HHS Public Access

Author manuscript

J Mol Endocrinol. Author manuscript; available in PMC 2018 April 01.

Published in final edited form as:

J Mol Endocrinol. 2017 April ; 58(3): 127–139. doi:10.1530/JME-16-0202.

Effects of *G6pc2* Deletion on Body Weight and Cholesterol in Mice

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Abstract

Genome wide association study (GWAS) data have linked the *G6PC2* gene to variations in fasting blood glucose (FBG). *G6PC2* encodes an islet-specific glucose-6-phosphatase catalytic subunit that forms a substrate cycle with the beta cell glucose sensor glucokinase. This cycle modulates the glucose sensitivity of insulin secretion and hence FBG. GWAS data have not linked *G6PC2* to variations in body weight but we previously reported that female C57BL/6J *G6pc2* knockout (KO) mice were lighter than wild-type littermates on both a chow and high fat diet. The purpose of this study was to compare the effects of *G6pc2* deletion on FBG and body weight in both chow fed and high fat fed mice on two other genetic backgrounds. FBG was reduced in *G6pc2* KO mice largely independently of gender, genetic background or diet. In contrast, the effect of *G6pc2* deletion on body weight was markedly influenced by these variables. Deletion of *G6pc2* conferred a marked protection against diet-induced obesity in male mixed genetic background mice whereas in 129SvEv mice deletion of *G6pc2* had no effect on body weight. *G6pc2* deletion also reduced plasma cholesterol levels in a manner dependent on gender, genetic background and diet. An association between *G6PC2* and plasma cholesterol was also observed in humans through electronic health record-derived phenotype analyses. These observations suggest that the action of G6PC2 on FBG is largely independent of the influences of environment, modifier genes or

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Author Contributions

K. A. B. performed most of the mouse phenotyping studies and manuscript writing.
K. E. S. performed some of the mouse studies.
L. D. P. performed some of the mouse studies.
H. M. performed association studies with EHR data and manuscript editing.
L. B. performed association studies with EHR data.
J. K. O. performed some of the mouse studies.
O. P. M. contributed to the design of experiments and manuscript editing.
J. C. D. contributed to design of EHR studies and manuscript editing.
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None of the authors have a conflict of interest relating to this study.

epigenetic events whereas the action of G6PC2 on body weight and cholesterol are influenced by unknown variables.

Introduction

The glucose-6-phosphatase (G6Pase) enzyme system is located in the endoplasmic reticulum and catalyzes the hydrolysis of glucose-6-phosphate (G6P) to glucose and inorganic phosphate (Hutton and O'Brien 2009; O'Brien 2013). In addition to a catalytic subunit, which can be one of three isoforms, G6PC1, G6PC2 or G6PC3, the G6Pase system is composed of a glucose transporter and a G6P/Pi transporter, encoded by the *SLC37A4* gene (Hutton and O'Brien 2009; O'Brien 2013). *G6PC2* is thought to be expressed exclusively in pancreatic islet beta cells (Hutton and O'Brien 2009; O'Brien 2013). Experiments comparing wild type (WT) and *G6pc2* knockout (KO) mouse islets suggest that G6pc2 opposes the action of the beta cell glucose sensor, glucokinase, which catalyzes the formation of G6P from glucose (Iynedjian 2009; Matschinsky 2005). In isolated *G6pc2* KO islets G6Pase activity (Pound, et al. 2013) and glucose cycling (Wall, et al. 2015) are abolished. This results in leftward shift in the dose response curve for glucose-stimulated insulin secretion (GSIS) (Pound et al. 2013). Under fasting conditions, where insulin levels are the same in WT and *G6pc2* KO mice, this shift results in reduced fasting blood glucose (FBG) in KO mice (Boortz, et al. 2016a; Pound et al. 2013; Wang, et al. 2007). In contrast, under stimulatory conditions using a sub-maximal concentration of glucose this shift results in increased GSIS from *G6pc2* KO relative to WT mouse islets (Pound et al. 2013). As predicted from a parallel shift in the dose response curve for GSIS, under stimulatory conditions using a high concentration of glucose, this shift results no difference in GSIS between *G6pc2* KO and WT as assessed in either isolated islets *in situ* (Pound et al. 2013) or mice *in vivo* using hyperglycemic clamps (Wang et al. 2007).

Consistent with these mouse studies, genome wide association studies (GWAS) have linked the rs560887 single nucleotide polymorphism (SNP) in the *G6PC2* gene to variations in FBG (Bouatia-Naji, et al. 2008; Chen, et al. 2008). Molecular studies have shown that the rs560887-G allele represents a gain of function that is associated with increased *G6PC2* RNA splicing which is predicted to lead to increased full length G6PC2 protein expression and elevated glucose cycling (Baerenwald, et al. 2013). Since GWAS data show that the rs560887-G allele is associated with elevated FBG (Bouatia-Naji et al. 2008; Chen et al. 2008), the combination of these splicing (Baerenwald et al. 2013) and *G6pc2* KO mouse (Pound et al. 2013; Wang et al. 2007) studies suggest that rs560887 is a potentially causative variant. The association between *G6PC2* and FBG has been confirmed in multiple GWAS and in different populations (Bouatia-Naji, et al. 2009; Dupuis, et al. 2010; Hu, et al. 2009; Hu, et al. 2010; Prokopenko, et al. 2008; Reiling, et al. 2009; Tam, et al. 2010; Wang, et al. 2013).

Numerous GWAS have also examined the genes that are associated with variations in body weight, fat mass and fat distribution and have shown that greater than 160 loci are linked to these parameters (Lu, et al. 2016). While *G6PC2* was not one of the loci identified (Lu et al. 2016) we previously observed that female C57BL/6J *G6pc2* KO mice were lighter than

wild-type (WT) littermates on both a chow fed and high fat fed diet (Pound et al. 2013). This observation prompted us to examine whether genetic background influences the effect of *G6pc2* deletion on body weight and the response to diet-induced obesity (DIO). The results show that the effect of *G6pc2* deletion on FBG is largely independent of gender, genetic background and diet whereas the effect of *G6pc2* deletion on body weight is highly dependent on these variables. We also found that deletion of *G6pc2* reduced plasma cholesterol levels in a manner dependent on gender, genetic background and diet. These observations suggest that the action of G6PC2 on FBG is largely unaffected by the influences of environment, gender, modifier genes or epigenetic events whereas the action of G6PC2 on body weight and cholesterol are influenced by unknown variables.

Materials and Methods

Animal Care

The Vanderbilt University Medical Center Animal Care and Use Committee approved all protocols used. Mice were maintained on either a standard rodent chow diet (calorie contributions: 28% protein, 12% fat, 60% carbohydrate (14% disaccharides); LabDiet 5001; PMI Nutrition International) or a high-fat diet (calorie contributions: 15% protein, 59% fat, 26% carbohydrate (42% disaccharides); Mouse Diet F3282; BioServ). High-fat feeding studies were initiated at 8 weeks of age and mice were maintained on the diet for 8–14 weeks as indicated. Food and water were provided *ad libitum*.

