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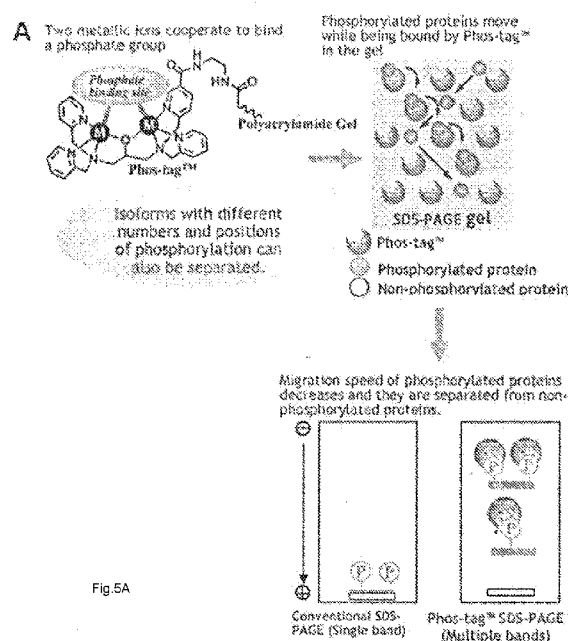


Fig.5A

(57) Abstract: The present invention provides methods for reducing a subject's hepatic and plasma triglyceride levels comprising administering' to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that decreases KCTD17 expression in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

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METHOD FOR PREVENTING OBESITY-INDUCED FATTY LIVER BY INHIBITING KCTD17

This application claims the priority of U.S. Patent Application No. 15/143,305, filed April 29, 2016, the contents of which are hereby
5 incorporated by reference.

This invention was made with government support under grant number DK093604 awarded by the National Institutes of Health. The government
has certain rights in the invention.

10 This application incorporates-by-reference nucleotide and/or amino acid sequences which are present in the file named "170428_0575_89275_A_PCT_SequenceListing_MW.txt," which is 28 kilobytes in size, and which was created April 28, 2017 in the IBM-PC
15 machine format, having an operating system compatibility with MS-Windows, which is contained in the text file filed April 28, 2017 as part of this application.

Throughout this application various publications are referred to by
20 first author and year of publication. Full citations of these references can be found following the Examples. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

BACKGROUND OF THE INVENTION

Obesity has reached epidemic status in the United States – the Centers for Disease Control has stated that more than 1/3 of American adults are obese, and estimated the medical costs attributable to obesity at
30 \$147 billion in 2008, a number that is likely to be significantly higher today and in the future.

Obesity-induced metabolic dysfunction manifests as multiple chronic medical conditions, including Type 2 Diabetes (T2D) and Nonalcoholic
35 fatty liver disease (NAFLD) (Ford et al., 2002). NAFLD is the result of compensatory hyperinsulinemia and hepatic de novo lipogenesis. NAFLD contributes to the overall cardiovascular risk of obesity

(Villanova et al., 2005), but is also the most common chronic liver disease, predisposing to cirrhosis and hepatocellular carcinoma (Dowman et al., 2011).

5 Obesity leads to insulin resistance, which begets the hyperglycemia of T2D. In a parallel but poorly understood process, compensatory hyperinsulinemia drives hepatic de novo lipogenesis, mediated in part by the nutrient-sensitive mechanistic target of rapamycin (mTOR) pathway, which predisposes excessive liver fat or NAFLD. The presence
10 of NAFLD increases underlying insulin resistance - this vicious cycle results in exacerbations of both T2D and NAFLD, which show independent associations with cardiovascular disease and all-cause mortality.

Aging and obesity are well-established risk factors for NAFLD (Slawik
15 and Vidal-Puig, 2006), but the molecular mechanism underlying this risk is poorly defined, precluding specific pharmacologic strategies to target excess hepatic triglycerides (TG).

There is no approved pharmacologic therapy for NAFLD - the only
20 clinical recourse is liver transplantation; current projections suggest that NAFLD will be the leading cause for liver transplantation by 2020, a conundrum as available organs are already limiting. Novel pathways are sought to both further our understanding of the pathophysiology, as well as provide new pharmacologic targets to
25 assist in our management of obesity-related morbidity and mortality.

Thus, new therapies are needed.

SUMMARY OF THE INVENTION

The present invention provides a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that decreases KCTD17 expression
5 in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

The present invention provides a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a
10 subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that increases PHLPP2 in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

15 The present invention also provides a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that increases free Raptor in
20 liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

The present invention also provides a process for determining the amount of free Raptor in a subject's liver comprising:

- 25 a) obtaining a biological sample comprising liver cells of the subject;
- b) separating free Raptor and mTORC1-associated Raptor in the sample; and
- c) determining the amount of free Raptor in the sample.

30 The present invention also provides a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that prevents PHLPP2 degradation
35 in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

The present invention also provides a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that inhibits Glucagon signaling
5 in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

The present invention also provides a process for determining the amount of KCTD17 expression in a subject's liver comprising:

- 10 a) obtaining a biological sample comprising liver cells of the subject;
 b) determining the amount of KCTD17 mRNA in the sample.

The present invention also provides a process for diagnosing whether
15 a subject is afflicted with increased KCTD17 expression comprising:

- a) determining the amount of KCTD17 in the subject according to the process of claim 31;
 b) determining the amount of KCTD17 in a reference subject according to the process of claim 31; and
20 c) diagnosing the subject to be afflicted with increased KCTD17 expression if the amount of KCTD17 expression in step (a) is substantially increased compared to the amount of KCTD17 expression in step (b).

25 The present invention also provides a method of treating a subject afflicted with elevated triglyceride levels comprising administering to the subject a pharmaceutical composition comprising a pharmaceutical carrier and a compound that decreases KCTD17 expression in liver cells in an amount effective to reduce the subject's hepatic
30 and plasma triglyceride levels.

The present invention also provides a method of treating a subject afflicted with elevated triglyceride levels comprising administering to the subject a pharmaceutical composition comprising a
35 pharmaceutical carrier and a compound that decreases PHLPP2 phosphorylation at Serine 1119 and Serine 1210 residues in liver cells

in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

5 The present invention also provides a method of treating a subject at risk of developing elevated triglyceride levels comprising administering to the subject a pharmaceutical composition comprising a pharmaceutical carrier and a compound that decreases KCTD17 expression in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

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The present invention also provides a method of treating a subject at risk of developing elevated triglyceride levels comprising administering to the subject a pharmaceutical composition comprising a pharmaceutical carrier and a compound that increases PHLPP2
15 phosphorylation at Serine 1119 and Serine 1210 residues in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Identification of PHLPP2 protein modification. C57BL/6 mice were injected with GFP (control) or HA/Flag/PHLPP2 adenovirus and sacrificed on day 5 after overnight fast, 2 hour re-fed, or 12 hour re-fed. Immunoprecipitation (IP) with anti-Flag (harsher condition) and liquid chromatography-tandem mass spectrometry (LC/MS-MS) were performed.

10 Figure 2. Phosphorylation sites identified in PHLPP2.

Figure 3. BasePeak Chromatogram. Overlay if base peak chromatogram of trypsin digest of the bottom band (enriched in PHLPP2). To no surprise the dominant peaks are identical between the three samples.

15 Figure 4. Amino acid sequence alignment of PHLPP2 among various species. Site-directed mutagenesis of Ser/Thr to Ala was performed and the phosphorylation of each mutant was tested.

20 Fig. 5A. Schematic of phos-tag-based mobility shift detection of phosphorylated proteins.

Fig. 5B. Phos-tag-based mobility shift detection of phosphorylated proteins in the absence of phos-tag.

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Fig. 5C. Phos-tag-based mobility shift detection of phosphorylated proteins in the presence of 25 μ M phos-tag.

30 Figure 6. Identification of upstream kinases for PHLPP2 phosphorylation.

Fig. 7A. Identification of phosphorylation sites in PHLPP2. Each identified Ser/Thr site from LC/MS-MS-based phospho-peptide mapping results was individually mutated to Alanine, then transfected WT or Ala-mutant PHLPP2 in hepatocytes. Results in the presence of serum.

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Fig. 7B. Identification of phosphorylation sites in PHLPP2. Each identified Ser/Thr site from LC/MS-MS-based phospho-peptide mapping results was individually mutated to Alanine, then transfected WT or Ala-mutant PHLPP2 in hepatocytes. Results in the absence of serum.

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Fig. 8A. Forskolin induced phosphorylation overexpressed PHLPP2 in primary hepatocytes.

Fig. 8B. cAMP activator induces PHLPP2 phosphorylation on two Serine residues. WT, S1119A, and S1210A mutant of PHLPP2 were all phosphorylated with forskolin treatment.

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Fig. 8C. cAMP activator induces PHLPP2 phosphorylation on two Serine residues. Ablation of both sites rendered the S1119A/S1210A mutant insensitive to forskolin-induced phosphorylation.

Fig. 9A. Identification of phosphorylation sites in PHLPP2 by Glucagon/PKA signaling. Forskolin-mediated PHLPP2 phosphorylation.

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Fig. 9B. Identification of phosphorylation sites in PHLPP2 by Glucagon/PKA signaling. Forskolin-mediated PHLPP2 phosphorylation was reduced by pretreatment of PKA inhibitor, H89.

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Fig. 10A. Inhibition of Glucagon signaling increases PHLPP2 protein levels in *db/db* mice. *Glucagon receptor (Gcgr)* expression from livers of *db/db* mice transduced with AAV8-shControl or AAV8-shGcgr.

Fig. 10B. Inhibition of Glucagon signaling increases PHLPP2 protein levels in *db/db* mice. Western blots from livers of *db/db* mice transduced with AAV8-shControl or AAV8-shGcgr.

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Fig. 10C. Inhibition of Glucagon signaling increases PHLPP2 protein levels in *db/db* mice. Quantification of Western blots in Fig. 43B, normalized to either total PHLPP2 or β -actin.

Fig. 11A. Inhibition of Glucagon signaling increases PHLPP2 protein

levels in DIO mice. Western blots from livers of diet-induced obese (DIO) C57BL6 mice transduced with either AAV8-shControl or AAV8-shGcgr.

Fig. 11B. Inhibition of Glucagon signaling increases PHLPP2 protein levels in DIO mice. Quantification of PHLPP2 levels, normalized to β -actin.

Fig. 12A. Generation of PHLPP2 KO cells using Genome-scale CRISPR Knock-Out (GeCKO) system. PHLPP2 KO HepG2 hepatoma cells were generated using the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas9 system. Using three different single guide RNA (sgRNA) for PHLPP2, significantly reduced PHLPP2 protein and mRNA levels

Fig. 12B. Generation of PHLPP2 KO cells using Genome-scale CRISPR Knock-Out (GeCKO) system. Quantification of PHLPP2 levels in Fig. 45A.

Fig. 12C. Generation of PHLPP2 KO cells using Genome-scale CRISPR Knock-Out (GeCKO) system. Quantification of off-target gene expression.

Fig. 13A. Generation of PHLPP2 KO cells using Genome-scale CRISPR Knock-Out (GeCKO) system. HepG2 stable cell lines were generated expressing the most efficient PHLPP2 sgRNA (#3) sgRNA3.

Fig. 13B. Generation of PHLPP2 KO cells using Genome-scale CRISPR Knock-Out (GeCKO) system. Mouse hepatoma cell lines, Hepalclc7 cells, were generated.

Fig. 14A. Characterization of PHLPP2 KO cells. PHLPP2-deficient cells showed sustained insulin signaling.

Fig. 14B. Characterization of PHLPP2 KO cells. PHLPP2 levels "rescued" in CRISPR-induced knockout cell lines with either WT or S1119/1210A PHLPP2.

Figure 15. Identification of novel interaction partners with PHLPP2.

Fig. 16A. Identification of novel interaction partners with PHLPP2 - KCTD17 interaction with PHLPP2. PHLPP2 associated with KCTD17 in a proteasome-dependent manner.

5 Fig. 16B. Identification of novel interaction partners with PHLPP2 - KCTD17 interaction with PHLPP2. Association of endogenous PHLPP2 with KCTD17 was significantly enhanced by treatment of forskolin and simultaneous treatment with forskolin and the proteasome inhibitor, MG132.

10 Figure 17. Identification of novel interaction partners with PHLPP2 - KCTD17 interaction with PHLPP2, phosphorylation-dependent manner.

15 Fig. 18A. Loss of *Phlpp2* causes fatty liver. Hepatic *Phlpp2* expression negatively correlates with hepatic lipid across different BXD populations (Spearman $\rho = -0.60$, $p = 0.04$).

20 Fig. 18B. Loss of *Phlpp2* causes fatty liver. Normalized enrichment score (ES) of GSEA indicating gene sets that show the most significant negative correlation with hepatic *Phlpp2* expression.

Fig. 18C. Loss of *Phlpp2* causes fatty liver. Heat map showing co-regulation of *Phlpp2* and lipogenic genes in BXD strains.

25 Fig. 18D. Loss of *Phlpp2* causes fatty liver. Liver weight in Cre-control and liver-specific *PHLPP2* knockout (*L-PHLPP2*) mice fasted for 18 h and then refed for 8 h (n=9-10/group).

30 Fig. 18E. Loss of *Phlpp2* causes fatty liver. Triglycerides in Cre-control and liver-specific *PHLPP2* knockout (*L-PHLPP2*) mice fasted for 18 h and then refed for 8 h (n=9-10/group).

35 Fig. 18F. Loss of *Phlpp2* causes fatty liver. Western blots of liver lysates in Cre-control and liver-specific *PHLPP2* knockout (*L-PHLPP2*) mice fasted for 18 h and then refed for 8 h (n=9-10/group)

Fig. 18G. Loss of *Phlpp2* causes fatty liver. Western blots of hepatic gene expression in Cre- control and liver-specific *PHLPP2* knockout (*L-PHLPP2*) mice fasted for 18 h and then refed for 8 h (n=9-10/group).

5 Fig. 18H. Loss of *Phlpp2* causes fatty liver. Western blots from control, *PHLPP2* KO, or *PHLPP2*-reconstituted *PHLPP2* KO cells "pulsed" with 10 nM insulin for 30 min, with or without a 2 h "chase" in insulin-free medium. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 as compared to the indicated control by two-way ANOVA. All data are shown as the
10 means $\pm$ s.e.m.

Fig. 19A. *Phlpp2* expression is inversely correlated with lipogenic gene expression. Geneset enrichment analysis (GSEA) using all hepatic transcriptomes (GSE60149) of BXD lines, highlighting the strains
15 showing lowest (left) or highest (right) *Phlpp2* expression.

Fig. 19B. *Phlpp2* expression is inversely correlated with lipogenic gene expression. Normalized enrichment score of GSEA indicating genesets that show negative correlation with hepatic *Phlpp2*
20 expression.

Fig. 20A. Generation of conditional *PHLPP2* knockout mice. Targeting strategy to generate *PHLPP2* flox/flox allele.

25 Fig. 20B. Generation of conditional *PHLPP2* knockout mice. Genotyping analysis of successfully target allele.

Fig. 20C. Generation of conditional *PHLPP2* knockout mice. Western blots from liver, epididymal (eWAT) or inguinal white adipose tissue (iWAT), brown adipose tissue (BAT), and whole pancreas in *PHLPP2*
30 floxed mice transduced with AAV8-TBG-GFP or AAV8-TBG-Cre.

Fig. 20D. Generation of conditional *PHLPP2* knockout mice. Western blots in isolated primary hepatocytes from *PHLPP2* flox/flox mice,
35 transduced with Ad-GFP or Ad-Cre.

Fig. 21A. Prolonged Akt phosphorylation in L-PHLPP2 mice leads to fatty liver. Body weight in Cre- or L- PHLPP2 mice, sacrificed after a 18h fast followed by 8h refeeding (n=9-10/group).

5 Fig. 21B. Prolonged Akt phosphorylation in L-PHLPP2 mice leads to fatty liver. Normalized epidydimal (eWAT) oringuinal (iWAT) white adipose tissue weight in Cre- or L- PHLPP2 mice, sacrificed after a 18h fast followed by 8h refeeding (n=9-10/group).

10 Fig. 21C. Prolonged Akt phosphorylation in L-PHLPP2 mice leads to fatty liver. Liver cholesterol in Cre- or L- PHLPP2 mice, sacrificed after a 18h fast followed by 8h refeeding (n=9-10/group).

15 Fig. 21D. Prolonged Akt phosphorylation in L-PHLPP2 mice leads to fatty liver. Plasma cholesterol in Cre- or L- PHLPP2 mice, sacrificed after a 18h fast followed by 8h refeeding (n=9-10/group).

20 Fig. 21E. Prolonged Akt phosphorylation in L-PHLPP2 mice leads to fatty liver. Non-esterified fatty acid (NEFA) in Cre- or L- PHLPP2 mice, sacrificed after a 18h fast followed by 8h refeeding (n=9-10/group).

25 Fig. 21F. Prolonged Akt phosphorylation in L-PHLPP2 mice leads to fatty liver. Plasma triglyceride in Cre- or L- PHLPP2 mice, sacrificed after a 18h fast followed by 8h refeeding (n=9-10/group).

Fig. 69G. Prolonged Akt phosphorylation in L-PHLPP2 mice leads to fatty liver. Western blots from primary hepatocyte sisolated from PHLPP2 flox/flox mice, transduced with Ad-GFP or Ad-Cre, then exposed to medium with or without 10nM insulin for 6h.

