Hepatic hexokinase domain containing 1 (HKDC1) improves whole body glucose tolerance and insulin sensitivity in pregnant mice

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Abstract

Hexokinase domain containing 1, a recently discovered putative fifth hexokinase, is hypothesized to play key roles in glucose metabolism. Specifically, during pregnancy in a recent genome wide association study (GWAS), a strong correlation between HKDC1 and 2-hr plasma glucose in pregnant women from different ethnic backgrounds was shown. Our earlier work also reported diminished glucose tolerance during pregnancy in our whole body HKDC1 heterozygous mice. Therefore, we hypothesized that HKDC1 plays important roles in gestational metabolism, and designed this study to assess the role of hepatic HKDC1 in whole body glucose utilization and insulin action during pregnancy. We overexpressed human HKDC1 in mouse liver by injecting a human HKDC1 adenoviral construct; whereas, for the liver-specific HKDC1 knockout model, we used AAV-Cre constructs in our HKDC1$^{fl/fl}$ mice. Both groups of mice were subjected to metabolic testing before and during pregnancy on gestation day 17-18. Our results indicate that hepatic HKDC1 overexpression during pregnancy leads to improved whole-body glucose tolerance and enhanced hepatic and peripheral insulin sensitivity while hepatic HKDC1 knockout results in diminished glucose tolerance. Further, we observed reduced gluconeogenesis with hepatic HKDC1 overexpression while HKDC1 knockout led to increased gluconeogenesis. These changes were associated with significantly enhanced ketone body production in HKDC1 overexpressing mice, indicating that these mice shift their metabolic needs from glucose reliance to greater fat oxidation and ketone utilization during fasting. Taken together, our results indicate

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that hepatic HKDC1 contributes to whole body glucose disposal, insulin sensitivity, and aspects of nutrient balance during pregnancy.

Keywords

Gestational diabetes; Hexokinase; insulin resistance

Introduction

Pregnancy brings forth significant metabolic reprogramming that induces a number of metabolic changes to ensure that the nutrient requirements of the mother and fetus are met (1–3). These adaptations change radically over the period of pregnancy depending on the anabolic or catabolic demand (1–3). The overall focus of the maternal metabolic response is focused on conserving and storing nutrients to sustain the demands of continuous supply of nutrients to the fetus even during periods of food unavailability to preserve fetal development and maternal survival (4–5). Understanding these shifts of nutrients is integral to the health of the mother and developing fetus.

Glucose is the major source of energy and macromolecular building blocks during pregnancy, and thus, understanding its regulation and flux during pregnancy is important under physiological and pathophysiological conditions. Overall, insulin controls glucose homeostasis by lowering plasma glucose by increasing cellular uptake and inhibiting hepatic glucose production. In contrast, reduced insulin action promotes lipolysis, fatty acid oxidation and gluconeogenesis. A balance between insulin production by the pancreas and its subsequent action on maternal muscle, liver and fat tissues mediates the regulation of gestational glucose levels with unique differences from this balance in the nonpregnant state (6).

Early pregnancy is characterized by heightened insulin sensitivity that leads to maternal lipid accumulation; whereas, later stages of pregnancy show increased resistance to insulin with increased mobilization of the stored lipid depots (2–3,7–15). In early gestation, progressive accumulation of maternal adipose tissue lipids is due to enhanced food intake, extrahepatic tissue lipoprotein lipase (LPL) activity, and adipose tissue lipogenesis (16). During the later stages of pregnancy, insulin resistance is characterized by reduced maternal glucose utilization, increased lipolysis and increased levels of hepatic glucose production (7–15). In late gestation, there is increasing insulin resistance contributing to maternal hypertriglyceridemia and also decreased fat synthesis accompanied by enhanced adipose tissue lipolytic activity, which depletes maternal fat depots (7–15,17–19). Additionally, in late gestation, the maternal-fetal glucose transfer results in a lowering of the maternal glucose set point especially during fasting and/or starvation which further activates maternal gluconeogenesis (4–5).

Recently, in a genome-wide association (GWA) study that focused on human gestational glucose metabolism, we discovered that HKDC1 (hexokinase domain containing 1), a novel fifth hexokinase, is associated with glucose tolerance in pregnant mothers at 24–28 weeks of gestation (20), through polymorphisms in regulatory variants (21). These data suggest that

HKDC1 may play a specific role during pregnancy (20–24). HKDC1, based on sequence, was suggested to be a novel hexokinase, and has since been shown to have hexokinase activity (21). The primary function of a hexokinase is to phosphorylate and thus trap glucose in the cell for further processing (25). Previously, the hexokinase (HK) family was comprised of four known members, each having distinct expression patterns and enzyme activities (25–26). HK 1-3 have a high enzymatic activity in phosphorylating glucose (represented by low Km values) and are responsible for glucose metabolism and energy production. In contrast, HK 4, known as glucokinase (GCK), has a high Km value and is considered the glucose sensor of the body due to its expression being restricted to tissues that participate in whole-body glucose sensing (pancreas and liver) (27). These four HKs have been thought to be the only HKs in humans to mediate this pivotal step of glucose metabolism, until the discovery of HKDC1. Our data, and others, have now confirmed HKDC1 is a 5th hexokinase; however, its enzymatic properties have yet to be fully reported (20–21, 28).

Studies conducted in our laboratory, thus far, have shown that HKDC1 is broadly expressed (28). Using the first developed HKDC1 mouse model, we found that complete loss of HKDC1 function is embryonic lethal, similar to other HKs (29–30). Further studies using HKDC1 heterozygous knockout mice showed normal insulin production, insulin sensitivity, gluconeogenesis and glucose homeostasis, although there was a mild impairment of glucose tolerance in aged mice (28). We also did observe impaired glucose tolerance in pregnant mice, which supports the GWAS-derived observation that HKDC1 is associated with maternal 2-h glucose levels after an oral glucose challenge at 28 weeks' gestation in humans (20). Therefore, in our initial report, we concluded that only under conditions of metabolic stress (i.e., pregnancy or aging) does reduced HKDC1 expression affect glucose homeostasis. The liver plays important roles in nutrient management, including glucose production, ketone synthesis, and lipid handling that are important for maternal health and fetal growth (31). As HKDC1 is expressed in the liver (32), we have examined, in this study, the role of hepatic HKDC1 in late term pregnancy. To do this, we have developed mouse models to delete or over-express HKDC1 in the liver and used them to understand the role of HKDC1 in the control of nutrient metabolism, in particular glucose, during pregnancy.

Materials and Methods

Materials.

The complete list of primers used for quantitative PCR (qPCR) can be found in Supplementary Table 1 and the list of primary antibodies used with dilutions is in Supplementary Table 2.

Adenovirus Construction.

