a secondary metabolic stressor, such as HFD feeding, would trigger the development of GDM. Our findings should also be validated with a larger sample size in future studies to increase the statistical power of our analysis.

To date, epidemiological studies investigating the association between POP exposure during pregnancy and diabetes incidence have been inconsistent. Some studies reported a positive association between GDM and serum POP levels, including polychlorinated biphenyls (PCB) [23–25], polyfluoroalkyl (PFAS) [24], and polybrominated diphenyl ethers (PBDEs) [24,26]. A positive association between GDM and self-reported pesticide exposure during pregnancy was also reported [30]. However, other studies showed no association between GDM incidence and serum organochloride pesticides [26,27] or PCB levels [27,28]. Inconsistencies between human studies can result from the inability to control confounding variables, such as varying POP concentrations between participants, differences in timing of POP measurements, method of diabetes diagnosis, genetic variations, and environmental factors. Using a controlled mouse model, we demonstrated that low-dose TCDD exposure did not impair body weight, basal blood glucose, plasma insulin levels, or beta cell plasticity acutely during pregnancy, but rather caused long-term metabolic dysregulation long after TCDD had been excreted. Of note, we did not have non-pregnant controls in this study and therefore we cannot determine whether the long-term effects of TCDD on metabolism were influenced by the timing of exposure during pregnancy/lactation. Future studies should include a head-to-head comparison of the effects of TCDD on metabolism in pregnant vs non-pregnant females. Regardless, our study highlights the need to track the metabolic health of mothers long-term following pollutant exposure during pregnancy.

The most persistent consequence of transient low-dose TCDD exposure during pregnancy and lactation in our model was excessive weight gain later in life. TCDD-exposed dams showed modest weight gain in the absence of a metabolic stressor, but rapidly developed pronounced obesity following a transition to HFD feeding. These results were interesting given that acute high-dose TCDD exposure is known to cause wasting syndrome [8,31]. To our knowledge, this is the first study to report an increase in weight gain following low-dose TCDD exposure. In a separate study, we found that female mice with prolonged low-dose TCDD exposure and simultaneously fed chow or HFD did not display chemical-induced weight gain [10], suggesting that TCDD exposure specifically during pregnancy may promote weight gain. However, this hypothesis must be verified with a head-to-head comparison between pregnant and non-pregnant female mice. Obesity in TCDDHFD dams was also associated with abnormal energy

homeostasis. TCDDHFD dams had significantly reduced circulating leptin levels and hypothalamic *Pomc* levels compared to COHFD dams. Leptin is essential for regulating energy homeostasis and can activate POMC neurons in the hypothalamus [32,33]. *Pomc* expression, especially in LepR-expressing POMC neurons, is critical for maintaining normal energy balance by providing anorexigenic drive [34]. Melanocortins produced by POMC neurons [36] and leptin [35] can directly regulate peripheral lipid metabolism, and in effect, leptin- and POMC-deficiency leads to obesity by increasing food intake, limiting energy expenditure, and promoting fat storage [37–39]. We did not observe significant changes in food intake between TCDDHFD and COHFD dams but did find that TCDDHFD dams had significantly higher % fat mass than COHFD mice, suggesting an adaptation in energy expenditure and/or energy storage that promotes obesity. Studies using metabolic chambers would provide additional insight into the mechanisms underlying TCDD-induced weight gain. Regardless, our mouse model suggests that transient low-dose TCDD exposure during pregnancy and lactation contribute to obesity pathogenesis. Transient TCDD exposure also accentuated the development of diet-induced glucose intolerance, which appears to be primarily driven by impaired beta cell plasticity and function. The rapid onset of hyperglycemia in TCDD-exposed mice after 2.5 weeks of HFD feeding coincided with suppressed glucose-induced plasma insulin levels *in vivo*, but no change in peripheral insulin sensitivity. Interestingly, hypoinsulinemia in TCDDHFD dams transitioned to hyperinsulinemia before eventually re-equilibrating back to COHFD levels, demonstrating an initial defect in insulin secretion at the beta cell level. Although TCDD-induced exacerbation of glucose intolerance and dysregulation of glucose-induced plasma insulin levels eventually returned to COHFD levels, TCDDHFD dams showed lasting defects in islet morphology, beta cell maturity, and proinsulin processing that could have long-term implications on metabolism. TCDDHFD dams had decreased islet size compared to COHFD dams, suggesting that TCDD-exposed beta cells lack the adaptive proliferative response to the metabolic demand of HFD feeding. However, without tissues from different timepoints, we cannot rule out the possibility that TCDDHFD dams experienced an appropriate increase in beta cell proliferation earlier followed by an increase in beta cell apoptosis. We also found that TCDDHFD dams had a modest but significant increase in MAFA<sup>ve</sup> beta cells. Similarly, non-pregnant females from our second model with simultaneous TCDD and HFD exposure had reduced *Mafa*, *Slc2a2*, and *Nkx6.1* expression relative to TCDDChow females. MAFA is a transcription factor essential for maintaining beta cell maturity and function. Reduced MAFA expression is associated with beta cell dedifferentiation and beta cell dysfunction during diabetes progression [40,41]; MAFA is also commonly suppressed in beta cells from humans with type 2 diabetes [42,43]. Likewise, impaired *Slc2a2* and *Nkx6.1* expression is characteristic of immature beta cells [44–47]. Lastly, TCDDHFD dams also had increased proinsulin accumulation, indicating impaired proinsulin synthesis and/or processing. Interestingly, in the non-pregnancy model, *Pcsk1* expression was increased in TCDDHFD islets compared to COHFD islets, which could reflect an adaptive response to proinsulin accumulation, and supports the idea that TCDD alters insulin processing; however, this hypothesis requires further investigating. Taken together, our results suggest that TCDD exposure promotes diet-induced beta cell dedifferentiation in female mice, irrespective of pregnancy, which could contribute to increased diabetes risk. In summary, epidemiological studies consistently show an association between exposure to dioxin/dioxin-like compounds and diabetes

