RESEARCH NOTE

REVISED GPR21 KO mice demonstrate no resistance to high fat diet induced obesity or improved glucose tolerance [version 2; referees: 1 approved, 2 approved with reservations]

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Abstract

Gpr21 KO mice generated with *Gpr21* KO ES cells obtained from Deltagen showed improved glucose tolerance and insulin sensitivity when fed a high fat diet. Further mRNA expression analysis revealed changes in *Rabgap1* levels and raised the possibility that *Rabgap1* gene may have been modified. To assess this hypothesis a new *Gpr21* KO mouse line using TALENS technology was generated. *Gpr21* gene deletion was confirmed by PCR and *Gpr21* and *Rabgap1* mRNA expression levels were determined by RT-PCR. The newly generated *Gpr21* KO mice when fed a normal or high fat diet chow did not maintain their improved metabolic phenotype. In conclusion, *Rabgap1* disturbance mRNA expression levels may have contributed to the phenotype of the originally designed *Gpr21* KO mice.

This article is included in the Preclinical Reproducibility and Robustness channel.

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Referee Status: ? ✓ ?

	Invited Referees 1	2	3
REVISED version 2 published 17 Jun 2016	? report		
version 1 published 04 Feb 2016	? report	✓ report	? report

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Competing interests: All authors were working at Amgen at the time the studies were performed.

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REVISED **Amendments from Version 1**

Materials and Methods: We added more details to the GPR21 KO mice generation paragraph. In 'Microarray and data analysis' we added: "Tissue samples from the Deltagen Gpr21 knockout mice were used for microarray analysis. These samples were collected by Gardner *et al.* during the course of their Gpr21 knockout mice study".

In both OGTT and serum insulin measurement paragraphs we have now added some details, and a statistical analyses paragraph.

Results: We added the analysis of liver and BAT Rabgap1 mRNA expression levels. In the manuscript it now reads: "Also, Rabgap1 mRNA expression levels were assessed using 2 Taqman probes (Table 1) that amplified different regions of Rabgap1 in liver and BAT of KO and their wildtype littermate mice. One primer/probe set spanned Rabgap1 exon 3 and 4 (Table 1), which is located upstream of the Gpr21 gene. Another primer/probe set spanned Rabgap1 exon 17 and 18, which is located downstream of the Gpr21 gene (Table 1). Rabgap1 mRNA expression levels in liver and BAT of Gpr21 KO mice were not changed compared to their wildtype littermate mice with the upstream primer/probe set (Figure 1C), however it was dramatically decreased in liver and BAT of Gpr21 KO mice compared to their wildtype littermate mice with the downstream primer/probe set (Figure 1C)".

Next Steps: we added in the text "In any work using genetically manipulated animal models, it is critical to demonstrate that the targeted manipulation behind the biological differences is being explored. While this should be obvious and generally assessed, this study illustrates one of the numerous ways in which scientists may be misled – changes in expression or function of other genes near the targeted gene. An analysis of the expression or function of nearby genes may be a general recommendation that could be made for all KO studies.

See referee reports

Introduction

The G-protein receptors (GPCRs) are the largest family of proteins targeted by drug discovery. GPCRs are crucial molecular sensors for many vital physiological processes. GPR21 is part of the GPCRs family and shares 71% identity to GPR52. It was identified along with GPR22 and GPR23 based on their homology to GPR20 (O'Dowd *et al.,* 1997). Originally, GPR21 was detected in regions of the brain and later, several other tissues as spleen, brown fat, and macrophages, were reported to express high levels of GPR21 mRNA (Gardner et al., 2012; Osborn et al., 2012). The natural ligand of GPR21 remains unknown; however, constitutive activity of the GPR21 receptor has been observed when it was co-transfected with Gα15/16 proteins in HEK293 cells (Gardner *et al.,* 2012; Xiao *et al.,* 2008). Also, GPR21 has been reported to activate the Gq pathway on calcium-sensitive CHO cells (Bresnick *et al.,* 2003).

In *Gpr21* KO mice generated with *Gpr21* KO ES cells obtained from Deltagen (Deltagen GPR21, Deltagen San Mateo, CA), Osborn *et al.* and Gardner *et al.* have reported that glucose tolerance and insulin sensitivity were improved when compared to their wildtype control mice (Gardner et al., 2012; Osborn et al., 2012). These *Gpr21* KO mice were leaner than their wildtype littermate control (Osborn *et al.*) and were resistant to diet-induced obesity (Gardner *et al.,* 2012), making GPR21 a potential drug target candidate for the treatment of diabetes and obesity. Reduced inflammation and macrophage infiltration were also observed in the KO mice (Osborn *et al.,* 2012).

Mouse *Gpr21* gene is located on chromosome 2 within the intron of *Rabgap1* gene, between exon 13 and 14 according to the UCSC GRCm38/mm10 assembly. *Strbp* gene is located on the opposite strand in the same region. Deltagen *Gpr21* KO mice contain a deletion in the gene of exon one with the insertion of a 5.3 kb lacZ/Neo cassette. After considering the location of the insertion of the neo cassette, we hypothesized that the gene structures, the expression and physiological functions of *Rabgap1* and Strbp may have been altered.

In brief, small RAB GTPases are essential for the coordination of vesicle budding, transport, and fusion of vesicles (Frasa *et al.,* 2012). RAB proteins are activated by guanine nucleotide exchange factors (GEFs) and inactivated by RAB GTPase activating proteins (RABGAPs). The TBC (TRE2-BUB2-CDC16) domain facilitates the RAB GTP hydrolysis from the GTP-bound active form to the GDP-bound inactive form. However, the physiological function of RABGAP1 (TBC1D11) is less understood. It may be implicated in microtubule and Golgi dynamics during cell cycle and regulation of spindle checkpoint (Cuif *et al.,* 1999; Miserey-Lenkei *et al.,* 2006). STRBP (SPNR) is a microtubule-associated RNA-binding protein localized in developing spermatids and plays an important role in normal spermatogenesis and sperm function (Pires-daSilva *et al.,* 2001). *Strbp* deficient mice are smaller, have neurological defects, a high premature mortality rate, show reduced fertility and mating drive as well as abnormal sperm motility.