Adenovirus containing the human HKDC1 cDNA was generated as previously described (21). In brief, fragment containing the 2,750 bp ORF of HKDC1 (RC221178 plasmid, Origene, Rockville, MD, USA), was cloned into the pShuttle-CMV adenoviral vector (Agilent, Santa Clara, CA, USA). The resulting HKDC1 adenoviral shuttles were then linearized and transformed into BJ5183-AD1 cells (Agilent) producing recombinant clones

which were isolated and digested. Recombinant HKDC1 adenoviruses were produced by transfecting HEK293 cells (Clontech, Mountainview, CA, USA) with PacI-digested recombinant plasmid DNA and FuGene36. After transfection was complete, the viral lysate was collected and further amplified in HEK293 cells. Infected cells were collected and lysed by two freeze/thaw cycles in 2 ml freeze/thaw buffer (lOmM Tris/HCl, pH 8.0, ImM MgC12). The virus was then purified by ultracentrifugation using a CsCl gradient. Purified virus was de-salted using a 7k MWCO column (Thermo Scientific, Waltham, MA, USA) and equilibrated with freeze/thaw buffer, after which glycerol was added to a final concentration of 10%. Viruses were titrated by measuring OD260 (1 OD260 = 1.1 Å~ 1012 virions ml-1) and by plaque assay in HEK293 cells.

Mice.

All mouse studies were approved by the IACUC of the University of Illinois at Chicago and performed in accordance with the Guide for the Care and Use of Laboratory Animals. Animals were housed in a temperature (22–24°C) and humidity-controlled specific pathogen-free barrier facility with 12-h light/12-h dark cycle (lights on at 06:00 h). Mice were fed a standard laboratory rodent chow diet (Envigo 7912, Madison WI, USA).

Adult-onset human HKDC1 overexpression model (aHepHKDC1-OE).—10-12
weeks old male and female C57B1/6J (The Jackson Laboratory) mice were mated and female mice were assessed for plug formation the next day and their body weights were followed for 10 days to ensure they were pregnant. 10-day pregnant females were injected in the lateral tail vein with 100 μL saline containing 1×10^9 Ad-HKDC1 constructs to generate the adult-onset hepatocyte-specific human HKDC1 overexpression mice (aHepHKDC1-OE). Pregnant females injected with Ad-GFP served as controls.

Adult-onset hepatocyte-specific Hkdc1 knock-out model (aHepHKDC1-KO).—
10-12 weeks old female HKDC1$^{fl/fl}$ mice (www.komp.org) were injected in the lateral tail vein with 100 μL saline containing 1.5×10^{11} genome copies of an adeno-associated virus serotype 8 (AAV8) bearing a thyroid-binding globulin (TBG) promoter driven Cre recombinase (AAV8-TBGp-Cre, Penn Vector Core, University of Pennsylvania) to generate the adult-onset hepatocyte-specific HKDC1-knockout mice (aHepHKDC1-KO). Female mice injected with a Null vector (AAV8-TBGp-Null) served as controls. One week after the AAV injections, mice were mated with C57B1/6J males. Female mice were assessed for plug formation the next day and their body weights were followed for 10 days to verify they were pregnant. When mice reached 17-18th day of pregnancy, they were either put through glucose/insulin/pyruvate tolerance tests (described below) or sacrificed after being anesthetized for collection of tissues and blood was collected to determine glucose, plasma insulin, TAG, and cholesterol levels. Tissues, including liver, gastrocnemius and sub-cutaneous fat sub-depots, were snap-frozen in liquid nitrogen and stored at −80°C.

Intraperitoneal glucose tolerance test (GTT).

After a 15-h fast, animals were administered 2 g glucose per kilogram of body weight (bwt) by intra-peritoneal (ip) injection. Blood was obtained from the tail vein and glucose levels monitored using an OneTouch UltraMini glucometer (LifeScan, Inc) at 0, 5, 15, 30, 45, 60,

and 120 minutes. Additionally, blood was collected at multiple points between 0 and 30 minutes in heparinized capillary tubes and centrifuged at 3500 rpm for 15 minutes and the plasma collected for insulin measurements. Insulin levels in these samples were determined by an ELISA kit (ALPCO).

Insulin tolerance test (ITT).

Mice were fasted for 4 hrs and then injected ip with 0.75 Units/kg bwt of Humalog insulin (Eli Lily & Co, Indianapolis, IN, USA). Blood glucose levels were determined in a similar manner to the aforementioned glucose tolerance tests.

Pyruvate tolerance test (PTT).

After a 15-h fast, animals were given ip 1 g/kg bwt of sodium pyruvate dissolved in saline. Blood glucose levels were determined in a similar manner to the aforementioned glucose tolerance tests.

Plasma/liver triglyceride and cholesterol.

Blood for lipid analyses was collected from sacrificed mice by heart puncture in EDTA coated collection tubes (Microtainer K2E, BD, Franklin Lakes, NJ, USA) and centrifuged at 3500 rpm for 15 minutes to separate plasma. Harvested livers were flash frozen in liquid nitrogen. Livers were homogenized in isopropanol and triglyceride levels were measured using reagents obtained from Wako Diagnostics according to the manufacture's protocol (Wako Diagnostics, Richmond, VA, USA). Liver and serum cholesterol levels were determined using the Infinity cholesterol solution (ThermoFisher Scientific).

NEFAs and ketones.

Plasma NEFAs and total ketones were quantified using reagents obtained from Wako Diagnostics according to the manufacture's protocol.

Hexokinase activity.

Hexokinase activity was assayed in liver homogenates using the Hexokinase activity kit (MAK091, Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Activity was normalized to the amount of protein in the sample.

Insulin signaling studies.

Animals were fasted for 15h and either saline or insulin (2U/kg bwt) was ip injected. 20 mins after injection, animals were sacrificed, and blood, liver, sub-cutaneous fat and soleus was harvested and flash-frozen for further analysis of the insulin signaling pathway.

Immunoblotting.

Livers were homogenized in lysis in buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1% Triton X-100 with protease/phosphatase inhibitor mixture (Complete, Roche). Protein concentration was determined using Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA). 30 μg of denatured proteins were separated by SDS-PAGE (Mini-PROTEAN TGX Gels 10%, Bio-Rad Laboratories) and transferred to

0.45 μm nitrocellulose membranes. Membranes were blocked with 5% nonfat, dried milk in Tris-buffered saline with 0.1% Tween-20 for 1 h at 25°C, then incubated with primary antibodies overnight at 4°C washed and incubated with secondary antibodies for 1 hr at 25°C. After washing, Clarity Western ECL Substrate (Bio-Rad) was added and the light signal was detected and analyzed using a ChemiDoc MP and Image Lab Ver 6.0 (Bio-Rad). Protein levels were quantified by signal intensity using the Image Lab Ver 6.0 (Bio-Rad) and normalized to either GAPDH (Fig 1) or β-actin (Fig 4) for quantification purposes.

TNF-α measurements.

TNF-α was quantified in plasma, liver and adipose tissue collected from mice described in insulin signaling studies by a ELISA kit (Cat No. KE10002, Proteintech, Rosemont, IL, USA) according to the manufacturers protocol. TNF-α levels in liver and adipose tissues were normalized to amount of protein in the respective homogenates.

