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Chronic hyperprolactinemia evoked by disruption of lactotrope dopamine D2 receptors impacts on liver and adipocyte genes related to glucose and insulin balance

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Itemized list of how each author contributed to the study:

GML and FLV: performed most experiments, and participated in writing the manuscript.

AMO: participated in the characterization of the mutant model, and performed GTTs.

CDW: performed RIAs and helped in the characterization of the model.

GM and EF performed in vitro experiments with cultured hepatocytes.

BB: performed real time PCRS in liver, and helped in the discussion of the manuscript.

MIPM and MR: participated in the generation and characterization of the mutant model, and in the Discussion of the manuscript.

DBV: participated in the design, correction, discussion and writing of the manuscript.

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Abstract

We studied the impact of high prolactin titers on liver and adipocyte gene expression related to glucose and insulin homeostasis, in correlation with obesity onset. To that end we used mutant female mice that selectively lack dopamine type 2 receptors (D2Rs) from pituitary lactotropes (lacDrd2KO) which have chronic high prolactin levels associated with increased body weight, marked increments in fat depots, adipocyte size, and serum lipids, a metabolic phenotype which intensifies with age. LacDrd2KO mice of two developmental ages, 5 and 10 months were used. In the first time point, obesity and increased body weight are marginal even though mice are hyperprolactinemic, while at 10 months there is marked adiposity with a 136 % increase in gonadal fat, and a 36 % increase in liver weight due to lipid accumulation. LacDrd2KO mice had glucose intolerance, hyperinsulinemia, and impaired insulin response to glucose, already in early stages of obesity, but changes in liver and adipose tissue transcription factors were time and tissue dependent. In chronic hyperprolactinemic mice liver *Prlr* were upregulated, there was liver steatosis, altered expression of the lipogenic transcription factor *Chrebp* and blunted response of *Srebp-1c* to refeeding at 5 months of age, while no effect was observed in the glycogenesis pathway. On the other hand, in adipose tissue a marked decrease in lipogenic transcription factor expression was observed when morbid obesity was already settled. These adaptive changes underscore the role of prolactin signaling in different tissues to promote energy storage.

Introduction

The actions of prolactin go far beyond its well established role in lactation and pregnancy. It is involved in behavior, migrations, water and electrolyte balance, growth, development, immune regulation, gonadal suppression, and strong evidence points to prolactin as a metabolic hormone (27; 33). A large-scale tissue array method has identified several tissues that respond acutely to prolactin administration (42), including those which participate in metabolic regulation such as liver, pancreas, adipose tissue and brain; and concordantly, prolactin receptors (PRLR) are present in these tissues (13; 24; 45; 51; 61). On the other hand, prolactin and *Prlr* knockout mice do not exhibit a prominently altered metabolic phenotype (41) (even though there is lack of lactation and altered fertility in females), indicating that many of the reported metabolic actions of prolactin are redundant or overlap with other physiological effectors. Nevertheless, understanding the role of prolactin becomes relevant in explaining many symptoms and manifestations which occur in prolactin overproduction, such as during pharmacological psychiatric treatments or in patients with prolactinomas.

The liver is a complex organ with multifaceted functions and paramount importance in glucose metabolism. It participates in maintaining long-term energy stores by conversion of carbohydrates to fat. Enzymes involved in these pathways are regulated by post translational mechanisms and respond to availability of nutrients and hormone levels. Long term exposure of the liver to high levels of insulin and glucose results in alterations in these key enzymes. Insulin increases glycogen stores and lipogenesis (15; 71), and inhibits gluconeogenesis and glucose secretion (15) partially by induction of glucokinase (64).

In the synthesis of fatty acids two important transcription factors participate regulating genes that encode enzymes for glucose metabolism or lipogenesis in the liver: sterol regulatory element binding protein-1c (SREBP-1c) and carbohydrate responsive element binding protein (ChREBP). Both transcription factors may coordinately or independently regulate *de novo* lipogenesis, and are differentially regulated by insulin and glucose (39; 74). *SREBP-1c* expression is stimulated by insulin via a PI3K pathway (48), downregulates phosphoenolpyruvate carboxykinase, an enzyme involved in liver gluconeogenesis, and stimulates glucokinase which is involved in glycolysis and lipogenesis (21; 30). ChREBP, also fundamental in *de novo* lipogenesis in the liver (38; 72), is not directly stimulated by insulin, but requires glucose phosphorylation via glucokinase to activate the expression of glycolytic and lipogenic genes (14; 37). It has been shown that *Chrebp* overexpression in liver induces hepatic steatosis (6), and high levels of liver *Chrebp* are found in obese mice (63).

Nevertheless, ChREBP overexpression generates beneficial lipid signals that dissociate hepatic steatosis from insulin resistance, which positions ChREBP regulated genes as therapeutic targets in the treatment of obesity related diabetes (16; 37).

An effect of prolactin on liver gene expression is inferred by high *PRLR* mRNA levels found in hepatocytes (10; 13; 51), and, within the liver prolactin activates numerous signaling pathways (42) and growth-related or -unrelated genes (7; 62; 70).

Prlr mRNA has also been documented in adipocytes, deducing a regulatory role for prolactin also in adipose tissue (46; 76). It participates in adipogenesis and adipocyte differentiation (18; 27), inhibits adiponectin (2), lipid protein lipase expression (59) and activity (46), and has been described to stimulate (29; 73) or inhibit (8) leptin. Prolactin enhances the expression of master genes of adipogenesis (52), and lack or *Prlr* in mice impairs parametrial, abdominal and subcutaneous adipose tissues (19), and protects mice from high-fat diet-induced obesity (3). These metabolic actions are adaptive in pregnancy and lactation, physiological periods during which prolactin promotes fat deposition or mobilization, respectively, to ensure optimal nutrition for the offspring (27). In humans, pathological hyperprolactinemia as in patients bearing prolactinomas may induce weight gain (28; 58).

Nevertheless, opposite functions have also been proposed for prolactin in adipogenesis. For example, a small reduction in retroperitoneal fat mass was found in transgenic mice that over-express prolactin (45), abdominal adipose tissue is decreased during lactation (20), and prolactin has been found to suppress malonyl-coA expression suggesting that it inhibits lipogenesis (54). Therefore, the role of prolactin in adipocyte function is far from being settled.

Adipocyte SREBP-1c is involved in the regulation of genes associated to lipid metabolism, even though its role in this tissue has not been clearly ascertained (4). On the other hand, adipocyte ChREBP is involved in *de novo* lipogenesis, and is expressed at lower levels than in liver (37).

Finally, prolactin is also involved in pancreatic islet cell biology. It stimulates insulin expression and release, β-cell expansion (9; 27; 68), STAT5 tyrosine phosphorylation (42), glucose transporter 2 (GLUT-2) expression and therefore promotes glucose entry into the β-cells (60), consistent with the presence of *Prlr* mRNA in islet cells (57). The effects of prolactin on glucose homeostasis during pregnancy promote glucose transfer to the fetus, and these peripheral actions of prolactin on metabolic homeostasis are reinforced by its action at the central nervous system.

But even though prolactin is considered a metabolic hormone, its role on liver and adipose tissue gene expression, and particularly the expression of transcription factors involved in lipogenesis has not been described. In a previous work we showed that chronic high prolactin levels in 11 month-old female mice that selectively lack dopamine type 2 receptors (D2Rs) from pituitary lactotropes (lacDrd2KO) (55) are associated with increased body weight beginning at 5 months of age (59). In correlation, marked increments in fat depots, adipocyte size, serum triglycerides, and nonesterified fatty acid levels were found, a metabolic phenotype which intensified with age. Furthermore, 7 month-old female lacDrd2KO mice had glucose intolerance but a preserved glucose response to insulin (59). We have therefore used this experimental model to study the role of high prolactin titers on liver and adipocyte gene expression related to glucose and insulin homeostasis, and in correlation with the development of hyperprolactinemia and obesity. To that end, we used lacDrd2KO mice of two developmental ages, 5 and 10 months. In the first time point, obesity is not evident and body weight is marginally increased even though mice are hyperprolactinemic, while at 10 months there is a 136 % increase in gonadal fat, and a 36 % increase in liver weight due to lipid accumulation (59). Our present data highlight adaptive changes in chronic hyperprolactinemia that are associated to glucose intolerance, hyperinsulinemia, and impaired insulin response to glucose, which are already evident in early stages of obesity, and underscore the role of prolactin signaling in different tissues to promote energy storage.

Materials and Methods

Animals. Mice lacking expression of D2Rs in pituitary lactotropes were generated by crossing *Drd2*$^{loxP/loxP}$ mice (5) with transgenic mice expressing *Cre* recombinase driven by the mouse prolactin promoter (Tg(Prl-cre)^{1Mrub} (55)) for two generations. Tissue specificity of *Cre* expression in (Tg(Prl-cre)^{1Mrub} transgenic mice was analyzed by real time PCR and *Cre* mRNA levels were highly expressed in the pituitary and very low or almost absent in the hypothalamus, liver, kidney, ovary and lung (59). Functional Cre recombinase activity was evaluated in the pituitary and it was present in most prolactin producing cells of the anterior pituitary in a highly selective manner as described (55). LacDrd2KO and their *Drd2*$^{loxP/loxP}$ control littermates were congenic to C57BL/6J (n= 10).

Breeding pairs of female *Drd2*$^{loxP/loxP}$ and male *Drd2*$^{loxP/loxP}$.Tg(Prl-cre) mice were used to generate *Drd2*$^{loxP/loxP}$(control) and *Drd2*$^{loxP/loxP}$.Tg(Prl-cre) (lacDrd2KO) littermates, which were included in each experiment. Mice of mixed genotypes were housed in groups of 4 or 5 in a temperature-controlled room with lights on at 0700 h and off at 1900 h, and had free access to tap water and laboratory chow except when is indicated.

Because in male mice there was a marginal increase in prolactin levels, and no differences in body or pituitary weight, fat mass depots or food intake (59), we used female mice in our experiments.

Mice were euthanized by decapitation at defined ages. Sera were collected for cholesterol, triglycerides and insulin measurements. Liver, adipose tissue, pancreas and adrenals were processed for real time PCR or immunohistochemistry as detailed below.

All experimental procedures were carried according to guidelines of the institutional animal care and use committee of the Instituto de Biología y Medicina Experimental, Buenos Aires (in accordance with the Animal Welfare Assurance for the Instituto de Biología y Medicina Experimental , Office of Laboratory Animal Welfare, NIH, A#5072-01).

Fasting and refeeding. Female lacDrd2KO and *Drd2*$^{loxP/loxP}$ mice at 5 or 10 months of age were housed individually and fasted for 12 h, removing the laboratory chow at 1900 h. Body weight was registered before and after fasting. After 12 h of fasting, one group was refed for 1 h (Refed Group), while the other one was fasted for 1 more h (Fasted Group). Finally, both groups were euthanized and samples collected.

Glucose tolerance test (GTT). GTT was performed in conscious female lacDrd2KO and *Drd2*$^{loxP/loxP}$ mice at 5 and 10 months of age. Briefly, after overnight fasting (8 h), ip glucose solution (2 mg/g body weight) was administered. Blood glucose levels (2 µl of blood obtained from the tail of each mouse) were measured at 0, 15, 30, 60 and 120 min after glucose injection with a hand-held glucose monitor (Dex-II, Bayer).

Glucose-stimulated insulin secretion (GSIS). Eight-h-fasted 5 and 10 month-old female mice were used. Tail blood glucose levels were measured before (0 min), and 30 min after glucose ip administration (3 mg/g). Serum samples were immediately obtained by centrifugation at 3,000 rpm for 10 min and stored at -20 C. Insulin secretion levels were assessed by a sensitive mouse insulin ELISA kit as described below.

Insulin ELISA. Mouse serum insulin levels were assessed by a sensitive mouse insulin ELISA kit (Crystal Chem, Chicago, IL) following the manufacturer's instructions. Aliquots of 5 µl serum were used in duplicate. The lower limit of the assay sensitivity was 0.1 ng/ml.