Generation of *G6pc2* Knockout (KO) Mice

Previous studies have described the generation of *G6pc2* KO mice on a mixed 129/SvEv × C57BL/6J (Wang et al. 2007), C57BL/6J (Pound et al. 2013) and 129SvEv (Boortz et al. 2016a) genetic background. The targeting vector used to generate the KO allele replaced exons 1–3 of the *G6pc2* gene with a LacZ/Neo cassette leaving exons 4 and 5 intact (Wang et al. 2007). Exon 1 contains the translation initiation methionine (Ebert, et al. 1999). As such, the design of the targeting vector completely abolishes *G6pc2* expression (Wang et al. 2007). All the mice examined in these studies were littermates generated by interbreeding of heterozygous (HET) mice.

Intraperitoneal Glucose Tolerance Tests

Intraperitoneal glucose tolerance tests (IPGTTs) were performed on ~22 week old male mice as previously described (Pound, et al. 2012).

Phenotypic Analysis of Fasted *G6pc2* KO Mice

Mice were fasted for 5 hours and then weighed. After an additional hour of fasting, mice were anesthetized using isoflurane and blood samples were isolated from the retro-orbital venous plexus. Glucose concentrations were measured in whole blood using a glucose monitor (Accu-Check Advantage; Roche, Indianapolis, USA). EDTA (5 μl; 0.5 M) was then added to blood samples prior to isolation of plasma by centrifugation. Insulin samples were assayed using RIA (Morgan and Lazarow 1963) by the Vanderbilt Hormone Assay and Analytical Services Core. Cholesterol was assayed using a cholesterol reagent kit (Raichem, San Diego, CA, USA), while triacylglycerol was assayed using a serum triacylglycerol

determination kit (Sigma, St Louis, MO, USA). Body composition was assessed using an mq10 NMR analyzer (Bruker Optics).

Analysis of Gene Expression in Mouse Pancreas

Pancreatic gene expression was analyzed as previously described (Boortz et al. 2016a). The following mouse primer pairs were used for the analysis of gene expression:

G6pc2 Forward	5′-CCCTGATGGTGGTGGCTCTA-3′
G6pc2 Reverse	5′-GTCTGTGGGTGGAGCAGGAC-3′
Ins2 Forward	5′-CACCCAGGCTTTTGTCAAGC-3′
Ins2 Reverse	5′-CCAGTGCCAAGGTCTGAAGG-3′
Mouse *Ppia* Forward	5′-GGCCGATGACGAGCCC-3′
Mouse *Ppia* Reverse	5′-TGTCTTTGGAACTTTGTCTGCAA-3′

Electronic Health Record (EHR)-Based Phenotyping of Human Research Subjects

EHR-based phenotyping was conducted using data on human subjects in the Vanderbilt University Medical Center (VUMC) BioVU DNA databank. Genotyping data in BioVU is linked to the Synthetic Derivative (SD), a de-identified version of the VUMC EHR repository. Detailed descriptions of program operations, ethical considerations, and continuing oversight and patient engagement have been published (Pulley, et al. 2010; Roden, et al. 2008). For these studies we used a previously genotyped cohort of 29,722 European descendants from VUMC with longitudinal medical care. Genotyping was performed on the Illumina Human Exome BeadChip platform. For this study, we specifically analyzed the intronic *G6PC2* SNP rs560887. Lipid measurements utilized routine clinical laboratory testing values present in the EHR.

Statistical Analyses

Other than IPGTTs, data were analyzed using a Student's t-test: two sample assuming equal variance. The level of significance was as indicated (two-sided Student's t-test). IPGTT data were analyzed using a two-way ANOVA assuming normal distribution and equal variance. A post hoc analysis was performed using the Bonferroni correction for multiple comparisons. The level of significance was as indicated.

To analyze genetic associations with lipids in BioVU, we used the median value for each individual. The associations between the genotypes and the aggregated laboratory values (as continuous variables) were performed on R with linear model, adjusted for age, sex, and body mass index (BMI). We report beta values, 95% confidence intervals (CI), and p values. $P < 0.05$ was considered to be significant. All tests assumed a two-tailed distribution.

Results

Analysis of the Effect of *G6pc2* Deletion on Body Weight and Composition in Chow and High Fat Fed 129SvEv Mice

We have previously shown that 16 week old chow fed female, but not male, C57BL/6J *G6pc2* knockout (KO) mice are slightly lighter than wild type (WT) littermates and have

reduced body fat (Pound et al. 2013). These differences were also observed following 12 weeks of high fat feeding in female, but not male, C57BL/6J *G6pc2* KO mice (Pound et al. 2013). In this study we repeated these analyses with *G6pc2* KO mice on a 129SvEv or mixed genetic background.

In 16 week old chow fed 129SvEv *G6pc2* mice no differences in weight or body fat were observed between female WT or KO mice (Table 1). However, male chow fed 129SvEv *G6pc2* KO mice were slightly lighter than WT littermates and female chow fed 129SvEv *G6pc2* KO mice had slightly increased muscle mass (Table 1).

High-fat feeding is a standard nutritional challenge in the field of obesity and diabetes research that induces insulin resistance and is considered to model human disease (Young and Kirkland 2007). High fat feeding of 129SvEv mice was started at 8 weeks of age and continued for 12 weeks. In contrast to C57BL/6J mice that markedly increase their body weight in response to high fat feeding (Pound et al. 2013; Surwit, et al. 1988; Winzell and Ahren 2004) almost no difference in body weight was observed between 16 week old chow fed female and male 129SvEv mice (Table 1) versus 20 week old high fat fed female and male 129SvEv mice (Table 2). Weekly measurements of body weight in non-fasted high fat fed mice during the 12 weeks of high fat feeding showed no evidence for a biphasic change in weight, that would have been suggestive of a toxic effect of prolonged high fat feeding, in either female (Fig. 1A) or male (Fig. 1B) WT and KO mice. These data are consistent with previous studies that have observed that 129SvEv mice are resistant to DIO (Almind and Kahn 2004).

Despite the lack of weight gain, high fat fed female and male 129SvEv mice showed a marked increase in body fat (%) relative to chow fed mice (Fig. 1C). This was associated with a reduction in body muscle (%) in both high fat fed female and male 129SvEv mice (Table 2) relative to chow fed female and male mice (Table 1) ($p<0.05$).

In 20 week old high fat fed 129SvEv *G6pc2* mice no differences in body fat were observed between female or male WT versus KO mice (Table 2). However, male high fat fed 129SvEv *G6pc2* KO mice were slightly heavier than WT littermates and free fluid was reduced in female high fat fed 129SvEv *G6pc2* KO mice (Table 2).

Analysis of the Effect of *G6pc2* Deletion on Fasting Blood Glucose (FBG) and Fasting Plasma Insulin (FPI) in High Fat Fed 129SvEv Mice

We have previously shown that male 129SvEv *G6pc2* KO mice have reduced FBG but no change in FPI relative to WT littermates (Boortz et al. 2016a). When this analysis was repeated using female 129SvEv chow fed mice this same reduction in FBG was observed in *G6pc2* KO mice (Fig. 1D) with no change in FPI (Fig. 1E).