RNA isolation and qPCR.

RNA was extracted from 10mg of liver tissue using the Quick-RNA Miniprep Kit (Zymo Research, Irvine, CA, USA). 1μg of purified RNA was reverse transcribed using qScript Reverse Transcriptase (Quanta Biosciences, Beverly, MA, USA) and quantified via qPCR using PerfeCTa SYBR Green SuperMix (Quanta Biosciences) with final primer concentrations of 0.625 μM for each reaction. Data were analyzed using the CFX Connect Real-Time PCR Detection System (BioRad).

Statistics

All statistical analyses and calculations were performed on the GraphPad Prism. For GTT, PTT and ITT protocols, the changes in glucose concentration were analyzed by two-way ANOVA (Bonferroni post-hoc) with time as a repeated measure. The area under the curve (AUC) in the GTT, ITT and PTT for the changes in glucose concentrations were calculated by Graph Pad Prism software. For most of the data, differences between Ad-GFP vs Ad-HKDC1 and AAV-Null vs AAV-Cre were analyzed by an unpaired two-tailed students *t*-test (Mann-Whitney post-hoc).

Results

Hepatic HKDC1 expression is significantly elevated during pregnancy.

We first determined if the expression of hepatic HKDC1 during pregnancy is altered, where we analyzed wildtype C57Bl/6J mice at day 17-18 of gestation, a point of heightened insulin resistance in mice, and compared them to non-pregnant mice. Pregnant mice exhibited a significant increase in the HKDC1 mRNA and protein levels in the liver (Fig. 1A-B). Considering these data and the key role the liver plays in gestational metabolism, we next set to explore the possible role(s) of hepatic expression of HKDC1 in gestational glucose metabolism. Thus, we developed mouse models for hepatic specific overexpression (aHepHKDC1-OE) and knockout (aHepHKDC1-KO) of HKDC1 (Fig. 1C). For the aHepHKDC1-OE model, we induced pregnancy in 10-12 weeks old C57BL/6J mice and on the tenth day of gestation delivered the human HKDC1 expressing adenovirus via tail vein injection (Ad-HKDC1) or a control GFP-expressing adenovirus (Ad-GFP). For our

aHepHKDC1-KO model, we used a Cre expressing adeno-associated virus (AAV-Cre) infection of HKDC1$^{fl/fl}$ females, with control mice receiving an AAV-null. The expected changes in HKDC1 expression were observed by quantitative PCR (for mRNA expression) and immunoblot (for protein expression) in both models (Fig. 1D-E).

Using these models, we first followed their body weight from gestation day 0 to day 17-18, observing that aHepHKDC1-KO mice gained significantly more body weight compared to pregnant controls at day 17-18 of gestation, while there was no difference in body weight in the aHepHKDC1-OE model relative to its control (Fig. 2A-B). We did not observe any changes in fasting plasma triglycerides or cholesterol in either the aHepHKDC1-OE or -KO model (Fig. 2C). Liver weights, hepatic triglycerides and hepatic glycogen also remained unchanged in all groups however, we did observe an increasing trend in liver triglycerides in aHepHKDC1-OE although the change did not reach statistical significance (Fig. 2D-E).

However, there was a significant decrease in liver cholesterol levels in aHepHKDC1-KO mice (Fig. 2D). In these models at day 17-18 of gestation, we did observe that liver specific HKDC1 OE led to a significant increase in total hexokinase activity (Fig. 2F) and on the other hand hepatic HKDC1-KO resulted in a significant decrease in hexokinase activity (Fig. 2F). Thus, we next used these models to more extensively explore the metabolic role of hepatocyte-specific HKDC1 in pregnancy.

Hepatic HKDC1 expression is positively associated with glucose tolerance and insulin sensitivity in pregnant females

To examine glucose metabolism, we first performed glucose tolerance tests in our HKDC1 models before and at day 17-18 of pregnancy. Alteration of hepatic HKDC1 expression in non-pregnant females did not impact the response to glucose or insulin injections (Supplementary Fig. 1A-D). However, in pregnant females, HKDC1 expression was positively associated with an improved response to glucose tolerance (Fig. 3A-B). We also measured insulin levels during the IPGTT and found that aHepHKDC1-OE mice had lower fasting insulin levels and secreted less insulin in response to the glucose challenge compared to their controls (Fig. 3C); however, they were still able to efficiently dispose of the glucose. aHepHKDC1-KO mice, on the other hand, had similar levels of insulin as that of their controls, both after fasting and in response to the glucose challenge (Fig. 3D). These data suggested that increased hepatic HKDC1 expression may serve to reduce insulin demands to control glucose levels, and reduced HKDC1 expression may increase these demands.

Considering these data, we assessed whole-body insulin-mediated glucose disposal (by ITT) and tissue-specific insulin sensitivity (by acute insulin injections) in pregnant females with altered hepatic HKDC1 expression. Intriguingly, hepatic overexpression of HKDC1 led to profound increases in insulin sensitivity in pregnant mice compared to their controls (Fig. 4A). On the other hand, the aHepHKDC1-KO model showed a slight loss of insulin sensitivity compared to controls, but the results did not reach statistical significance (Fig. 4B). Exploring this further, we harvested the key insulin responsive tissues (muscle, liver and adipose) from 15 h fasted mice, 20 mins after they received an insulin injection and immunoblotted for phosphorylation of Serine 473 (S473) and Threonine 308 (T308) residues of protein kinase B (Akt), a key mediator of insulin signaling. Pregnancy had a

profound negative impact on the insulin sensitivity in liver and adipose tissue, as we did not observe any significant phosphorylation of Akt in control animals (Fig. 4C-D). However, importantly, HKDC1 overexpression enhanced phosphorylation of Akt in liver, adipose and skeletal muscle, further strengthening the observation that liver specific overexpression of HKDC1 enhances whole-body insulin sensitivity (Fig. 4C). In contrast, deletion of hepatic HKDC1 had no effect upon Akt activation and in skeletal muscle, and actually resulted in decreased inactivation relative to controls (Fig. 4D).

As the pro-inflammatory cytokine TNF-α, has been associated with IR during pregnancy (33), we next explored TNF-α levels in the blood and tissue depots. While there were no significant changes in plasma levels of TNF-α (Fig. 4E), there was a significant reduction in both adipose and liver TNF-α levels with hepatic HKDC1 overexpression during pregnancy (Fig. 4F-G). Conversely, we observed a significant increase in TNF-α in the adipose tissue of aHepHKDC1-KO mice (Fig. 4G). Thus, TNF-α changes could be the result of the role of hepatic HKDC1 in altering insulin sensitivity in those tissues during pregnancy. Taken together, these results show that expression of hepatocyte-specific HKDC1 is positively associated with improved control of glucose homeostasis and this could be due in part to improved hepatic and peripheral tissue insulin sensitivity.