Intraperitoneal insulin tolerance test (ITT). Five and 10 month-old mice were fasted for 2 h and then injected ip with human insulin (Humulin 1 U/kg body weight; Eli Lilly, Toronto, Canada). Tail-

blood glucose levels were measured 0, 15, 30, 60 and 120 min thereafter with a hand-held glucose monitor (Dex-II, Bayer).

Serum lipid profile. Triglycerides and total cholesterol were measured by Trinder colorimetric assay in 30 µl of diluted serum (1/2). The dilution was made with saline solution.

Prolactin RIA: Aliquots (10 ul) of serum obtained by decapitation of 5 or 10 month-old mice were used to assay prolactin by RIA using a kit provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK; Dr. A.F. Parlow, National Hormone and Pituitary Program (NHPP), Torrance, CA). Results are expressed in terms of mouse prolactin standard RP3. Intra- and inter-assay coefficients of variation were 7.2% and 12.8%.

Hepatocyte culture: Hepatocytes were isolated from adult male 5 month-old *Drd2*$^{loxP/loxP}$ mice as previously described (17). Briefly, the liver was washed with washing buffer (Hank´s balance salt solution) and digested with collagenase V (Sigma-Aldrich Co.) by *in situ* perfusion. Then, liver cells were collected, centrifuged at 200 g for 10 min, washed and plated in DMEM/F12 supplemented with 10% FBS (Invitrogen) at a density of 2,5x10^4 cells/cm^2. After 3 h, medium was replaced. Two days after plating, hepatocytes were incubated for 6, 12 and 24 h with DMEM as a control, or DMEM supplemented with 100, or 200 ng/ml prolactin (recombinant ovine prolactin from the National Hormone and Peptide Program (NHPP), Torrance, CA). The concentrations used were selected from (31). Cells were then collected in TRIzol reagent (Sigma-Aldrich Co) for total RNA extraction and real time PCR analysis as described below.

RNA extraction and cDNA synthesis. Gonadal adipose tissue and liver samples from *Drd2*$^{loxP/loxP}$ and lacDrd2KO were obtained and processed for recovery of total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA). The RNA concentration was determined on the basis of absorbance at 260 nm, its purity was evaluated by the ratio of absorbance at 260/280 nm (>1.8), and its integrity by agarose gel electrophoresis. RNAs were kept frozen at –80 C until analyzed. RNA (1 µg) was reversed transcribed in 20 µl volume in the presence of 10 mM $MgCl_2$, 50 mM Tris·HCl (pH 8.6), 75 mM KCl, 0.5 mM deoxy-NTPs, 4 mM DTT, 0.5 µg oligo(dT)$_{15}$ primer (Biodynamics, Buenos Aires, Argentina), and 20 U of MMLV reverse transcriptase (Epicentre, Madison, WI). The reverse transcriptase was omitted in control reactions; the absence of PCR-amplified DNA fragments in these samples indicated the isolation of RNA free of genomic DNA.

Real time PCR. Measurements were performed as previously described in (25). Sense and antisense oligonucleotide primers were designed on the basis of the published cDNA or by the use of PrimerBlast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Oligonucleotides were obtained from Invitrogen. The sequences are described in Table 1.

Briefly, the reactions were performed by kinetic PCR using TAQurate^{TM} GREEN Real-Time PCR MasterMix (9.4 µl containing 10 mM Tris·HCl, 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM deoxy-NTPs and 1.25 U *Taq* polymerase), 100 ng cDNA and 0.3 µM primers in a final volume of 10 µl. After denaturation at 95 C for 15 min, the cDNA products were amplified with 40 cycles. Cycle conditions (denaturation, annealing and extension) for each gene are detailed in Table 2, and optical reading stage was performed at 80 C for 33 s. The accumulating DNA products were monitored by the ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA), and data were stored continuously during the reaction. The results were validated on the basis of the quality of dissociation curves (25) generated at the end of the PCR runs by ramping the temperature of the samples from 60 to 95 C, while continuously collecting fluorescence data. Product purity was confirmed by agarose gel electrophoresis. Each sample was analyzed in duplicate. Relative gene expression levels were calculated according to the comparative cycle threshold (CT) method. Normalized target gene expression relative to cyclophilin was obtained by calculating the difference in CT values, the relative change in target transcripts being computed as $2^{-\Delta CT}$. To validate the comparative CT method of relative quantification, the efficiencies of each target and housekeeping gene amplification (endogenous cyclophilin) were measured and shown to be approximately equal.

Insulin RIA. Forty mg of pancreatic tissue were homogenized in 1 ml ice-cold acidic alcohol solution (12.5 % v/v HCl + 87.5 % v/v ethanol) and incubated for 1 h at 4 C. Samples were centrifuged at 800 g for 10 min at 4 C and 100 µl of the supernatant was neutralized with 0.855 M Tris, to later determinate insulin concentration by RIA. Protein content of samples was previously identified by Qubit Quant it protein assay kit (Invitrogen, Buenos Aires, Argentina) following manufacturer's instructions, and RIA was performed with 50 ng of total protein. A specific insulin RIA was used as described previously (26) using human insulin for iodination and standard (Beta Laboratories, Buenos Aires), and guinea pig anti-mouse insulin antibody (Sigma). The minimum detectable concentration was 0.002 ng/ml, and the intra- and interassay coefficients of variation were 6.8 and 9.1%, respectively.

Immunohistochemistry.

Pancreas from 5 month-old animals were fixed in formalin and embedded in paraffin. Immunohistochemistry was performed using avidin-biotin peroxidase method as previously

described (47). Each immunohistochemical assay included negative controls replacing the primary antibody with PBS. Antibodies for insulin (polyclonal guinea pig anti-insulin antibody, 1:200 dilution; Abcam, Cambridge, MA) and glucagon (rabbit anti-glucagon, 1:200 dilution; Santa Cruz Biotechnologies, Santa Cruz, CA) were used. Appropriate secondary antibodies were chosen. Samples were counterstained with hematoxylin and mounted with permanent mounting medium.

Morphometric analysis. Pancreatic digital images were captured using 5X or 40X magnification objective, a Zeiss Axiostar Plus microscope and a Canon PowerShot G6 digital camera. To determine total pancreas area, the necessary images covering all the tissue with 5X magnification were taken. Images of each islet were captured using 40X magnification. Morphometric analysis was performed with the aid of ImageJ software (NIH). The number of islets (defined as insulin-positive aggregates of at least 20 μm diameter) was scored and used to calculate the islet numerical density (number of islets per square centimeter of tissue). Islets less than 5000 μm^2 in size were defined as small, and those exceeding 5000 μm^2 as medium – large, and they were expressed as percentage of islets in each group. Mean islet size was calculated as the ratio of the total islet area to the total islet number on the sections. The relative areas occupied by islets and β-cells were also calculated as the ratio of islet or insulin-positive cell area to the total tissue area on the entire section, respectively. Counting the number of cells that showed insulin and glucagon staining in relation with total nuclei in islets (hematoxylin counterstaining) we determined the percentage of β- and α-cells, respectively. Data were calculated from three sections of each pancreas, representing the entire pancreas for each animal (head, body, and tail). Approximately 70–120 islets per section were analyzed. Six and 7 animals were studied per genotype (*Drd2*$^{loxP/loxP}$ and lacDrd2KO, respectively).

Hepatic glycogen content. Twenty to 25 mg of hepatic tissue was collected (the exact tissue weight was registered) from female mice of 5 and 10 month-old. Samples were incubated with 400 μl of 30% KOH in a boiling bath for 20 min. After cooling the samples, 50 μl of a saturated solution of sodium sulfate (5%) was added. Ethanol (50%) was used to precipitate glycogen. A 0.15% Antrona (Merck Millipore) solution was added, and after 20 min of incubation at 90 C the samples were measured at 620 nm of absorbance (Multiskan FC, Thermo Scientific ELISA lector). Gycogen standard (Sigma) was used to perform calibration curves.

Statistical analysis. Results are expressed as means ± SEM. The differences between means were analyzed by the unpaired Student's t-test (in the case of only two groups). Two-way ANOVA with repeated-measures design was used to analyze GTT, ITT, GSIS and the effect of prolactin on hepatocytes *in vitro*. Two-way ANOVA for independent measures was used to analyze gene mRNA

expression, glucose and insulin levels (for the effects of: experimental condition x genotype, experimental condition x age, or genotype x age). Post-hoc Tukey's test was employed when necessary. Percentages were analyzed by Chi-square test. $P<0.05$ was considered significant. Parametric or nonparametric comparisons were used as dictated by data distribution.

Results

Glucose intolerance in 5 and 10 month-old lacDrd2KO and *Drd2*$^{loxP/loxP}$ female mice

Prolactin levels and body weight were increased at 5 and 10 months of age in lacDrd2KO mice compared to age-matched *Drd2*$^{loxP/loxP}$ mice (Figure 1A), but body weight differences between genotypes were greater at 10 months. Basal glucose levels were similar between genotypes at 5 and 10 months in *ad libitum* fed or fasted females (Figure 1B). At 10 months of age in *ad libitum* condition both genotypes had higher glucose levels than at 5 months (Figure 1B). Glucose intolerance after 2 mg/g glucose ip injection was evident in 5 and 10 month-old lacDrd2KO mice (Figure 1C) compared to *Drd2*$^{loxP/loxP}$ mice, while no differences were found in glucose response to administration of 1 U/kg insulin (Figure 1D).

Increased serum and pancreatic insulin levels in 5 and 10 month-old lacDrd2KO females compared to *Drd2*$^{loxP/loxP}$ control mice

LacDrd2KO female mice at 5 and 10 months of age were hyperinsulinemic in *ad libitum* condition (Figure 2A); and in fasted lacDrd2KO mice there was a strong tendency to higher insulin levels (P= 0.057 lacDrd2KO vs. *Drd2*$^{loxP/loxP}$ mice, Figure 2B). This correlated with increased insulin content in pancreas from lacDrdKO compared to *Drd2*$^{loxP/loxP}$ mice at 5 and 10 months of age, both in *ad libitum* (P= 0.000043 lacDrd2KO vs. *Drd2*$^{loxP/loxP}$ mice, Figure 2C) and fasted conditions (P= 0.000040 lacDrd2KO vs. *Drd2*$^{loxP/loxP}$ mice, Figure 2D).

Pancreas sections were analyzed by immuhistochemistry in 5 month-old mice. There was an increase in the relative β-cell area in pancreatic tissue, as well as in the percentage of β-cell fraction within islets (Figure 3A, D and Table 3), and even though islet number or density was similar in both genotypes (Figure 3B) there was shift from small (< 5000 μm^2) to medium-large (>5,000 μm^2) islets in lacDrd2KO compared to *Drd2*$^{loxP/loxP}$ mice (Figure 3C and D). In concordance, percentage of islet area, mean β-cell size, and the ratio of insulin to glucagon immunoreactive areas was increased in lacDrd2KO mice (Table 3).

Impaired insulin response to glucose administration

In spite of the increased pancreatic insulin concentration, *in vivo* insulin response to glucose administration (3mg/g BW) was impaired at both ages in lacDrd2KO mice (P ≤ 0.02 lacDrd2KO vs. $Drd2^{loxP/loxP}$, Figure 3E). Altered insulin response was also inferred from experiments comparing genotypes after a fast-refed protocol: which consisted of a 12 h fast for all animals, followed by refeeding (Refed Group) or fasting (Fasted Group) for an extra hour. Serum insulin was marginally increased in response to refeeding in 5 and 10 month-old $Drd2^{loxP/loxP}$ mice (*P*= 0.069 for the effect of refeeding, Figure 3F left), and not in lacDrd2KO mice (*P*= 0.37; Figure 3F right).