We next analyzed the effect of high fat feeding on FBG and FPI in 129SvEv mice. Despite 13 weeks of high fat feeding a comparison between female (Fig. 1D) and male (Boortz et al. 2016a) chow fed with female (Fig. 1F) and male (Fig. 1G) high fat fed 129SvEv WT mice revealed surprisingly no increase in FBG. Similarly, a comparison between female (Fig. 1E)

and male (Boortz et al. 2016a) chow fed with female (Fig. 1H) and male (Fig. 1I) high fat fed 129SvEv WT mice revealed surprisingly no increase in FPI.

After 13 weeks of high fat feeding a reduction in FBG was observed in both female (Fig. 1F) and male (Fig. 1G) *G6pc2* KO relative to WT mice with no differences in FPI in either female (Fig. 1H) or male (Fig. 1I) mice relative to WT.

Analysis of the Effect of High Fat Feeding on Glucose Tolerance in 129SvEv WT and *G6pc2* KO Mice

We have previously shown that deletion of *G6pc2* does not effect glucose tolerance in chow fed C57BL/6J (Pound et al. 2013) and 129SvEv (Boortz et al. 2016a) mice, consistent with human GWAS data showing no association between *G6PC2* SNPs and variations in glucose tolerance (Heni, et al. 2010; Ingelsson, et al. 2010; Li, et al. 2009; Rose, et al. 2009). Although high fat feeding did not result in weight gain in male 129SvEv mice (Table 2) relative to chow fed mice (Table 1), intraperitoneal glucose tolerance tests (IPGTTs) revealed a clear impairment in glucose tolerance in both WT ($p<0.0002$) and *G6pc2* KO ($p<0.0001$) high fat fed 129SvEv mice relative to chow fed mice (Fig. 1J), suggesting the presence of either insulin resistance and/or impaired GSIS in high fat fed 129SvEv mice. However, even in high fat fed mice, deletion of *G6pc2* did not affect glucose tolerance (Fig. 1H).

Analysis of the Effect of *G6pc2* Deletion on Body Weight, FBG and FPI in High Fat Fed Mixed Genetic Background Mice

A comparison of data derived from studies on C57BL/6J (Pound et al. 2013) and 129SvEv (Fig. 1; Tables 1 & 2) mice suggest that the effect of *G6pc2* deletion on body weight varies with gender and genetic background. We therefore repeated these high fat feeding analyses in mice with a mixed C57BL/6J X 129SvEv genetic background. We have previously shown that FBG is reduced in both female and male mixed C57BL/6J X 129SvEv genetic background *G6pc2* KO mice relative to WT with no differences in body weight or FPI (Wang et al. 2007). After starting high fat feeding at 8 weeks of age and continuing for 8 weeks we observed no differences in body weight between female mixed genetic background WT and KO mice (Fig. 2A). In contrast, male mixed genetic background *G6pc2* KO mice exhibited a striking protection against DIO (Fig. 2B).

No reduction in FBG was observed in high fat fed female KO mice relative to WT mice (Fig. 2C) whereas FBG was markedly reduced in high fat fed male KO mice relative to WT mice (Fig. 2D). Similarly, while no difference in FPI was observed between high fat fed female KO mice relative to WT mice (Fig. 2E), FPI was markedly reduced in high fat fed male KO mice relative to WT mice (Fig. 2F).

Comparison of Pancreatic *G6pc2* Expression in 129SvEv and C57BL/6J Mice

The data derived from studies on C57BL/6J, 129SvEv and mixed genetic background mice reveal that the effect of *G6pc2* deletion on body weight varies with gender and genetic background. In addition, FBG is lower in both male chow fed (Boortz et al. 2016a) and high fat fed (Figs. 1F) 129SvEv mice than C57BL/6J mice (Goren, et al. 2004; Mazzaccara, et al.

2008; Pound et al. 2013). While there are likely multiple factors that account for these differences, one potential contributing factor could be variations in *G6pc2* gene expression between C57BL/6J and 129SvEv mice. To address this possibility we compared pancreatic *G6pc2* and *Ins2* gene expression in both mouse strains. There was no difference in the ratio of *G6pc2* to *Ins2* gene expression between female and male chow fed 129SvEv mice (Fig. 3A) or between female and male chow fed C57BL/6J mice (Fig. 3B). There was also no difference in the ratio of *G6pc2* to *Ins2* gene expression between chow fed female 129SvEv and C57BL/6J mice (Fig. 3C) or between chow fed male 129SvEv and C57BL/6J mice (Fig. 3D). In contrast, while there was no difference in the ratio of *G6pc2* to *Ins2* gene expression between female and male high fat fed 129SvEv mice (Fig. 3E) there was a difference between female and male high fat fed C57BL/6J mice (Fig. 3F). Similarly, while there was no difference in the ratio of *G6pc2* to *Ins2* gene expression between high fat fed female 129SvEv and C57BL/6J mice (Fig. 3G) there was a difference between high fat fed male 129SvEv and C57BL/6J mice (Fig. 3H). These data suggest that *G6pc2* expression is induced by high fat feeding relative to *Ins2* expression in male C57BL/6J mice, which may contribute to differences versus high fat fed female C57BL/6J mice and male 129SvEv mice.

Analysis of the Effect of *G6pc2* Deletion on Plasma Cholesterol in 129SvEv, C57BL/6J and Mixed Genetic Background Mice

Since multiple plasma lipids change in response to high fat feeding (Eisinger, et al. 2014) we also compared plasma cholesterol levels in *G6pc2* KO mice on different genetic backgrounds. We previously observed no change in cholesterol levels in male or female mixed genetic background *G6pc2* KO mice relative to WT (Wang et al. 2007). But when we repeated these analyses in chow fed 129SvEv and C57BL/6J mice along with high fat fed 129SvEv, C57BL/6J and mixed genetic background mice we observed that plasma cholesterol levels were reduced in chow fed male C57BL/6J KO mice (Fig. 4D), high fat fed female (Fig. 4G) and male (Fig. 4H) C57BL/6J KO mice, and high fat fed mixed genetic background male KO mice (Fig. 4J).

Analysis of the Relationship Between *G6PC2* SNPs and Metabolic Parameters in Humans using BioVU

Our results in mice demonstrate that the effect of *G6pc2* deletion on cholesterol levels varies with gender and genetic background. We next used Vanderbilt's BioVU DNA databank to determine whether G6PC2 affects these parameters in humans. BioVU individuals with extant genotyping at the intronic *G6PC2* SNP rs560887 were screened to identify associations with cholesterol and triglyceride measurements. The rs560887-G allele, which enhances *G6PC2* pre-mRNA splicing (Baerenwald et al. 2013), was associated with increased cholesterol (total cholesterol: $\beta = 1.0$, $p = 0.039$; LDL-C: $\beta = 1.1$, $p = 0.006$), but not triglyceride levels ($\beta = 0.90$, $p = 0.46$) or HDL-C ($\beta = -0.07$, $p = 0.75$) (Table 3). We further analyzed the population by sex and found that rs560887-G significantly associated with increased LDL-C in males ($p = 0.009$) but not in females ($p=0.15$), although SNP and sex interaction is not significant ($p=0.30$) (Table 3). Rs560887 did not associate with diabetes status ($p=0.37$). Thus, as in mice, the impact in humans of modulating *G6PC2* expression on plasma lipids is dependent on gender.