Hepatic HKDC1 overexpression modulates energy metabolism during fasting in pregnancy

Insulin resistance in pregnancy leads to enhanced gluconeogenesis even under fasting conditions to meet the requirements of the mother and the growing fetus as glucose is the major fuel which crosses the placental barrier (4). As hepatic HKDC1 expression influences insulin sensitivity, we next measured hepatic gluconeogenesis using the pyruvate tolerance test in each model. Interestingly, during pregnancy, we found that aHepHKDC1-OE pregnant mice were producing significantly less glucose after the pyruvate challenge with a corresponding significant reduction in the hepatic expression of gluconeogenic genes G6Pc and PCK1 (Fig. 5A-B). On the other hand, pregnant aHepHKDC1-KO mice produced more glucose in response to the pyruvate challenge than their pregnant controls and had increased hepatic expression of gluconeogenic genes (Fig. 5C-D).

Insulin resistance in pregnancy particularly in the later stages is involved in the regulation of lipolysis (by mobilizing the fat depots) thereby increasing the plasma NEFA levels. Interestingly, we found that aHepHKDC1-OE pregnant females showed increased levels of circulating NEFA (Fig. 5E), and plasma ketones when they were subjected to an overnight fast (Fig. 5F). This suggests that adipose tissue-derived NEFA may be taken up by the liver and getting oxidized in the liver to generate ketones as an alternative source of fuel for the mother and fetus during prolonged starvation in place of gluconeogenesis which was found to be suppressed in aHepHKDC1-OE pregnant mice (Fig. 5A). We measured the levels of NEFAs in the plasma after administration of insulin and we found that only in aHepHKDC1-OE mice, could insulin administration suppress serum NEFA levels, by approximately 50% (Fig. 5G) confirming that aHepHKDC1-OE mice are more insulin sensitive. However, aHepHKDC1-KO pregnant fasted females did not influence circulating NEFA (Fig. 5E) or plasma ketones levels (Fig. 5E-F).

A main question that arises from our data was the source that increased plasma ketones in pregnant females that overexpress hepatic HKDC1. We hypothesized that hepatocytes of aHepHKDC1-OE females are consuming more NEFA thereby producing more ketones and, therefore, we quantified the expression of hepatic genes involved in ketone synthesis (HMGCS) and β-oxidation (CPT2). The expression of these genes was positively associated with the expression of HKDC1 in OE and KO models (Fig. 5H). In addition, we found that expression of genes involved in de novo lipogenesis: transcriptional regulators of lipogenesis (ChREBP and SREBP1c) and lipogenic enzymes (ACC1 and FASN) were down regulated in aHepHKDC1-OE mice (Fig. 5H). Finally, we found that PDK2, a regulator of PDH complex was also downregulated in aHepHKDC1-OE females. This may impact the regulation of anaplerotic pathways in hepatocytes that may lead to enhanced ketogenesis as shown by *Go et al* in high fat diet-induced obese mice (34). Taken together, these data suggest that during pregnancy HKDC1 expression in hepatocytes is associated with changes in metabolism that promotes fatty acid utilization (likely oxidation) and prevents the production of new fatty acids by DNL during prolonged starvation resulting in production of ketone bodies that may serve as fuel for the mother and fetus.

DISCUSSION

Pregnancy is characterized by a plethora of biochemical changes that work together to allocate nutrients between the growing fetus and the mother. These finely tuned mechanisms are tightly regulated, and a more complete understanding is required to develop better treatment avenues for pregnancy in pathophysiological conditions such as diabetes and obesity. For reasons noted above, this study focused on the role of hepatic specific gain of function and loss of function of HKDC1 during the later stages of pregnancy. Collectively, these data reveal that HKDC1 plays previously unidentified roles in gestational glucose metabolism through its action in the liver. Moreover, its hepatic expression influences not only maternal glucose but also other nutrients balance (fatty acids and ketone levels), through its modulation of whole-body insulin resistance, hepatic gluconeogenesis and ketoneogenesis. In humans, regulatory regions influence HKDC1 expression, and through the large human population studies, was associated with maternal glucose levels (20). Our new data provide insight into the influence of the expression of this gene on maternal glucose levels and its downstream effects on maternal nutrients, a fundamental important balance for the health of the mother and fetus.

It has been well known that in late pregnancy, maternal IR develops thereby shifting the nutrient balance, which supports the rapidly growing fetus (3,11–12,33). However, the molecular basis of IR during late pregnancy is still incompletely understood, where a number of maternal and fetal variables influence this. While insulin resistance was evident in our pregnant control dams by the insulin tolerance test, this effect was reversed in pregnant aHepHKDC1-OE mice. In contrast, aHepHKDC1-KO mice had evidence of further impaired insulin tolerance. Therefore, we found that in addition to modulating glucose disposal, hepatic expressed HKDC1 was altering whole body insulin sensitivity. Strengthening these findings, the phosphorylation state of protein kinase B confirmed these findings, not only in the liver, but in adipose and skeletal muscle. Future directions need to

explore how hepatic HKDC1 expression influences whole body insulin sensitivity during pregnancy.

Basal glucose levels undergo gradual changes over the entire course of pregnancy to meet the nutritional and anabolic demands of the mother and fetus. Fasting plasma glucose levels significantly decline despite increased hepatic glucose production over the course of pregnancy by complex mechanisms that are still poorly understood (13). We found that in line with enhanced insulin sensitivity in the hepatic aHepHKDC1-OE mice, significantly less glucose production after an overnight fast in a pyruvate challenge was observed. Moreover, aHepHKDC1-KO mice produced more glucose when challenged with pyruvate. Complimenting this, we found that genes involved in de novo gluconeogenesis were down-regulated in aHepHKDC1-OE and up-regulated in aHepHKDC1-KO liver. Next steps will be to explore how HKDC1 influences the hepatic glucose production.

Freinkel first reported in the late 1960s that maternal hypertriglyceridemia benefits the fetus during starvation by increasing maternal consumption of triglyceride for ketone body synthesis giving the basis for "accelerated starvation" theory (4,5). This maternal metabolic reprogramming during periods of inadequate food allows her to utilize her accumulated adipose depots for the synthesis of ketone bodies as alternative fuels which allows for the preservation of glucose and amino acids that are required for other essential processes (17). Furthermore, the fetus benefits from this as ketones can cross the placental barrier freely unlike lipids and may be used by the fetus either as fuels or as anabolic substrates (18–19). In the fasting state, plasma NEFAs levels were higher in aHepHKDC1-OE mice and are converted to acetyl-CoA by enhanced β-oxidation and ultimately ketone bodies, which act as an energy source for maternal and fetal tissues during fasting. Our data suggest that hepatic HKDC1 is influencing the 'early starvation of pregnancy' phenomenon. We need to next assess how hepatic HKDC1 may influence NEFA levels (possibly via adipose lipolysis) and hepatic ketogenesis pathways.