Liver *Prlr,* growth hormone receptor *(Ghr),* and glucocorticoid receptor *(Glucor)* mRNA expression

We next sought to analyze the impact of high prolactin levels, and impaired insulin and glucose homeostasis on mRNA expression of liver hormone receptors. There was an increase in total (short and long isoforms) *Prlr* mRNA expression both in 5 and 10 month-old lacDrdKO mice compared to $Drd2^{loxP/loxP}$ controls (*P*= 0.029 in 5 month- and *P*= 0.031 in 10 month-old lacDrd2KO vs. age-matched $Drd2^{loxP/loxP}$ mice. Figure 4A), which in 10 month-old mice was mainly driven by the *Prlr-s*3 isoform. Analysis of delta CTs pointed to a greater abundance of the *Prlr-s3* isoform (lower deltaCT), and an almost undetectable level of the *Prlr-s2* isoform (dCT $\pm$ SE in $Drd2^{loxP/loxP}$ mice= 6.2 $\pm$ 0.5; 6.7 $\pm$ 0.5; 11.8 $\pm$ 0.2; 4.2 $\pm$ 0.3 for *Prlr-l, Prlr-s1, Prlr-s2* and *Prlr-s3,* respectively*).*

On the other hand, there were no differences between genotypes in liver *Ghr* or *Glucor* mRNA expression at either age (Figure 4B and C).

Liver *Chrebp*, *Srebp-1c*, glucokinase, and *Gys* mRNA expression levels, and glycogen content

ChREBP and SREBP-1c are two transcription factors involved in glycolytic and lipogenic gene expression. Liver *Chrebp* mRNA expression was higher in *ad libitum* fed 5 month-old lacDrd2KO compared to $Drd2^{loxP/loxP}$ mice (P≤ 0.03, Figure 5A), while no differences between genotypes were observed in 10 month-old mice, or in *Srebp-1c* expression at both ages (Figure 5A).

After a 12 h fast followed by one hour of refeeding, *Srebp-1c* mRNA expression was increased in $Drd2^{loxP/loxP}$ but not in lacDrd2KO mice (Figure 5C), at 5 months of age. This effect was not evidenced in 10 month-old mice (Figure 5C). On the other hand, liver *Chrebp* mRNA expression was not significantly modified by refeeding (Figure 5B).

After glucose entry into hepatocytes it is phosphorylated on carbon 6 by glucokinase generating G-6P, which may be converted into glycogen by the action of glycogen synthase (Gys) or metabolized to be used in the synthesis of fatty acids, cholesterol and bile salts. In *ad libitum* fed mice liver glucokinase mRNA expression was increased in 10 month-old lacDrd2KO compared to $Drd2^{loxP/loxP}$

mice (Figure 6A, *P*= 0.0015), whereas no differences between genotypes were observed in *Gys* mRNA expression levels, or liver glycogen concentration (Figure 6A). After a 12 h fast followed by one hour of refeeding, glucokinase mRNA expression was increased in both genotypes and at both ages (Figure 6B), the magnitude of increase being higher in younger mice. Liver glycogen concentration also increased in response to refeeding in both genotypes and at both ages (Figure 6C). These results indicate that liver *de novo* lipogenesis was altered, while the glycogen synthesis pathway was not.

Prolactin enhances *Chrebp* and *Prlr* mRNA expression in cultured hepatocytes

Hepatocytes from *Drd2*$^{loxP/loxP}$ 5 month-old mice were cultured *in vitro* and challenged with prolactin to test a direct effect of the hormone. *Chrebp* and *Prlr* (all isoforms) mRNA expression increased after after 6, 12 and 24 h of 200 ng/ml prolactin treatment (for *Chrebp P* interaction= 0.615; 200 ng/ml vs. basal *P*= 0.013; for prolactin *P* interaction= 0.397; 200 ng/ml vs. basal *P*= 0.0025). No effect was evidenced for *Srebp-1c* or *Fas* expression (Figure 7).

Serum cholesterol and triglyceride levels in *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice

Serum cholesterol levels were not different between genotypes at 5 and 10 months of age in *ad libitum* conditions, while serum triglyceride levels were prominently increased in 10 month-old *ad libitum* fed but not fasted mice (Table 4).

Normal adipose tissue *Prlr, Ghr,* and *Glucor* mRNA expression

No significant differences between genotypes were found in *Prlr, Ghr* or *Glucor* mRNA expression in gonadal white adipose tissue (Figure 8A-C). In this tissue, the long isoform of the *Prlr* (*Prlr-l*) was predominant (lower dCT), while the *Prlr-s1* and *-s2* isoforms were barely detectable (dCT $\pm$ SE in *Drd2*$^{loxP/loxP}$ mice= 4.7 $\pm$ 0.2; 13.8 $\pm$ 0.9; ND; and 9.4 $\pm$ 0.3 for *Prlr-l, Prlr-s1, Prlr-s2* and *Prlr-s3,* respectively*).*

Similar *Glucor* mRNA levels in liver and adipose tissue pointed to a conserved adrenal axis. In accordance, we found no differences between genotypes in adrenal weight and histolo-morphology in 5 month-old mice (data not shown).

Altered adipose tissue *Chrebp* and *Srebp-1c* mRNA expression levels

In 10 month-old *ad libitum*, fasted and refed mice *Chrebp* was lower in lacDrd2KO compared to condition-matched *Drd2*$^{loxP/loxP}$ mice (Figure 9A and B). *Srebp-1c* was also lower in the lacDrd2KO genotype in *ad libitum* 10 month-old fed mice (Figure 9A). Acutely refeeding mice after a fasting period induced *Chrebp* mRNA expression in both genotypes and at both ages (Figure 9B), while

Srebp-1c mRNA expression increased in adipose tissue from 5 month- but not 10 month-old fasted mice of both genotypes (Figure 9C).

Discussion

Prolactin is critical during two major physiological periods, pregnancy and lactation, favoring lipid storage and glucose availability, vital requisites to meet energy needs of mother and offspring. Prolactin signaling orchestrates several organs to this end, including the brain where it exerts an orexigenic action (59; 65). If prolactin is overproduced at an inappropriate time, metabolic disorders could be envisaged. For example, patients with hyperprolactinemia are prone to excessive weight gain, and normalization of prolactin levels with dopamine agonists correlates with weight loss (28; 58). There is a genetic association between prolactin and obesity, and genome wide association studies revealed a linkage of obesity to a common variant adjacent to the prolactin gene (49; 53) suggesting abnormalities in prolactin signaling may contribute to human obesity.

We previously conducted a cell-specific genetic dissection study using conditional mutant mice that selectively lack D2Rs from pituitary lactotropes (lacDrd2KO) to evaluate the role of elevated prolactin levels on metabolism, and demonstrated that 11 month-old lacDrd2KO female mice have increased body weight, fatty liver and adiposity accretion (59). High lipid content in the liver correlated with increased triglyceride content and liver weight, but no alterations in the lipolytic (adipose triglyceride lipase, hormone-sensitive lipase) or lipogenic (fatty acid synthase, lipoprotein lipase) enzymes were found, indicating that other mechanisms may be involved in increased lipid content in the liver. On the other hand, gross adiposity correlated with marked increments in fat depots, adipocyte size, serum triglycerides and nonesterified fatty acid levels. In adipose tissue decreased expression of lipolytic enzymes (adipose triglyceride lipase, and hormone-sensitive lipase) could explain increased lipid droplets, but there was no evidence of increased lipogenic enzymes. In the present study we aimed at establishing the role of hyperprolactinemia on the expression of transcription factors and enzymes involved in lipogenesis in the liver and adipose tissue during the development of obesity in the mutant model.

Prolactin receptors exist in various isoforms, short and long, depending on the length of the cytoplasmic domain (12; 51). They are widely distributed in tissues and hence if prolactin levels increase supra-physiologically there is a potential risk for a wide variety of systems to be

influenced. We found increased liver *Prlr* mRNA levels in lacDrd2KO, with predominance of the -s3 short isoform, furthermore, a direct effect of prolactin on liver gene expression could be inferred from *in vitro* experiments. To this regard, it has been demonstrated that prolactin may upregulate its cognate receptor in epididymal adipocytes (8), and breast cancer cells (40). On the other hand, in adipose tissue there was a predominance of the long isoform of the *Prlr* mRNA and no significant modification of *Prlr* mRNA levels was observed between genotypes. To this respect it has been documented that adipocyte responsiveness to prolactin is moderate, compared to the strong responsiveness to GH (18; 42).

Even though marked obesity was not evident in 5 month-old mice, glucose intolerance and hyperinsulinemia were already present at this age, as previously described in 7 month-old mice (59), and our present results indicate that both glucose intolerance and hypersinulinemia persisted life-long. Glucose intolerance at 5 months could not be explained by increased body weight, but may be related to the impaired response of insulin secretion to glucose overload found in lacDrd2KO mice. Furthermore, the increased insulin and decreased glucagon pancreatic content described, favor a role for prolactin at the pancreas, and is consistent with the fact that prolactin promotes islet growth and function (9; 23). These actions are of paramount importance in the adaptive metabolic response during pregnancy (35), and should be tightly regulated to prevent gestational diabetes.

Long term exposure of the liver to elevated glucose and insulin levels modifies transcription and translation of key enzymes involved in lipogenesis. SREBP-1c and ChREBP are transcription factors of the basic helix-loop-helix leucine zipper family (34; 78) involved in fatty acid, glucose and cholesterol metabolism (16), and which are differentially regulated by insulin and glucose (16; 39).

In the liver SREBP-1c and its lipogenic target genes are transcriptionally stimulated by insulin (21) and repressed by glucagon (22). ChREBP is abundantly expressed in liver, white and brown adipose tissues (38), active sites of *de novo* lipogenesis, and together with SREBP-1c is a crucial modulator of transcriptional control of lipogenic genes (16). Hepatic *Chrebp* expression in mainly induced by glucose and high carbohydrate diet. During fasting the actions of the both lipogenic transcription factors ChREBP and SREBP-1c are suppressed and refeeding produces hyperglycemia and insulin release which cause activation of ChREBP (38) and SREBP-1c, respectively, in order to initiate lipogenesis (72).

A relation of prolactin or PRLRs with liver or adipocyte *Srebp-1c* and *Chrebp* expression has not been explored. It has been reported that *Prlr* deficient mice were highly resistant to high fat diet-induced obesity with improved glucose homeostasis, insulin resistance and conservation of insulin secretion (3). These *Prlr* disrupted mice had lower *Srebp-1c* in pre-renal and not subcutaneous white adipose tissue (3) in standard fed conditions, suggesting an effect of prolactin on *Srebp-1c* expression in adipose tissue, while the effect on the liver was not studied.

Our results point to tissue and age-specific alteration in liver *Srebp-1c* and *Chrebp* expression in hyperprolactinemic mice. Refeeding mice after an overnight fast induced *Srebp-1c* mRNA expression in the liver of 5 month-old $Drd2^{loxP/loxP}$ mice, but there was marked loss of response in age-matched lacDrd2KO mice, and a desensitization of the effect in older mice. Refeeding induces *de novo* lipogenesis, associated to *Srebp-1c* expression (34), and *Srebp-1c* is regulated by insulin. As pointed above, the insulin response to refeeding, or to glucose administration, was impaired in lacDrd2KO mice. Therefore, failure of liver *Srebp-1c* gene induction in hyperprolactinemic mice may be associated to inappropriate insulin release by refeeding, or an early desensitization of the response, and identifies an alteration of *de novo* lipogenesis pathways in liver already at 5 months of age, when obesity is not fully settled. We could not detect a direct role of prolactin on hepatocyte *Srebp-1c* mRNA levels, also pointing to insulin as the cause of the inadequate response.