Analysis of the Effect of *G6pc2* Deletion on Food Intake

A key question that arises from these studies is how *G6PC2*, which is thought to be expressed exclusively in pancreatic islet beta cells (Arden, et al. 1999; Martin, et al. 2001), could be affecting body weight. One possible explanation for the link between G6PC2 and body weight is that G6PC2 affects satiety. Thus the leftward shift in the dose response curve for GSIS observed in *G6pc2* KO mice (Pound et al. 2013) might result in a faster rise in plasma insulin levels after eating or glucose injection in an IPGTT. Since insulin is a satiety factor (Woods, et al. 2006), this faster rise in insulin could promote a quicker cessation of feeding and ultimately reduced food intake. To address this hypothesis we measured the intake of high fat food in female C57BL/6J WT and *G6pc2* KO mice. While female C57BL/6J *G6pc2* KO mice are lighter than wild-type (WT) littermates on both a chow fed and high fat fed diet (Pound et al. 2013) no difference in food intake was detected (Fig. 5).

Discussion

Our results demonstrate that the effect of *G6pc2* deletion in mice on FBG closely parallels human GWAS data in that the effect of *G6pc2* deletion on FBG is largely independent of gender and genetic background. We previously showed that, relative to WT mice, FBG is reduced in both female and male chow fed and high fat fed *G6pc2* KO on a pure C57BL/6J genetic background (Pound et al. 2013), female and male chow fed *G6pc2* KO mice on a mixed genetic background (Wang et al. 2007) and male mice on a 129SvEv genetic background (Boortz et al. 2016a). We show here that FBG is also reduced in 129SvEv chow fed female mice (Fig. 1D) and high fat fed female (Fig. 1F) and male (Fig. 1G) mice. Similarly, FBG is reduced in male high fat fed mixed genetic background *G6pc2* KO relative to WT mice (Fig. 2D). FBG was not reduced in female high fat fed mixed genetic background *G6pc2* KO mice (Fig. 2C), though the n value in this study was relatively low. These observations are largely consistent with human GWAS data showing an association between *G6PC2* and FBG in multiple different populations (Bouatia-Naji et al. 2009; Dupuis et al. 2010; Hu et al. 2009; Hu et al. 2010; Prokopenko et al. 2008; Reiling et al. 2009; Tam et al. 2010; Wang et al. 2013).

With respect to FPI, we previously showed that, relative to WT mice, FPI is unchanged in both female and male chow fed and high fat fed *G6pc2* KO mice on a pure C57BL/6J genetic background (Pound et al. 2013), female and male chow fed *G6pc2* KO mice on a mixed genetic background (Wang et al. 2007) and male mice on a 129SvEv genetic background (Boortz et al. 2016a). We show here that FBG is also unchanged in 129SvEv chow fed female mice (Fig. 1E) and high fat fed female (Fig. 1H) and male (Fig. 1I) mice. Similarly, FPI is unchanged in female high fat fed mixed genetic background *G6pc2* KO mice (Fig. 2E). A reduction in FPI was observed in male high fat fed mixed genetic background *G6pc2* KO mice (Fig. 2F) but this is presumably secondary to the marked effect of *G6pc2* deletion on body weight in males (Fig. 2B). These observations are consistent with human GWAS data showing no association between *G6PC2* and FPI in multiple different populations (Bouatia-Naji et al. 2008; Chen et al. 2008; Mahajan, et al. 2015; Wessel, et al. 2015).

In contrast to these data showing largely consistent effects of *G6pc2* deletion on FBG and FPI regardless of gender, diet and genetic background, the effect of *G6pc2* deletion on body weight and body composition is highly dependent on these variables. We previously showed that female, but not male, *G6pc2* KO mice on a pure C57BL/6J genetic background had reduced body weight and body fat on both a chow and high fat diet relative to WT mice (Pound et al. 2013). In contrast, we show here that deletion of *G6pc2* in female mice on the 129SvEv genetic background has no effect on body weight or body fat on either a chow (Table 1) or high fat (Table 2) diet relative to WT mice. Similarly, deletion of *G6pc2* in female mice on a mixed 129SvEv X C57BL/6J genetic background has no effect on body weight on either a chow (Wang et al. 2007) or high fat (Fig. 2A) diet relative to WT mice. In males deletion of *G6pc2* on the 129SvEv genetic background was associated with reduced body weight on a chow diet (Table 1) but increased body weight on a high fat diet (Table 2). In contrast, deletion of *G6pc2* in male mice on a mixed 129SvEv X C57BL/6J genetic background had no effect on body weight on a chow diet (Wang et al. 2007) whereas this conferred a marked protection against DIO on a high fat diet (Fig. 2B). Overall our results suggest that FBG is a much more tightly regulated variable than body weight. Thus while FBG levels are relatively similar in chow fed C57BL/6J (Pound et al. 2013), 129SvEv (Boortz et al. 2016a) and mixed (Wang et al. 2007) genetic background mice, the increase in body weight and body fat in response to high fat feeding is markedly different in C57BL/6J (Pound et al. 2013; Surwit et al. 1988; Winzell and Ahren 2004) and 129SvEv mice (Fig. 1) (Almind and Kahn 2004). Interestingly, the response to DIO varies remarkably even within inbred mice through poorly understood epigenetic mechanisms (Burcelin, et al. 2002; Koza, et al. 2006; Oey, et al. 2015) though whether such mechanisms and/or environmental factors or modifier genes contribute to the variable effects of *G6pc2* deletion on body weight and composition is unknown.

In humans a GWAS performed in a cohort of Mexican Americans linked the *G6PC2* rs560887-A allele with a small decrease in BMI and adiposity in this population (Li et al. 2009), however, other GWAS have not associated *G6PC2* with variations in body mass index, fat mass or fat distribution (Lu et al. 2016). Similarly, a strong association between *G6PC2* and cholesterol was not detected using GWAS (Aulchenko, et al. 2009; Kathiresan, et al. 2009), though a weak association can be detected using BioVU (Table 3). These observations suggest that either the effects of *G6pc2* on body mass and cholesterol are quantitatively only easily detected in mice on specific genetic backgrounds or that there is a threshold effect such that *G6PC2* will markedly affect these parameters in some human populations but only after a substantial change in expression rather than the subtle changes associated with common SNPs (Baerenwald et al. 2013; Bouatia-Naji, et al. 2010). If correct, the association observed by GWAS between common SNPs in *G6PC2* and subclinical atherosclerosis (Rasmussen-Torvik, et al. 2011) is likely secondary to the effect of G6PC2 on FBG rather than a direct effect of G6PC2 on cholesterol metabolism.