incidence, but limited studies have examined dioxin exposure during pregnancy. Our study shows that transient low-dose TCDD exposure during pregnancy was insufficient to induce GDM but eventually led to impaired metabolic adaptability to HFD feeding, including glucose intolerance, beta cell dysfunction, and increased weight gain in mice. Our study suggests that background exposure to dioxin or dioxin-like chemicals could contribute to obesity and diabetes incidence. The effects of low-dose pollutant exposure on metabolism, particularly in females, should be further investigated. More research is also required to determine the underlying mechanisms involved in dioxin-induced alterations in glucose homeostasis and beta cell function.

## AUTHOR CONTRIBUTIONS

J.E.B. and M.P.H. conceived the experimental design and wrote the manuscript. M.P.H., R.C.M., H.L.B., C.D.S., M.A.P., D.I.M.A., M.Z., G.M., M.J.C., and J.E.B. were involved with the acquisition, analysis, and interpretation of the data. All authors contributed to manuscript revisions and approved the final version of the article.

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## CONFLICT OF INTEREST

The authors declare no competing interests.

## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molmet.2020.101104>.

## REFERENCES

- [1] IDF, 2019. IDF diabetes atlas, 9th ed. <https://www.diabetesatlas.org/en/>.
- [2] Wu, Y., Ding, Y., Tanaka, Y., Zhang, W., 2014. Risk factors contributing to type 2 diabetes and recent advances in the treatment and prevention. *International Journal of Medical Sciences* 11(11):1185–1200. <https://doi.org/10.7150/ijms.10001>.
- [3] Taylor, K.W., Novak, R.F., Anderson, H.A., Birnbaum, L.S., Blystone, C., Devito, M., et al., 2013. Evaluation of the association between persistent organic pollutants (POPs) and diabetes in epidemiological studies: a national toxicology program workshop review. *Environmental Health Perspectives* 121(7):774–783. <https://doi.org/10.1289/ehp.1205502>.
- [4] Sun, W., Clark, J.M., Park, Y., 2017. Environmental pollutants and type 2 diabetes: a review of human studies. *Toxicology & Environmental Chemistry* 99(9–10):1283–1303. <https://doi.org/10.1080/02772248.2017.1393818>.