On the other hand, before morbid adiposity onset, (i.e. at 5 months) liver *Chrebp* mRNA expression was increased in lacDrd2KO mice in *ad libitum* condition compared to $Drd2^{loxP/loxP}$ mice. Increased liver *Chrebp* at this age may result from a direct effect of prolactin, as we demonstrate *in vitro*, but we cannot rule out the participation of high glucose or insulin levels found in our experimental model (39). Increased *Chrebp*, indicates *de novo* lipogenesis, in correlation with increased liver weight in this genotype already at this age (59). The lack of liver *Chrebp* increase in lacDrd2KO mice at 10 months of age may indicate an adaptative response aimed at limiting further expansion of fat storage, and suggests that lipogenesis is a dynamic process which varies according to the development of obesity. On the other hand, liver *Chrebp* mRNA levels were not significantly modified by refeeding in either genotype, consistent with data which suggest that *Chrebp* mRNA levels in liver and adipose tissue *in vivo* are barely responsive to changes in nutrient status (43). On the other hand, in contradiction to published data (43) our results point to a striking tissue specific response of *Chrebp* to refeeding, as in adipose tissue and not in liver a consistent upregulation was observed. Furthermore, our results suggest that acute *Chrebp* response to refeeding after prolonged fasting, is not comparable to an *ad libitum* condition, in which continuous high prolactin, insulin and glucose levels maintained high liver *Chrebp* expression at 5 months.

514

515 Even though higher levels of liver glucokinase were observed in *ad libitum* lacDrd2KO mice at 10
516 months of age, *Gys* mRNA levels and glycogen content were not significantly altered. The tendency
517 observed for lower glycogen in 10 month-old obese animals might be the result of increased lipid
518 content per mg tissue. Furthermore, similar glucokinase, and glycogen responses to refeeding were
519 obtained in both genotypes. One hour refeeding did not increase glucokinase levels to those in *ad*
520 *libitum* fed lacDrd2KO mice at 5 months of age.
521 Therefore results obtained in liver tissue suggest that the lipogenic signaling pathway was altered
522 during chronic hyperprolactinemia, while no marked evidence of alterations in the glycogenic
523 pathway was found.
524

525 On the other hand, the role of SREBP-1c and ChREBP on lipogenesis in adipose tissue has not been
526 conclusively settled (75). White adipose tissue was not significantly decreased in *Srebp-1c* disrupted
527 mice (67), and double *Srebpc-1c* and *ob/ob* knockout mice showed that *Srebp-1*c was not
528 determinant in obesity outcome, even though improved fatty livers were evidenced in the double
529 knockout (77). *Chrebp* is expressed in rat and human adipose tissue (44), and activated during
530 differentiation of pre-adipocytes to adipocytes (37), suggesting its participation in adipocyte
531 adipogenesis. Nevertheless, the physiological role of ChREBP and SREBP-1c in adipose tissue
532 warrants clarification.
533

534 In the present work *Srebp-1c* and *Chrebp* mRNA expression levels were markedly altered in
535 adipocytes from lacDrd2KO mice. In *ad libitum* condition, *Srebp-1c* mRNA expression was lower in
536 10 but not in 5 month-old lacDrd2KO mice. These results resemble *Srebp-1c* decrease in adipose
537 tissue observed in a model of induced obesity (77), and in adipocytes of patients with morbid
538 obesity (11), indicating that the decrease in adipose tissue *Srebp-1c* expression observed in
539 lacDrd2KO mice may be associated with the adiposity accretion at this age. Furthermore, it has
540 been suggested that increased leptin levels may downregulate *Srebp-1c* expression (56).
541 Nevertheless, adipocyte *Srebp-1c* expression was induced by feeding at 5 months of age in both
542 genotypes, denoting a preserved response to a feeding stimulus. Present results point to tissue
543 specific regulation of *Srebp-1c*, and suggest that its expression in adipocytes may be less dependent
544 on insulin levels, compared to hepatocytes. Furthermore, adipose tissue *Chrebp* expression was
545 decreased in obese 10 month-old mice not only in *ad libitum* condition, but also in fasted and refed
546 conditions. It has been demonstrated that genetically altering adipose tissue glucose flux regulates
547 the expression of ChREBP and its lipogenic targets (32). Nevertheless, *Chrebp* mRNA levels in
548 response to refeeding were similar between genotypes. These data indicate a preserved induction

549 of this transcription factor to refeeding, but a tissue and age specific alteration of *Chrebp*. A similar
550 decrease has been found in adipose tissue from obese subjects (36) and in high fat diet fed mice
551 (32).
552
553 Contrary to the initial expectations, in obese mice due to leptin deficiency adipocyte expression of
554 numerous genes involved in cell differentiation and adipogenesis, including *SREBP,* was actually
555 downregulated, suggesting a process of loss of adipocyte phenotype or desensitization of nutrient
556 sensors with advancing obesity (50). In early stages of adipocyte differentiation *in vitro* and *in vivo*
557 many of these genes are upregulated (69) indicating ongoing adipocyte hypertrophy and fat
558 accumulation, and at later stages expression markedly decreases, as in our present mouse model.
559 The finding that obesity leads to a downregulation of markers that characterize mature,
560 metabolically active, adipose cells, suggests that adipocytes from obese mice and humans have a
561 decreased lipogenic capacity, and underscore the complex process of lipid accumulation in which
562 gene expression profiles in adipocytes vary according to the different stages of development of
563 obesity (1).
564
565 Prolactin receptors have been found in the adrenal gland, and particularly prolactin activates STAT
566 phosphorylation in the adrenal cortex and not in the medulla (42), nevertheless we did not find
567 differences in adrenal weight, or *Glucor* in adipose tissue and liver in lacDrd2KO compared to
568 $Drd2^{loxP/loxP}$ mice which is indicative of absence of altered secretion of glucocorticoids.
569
570 In conclusion, we propose that chronic hyperprolactinemia upregulates liver *Prlr*, and evokes liver
571 steatosis, enhancement of the lipogenic transcription factor *Chrebp* and alteration in the *Srebp-1c*
572 response to refeeding; while in adipose tissue marked adiposity is associated to a decrease in both
573 transcription factors. These adaptive changes may be linked with glucose intolerance,
574 hyperinsulinemia, and impaired insulin response to glucose, which are already evident in early
575 stages of obesity, while a direct effect of prolactin on hepatocyte function cannot be ruled out, and
576 underscore the role of prolactin signaling in different tissues to promote energy storage. In
577 humans, hyperprolactinemia may be associated with hyperinsulinemia and insulin resistance (66;
578 79), and accumulation of toxic lipids is the most common etiology of insulin resistance in type 2
579 diabetes and associated metabolic disorders such as obesity and non-alcoholic fatty liver disease.
580 Therefore, understanding of the underlying mechanisms of metabolic manifestations during
581 untimely prolactin overproduction may reveal opportunities to target key regulators in lipid
582 metabolic pathways for the treatment of metabolic diseases.
583

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596 **Table 1**. Primers used for real-time PCR.

Gene	Strand	Primer Sequence (5' - 3')	Source
Adiponectin	Sense	ATCCTGGCCACAATGGCACA	Primer Blast
	Antisense	CAAGAAGACCTGCATCTCCT	
Atgl	Sense	AGGACAGCTCCACCAACATC	Primer Blast
	Antisense	TGGTTCAGTAGGCCATTCCT	
Chrebp	Sense	TCGATCCGACACTCACCCA	Primer Blast
	Antisense	CCAGGCTCTCCAGATGGCGT	
Cyclophilin	Sense	TTCTTCATAACCACAGTCAAGACC	Primer Blast
	Antisense	ACCTTCCGTACCACATCCAT	
Fas	Sense	AAGTTGCCCGAGTCAGAGAA	Primer Blast
	Antisense	CGTCGAACTTGGAGAGATCC	
Ghr	Sense	CCAACTCGCCTCTACACCG	Primer Blast
	Antisense	GGGAAAGGACTACACCACCTG	
Glucokinase	Sense	CCGTGATCCGGGAAGAGAA	Primer Blast
	Antisense	GGGAAACCTGACAGGGATGAG	
Glucor	Sense	CGGGACCACCTCCCAAA	Primer Blast
	Antisense	CCCCATAATGGCATCCCGAA	
Gys2	Sense	CCAGCTTGACAAGTTCGA	Primer Blast
	Antisense	ATCAGGCTTCCTCTTCAG	
Hsl	Sense	TCTGCTGGCCCCTGACA	Primer Blast
	Antisense	AGAGCGCAAGCCACAAGGT	
Lpl	Sense	CCCTACAAAGTGTTCCATTACCAA	Primer Blast
	Antisense	TTGTGTTGCTTGCCATCCTCA	
*Prlr**	Sense	CACAGTAAATGCCACGAACG	Primer Blast
	Antisense	GGCAACCATTTTACCCACAG	
Prlr-l	Sense	CTGGGCAGTGGCTTTGAAG	Primer Blast
	Antisense	CCAAGGCACTCAGCAGTTCT	
Prlr-s1	Sense	CCTGCATCTTTCCACCAGTTC	Primer Blast
	Antisense	GGGAAGTCAACTGGAGAATAGAACA	
Prlr-s2	Sense	CCTGCATCTTTCCACCAGTTC	Primer Blast
	Antisense	TTTTCAAGTTGCTCTTTGTTGTGAA	
Prlr-s3	Sense	CCTGCATCTTTCCACCAGTTC	Primer Blast
	Antisense	GATCCACCTTGTATTTGCTTGGAG	

Srebp-1c	Sense	CAGCGGCCCTGAGGGTCAAA	Primer Blast
	Antisense	TGCATGGCAAGAGGCACCGA	

597

598 ** Prolactin receptor (PRLR) primers will potentially amplified four different Prlr mRNAs (if expressed)*

599 *Prlr-l, Prlr-s1, Prlr-s2 and Prlr-s3.*

600

Table 2: PCR conditions for different genes.

Gene	Denaturation	Annealing	Extension
Adiponectin	95°C for 30 s	61°C for 1 min	72°C for 30 s
Atgl	95°C for 15 s	55°C for 20 s	72°C for 20 s
Chrebp	95°C for 20 s	60°C for 1 min	60°C for 1 min
Cyclophilin	95°C for 30 s	61°C for 1 min	72°C for 30 s
Fas	95°C for 15 s	55°C for 20 s	72°C for 20 s
Ghr	95°C for 20 s	60°C for 1 min	60°C for 1 min
Glucokinase	95°C for 20 s	60°C for 1 min	60°C for 1 min
Gr	95°C for 30 s	61°C for 1 min	72°C for 30 s
Gys2	95°C for 20 s	60°C for 1 min	60°C for 1 min
Hsl	95°C for 15 s	58°C for 20 s	72°C for 20 s
Lpl	95°C for 15 s	58°C for 20 s	72°C for 20 s
*Prlr**	95°C for 30 s	61°C for 1 min	72°C for 30 s
Prlr-l	95°C for 30 s	61°C for 1 min	72°C for 30 s
Prlr-s1	95°C for 30 s	61°C for 1 min	72°C for 30 s
Prlr-s2	95°C for 30 s	61°C for 1 min	72°C for 30 s
Prlr-s3	95°C for 30 s	61°C for 1 min	72°C for 30 s
Srebp-1c	95°C for 15 s	60°C for 1 min	60°C for 1 min

604 **Table 3**. Pancreas insulin and glucagon content assessed by immunohistochemistry, in *Drd2*$^{loxP/loxP}$

605 and lacDrd2KO female mice.

	Drd2$^{loxP/loxP}$	lacDrd2KO	*P*
Pancreas weight (g)	0.298± 0.020	0.385± 0.027	0.063
Pancreas weight/BW	0.014± 0.001	0.015± 0.001	0.55
Mean islet size (µm^2)	7316± 1340	11613± 1582	0.081
Islets area/total area (%)	0.602± 0.123	0.999± 0.104	0.048*
β-cell fraction (%)	67.21± 1.61	78.65± 2.89	0.0071*
Mean β-cell size (µm^2)	84.75± 3.16	94.88± 2.30	0.014*
Ratio insulin/glucagon area	2.095± 0.130	4.093± 0.517	0.0036*

606

607 *Drd2*$^{loxP/loxP}$ n= 7; lacDrd2KO n= 6 ; * *P*< 0.05 vs. *Drd2*$^{loxP/loxP}$ mice. Values are means ± SEM.

Table 4. Serum cholesterol and triglycerides levels in *Drd2*$^{loxP/loxP}$ and lacDrd2KO female mice.