After further analysis of the Deltagen *Gpr21* KO mice, we observed that *Rabgap1* mRNA expression levels were modified. To assess if the metabolic phenotype observed in these KO mice (Gardner *et al.,* 2012; Osborn et al., 2012) was solely related to knocking out the *Gpr21* gene, we generated a new line of *Gpr21* KO mice using the TALENS technology. We created a 29 bp deletion within the coding exon of *Gpr21* (*Gpr21* TAL 29bp), a location very close to the ATG, an out of frame mutation and an early termination of *Gpr21*. The phenotypic analysis of our new *Gpr21* TAL 29bp KO mice showed no improvement of the previously observed metabolic parameters that were identified in Deltagen *Gpr21* KO mice. The originally published improved metabolic phenotype of the Deltagen *Gpr21* KO mice was not solely due to the deletion of the *Gpr21* gene, *Rabgap1*may have been implicated.

Materials and Methods

Animals and *Gpr21* (*Gpr21* TAL 29bp) KO mice generation

All animal experiments were approved by the Institutional Animal Care and Use Committee of Amgen. Mice were housed in a pathogen-free facility with a 12 h light-dark cycle at 22°C. Mice were allowed ad libitum access to water and food. Single housed male *Gpr21* KO (*Gpr21* TAL 29bp) and their male littermate mice were used in this study. Mice were fed a normal chow (Harlan 2920) until they were 11 weeks old and then a high fat diet (Research Diets D12451,45 kcal % fat) for the next 15 weeks.

GPR21 KO mice were created using a pair of transcription activator-like effector nucleases (TALENs) from Life Technologies targeting exon 2 of mouse GPR21. TALEN binding sites are underlined below with a 15 base pair spacer between the 2 sites.

5' - TGAACTCCACCTGGGATGG **TAATCAGAGCAGCCA** TCCTTTCTGTCTTCTGGCA

ACTTGAGGTGGACCCTACC **ATTAGTCTCGTCGGT** AGGAAAGACAGAAGACCGT – 5'

Design, cloning and validation of the TALENs were performed by Life Technologies. Messenger RNA (provided from Life Tech) for each of the TALENs were diluted in RNAse free microinjection buffer to a final concentration of 4.0 ng/µl for each TALEN (8.0 ng/µl total concentration). The TALENs were microinjected into the pronucleus of fertilized one-cell embryos (0.5 days post coitus) obtained from the mating of C57BL/6 (Taconic) males to superovulated C57BL/6 (Taconic) female mice. Microinjected eggs were transferred to pseudopregnant Swiss Webster recipients. Founder pups were screened for TALEN induced mutations in GPR21 by sequencing across exon 2. Two founders, one with a 5 bp deletion and the other with a 29 bp deletion were expanded for further analysis.

Genomic DNA preparation and PCR genotyping

Genomic DNA was prepared from liver, BAT and spleen using DNeasy blood and tissue kit (Qiagen, Valencia, CA) following manufacturer's instruction. PCR were carried out using the primers 5'-CAGCATGAAGTGAGAGCCAG-3' and 5'-CAAGTAGCCCAGTGCCAGAAG-3'.

Microarray and data analysis

Tissue samples from the Deltagen *Gpr21* knockout mice were used for microarray analysis. These samples were collected by Gardner *et al.* during the course of their *Gpr21* knockout mice study. mRNA was isolated from 6 animals for each group using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) and processed following the protocols described in section 2 (Eukaryotic Sample and Array Processing; 701024 rev 1) of the Affymetrix Technical manual. Briefly, 5 µg total RNA was used to synthesize cDNA (10 pmol of T7-(dT)24 primer, and Superscript II (Invitrogen, Carlsbad, CA). Purified double-stranded cDNA (MinElute Reaction Cleanup Kit, Qiagen, Valencia, CA) was used to generate biotinylated cRNA using Bioarray HighYield RNA Transcript labeling Kit (Enzo Diagnostics, Farmingdale, NY) followed by purification with Qiagen RNeasy Mini kit and hybridization to the Affymetrix HT MG 430 PM array. Arrays were washed on a GeneChip Fluidic Station 450 (EukGE_WS2v4_450 protocol) and scanned using the Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA). Data analysis was conducted with R (version 2.15, http://r-project.org) with Bioconductor (version 2.10, http://bioconductor.org/) and ArrayStudio (Omicsoft, version 8.0). Briefly, Affymetrix CEL files were normalized in Bioconductor using the GCRMA method. Differentially regulated genes were identified using a moderated t-test. False discovery rate adjusted P-values were calculated using the method of Benjamini and Hochberg.

RNA isolation and expression assays

Total RNA was isolated using Qiagen midi RNA preparation kit (Qiagen, Valencia, CA). The total RNA concentration was determined with a Nanodrop (ThermoFisher Scientific, Wilmington, DE). QPCR was performed using 10 ng RNA per well, Taqman master mix (Applied Biosystems, Foster City, CA). *Rabgap1* gene expression was assessed using Taqman Probes from Applied Biosystems (Mm01327207_m1 and Mm01327199_m1). Gpr21 mRNA level was measured using the forward primer 5'-CACCTGGGATGGTAATCAGAG-3', reverse primer 5'- TCACAATGATGTTGCCAGAAAT-3' and probe 5'

FAM/TTCTGGCAC/Zen/TGGGCTACTTGGAAA/IABkFQ-3' from Integrated DNA Technologies (Coralville, IA). Results were evaluated using the $\Delta\Delta C_T$ method and normalized relative to the expression of glyceraldehyde-3-phosphate dehydrogenase *(Gapdh)*.

Oral glucose (OGTT) tolerance test

17 *Gpr21* TAL 29bp knockout and 17 wildtype littermate mice were used in this experiment. Glucose, body weight and OGTT were measured on 11 week old mice fed a normal chow diet. Then, mice were switched to high fat diet (HFD) feeding. Two more glucose, body weight and OGTT measurements were performed on 15 and 26 week-old mice (fed HFD for 4 and 15 weeks, respectively). At 6 am, mice were fasted for 4 hr. Glucose levels were measured and blood samples were taken from the tail vein before oral glucose tolerance test (OGTT) was initiated. GTT was performed by oral administration of a bolus glucose (2g/kg body weight). Glucose levels were measured at 20, 40 and 60 min after glucose administration by using AlphaTrak blood glucose meter (Abbott, Chicago, IL).