- [5] Breivik, K., Alcock, R., Li, Y.F., Bailey, R.E., Fiedler, H., Pacyna, J.M., 2004. Primary sources of selected POPs: regional and global scale emission inventories. *Environmental Pollution* 128(1–2):3–16. <https://doi.org/10.1016/j.envpol.2003.08.031>.
- [6] White, S.S., Birnbaum, L.S., 2009. An overview of the effects of dioxins and dioxin-like compounds on vertebrates, as documented in human and ecological epidemiology. *Journal of Environmental Science and Health - Part C: Environmental Carcinogenesis & Ecotoxicology Reviews*. <https://doi.org/10.1080/10590500903310047>.
- [7] Mimura, J., Fujii-Kuriyama, Y., 2003. Functional role of AhR in the expression of toxic effects by TCDD. *Biochimica et Biophysica Acta (BBA) - General Subjects* 1619(3):263–268. [https://doi.org/10.1016/S0304-4165\(02\)00485-3](https://doi.org/10.1016/S0304-4165(02)00485-3).
- [8] Ibrahim, M., MacFarlane, E.M., Matteo, G., Hoyeck, M.P., Rick, K.R.C., Boroujeni, S.F., et al., 2020. Functional cytochrome P450 1a enzymes are induced in mouse and human islets following pollutant exposure. *Diabetologia* 63:162–178. <https://doi.org/10.1007/s00125-019-05035-0>.
- [9] Hoyeck, M.P., Blair, H., Ibrahim, M., Solanki, S., Elsayy, M., Prakash, A., et al., 2020. Long-term metabolic consequences of acute dioxin exposure differ between male and female mice. *Scientific Reports* 10(1):1448. <https://doi.org/10.1101/762476>.
- [10] Matteo, G., Hoyeck, M.P., Blair, H.L., Zebarth, J., Rick, K.R.C., Williams, A., et al., 2020. Chronic low-dose dioxin exposure accelerates high fat diet-induced hyperglycemia in female mice. *BioRxiv*. <https://doi.org/10.1101/2020.09.12.294587>.
- [11] Sorenson, R.L., Brejle, T.C., 1997. Adaptation of islets of Langerhans to pregnancy:  $\beta$ -cell growth, enhanced insulin secretion and the role of lactogenic hormones. *Hormone and Metabolic Research* 29(6):301–307. <https://doi.org/10.1055/s-2007-979040>.
- [12] Karnik, S.K., Chen, H., McLean, G.W., Heit, J.J., Gu, X., Zhang, A.Y., et al., 2007. Menin controls growth of pancreatic  $\beta$ -cells in pregnant mice and promotes gestational diabetes mellitus. *Science* 318:806–809. <https://doi.org/10.1126/science.1146812>.
- [13] Van Assche, F.A., Aerts, L., Prins, F. De., 1978. A morphological study of the endocrine pancreas in human pregnancy. *British Journal of Obstetrics and Gynaecology* 85:818–880. <https://doi.org/10.1111/j.1471-0528.1978.tb15835.x>.
- [14] Butler, A.E., Cao-Minh, L., Galasso, R., Rizza, R.A., Corradin, A., Cobelli, C., et al., 2010. Adaptive changes in pancreatic beta cell fractional area and beta cell turnover in human pregnancy. *Diabetologia* 53(10):2167–2176. <https://doi.org/10.1007/s00125-010-1809-6>.
- [15] Moyce, B.L., Dolinsky, V.W., 2018. Maternal  $\beta$ -Cell adaptations in pregnancy and placental signalling: implications for gestational diabetes. *International Journal of Molecular Sciences* 19(11):3467. <https://doi.org/10.3390/ijms19113467>.
- [16] Consonni, D., Pesatori, A.C., Zocchetti, C., Sindaco, R., D'Oro, L.C., Rubagotti, M., et al., 2008. Mortality in a population exposed to dioxin after the Seveso, Italy, accident in 1976: 25 years of follow-up. *American Journal of Epidemiology* 167(7):847–858. <https://doi.org/10.1093/aje/kwm371>.
- [17] Vasilii, O., Cameron, L., Gardiner, J., DeGuire, P., Karmaus, W., 2006. Polychlorinated biphenyls, polychlorinated biphenyls, body weight, and incidence of adult-onset diabetes mellitus. *Epidemiology* 17:352–359. <https://doi.org/10.1097/01.ede.0000220553.84350.c5>.
- [18] Wang, S.L., Tsai, P.C., Yang, C.Y., Guo, Y.L., 2008. Increased risk of diabetes and polychlorinated biphenyls and dioxins: a 24-year follow-up study of the Yucheng cohort. *Diabetes Care* 31(8):1574–1579. <https://doi.org/10.2337/dc07-2449>.
- [19] Steenland, K., Piacitelli, L., Deddens, J., Fingerhut, M., Chang, L.I., 1999. Cancer, heart disease, and diabetes in workers exposed to 2,3,7,8-tetrachlorodibenzodioxin. *Journal of the National Cancer Institute* 91:779–786. <https://doi.org/10.1093/jnci/91.9.779>.