	5 months			*10 months*		
	Drd2$^{loxP/loxP}$	lacDrd2KO	*P*	*Drd2*$^{loxP/loxP}$	lacDrd2KO	*P*
Serum cholesterol (*ad libitum*)	59.0 ± 15.7	61.3 ± 18.9	0.93	62.0 ± 10.9	71.3 ± 10.0	0.58
Serum triglycerides (fasted)	104.0 ± 17.6	88.0 ± 16.9	0.57	99.0 ± 16.3	80.0 ± 20.1	0.53
Serum triglycerides (*ad libitum*)	125.0 ± 13.0	95.7 ± 8.8	0.11	141.3 ± 13.0	248.0 ±40.8	0.046*

n= between 5 and 7 for each group.

* *P*< 0.05 vs. condition-matched *Drd2*$^{loxP/loxP}$ mice. Values are means ± SEM.

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Figure Captions

Figure 1:

High serum prolactin, body weight and glucose intolerance in lacDrd2KO female mice. A) Left: Serum prolactin levels (ng/ml) in *ad libitum* 5 and 10 month-old female mice; n= 10 for each group, * $P< 0.0001$ vs. age-matched mice. Right: Body weight in *ad libitum* 5 and 10 month-old female mice; n= 10 for each group, * $P< 0.001$ vs. $Drd2^{loxP/loxP}$ age-matched mice, and # $P< 0.01$ vs. 5 months genotype-matched mice; **B)** glucose levels (mg/dl) in *ad libitum* condition (left) and after an 8 h fast (Fasted, right panel) in 5 and 10 month-old female $Drd2^{loxP/loxP}$ and lacDrd2KO mice, n *ad libitum*= 5 and 7 (5 months) and 5 and 5 (10 months); n fasted= 6 and 6 (5 months), 7 and 8 (10 months) for $Drd2^{loxP/loxP}$ and lacDrd2KO mice, respectively: # $P< 0.05$ vs. 5 months genotype-matched mice. **C**) Intraperitoneal glucose tolerance test (GTT, 2 mg/g) in fasted $Drd2^{loxP/loxP}$ and lacDrd2KO female mice (n= 9 and 9 (5 months), 4 and 6 (10 months) $Drd2^{loxP/loxP}$ and lacDrd2KO mice, respectively). Two way ANOVA with repeated-measures design; * $P< 0.05$ vs. time-matched $Drd2^{loxP/loxP}$ mice. **D)** Insulin tolerance test (ITT) in 5 month- and 10 month- old $Drd2^{loxP/loxP}$ and lacDrd2KO females: mice were injected with 1 U/kg BW human insulin, and blood glucose was measured at different times; no significant differences were found; n= 13 and 12, and 6 and 7 for 5 and 10 months, $Drd2^{loxP/loxP}$ and lacDrd2KO female mice respectively.

Figure 2:

Hyperinsulinemia in lacDrd2KO female mice. A) Insulin levels (ng/ml) in *ad libitum* condition, and **B)** after an 8 h fast in 5 and 10 month-old female $Drd2^{loxP/loxP}$ and lacDrd2KO mice, n *ad libitum*= 4 and 4 (5 months) and 4 and 7 (10 months); fasted n= 4 and 5 (5 months), 4 and 4 (10 months) for $Drd2^{loxP/loxP}$ and lacDrd2KO mice, respectively; * $P< 0.05$ vs. age-matched $Drd2^{loxP/loxP}$ mice: & $P= 0.057$ vs. age-matched $Drd2^{loxP/loxP}$ mice. **C)** Pancreatic insulin concentration (µg/ng protein) in *ad libitum* condition, and **D)** after an 8 h fast in 5 and 10 month-old female $Drd2^{loxP/loxP}$ and lacDrd2KO mice, n *ad libitum*= 5 and 5 (5 months) and 12 and 14 (10 months), and n fasted= 6 and 6 (5 months), 5 and 5 (10 months) for $Drd2^{loxP/loxP}$ and lacDrd2KO mice, respectively; * $P< 0.0005$ vs. age-matched $Drd2^{loxP/loxP}$ mice.

Figure 3:

Increased β-cell area and medium-large islets in pancreas from lacDrd2KO females, but impaired insulin response to glucose. A) β-cell area (relative insulin positive area/total tissue area); **B**) islet density (defined as insulin-positive aggregates of at least 20 μm diameter per square centimeter of tissue), and **C**) percentage of small and medium-large islets (islets less than 5000 μm^2 in size were defined as small, and those exceeding 5000 μm^2 as medium–large). N= 7 and 6, *Drd2*$^{loxP/loxP}$ and lacDrd2KO 5 month-old mice; * $P< 0.05$ vs. age-matched *Drd2*$^{loxP/loxP}$ mice**; D**) representative consecutive images of insulin (left) and glucagon (right) staining in pancreatic tissue from lacDrd2KO and *Drd2*$^{loxP/loxP}$ mice; specific immunolabeling is detected in brown corresponding to the DAB dye, and cell nuclei are visualized in blue (hematoxylin counterstaining). **E)** Insulin response to 3 mg/g ip glucose stimulation expressed as Δ insulin= insulin levels after stimulation - insulin basal levels in ng/ml in 5 and 10 month-old *Drd2*$^{loxP/loxP}$ and lacDrd2 female mice; n= 6 and 6 (5 months) and 4 and 6 (10 months), for *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice, respectively, * $P< 0.02$ vs. age-matched *Drd2*$^{loxP/loxP}$ mice; **F)** serum insulin levels (ng/ml) in 12 h fasted or 12 h fasted followed by one hour of refeeding (refed) *Drd2*$^{loxP/loxP}$ (left panel) at 5 or 10 months of age; n= 4 and 4 (5 month-old *Drd2*$^{loxP/loxP}$ fasted and refed); 4 and 5 (10 month-old *Drd2*$^{loxP/loxP}$ fasted and refed); $P= 0.069$ fasted vs. refed in *Drd2*$^{loxP/loxP}$ mice; right: Serum insulin levels (ng/ml) in 12 h fasted or 12 h fasted followed by one hour of refeeding (refed) lacDrd2KO mice at 5 or 10 months of age: n= 4 and 4 (5 month-old lacDrd2KO fasted and refed); 7 and 6 (10 month-old lacDrd2KO fasted and refed).

Figure 4:

Increased *Prlr* mRNA levels in livers from lacDrd2KO females. A) *Prlr* mRNA levels in 5 and 10 month-old female *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice; *Prlr*: all isoforms; *Prlr-l*: long subtype; *Prlr-s1-3*: short isoforms 1-3; n= 5 and 4 (5 months), and 6 and 7 (10 months) *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice, respectively; * $P< 0.05$ vs. age-matched *Drd2*$^{loxP/loxP}$ mice. **B)** Liver *Ghr* mRNA levels; n= 5 and 4 (5 months), and 7 and 7 (10 months) *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice,

respectively; and **C)** liver *Glucor* mRNA levels; n= 5 and 4 (5 months), and 7 and 8 (10 months) *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice, respectively.

Figure 5:

Altered liver *Chrebp and Srebp-1c* mRNA levels in lacDrd2KO females in *ad libitum*, fasted and refed conditions. A) *Chrebp* and *Srebp-1c* mRNA levels in *ad libitum* condition in 5 and 10 month-old female *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice; n= 5 and 4 (5 months), and 6 and 7 (10 months) for *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice, respectively; * $P< 0.05$ vs. age-matched *Drd2*$^{loxP/loxP}$ mice; for this and subsequent panels percentage of target mRNA levels normalized *Cyclophilin* mRNA levels in relation to age matched *Drd2*$^{loxP/loxP}$ mice (100%) is represented in the Y-axis. **B)** *Chrebp* mRNA levels in 12 h fasted or 12 h fasted followed by one hour refeeding (refed) *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice at 5 or 10 months of age; n= 8 and 5 (5 month-old *Drd2*$^{loxP/loxP}$ fasted and refed); 8 and 8 (5 month-old lacDrd2KO fasted and refed); 5 and 5 (10 month-old *Drd2*$^{loxP/loxP}$ fasted and refed); 4 and 5 (10 month-old lacDrd2KO fasted and refed); **C)** *Srebp-1c* mRNA levels in 12 h fasted or 12 h fasted followed by one hour of refeeding (refed) *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice at 5 or 10 months of age (n= 7 and 6 (5 month-old *Drd2*$^{loxP/loxP}$ fasted and refed); 8 and 8 (5 month-old lacDrd2KO fasted and refed); 5 and 5 (10 month-old *Drd2*$^{loxP/loxP}$ fasted and refed) ; 4 and 5 (10 month-old lacDrd2KO fasted and refed); * $P< 0.05$ vs. fasted *Drd2*$^{loxP/loxP}$ mice, # $P< 0.05$ refed lacDrd2KO mice.

Figure 6:

Liver glucokinase and *Gys* mRNA levels, and glycogen concentration in *ad libitum*, fasted and refed conditions. A) Glucokinase and *Gys* mRNA levels, and glycogen content (µg/mg) in *ad libitum* condition in 5 and 10 month-old female *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice; n= 5 and 4 (5 months), and 6 and 7 (10 months) for *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice, respectively; and 5 and 5 (5 months), 9 and 7 (10 months) for glycogen concentration for *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice, respectively ; * $P< 0.01$ vs. age-matched *Drd2*$^{loxP/loxP}$ mice; **B)** glucokinase mRNA levels in 12 h fasted or 12 h fasted followed by one hour refeeding (refed) *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice at 5 or

10 months of age; n= 7 and 6 (5 month-old *Drd2*$^{loxP/loxP}$ fasted and refed); 8 and 8 (5 month-old lacDrd2KO fasted and refed); 5 and 4 (10 month-old *Drd2*$^{loxP/loxP}$ fasted and refed); 5 and 5 (10 month-old lacDrd2KO fasted and refed); for A and B panels percentage of target mRNA levels normalized *Cyclophilin* mRNA levels in relation to age matched fasted *Drd2*$^{loxP/loxP}$ mice (100%) is represented in the Y-axis. **C)** Glycogen content in 12 h fasted or 12 h fasted followed by one hour refeeding (refed) *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice at 5 or 10 months of age; n= 6 and 4 (5 month-old *Drd2*$^{loxP/loxP}$ fasted and refed); 5 and 4 (5 month-old lacDrd2KO fasted and refed); 5 and 4 (10 month-old *Drd2*$^{loxP/loxP}$ fasted and refed); 5 and 5 (10 month-old lacDrd2KO fasted and refed); * $P<$ 0.05 vs. fasted *Drd2*$^{loxP/loxP}$ mice, # $P<$ 0.05 refed lacDrd2KO mice.