Serum insulin measurement

Blood samples collected before OGTT, were centrifuged at 10000rpm. Serum insulin levels were determined by using Insulin (mouse) ultra-sensitive EIA kit 80-INSMSU-E10 or mouse high range insulin ELISA 80-INSMSH-E01 (ALPCO Diagnostics, Salem, NH).

Statistical analyses

Two-way ANOVA followed by Bonferroni was used to compare more than two groups. For comparison between two groups, unpaired two-tailed t test was performed. All tests used the software GraphPAD Prism (GraphPad, San Diego, CA). Significance was defined as *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

Results and discussion

Rabgap1 expression was changed in Deltagen *Gpr21* KO mice

We isolated RNA from spleen, liver, perirenal fat (WAT) and brown fat (BAT) from Deltagen *Gpr21* KO mice and their wildtype littermate control mice. Microarray results identified that *Rabgap1* was the only gene that was changed in all the tissues analyzed. Rabgap1 mRNA levels were increased by 1–4 fold when using two independent *Rabgap1* probes (Figure 1A, Figure 1B, Table 1) located upstream of *Gpr21* gene and were down regulated by

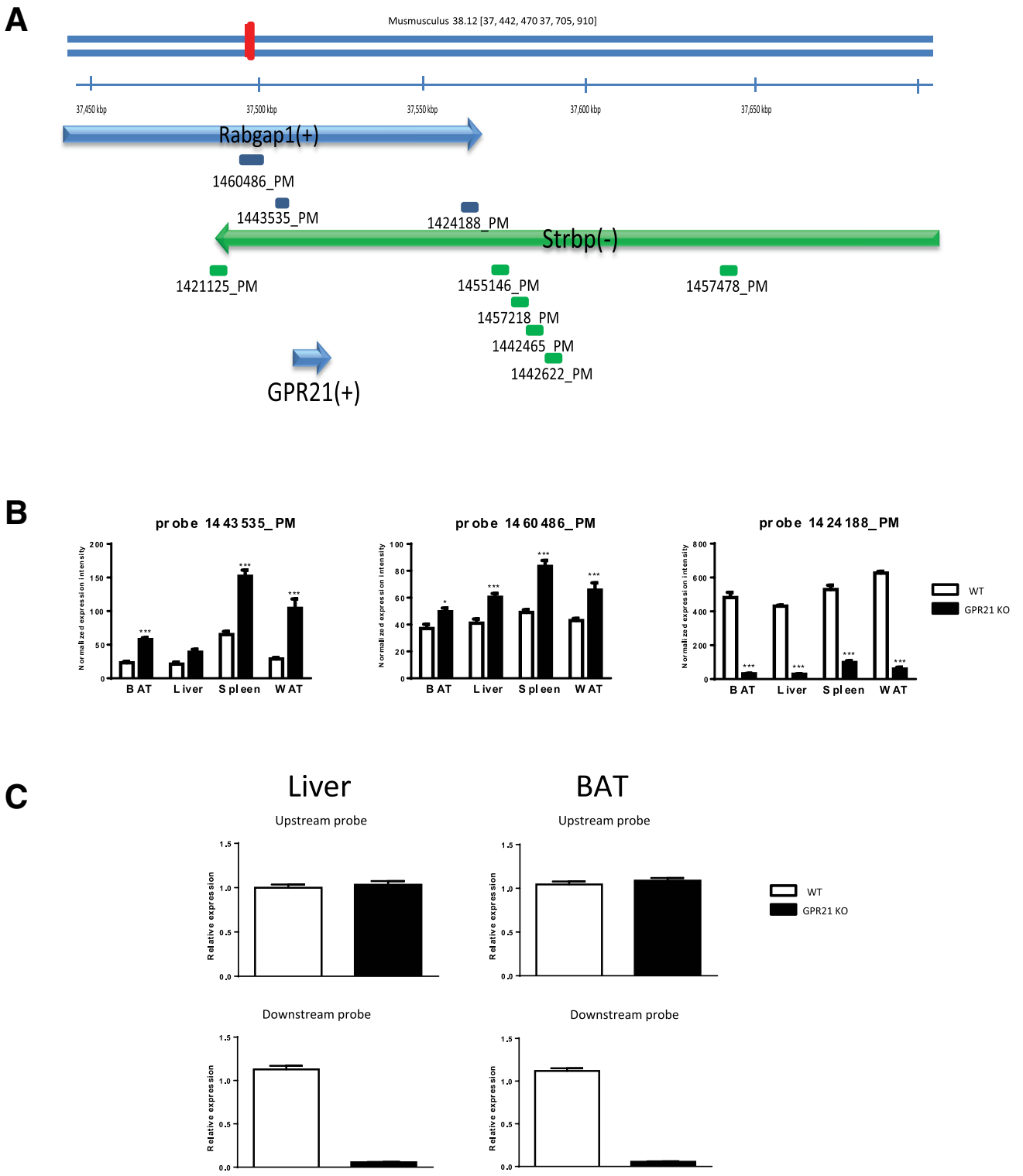

Figure 1. (**A**) Mouse *Gpr21* is located on Chromosome 2 within the intron of *Rabgap1* gene between exon 13 and 14 on the positive strand according to UCSC GRCm38/mm10 assembly. *Strpb* gene is on the opposite strand in the same region. The blue arrow represents the positive strand while the green one the negative strand. The bars under the genes represent microarray probe sets from Affymetrix mouse array HT MG-430PM platform. There is no probe set covering *Gpr21* gene. The closest probe set 1421125_PM is located at 2,866 bases upstream of *Gpr21*. (**B**) The level of *Rabgap1* transcript was shown as normalized expression intensity. RNA was prepared from BAT, liver, spleen and WAT of Deltagen *Gpr21* KO mice and their WT littermate controls. Probe 1443535_PM, 1460486_PM and 1424188_PM allow detection of *Rabgap1* mRNA expression levels. (**C**) *Rabgap1* mRNA expression levels were assessed using 2 Taqman probes in liver (left panel) and BAT (right panel) of GPR21 KO and their wildtype littermate mice.

Table 1. Sequences of the different probes used.