A key question that remains to be addressed is how *G6PC2*, which is thought to be expressed exclusively in pancreatic islet beta cells (Arden et al. 1999; Martin et al. 2001), could be affecting body weight. One possibility, as proposed by Li et al. (Li et al. 2009), is that the differences in body weight they observed in humans are secondary effects due to altered insulin signaling efficacy that arise due to an effect of G6PC2 on the pulsatility of

insulin secretion. Another possibility is that *G6PC2* expression in other tissues that affect body weight has been overlooked. Indeed while RNA blotting showed no evidence for *G6PC2* expression in brain (Martin et al. 2001) and transgenic mouse studies gave inconsistent results (Frigeri, et al. 2004; Wang, et al. 2008), one group has reported *G6pc2* expression in the mouse hypothalamus (Goh, et al. 2006), a region critical for the control of body weight (Morton, et al. 2006). However, this expression was only detected using very high template concentrations and PCR cycles (Goh et al. 2006). Moreover, while low levels of expression were detected, it is unlikely to be biologically consequential since expression of the *G6pc3* isoform was detected at much higher levels (Goh et al. 2006) and G6pc3 is enzymatically more active than G6pc2 (Petrolonis, et al. 2004; Shieh, et al. 2003). One other potential explanation for the link between G6PC2 and body weight is that G6PC2 affects satiety. Thus the leftward shift in the dose response curve for GSIS observed in *G6pc2* KO mice (Pound et al. 2013) might result in a faster rise in plasma insulin levels after eating. Since insulin is a satiety factor (Woods et al. 2006), this faster rise in insulin could promote a quicker cessation of feeding and ultimately reduced food intake. However, an analysis of food intake did not detect a difference between female C57BL/6J WT and *G6pc2* KO mice (Fig. 5). Future studies will examine the alternate possibility, specifically whether deletion of *G6pc2* affects energy expenditure (Ellacott, et al. 2010). While food intake did not differ between C57BL/6J WT and *G6pc2* KO mice, an effect of G6PC2 on the timing of GSIS during glucose tolerance tests could explain the counterintuitive observation that the rs560887-G allele, which confers elevated *G6PC2* expression (Baerenwald et al. 2013), is associated with elevated FBG but also higher insulin levels at the 30 minute time point in a glucose tolerance test (Li et al. 2009). We hypothesize that insulin levels may peak earlier in individuals with the rs560887-A allele, which confers lower *G6PC2* expression (Baerenwald et al. 2013).

Acknowledgments

We thank Susan Hajizadeh for performing insulin assays and Devin A. Baerenwald for assistance with the food intake study. This research was supported by the following grants: R.O'B., DK92589; O. P. M., DK043748 and DK078188; J.C.D., LM010685. The measurement of plasma insulin by the Vanderbilt Hormone Assay & Analytical Services Core was supported by NIH grant P60 DK20593, to the Vanderbilt Diabetes Research Training Center. The measurement of body composition using the Vanderbilt Mouse Metabolic Phenotyping Center mq10 NMR analyzer was supported by NIH grant DK59637. K. E. S. and L. D. P. were supported by the Vanderbilt Molecular Endocrinology Training Program grant 5T32 DK07563.

R.O'B. is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.

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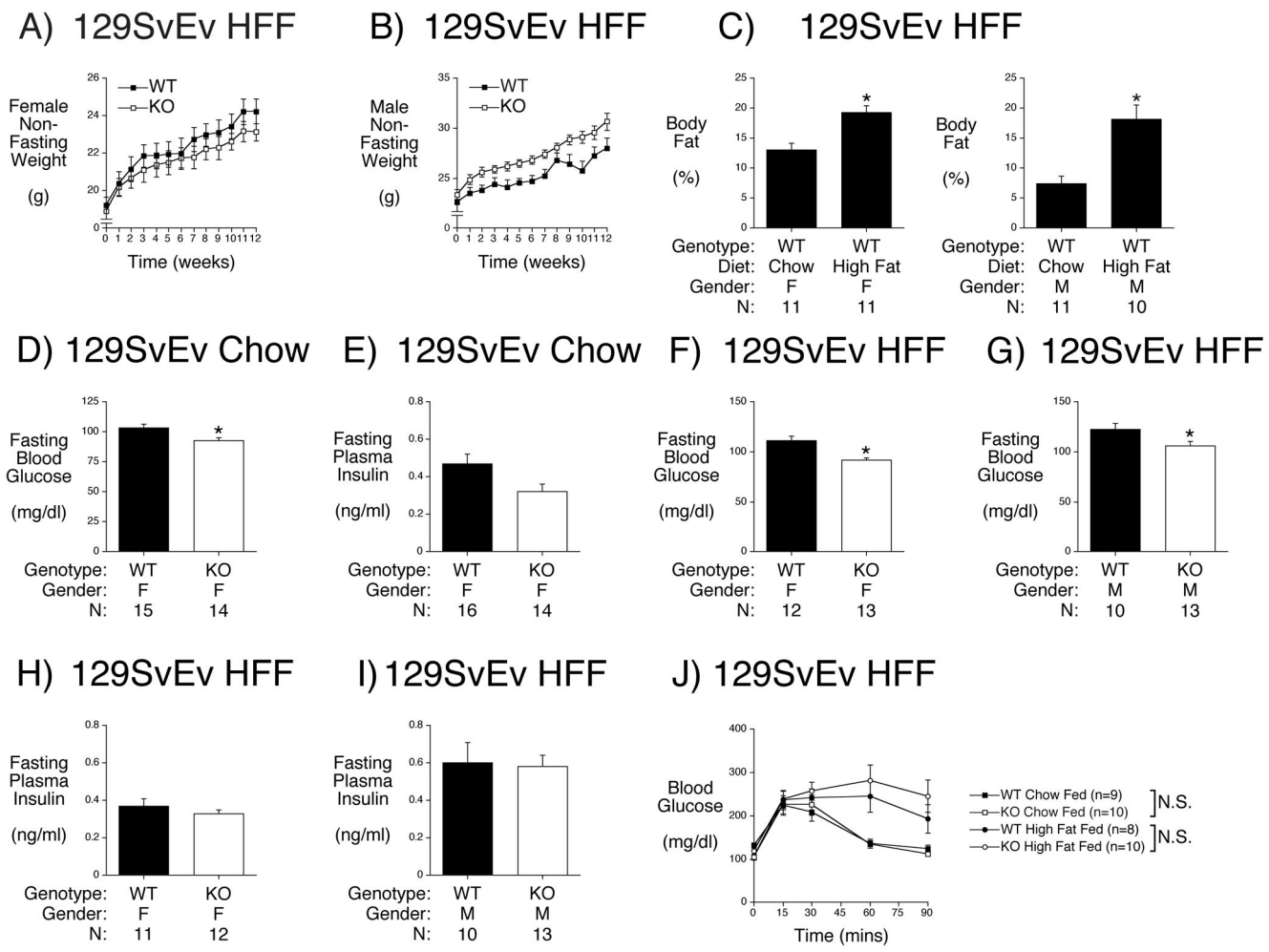

Figure 1. Effect of *G6pc2* Deletion on Body Weight, Composition and Metabolic Parameters in High Fat Fed 129SvEv Mice

Panels A & B: Starting at 8 weeks of age, female (**Panel A**) and male (**Panel B**) mice were fed a high-fat diet with non-fasting body weights measured weekly. Results are the mean ± S.E.M. of data from the following number of animals: female WT n=11; male WT n=11.