30

Fig. 21H. Prolonged Akt phosphorylation in L-PHLPP2 mice leads to fatty liver. Blood glucose in Cre- and L- PHLPP2 mice. *P<0.05 as compared to the indicated control by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

35

Fig. 21I. Prolonged Akt phosphorylation in L-PHLPP2 mice leads to fatty liver. Insulin in Cre- and L- PHLPP2 mice. *P<0.05 as compared to the indicated control by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

5

Fig. 21J. Prolonged Akt phosphorylation in L-PHLPP2 mice leads to fatty liver. Intraperitoneal glucose tolerance test (GTT) in Cre- and L- PHLPP2 mice. *P<0.05 as compared to the indicated control by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

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Fig. 22A. HFD-fed L-PHLPP2 mice show normal glucose/lipid homeostasis. Liver weight in HFD-fed Cre- or L-PHLPP2 mice, sacrificed after a 18h fast followed by 8h refeeding. (n=9-10/group). ***P<0.001 as compared to Cre-mice by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

15

Fig. 22B. HFD-fed L-PHLPP2 mice show normal glucose/lipid homeostasis. Triglyceride in HFD-fed Cre- or L-PHLPP2 mice, sacrificed after a 18h fast followed by 8h refeeding. (n=9-10/group). ***P<0.001 as compared to Cre-mice by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

20

Fig. 22C. HFD-fed L-PHLPP2 mice show normal glucose/lipid homeostasis. Body weight in HFD-fed Cre- or L-PHLPP2 mice, sacrificed after a 18h fast followed by 8h refeeding. (n=9-10/group). ***P<0.001 as compared to Cre-mice by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

25

Fig. 22D. HFD-fed L-PHLPP2 mice show normal glucose/lipid homeostasis. Blood glucose in HFD-fed Cre- or L-PHLPP2 mice, sacrificed after a 18h fast followed by 8h refeeding. (n=9-10/group). ***P<0.001 as compared to Cre-mice by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

30

Fig. 22E. HFD-fed L-PHLPP2 mice show normal glucose/lipid homeostasis. eWAT weight in HFD-fed Cre- or L-PHLPP2 mice, sacrificed after a 18h fast followed by 8h refeeding. (n=9-10/group). ***P<0.001 as compared to Cre-mice by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

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Fig. 22F. HFD-fed L-PHLPP2 mice show normal glucose/lipid homeostasis. Liver protein in HFD-fed Cre- or L-PHLPP2 mice, sacrificed after a 18h fast followed by 8h refeeding. (n=9-10/group). ***P<0.001 as compared to Cre-mice by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

Fig. 22G. HFD-fed L-PHLPP2 mice show normal glucose/lipid homeostasis. Plasma triglyceride in HFD-fed Cre- or L-PHLPP2 mice, sacrificed after a 18h fast followed by 8h refeeding. (n=9-10/group). ***P<0.001 as compared to Cre-mice by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

Fig. 22H. HFD-fed L-PHLPP2 mice show normal glucose/lipid homeostasis. Cholesterol in HFD-fed Cre- or L-PHLPP2 mice, sacrificed after a 18h fast followed by 8h refeeding. (n=9-10/group). ***P<0.001 as compared to Cre-mice by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

Fig. 22I. HFD-fed L-PHLPP2 mice show normal glucose/lipid homeostasis. NEFA levels in HFD-fed Cre- or L-PHLPP2 mice, sacrificed after a 18h fast followed by 8h refeeding. (n=9-10/group). ***P<0.001 as compared to Cre-mice by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

Fig. 22J. HFD-fed L-PHLPP2 mice show normal glucose/lipid homeostasis. Liver mRNA in HFD-fed Cre- or L-PHLPP2 mice, sacrificed after a 18h fast followed by 8h refeeding. (n=9-10/group). ***P<0.001 as compared to Cre-mice by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

Fig. 23A. PHLPP2KO cells have prolonged insulin-mediated lipogenic gene expression. Generation of PHLPP2 KO cells using CRISPR/Cas9 by transducing HepG2 hepatoma cells with lentivirus expressing three different single guide RNA (sgRNA

Fig. 23B. PHLPP2KO cells have prolonged insulin-mediated lipogenic gene expression. Generation of PHLPP2 KO cells using CRISPR/Cas9 by transducing HepG2 hepatoma cells with lentivirus expressing three different single guide RNA (sgRNA).

Fig. 23C. PHLPP2KO cells have prolonged insulin-mediated lipogenic gene expression. qPCR analysis of predicted potential off-target genes for sgRNAs. *P<0.05 and **P<0.01 as compared to the indicated control by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

5

Fig. 23D. PHLPP2KO cells have prolonged insulin-mediated lipogenic gene expression. qPCR analysis in control and PHLPP2 KO cells after insul in exposure for 6h. *P<0.05 and **P<0.01 as compared to the indicated control by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

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Fig. 24A. PHLPP2 is phosphorylated on Ser1119 and Ser1210 by glucagon/PKA action. Measured and theoretical mass (singly charged) of phosphopeptides of PHLPP2 isolated from Ad-PHLPP-transduced liver via immunoprecipitation and analyzed by LC-MS/MS, with the phosphorylated serine residue indicated with

15

Fig. 24B. PHLPP2 is phosphorylated on Ser1119 and Ser1210 by glucagon/PKA action. PHLPP2 contains Ras-association (RA), pleckstrin homology (PH), hydrophobic leucine-rich repeat (LRR), protein phosphatase 2C (PP2C) domains; amino acid sequence comparison from selected mammalian species with PKA targeting sequence (red) and phosphorylated Ser (blue) residues highlighted.

20

Fig. 24C. PHLPP2 is phosphorylated on Ser1119 and Ser1210 by glucagon/PKA action. Western blots from primary hepatocytes exposed to 100 nM glucagon, using Phos-tag to separate phosphorylated from unphosphorylated PHLPP2.

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Fig. 24D. PHLPP2 is phosphorylated on Ser1119 and Ser1210 by glucagon/PKA action. Western blots from primary hepatocytes exposed to 100 μ M cAMP or 10 μ M forskolin for 1 h, using Phos-tag to separate phosphorylated from unphosphorylated PHLPP2.

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Fig. 24E. PHLPP2 is phosphorylated on Ser1119 and Ser1210 by glucagon/PKA action. Western blots from primary hepatocytes exposed to glucagon for the indicated times with or without forskolin.

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Fig. 24F. PHLPP2 is phosphorylated on Ser1119 and Ser1210 by glucagon/PKA action. Western blots from HepG2 cells stably expressing two different shRNA directed at PKA or shControl (-) with or without forskolin.

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Fig. 24G. PHLPP2 is phosphorylated on Ser1119 and Ser1210 by glucagon/PKA action. Western blots from primary hepatocytes exposed to Co-immunoprecipitation (CoIP) of PHLPP2 (WT) but not nonphosphorylatable mutant PHLPP2 (2A) with phospho-PKA substrate in response to forskolin, sensitive to the PKA inhibitor H-89, in primary hepatocytes.

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Fig. 25A. Glucagon/PKA-induced phosphorylation of PHLPP2 at Ser1119 and Ser1210. Primary hepatocytes were incubated with insulin or the indicated kinase inhibitors prior to Phos-tag Western blot.

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Fig. 25B. Glucagon/PKA-induced phosphorylation of PHLPP2 at Ser1119 and Ser1210. Transfected with PHLPP2-WT, S1119A, S1210A with or without forskolin, prior to Phos-tag Western blot.

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Fig. 25C. Glucagon/PKA-induced phosphorylation of PHLPP2 at Ser1119 and Ser1210. Validation of novel PHLPP2 Ser1119- or 1210- specific antibodies in hepatocytes transfected with empty vector (EV), PHLPP2 (WT), or nonphosphorylatable mutant PHLPP2 (2A) with or without forskolin.

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Fig. 25D. Glucagon/PKA-induced phosphorylation of PHLPP2 at Ser1119 and Ser1210. Validation of novel PHLPP2 Ser1119- or 1210- specific antibodies in hepatocytes transfected with EV, WT, S1119A, S1210A, or 2A.

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Fig. 25E. Glucagon/PKA-induced phosphorylation of PHLPP2 at Ser1119 and Ser1210. CoIP of WT but not 2A with phospho-PKA substrate antibody in response to glucagon or forskolin in primary hepatocytes.

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Fig. 26A. Glucagon/PKA-induced phosphorylation of PHLPP2 at Ser1119 and Ser1210 in vivo. Western blots of liver lysate of p-PHLPP2/total PHLPP2 and PHLPP2/b-actin. *P<0.05 and **P<0.01 as compared to fasted mice by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

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Fig. 26B. Glucagon/PKA-induced phosphorylation of PHLPP2 at Ser1119 and Ser1210 in vivo. Quantification of p-PHLPP2/total PHLPP2 and PHLPP2/b-actin. *P<0.05 and **P<0.01 as compared to fasted mice by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

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Fig. 27A. Glucagon-mediated PHLPP2 phosphorylation and destabilization in obesity. Western blots of liver lysate of p-PHLPP2, total PHLPP2 and β -actin, and corresponding liver Phlpp2 expression in C57BL/6 wild-type mice fed normal chow or HFD for 16 weeks.

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Fig. 27B. Glucagon-mediated PHLPP2 phosphorylation and destabilization in obesity. Western blot quantification of p-PHLPP2/total PHLPP2 and PHLPP2/ β -actin in C57BL/6 wild-type mice fed normal chow or HFD for 16 weeks.

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Fig. 27C. Glucagon-mediated PHLPP2 phosphorylation and destabilization in obesity. Western blot quantification of corresponding liver Phlpp2 expression in C57BL/6 wild-type mice fed normal chow or HFD for 16 weeks.

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Fig. 27D. Glucagon-mediated PHLPP2 phosphorylation and destabilization in obesity. Western blots of liver lysate of p-PHLPP2, total PHLPP2 and β -actin, and corresponding liver Phlpp2 expression 8-week-old C57BL/6J wild-type as compared to db/db mice.

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Fig. 27E. Glucagon-mediated PHLPP2 phosphorylation and destabilization in obesity. Western blot quantification of p-PHLPP2/total PHLPP2 and PHLPP2/ β -actin in 8-week-old C57BL/6J wild-type as compared to db/db mice.

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Fig. 27F. Glucagon-mediated PHLPP2 phosphorylation and destabilization in obesity. Western blot quantification of corresponding liver Phlpp2 expression in C57BL/6 wild-type mice fed normal chow or HFD for 16 weeks.

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Fig. 27G. Glucagon-mediated PHLPP2 phosphorylation and destabilization in obesity. Western blot of PHLPP2 in cycloheximide (CHX, 50 mg ml⁻¹)-treated primary hepatocytes, with or without forskolin, with quantification of PHLPP2/ α -tubulin relative to time

10 0.

Fig. 27H. Glucagon-mediated PHLPP2 phosphorylation and destabilization in obesity. qPCR from AAV8-shGcgr-transduced, HFD-fed C57BL/6 and db/db mice. *P < 0.05, **P < 0.01, ***P < 0.001 as compared to the indicated control by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

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Fig. 27I. Glucagon-mediated PHLPP2 phosphorylation and destabilization in obesity. Western blots from AAV8-shGcgr-transduced, HFD-fed C57BL/6 and db/db mice. *P < 0.05, **P < 0.01, ***P < 0.001 as compared to the indicated control by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

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Fig. 28A. KCTD17 binds phosphorylated PHLPP2 to induce its degradation in obese liver. Liver Kctd17 expression in C57BL/6 wild-type mice fed normal chow vs. HFD for either 2 or 4 months or C57BL/6J wild-type vs. db/db mice.

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Fig. 28B. KCTD17 binds phosphorylated PHLPP2 to induce its degradation in obese liver. Endogenous CoIP of KCTD17 and PHLPP2 in liver lysate from HFD-fed mice, fasted for 18 h and then refed for 8 h.

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Fig. 28C. KCTD17 binds phosphorylated PHLPP2 to induce its degradation in obese liver. CoIP of KCTD17 by PHLPP2 in primary hepatocytes is increased by MG-132

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Fig. 28D. KCTD17 binds phosphorylated PHLPP2 to induce its degradation in obese liver. CoIP of KCTD17 by PHLPP2 in primary hepatocytes is increased synergistically by forskolin.

5 Fig. 28E. KCTD17 binds phosphorylated PHLPP2 to induce its degradation in obese liver. KCTD17 facilitates the interaction of PHLPP2 (WT), with the E3-ligase, Cullin3.

Fig. 28F. KCTD17 binds phosphorylated PHLPP2 to induce its degradation
10 in obese liver. KCTD17 does not facilitate the interaction of the nonphosphorylatable mutant PHLPP2 (2A), with the E3-ligase, Cullin3.

Fig. 28G. KCTD17 binds phosphorylated PHLPP2 to induce its degradation in obese liver. Western blots of liver lysate from HFD-fed C57BL/6
15 mice transduced with Ad-GFP, Ad-PHLPP2 (WT), or Ad-PHLPP2 (2A), fasted for 18 h and then refed for 8 h prior to sacrifice.

Fig. 28H. KCTD17 binds phosphorylated PHLPP2 to induce its degradation in obese liver. Liver weight from HFD-fed C57BL/6 mice transduced with
20 Ad-GFP, Ad-PHLPP2 (WT), or Ad-PHLPP2 (2A), fasted for 18 h and then refed for 8 h prior to sacrifice. (n=6-7/group). *P < 0.05, **P < 0.01 as compared to the indicated control by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

25 Fig. 28I. KCTD17 binds phosphorylated PHLPP2 to induce its degradation in obese liver. Triglycerides from HFD-fed C57BL/6 mice transduced with Ad-GFP, Ad-PHLPP2 (WT), or Ad-PHLPP2 (2A), fasted for 18 h and then refed for 8 h prior to sacrifice. (n=6-7/group). *P < 0.05, **P < 0.01 as compared to the indicated control by two-way ANOVA. All data
30 are shown as the means $\pm$ s.e.m.

Fig. 28J. KCTD17 binds phosphorylated PHLPP2 to induce its degradation in obese liver. Plasma triglycerides and (k) hepatic gene expression from HFD-fed C57BL/6 mice transduced with Ad-GFP, Ad-PHLPP2 (WT), or
35 Ad-PHLPP2 (2A), fasted for 18 h and then refed for 8 h prior to sacrifice. (n=6-7/group). *P < 0.05, **P < 0.01 as compared to the indicated control by two-way ANOVA. All data are shown as the means

± s.e.m.

Fig. 28K. KCTD17 binds phosphorylated PHLPP2 to induce its degradation in obese liver. Hepatic gene expression from HFD-fed C57BL/6 mice transduced with Ad-GFP, Ad-PHLPP2 (WT), or Ad-PHLPP2 (2A), fasted for 18 h and then refed for 8 h prior to sacrifice. (n=6-7/group). *P < 0.05, **P < 0.01 as compared to the indicated control by two-way ANOVA. All data are shown as the means ± s.e.m.

Fig. 29A. Similar expression of WT and 2A in obese mice. Hepatic Phlpp2 expression in HFD-fed C57BL/6 mice transduced with Ad-GFP, Ad-PHLPP2 (WT), or Ad-PHLPP2(2A), fasted for 18h and then refed for 8h prior to sacrifice. (n = 6-7/group). ***P<0.001 as compared to Ad-GFP-transduced mice by two-way ANOVA. All data are shown as the means ± s.e.m.

Fig. 29B. Similar expression of WT and 2A in obese mice. Body weight in HFD-fed C57BL/6 mice transduced with Ad-GFP, Ad-PHLPP2 (WT), or Ad-PHLPP2(2A), fasted for 18h and then refed for 8h prior to sacrifice. (n = 6-7/group). ***P<0.001 as compared to Ad-GFP-transduced mice by two-way ANOVA. All data are shown as the means ± s.e.m.

Fig. 29C. Similar expression of WT and 2A in obese mice. Normalized eWAT weight in HFD-fed C57BL/6 mice transduced with Ad-GFP, Ad-PHLPP2 (WT), or Ad-PHLPP2(2A), fasted for 18h and then refed for 8h prior to sacrifice. (n = 6-7/group). ***P<0.001 as compared to Ad-GFP-transduced mice by two-way ANOVA. All data are shown as the means ± s.e.m.

Fig. 29D. Similar expression of WT and 2A in obese mice. Blood glucose levels in HFD-fed C57BL/6 mice transduced with Ad-GFP, Ad-PHLPP2 (WT), or Ad-PHLPP2(2A), fasted for 18h and then refed for 8h prior to sacrifice. (n = 6-7/group). ***P<0.001 as compared to Ad-GFP-transduced mice by two-way ANOVA. All data are shown as the means ± s.e.m.

Fig. 29E. Similar expression of WT and 2A in obese mice. Insulin levels in HFD-fed C57BL/6 mice transduced with Ad-GFP, Ad-PHLPP2 (WT), or Ad-PHLPP2(2A), fasted for 18h and then refed for 8h prior to sacrifice. (n = 6-7/group). ***P<0.001 as compared to Ad-GFP-transduced mice by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

Fig. 30A. Knockdown of hepatic KCTD17 prevents diet-induced hepatic steatosis. qPCR from primary hepatocytes transduced with Ad-shKctd17.

Fig. 30B. Knockdown of hepatic KCTD17 prevents diet-induced hepatic steatosis. Western blots from primary hepatocytes transduced with Ad-shKctd17.

Fig. 30C. Knockdown of hepatic KCTD17 prevents diet-induced hepatic steatosis. Knockdown of Kctd17 reduces endogenous PHLPP2 ubiquitination in HA/Ub-transfected primary hepatocytes.

Fig. 30D. Knockdown of hepatic KCTD17 prevents diet-induced hepatic steatosis. Western blots from liver lysate in HFD-fed C57BL/6 mice transduced with Ad-shControl or Ad-shKctd17, fasted for 18 h and then refed for 8 h prior to sacrifice (n=6-7/group). *P < 0.05, **P < 0.01, ***P<0.001 as compared to the indicated control by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

Fig. 30E. Knockdown of hepatic KCTD17 prevents diet-induced hepatic steatosis. Liver weight in HFD-fed C57BL/6 mice transduced with Ad-shControl or Ad-shKctd17, fasted for 18 h and then refed for 8 h prior to sacrifice (n=6-7/group). *P < 0.05, **P < 0.01, ***P<0.001 as compared to the indicated control by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

Fig. 30F. Knockdown of hepatic KCTD17 prevents diet-induced hepatic steatosis. Triglycerides in HFD-fed C57BL/6 mice transduced with Ad-shControl or Ad-shKctd17, fasted for 18 h and then refed for 8 h prior to sacrifice (n=6-7/group). *P < 0.05, **P < 0.01, ***P<0.001 as compared to the indicated control by two-way ANOVA. All data are shown

as the means $\pm$ s.e.m.

Fig. 30G. Knockdown of hepatic KCTD17 prevents diet-induced hepatic steatosis. Liver H&E and Oil-Red-O staining in HFD-fed C57BL/6 mice transduced with Ad-shControl or Ad-shKctd17, fasted for 18 h and then refed for 8 h prior to sacrifice (n=6-7/group). *P < 0.05, **P < 0.01, ***P<0.001 as compared to the indicated control by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

Fig. 30H. Knockdown of hepatic KCTD17 prevents diet-induced hepatic steatosis. Hepatic gene expression in HFD-fed C57BL/6 mice transduced with Ad-shControl or Ad-shKctd17, fasted for 18 h and then refed for 8 h prior to sacrifice (n=6-7/group). *P < 0.05, **P < 0.01, ***P<0.001 as compared to the indicated control by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

Fig. 31A. Knockdown of KCTD17 prevents hepatic steatosis. Western blots from Hepalclc7 cells transduced with Ad-shControl or Ad-shKctd17.