Primer/probe	Sequence (5'-3') or product No
genotyping forward primer	CAGCATGAAGTGAGAGCCAG
genotyping reverse primer	CAAGTAGCCCAGTGCCAGAAG
Gpr21 qPCR forward primer	CACCTGGGATGGTAATCAGAG
Gpr21 qPCR reverse primer	TCACAATGATGTTGCCAGAAAT
Gpr21 qPCR probe	FAM/TTCTGGCAC/Zen/TGGGCTACTTGGAAA/IABkFQ
Rabgap1 primer/probe set 1	Applied Biosystems, Mm01327207_m1
Rabgap1 primer/probe set 2	Applied Biosystems, Mm01327199_m1
Strbp primer/probe set	Applied Biosystems, Mm00486379_m1
microarray probe 1460486_PM	**Probe sequence (5'-3')**
	GACAAAAGTTCGAGTGTGCTCACCT
	GTTCGAGTGTGCTCACCTAATGAAA
	GTGTGCTCACCTAATGAAAGGTTAT
	CCCTTCAGCAAACGAAGCACTACTG
	AGCACTACTGAAAACTTCTTTCTGA
	ATATGAAGTTGTGTGTTTGGAGAGT
	GAAAACCACAGCCAGTCCTTCAGTT
	TTCAGTTCGCCTGCCACAGTCTGGA
	GATAATGATGAACCTCTCTTGAGTG
	TGAACCTCTCTTGAGTGGATTTGGG
	GGGATGTATCCAAAGAATGTGCAGA
microarray probe 1443535_PM	**Probe sequence (5'-3')**
	GAAATTAAAGCTATGTGACCACCCC
	AAACATTTCCATTCCATCTGTCAAA
	GGCTAAGAAGTTCCAGGGTTTCCTG
	CAGGGTTTCCTGCATTCCAAGAATG
	TTGTTTAACCCACAGAAGTTTTATG
	GTTTTATGTCATTTAGCCTGGTCTA
	AAAAGCTTGGGATCAGAACTGTTTC
	ATGGTTTTGTCTGTCTTGGTTTGAT
	TGTTGACTTATCAGTTAAACCACCA
	GAAACCTTAGGCTATTGCAAGACTT
	ATGCATACCTAGTTATTGCAGCTTC

Primer/probe	Sequence (5'-3') or product No
microarray probe 1424188_PM	**Probe sequence (5'-3')**
	GAAAGTCCCTACACACTGTAAAGTC
	TAAAGTCCTACTTTCCTGGCTGGAT
	GGCTGGATCTCTGTCAGGCCTCTGA
	CAGGTGTACATCTCACTGGTCAGGT
	GAAAATGGCAGTTTTAGCACCTTTT
	AGTGGTGTCACAAGTGGCTCATCCT
	CTCTGTGTGCAGGTAGCTTGGGTTT
	GTTGGCTTTTCTAATGCTTGATGAG
	TCTGTCTCGTTCAGTTAACCCAAAC
	AACCCAAACAGTATAAGCCCATCTT
	TGGACATTGTGTGCTAGGGTAGTTT

5–15 fold when using one *Rabgap1* probe located downstream of *Gpr21* gene. This result indicated that the genetic modifications in the original KO line modified Rapgap1 mRNA expression levels (Figure 1B). *Strbp* mRNA expression levels were not changed when using multiple probes that were located downstream of the *GPR21* gene (Figure 1A and data not shown). Also, *Rabgap1* mRNA expression levels were assessed using 2 Taqman probes (Table 1) that amplified different regions of *Rabgap1* in liver and BAT of KO and their wildtype littermate mice. One primer/probe set spanned *Rabgap1* exon 3 and 4 (Table 1), which is located upstream of the *Gpr21* gene. Another primer/probe set spanned *Rabgap1* exon 17 and 18, which is located downstream of the *Gpr21* gene (Table 1). *Rabgap1* mRNA expression levels in liver and BAT of *Gpr21* KO mice were not changed compared to their wildtype littermate mice with the upstream primer/probe set (Figure 1C), however it was dramatically decreased in liver and BAT of *Gpr21* KO mice compared to their wildtype littermate mice with the downstream primer/probe set (Figure 1C).

Generation of *Gpr21* specific KO (*Gpr21* TAL 29bp) mice

A new line of *Gpr21* KO mice (*Gpr21* TAL 29bp) was created by deleting a 29 bp within the coding exon of *Gpr21* gene. Using the TALENS technology, a 29 bp very close to the ATG codon was deleted, thus causing an out of frame mutation and early termination of *Gpr21* gene (Figure 2A). Homozygous *Gpr21* KO mice genotype was confirmed by PCR using primers located upstream and downstream of the 29bp deletion from genomic DNA of several tissues (Figure 2B). As predicted, a 100 bp band was identified for the wildtype mice and a 71 bp band for the *Gpr21* TAL 29bp homozygous KO mice (Figure 2C). The PCR fragment was sequenced and a 29 bp deletion was confirmed. From RNA isolated out of BAT and liver, no detectable *Gpr21* mRNA levels were identified in *Gpr21* TAL 29bp KO mice using qPCR and *Gpr21* probes that are located around the 29 bp deletion region (Figure 2D). Similar mRNA levels were detected using primer/probe that were located downstream of 29bp deletion in exon 2 (data not shown). The result confirmed that *Gpr21* transcripts in *Gpr21* TAL 29bp KO mice had the 29bp deletion.

Rabgap1 and *Strbp* expression levels are not affected in *Gpr21* TAL 29bp KO mice

Rabgap1 mRNA expression levels were assessed using 2 Taqman probes (Table 1) that amplified different regions of *Rabgap1* in liver and BAT of KO and their wildtype littermate mice. One primer/probe set spanned *Rabgap1* exon 3 and 4 (Table 1), which is located upstream of the *Gpr21* gene. Another primer/probe set spanned *Rabgap1* exon 17 and 18, which is located downstream of the *Gpr21* gene (Table 1). *Rabgap1* mRNA expression levels in *Gpr21* TAL 29bp KO mice were not changed compared with their wildtype littermate mice with both primer/probe sets (Figure 3A). Liver and BAT *Strbp* mRNA expression levels were also not changed between *Gpr21* TAL 29bp KO mice and their wildtype littermate mice (Figure 3B). One of the limitations of our study is that we have not measured GPR21 protein levels.