Whether acetyl-CoA produced by β-oxidation forms ketone bodies or enters the citric acid (TCA) cycle is determined by anaplerotic influx of TCA cycle intermediates (34). The conversion of pyruvate to oxaloacetate (OAA) by pyruvate carboxylase (PC) is one of the most important sources of anaplerosis in the liver (34). Pyruvate can also be converted to acetyl-CoA by oxidative decarboxylation mediated by the pyruvate dehydrogenase complex (PDC). PDC activity is inhibited via phosphorylation of the pyruvate dehydrogenase by increased expression of pyruvate dehydrogenase kinases (PDKs) during fasting or in IR states (35–37). We found that PDK2 levels which is the major PDK responsible for regulation of PDC activity in the liver (38–40) was significantly decreased in aHepHKDC1-OE mice. It has been reported that decreased PDC activity and enhanced pyruvate carboxylation due to hepatic IR contributes to increased gluconeogenesis in obese subjects with hepatic steatosis (41,42). Because of competition for pyruvate, the balance between PDC and PC activity may play a critical role in metabolic dysfunction caused by IR. We found that liver PDK2 inhibition may reduce the anaplerotic flux of pyruvate into the TCA cycle thereby channeling more ketogenesis which serves as fuel in the fasting state (Fig. 6).

Conclusion

In sum, we observed that hepatic HKDC1 influences maternal glucose tolerance, where it also affects whole body insulin sensitivity, hepatic gluconeogenesis, and ketone production during pregnancy (Fig. 6). These changes, through hepatic expression of this novel hexokinase, collectively alter maternal nutrient balance. Next steps will be important to elucidate how HKDC1 mechanistically drives each of these metabolic changes, as we suggest one proposed mechanism for ketone production, and what the influence on fetal outcomes is.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements:

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Research Highlights.

- Hexokinase domain containing 1 (HKDC1) is a recently discovered putative 5th hexokinase.

- HKDC1 modulates gestational glucose metabolism and insulin sensitivity.

- Liver specific HKDC1 overexpression improves gestational whole-body insulin sensitivity.