Panel C: Body composition was assessed in chow fed 129SvEv mice at 16 weeks of age and in high fat fed 129SvEv mice at 20 weeks of age following 12 weeks of high fat feeding. Mice were fasted for 5 hours and then weighed. One hour later body fat was determined by NMR. Results are the mean ± S.E.M. of data with the genotype, gender and number of animals indicated. WT=wild type; F=female; M= male. *p < 4.61E-10 high fat fed vs chow fed females; *p < 3.94E-05 high fat fed vs chow fed males.

Panels D & E: At 17 weeks of age chow fed mice were fasted for 5 hours and then weighed. One hour later mice were anesthetized and blood isolated. Blood glucose (**Panel D**) and plasma insulin (**Panel E**) were determined as described in Methods. Results are the mean ± S.E.M. of data with the genotype, gender and number of animals indicated. WT=wild type; KO=knockout; F=female. *p < 0.014 WT *versus* KO (**Panel D**).

Panels F – I: Metabolic parameters were assessed in high fat fed 129SvEv mice at 21 weeks of age following 13 weeks of high fat feeding. Mice were fasted for 5 hours and then

weighed. One hour later mice were anesthetized and blood isolated. Blood glucose (**Panels F & G**) and plasma insulin (**Panels H & I**) were determined as described in Methods. Results are the mean ± S.E.M. of data with the genotype, gender and number of animals indicated. WT=wild type; KO=knockout; F=female; M= male. *p < 0.001 female WT *versus* KO (**Panel F**); *p < 0.037 male WT *versus* KO (**Panel G**).

Panel J: Glucose tolerance was assessed in chow fed 129SvEv at ~22 weeks of age and in high fat fed 129SvEv mice at 22 weeks of age following 13 weeks of high fat feeding. IPGTTs using 2.0 g/kg glucose were performed on 6 hr fasted, conscious, chow or high fat fed wild type (WT) and *G6pc2* knockout (KO) male mice as described in Methods. The results show the mean glucose concentrations ± S.E.M.

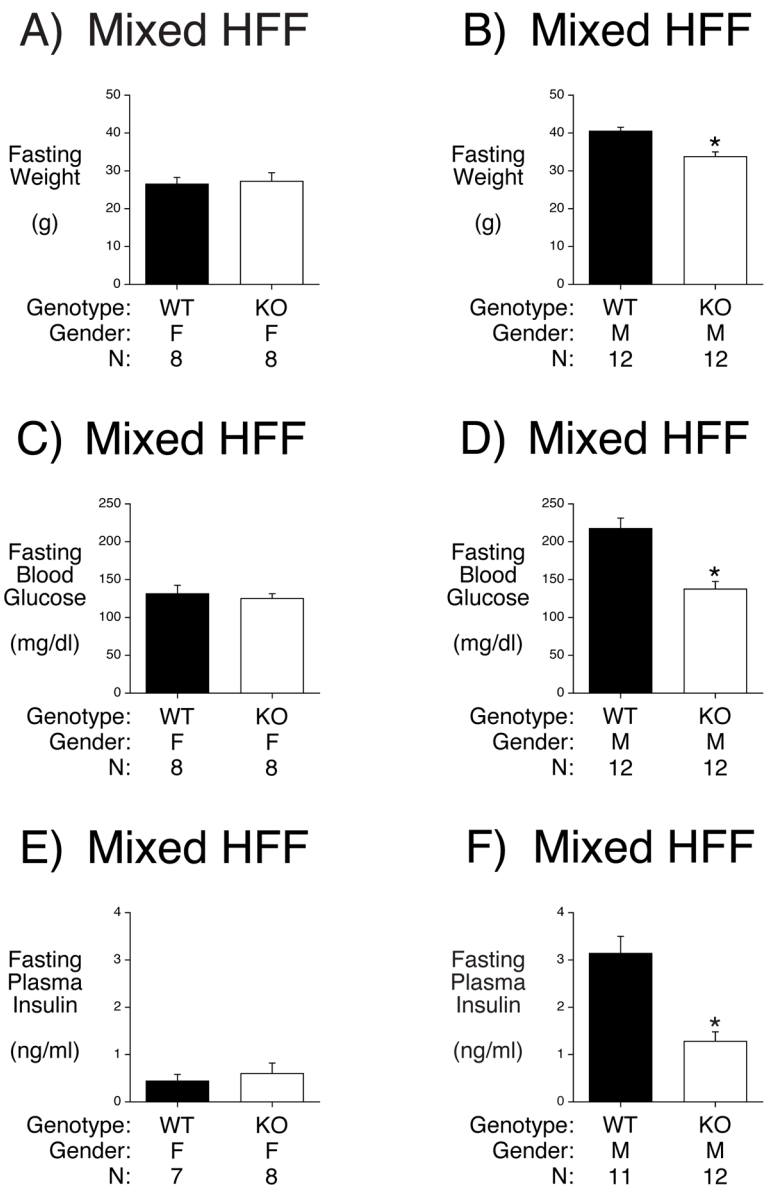

Figure 2. Effect of *G6pc2* Deletion on Body Weight and Metabolic Parameters in High Fat fed Mixed Background Mice

Metabolic parameters were assessed in high fat fed mixed genetic background mice at 16 weeks of age following 8 weeks of high fat feeding. Mice were fasted for 5 hours and then weighed (**Panels A & B**). One hour later mice were anesthetized and blood isolated. Blood glucose (**Panels C & D**) and plasma insulin (**Panels E & F**) were determined as described in Methods. Results are the mean ± S.E.M. of data with the genotype, gender and number of animals indicated. WT=wild type; KO=knockout; F=female; M= male.

*p < 8.32E-05 WT *versus* KO (Panel B); *p < 3.45E-05 WT *versus* KO (Panel D); *p < 6.84E-05 WT *versus* KO (Panel F).