Gpr21 TAL 29bp did not show improvements in glucose and insulin metabolism

The body weight, OGTT and insulin levels of *Gpr21* TAL 29bp KO mice fed a normal chow were not different from the ones of their wildtype littermates (Figure 4 A–C). Mice were then fed with a 45% high fat diet to induce obesity and insulin resistance. After 4 weeks and 15 weeks of high-fat feeding, *Gpr21* TAL 29bp KO mice gained similar body weight to that of their wildtype littermates, showed no difference on glucose tolerance and fasting blood glucose and insulin levels were not different from their wildtype littermates, Figures 4 D–I, respectively.

Next Steps

The results of Osborn and Gardner suggest that GPR21 may play an important role in regulating body weight and glucose

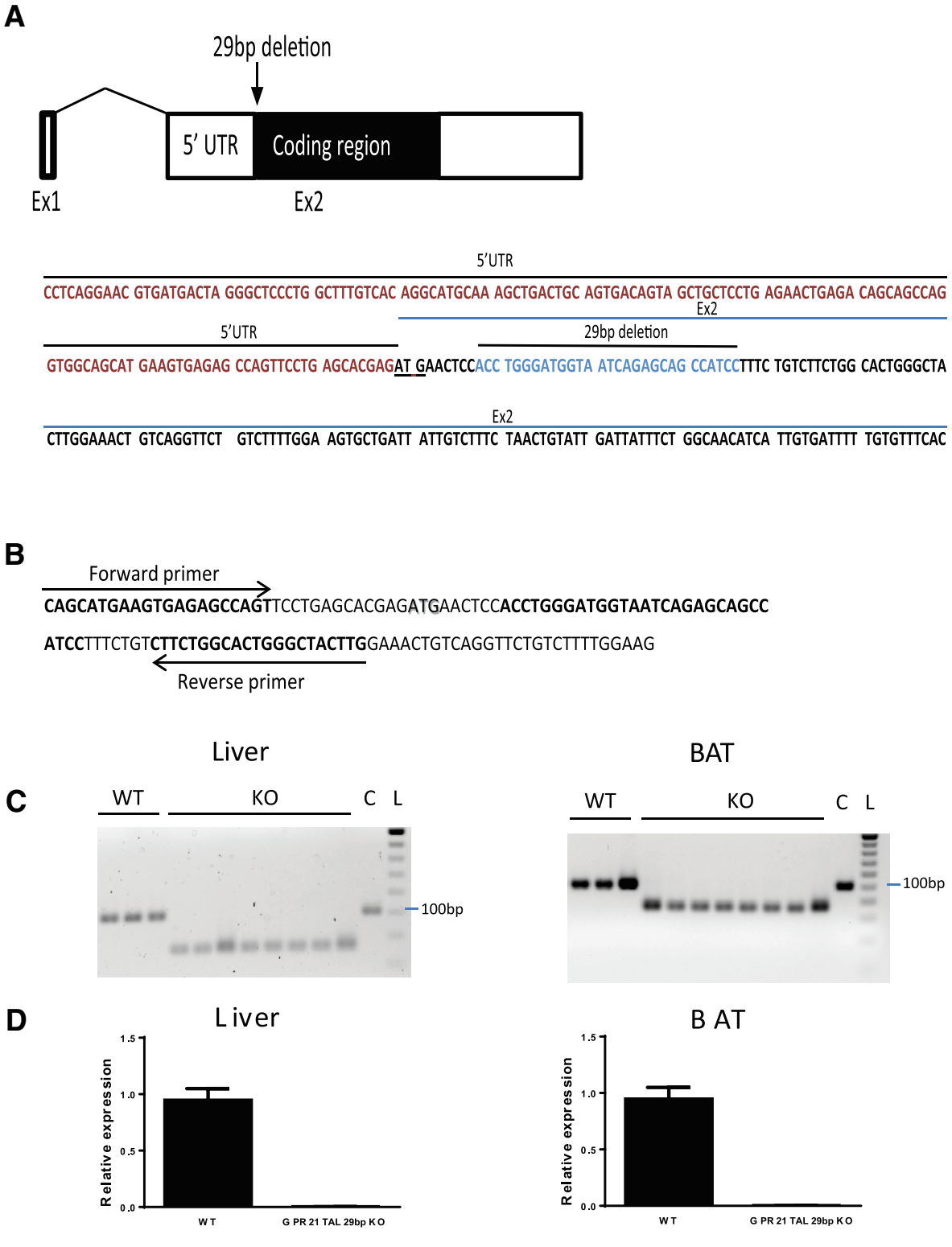

Figure 2. (**A**). Sequence and location of the 29 bp deletion in *Gpr21* TAL 29bp KO mice. (**B**). sequence and location of genotyping primers. (**C**). Genotyping of *Gpr21* TAL 29bp KO mice. Genomic DNA was generated from liver and BAT. PCR with genotyping primers amplified a 100 bp fragment from the genome of WT littermate mice and a 71 bp fragment from homozygous *Gpr21* TAL 29bp KO mice. C: commercial mouse genomic DNA. L: 20 bp DNA ladder. (**D**). No wildtype *Gpr21* transcript were detected. qPCR analysis of *Gpr21* gene in liver and BAT using primer/probe set that located in the 29 bp region and only detect wildtype *Gpr21* transcript.