- HKDC1 reprograms substrate utilization to ketoneogenesis instead of gluconeogenesis during prolonged starvation in pregnancy.
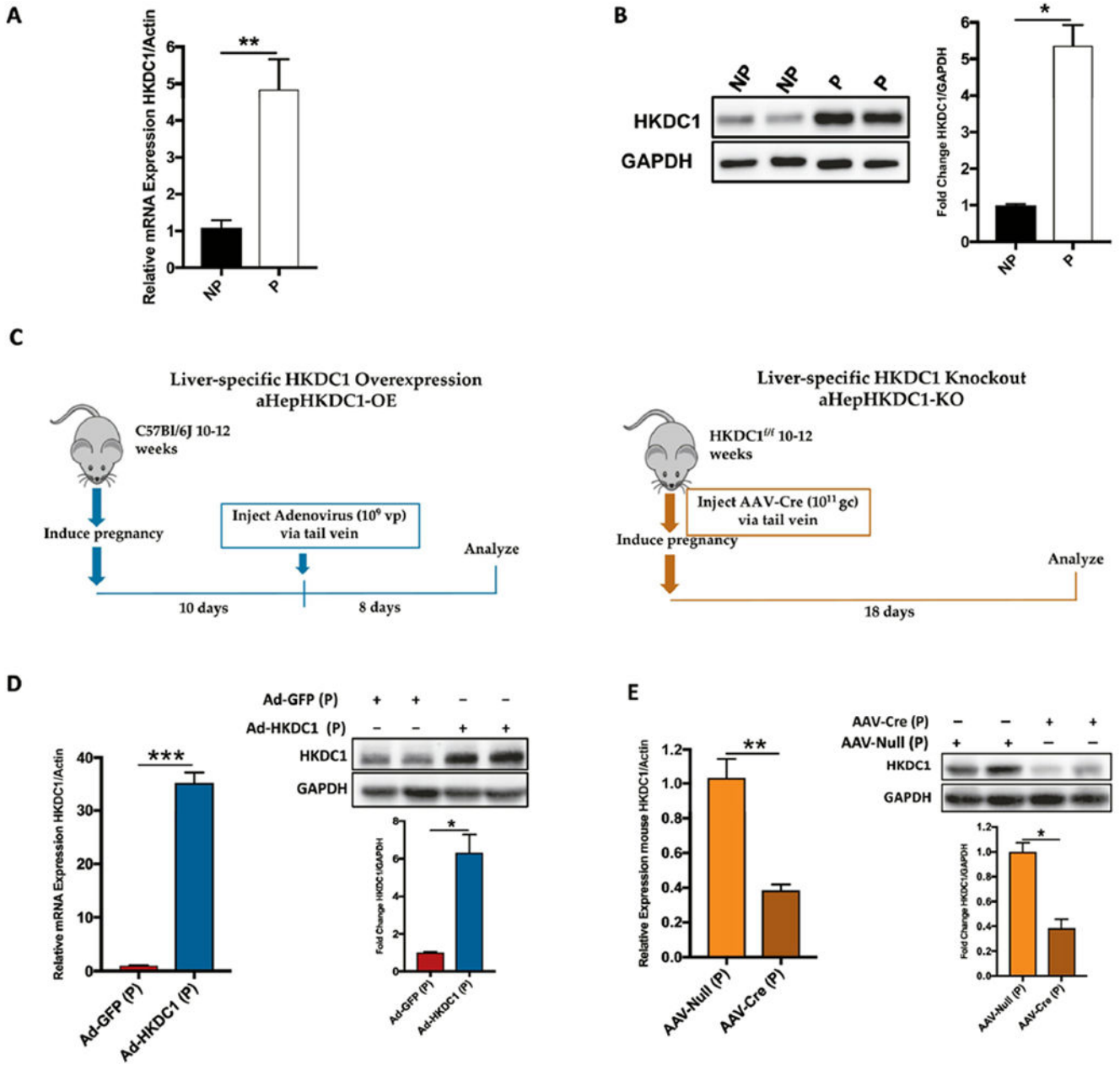
Fig. 1. HKDC1 is preferentially expressed in the liver during pregnancy.
HKDC1 mRNA (A) and protein expression (B) was quantified in day 18 pregnant dams in respect to non-pregnant females. Overall experimental approach (C). HKDC1 mRNA and protein expression after overexpression (D) and knockdown (E) was quantified in day 17-18 pregnant dams in respect to controls. n=5, all values are mean±SEM *p<0.05, **p<0.01 by student's *t*-test (Mann-Whitney post hoc analyses).
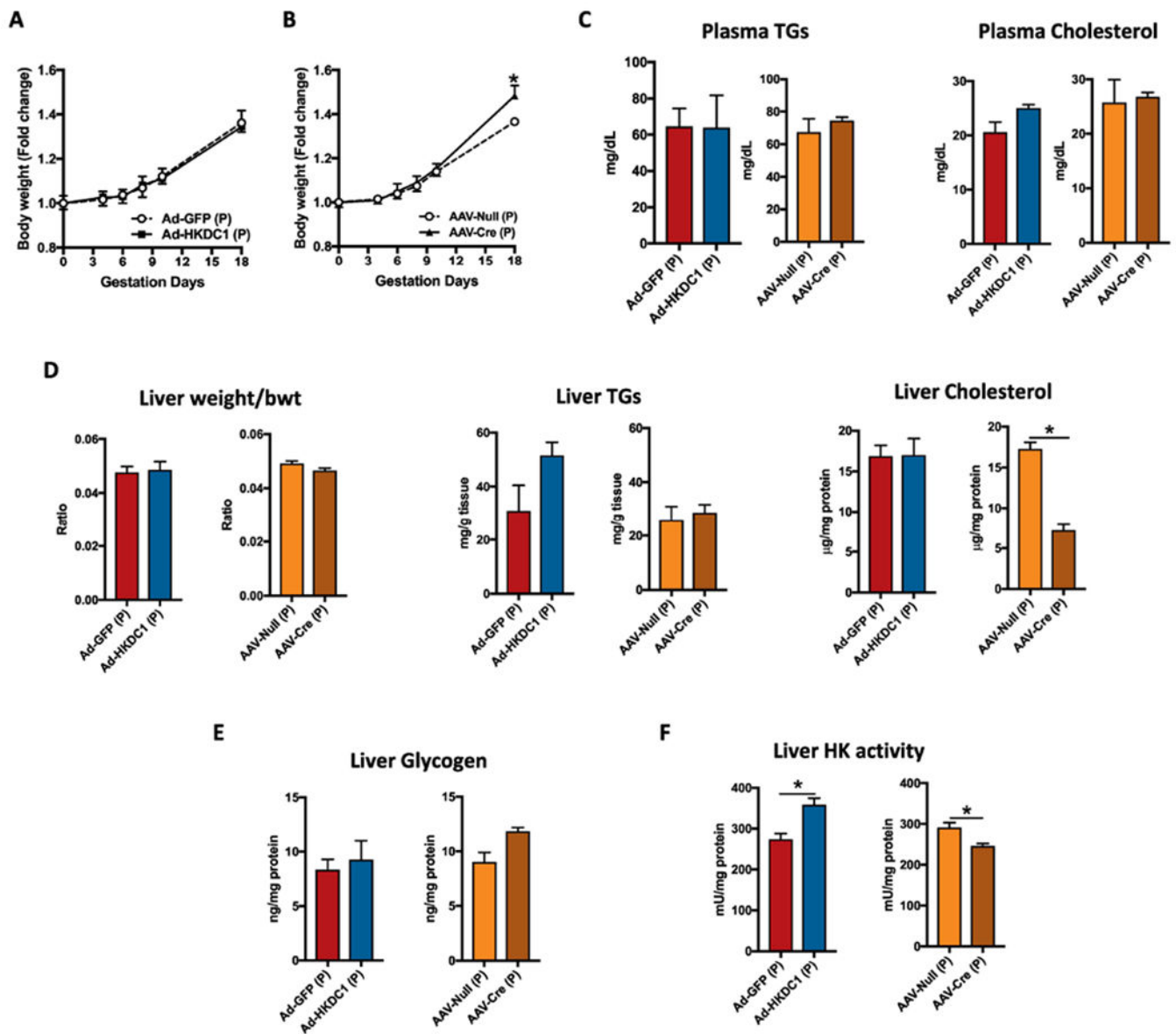
Fig. 2. Characterization of working models.
Body weights were recorded throughout the course of pregnancy till day 17-18 for both working models and the fold change respective to day 0 of gestation was calculated (A-B). Plasma TGs and cholesterol were assayed in day 17-18 pregnant dams after overnight fast (C). Liver weight was recorded relative to body weight and levels of TGs, cholesterol (E). Liver glycogen (F) and hexokinase activity (G) were estimated. n=5, all values are mean ±SEM *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by student's *t*-test (Fig. 2C-F) or one-way ANOVA with Bonferroni post hoc analyses (Fig. 2A-B).
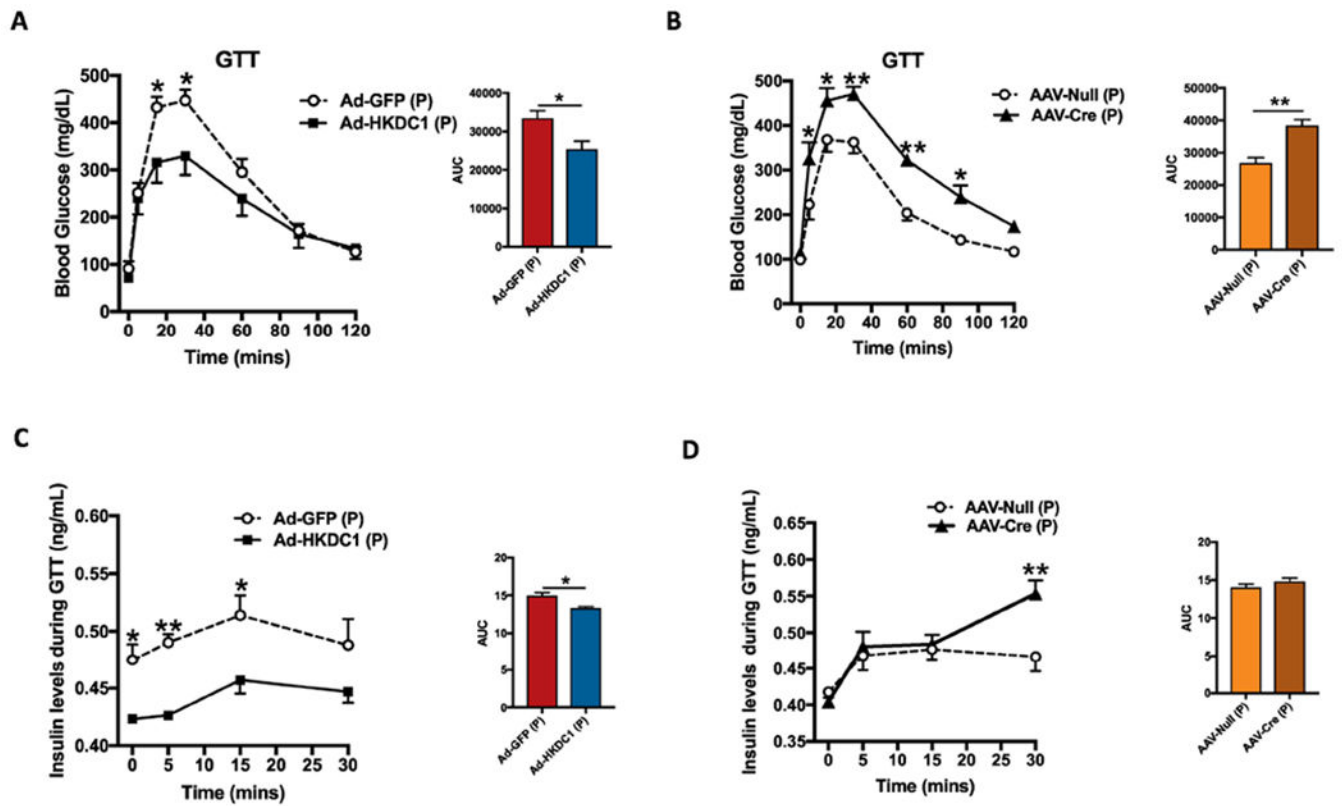
Fig. 3. Hepatic HKDC1 overexpression improves and knockout impairs whole-body glucose disposal.