Figure 7:

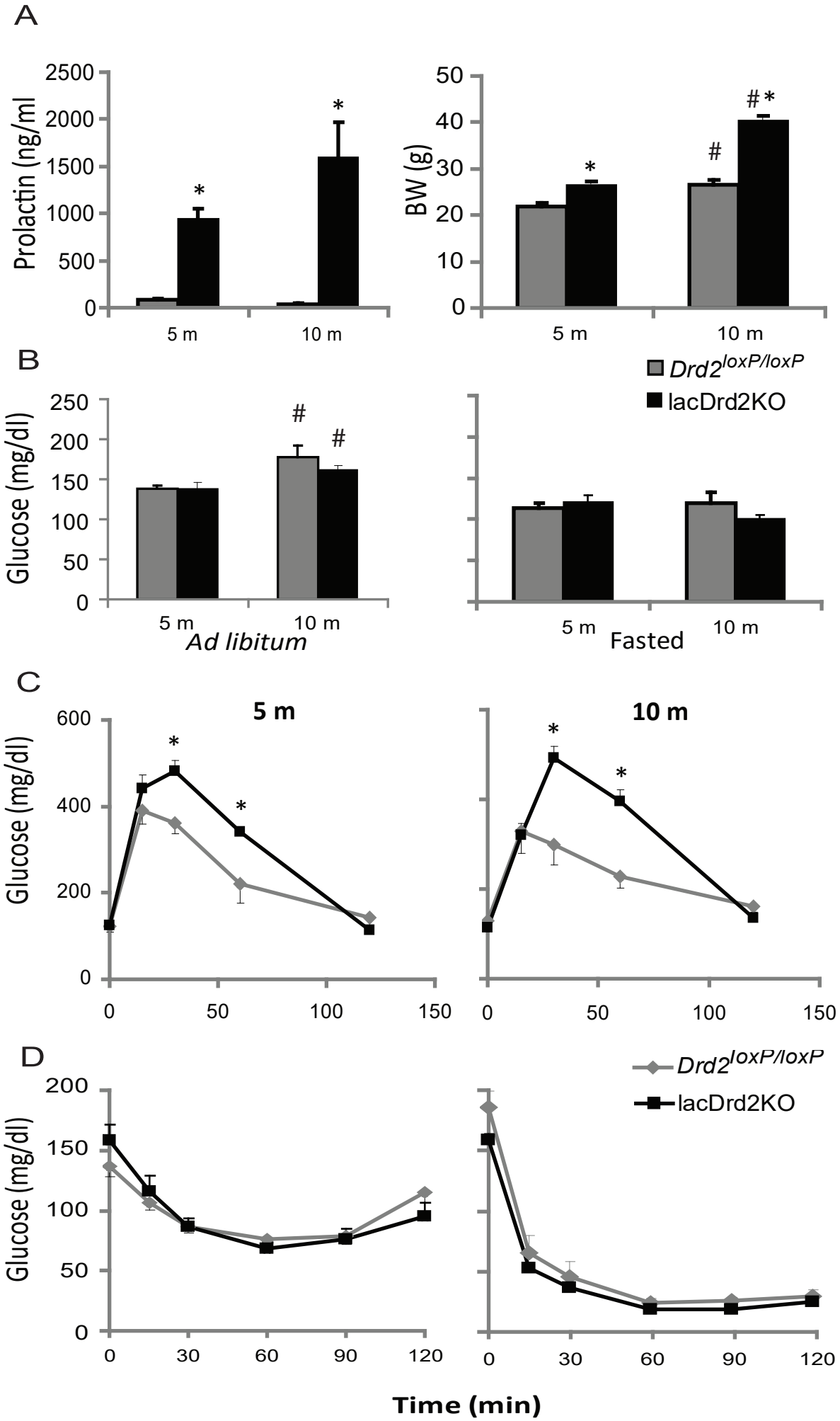
Prolactin enhances *Chrebp* and *Prlr* mRNA expression in cultured hepatocytes. Effect of prolactin (0, 100 or 200 ng/ml) on *Chrebp, Srebp-1c, Prlr* and *Fas* mRNA levels after 6, 12 or 24 h of incubation. Results are expressed as the percentage expression of target genes normalized to *Cyclophilin* mRNA levels to expression in cells incubated without prolactin (basal) at the same time points, which was considered 100%. * $P<$ 0.05 vs. basal incubated cells; n= 3 cell cultures, replicate measures in each culture.

Figure 8:

Adipose tissue *Prlr, Ghr and Glucor* mRNA levels are not modified . A) *Prlr* mRNA levels in 5 and 10 month-old *Drd2*$^{loxP/loxP}$ and lacDrd2KO female mice; *Prlr*: all isoforms; *Prlr-l*: long subtype; *Prlr-s1-3*: short isoforms 1-3; n= 5 and 5 (5 months), and 11 and 11 (10 months) *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice, respectively; ; for this and subsequent panels percentage of target mRNA levels normalized *Cyclophilin* mRNA levels in relation to age matched *Drd2*$^{loxP/loxP}$ mice (100%) is represented in the Y-axis. **B**) Adipose tissue *Ghr* mRNA levels; n=5 and 4, (5 months), and 11 and 11 (10 months) *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice, respectively; and **C)** adipose tissue *Glucor* mRNA levels; n= 5 and 4, (5 months), and 11 and 11 (10 months) *Drd2*$^{loxP/loxP}$ and lacDrd2KO mice, respectively.

Figure 9:

Adipose tissue *Chrebp* and *Srebp-1c* mRNA levels in *ad libitum*, fasted and refed conditions. A) *Chrebp* and *Srebp-1c* mRNA levels in *ad libitum* condition in 5 and 10 month-old female $Drd2^{loxP/loxP}$ and lacDrd2KO mice; n= 5 and 4 (5 months), and 10 and 9 (10 months) for $Drd2^{loxP/loxP}$ and lacDrd2KO mice, respectively; * $P< 0.05$ vs. age-matched $Drd2^{loxP/loxP}$ mice; ; for this and subsequent panels percentage of target mRNA levels normalized *Cyclophilin* mRNA levels in relation to age matched fasted $Drd2^{loxP/loxP}$ mice (100%) is represented in the Y-axis. **B)** *Chrebp* mRNA levels in 12 h fasted or 12 h fasted followed by one hour refeeding (refed) $Drd2^{loxP/loxP}$ and lacDrd2KO mice at 5 or 10 months of age; n= 8 and 6 (5 month-old $Drd2^{loxP/loxP}$ fasted and refed); 8 and 8 (5 month-old lacDrd2KO fasted and refed); 5 and 4 (10 month-old $Drd2^{loxP/loxP}$ fasted and refed); 4 and 4 (10 month-old lacDrd2KO fasted and refed); * $P< 0.05$ vs. genotype and age-matched fasted mice, # $P< 0.05$ condition-matched mice (lacDrd2KO vs.$Drd2^{loxP/loxP}$); **C)** *Srebp-1c* mRNA levels in 12 h fasted or 12 h fasted followed by one hour of refeeding (refed) $Drd2^{loxP/loxP}$ and lacDrd2KO mice at 5 or 10 months of age; n= 6 and 4 (5 month-old $Drd2^{loxP/loxP}$ fasted and refed); 6 and 6 (5 month-old lacDrd2KO fasted and refed); 5 and 4 (10 month-old $Drd2^{loxP/loxP}$ fasted and refed); 4 and 4 (10 month-old lacDrd2KO fasted and refed); * $P< 0.05$ vs. fasted $Drd2^{loxP/loxP}$ mice.



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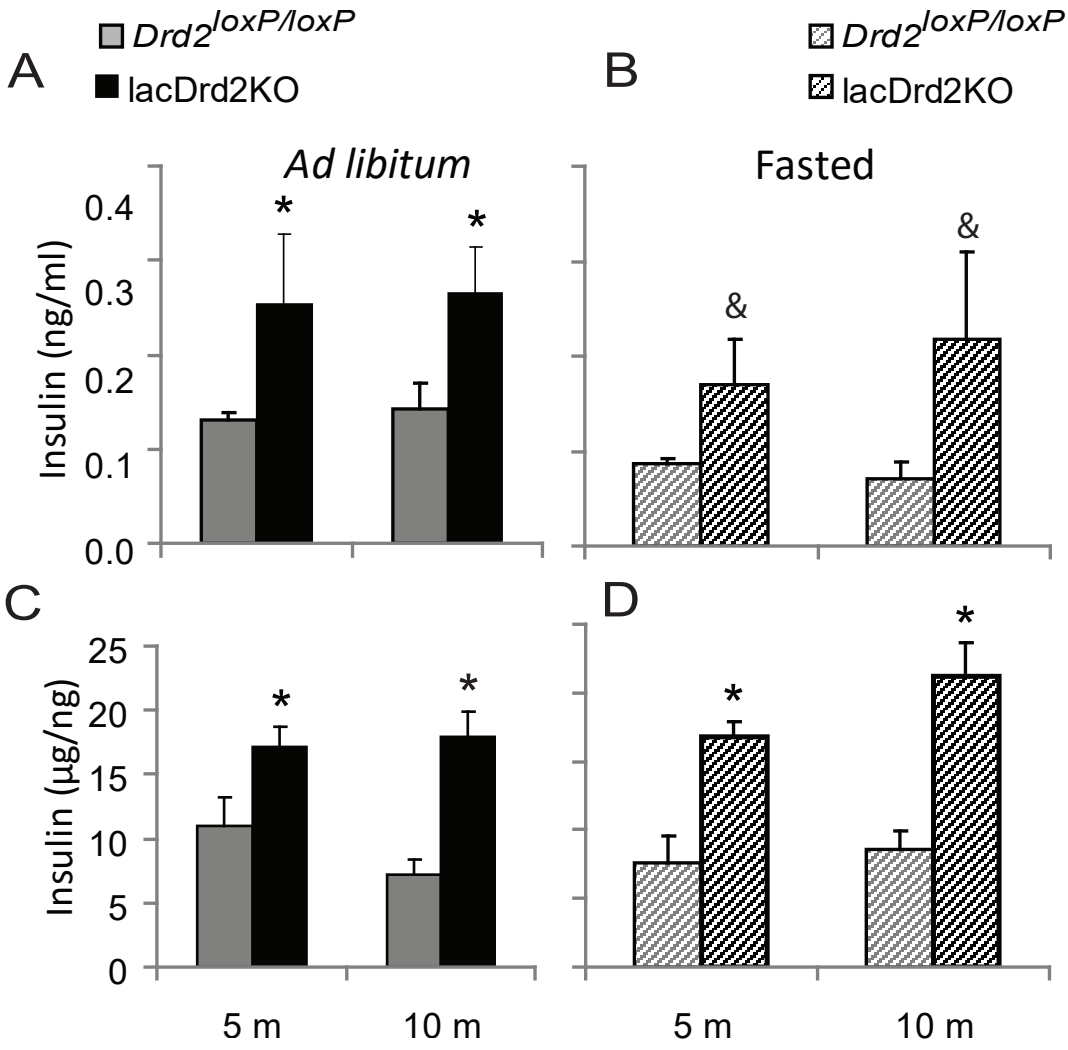

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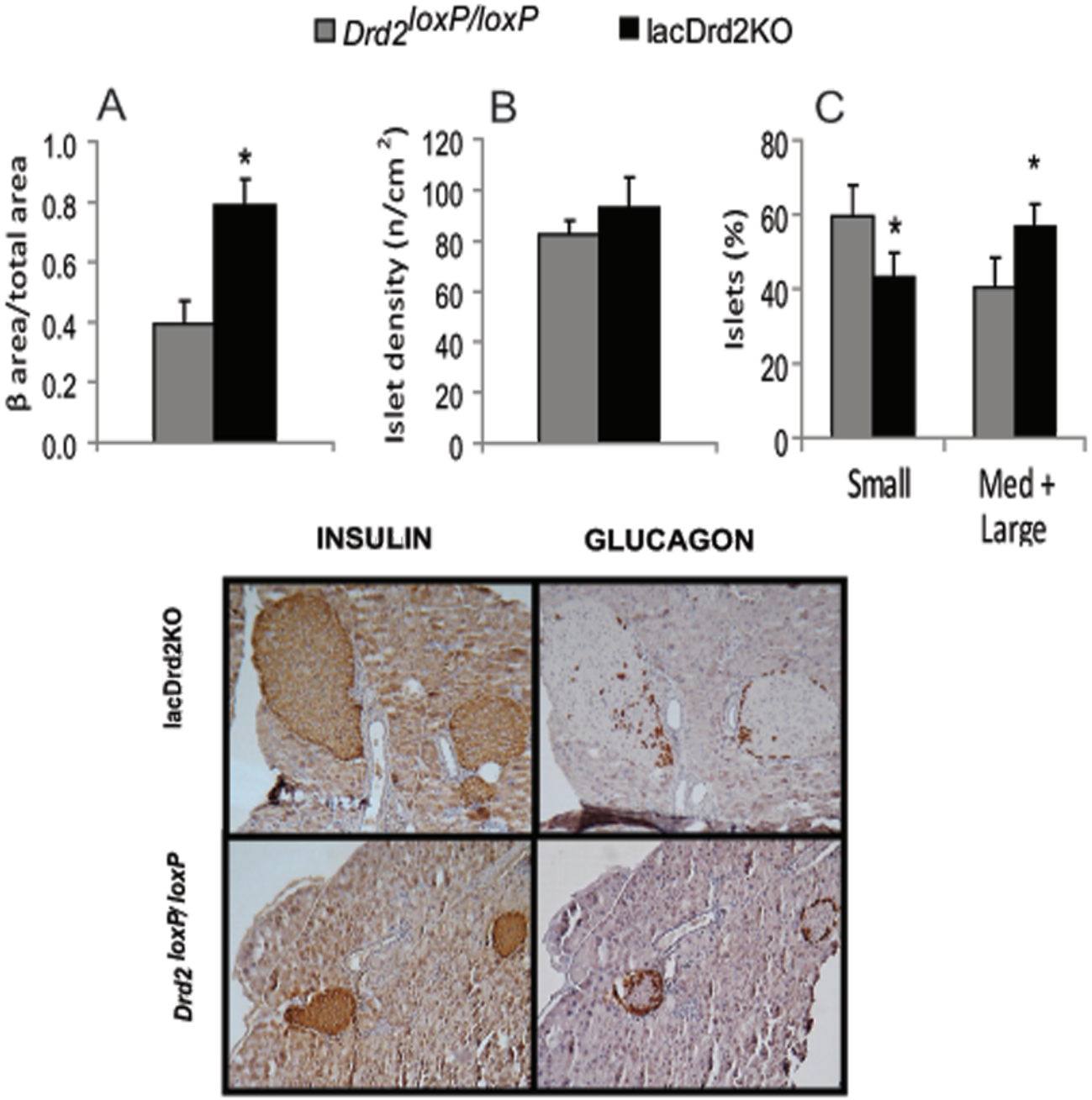