- [20] Rylander, L., Rignell-Hydbom, A., Hagmar, L., 2005. A cross-sectional study of the association between persistent organochlorine pollutants and diabetes. *Environmental Health: A Global Access Science Source* 4:28. <https://doi.org/10.1186/1476-069X-4-28>.
- [21] Airaksinen, R., Rantakokko, P., Eriksson, J.G., Blomstedt, P., Kajantie, E., Kiviranta, H., 2011. Association between type 2 diabetes and exposure to persistent organic pollutants. *Diabetes Care* 34(9):1972–2303. <https://doi.org/10.2337/dc10-2303>.
- [22] Glynn, A.W., Granath, F., Aune, M., Atuma, S., Darnerud, P.O., Bjerselius, R., et al., 2003. Organochlorines in Swedish women: determinants of serum concentrations. *Environmental Health Perspectives* 111:349–355. <https://doi.org/10.1289/ehp.5456>.
- [23] Longnecker, M.P., Klebanoff, M.A., Brock, J.W., Zhou, H., 2001. Polychlorinated biphenyl serum levels in pregnant subjects with diabetes. *Diabetes Care* 24:1099–1101. <https://doi.org/10.2337/diacare.24.6.1099>.
- [24] Rahman, M.L., Zhang, C., Smarr, M.M., Lee, S., Honda, M., Kannan, K., et al., 2019. Persistent organic pollutants and gestational diabetes: a multi-center prospective cohort study of healthy US women. *Environment International* 124:249–258. <https://doi.org/10.1016/j.envint.2019.01.027>.
- [25] Vafeiadi, M., Roumeliotaki, T., Chalkiadaki, G., Rantakokko, P., Kiviranta, H., Fthenou, E., et al., 2017. Persistent organic pollutants in early pregnancy and risk of gestational diabetes mellitus. *Environment International* 98:89–95. <https://doi.org/10.1016/j.envint.2016.10.005>.
- [26] Smarr, M.M., Grantz, K.L., Zhang, C., Sundaram, R., Maisog, J.M., Barr, D.B., et al., 2016. Persistent organic pollutants and pregnancy complications. *The Science of the Total Environment* 151(152):285–291. <https://doi.org/10.1016/j.scitotenv.2016.02.030>.
- [27] Shapiro, G.D., Dodds, L., Arbuckle, T.E., Ashley-Martin, J., Ettinger, A.S., Fisher, M., et al., 2016. Exposure to organophosphorus and organochlorine pesticides, perfluoroalkyl substances, and polychlorinated biphenyls in pregnancy and the association with impaired glucose tolerance and gestational diabetes mellitus: the MIREC Study. *Environmental Research* 147:71–81. <https://doi.org/10.1016/j.envres.2016.01.040>.
- [28] Jaacks, L.M., Barr, D.B., Sundaram, R., Maisog, J.M., Zhang, C., Buck Louis, G.M., 2016. Pre-pregnancy maternal exposure to polybrominated and polychlorinated biphenyls and gestational diabetes: a prospective cohort study. *Environmental Health: A Global Access Science Source* 15:11. <https://doi.org/10.1186/s12940-016-0092-5>.
- [29] Scheepers, H.C.J., De Jong, P.A., Essed, G.G.M., Kanhai, H.H.H., 2001. Fetal and maternal energy metabolism during labor in relation to the available caloric substrate. *Journal of Perinatal Medicine* 29:457–464. <https://doi.org/10.1515/JPM.2001.064>.
- [30] Saldana, T.M., Basso, O., Hoppin, J.A., Baird, D.D., Knott, C., Blair, A., et al., 2007. Pesticide exposure and self-reported gestational diabetes mellitus in the agricultural health study. *Diabetes Care* 30(3):529–534. <https://doi.org/10.2337/dc06-1832>.
- [31] Uno, S., Dalton, T.P., Sinclair, P.R., Gorman, N., Wang, B., Smith, A.G., et al., 2004. Cyp1a1(-/-) male mice: protection against high-dose TCDD-induced lethality and wasting syndrome, and resistance to intrahepatocyte lipid accumulation and uroporphyrin. *Toxicology and Applied Pharmacology* 196(3):410–421. <https://doi.org/10.1016/j.taap.2004.01.014>.
- [32] Cowley, M.A., Smart, J.L., Rubinstein, M., Cerdán, M.G., Diano, S., Horvath, T.L., et al., 2001. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 24(411):480–484. <https://doi.org/10.1038/35078085>.
- [33] Mercer, R.E., Michaelson, S.D., Chee, M.J.S., Atallah, T.A., Wevrick, R., Colmers, W.F., 2013. Magel2 is required for leptin-mediated depolarization of POMC neurons in the hypothalamic arcuate nucleus in mice. *PLoS Genetics* 9(1):e1003207. <https://doi.org/10.1371/journal.pgen.1003207>.
- [34] Lam, D.D., Attard, C.A., Mercer, A.J., Myers, M.G., Rubinstein, M., Low, M.J., 2015. Conditional expression of Pomc in the lepr-positive