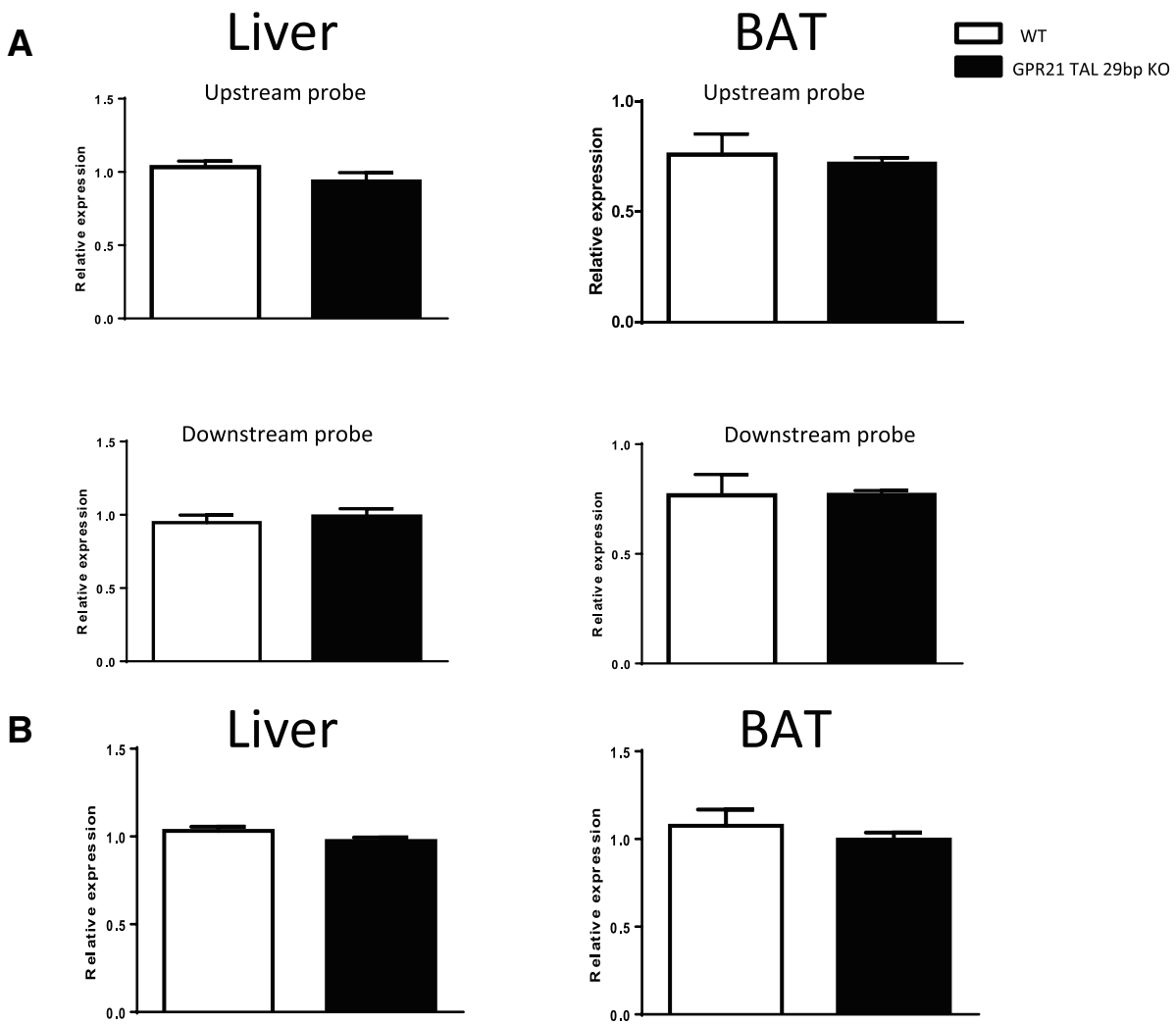

Figure 3. (**A**) *Rabgap1* mRNA expression levels were assessed using 2 Taqman probes in liver (top left panel) and BAT (top right panel) of GPR21 TAL 29 bp KO and their wildtype littermate mice. (**B**) Liver (bottom left panel) and BAT (bottom right panel) strbp mRNA expression levels were assessed in wildtype and *Gpr21* TAL 29bp KO mice.