Glucose tolerance test was conducted on day 18 pregnant dams for aHepHKDC1-OE model (A) and aHepHKDC1-KO model (B) after 15h fast with 2g/kg ip glucose and insulin levels were quantified during the GTT for aHepHKDC1-OE model (C) and aHepHKDC1-KO model (D). n=5, all values are mean±SEM *p<0.05, **p<0.01, by 2-way ANOVA with Bonferroni post hoc analyses and student's *t*-test (for AUCs).
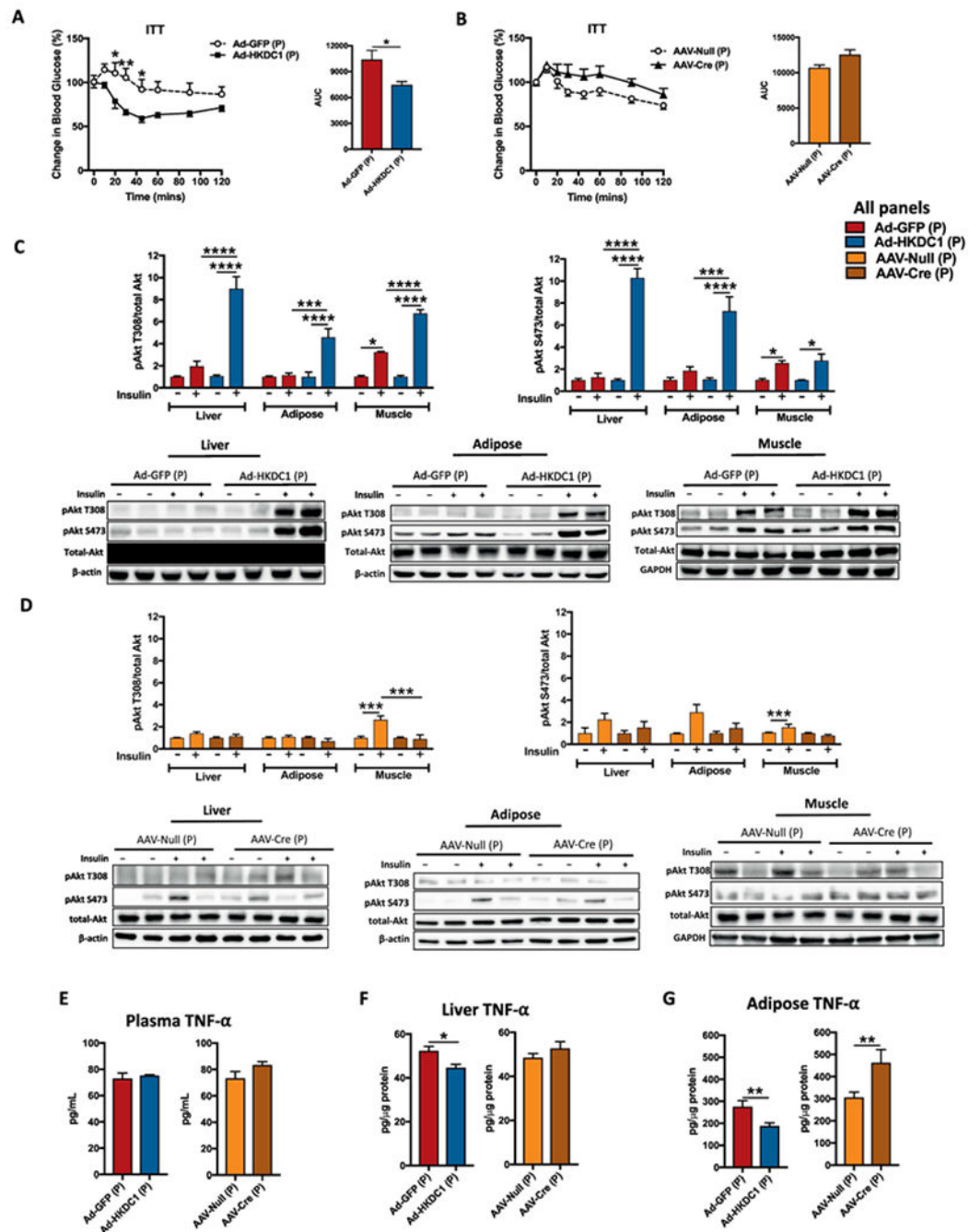
Fig. 4. Hepatic HKDC1 contributes to systemic insulin sensitivity in pregnant mice.
Insulin tolerance test was conducted on day 17-18 pregnant dams for aHepHKDC1-OE model (A) and aHepHKDC1-KO model (B) after 4h fast with 0.75mU/g ip insulin. Mice were fasted overnight and either saline or 2mU/g bwt insulin was ip injected. 20 mins after the injection mice were sacrificed and blood was collected. Liver, sub-cutaneous adipose and soleus tissues extracts were used for immunoblotting analysis (C-D) and quantification of TNF-α (E-G). n=5, all values are mean±SEM *p<0.05, **p<0.01, ***p<0.001,