Fig. 31B. Knockdown of KCTD17 prevents hepatic steatosis. qPCR from Hepalclc7 cells transduced with Ad-shControl or Ad-shKctd17.

Fig. 31C. Knockdown of KCTD17 prevents hepatic steatosis. Lipogenic gene expression in primary hepatocytes transduced with Ad-shControl or Ad-shKctd17 after insulin exposure for 6h (n=4 biologic replicates).

Fig. 31D. Knockdown of KCTD17 prevents hepatic steatosis. qPCR analysis in HFD-fed C57BL/6 mice transduced with Ad-shControl or Ad-shKctd17, fasted for 18h and then refed for 8h prior to sacrifice (n=6-7/group). *P<0.05, **P<0.01, and ***P<0.001 as compared to the indicated control by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

Fig. 31E. Knockdown of KCTD17 prevents hepatic steatosis. Body weight in HFD-fed C57BL/6 mice transduced with Ad-shControl or Ad-shKctd17, fasted for 18h and then refed for 8h prior to sacrifice (n=6-7/group). *P<0.05, **P<0.01, and ***P<0.001 as compared to the indicated control by two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

Fig. 32A. KCTD17 correlates with lipogenic gene expression and hepatic steatosis in NASH. KCTD17 expression in liver biopsy specimens (n=158) as related to steatosis subscore.

Fig. 32B. KCTD17 correlates with lipogenic gene expression and hepatic steatosis in NASH. KCTD17 expression in liver biopsy specimens (n=158) as related to total NAFLD activity score (NAS).

Fig. 32C. KCTD17 correlates with lipogenic gene expression and hepatic steatosis in NASH. Heat map of hepatic KCTD17 expression and de novo lipogenesis (DNL) gene set in hepatic transcriptomes of healthy controls and NASH patients before and after bariatric surgery (n=5-10/group), with the depth of shading of correlogram according to the magnitude of the correlation and positive and negative correlations.

Fig. 32D. KCTD17 correlates with lipogenic gene expression and hepatic steatosis in NASH. Volcano plot of hepatic KCTD17 expression and de novo lipogenesis (DNL) gene set in hepatic transcriptomes of healthy controls and NASH patients before and after bariatric surgery (n=5-10/group), with the depth of shading of correlogram according to the magnitude of the correlation and positive and negative correlations represented in blue and red, respectively.

Fig. 32E. KCTD17 correlates with lipogenic gene expression and hepatic steatosis in NASH. Correlogram of hepatic KCTD17 expression and de novo lipogenesis (DNL) gene set in hepatic transcriptomes of healthy controls and NASH patients before and after bariatric surgery (n=5-10/group), with the depth of shading of correlogram according to the magnitude of the correlation and positive and negative correlations.

Fig. 32F. KCTD17 correlates with lipogenic gene expression and hepatic steatosis in NASH. Model representing the parallel effects of obesity to increase glucagon-mediated PHLPP2 phosphorylation and KCTD17 expression/activity, which synergistically induce PHLPP2 degradation and prolongation of Akt-induced Srebp1c-mediated DNL, and fatty liver.

Fig. 33A. KCTD17 expression is correlated with multiple potentially pathogenic pathways in patients with NASH. Correlogram indicating Spearman's correlation between KCTD17 and gene sets of indicated pathways from NASH subjects.

Fig. 33B. KCTD17 expression is correlated with multiple potentially pathogenic pathways in patients with NASH. Interaction network showing KCTD17 expression association with genes sets involved in DNL, fatty acid esterification, fatty acid oxidation, ER stress, and inflammation, with black and grey connecting lines indicating positive and negative correlations, respectively. Only correlations with Spearman's $Rho > 0.5$ or < -0.5 are shown.

DETAILED DESCRIPTION OF THE INVENTIONEmbodiments of the Invention

The present invention provides a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that decreases KCTD17 expression in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

The present invention provides a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that increases PHLPP2 in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

The present invention also provides a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that increases free Raptor in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

In some embodiments, the pharmaceutical composition increases Raptor expression, thereby increasing free Raptor in the liver cells.

In some embodiments, the pharmaceutical composition inhibits interaction of Raptor and mTORC1, thereby increasing free Raptor in the liver cells.

In some embodiments, the compound reduces the expression of at least one lipogenic gene.

In some embodiments, the at least one lipogenic gene is Srebp1c, Fasn, Accl, or Scd1.

In some embodiments, the subject is afflicted with a metabolic disease.

5 In some embodiments, the pharmaceutical composition comprises a polynucleotide.

In some embodiments, the pharmaceutical composition is targeted to the liver of the subject.

10

In some embodiments, the metabolic disease is obesity.

In some embodiments, the metabolic disease is hypertriglyceridemia.

15 In some embodiments, the metabolic disease is hyperinsulinemia.

In some embodiments, the metabolic disease is Type 2 Diabetes.

In some embodiments, the metabolic disease is fatty liver disease.

20

In some embodiments, the fatty liver disease is nonalcoholic fatty liver disease or nonalcoholic steatohepatitis.

In some embodiments, the subject is afflicted with cirrhosis or
25 hepatocellular carcinoma.

In some embodiments, the subject is a human.

In some embodiments, the subject's hepatic or plasma triglyceride
30 levels are >150 mg/dL.

In some embodiments, the subject's hepatic or plasma triglyceride levels are >500 mg/dL, about 200 to 499 mg/dL, or about 150 to 199 mg/dL.

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In some embodiments, the subject's hepatic or plasma triglyceride levels are reduced by at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, or at least 75%, relative to
40 the level prior to the administration.

The present invention also provides a process for determining the amount of free Raptor in a subject's liver comprising:

- 5 d) obtaining a biological sample comprising liver cells of the subject;
- e) separating free Raptor and mTORC1-associated Raptor in the sample; and
- f) determining the amount of free Raptor in the sample.

10 The present invention also provides a process for diagnosing whether a subject is afflicted with decreased free Raptor comprising:

- a) determining the amount of free Raptor in the subject according to the process of the claimed invention;
- 15 b) determining the amount of free Raptor in a reference subject according to the process of the claimed invention; and
- d) diagnosing the subject to be afflicted with decreased free Raptor if the amount of free Raptor in step (a) is substantially decreased compared to the amount of free
- 20 Raptor in step (b).

The present invention also provides a method of treating a subject diagnosed to be afflicted with decreased free Raptor according to the process of the claimed invention comprising reducing the subject's

25 hepatic and plasma triglyceride levels according to the method of the claimed invention.

The present invention also provides a process for determining the amount of KCTD17 expression in a subject's liver comprising:

- 30 a) obtaining a biological sample comprising liver cells of the subject;
- b) determining the amount of KCTD17 mRNA in the sample.

The present invention also provides a process for diagnosing whether

35 a subject is afflicted with increased KCTD17 expression comprising:

- a) determining the amount of KCTD17 in the subject according to the process of claim 31;

b) determining the amount of KCTD17 in a reference subject according to the process of claim 31; and

c) diagnosing the subject to be afflicted with increased KCTD17 expression if the amount of KCTD17 expression in step (a) is substantially increased compared to the amount of KCTD17 expression in step (b).

The present invention also provides a method of treating a subject diagnosed to be afflicted with increased KCTD17 expression according to the process of claim 32 comprising reducing the subject's hepatic and plasma triglyceride levels according to a method of the present invention.

The present invention also provides a method of treating a subject afflicted with elevated triglyceride levels comprising administering to the subject a pharmaceutical composition comprising a pharmaceutical carrier and a compound that decreases KCTD17 expression in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

The present invention also provides a method of treating a subject afflicted with elevated triglyceride levels comprising administering to the subject a pharmaceutical composition comprising a pharmaceutical carrier and a compound that decreases PHLPP2 phosphorylation at Serine 1119 and Serine 1210 residues in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

The present invention also provides a method of treating a subject at risk of developing elevated triglyceride levels comprising administering to the subject a pharmaceutical composition comprising a pharmaceutical carrier and a compound that decreases KCTD17 expression in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

The present invention also provides a method of treating a subject at risk of developing elevated triglyceride levels comprising

administering to the subject a pharmaceutical composition comprising a pharmaceutical carrier and a compound that increases PHLPP2 phosphorylation at Serine 1119 and Serine 1210 residues in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

In some embodiments, the pharmaceutical composition reduces β -TrCP-mediated degradation of PHLPP2, thereby increasing free Raptor in the liver cells.

In some embodiments, the pharmaceutical composition comprises a Notch antagonist.

In some embodiments, the Notch antagonist comprises a γ -secretase inhibitor, anti-DLL4 mAB, or a DLK peptide.

In some embodiments, the pharmaceutical composition decreases PHLPP2 phosphorylation at Serine 1119 or Serine 1210 residues.

In some embodiments, the pharmaceutical composition inhibits interaction of PHLPP2 and KCTD17.

In some embodiments, the pharmaceutical composition decreases Akt signaling.

In some embodiments, the pharmaceutical composition decreases Akt phosphorylation at Serine 473 residue.

In some embodiments, the pharmaceutical composition prevents PHLPP2 degradation by inhibiting Glucagon signaling.

In some embodiments, the pharmaceutical composition reduces PHLPP2 degradation, thereby increasing free Raptor in the liver cells.

In some embodiments, the pharmaceutical composition prevents PHLPP2 degradation, thereby increasing free Raptor in the liver cells.

Methods of Inhibiting Glucagon Signaling

As used herein, "inhibiting glucagon signaling" includes reducing glucagon receptor expression, reducing glucagon receptor activation, blocking glucagon receptor activation, reducing endogenous glucagon action, or blocking glucagon action.

As used herein, "prevents PHLPP2 degradation" includes inhibiting PHLPP2 phosphorylation, thereby increasing PHLPP2 levels compared to controls.

As used herein, "reduces PHLPP2 degradation" includes inhibiting PHLPP2 phosphorylation, thereby increasing PHLPP2 levels compared to controls.

In some embodiments, the pharmaceutical composition inhibits Glucagon signaling, thereby reducing PHLPP2 degradation.

In some embodiments, the pharmaceutical composition comprises a Glucagon receptor (Gcgr) antagonizing antibody.

In some embodiments, the pharmaceutical composition comprises a Glucagon receptor (Gcgr) antagonist.

In some embodiments, the Glucagon receptor antagonist reduces PHLPP2 degradation.

In some embodiments, the Glucagon receptor antagonist is des-His¹-[Glu⁹]-Glucagon (1-29) amide, or L-168,049.

In some embodiments, the Glucagon receptor antagonist is 4-[3-(5-Bromo-2-propoxyphenyl)-5-(4-chlorophenyl)-1H-pyrrol-2-yl]pyridine.

In some embodiments, glucagon is suppressed by somatostatin infusion.

The present invention also provides a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a

pharmaceutical carrier and a compound that prevents PHLPP2 degradation in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

5 The present invention also provides a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that inhibits Glucagon signaling in liver cells in an amount effective to reduce the subject's hepatic
10 and plasma triglyceride levels.

In some embodiments, the pharmaceutical composition prevents PHLPP2 degradation, thereby increasing free Raptor in the liver cells.

15 In some embodiments, each compound administered to the subject is, independently, an organic compound having a molecular weight less than 1000 Daltons, a DNA aptamer, an RNA aptamer, a polypeptide, an antibody, an oligonucleotide, an interfering RNA (RNAi) molecule, a ribozyme, or a small molecule inhibitor.

20 In some embodiments, a compound that is capable of inhibiting glucagon signaling is administered to the subject.

In some embodiments, the compound which is capable of inhibiting
25 glucagon signaling is an organic compound having a molecular weight less than 1000 Daltons.

In some embodiments, the oligonucleotide is an antisense oligonucleotide, an RNA-interference inducing compound, or a ribozyme.

30 In some embodiments, the oligonucleotide is targeted to hepatocytes.

In some embodiments, the oligonucleotide comprises 1, 2, 3, 4, or 5 or more stretches of nucleotides in a sequence that is complementary
35 to glucagon-encoding mRNA or glucagon receptor-encoding mRNA, wherein each stretch of complementary contiguous nucleotides is at least at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more nucleotides in length.

In some embodiments, the oligonucleotide is modified to increase its stability *in vivo*.

5 Aspects of the present invention relate to the use of compounds effective to increase free RAPTOR can be used treat metabolic diseases such as hypertriglyceridemia, hyperinsulinemia, Type 2 Diabetes, or fatty liver disease.

10 Aspects of the present invention relate to the use of compounds effective to increase PHLPP2 can be used treat metabolic diseases such as hypertriglyceridemia, hyperinsulinemia, Type 2 Diabetes, or fatty liver disease.

15 The present invention also provides a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that prevents PHLPP2 degradation in liver cells in an amount effective to reduce the subject's hepatic
20 and plasma triglyceride levels.

In some embodiments, the pharmaceutical composition inhibits Glucagon signaling.

25 The present invention also provides a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that inhibits Glucagon signaling in liver cells in an amount effective to reduce the subject's hepatic
30 and plasma triglyceride levels.

In some embodiments, the pharmaceutical composition reduces PHLPP2 degradation.

35 In some embodiments, the pharmaceutical composition increases free Raptor in the liver cells.

The present invention also provides a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that decreases KCTD17 expression in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

The present invention also provides a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that decreases PHLPP2 phosphorylation at Serine 1119 and Serine 1210 residues in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

In some embodiments, the pharmaceutical composition decreases KCTD17 expression in liver cells.

The present invention also provides a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that decreases KCTD17 expression in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

The present invention also provides a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that decreases PHLPP2 phosphorylation at Serine 1119 and Serine 1210 residues in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

In some embodiments, the pharmaceutical composition decreases KCTD17 expression in liver cells.

In some embodiments, a compound that is capable of inhibiting a Glucagon signaling is administered to the subject.

Inhibiting Srebp1c

5 As used herein, "inhibiting Srebp1c" includes inhibiting the translocation of SREBP, reducing SREBP activation, inhibiting SREBP processing, or reducing the expression of SREBP. Further examples may be found in Kamisuki et al., 2009; Tang et al., 2011; Zhao et al., 2014; Hawkins et al., 2008; and Wu et al., 2014.

10

In some embodiments, the pharmaceutical composition comprises an Srebp1c inhibitor, thereby decreasing the expression of genes involved in lipid metabolism.

15 In some embodiments, the pharmaceutical composition comprises an Srebp1c inhibitor, thereby reducing the subject's hepatic and plasma triglyceride levels.

20 In some embodiments, the Srebp1c inhibitor is 24, 25-expoxycholesterol, and 25-HC.

In some embodiments, the Srebp1c inhibitor is Fatostatin, Betulin, BF-175, PF-429242, or BioE-1115.

25 In some embodiments, the compound that is capable of inhibiting Srebp1c is an antisense oligonucleotide, an RNAi molecule, or a ribozyme that targets Srebp1c mRNA and reduces Srebp1c expression.

30 In some embodiments, a compound that is capable of inhibiting Srebp1c is administered to the subject.

Inhibiting KCTD17

35 As used herein, "inhibiting KCTD17" includes inhibiting the translocation of KCTD17, reducing KCTD17 activation, inhibiting KCTD17 processing, or reducing the expression of KCTD17.

In some embodiments, the pharmaceutical composition comprises an KCTD17 inhibitor, thereby decreasing the expression of genes involved in lipid metabolism.

- 5 In some embodiments, the pharmaceutical composition comprises an KCTD17 inhibitor, thereby reducing the subject's hepatic and plasma triglyceride levels.

10 In some embodiments, the compound that is capable of inhibiting KCTD17 is an antisense oligonucleotide, an RNAi molecule, or a ribozyme that targets KCTD17 mRNA and reduces KCTD17 expression.

In some embodiments, a compound that is capable of inhibiting KCTD17 is administered to the subject.

15

Threshold information for diagnostic utility of KCTD17

Steatosis scores >2 correspond to relatively severe steatosis; further, NAFLD activity score (NAS) >3 indicates a very high risk of NASH. Evaluation of qPCR data from patients undergoing liver biopsy
20 for suspected NAFLD/NASH revealed a significant positive correlation between KCTD17 expression and NASH as well as the steatosis sub-score (Fig. 80A and 80B). Further, a 1.9-fold increase in hepatic KCTD17 expression (as compared to the mean for the population) predicted a steatosis score >2 and NAS >3 , and may define a diagnostic threshold
25 in the evaluation of patients for advanced steatosis and high risk for NASH.

In some embodiments, the subject afflicted with elevated triglyceride levels has increased hepatic KCTD17 expression.

30

In some embodiments, the subject at risk of developing elevated triglyceride levels has increased hepatic KCTD17 expression.

In some embodiments, the increased hepatic KCTD17 expression is about
35 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.2, 2.3, 2.3 fold or more higher than the KCTD17 expression in a reference subject.

In some embodiments, the increased hepatic KCTD17 expression is about 1.9 fold or more higher than the KCTD17 expression in a reference subject.

- 5 In some embodiments, the increased hepatic KCTD17 expression is about 1.9 fold higher than the KCTD17 expression in a reference subject.

Each embodiment disclosed herein is contemplated as being applicable to each of the other disclosed embodiments. Thus, all combinations of
10 the various elements described herein are within the scope of the invention.

It is understood that where a parameter range is provided, all integers within that range, and tenths thereof, are also provided by the
15 invention. For example, "0.2-5 mg/kg/day" is a disclosure of 0.2 mg/kg/day, 0.3 mg/kg/day, 0.4 mg/kg/day, 0.5 mg/kg/day, 0.6 mg/kg/day etc. up to 5.0 mg/kg/day.

This invention will be better understood by reference to the
20 Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

25

Experimental Details

Methods

Experimental animals

5 Male wild-type C57/BL/6 mice, fed on standard chow or HFD (Harlan Laboratories TD.06414), were purchased from Jackson Labs. We injected AAV8-TBG-GFP or AAV8-TBG-Cre (Penn Vector Core) into male *Raptorfl/fl* (Jackson Laboratory; stock number 013188) mice, and characterized the mice 2 weeks after adeno-associated virus (AAV) injection. Number of
10 animals used in experiments was chosen to ensure adequate power to detect experimental difference with alpha set to 0.05. All animal experiments were conducted in accordance with guidelines of the Columbia University Institutional Animal Care and Use Committee.