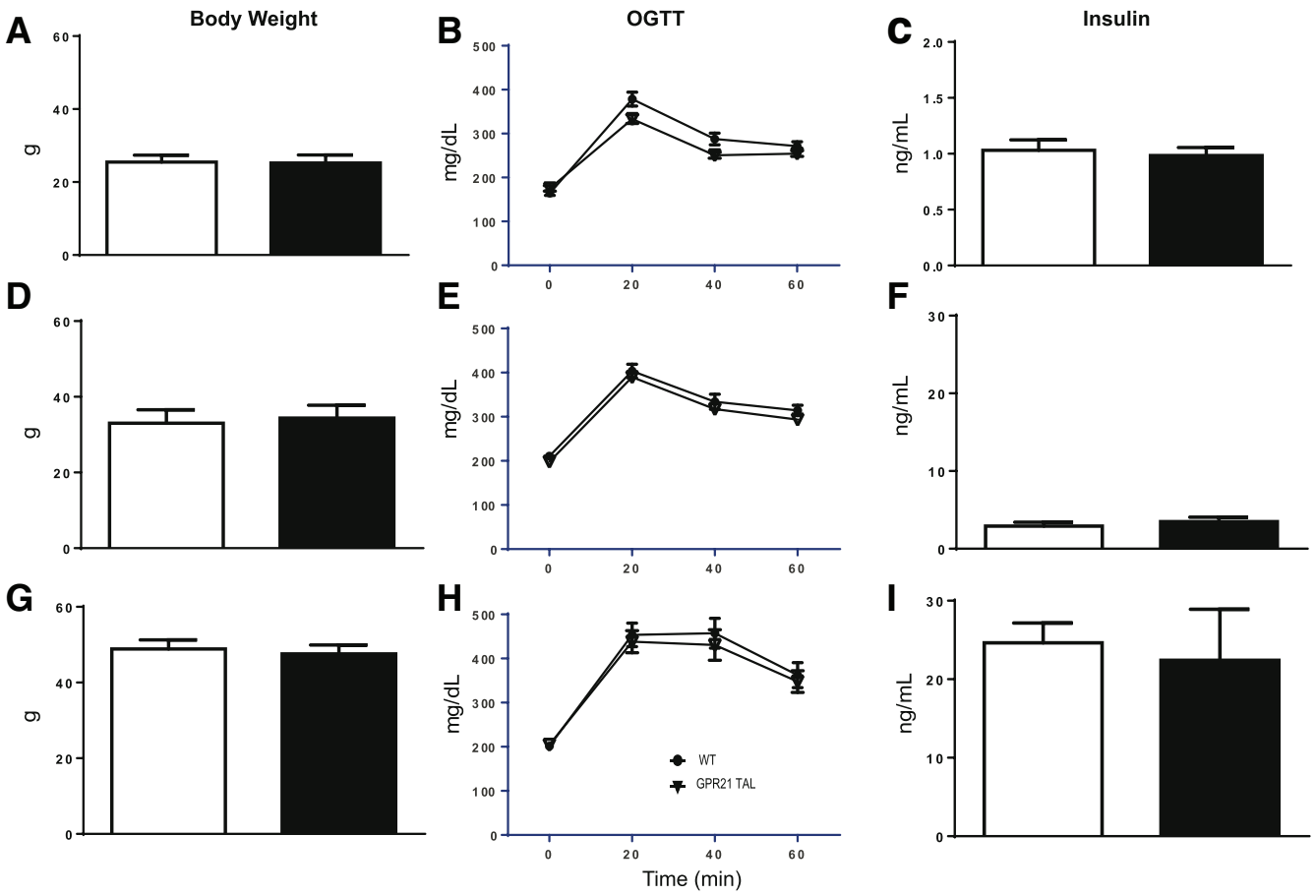

Figure 4. At 11 weeks of age, body weight (Figure **4A**), OGTT (Figure **4B**) and insulin levels (Figure **4C**) were measured in wildtype (open bar and open circle) and *Gpr21* TAL 29bp KO Mice (filled bar and open triangle) fed a normal chow diet. At 15 weeks of age and at 26 weeks of age body weight (Figure **4D** & **4G**), OGTT (Figure **4E** & **4H**) and insulin levels (Figure **4F** & **4I**) were measured in wildtype (open bar and open circle) and *Gpr21* TAL 29bp KO Mice (filled bar and open triangle) fed a high fat diet for 4 weeks and 15 weeks, respectively.

metabolism. However, in our attempts presented here to confirm their findings we didn't see the same effect. We would therefore like to encourage an open discussion and collaborate with Osborn and Gardner as well as others in the wider community to further elucidate the potential effectiveness of pharmacologically inhibiting GPR21. In any work using genetically manipulated animal models, it is critical to demonstrate that the targeted manipulation behind the biological differences is being explored. While this should be obvious and generally assessed, this study illustrates one of the numerous ways in which scientists may be misled – changes in expression or function of other genes near the targeted gene. An analysis of the expression or function of nearby genes may be a general recommendation that could be made for all KO studies.

Author contributions

JW: study design, performed some experiments, wrote the manuscript; ZP: study design, performed some experiments; HB: study design; DC: generated TALENS KO mice, edited manuscript; CG: performed some experiments; MV: study design, wrote and edited the manuscript.

Competing interests

All authors were working at Amgen at the time the studies were performed.

Grant information

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Current Referee Status: ? ✓ ?

Version 2

Referee Report 24 June 2016

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Mary Pelleymounter
Office of Translational Research Program (OTR), National Institute of Neurological Disorders and Stroke (NINDS), Bethesda, MD, USA

In this first revision of the manuscript, “GPR21 KO mice demonstrate no resistance to high fat diet induced obesity or improved glucose tolerance”, the authors have addressed many of the concerns related to the addition of detail to the Materials and Methods section. However, the authors have not addressed the potential impact of differences in methodology between their physiological assessments and those of Gardner, *et al* and Osborne, *et al*. For example, they did not verify GPR21 KO in hypothalamic or brain tissue and they did not attempt to replicate the gender, diet or even OGTT methods from either study. Instead their methods were somewhat of a hybrid of the Gardner and Osborne studies and did not conduct any form of in-depth metabolic analysis. Therefore, although their data certainly does suggest that the Deltagen KO strategy did involve changes in Rabgap1 expression, it is difficult to claim that Rabgap1 reduction explained the phenotype of the Deltagen GPR21 KO mice if the physiological measures were not obtained in the same way as the two studies in question. For these reasons, it would seem that the authors should discuss the caveats to their own study in the Next Steps section along with the suggestion to other investigators that GPR21 KO may not have been responsible for the metabolic phenotype observed by Gardner *et al* and Osborne, *et al*.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Version 1

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The article by Wang and colleagues addresses the resistance of GPR21 knockout mice to insulin resistance and high-fat diet induced obesity and metabolic dysregulation. Two previous publications using a different GPR21 knockout mouse model generated with ES cells from Deltagen have been previously published: Osborn *et al*. (2012) JCI 122: 2444-2453; and Gardner *et al*. (2012) BBRC 418: 1-5. Osborn *et al*. demonstrated that, at a baseline of 8 weeks, GPR21 KO mice were significantly lighter than their wild-type controls despite similar food intake, and after 11 weeks on normal chow had significantly improved insulin sensitivity and glucose tolerance. In addition, GPR21 KO mice fed a 60 kcal% HFD for 12 weeks showed no differences in % weight gained or adiposity on the diet, but maintained a significantly improved glucose tolerance and insulin sensitivity. Gardner *et al*. demonstrated that, at baseline of 120 days, GPR21 KO mice were lighter, again despite similar food intake, and after 12 weeks on a 45 kcal% diet, gained significantly less weight and adiposity and maintained better insulin sensitivity and glucose tolerance than the wild-type controls. In contrast, Wang and colleagues find no differences in body weight, adiposity, or glucose and insulin tolerance between their GPR21 knockout mice (generated using TALEN technology) and wild-type controls; an important finding they suggest is due to unintentional changes in RabGAP1 gene expression in the original Deltagen GPR21 knockout mice.

General Comments:
1) The aim of the current work, as stated in the last paragraph of the introduction, was to show that the effects observed in Osborn and Gardner were not solely due to loss of GPR21. I believe the authors have essentially demonstrated this, and the title of the article is appropriate and reflective of this. Yet, much of the abstract, introduction and results sections focus on RabGAP1 expression. More confirmatory experiments seem necessary to implicate changes in RabGAP1 in the Deltagen GPR21 knockout phenotype (see specific comment 2).

2) As a pure replicative paper, the authors did not directly replicate either of the original HFD studies performed in Osborn or Gardner. The 45 kcal% HFD protocol was closer to that described in Gardner, yet the mice were started on the HFD 6 weeks earlier (presumably at 11 weeks based on figure legends instead of ~17 weeks in Gardner) and continued on the diet 3 weeks longer. The statement in the "Next Steps" should be modified as such.

Specific Comments:
1) In the Materials and Methods section, the description of the high-fat diet experiments are not detailed enough. This is important as the high fat diet experiments in the original referenced publications are actually quite different from each other. Furthermore, this would allow other investigators to more easily replicate the diet study.

2) The authors propose that changes in RabGAP1 expression might be responsible for the phenotypes observed in Gardner and Osborn. This conclusion would be stronger with qPCR analysis of the original Deltagen mouse tissues (such as in Figure 3, which was performed for the GPR21 TALEN KO mice) instead of reliance on microarray probe data. In such a case, a direct comparison of RabGAP1 expression could be made between the Deltagen and TALEN GPR21 knockout mice.