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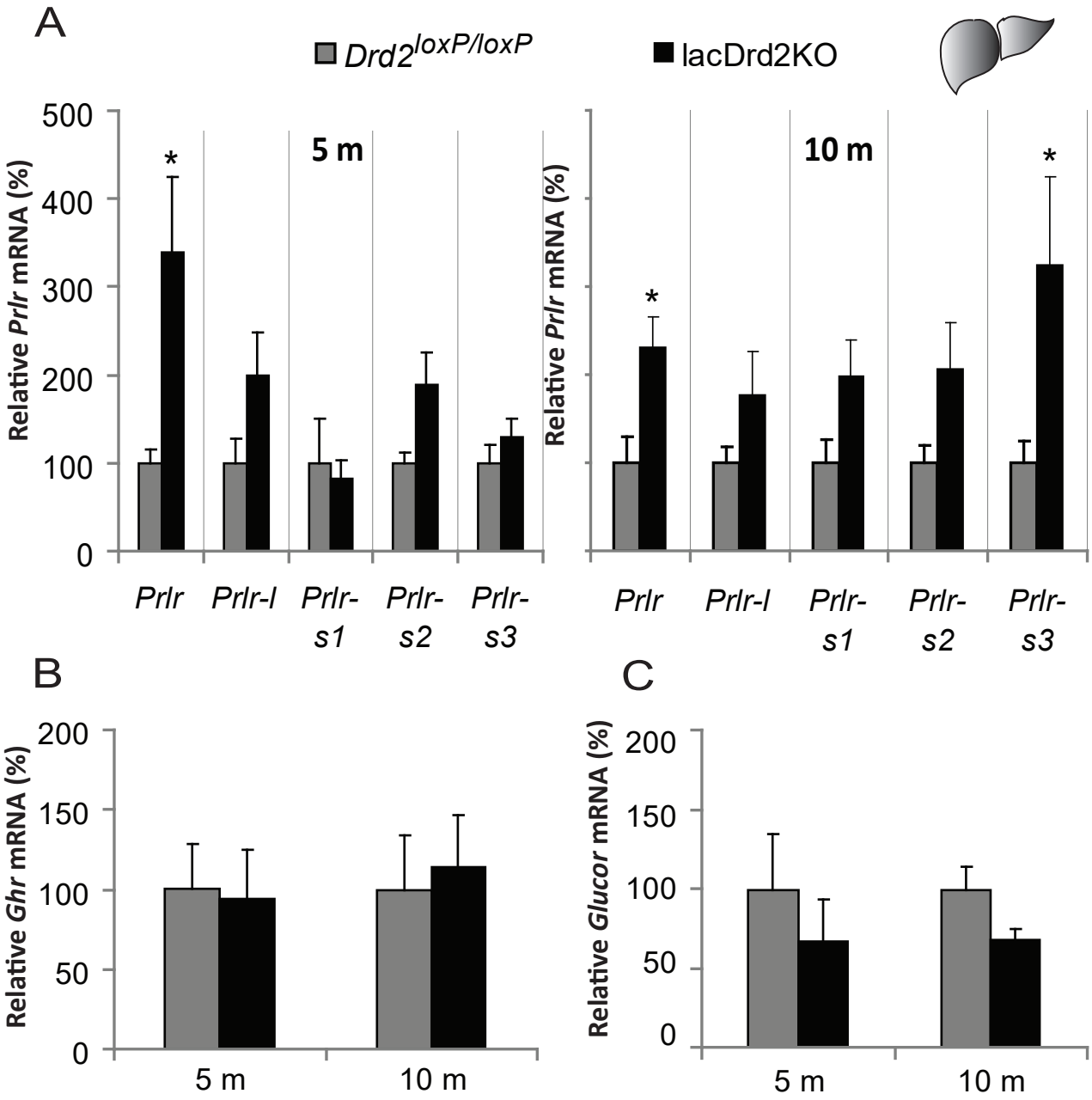

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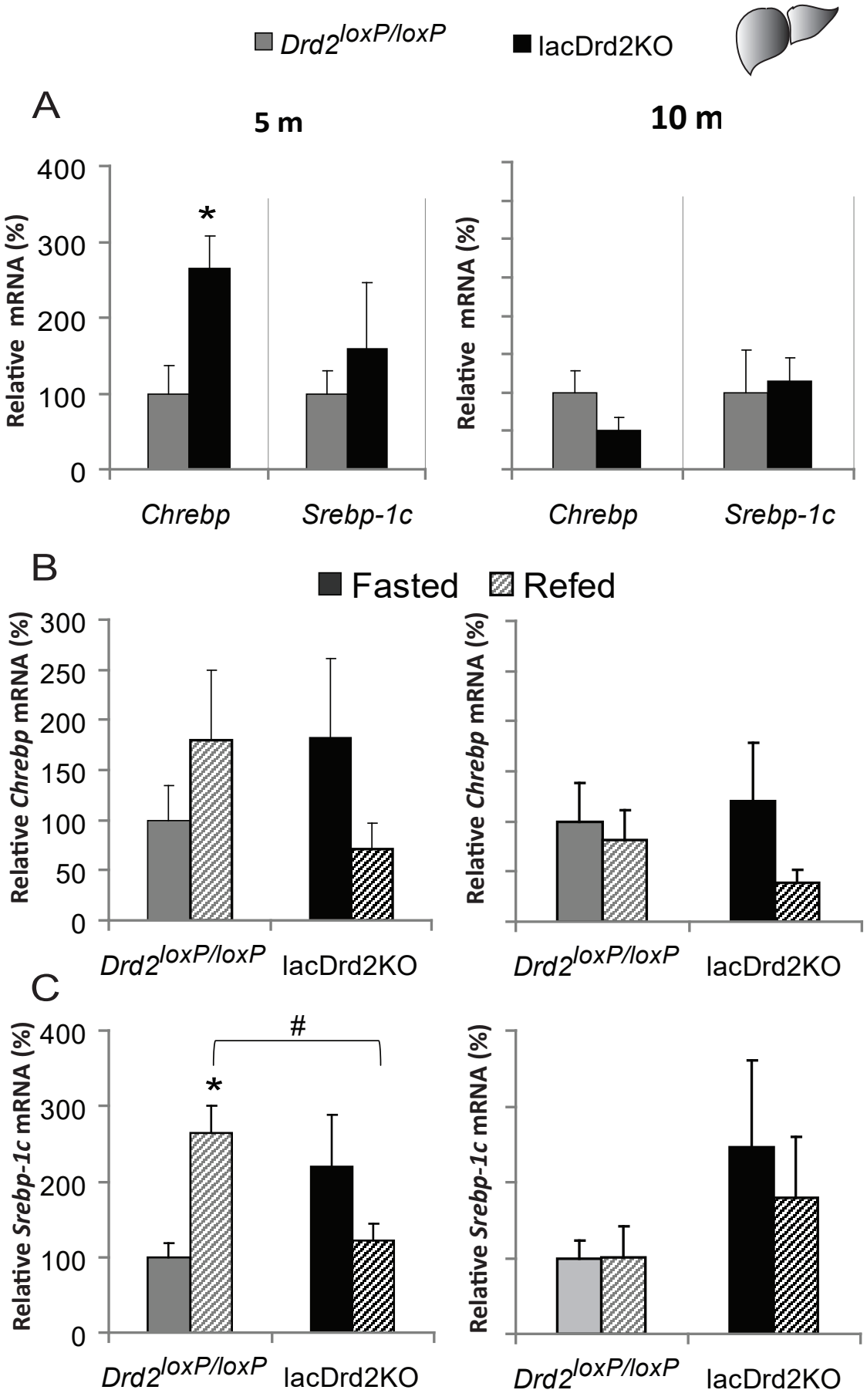
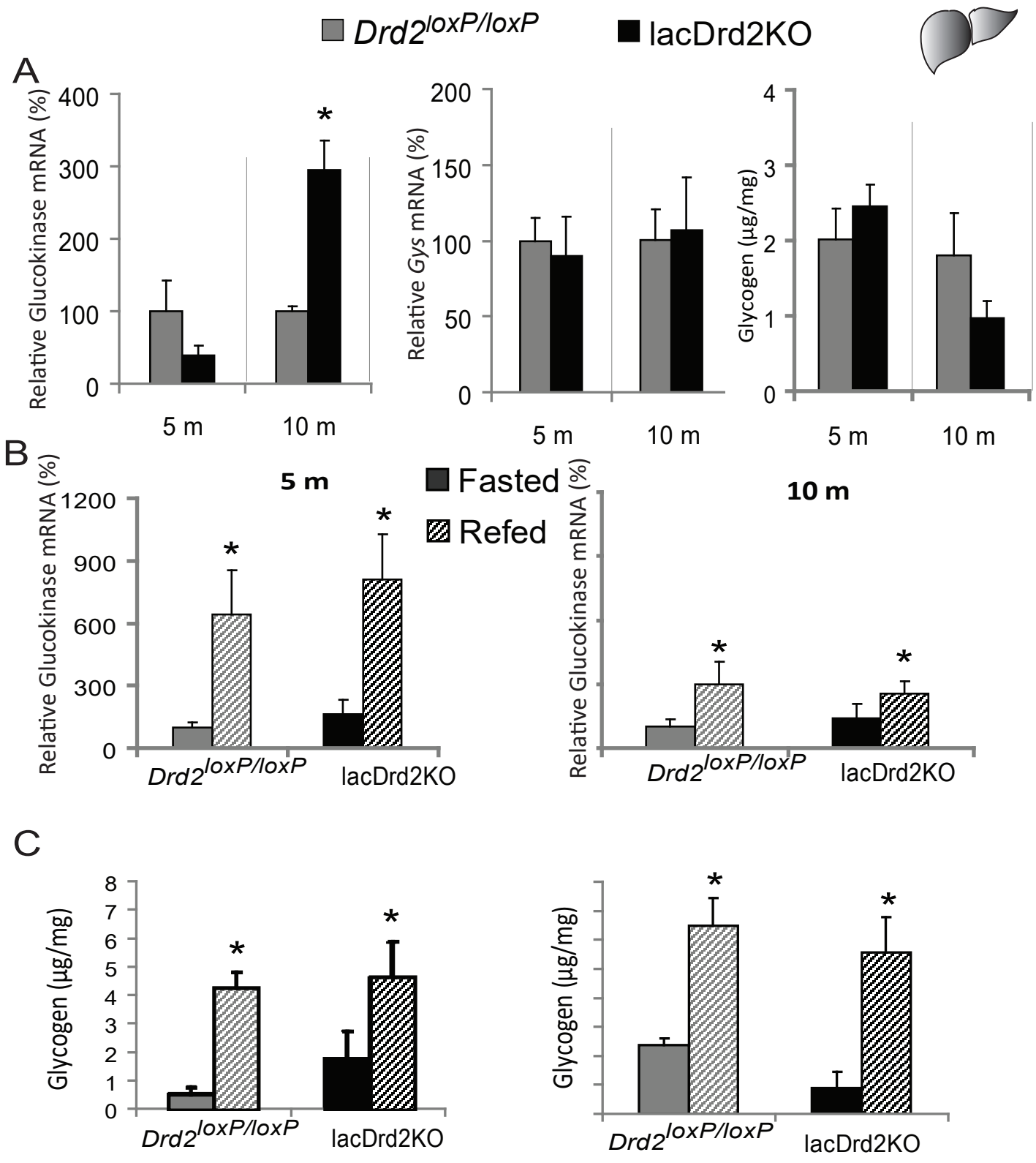