15 Metabolic analyses

Blood glucose was measured using a glucose meter (OneTouch), and plasma insulin by mouse insulin ELISA kit (Mercodia). Glucose tolerance tests were performed by intraperitoneal injection of 2 g per kg body weight glucose after a 16 h fast. Hepatic lipids were
20 extracted (Folch et al., 1957), and plasma and hepatic triglyceride, Cholesterol E and NEFA were measured using a colorimetric assay from Thermo or Wako Chemicals, according to the manufacturer's protocol. We measured *de novo* lipogenesis as previously described (Zhang et al., 2006).

25

Adenovirus studies

The GFP, Raptor and PHLPP1/2 adenoviruses have been described (Miyamoto et al., 2010; Pajvani et al., 2013). The HA-Flag-PHLPP2 adenovirus was constructed by inserting a Flag sequence into HA-PHLPP2
30 vector (Addgene #22403, courtesy of Alexandra Newton) and adenoviruses encoding HA-Flag-PHLPP2 generated by Welgen, Inc. (Worcester, MA). Mouse shPhlpp1 or 2 target sequences were selected among three candidate sequences, respectively and adenoviruses encoding shPhlpp1 or 2 generated by Welgen, Inc. For *in vivo* studies, we injected 5 X
35 10⁸ or 2.5 X 10⁸ purified viral particles per g body weight; we performed metabolic analysis on days 3-5 and euthanized the mice at day 8 or 10 after injection. Infection with adenovirus in primary

hepatocytes or Hepalclc7 cells was performed at 2.5, 5, or 10 multiplicity of infection (MOI).

Primary hepatocyte cultures

5 We isolated and cultured primary mouse hepatocytes as previously described (Kim et al., 2012). For inhibitor experiments, we treated hepatocytes with 20 nM rapamycin (Cell Signaling), 250 nM Torin1, or vehicle for either 1 h or 24 h.

10 Western blotting and immunoprecipitation

Tissues and cells were lysed in 0.3% CHAPS lysis buffer (Kim et al., 2002) unless otherwise stated, and whole cell lysates obtained by centrifugation. Immunoblots were conducted on samples randomly chosen within each experimental cohort with antibodies against Raptor, Akt, 15 p-Akt (S473), p-Akt (T308), p-Akt1 (S473), p-Akt2 (S474), p-Akt (T450), p-Akt substrate, PKC α , p-GSK3 β (S9), GSK3 β , mTOR, Rictor, G β L, p-S6 (S240/244), S6, β -TrCP and β -actin from Cell Signaling; mTOR and α -tubulin from SantaCruz Biotechnology Inc.; PHLPP1 and PHLPP2 from Bethyl Laboratories Inc.; as well as a polyclonal antibody against 20 Raptor from Invitrogen. For immunoprecipitation (IP) experiments, liver or cellular lysate was incubated with anti-mTOR or PHLPP2 antibody immobilized on Protein A or G-Sepharose (Invitrogen).

Gel filtration chromatography

25 Livers from three mice per group were pooled and lysed in hypotonic buffer (40 mM Tris-HCl, pH 7.5) with protease inhibitor (Pierce) by repeated passage in a 27-gauge needle. Resultant lysates were repeatedly centrifuged at 14,000 rpm for 15 min, and the supernatant fraction filtered through a PVDF membrane prior to application to a 30 Superose 6 column (Amersham Biosciences) calibrated with the Gel Filtration Marker Kit (Sigma), and eluted with 40 mM Tris-HCl (pH 7.5) and 150 mM NaCl.

Crosslinking assays

35 Liver was lysed in 1% Triton X-100 lysis buffer (Kim et al., 2002) with or without 2.5 mg/ml DSP or DSS, and incubated for 2 h on ice. Crosslinking was quenched with 1 M Tris-HCl (pH 7.5) for 30 min on

ice. For *in vitro* crosslinking assays, DSP or DSS were added to a final concentration of 1 mg/ml culture medium, and quenched by Tris-HCl as above. Equal protein (BCA assay, Thermo) was then subjected to Western blot, with or without IP.

5

Quantitative RT-PCR

We isolated RNA with TRIzol (Invitrogen) or an RNeasy Mini kit (Qiagen), synthesized cDNA with High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) and performed quantitative RT-PCR with a GoTaq SYBR Green qPCR kit (Promega) in a CFX96 Real-Time PCR detection system (Bio-Rad).

10

***In vitro* Akt kinase assay**

Liver lysates were immunoprecipitated with anti-Akt antibody (sepharose bead conjugated); bound protein was incubated with a recombinant GSK-3 fusion protein substrate in kinase buffer for 30 min at 30°C, prior to termination with SDS loading buffer and SDS-PAGE, with p-GSK3 β (S9) detected by immunoblot.

15

20 Statistical analysis

We performed comparisons using two-way ANOVA. All data are shown as the means $\pm$ s.e.m.

TERMS

As used herein, "about" in the context of a numerical value or range means $\pm 10\%$ of the numerical value or range recited or claimed, unless the context requires a more limited range.

5

As used herein, "a subject afflicted with" a disease, e.g. nonalcoholic fatty liver disease, means a human patient who was been affirmatively diagnosed to have the disease.

10

As used herein, "effective" when referring to an amount of a compound or compounds refers to the quantity of the compound or compounds that is sufficient to yield a desired therapeutic response without undue adverse side effects (such as toxicity, irritation, or allergic response) commensurate with a reasonable benefit/risk ratio when used in the manner of this invention. The specific effective amount will vary with such factors as the physical condition of the patient, the type of subject being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed and the structure of the compounds or its derivatives.

20

In some embodiments, "a subject in need" includes a subject with elevated triglyceride levels, e.g., a subject with hepatic or plasma or serum triglyceride levels greater than 150 mg/dL, greater than 200 mg/dL, or to greater than 500 mg/dL.

25

In some embodiments, "a subject in need" includes a subject with decreased free Raptor, e.g., a subject afflicted with obesity.

In some embodiments, "a subject in need" encompasses, e.g., a subject with plasma or serum triglyceride level greater than 150 mg/dL, greater than 200 mg/dL, or to greater than 500 mg/dL.

30

In some embodiments, a subject afflicted with a metabolic disease, such as obesity, tends to have elevated triglyceride levels.

35

In some embodiments, a pharmaceutical composition comprises a pharmaceutical carrier and a compound.

In some embodiments, the pharmaceutical composition is manufactured, wherein the pharmaceutical composition comprises an isolated or purified naturally occurring compound.

5

In some embodiments, the pharmaceutical composition is manufactured, wherein the pharmaceutical composition comprises a compound which was not produced by a process in nature.

10 In some embodiments, a reference subject includes a subject with hepatic or plasma or serum triglyceride levels less than 150 mg/dL.

As described herein, young mice (9-week old, equivalent to an immediately post-pubertal human) tend to have "normal" Raptor levels,
15 which decline by 24-weeks (equivalent to adulthood).

In some embodiments, a reference subject includes a subject that has not reached adulthood.

20 In some embodiments, a reference subject includes a subject younger than 18 years old.

In some embodiments, a reference subject is not afflicted with a metabolic disease and is younger than 18 years old.

25

Methods of increasing Raptor or PHLPP2

In some embodiments, each compound administered to the subject is, independently, an organic compound having a molecular weight less than 1000 Daltons, a polypeptide, an oligonucleotide, or a small molecule.

30

In some embodiments, the pharmaceutical composition which is capable of increasing PHLPP2 or free Raptor enhances expression of a gene or enhances transcription.

35 In some embodiments, the pharmaceutical composition which is capable of increasing free Raptor inhibits interaction of Raptor and mTORC1.

In some embodiments, a pharmaceutical composition that is capable of increasing free Raptor is administered to the subject.

5 In some embodiments, a pharmaceutical composition that is capable of increasing PHLPP2 is administered to the subject.

In some embodiments, a pharmaceutical composition that is capable of increasing Raptor expression is administered to the subject.

10 In some embodiments, a pharmaceutical composition that is capable of increasing PHLPP2 expression is administered to the subject.

Small Molecule

15 A small molecule may be administered herein to increase free Raptor or increase activity of Raptor.

A small molecule may be administered herein to increase PHLPP2 or increase activity of PHLPP2.

20 Non-limiting examples of PHLPP2 activators such as anti-miRNA oligonucleotides to miR-190, or small-molecular or other inhibitors of the B-TrCP degradative pathway are described, for example, in the following publications: Beezhold, et al, Toxicological Sciences, 2011, and Li, Liu and Gao, Mol Cell Biology, 2009, which are hereby
25 incorporated by reference in their entirety.

Oligonucleotide

Non-limiting examples of oligonucleotides capable of increasing Raptor or PHLPP2 expression include antisense oligonucleotides,
30 polynucleotides, and adenoviral vectors.

The amino acid sequence of Raptor, or KIAA1303, is accessible in public databases by the GenBank accession number Q8N122.1, and is set forth herein as SEQ ID NO: 1.

35

The amino acid sequence of, PH domain leucine-rich repeat-containing protein phosphatase 2 (PHLPP2), is accessible in public databases by

the GenBank accession number Q6ZVD8.3, and is set forth herein as SEQ ID NO: 2.

5 In some embodiments, the pharmaceutical composition which is capable of increasing free Raptor or PHLPP2 comprises a polynucleotide or an adenovirus.

10 In some embodiments, the pharmaceutical composition which is capable of increasing Raptor or PHLPP2 expression comprises a polynucleotide or an adenovirus.

15 Non-limiting examples of oligonucleotides capable of decreasing Glucagon signaling include antisense oligonucleotides, polynucleotides, and adenoviral vectors.

The sequence of, Glucagon (GCG), is accessible in public databases by the GenBank accession number NM_002054.4, and is set forth herein as SEQ ID NO: 3.

20 The amino acid sequence of Glucagon Receptor (gcgr) is accessible in public databases by the GenBank accession number L20316.1, and is set forth herein as SEQ ID NO: 4.

25 As used herein, "a subject afflicted with" a disease, e.g. nonalcoholic fatty liver disease, means a human patient who was been affirmatively diagnosed to have the disease.

30 As used herein, "a subject afflicted with" a disease, e.g. nonalcoholic fatty liver disease, means a human patient who was been affirmatively diagnosed to have the disease.

Antisense Oligonucleotide

35 Antisense oligonucleotides are nucleotide sequences which are complementary to a specific DNA or RNA sequence. Once introduced into a cell, the complementary nucleotides combine with natural sequences produced by the cell to form complexes and block either transcription or translation. Preferably, an antisense oligonucleotide is at least

11 nucleotides in length, but can be at least 12, 15, 20, 25, 30, 35, 40, 45, or 50 or more nucleotides long. Longer sequences also can be used. Antisense oligonucleotide molecules can be provided in a DNA construct and introduced into a cell as described above to decrease
5 the level of target gene products in the cell.

Antisense oligonucleotides can be deoxyribonucleotides, ribonucleotides, or a combination of both. Oligonucleotides can be synthesized manually or by an automated synthesizer, by covalently
10 linking the 5' end of one nucleotide with the 3' end of another nucleotide with non-phosphodiester internucleotide linkages such as alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, alkylphosphonates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters,
15 carbonates, and phosphate triesters.

Modifications of gene expression can be obtained by designing antisense oligonucleotides which will form duplexes to the control, 5', or regulatory regions of the gene. Oligonucleotides derived from
20 the transcription initiation site, e.g., between positions -10 and +10 from the start site, are preferred. Similarly, inhibition can be achieved using "triple helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases,
25 transcription factors, or chaperons. Therapeutic advances using triplex DNA have been described in the literature (Nicholls et al., 1993, J Immunol Meth 165:81-91). An antisense oligonucleotide also can be designed to block translation of mRNA by preventing the transcript from binding to ribosomes.

30 Precise complementarity is not required for successful complex formation between an antisense oligonucleotide and the complementary sequence of a target polynucleotide. Antisense oligonucleotides which comprise, for example, 1, 2, 3, 4, or 5 or more stretches of contiguous
35 nucleotides which are precisely complementary to a target polynucleotide, each separated by a stretch of contiguous nucleotides which are not complementary to adjacent nucleotides, can provide

sufficient targeting specificity for a target mRNA. Preferably, each stretch of complementary contiguous nucleotides is at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more nucleotides in length. Noncomplementary intervening sequences are preferably 1, 2, 3, or 4 nucleotides in length. One skilled in the art can easily use the calculated melting point of an antisense-sense pair to determine the degree of mismatching which will be tolerated between a particular antisense oligonucleotide and a particular target polynucleotide sequence. Antisense oligonucleotides can be modified without affecting their ability to hybridize to a target polynucleotide. These modifications can be internal or at one or both ends of the antisense molecule. For example, internucleoside phosphate linkages can be modified by adding cholesteryl or diamine moieties with varying numbers of carbon residues between the amino groups and terminal ribose. Modified bases and/or sugars, such as arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide in which the 3' hydroxyl group or the 5' phosphate group are substituted, also can be employed in a modified antisense oligonucleotide. These modified oligonucleotides can be prepared by methods well known in the art.

Ribozymes

Ribozymes are RNA molecules with catalytic activity (Uhlmann et al., 1987, Tetrahedron. Lett. 215, 3539-3542). Ribozymes can be used to inhibit gene function by cleaving an RNA sequence, as is known in the art. The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Examples include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of specific nucleotide sequences. The coding sequence of a polynucleotide can be used to generate ribozymes which will specifically bind to mRNA transcribed from the polynucleotide. Methods of designing and constructing ribozymes which can cleave other RNA molecules in trans in a highly sequence specific manner have been developed and described in the art. For example, the cleavage activity of ribozymes can be targeted to specific RNAs by engineering a discrete "hybridization" region into

the ribozyme. The hybridization region contains a sequence complementary to the target RNA and thus specifically hybridizes with the target RNA.

5 Specific ribozyme cleavage sites within an RNA target can be identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences: GUA, GUU, and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target RNA containing the cleavage
10 site can be evaluated for secondary structural features which may render the target inoperable. Suitability of candidate RNA targets also can be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays. Longer complementary sequences can be used to increase the affinity
15 of the hybridization sequence for the target. The hybridizing and cleavage regions of the ribozyme can be integrally related such that upon hybridizing to the target RNA through the complementary regions, the catalytic region of the ribozyme can cleave the target.

20 Ribozymes can be introduced into cells as part of a DNA construct. Mechanical methods, such as microinjection, liposome-mediated transfection, electroporation, or calcium phosphate precipitation, can be used to introduce a ribozyme-containing DNA construct into cells in which it is desired to decrease target gene expression.
25 Alternatively, if it is desired that the cells stably retain the DNA construct, the construct can be supplied on a plasmid and maintained as a separate element or integrated into the genome of the cells, as is known in the art. A ribozyme-encoding DNA construct can include transcriptional regulatory elements, such as a promoter element, an
30 enhancer or VAS element, and a transcriptional 45on alcohol signal, for controlling transcription of ribozymes in the cells (U.S. 5,641,673). Ribozymes also can be engineered to provide an additional level of regulation, so that destruction of mRNA occurs only when both a ribozyme and a target gene are induced in the cells.

35

RNA Interference

An interfering RNA (RNAi) molecule involves mRNA degradation. The use

of RNAi has been described in Fire et al., 1998, Carthew et al., 2001, and Elbashir et al., 2001, the contents of which are incorporated herein by reference.

5 Interfering RNA or small inhibitory RNA (RNAi) molecules include short interfering RNAs (siRNAs), repeat-associated siRNAs (rasiRNAs), and micro-RNAs (miRNAs) in all stages of processing, including shRNAs, pri-miRNAs, and pre-miRNAs. These molecules have different origins: siRNAs are processed from double-stranded precursors (dsRNAs) with
10 two distinct strands of base-paired RNA; siRNAs that are derived from repetitive sequences in the genome are called rasiRNAs; miRNAs are derived from a single transcript that forms base-paired hairpins. Base pairing of siRNAs and miRNAs can be perfect (i.e., fully complementary) or imperfect, including bulges in the duplex region.

15 Interfering RNA molecules encoded by recombinase-dependent transgenes of the invention can be based on existing shRNA, siRNA, piwi-interacting RNA (piRNA), micro RNA (miRNA), double-stranded RNA (dsRNA), antisense RNA, or any other RNA species that can be cleaved
20 inside a cell to form interfering RNAs, with compatible modifications described herein.

As used herein, an "shRNA molecule" includes a conventional stem-loop shRNA, which forms a precursor miRNA (pre-miRNA). "shRNA" also
25 includes micro-RNA embedded shRNAs (miRNA-based shRNAs), wherein the guide strand and the passenger strand of the miRNA duplex are incorporated into an existing (or natural) miRNA or into a modified or synthetic (designed) miRNA. When transcribed, a shRNA may form a primary miRNA (pri-miRNA) or a structure very similar to a natural
30 pri-miRNA. The pri-miRNA is subsequently processed by Drosha and its cofactors into pre-miRNA. Therefore, the term "shRNA" includes pri-miRNA (shRNA-mir) molecules and pre-miRNA molecules.

A "stem-loop structure" refers to a nucleic acid having a secondary
35 structure that includes a region of nucleotides which are known or predicted to form a double strand or duplex (stem portion) that is linked on one side by a region of predominantly single-stranded

nucleotides (loop portion). The terms "hairpin" and "fold-back" structures are also used herein to refer to stem-loop structures. Such structures are well known in the art and the term is used consistently with its known meaning in the art. As is known in the art, the secondary structure does not require exact base-pairing. Thus, the stem can include one or more base mismatches or bulges. Alternatively, the base-pairing can be exact, i.e. not include any mismatches.

"RNAi-expressing construct" or "RNAi construct" is a generic term that includes nucleic acid preparations designed to achieve an RNA interference effect. An RNAi-expressing construct comprises an RNAi molecule that can be cleaved *in vivo* to form an siRNA or a mature shRNA. For example, an RNAi construct is an expression vector capable of giving rise to a siRNA or a mature shRNA *in vivo*. Non-limiting examples of vectors that may be used in accordance with the present invention are described herein and will be well known to a person having ordinary skill in the art. Exemplary methods of making and delivering long or short RNAi constructs can be found, for example, in WO01/68836 and WO01/75164.

Use of RNAi

RNAi is a powerful tool for *in vitro* and *in vivo* studies of gene function in mammalian cells and for therapy in both human and veterinary contexts. Inhibition of a target gene is sequence-specific in that gene sequences corresponding to a portion of the RNAi sequence, and the target gene itself, are specifically targeted for genetic inhibition. Multiple mechanisms of utilizing RNAi in mammalian cells have been described. The first is cytoplasmic delivery of siRNA molecules, which are either chemically synthesized or generated by DICER-digestion of dsRNA. These siRNAs are introduced into cells using standard transfection methods. The siRNAs enter the RISC to silence target mRNA expression.