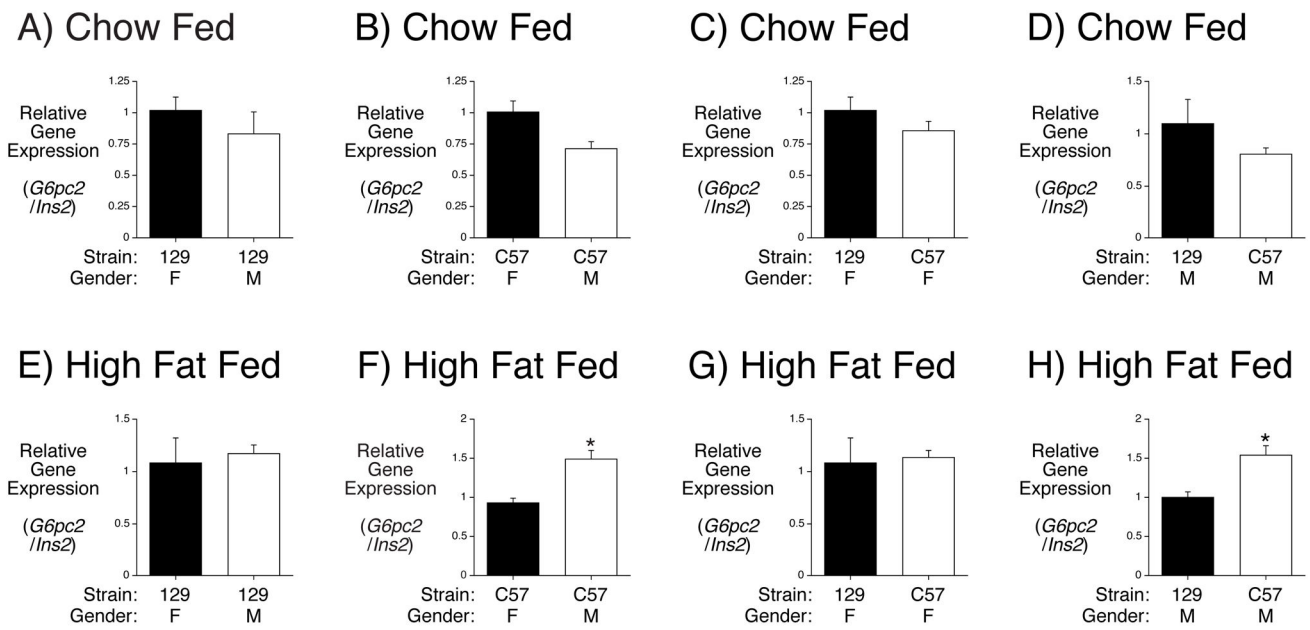

Figure 3. Comparison of Pancreatic *G6pc2* Expression in 129SvEv and C57BL/6J Mice

Pancreatic RNA was isolated following a 6 hr fast from chow fed (**Panels A-D**) 129SvEv (129) or C57BL/6J (C57) mice or mice fed a high fat diet for 2 weeks (**Panels E-H**). *G6pc2* and *Ins2* expression were quantitated by Real Time PCR. Results show the ratio of *G6pc2* to *Ins2* expression ± S.E.M. in 3–5 pancreata. *p < 0.05 *versus* control.

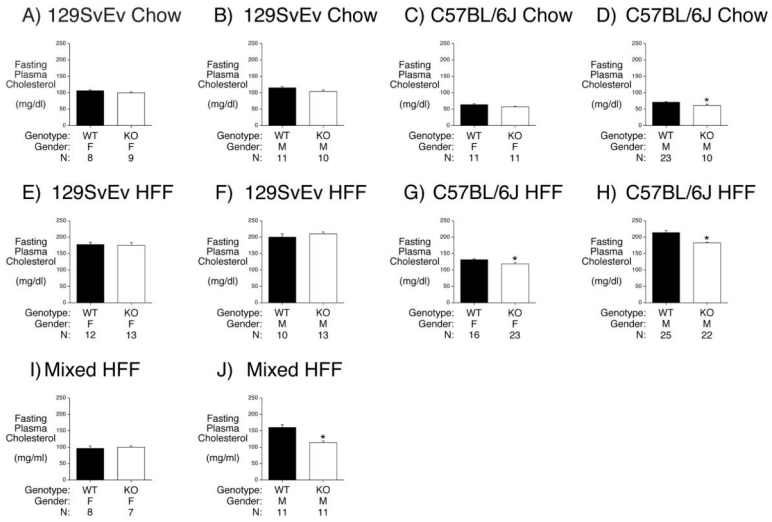

Figure 4. Effect of *G6pc2* Deletion on Plasma Cholesterol in 129SvEv, C57BL6J and Mixed Genetic Background Mice

Panels A – D: At 17 weeks of age chow fed 129SvEv or C57BL/6J mice were fasted for 5 hours and then weighed. One hour later mice were anesthetized and blood isolated.

Panels E - H: At 21 weeks of age, following 13 weeks of high fat feeding, 129SvEv and C57BL/6J mice were fasted for 5 hours and then weighed. One hour later mice were anesthetized and blood isolated.

Panels I & J: At 16 weeks of age, following 8 weeks of high fat feeding, mixed genetic background mice mice were fasted for 5 hours and then weighed. One hour later mice were anesthetized and blood isolated.

Plasma cholesterol was determined as described in Methods. Results are the mean ± S.E.M. of data with the genotype, gender and number of animals indicated. WT=wild type; KO=knockout; F=female; M= male. *p < 0.0069 WT *versus* KO (**Panel D**); *p < 0.02 female WT *versus* KO (**Panel G**); *p < 7.01E-06 male WT *versus* KO (**Panel H**); *p < 0.001 WT *versus* KO (**Panel J**).

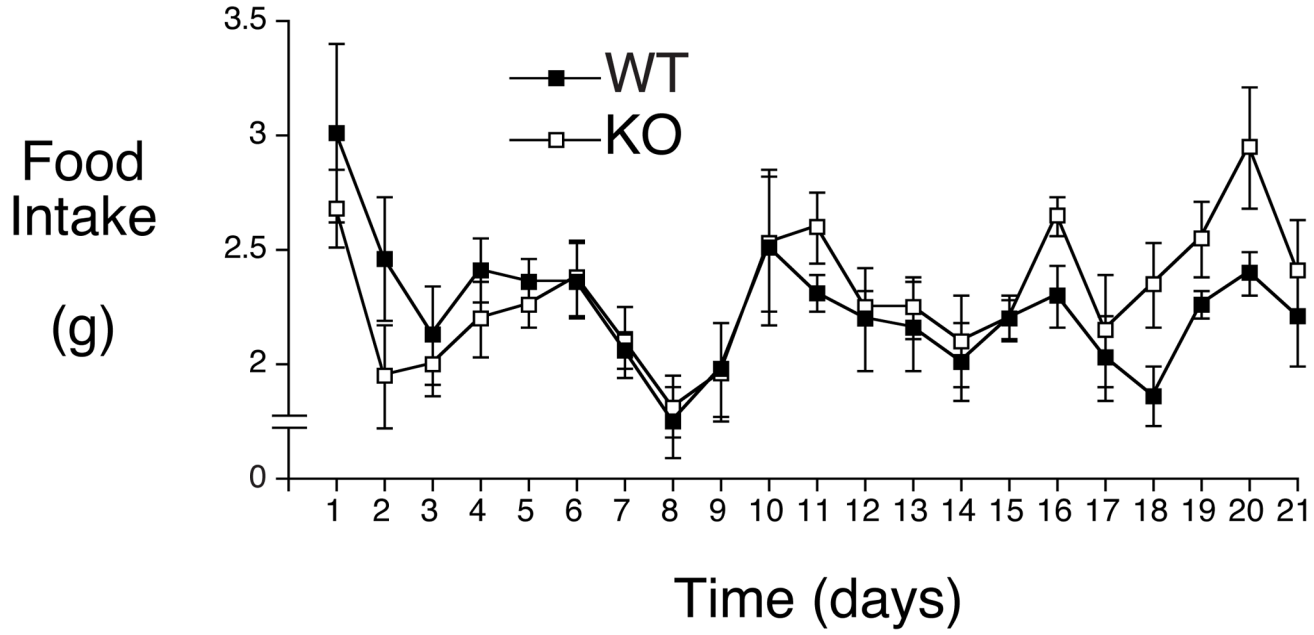

Figure 5. Effect of *G6pc2* Deletion on High Fat Food Intake in Female C57BL/6J WT and *G6pc2* KO Mice

Female C57BL/6J WT and *G6pc2* KO mice were switched from chow food to high fat food at 8 weeks of age and food intake measured daily for 21 days. Results show the mean food intake ± S.E.M. in 6 WT and 6 *G6pc2* KO mice.