- subpopulation of POMC Neurons is sufficient for normal energy homeostasis and metabolism. *Endocrinology* 156(4):1292–1302. <https://doi.org/10.1210/en.2014-1373>.
- [35] Prieur, X., Tung, Y.C.L., Griffin, J.L., Farooqi, I.S., O'Rahilly, S., Coll, A.P., 2008. Leptin regulates peripheral lipid metabolism primarily through central effects on food intake. *Endocrinology* 148(11):5432–5439. <https://doi.org/10.1210/en.2008-0498>.
- [36] Nogueiras, R., Wiedmer, P., Perez-Tilve, D., Veyrat-Durebex, C., Keogh, J.M., Sutton, G.M., et al., 2007. The central melanocortin system directly controls peripheral lipid metabolism. *Journal of Clinical Investigation* 117(11):3475–3488. <https://doi.org/10.1172/JCI31743>.
- [37] Klok, M.D., Jakobsdottir, S., Drent, M.L., 2007. The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review. *Obesity Reviews* 8:21–34. <https://doi.org/10.1111/j.1467-789X.2006.00270.x>.
- [38] Ahima, R.S., 2008. Revisiting leptin's role in obesity and weight loss. *Journal of Clinical Investigation* 118:2380–2383. <https://doi.org/10.1172/JC136284>.
- [39] Coll, A.P., Farooqi, I.S., Challis, B.G., Yeo, G.S.H., O'Rahilly, S., 2004. Proopiomelanocortin and energy balance: insights from human and murine genetics. *Journal of Clinical Endocrinology & Metabolism* 89:2557–2562. <https://doi.org/10.1210/jc.2004-0428>.
- [40] Nishimura, W., Takahashi, S., Yasuda, K., 2015. MafA is critical for maintenance of the mature beta cell phenotype in mice. *Diabetologia* 58:566–574. <https://doi.org/10.1007/s00125-014-3464-9>.
- [41] Talchai, C., Xuan, S., Lin, H.V., Sussel, L., Accili, D., 2012. Pancreatic  $\beta$  cell dedifferentiation as a mechanism of diabetic  $\beta$  cell failure. *Cell* 150(6):1223–1234. <https://doi.org/10.1016/j.cell.2012.07.029>.
- [42] Butler, A.E., Robertson, R.P., Hernandez, R., Matveyenko, A.V., Gurlo, T., Butler, P.C., 2012. Beta cell nuclear musculoaponeurotic fibrosarcoma oncogene family A (MafA) is deficient in type 2 diabetes. *Diabetologia* 55(11):2985–2988. <https://doi.org/10.1007/s00125-012-2666-2>.
- [43] Guo, S., Dai, C., Guo, M., Taylor, B., Harmon, J.S., Sander, M., et al., 2013. Inactivation of specific  $\beta$  cell transcription factors in type 2 diabetes. *Journal of Clinical Investigation* 123(8):3305–3316. <https://doi.org/10.1172/JCI65390>.
- [44] Schaffer, A.E., Taylor, B.L., Benthuyzen, J.R., Liu, J., Thorel, F., Yuan, W., et al., 2013. Nkx6.1 controls a gene regulatory network required for establishing and maintaining pancreatic beta cell identity. *PLoS Genetics* 9(1):e1003274. <https://doi.org/10.1371/journal.pgen.1003274>.
- [45] Taylor, B.L., Liu, F.F., Sander, M., 2013. Nkx6.1 is essential for maintaining the functional state of pancreatic beta cells. *Cell Reports* 4(6):1262–1275. <https://doi.org/10.1016/j.celrep.2013.08.010>.
- [46] Jonas, J.C., Sharma, A., Hasenkamp, W., Ilkova, H., Patané, G., Laybutt, R., et al., 1999. Chronic hyperglycemia triggers loss of pancreatic  $\beta$  cell differentiation in an animal model of diabetes. *Journal of Biological Chemistry* 274:14112–14121. <https://doi.org/10.1074/jbc.274.20.14112>.
- [47] Nordmann, T.M., Dror, E., Schulze, F., Traub, S., Berishvili, E., Barbieux, C., et al., 2017. The role of inflammation in  $\beta$ -cell dedifferentiation. *Scientific Reports* 7:6285. <https://doi.org/10.1038/s41598-017-06731-w>.