Another mechanism is nuclear delivery, via viral vectors, of gene expression cassettes expressing a short hairpin RNA (shRNA). The shRNA is modeled on micro interfering RNA (miRNA), an endogenous trigger of the RNAi pathway (Lu *et al.*, 2005, *Advances in Genetics*

54: 117-142, Fewell *et al.*, 2006, *Drug Discovery Today* 11: 975-982). Conventional shRNAs, which mimic pre-miRNA, are transcribed by RNA Polymerase II or III as single-stranded molecules that form stem-loop structures. Once produced, they exit the nucleus, are cleaved by
5 DICER, and enter the RISC as siRNAs.

Another mechanism is identical to the second mechanism, except that the shRNA is modeled on primary miRNA (shRNAmir), rather than pre-miRNA transcripts (Fewell *et al.*, 2006). An example is the miR-30
10 miRNA construct. The use of this transcript produces a more physiological shRNA that reduces toxic effects.

The shRNAmir is first cleaved to produce shRNA, and then cleaved again by DICER to produce siRNA. The siRNA is then incorporated into the
15 RISC for target mRNA degradation. However, aspects of the present invention relate to RNAi molecules that do not require DICER cleavage. See, e.g., U.S. Patent No. 8,273,871, the entire contents of which are incorporated herein by reference.

20 For mRNA degradation, translational repression, or deadenylation, mature miRNAs or siRNAs are loaded into the RNA Induced Silencing Complex (RISC) by the RISC-loading complex (RLC). Subsequently, the guide strand leads the RISC to cognate target mRNAs in a sequence-specific manner and the Slicer component of RISC hydrolyses the
25 phosphodiester bond coupling the target mRNA nucleotides paired to nucleotide 10 and 11 of the RNA guide strand. Slicer forms together with distinct classes of small RNAs the RNAi effector complex, which is the core of RISC. Therefore, the "guide strand" is that portion of the double-stranded RNA that associates with RISC, as opposed to
30 the "passenger strand," which is not associated with RISC.

It is not necessary that there be perfect correspondence of the sequences, but the correspondence must be sufficient to enable the RNA to direct RNAi inhibition by cleavage or blocking expression of the
35 target mRNA. In preferred RNA molecules, the number of nucleotides which is complementary to a target sequence is 16 to 29, 18 to 23, or 21-23, or 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25.

Isolated RNA molecules can mediate RNAi. That is, the isolated RNA molecules of the present invention mediate degradation or block expression of mRNA that is the transcriptional product of the gene.

5 For convenience, such mRNA may also be referred to herein as mRNA to be degraded. The terms RNA, RNA molecule(s), RNA segment(s) and RNA fragment(s) may be used interchangeably to refer to RNA that mediates RNA interference. These terms include double-stranded RNA, small interfering RNA (siRNA), hairpin RNA, single-stranded RNA, isolated
10 RNA (partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA), as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the RNA
15 or internally (at one or more nucleotides of the RNA). Nucleotides in the RNA molecules of the present invention can also comprise nonstandard nucleotides, including non-naturally occurring nucleotides or deoxyribonucleotides. Collectively, all such altered RNAi molecules are referred to as analogs or analogs of naturally-
20 occurring RNA. RNA of the present invention need only be sufficiently similar to natural RNA that it has the ability to mediate RNAi.

As used herein the phrase "mediate RNAi" refers to and indicates the ability to distinguish which mRNA molecules are to be afflicted with
25 the RNAi machinery or process. RNA that mediates RNAi interacts with the RNAi machinery such that it directs the machinery to degrade particular mRNAs or to otherwise reduce the expression of the target protein. In one embodiment, the present invention relates to RNA molecules that direct cleavage of specific mRNA to which their
30 sequence corresponds. It is not necessary that there be perfect correspondence of the sequences, but the correspondence must be sufficient to enable the RNA to direct RNAi inhibition by cleavage or blocking expression of the target mRNA.

35 In some embodiments, an RNAi molecule of the invention is introduced into a mammalian cell in an amount sufficient to attenuate target gene expression in a sequence specific manner. The RNAi molecules of the

invention can be introduced into the cell directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to the cell. In certain embodiments the RNAi molecule can be a synthetic RNAi molecule, including RNAi molecules incorporating modified nucleotides, such as those with chemical modifications to the 2'-OH group in the ribose sugar backbone, such as 2'-O-methyl (2'Ome), 2'-fluoro (2'F) substitutions, and those containing 2'Ome, or 2'F, or 2'-deoxy, or "locked nucleic acid" (LNA) modifications. In some embodiments, an RNAi molecule of the invention contains modified nucleotides that increase the stability or half-life of the RNAi molecule in vivo and/or in vitro. Alternatively, the RNAi molecule can comprise one or more aptamers, which interact(s) with a target of interest to form an aptamer:target complex. The aptamer can be at the 5' or the 3' end of the RNAi molecule. Aptamers can be developed through the SELEX screening process and chemically synthesized. An aptamer is generally chosen to preferentially bind to a target. Suitable targets include small organic molecules, polynucleotides, polypeptides, and proteins. Proteins can be cell surface proteins, extracellular proteins, membrane proteins, or serum proteins, such as albumin. Such target molecules may be internalized by a cell, thus effecting cellular uptake of the shRNA. Other potential targets include organelles, viruses, and cells.

As noted above, the RNA molecules of the present invention in general comprise an RNA portion and some additional portion, for example a deoxyribonucleotide portion. The total number of nucleotides in the RNA molecule is suitably less than in order to be effective mediators of RNAi. In preferred RNA molecules, the number of nucleotides is 16 to 29, more preferably 18 to 23, and most preferably 21-23.

Adenoviral Vector

An adenoviral vector encodes an oligonucleotide. The use of adenoviral vectors in gene therapy and tissue-specific targeting has been described in Beatty and Curiel, 2012, Barnett et al., 2002, and Rots et al., 2003, the contents of which are incorporated herein by reference.

Methods of Administration

"Administering" compounds in embodiments of the invention can be effected or performed using any of the various methods and delivery systems known to those skilled in the art. The administering can be, for example, intravenous, oral, intramuscular, intravascular, intra-arterial, intracoronary, intramyocardial, intraperitoneal, and subcutaneous. Other non-limiting examples include topical administration, or coating of a device to be placed within the subject.

10 *Injectable Drug Delivery*

Injectable drug delivery systems may be employed in the methods described herein include solutions, suspensions, gels.

Oral Drug Delivery

15 Oral delivery systems include tablets and capsules. These can contain excipients such as binders (e.g., hydroxypropylmethylcellulose, polyvinyl pyrrolidone, other cellulosic materials and starch), diluents (e.g., lactose and other sugars, starch, dicalcium phosphate and cellulosic materials), disintegrating agents (e.g., starch polymers and cellulosic materials) and lubricating agents (e.g., stearates and talc). Solutions, suspensions and powders for reconstitutable delivery systems include vehicles such as suspending agents (e.g., gums, xanthans, cellulose and sugars), humectants (e.g., sorbitol), solubilizers (e.g., ethanol, water, PEG and propylene glycol),
20 surfactants (e.g., sodium lauryl sulfate, Spans, Tweens, and cetyl pyridine), preservatives and antioxidants (e.g., parabens, vitamins E and C, and ascorbic acid), anti-caking agents, coating agents, and chelating agents (e.g., EDTA).

30 For oral administration in liquid dosage form, a PHLPP2 or Raptor activator may be combined with any oral, non-toxic, pharmaceutically acceptable inert carrier such as ethanol, glycerol, water, and the like.

35 *Pharmaceutically Acceptable Carrier*

The compounds used in embodiments of the present invention can be administered in a pharmaceutically acceptable carrier. As used herein,

a "pharmaceutically acceptable carrier" is a pharmaceutically acceptable solvent, suspending agent or vehicle, for delivering the compounds to the subject. The carrier may be liquid or solid and is selected with the planned manner of administration in mind. Liposomes
5 such as small unilamellar vesicles, large unilamellar vesicles, and multilamellar vesicles are also a pharmaceutically acceptable carrier. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine, or phosphatidylcholines. The compounds may be administered as components of tissue-targeted emulsions. Examples
10 of lipid carriers for antisense delivery are disclosed in U.S. Patent Nos. 5,855,911 and 5,417,978, which are incorporated herein by reference. The compounds used in the methods of the present invention can be administered in admixture with suitable pharmaceutical diluents, extenders, excipients, or carriers (collectively referred
15 to herein as a pharmaceutically acceptable carrier) suitably selected with respect to the intended form of administration and as consistent with conventional pharmaceutical practices. The unit will be in a form suitable for oral, rectal, topical, intravenous or direct injection or parenteral administration. The compounds can be administered alone
20 or mixed with a pharmaceutically acceptable carrier. This carrier can be a solid or liquid, and the type of carrier is generally chosen based on the type of administration being used. The active agent can be co-administered in the form of a tablet or capsule, liposome, as an agglomerated powder or in a liquid form. Examples of suitable solid
25 carriers include lactose, sucrose, gelatin and agar. Capsule or tablets can be easily formulated and can be made easy to swallow or chew; other solid forms include granules, and bulk powders. Tablets may contain suitable binders, lubricants, diluents, disintegrating agents, coloring agents, flavoring agents, flow-inducing agents, and
30 melting agents. Examples of suitable liquid dosage forms include solutions or suspensions in water, pharmaceutically acceptable fats and oils, alcohols or other organic solvents, including esters, emulsions, syrups or elixirs, suspensions, solutions and/or suspensions reconstituted from non-effervescent granules and
35 effervescent preparations reconstituted from effervescent granules. Such liquid dosage forms may contain, for example, suitable solvents, preservatives, emulsifying agents, suspending agents, diluents,

sweeteners, thickeners, and melting agents. Oral dosage forms optionally contain flavorants and coloring agents. Parenteral and intravenous forms may also include minerals and other materials to make them compatible with the type of injection or delivery system chosen.

A compound of the invention can be administered in a mixture with suitable pharmaceutical diluents, extenders, excipients, or carriers (collectively referred to herein as a pharmaceutically acceptable carrier) suitably selected with respect to the intended form of administration and as consistent with conventional pharmaceutical practices. The unit will be in a form suitable for oral, rectal, topical, intravenous or direct injection or parenteral administration. The compounds can be administered alone but are generally mixed with a pharmaceutically acceptable carrier. This carrier can be a solid or liquid, and the type of carrier is generally chosen based on the type of administration being used. In one embodiment the carrier can be a monoclonal antibody. The active agent can be co-administered in the form of a tablet or capsule, liposome, as an agglomerated powder or in a liquid form. Examples of suitable solid carriers include lactose, sucrose, gelatin and agar. Capsule or tablets can be easily formulated and can be made easy to swallow or chew; other solid forms include granules, and bulk powders. Tablets may contain suitable binders, lubricants, diluents, disintegrating agents, coloring agents, flavoring agents, flow-inducing agents, and melting agents. Examples of suitable liquid dosage forms include solutions or suspensions in water, pharmaceutically acceptable fats and oils, alcohols or other organic solvents, including esters, emulsions, syrups or elixirs, suspensions, solutions and/or suspensions reconstituted from non-effervescent granules and effervescent preparations reconstituted from effervescent granules. Such liquid dosage forms may contain, for example, suitable solvents, preservatives, emulsifying agents, suspending agents, diluents, sweeteners, thickeners, and melting agents. Oral dosage forms optionally contain flavorants and coloring agents. Parenteral and intravenous forms may also include minerals and other materials to make them compatible with the type of injection or delivery system chosen.

Specific examples of pharmaceutical acceptable carriers and excipients that may be used to formulate oral dosage forms of the present invention are described in U. S. Pat. No. 3,903,297, issued Sept. 2, 1975.

Tablets

Tablets may contain suitable binders, lubricants, disintegrating agents, coloring agents, flavoring agents, flow-inducing agents, and melting agents. For instance, for oral administration in the dosage unit form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic, pharmaceutically acceptable, inert carrier such as lactose, gelatin, agar, starch, sucrose, glucose, methyl cellulose, magnesium stearate, dicalcium phosphate, calcium sulfate, mannitol, sorbitol and the like. Suitable binders include starch, gelatin, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth, or sodium alginate, carboxymethylcellulose, polyethylene glycol, waxes, and the like. Lubricants used in these dosage forms include sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride, and the like. Disintegrators include, without limitation, starch, methyl cellulose, agar, bentonite, xanthan gum, and the like.

Specific Administration To Liver

Embodiments of the invention relate to specific administration to the liver or hepatocytes.

In some embodiments, a compound may specifically target the liver.

In some embodiments, a compound may specifically target hepatocytes.

In some embodiments, a compound may be specifically targeted to the liver by coupling the compound to ligand molecules, targeting the compound to a receptor on a hepatic cell, or administering the compound by a bio-nanocapsule.

A compound of the invention can also be administered by coupling of ligand molecules, such as coupling or targeting moieties on preformed nanocarriers, such as (PGA-PLA nanoparticles, PLGA nanoparticles, cyclic RGD-doxorubicin-nanoparticles, and poly(ethylene glycol)-coated biodegradable nanoparticles); by the post-insertion method, by the Avidin-Biotin complex, or before nanocarriers formulation, or by targeting receptors present on various hepatic cell, such as Asialoglycoprotein receptor (ASGP-R), HDL-R, LDL-R, IgA-R, Scavenger R, Transferrin R, and Insulin R, as described in: Mishra et al., (2013) Efficient Hepatic Delivery of Drugs: Novel Strategies and Their Significance, BioMed Research International 2013: 382184, dx.doi.org/10.1155/2013/382184, the entire contents of which are incorporated herein by reference.

A compound of the invention can also be administered by bio-nanocapsule, as described in: Yu et al., (2005) The Specific delivery of proteins to human liver cells by engineered bio-nanocapsules, FEBS Journal 272: 3651-3660, dx.doi.org/10.1111/j.1742-4658.2005.04790.x, the entire contents of which are incorporated herein by reference.

In some embodiments, an oligonucleotide specifically targets the liver.

In some embodiments, an oligonucleotide specifically targets hepatocytes.

Antisense oligonucleotides of the invention can also be targeted to hepatocytes, as described in: Prakash et al., (2014) Targeted delivery of antisense oligonucleotides to hepatocytes using triantennary N-acetyl galactosamine improves potency 10-fold in mice, Nucleic Acids Research 42(13): 8796-8807, dx.doi.org/10.1093/nar/gku531, the entire contents of which are incorporated herein by reference.

As used herein, the term "effective amount" refers to the quantity of a component that is sufficient to treat a subject without undue adverse side effects (such as toxicity, irritation, or allergic response)

commensurate with a reasonable benefit/risk ratio when used in the manner of this invention, i.e. a therapeutically effective amount. The specific effective amount will vary with such factors as the particular condition being treated, the physical condition of the patient, the type of subject being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed and the structure of the compounds or its derivatives.

- Techniques and compositions for making dosage forms useful in the present invention are described in the following references: 7 Modern Pharmaceutics, Chapters 9 and 10 (Banker & Rhodes, Editors, 1979); Pharmaceutical Dosage Forms: Tablets (Lieberman et al., 1981); Ansel, Introduction to Pharmaceutical Dosage Forms 2nd Edition (1976); Remington's Pharmaceutical Sciences, 17th ed. (Mack Publishing Company, Easton, Pa., 1985); Advances in Pharmaceutical Sciences (David Ganderton, Trevor Jones, Eds., 1992); Advances in Pharmaceutical Sciences Vol. 7. (David Ganderton, Trevor Jones, James McGinity, Eds., 1995); Aqueous Polymeric Coatings for Pharmaceutical Dosage Forms (Drugs and the Pharmaceutical Sciences, Series 36 (James McGinity, Ed., 1989); Pharmaceutical Particulate Carriers: Therapeutic Applications: Drugs and the Pharmaceutical Sciences, Vol 61 (Alain Rolland, Ed., 1993); Drug Delivery to the Gastrointestinal Tract (Ellis Horwood Books in the Biological Sciences. Series in Pharmaceutical Technology; J. G. Hardy, S. S. Davis, Clive G. Wilson, Eds.); Modern Pharmaceutics Drugs and the Pharmaceutical Sciences, Vol 40 (Gilbert S. Banker, Christopher T. Rhodes, Eds.). All of the aforementioned publications are incorporated by reference herein.
- The dosage of a compound of the invention administered in treatment will vary depending upon factors such as the 56on alcoholic56ics characteristics of the compound and its mode and route of administration; the age, sex, metabolic rate, absorptive efficiency, health and weight of the recipient; the nature and extent of the symptoms; the kind of concurrent treatment being administered; the frequency of treatment with; and the desired therapeutic effect.

A dosage unit of the compounds of the invention may comprise a compound alone, or mixtures of a compound with additional compounds used to treat cancer. The compounds can be administered in oral dosage forms as tablets, capsules, pills, powders, granules, elixirs, tinctures, suspensions, syrups, and emulsions. The compounds may also be administered in intravenous (bolus or infusion), intraperitoneal, subcutaneous, or intramuscular form, or introduced directly, e.g. by injection or other methods, into the eye, all using dosage forms well known to those of ordinary skill in the pharmaceutical arts.

In an embodiment, the pharmaceutical composition may be administered once a day, twice a day, every other day, once weekly, or twice weekly.

A subject's triglyceride level may be expressed herein as hepatic triglyceride or plasma triglyceride or serum triglyceride.

Where a range is given in the specification it is understood that the range includes all integers and 0.1 units within that range, and any sub-range thereof. For example, a range of 1 to 5 is a disclosure of 1.0, 1.1, 1.2, etc.

This invention will be better understood by reference to the Examples which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

Combination Therapy

The administration of two drugs to treat a given condition, such as non-alcoholic fatty liver disease (NAFLD), raises a number of potential problems. In vivo interactions between two drugs are complex. The effects of any single drug are related to its absorption, distribution, and elimination. When two drugs are introduced into the body, each drug can affect the absorption, distribution, and elimination of the other and hence, alter the effects of the other. For instance, one drug may inhibit, activate or induce the production of enzymes involved in a metabolic route of elimination of the other drug. (Guidance for Industry, 1999) Thus, when two drugs are administered to treat the same condition, it is unpredictable whether each will complement, have no effect on, or interfere with the therapeutic activity of the other in a human subject.

Not only may the interaction between two drugs affect the intended therapeutic activity of each drug, but the interaction may increase the levels of toxic metabolites (Guidance for Industry, 1999). The interaction may also heighten or lessen the side effects of each drug. Hence, upon administration of two drugs to treat a disease, it is unpredictable what change will occur in the negative side effect profile of each drug.