Table 1

NMR Analysis of Chow Fed 129SvEv *G6pc2* KO Mouse Body Composition

Body composition of 6 hour fasted, 16 week old animals was assessed using a mq10 NMR analyzer. Results are means ± S.E.M. obtained from the number of animals indicated in parentheses. WT=wild type; KO=knockout.

Gender & Genotype	Body Weight (g)	Fat (g)	Muscle (g)	Free Fluid (g)	Fat (%)	Muscle (%)	Free Fluid (%)
Female WT	22.23 ± 0.26 (15)	2.76 ± 0.26 (11)	13.94 ± 0.27 (11)	0.58 ± 0.05 (11)	13.04 ± 1.15 (11)	66.32 ± 0.94 (11)	2.78 ± 0.26 (11)
Female KO	22.11 ± 0.54 (14)	3.09 ± 0.46 (9)	14.69 ± 0.25 (9), *	0.60 ± 0.03 (9)	13.48 ± 1.62 (9)	66.05 ± 1.34 (9)	2.71 ± 0.15 (9)
Male WT	28.47 ± 0.34 (16)	2.02 ± 0.35 (11)	19.23 ± 0.37 (11)	0.82 ± 0.10 (11)	7.40 ± 1.29 (11)	69.90 ± 0.91 (11)	2.96 ± 0.25 (11)
Male KO	26.93 ± 0.54 (12), *	1.51 ± 0.25 (9)	18.21 ± 0.38 (9)	0.75 ± 0.12 (9)	6.03 ± 1.00 (9)	72.45 ± 0.96 (9)	3.01 ± 0.51 (9)

1. Weight: *p < 0.009 male WT vs KO
2. Muscle: *p < 0.04 female WT vs KO

Table 2

NMR Analysis of High Fat Fed 129SvEv *G6pc2* KO Mouse Body Composition

Body composition of 6 hour fasted, 20 week old animals following 12 weeks of high fat feeding was assessed using a mq10 NMR analyzer. Results are means ± S.E.M. obtained from the number of animals indicated in parentheses. WT=wild type; KO=knockout.

Gender & Genotype	Body Weight (g)	Fat (g)	Muscle (g)	Free Fluid (g)	Fat (%)	Muscle (%)	Free Fluid (%)
Female WT	23.06 ± 0.69 (11)	4.46 ± 0.34 (11)	14.80 ± 0.32 (11)	0.58 ± 0.06 (11)	19.26 ± 1.23 (11)	64.46 ± 144 (11)	2.48 ± 0.23 (11)
Female KO	22.65 ± 0.58 (11)	4.30 ± 0.32 (11)	14.37 ± 0.37 (11)	0.50 ± 0.04 (11)	18.88 ± 1.11 (11)	63.55 ± 1.02 (11)	2.17 ± 0.15 (11), *
Male WT	26.80 ± 0.93 (10)	5 ± 0.78 (10)	16.72 ± 0.50 (10)	0.55 ± 0.07 (10)	18.18 ± 2.34 (10)	62.68 ± 1.63 (10)	2.02 ± 0.19 (10)
Male KO	30.20 ± 0.82 (13), *	6.98 ± 0.65 (13)	17.51 ± 0.35 (13)	0.60 ± 0.03 (13)	22.67 ± 1.75 (13)	58.35 ± 1.58 (13)	2.00 ± 0.09 (13)

1. Weight: *p < 0.0124 WT vs KO

Table 3

Association Between *G6PC2* SNP rs560887 and Plasma Lipid Measurements Using Electronic Health Record (EHR)-Derived Phenotype Analyses

Plasma lipid measurements were obtained from routine lipid panels in Vanderbilt University Medical Center's EHR repository. For each laboratory of each individual, the associations are tested against the median of all lab results for that test. All associations were adjusted for age, sex, and body mass index using linear regression. Cholesterol: total cholesterol; LDL-C: calculated low-density lipoprotein; HDL-C: calculated high-density lipoprotein. The unit for all measurements is mg/dL.

Lab	Population	N	Beta (G)	95% CI	P-value	Allele		
						GG	GA	AA
LDL-C	All	13087	1.15	0.33 ~ 1.96	0.006	102.13 ± 31.43	101.58 ± 31.33	99.08 ± 31.25
LDL-C	Male	5863	1.60	0.4 ~ 2.8	0.009	97.5 ± 31.23	95.77 ± 29.41	94.29 ± 32.15
LDL-C	Female	7224	0.82	−0.29 ~ 1.93	0.148	105.9 ± 31.09	106.33 ± 32.05	102.73 ± 30.07
Cholesterol	All	14349	1.00	0.05 ~ 1.95	0.039	183.62 ± 38.55	183.25 ± 38.96	181.18 ± 40.03
Cholesterol	Male	6412	1.49	0.09 ~ 2.87	0.037	173.36 ± 37.46	171. 53 ± 36.79	170.63 ± 38.45
Cholesterol	Female	7937	0.68	−0.59 ~ 1.95	0.29	191.97 ± 37.39	192.81 ± 38.06	189.05 ± 39.39
Triglycerides	All	14213	0.90	−1.48 ~ 3.28	0.459	149.92 ± 98.13	149.41 ± 95.16	148.37 ± 99.99
Triglycerides	Male	6398	−0.08	−4.07 ~ 3.9	0.967	157.72 ± 109.51	157.17 ± 105.84	157.87 ± 107.95
Triglycerides	Female	7815	1.80	−1.02 ~ 4.62	0.211	143.49 ± 87.14	143.00 ± 84.82	141.09 ± 92.86
HDL-C	All	13457	−0.07	−0.46 ~ 0.33	0.746	51.42 ± 17.11	51.36 ± 17.47	51.76 ± 17.56
HDL-C	Male	6006	0.28	−0.22 ~ 0.78	0.269	44.00 ± 13.49	43.54 ± 12.93	43.72 ± 13.33
HDL-C	Female	7451	−0.35	−0.94 ~ 0.24	0.245	57.43 ± 17.38	57.70 ± 18.09	57.85 ± 17.93