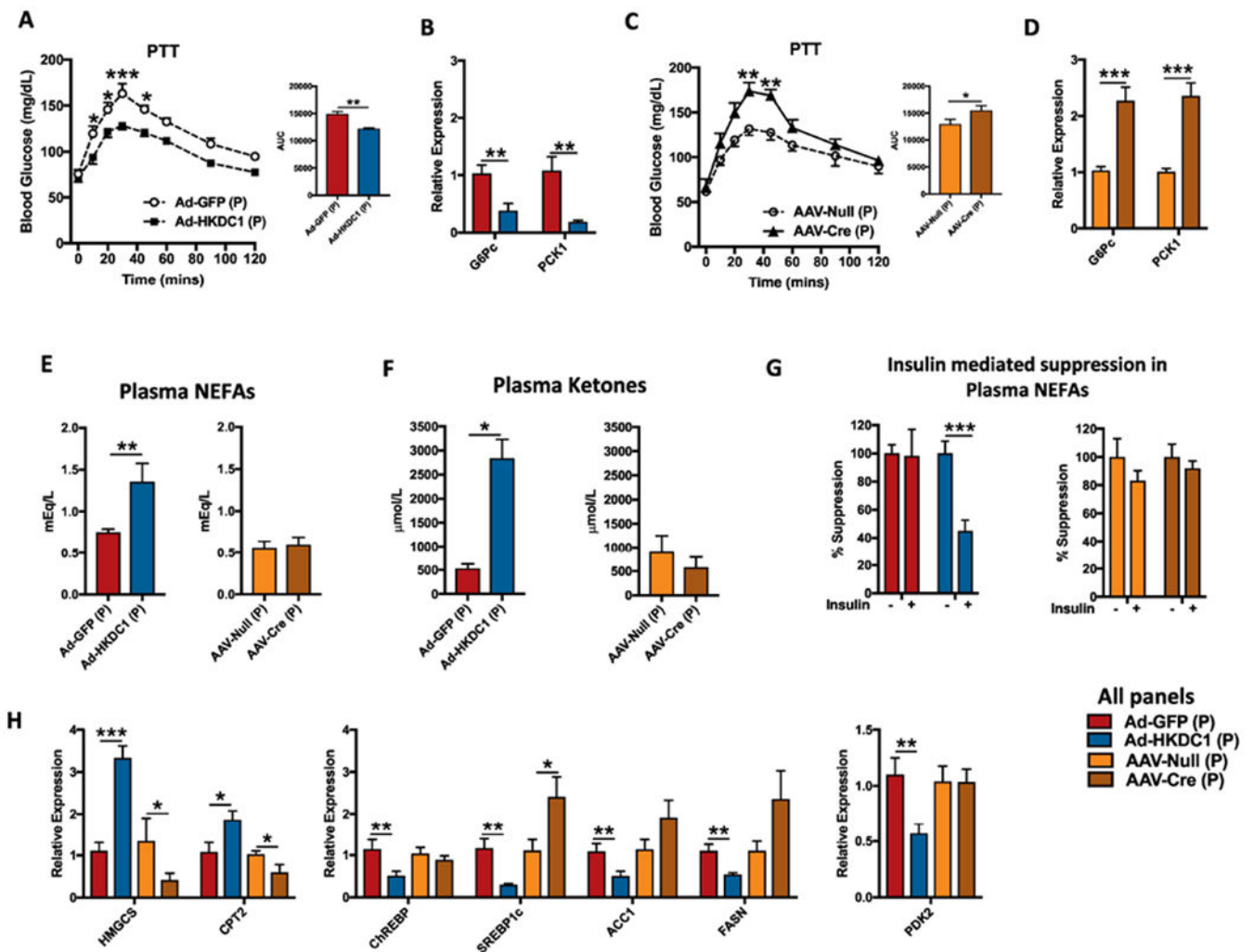****p<0.0001 by 2-way ANOVA with Bonferroni post hoc analyses (Fig. 4A-B), student's *t*-test (for AUCs and 4C-G)

Fig. 5. Hepatic HKDC1 contributes to gluconeogenesis and ketone production during fasting.
Pyruvate tolerance test was conducted on day 18 pregnant dams for aHepHKDC1-OE model
(A) and aHepHKDC1-KO model ((C) after 15h fast with 1g/kg ip sodium pyruvate. Livers
were used to quantify the mRNA expression of gluconeogenic genes (B, D). Plasma was
used to quantify NEFAs (E) and ketones (F) and effect of insulin on NEFA (G). Livers were
used to quantify the mRNA expression of various genes involved in regulation of
ketogenesis, lipid oxidation and lipogenesis (H). n=6, all values are mean±SEM *p<0.05,
**p<0.01, ***p<0.001 by 2-way ANOVA with Bonferroni post hoc analyses (Fig. 5A, C),
student's *t*-test (for AUCs, Fig. 5 B, D, E-H)

In Pregnancy During Fasting Conditions
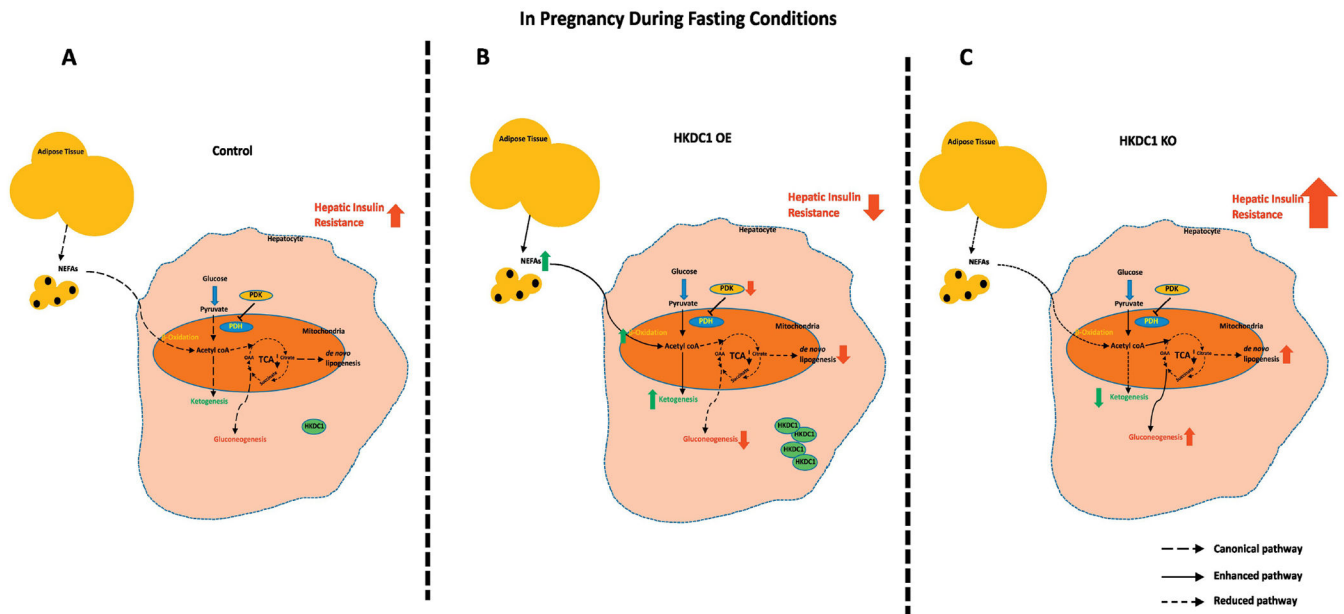
Fig. 6. Model of the influence of HKDC1 on metabolic change during pregnancy.
Canonical pathways involving the metabolism of glucose during fasting conditions in pregnancy (A). HKDC1 expression in the liver contributes to whole body glucose homeostasis, insulin sensitivity, and gluconeogenesis. We also hypothesize that during fasting when HKDC1 is over-expressed in the liver it leads to more lipolysis which is taken up by the liver and converted to ketones shifting the balance from gluconeogenesis to ketoneogenesis during the fasting state (B) while hepatic HKDC1 knockdown leads to heightened insulin resistance (C).