Additionally, it is difficult to accurately predict when the effects of the interaction between the two drugs will become manifest. For example, metabolic interactions between drugs may become apparent upon the initial administration of the second drug, after the two have reached a steady-state concentration or upon discontinuation of one of the drugs. (Guidance for Industry, 1999)

Example 1**Identification of post-translational modifications (PTMs) on PHLPP2 protein**

Reduced hepatic PHLPP2 protein levels, but not *Phlpp2* gene expression, were observed in aging/obesity and short-term refeeding, suggesting altered PHLPP2 stability in the insulin-resistant or insulin-rich liver, consistent with data showing regulated PHLPP2 ubiquitination and proteasomal degradation. It was next hypothesized that PHLPP2 protein stability was affected by nutrient- or hormone-mediated post-translational modifications (PTMs), and an *in vivo* unbiased liquid chromatography-tandem mass spectrometry (LC/MS-MS) screen by transducing C57BL/6 wild-type (WT) mice with Ad-HA-Flag-PHLPP2 followed by sequential anti-HA→anti-FLAG IP was performed (Figure 1). With this approach, five novel phosphorylation sites were identified at the functionally undefined C-terminal region between the PP2C domain and PDZ-binding motif of PHLPP2 (Figure 2-4).

PHLPP2 Ser1119 and Ser 1210 are phosphorylated in response to glucagon

To identify the signal transduction pathways involved in PHLPP2 phosphorylation, a Phos-tag-based mobility shift assay was used, which magnifies the difference in migration speed of phosphorylated and non-phosphorylated proteins (Fig. 5A-C), and it was found that activation of protein kinase A (PKA) signaling by treatment with either cAMP, or forskolin which activates adenylate cyclase to stimulates cAMP production, increased PHLPP2 phosphorylation (Figure 6). Next, to validate LC/MS-MS-based phospho-peptide mapping results, each identified Ser/Thr site was individually mutated to Alanine, then transfected WT or Ala-mutant PHLPP2 in hepatocytes, and Ser1119 and Ser1210 phosphorylations was confirmed (Fig. 7A-B). Next, it was found that forskolin could similarly induce phosphorylation overexpressed PHLPP2 in primary hepatocytes (Fig. 8A), essential to test the effects of Ala-mutant PHLPP2 *in vitro*. Interestingly, WT, S1119A, and S1210A mutant of PHLPP2 were all phosphorylated with forskolin treatment, suggesting that both S1119 and S1210 sites might be phosphorylated by PKA (Fig. 8B). Thus, double mutants were generated, such as S1119/1210

and it was found that ablation of both sites rendered the S1119A/S1210A mutant insensitive to forskolin-induced phosphorylation (Fig. 8C). These data matched *in silico* analysis of consensus substrate sites for mammalian kinases, as both Ser1119 and Ser1210 are exact matches for the cyclic AMP-PKA consensus site. To investigate the effect of PKA on PHLPP2 phosphorylation in primary hepatocytes, either WT or S1119/1210A mutant of PHLPP2 was immunoprecipitated from cell extracts treated with glucagon or forskolin, and then subjected to immunoblot analysis with an antibody specific for phosphorylated PKA substrate proteins. PKA-mediated phosphorylation of WT, but not S1119A/S1210A mutant PHLPP2 was robustly increased following glucagon or forskolin treatment of primary hepatocytes. In addition, forskolin-mediated PHLPP2 phosphorylation was reduced by pretreatment of PKA inhibitor, H89, proving that PKA stimulates PHLPP2 phosphorylation (Fig. 9A-B).

These data suggest that glucagon may induce PHLPP2 phosphorylation and thus degradation, and that inhibitors of glucagon signaling would increase PHLPP2 levels. To test this concept *in vivo*, adenoviruses encoding shRNA were transduced to the glucagon receptor (or a control shRNA) to either genetically-induced (db/db) mice or diet-induced obese (DIO) mice. As hypothesized, knockdown of liver glucagon signaling rescues the lower PHLPP2 levels seen in obesity (Figs. 10A-C and Figs. 11A-B). This suggests that inhibitors of glucagon signaling will similarly increase the pathologically lower PHLPP2 levels in the obese state, and reduce hepatic and plasma triglyceride.

PHLPP2 knockout (KO) cells show prolonged insulin signaling.

To determine whether PHLPP2 is necessary to terminate insulin signaling, PHLPP2 KO HepG2 hepatoma cells were generated using the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas9 system. Using three different single guide RNA (sgRNA) for PHLPP2, significantly reduced PHLPP2 protein and mRNA levels were observed, without effects on off-target gene expression (Fig. 12A-C). Next, HepG2 stable cell lines were generated expressing the most efficient PHLPP2 sgRNA (#3) sgRNA3 (Fig. 13A) and, in parallel, a mouse hepatoma cell lines, Hepalclc7 cells (Fig. 13B). It was found that PHLPP2-deficient cells showed sustained insulin signaling as

compared to control cells, as judged by Akt phosphorylation (Fig. 14A). Next, PHLPP2 levels were "rescued" in these CRISPR-induced knockout cell lines with either WT or S1119/1210A PHLPP2, and found that only mutant PHLPP2 prevented acute insulin-induced Akt phosphorylation (Fig. 14B), indicating that PKA-mediated phosphorylation is likely responsible for decreased insulin-mediated Akt phosphorylation.

Identification of novel PHLPP2 interaction proteins

To further identify the regulatory mechanism by which PHLPP2 levels decrease in aging and obese liver through proteasomal degradation, LC/MS-MS analysis was repeated to determine novel PHLPP2 binding partners (Figure 15), and identified KCTD17 (potassium channel tetramerization domain containing 17) as a strong PHLPP2 interactor. Next, PHLPP2 was confirmed to associate with KCTD17, but in a proteasome-dependent manner (Fig. 16A). Consistent with the notion that S1119/S120 phosphorylation regulates PHLPP2 stability by proteasomal degradation, the association of endogenous PHLPP2 with KCTD17 was significantly enhanced by treatment of forskolin, and even more strongly with simultaneous treatment with forskolin and the proteasome inhibitor, MG132 (Fig. 16B). Finally, and as hypothesized, while WT PHLPP2 associates with KCTD17 with forskolin and MG132 treatment, S1119/1210A mutant PHLPP2 showed markedly lower KCTD17 binding (Figure 17). In sum, these data indicate that KCTD17 is a novel PHLPP2 interacting protein, which may regulate PHLPP2 degradation in a phosphorylation/proteasome-dependent manner.

Example 2

KCTD17 targets PHLPP2 for degradation to induce NAFLD

Obesity-induced fatty liver predisposes to non-alcoholic steatohepatitis (NASH), which has no approved pharmacotherapy, making it the fastest growing indication for liver transplantation. Fatty liver develops in part due to excess hepatic *de novo* lipogenesis (DNL), an insulin-stimulated cell-autonomous synthesis of fatty acids, a conundrum as obesity is commonly associated with insulin resistance and glucose intolerance. The serine/threonine phosphatase PHLPP2

terminates insulin action in the liver by dephosphorylating Akt Ser473 to repress DNL but not gluconeogenic pathways. Thus, BXD strains with lowest *Phlpp2* expression and liver-specific *PHLPP2* knockout (*L-PHLPP2*) mice show normal initiation of insulin signaling and intact glucose tolerance, but prolonged insulin action and hepatic steatosis. In obesity, endogenous *PHLPP2* is degraded - to understand this regulation, we performed LC-MS/MS which identified glucagon/PKA-dependent *PHLPP2* phosphorylations at Ser1119 and Ser1210, which recruit the adaptor *KCTD17* and ubiquitin E3 ligase Cullin3. Hepatic *Kctd17* expression is increased in murine obesity, and *KCTD17* is correlated with excess hepatic fat in patients. Knockdown of hepatic *Kctd17* in obese mice prevents *PHLPP2* degradation, allowing normal repression of DNL. These results demonstrate the first direct signaling interaction between insulin and glucagon pathways, and suggest that modulators of the *PHLPP2/KCTD17* axis can reverse obesity-induced hepatic steatosis to treat NASH.

KCTD17 targets PHLPP2 for degradation to induce NAFLD

The liver integrates nutrient state with the need to preserve glucose and lipids in a narrow physiologic range, with assistance from hormonal inputs (Pajvani and Accili, 2015). In normal physiology, fasting triggers the release of glucagon from pancreatic α -cells, which stimulates gluconeogenesis and glycogenolysis to maintain normoglycemia (Jelinek et al. 1993). In the postprandial state, pancreatic β -cells dial up insulin levels to block hepatic glucose production (Summers and Birnbaum, 1997; Puigserver et al., 2003; Petersen et al., 1998) and utilize excess glucose for long-term energy storage in the liver in the form of glycogen, and indirectly, triglycerides (Saltiel et al. 2001).

The elegance of this system is undermined by the chronic overnutrition associated with obesity, which leads to insulin resistance and an inability to restrain hepatic glucose production and the fasting hyperglycemia of Type 2 Diabetes (T2D). Nonetheless, compensatory hyperinsulinemia is apparently sufficient to activate sterol regulatory element-binding protein 1c (*Srebp1c*)-mediated lipogenesis (Shimomura et al., 2000; Matsumoto et al., 2006), which contributes

to the excess hepatic triglyceride (TG) accumulation that defines Non-Alcoholic Fatty Liver Disease (NAFLD) (Donnelly et al., 2005). The mechanism underlying this "selective" insulin resistance remains under debate, but we and others have shown that insulin signaling diverges at the critical molecular node, Akt, to activate mTORC1-dependent and -independent lipogenic pathways (Shi et al., 2010; Yecies et al., 2011; Kim et al., 2016). But insulin doesn't act alone - the lipogenic program is under complex control by nutrient state and other hormones. Of note in this cadre is glucagon (Kersten et al., 2001), postprandial plasma levels of which remain inappropriately high in T2D patients (Butler et al., 1991; Dunning et al., 2007; Ali and Drucker, 2009). The interplay between insulin and glucagon pathways is further highlighted by the unique shared phenotype of reduced hepatic and plasma TG in liver-specific insulin receptor knockout (Biddinger et al., 2008) and glucagon receptor knockout mice (Conarello et al., 2007), but the potential role of glucagon in the selective insulin resistance seen in obesity has not been explored.

Pleckstrin homology (PH) domain leucine-rich repeat protein phosphatases (PHLPPs) have been shown to dephosphorylate the key regulatory Ser473 residue of Akt, thereby terminating growth factor-stimulated Akt activity and suppress tumor growth in cancer models (Gao et al., 2005; Grzechnik and Newton, 2016; Newton and Trotman, 2014). Recent work has demonstrated that PHLPPs, specifically the PHLPP2 isoform, similarly terminates insulin action in obesity (Kim et al., 2016). It was observed that diet-induced (HFD-feeding) or genetic (leptin-signaling deficient *ob/ob* or *db/db* mice) mouse models of obesity have lower PHLPP2 protein levels, which renders these mice unable to terminate insulin signaling, ultimately resulting in increased DNL and fatty liver (Kim et al., 2016). Consistently, exogenous "rescue" of hepatic PHLPP2 levels in obese mice reduced inappropriately elevated Akt Ser473 phosphorylation in the "late" refed state, and reversed fatty liver, but surprisingly, did not affect glucose tolerance. These data suggested that PHLPP2 is necessary to prevent insulin-potentiated DNL, but does not affect early post-prandial events such as insulin/Akt repression of FoxO-dependent hepatic gluconeogenesis in the bifurcation model of hepatic

insulin signaling (Shi et al., 2010).

Several important questions remained from these studies, however - 1) is chronic reduction of hepatic PHLPP2 levels sufficient to induce hepatic steatosis; 2) how is hepatic PHLPP2 lost in obesity, as *Phlpp2* mRNA is unaffected in HFD-fed and genetic mouse models; and, 3) whether these data may have clinical and/or therapeutic implications. Here, we generate and characterize hepatocyte-specific PHLPP2 knockout (*L-PHLPP2*) mice, which show prolonged insulin-stimulated Akt Ser473 phosphorylation and excess DNL and fatty liver. To understand the endogenous regulatory machinery that mediates PHLPP2 degradation in obese liver, we performed sequential LC/MS-MS-based screens, which identified novel glucagon/PKA-mediated PHLPP2 phosphorylation at Ser1119 and Ser1210, which augments PHLPP2 interaction with KCTD17, an adaptor protein for Cul3-RING ubiquitin ligases (Kasahara et al., 2014). Interestingly, *KCTD17* expression is increased in livers from obese mice and patients with NASH, linking glucagon-induced PHLPP2 phosphorylation with degradation. Thus, knockdown of KCTD17 in obese mice increases endogenous PHLPP2 and ameliorates obesity-induced fatty liver, revealing a novel strategy in the treatment of obesity-mediated NAFLD/NASH.

PHLPP2 loss-of-function induces fatty liver but does not affect glucose tolerance

To explore endogenous differences in hepatic PHLPP2 levels and relationship to metabolism, 41 strains of genetically diverse BXD mice descendant from sequential C57BL/6J and DBA/2J crosses were examined - BXD mice have ~5 million diverged sequence variants and represents, at present, the largest and best-characterized mouse genetic reference population (Williams et al., 2016). A significant, inverse correlation was found between *Phlpp2* expression and liver TG across these strains (Fig. 18a). As a first step to understand the mechanism underlying this inverse correlation, gene set enrichment analysis (GSEA) was performed using hepatic transcriptomes of 14 BXD strains showing highest and lowest expression of *Phlpp2* (Fig. 19a). In this analysis, that *Phlpp2* was found to be negatively correlated with lipogenic gene sets [*Chrebp2* (NOM $p < 0.001$), adipogenesis (NOM $p < 0.02$),

triglyceride biosynthesis (NOM $p < 0.02$) and lipid biosynthesis (NOM $p < 0.02$) which all contain *Srebp1* and its target lipogenic genes (Fig. 18b, c and Fig. 19b). Consistent with our observation that PHLPP2 overexpression does not affect glucose tolerance (Kim et al., 2016), no correlation was observed between *Phlpp2* and gene sets associated with hepatic glucose production, or plasma levels of glucose (Spearman $\rho = 0.11$, $p = 0.567$), insulin (Spearman $\rho = -0.11$, $p = 0.502$) and HOMA-IR (Spearman $\rho = 0.10$, $p = 0.553$). These data show that across a genetically diverse mouse population, higher hepatic *Phlpp2* expression is associated with lower lipid biosynthesis and may distinguish insulin-mediated functions in hepatic glucose and lipid metabolism.

To mimic the pathologic loss of hepatic PHLPP2 seen in obese mice (Lim et al., 2016), which may mirror endogenous differences in BXD strains, we generated homozygous *PHLPP2^{flox/flox}* mice (Fig. 20a, b) and transduced with AAV8-TBG-Cre to produce hepatocyte-specific PHLPP2 knockout (*L-PHLPP2*) mice. Livers from *L-PHLPP2* mice, or adenoviral Cre-mediated deletion of loxP-flanked *PHLPP2* in primary hepatocytes, showed >80% PHLPP2 deletion without compensatory increase of the related isoform, PHLPP1 (Fig. 20c, d). *PHLPP2^{flox/flox}* mice develop normally, and post-AAV transduction, *L-PHLPP2* mice show comparable body weight, adiposity and cholesterol/non-esterified fatty acid (NEFA) as control *PHLPP2^{flox/flox}* mice transduced with AAV8-TBG-GFP (Fig. 21a-e). Nevertheless, at sacrifice, *L-PHLPP2* mice show increased liver weight and TG, with associated mild hypertriglyceridemia (Fig. 18d, e and Fig. 21f). Livers from *L-PHLPP2* mice show higher Akt Ser473 phosphorylation in "late" refeeding, similar to Ad-Cre-transduced *PHLPP2^{flox/flox}* hepatocytes after prolonged insulin stimulation *in vitro* (Fig. 18f and Fig. 21g). Prolonged activation of hepatic insulin signaling through Akt thus leads to higher expression of *Srebp1c* and its canonical targets (i.e., *Fasn*), but does not affect gluconeogenic gene expression (Fig. 18g). Consistently, we observed no differences in fasting or refeed blood glucose, plasma insulin or glucose tolerance (Fig. 19h-j).

Next, it was hypothesized that high-fat diet (HFD)-feeding to induce obesity and thus loss of endogenous PHLPP2 (Kim et al., 2016) would recapitulate the relative harm seen in chow-fed *L-PHLPP2* mice, and thus negate differences between *L-PHLPP2* and control mice. Indeed, HFD-fed *L-PHLPP2* mice showed unchanged liver weight and TG from Cre- controls (Fig. 22a, b), and in fact no discernible metabolic phenotype (Fig. 22c-i) as well as unchanged lipogenic gene expression (Fig. 22j). In combination, these data would suggest that early postprandial insulin action, necessary for nuclear FoxO exclusion and cessation of gluconeogenesis (Haeusler et al., 2014) is unimpeded, but that late postprandial insulin action on lipogenesis is heightened by either endogenous (HFD feeding) or artificial (*L-PHLPP2* mice) loss of PHLPP2.

To confirm that PHLPP2 terminates insulin-Akt signaling in a cell-autonomous manner, we generated *PHLPP2* knockout (KO) hepatoma cells using CRISPR/Cas9 (Fig. 23a-c). *PHLPP2* KO cells showed similar insulin-induced Akt phosphorylation as compared to control cells, but delayed Akt dephosphorylation in the "chase" period after the insulin pulse, which was normalized by PHLPP2 reconstitution (Fig. 18h). Similar to data from *L-PHLPP2* mice, *PHLPP2* KO cells show augmented insulin-stimulated *Srebp1c* and *Fasn* as compared with control cells (Fig. 23d). These data prove that PHLPP2 is required for termination of insulin/Akt signaling, which prevents excess *Srebp1c*-mediated DNL and hepatic lipid accumulation.