3) Hypothalamic GPR21 expression was proposed as responsible for phenotype of mice on normal chow observed in Osborn *et al*, and GPR21 is highly expressed in the hypothalamus. I would recommend adding this tissue to the panel analyzed by qPCR in Figure 3.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Referee Report 07 March 2016

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The title is appropriate.

The design, methods, and analysis are generally complete and appropriate with the following exceptions:

1. The methods to obtain data from the Deltagen GPR21 mutant mice are not sufficiently described. The source of the mice, genetic, background, and backcross history are not included in the methods. Since genetic background can profoundly change biological phenotypes, this information is essential to fully understand the differences between the Deltagen GPR21 KO and the new TALEN GPR21 KO mice described here.

2. The full sequence of the PCR product across the 29 bp deletion should be shown along with an alignment with the Gpr21 gene sequence to ensure that the deletion is indeed within the Gpr21 gene.

3. To definitively establish that the knockout does disrupt Gpr21, it would be helpful to additionally demonstrate reduced expression of the Gpr21 protein. This could be done by antibody detection methods, mass spec, or other functional assessments of Gpr21 activity.

Minor correction suggested:

1. Page 3 – "*Strbp* … probes that were located upstream and downstream of the *Strbp* gene … " should probably read "downstream of the *Gpr21* gene …".

The conclusions require modification and they should also be expanded to reflect broader implications of this study.

1. The description of results in the "Next steps" paragraph should be amended. It is important in our discussions of "replication" that scientists be precise. *The term replication should be used when the precise methods and reagents were used for the follow-on studies.* This work does not "replicate" the original study but attempts to "confirm" or "extend" it using a different approach. Clearly robust scientific results must not only be replicable but also must be able to be confirmed or extended to have real value in advancing our understanding of biological results or drug action. I suggest the following. Replace "replicate their findings" with "confirm their findings using a Gpr21 KO mouse generated with a different technology".

2. The limitation that the Gpr21 protein levels were not assessed should be included in the discussion unless data are shown to address that limitation.

3. It would also be valuable to note a more general conclusion from this study. In any work using genetically manipulated animal models, it is critical to demonstrate that the targeted manipulation is behind the biological differences being explored. While this should be obvious and generally assessed, this study illustrates one of the numerous ways in which scientists may be misled –

changes in expression or function of other genes near the targeted gene. An analysis of the expression or function of nearby genes may be a general recommendation that could be made for all KO studies.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

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Dr. Wang and colleagues have provided intriguing evidence to suggest that the metabolic phenotype of the Deltagen GPR21 KO mouse may not be completely due to deletion of the GPR21 gene. Rather, these authors suggest that the metabolic phenotype of the Deltagen GPR21 KO mouse could have been a function of the location of the 5.3 kb lacZ/Neo cassette within exon 1, which could have altered the expression and function of Rabgap1 and Strbp. Indeed, the authors did show that Rabgap1 was altered in brown and white adipose tissue, liver and spleen of the Deltagen GPR21 KO mice. In order to understand whether the metabolic phenotype of the Deltagen GPR21 KO mice was truly due to deletion of GPR21 or an artifact of the neo cassette location in the Deltagen mice, this group used a different strategy to delete GPR21 which did not involve Rabgap1 or Strbp. The KO mice generated as a result of this alternate GPR21 deletion strategy (GPR21 TAL29bp KO) did not demonstrate the high fat diet resistance phenotype observed in the Deltagen GPR21 KO mice. In addition, while there were no differences in Rabgap1 mRNA or strbp mRNA in the liver or brown adipose tissue of the GPR21 TAL 29bp KO mice, there was clear deletion of GPR21 in the same tissues of these mice.

Although this information is quite important for the research area of obesity and diabetes therapeutics in general, there are some aspects of the experimental design and manuscript narrative that could be strengthened in order to provide more confidence in the findings.

1. It is unclear why the authors chose to focus on liver and brown adipose tissue expression levels of Rabgap1 and GPR21. Tissue expression of GPR21 was much more prominent in brain and spleen in both the Gardner *et al*., and Osborn, *et al*., papers which described the high fat diet resistance phenotype in the Deltagen GPR21 KO mice. It seems that there would be even more confidence in the findings of the current manuscript if GPR21 was as dramatically knocked out in brain and spleen by the GPR21 TAL29bp deletion strategy as it was in liver and BAT.

2. The argument that the improved metabolic phenotype in the Deltagen GPR21 KO mice was an artifact of neo cassette location would have been more convincing if the current authors had compared the Deltagen GPR21 KO mice in a "head to head" fashion with the GPR21 TAL29bp KO mice when they evaluated resistance to diet-induced impairments in glucose tolerance. This would have shown that the authors were able to reproduce the metabolic improvements previously observed in the high fat diet-fed Deltagen mice even though the GPR21 TAL29bpKO mice did not show such an improvement under the same experimental conditions.

3. Since it is important to assure that the experimental conditions utilized to assess metabolic phenotype in the current study were similar to those utilized for the Deltagen GPR21 KO mice in other labs, it would have been helpful if the authors had provided more detail about their methodology in the current manuscript. For example, the following points are not clear from the narrative of the manuscript:

   a. What were the housing conditions of the animals (individual vs group-housed?, ambient temperature?
   b. Were the mice all one gender? (the Gardner paper used a mix of male and female, which could have influenced body weight, activity level, etc)
   c. What were the Ns for the OGTT, Insulin and BW data and how were they derived (basis for power analysis)?
   d. What statistical analysis strategy was used to evaluate the data?
   e. Was a baseline insulin level obtained under the conditions used for the OGTT?
   f. Were subsets of animals sacrificed after the respective OGTT and insulin measurements at each time point (11, 15 and 26 weeks)? If so, how was that taken into account in the statistical analysis for data collected over the entire time period (i.e., body weight)?

4. In the “Next Steps” section, the authors suggest that the follow up to their data will be the responsibility of those that read their article. However, their data only opens up the possibility that the metabolic phenotype of the Deltagen GPR21 KO mice is an artifact of neocassette location. In this reviewer’s opinion, the authors still have quite a bit of work to do in order to provide definitive evidence that the Deltagen GPR21 KO mouse phenotype is not correct. It would also be informative to hear the author’s view on the potential role of Rabgap1 in insulin sensitivity and resistance.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.