Luque Figure 5



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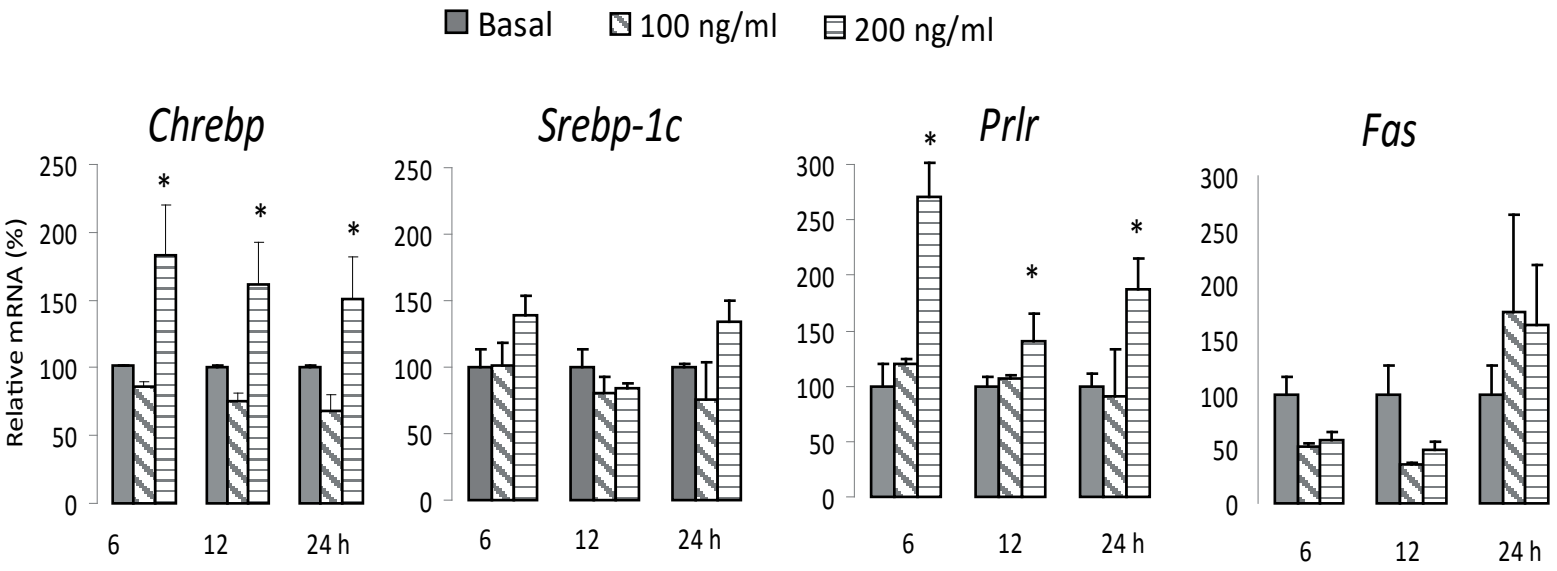

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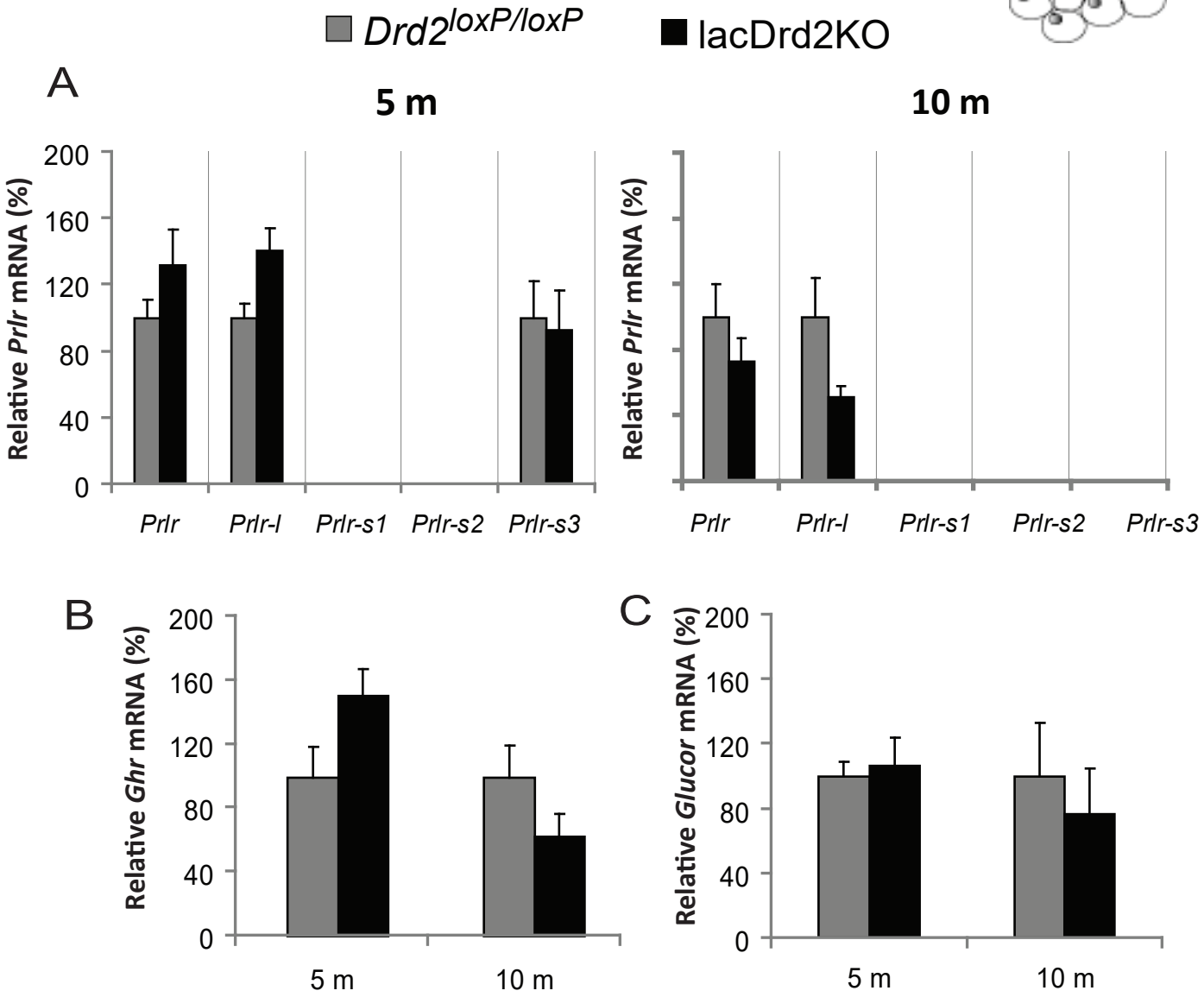
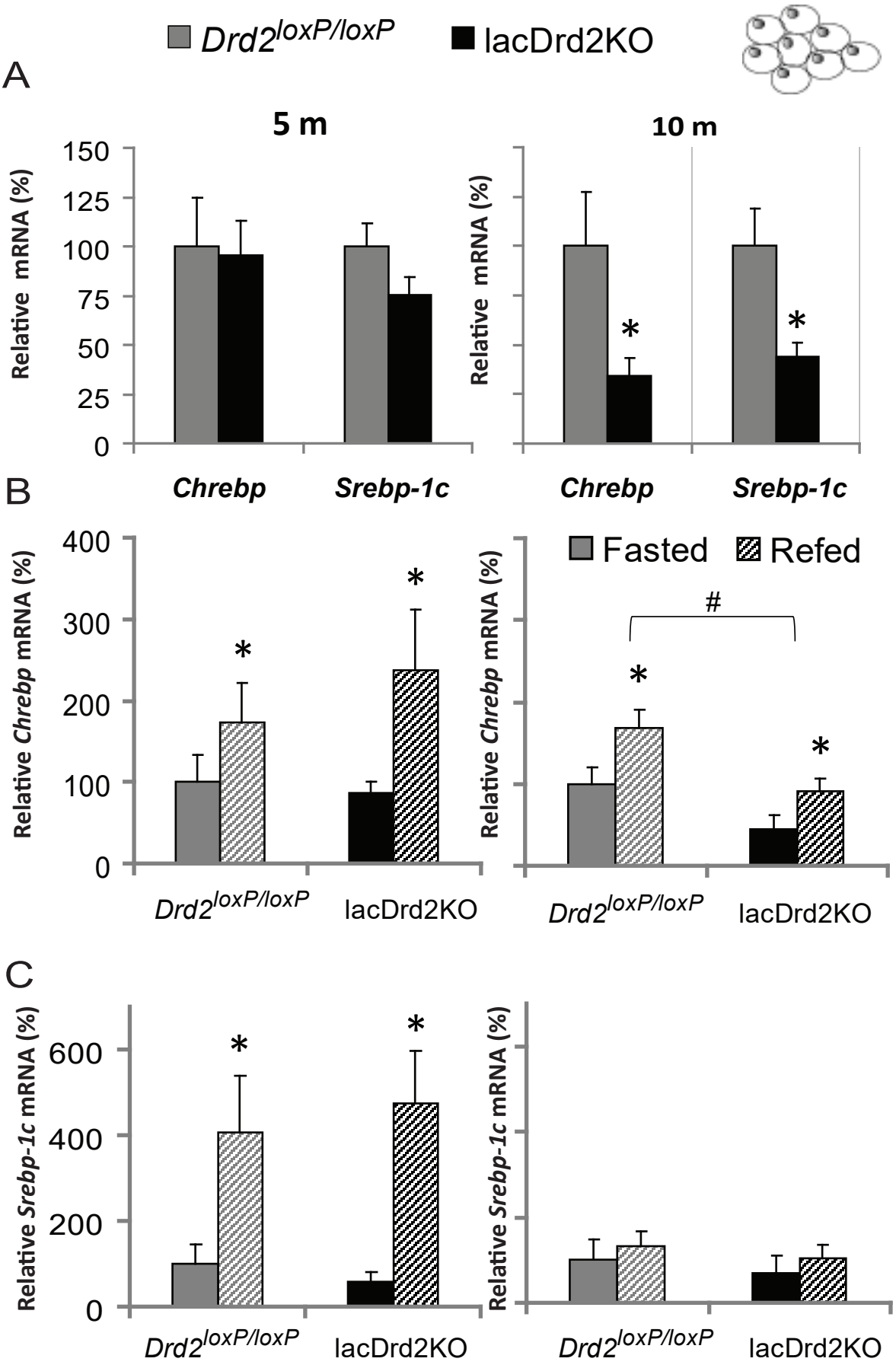


Luque Figure 8



Luque Figure 9