PHLPP2 is phosphorylated on Ser1119 and Ser1210 by glucagon/PKA action. Obese mice show lower hepatic PHLPP2 levels, but unchanged *Phlpp2* gene expression (Kim et al., 2016). Based on this finding, we hypothesized that PHLPP2 levels may be affected by hormone or nutrient-regulated post-translational modifications (PTMs). To test this hypothesis, we performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis in immunoprecipitated PHLPP2 from livers of C57BL/6 mice transduced with HA/Flag-tagged PHLPP2, and found 2 phosphopeptides - RCpSLHPTPTSGLFQR (Ser1119) and RQNpSVNSGMLLPMSK (Ser1210) - at evolutionarily-conserved PKA consensus [RRX(S/T)] sites (Fig. 24a, b). As glucagon is the major driver of PKA activity in liver (McKnight et al. 1991), we next tested whether glucagon can induce PHLPP2

phosphorylation. Indeed, using Phos-tag-based electrophoresis, we found that PHLPP2 is rapidly phosphorylated in primary hepatocytes exposed to glucagon (Fig. 24c), similar to treatment with potent PKA activators such as cAMP or forskolin (Fig. 24d), but not after
5 activation of other hormone/nutrient signaling (i.e. insulin/PI3K, MEK, GSK or mTOR) pathways (Fig. 25a). We next used site-directed mutagenesis to create Ser→Ala mutations at Ser1119 and Ser1210, but found that mutation of either site did not fully abolish forskolin-induced PHLPP2 phosphorylation (Fig. 25b). We next generated
10 polyclonal antibodies to both p-PHLPP2 (Ser1119) and p-PHLPP2 (Ser1210), and confirmed specificity of these antibodies in primary hepatocytes transfected with WT, single (S1119A or S1210A) or double (S1119A/S1210A) PHLPP2 Ser→Ala mutants (Fig. 25c, d). Using these novel reagents, we found that endogenous PHLPP2 phosphorylation at
15 Ser1119 and Ser1210 is increased with acute glucagon treatment of primary hepatocytes (Fig. 24e). As such, acute forskolin-induced phosphorylation at both Ser1119 and Ser1210 was reduced by knockdown of PKA signaling (Fig. 24f). Thus, mutation of both Serine sites (2A) led to near-absent PHLPP2 phosphorylation in primary hepatocytes
20 treated with glucagon or forskolin (Fig. 25e), recapitulating treatment with the PKA inhibitor H-89 (Fig. 24g). In sum, these data prove that glucagon-induced PKA activity phosphorylates hepatocyte PHLPP2 at Ser1119 and Ser1210.

25 *Glucagon-mediated PHLPP2 phosphorylation reduces its stability in obesity*

Glucagon is acutely increased in the fasted animal (Jelinek et al., 1993). Thus, it was found that hepatic PHLPP2 phosphorylation at both Ser1119 and Ser1210 is dynamically regulated by the fasting-refeeding
30 transition *in vivo* (Fig. 26a, b). Obesity and T2D are associated with persistent hyperglucagonemia, which may regulate hepatic lipogenesis (Horton et al., 2002; Strable and Ntambi, 2010; Perry et al., 2014). It was hypothesized that obesity may provoke “chronic” PHLPP2 phosphorylation. Indeed, markedly increased hepatic PHLPP2
35 phosphorylation and a simultaneous reduction in total PHLPP2 protein in HFD-fed were observed (Fig. 27a-c) and in leptin-receptor (*db/db*) deficient (Fig. 27d-f) mice, but strikingly, no change in *Phlpp2* mRNA.

These results suggested that PHLPP2 phosphorylation may directly affect its stability; to test this, cells were treated with cycloheximide to inhibit new protein translation, and markedly decreased PHLPP2 protein was found in the presence of forskolin (Fig. 27g). Next, it was examined whether inhibition of hepatic glucagon signaling, by means of AAV8 encoding shRNA to the glucagon receptor (AAV8-shGcgr), could prevent loss of PHLPP2 seen in HFD-fed or *db/db* mice. Indeed, as compared to AAV8-shControl-transduced mice, AAV8-shGcgr transduction robustly increase hepatic PHLPP2 without affecting *Phlpp2* mRNA (Fig. 27h, i). Similar data were obtained with use of glucagon receptor antagonists (GRAs) in primary hepatocytes. It was concluded that glucagon-induced hepatic PHLPP2 phosphorylation affects PHLPP2 stability.

KCTD17 binds PHLPP2 to induce its degradation in obese liver

Although glucagon induces PHLPP2 phosphorylation in both fasting and obesity, comparing Fig. 27b and 27e with Fig. 26b, only obesity reduced PHLPP2 protein levels. These data imply that PHLPP2 phosphorylation may be necessary but cannot be sufficient to induce putative PHLPP2 degradation, and that a "second hit" beyond hyperglucagonemia is necessary for the post-transcriptional loss of PHLPP2 seen in obese liver. To identify the regulatory mechanisms of glucagon-induced PHLPP2 degradation, LC/MS-MS was performed with adjusted purification conditions to isolate PHLPP2-binding proteins. I resultant list of PHLPP2 interactors for potential regulatory roles in protein stability and/or hepatic metabolism was sequentially queried, and for altered expression in obese liver. From this analysis, KCTD17 (potassium channel tetramerization domain containing 17) was identified as a novel PHLPP2-associated protein. Despite its name, KCTD17 has been shown to act as a substrate-adaptor for Cul3-RING ubiquitin ligases (CRL3s) to regulate proteosomal degradation of specific targets (Kasahara et al., 2014; Inaba et al., 2016; Ji et al., 2016). First, to explore its potential role in metabolic processes, hepatic *Kctd17* expression was examined and found to be robustly increased in HFD-fed and *db/db* mice (Fig. 28a). Next, to confirm our LC-MS/MS results, we immunoprecipitated endogenous KCTD17 in livers from HFD-fed mice using anti-PHLPP2 antibody, and vice versa (Fig. 28b). KCTD17-PHLPP2

interaction was increased by proteosomal inhibitors (Fig. 28c) and synergistically with forskolin co-treatment (Fig. 28d), suggesting that glucagon-mediated PHLPP2 phosphorylation and degradation are linked by KCTD17. Indeed, KCTD17 increased PHLPP2-Cul3 interaction (Fig. 28e), but far less efficiently facilitated the interaction of Cul3 with the nonphosphorylatable PHLPP2-2A mutant (Fig. 28f). We next hypothesized that the PHLPP2-2A mutant may be relatively resistant to degradation - to test this, we transduced HFD-fed mice with adenovirus encoding GFP, PHLPP2-WT or PHLPP2-2A. Despite similar mRNA induction as compared to Ad-GFP-transduced mice (Fig. 29a), PHLPP2-2A induced far greater increase in hepatic PHLPP2 levels than PHLPP2-WT, and thus greater decrease in refed hepatic Akt (Ser473) phosphorylation (Fig. 28g). Thus, we found a graded reduction in liver weight, liver TG and lipogenic gene expression as well as plasma TG from control→PHLPP2-WT→PHLPP2-2A-transduced mice (Fig. 28h-k), despite similar body weight/adiposity and glucose homeostasis among the groups (Fig. 29b-e). In sum, these data show that glucagon-induced phosphorylation and KCTD17-recruitment of Cul3 synergistically target PHLPP2 for degradation, which then increases DNL to provoke hepatic steatosis.

Knockdown of KCTD17 prevents diet-induced hepatic steatosis

It was next predicted that KCTD17 may be sufficient to protect PHLPP2 from degradation. shRNA directed against *Kctd17* was produced, and it was found that *Kctd17* knockdown increased PHLPP2 protein but not *Phlpp2* mRNA expression in primary hepatocytes (Fig. 30a, b) and in hepatoma cell lines (Fig. 31a, b). As hypothesized, acute *Kctd17* knockdown strongly reduced PHLPP2 ubiquitination (Fig. 78c), to completely block insulin-mediated induction of lipogenic genes (Fig. 31c). Based on these findings, it was predicted that reducing obesity-mediated increase of hepatic *Kctd17* would reproduce the lower liver TG seen with Ad-PHLPP2 or -2A-transduction, by "rescuing" lower PHLPP2 levels in obese mice. Consistent with *in vitro* data, Ad-sh*Kctd17*-transduced mice showed increased hepatic PHLPP2 protein levels and concomitantly reduced late refed Akt Ser473 phosphorylation (Fig. 30d), without change in *Phlpp2* expression (Fig. 31d). Remarkably, this acute reduction in *Kctd17* was sufficient to lower liver weight and TG

relative to Ad-shControl mice (Fig. 30e-g), without confounding changes in body weight/adiposity (Fig. 31e, f). Further, similar to Ad-PHLPP2 or -2A-transduced mice, we observed lower lipogenic but unchanged gluconeogenic gene expression in Ad-shKctd17-transduced
5 livers (Fig. 30h).

KCTD17 correlates with lipogenic gene expression and hepatic steatosis in NASH

These data show that KCTD17 regulates hepatic steatosis, at least in part by mediating PHLPP2 degradation. To begin to understand the
10 implication of these findings for human disease, liver *KCTD17* expression was analyzed in patients undergoing liver biopsy for suspected NASH (Table A), and a significant positive correlation was found between *KCTD17* and hepatic steatosis (Fig. 32a).

Table A. Demographic, clinical and histological features in a cross-sectional liver biopsy cohort.
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Liver biopsy cohort (n=158)	
Age, years	45±10
Sex, female	95 (60)
BMI, Kg/m ²	37.8±8.5
Type 2 Diabetes, yes	41 (26)
ALT, IU/l	28 [18-157]
AST, IU/l	22 [17-33]
PNPLA3, 148M/M	25 (16)
Histological steatosis grade	
0	17 (11)
1	50 (32)
2	49 (31)
3	42 (27)
Definite NASH, yes	31 (19)

Data represent means of ±SD, median [interquartile range], or number (%).
20

This positive correlation was the primary driver for a similar positive correlation between *KCTD17* and NAFLD Activity Score (NAS) (Fig. 32b), as higher hepatic *KCTD17* expression was no longer associated with increased NAS once adjusted for steatosis in
25 multivariate regression analysis (Table B).

Table B. Predictors of hepatic *KCTD17* expression in the cross-sectional liver biopsy cohort.

	Univariate analysis			Multivariate analysis		
	Beta	SE	<i>P</i> value	Beta	SE	<i>P</i> value
Age, years	+0.03	0.01	0.001	+0.02	0.01	0.012
Sex, Female	-0.24	0.09	0.008	-0.07	0.10	0.46
BMI, Kg/m ²	-0.03	0.01	0.011	-0.02	0.01	0.061
Type 2 diabetes, yes	+0.33	0.10	0.001	+0.12	0.11	0.25
<i>PNPLA3</i> I148M, alleles	-0.08	0.13	0.55	-0.19	0.12	0.13
Steatosis grade	+0.37	0.09	<0.0001	+0.28	0.11	0.008
Histological activity (Necroinflammation + ballooning)	+0.31	0.09	0.0008	+0.02	0.11	0.87

SE: standard error. Comparisons were made by fitting data to generalized linear models, unadjusted (univariate analyses), or considering as independent variables: age, sex, BMI, type 2 diabetes, *PNPLA3* I148M alleles, histological steatosis grade, and activity. Hepatic *KCTD17* mRNA levels were normalized for β -actin and log transformed before analyses to ensure a normal distribution.

These data suggest that the association with NASH observed at univariate analysis may be secondary to more severe steatosis. Interestingly, *KCTD17* expression was not associated with the *PNPLA3* I148M variant, the predominant determinant of hepatic fat accumulation in humans, which is consistent with our mouse data showing increased *Kctd17* is causative and not a simple consequence of hepatic steatosis.

These data derived from a cross-sectional cohort, which may limit its generalizability. To determine if *KCTD17* expression co-varies with NAFLD/NASH severity, we analyzed liver transcriptomes in patients with NASH before and after bariatric surgery, as well as normal controls (Ahrens et al., 2013), and found a striking co-regulation of *KCTD17* and lipogenic gene sets in patients with NASH (Fig. 32c). In paired analysis, *KCTD17* expression was significantly higher in patients with NASH as compared to normal controls, and significantly reduced after bariatric surgery (Fig. 32d). Thus, *KCTD17* showed a strong positive correlation with lipogenic and inflammatory gene expression (Fig. 32e and Fig. 33a), which closely clustered as a gene network (Fig. 33b), but weaker/indirect associations with other pathways associated with

NASH, such as β -oxidation, ER stress and fatty acid esterification (Fig. 33b). In combination with data from *L-PHLPP2* and the similar phenotypes of Ad-PHLPP2-2A and Ad-shKctd17 mice, these results establish the functional significance of glucagon-mediated KCTD17/Cul3 degradation of PHLPP2 in regulation of hepatic lipid accumulation.

DISCUSSION

U.S. Nonprovisional Application No. 15/143,305, filed April 29, 2016, describes Raptor exists in both the mTORC1-bound and -independent ("free") state in liver, and that free Raptor levels decline markedly in aging and obesity. Additionally, U.S. Nonprovisional Application No. 15/143,305, filed April 29, 2016, describes "free Raptor" as a regulator of hepatic PHLPP2, which promotes Akt Ser473 dephosphorylation and negatively regulates hepatic *de novo* lipogenesis without affecting hepatic glucose production. Accordingly, U.S. Nonprovisional Application No. 15/143,305, filed April 29, 2016, describes a role of free Raptor and PHLPP2 as a novel therapeutic targets for non-alcoholic fatty liver disease (NAFLD).

Furthermore, U.S. Nonprovisional Application No. 15/143,305, filed April 29, 2016, identifies glucagon induces phosphorylation of PHLPP2 at Ser1119 and Ser1210, and inhibitors of glucagon signaling increase the pathologically lower PHLPP2 levels in the obese state to effectively reduce hepatic and plasma triglyceride levels. U.S. Nonprovisional Application No. 15/143,305, filed April 29, 2016, also identifies KCTD17 is a novel PHLPP2 interacting protein, which may regulate PHLPP2 degradation in a phosphorylation/proteasome-dependent manner. The aforementioned disclosures of U.S. Nonprovisional Application No. 15/143,305, filed April 29, 2016, are hereby incorporated by reference into this disclosure.

Obesity causes insulin resistance and compensatory hyperinsulinemia, which then activates both mTORC1-dependent and -independent pathways to induce DNL (Shi et al., 2010; Yecies et al., 2011; Kim et al. 2016). Less appreciated is the possible effects of obesity-induced

hyperglucagonemia (Lefebvre et al., 1995), traditionally thought to antagonize insulin's beneficial effects on glucose homeostasis (Conarello et al., 2007). Our unbiased screen has revealed the first clear intersection between insulin and glucagon signaling pathways on DNL, through PHLPP2 (Fig. 32f). PHLPP2, previously shown by our group and others to dephosphorylate Akt at Ser473 to terminate insulin action, is rapidly phosphorylated by glucagon/PKA action to trigger PHLPP2 degradation. In fact, expression of a glucagon-resistant nonphosphorylatable PHLPP2 mutant prevents obesity-induced hyperinsulinemia from inducing DNL, and thus reverses fatty liver, echoing results from various glucagon antagonists in preclinical development (Gelling et al., 2003; Sloop et al., 2004; Sorensen et al., 2006). These results may explain the molecular mechanism underlying these observations, but also imply that other unrecognized molecular bridges between two critical hormone-regulated pathways to regulate DNL may exist. This may have implications even beyond metabolism, as DNL has emerged as a promising pathway for antineoplastic therapy.

L-PHLPP2 mice was found to show fatty liver, but normal glucose homeostasis. While this may appear paradoxical on face, this finding is actually quite consistent with normal signal transduction through $\text{InsR} \rightarrow \text{PI3K} \rightarrow \text{Akt}$ observed in response to feeding in *L-PHLPP2* mice, or following an insulin "pulse" in *PHLPP2* KO cells. In the absence of PHLPP2, however, prolonged Akt phosphorylation was observed during the "chase" period, and downstream increase in DNL due to a failure to terminate the insulin action. Conversely, adenovirus-mediated overexpression of PHLPP2 reduces liver TG, but also does not affect glucose homeostasis (Kim et al., 2016). These data suggest the bifurcation model of insulin signaling (Shi et al., 2010) may still explain the "selective" insulin resistance seen in the obese liver, but show that the kinetics of downstream signaling is as important as overall insulin levels - early post-prandial Akt activation is necessary to reduce hepatic glucose output, but prolonged Akt activity only increases DNL. These data suggest that this smoldering, chronic hepatic insulin signaling is partially responsible for increased liver TG in obesity without accompanying glycemic benefit.

LC/MS-MS screen identified PHLPP2 phosphorylations at both Ser1119 and Ser1210 in a previously undefined but evolutionarily-conserved domain of PHLPP2. Glucagon/PKA-mediated PHLPP2 phosphorylation at these sites is necessary but not sufficient to induce its degradation. Our second LC/MS-MS screen pointed to one potential mechanism for this by identifying KCTD17 as a novel PHLPP2-interacting protein. It was shown that the obesity-mediated increase in hepatic *Kctd17* expression is necessary to link PHLPP2 phosphorylation with degradation, as *Kctd17* knockdown in obese mice prevented PHLPP2 degradation, normalized Akt signaling and reduced liver TG. Studies to determine the molecular mechanism underlying increased *Kctd17* expression in obese liver are ongoing. *KCTD17* expression seems to preferentially predict steatosis. Additionally, KCTD17 may mediate "multiple hits" in NAFLD/NASH pathogenesis (Tilg and Moschen, 2010; Peverill et al., 2014; Buzzetti et al., 2016). Accordingly, further testing of KCTD17 effects in dietary mouse models of NASH (Wang et al., 2016) is needed.

In summary, obesity induces glucagon-mediated phosphorylation of PHLPP2, which targets its degradation by KCTD17/Cul3, which in turn increases insulin-mediated DNL and causes fatty liver. These data suggest that while glucagon receptor antagonists may have adverse effects (Kelly et al., 2015; Kazierad et al., 2016), inhibition of PHLPP2 phosphorylation, perhaps by dint of KCTD17 antagonism, may ameliorate obesity-induced fatty liver without affecting glucose homeostasis and stem the tide of liver disease in an increasingly obese population.

Example 3

Advantages of KCTD17 inhibition

Hepatic *de novo* lipogenesis (DNL) is the hepatocyte conversion of 2-carbon precursors derived from metabolism of sugars into fatty acids. DNL is increased in and contributes the pathogenesis of non-alcoholic fatty liver disease (NAFLD) (Donnelly et al., 2005), the most common chronic liver disease and the fastest-growing risk factor for liver transplantation, and often associated with type 2 diabetes (T2D). One

of the major stimulatory pathways for DNL is the sterol regulatory element binding protein 1c (SREBP1c)-mediated transcriptional regulation of enzymes which integral to fatty acid synthesis. SREBP1c in turn is activated by insulin signaling, via activation of the insulin receptor, and canonical signaling through the phosphoinositide-3 kinase (PI3K)/Akt pathway (Hegarty et al., 2005; Yellaturu et al., 2009). SREBP1c is also directly activated by the mammalian target of rapamycin complex 1 (mTORC1) (Ricoult and Manning, 2013), which in turn is also activated by insulin signaling, highlighting the intersection of hormones and nutrient to regulate DNL.

Accordingly, it was proposed that inhibition of these two pathways (insulin and mTORC1), or their final common mediator (SREBP1c) may reduce DNL, and thus ameliorate NAFLD. However, inhibition of liver insulin action by Akt deletion in mice has been shown to induce severe hyperglycemia, liver inflammation and hepatocellular carcinoma (HCC) (Lu et al., 2012; Wang et al., 2016). Further, use of the mTORC1 inhibitor, rapamycin, in clinical practice as an immunosuppressant often results in dyslipidemia (Wang et al., 2016), and chronic rapamycin treatment in rats causes insulin resistance and glucose intolerance, in part though inducing hepatic gluconeogenesis (Houde et al., 2010). Thus, inhibition of the upstream pathways (insulin and mTORC1) will have a poor risk/benefit ratio for chronic treatment of NAFLD. Similarly, investigators have determined that a small molecule inhibitor of SREBP1c, 75onolco, can ameliorate diet-induced fatty liver and insulin resistance (Tang et al., 2011), but in other studies, induces apoptosis through decreased mitochondrial membrane potential, activation of MAPK and modulation of NF- κ B in cancer cell lines (Gupta et al., 2010). Thus, inhibition of all major upstream pathways that regulate DNL (insulin and mTORC1), and even more specific inhibition of the effector (via the molecular activation of SREBP1c) are likely to be plagued with side-effects precluding application for chronic use in patients with NAFLD. Thus, new molecular targets that inhibit DNL without these side effects of glucose intolerance and liver damage/cancer are sorely needed. KCTD17 inhibition should reduce DNL and thus liver fat, without these side effects, as it bypasses the

need to fully inhibit insulin or mTORC1 signaling (and thus does not have glucose intolerance or liver inflammation/cancer as a side effect) and only modulates SREBP1c-induced DNL without full inhibition which should preclude off-target effects on cell death.

METHODS

Bioinformatics

For BXD analyses, unbiased GSEA (<http://www.broadinstitute.org/gsea>)
5 was performed using hepatic transcriptomes as previously described
(Ryu et al., 2014; Subramanian et al., 2005). Briefly, hepatic *Phlpp2*
was assessed in BXD mouse genetic reference populations fed normal
chow diet (GeneNetwork Accession No: GN432), and transcriptomes
corresponding to the top and bottom sextiles subjected to GSEA.

10 All raw transcriptomic data related to human NASH samples are publicly
available on Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo)
under accession numbers (GSE60149 and GSE48452) (Williams et al., 2016;
Ahrens et al., 2013). Heat maps were built using GENE-E
15 (www.broadinstitute.org/cancer/software/GENE-E). The depth of shading
at the correlation matrices (correlogram) indicates the magnitude of
the correlation (Spearman's Rho). Correlogram and interaction network
were generated using Rstudio (www.rstudio.com). Positive and negative
correlations are represented in blue and red, respectively. For the
20 interaction network, blue and red colored edge indicate positive and
negative correlations, respectively. Only correlations with
Spearman's $Rho > 0.5$ or < -0.5 are displayed in the interaction
network.

Animals

25 Male, wild-type C57BL/6J (strain #662) and male leptin-deficient *db/db*
(strain #642) mice were purchased from Jackson Labs. *PHLPP2* floxed
mice were generated using a targeting vector purchased from EUCOMM,
which was modified, linearized and electroporated into embryonic stem
30 (ES) cells. Antibiotic-resistant ES cell clones were microinjected
into C57BL/6 blastocysts to obtain chimeric mice. Founders that
exhibited germline transmission were bred with C57BL/6J mice
expressing flp recombinase (strain #016226) to remove the neo cassette
(*PHLPP2*^{fllox/+}) were then backcrossed >5 generations into C57BL/6J
35 background, then intercrossed to generate homozygous *PHLPP2*^{fllox/fllox}
mice. *L-PHLPP2* mice were created by transduction of male *PHLPP2*^{fllox/fllox}
mice with AAV8-TBG-Cre (Penn Vector Core). *L-PHLPP2* mice were fed

standard chow (Purina Mills 5053) or high-fat diet (18.4% calories/carbohydrates, 21.3% calories/protein and 60.3% calories/fat derived from lard; Harlan Laboratories, TD.06414). All mice were housed 3-5 animals per cage, with a 12 h light/dark cycle, in a temperature-controlled environment. Number of animals used in experiments was chosen to ensure adequate power to detect experimental difference with alpha set to 0.05. All animal experiments were approved by the Columbia University Institutional Animal Care and Utilization Committee.

CRISPR generation of PHLPP2-knockout (KO) cells and cell cultures studies

20 nucleotide single-guide RNA (sgRNA) sequences were designed using the CRISPR design tool at www.genome-engineering.org/crispr. sgRNAs were cloned into LentiCRISPRv2 from Addgene (plasmid #52961), and lentivirus encoding these sgRNAs was generated by co-transfection of 293T cells with the lentiviral vectors psPAX2 and pMD2.G. Viral supernatant was collected, and with polybrene (Sigma), applied to HepG2 or Hepalclc7 cells, which were selected with puromycin prior to expansion of single clones, screening by Western blot and genomic sequencing. Primary mouse hepatocytes were isolated as previously described (Kim et al., 2016; Pajvani et al., 2013).

Chemicals

The following chemicals were used in this study: puromycin, N-ethylmaleimide (ThermoFisher), polybrene, insulin, cAMP, forskolin, glucagon, Trichostatin A (Sigma), H-89 (Merck) or Phos-tag reagent (Wako).

Antibodies, Western blots and immunoprecipitation

Liver and cellular lysates were obtained by centrifugation as previously described (Kim et al., 2016). Immunoblots were conducted on 3-7 samples randomly chosen within each experimental cohort with antibodies against Akt (#9272), p-Akt (S473, #4060), p-Akt (T308, #13038), p-S6 (S240/244, #2215), S6 (#2217), p-(Ser/Thr) PKA substrate (#9621), PKA (#4782), DYKDDDDK-tag (#14793), HA-tag (#3724), myc-tag (#2276), and β -actin (#4970) from Cell Signaling; and PHLPP1 (A300-

660A) and PHLPP2 (A300-661A) from Bethyl Laboratories, Inc. Antibodies directed to novel PHLPP2 phosphorylations (S1119 or S1210, which is identical to S1116 or S1207 in mouse PHLPP2) were generated by immunizing rabbits with synthetic 79onalcoph-specific peptides, verified by ELISA and affinity purified (Lifetein). Specificity of anti-PHLPP2 (S1119) and anti-PHLPP2 (S1210) were confirmed in PHLPP2 KO cells.

Mass spectrometry

PHLPP2 was purified from livers of male, wild-type C57BL/6J mice transduced with Ad-HA-FLAG-PHLPP2. To identify PHLPP2 PTMs, liver lysates were prepared in stringent lysis buffer [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40, 0.05% SDS, 1% sodium deoxycholate, 10 mM α -glycerophosphate, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1.5 mM sodium orthovanadate, 30 μ M Trichostatin A, 20 mM sodium butyrate, 20 mM nicotinamide, 25 mM N-ethylmaleimide and protease inhibitor cocktail (Pierce)], prior to immunoprecipitation with anti-FLAG antibody-conjugated M2 agarose (Sigma) and separation of the enriched proteins by SDS-PAGE. The gel was stained with Coomassie blue, and the band corresponding to PHLPP2 excised, followed by protein extraction, trypsin digestion (Balasubramanian et al., 2016) and analysis by nanoflow liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. To examine PHLPP2 interacting proteins, liver lysates were extracted in less stringent lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1mM EDTA, 10% glycerol, 1% NP-40, 10 mM β -glycerophosphate, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1.5 mM sodium orthovanadate, and protease inhibitor cocktail (Pierce)], immunoprecipitated with anti-Flag M2 agarose, and bound polypeptides eluted with FLAG peptide (Sigma) prior to LC-MS/MS using the same procedure as above. Aliquots of each sample were loaded onto an Acclaim PepMap 100 precolumn (75 μ m $\times$ 2cm, C18, 3 μ m, 100 $\text{\AA}$, Thermo Scientific) in-line with an EASY-Spray, PepMap column (75 μ m $\times$ 50cm, C18, 2 μ m, 100 $\text{\AA}$ Thermo Scientific) using the autosampler of an EASY-nLC 1000 (Thermo Scientific). Peptides were gradient eluted into a Q Exactive mass spectrometer (Thermo Scientific) using a 60 min gradient from 2% solvent B to 40% solvent B. Solvent A was 2% acetonitrile in 0.5%

acetic acid and solvent B was 90% acetonitrile in 0.5% acetic acid. The Q Exactive mass spectrometer was set up to acquire high resolution full MS spectra with a resolution of 70,000 at m/z 200, an AGC target of 1e6, with a maximum ion time of 120ms, and scan range of 400 to 1500 m/z. Following each full MS twenty data-dependent high resolution HCD MS/MS spectra were acquired using the following instrument parameters: resolution of 17,500 at m/z 200, AGC target of 5e4, maximum ion time of 250 ms, one microscan, 2 m/z isolation window, fixed first mass of 150 m/z, and NCE of 27, dynamic exclusion 30 seconds. For the phosphorylation analysis the MS/MS spectra were searched against a PHLPP2 fasta file using Byonic™ (Bern et al., 2012) and the site of phosphorylation verified by manual inspection of the spectrum. For the binding partner analysis, the MS/MS spectra were searched against the uniprot mouse database using Sequest within Proteome Discoverer (ThermoFisher). The results were filtered using a <1%FDR (False Discovery Rate) searched against a decoy database and excluding proteins with less than two unique peptides.

Site-directed mutagenesis

Site-directed mutagenesis was performed as per the Q5 site-directed mutagenesis kit (NEB) to generate pcDNA3/HA/Flag/PHLPP2-S1119A, -S1210A or -S1119/1210A and resulting plasmids were sequence verified.

Adenovirus studies

Ad-GFP and Ad-HA/Flag/PHLPP2 adenoviruses have been described (Kim et al., 2016). Ad-HA/Flag/PHLPP2 (2A) and Ad-shKctd17 were generated by Welgen, Inc. (Worcester, MA). For *in vivo* studies, we injected between $2.5-5 \times 10^8$ purified viral particles per gram body weight, performed metabolic analysis on days 3-7 and killed the mice at day 7 or 10 post-injection.

Metabolic analyses

Blood tail vein glucose was measured using a glucose meter (Bayer). Glucose tolerance tests were performed by intraperitoneal injection of 1 or 2 g per kg body weight glucose after a 16 h fast. Hepatic lipids were extracted by the Folch method (Folch et al., 1957), and plasma and hepatic triglyceride (Thermo), Cholesterol E, or NEFA

(Wako) measured using colorimetric assays according to the manufacturer's protocol. Plasma insulin concentrations were measured using a mouse insulin ELISA kit (Mercodia).

5 *Cross-sectional analysis of KCTD17 in human liver biopsy specimens*

Liver gene expression were analyzed in individuals who underwent liver biopsy for suspected NASH, due to the presence of persistent elevations in liver enzymes or due to severe obesity (Mancina et al., 2016). The protocol was approved by the Ethical Committee of the
10 Fondazione IRCCS of Milan, and each patient signed a written informed consent. For statistical analysis, comparisons were made by fitting data to generalized linear models, unadjusted (univariate analyses), or considering as independent variables: age, sex, BMI, T2D, *PNPLA3* I148M alleles, histological steatosis grade, and activity. Hepatic
15 *KCTD17* mRNA levels were normalized for β -actin and log transformed before analyses to ensure a normal distribution.

RNA/quantitative PCR

RNA was isolated by TRIzol (Invitrogen) or NucleoSpin RNA (Clontech),
20 and cDNA synthesized with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) or SuperScript VILO cDNA synthesis kit (Life Technologies), which was followed by quantitative RT-PCR with Power SYBR Green PCR master mix (Applied Biosystems) in a CFX96 Real-Time PCR detection system (Bio-Rad). Primer sequences are available in
25 Table C..

Table C. Quantitative PCR primer sequences

Gene symbol	Sequences (5' to 3')	
	Forward	Reverse
<i>Mouse/Human 18s</i>	AAACGGCTACCACATCCAAG	CCTCCAATGGATCCTCGTTA
<i>Mouse 36b4</i>	GTTCTTGCCCATCAGCACC	AGATGCAGCAGATCCGCAT
<i>Mouse Fasn</i>	CTGACTCGGCTACTGACACG	TGAGCTGGGTTAGGGTAGGA
<i>Mouse G6pc</i>	GTCTGGATTCTACCTGCTAC	AAAGACTTCTTGTGTGTCTGTC
<i>Mouse Gcgr</i>	ACGGTACAGCCAGAAGATTG	TCTACCAGCAACCAGCAATAG
<i>Mouse Kctd17</i>	GAGCTCACACAGATGGTATCC	TGGTCCTCACTCCCATAGTT
<i>Mouse Pck1</i>	CCTGGAAGAACAAGGAGTGG	AGGGTCAATAATGGGGCACT
<i>Mouse Phlpp1</i>	AGGGTCCCGGAGACGATAAG	AGGGCGGAGATGTCTTTTGC
<i>Mouse Phlpp2</i>	GCCACAATCTTCTTACAGAGGTC	TCGAGGGGAATGTGCTCCA
<i>Mouse Srebp1c</i>	GAAGCTGTGCGGGTAGCGTCT	CTCTCAGGAGAGTTGGCACCTG
<i>Human ACTB</i>	CATGTACGTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
<i>Human DSCAML1</i>	CATCGGCTATCCCTACTACTC	TTGAGGGTCCCATTCTCAAAC
<i>Human GLIS3</i>	CAACCAGATCAGTCCTAGCTTAC	CCAAAGACTCACGCGAAATAAG
<i>Human JADE1</i>	CGGGAGCAGGATGTCTTATTT	GGTCACCATGTAAGTGAGGTT
<i>Human KCTD17</i>	AGAGAAGGACTACACGGTCA	CCATCAGACATGGTGGAGAC
<i>Human PHLPP2</i>	CATACAGGAGGAGGCTACAAATC	GGTCTTTCCTTGCGTACAT

5 Statistical analysis

All data shown as mean $\pm$ s.e.m. ANOVA was used for comparison of means among groups.

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What is claimed:

1. A method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that decreases KCTD17 expression in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.
2. A method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that prevents PHLPP2 degradation in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.
3. The method of claim 2, wherein the pharmaceutical composition inhibits Glucagon signaling.
4. A method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound that inhibits Glucagon signaling in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.
5. The method of claim 4, wherein the pharmaceutical composition reduces PHLPP2 degradation.
6. The method of claim 4 or 5, wherein the pharmaceutical composition increases free Raptor in the liver cells.

7. The method of claim 1-6, wherein the pharmaceutical composition decreases PHLPP2 phosphorylation at Serine 1119 and Serine 1210 residues in liver cells.
- 5 8. The method of claim 6, wherein the pharmaceutical composition prevents PHLPP2 degradation in liver cells.
9. The method of any one of claims 1-8, wherein the pharmaceutical composition increases PHLPP2 in liver cells.
- 10 10. The method of any one of claim 3-9, wherein the pharmaceutical composition prevents PHLPP2 degradation in liver cells.
- 15 11. The method of any one of claims 1-10, wherein the pharmaceutical composition decreases Akt phosphorylation at Serine 473 residue in liver cells.
12. The method of any one of claims 1-11, wherein the pharmaceutical composition decreases Akt signaling.
- 20 13. The method of any one of claims 1-12, wherein the pharmaceutical composition increases Raptor expression, thereby increasing free Raptor in the liver cells.
- 25 14. The method of any one of claims 1-13, wherein the pharmaceutical composition inhibits interaction of Raptor and mTORC1, thereby increasing free Raptor in the liver cells.
15. The method of any one of claims 1-14, wherein the compound reduces the expression of at least one lipogenic gene.
- 30 16. The method of claim 15, wherein the at least one lipogenic gene is Srebp1c, Fasn, Accl, or Scd1.
- 35 17. The method of any one of claims 1-16, wherein the subject is afflicted with a metabolic disease.

18. The method of any one of claims 1-17, wherein the pharmaceutical composition comprises a polynucleotide.
19. The method of any one of claims 1-18, wherein the pharmaceutical composition is targeted to the liver of the subject.
20. The method of claim 17, wherein the metabolic disease is obesity.
21. The method of claim 17, wherein the metabolic disease is hypertriglyceridemia.
22. The method of claim 17, wherein the metabolic disease is hyperinsulinemia.
23. The method of claim 17, wherein the metabolic disease is Type 2 Diabetes.
24. The method of claim 17, wherein the metabolic disease is fatty liver disease.
25. The method of claim 24, wherein the fatty liver disease is nonalcoholic fatty liver disease or nonalcoholic steatohepatitis.
26. The method of any one of claims 1-25, wherein the subject is afflicted with cirrhosis or hepatocellular carcinoma.
27. The method of any one of claims 1-26, wherein the subject is a human.
28. The method of any one of claims 1-27, wherein the subject's hepatic or plasma triglyceride levels are >150 mg/dL.
29. The method of any one of claims 1-28, wherein the subject's hepatic or plasma triglyceride levels are >500 mg/dL, about 200 to 499 mg/dL, or about 150 to 199 mg/dL.

30. The method of any one of claims 1-29, wherein the subject's hepatic or plasma triglyceride levels are reduced by at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, or at least 75%, relative to the level prior to the administration.
31. A process for determining the amount of KCTD17 expression in a subject's liver comprising:
- a) obtaining a biological sample comprising liver cells of the subject;
 - b) determining the amount of KCTD17 mRNA in the sample.
32. A process for diagnosing whether a subject is afflicted with increased KCTD17 expression comprising:
- a) determining the amount of KCTD17 in the subject according to the process of claim 31;
 - b) determining the amount of KCTD17 in a reference subject according to the process of claim 31; and
 - c) diagnosing the subject to be afflicted with increased KCTD17 expression if the amount of KCTD17 expression in step (a) is substantially increased compared to the amount of KCTD17 expression in step (b).
33. A method of treating a subject diagnosed to be afflicted with increased KCTD17 expression according to the process of claim 32 comprising reducing the subject's hepatic and plasma triglyceride levels according to the method of claims 1, 3, 6 or 7.
34. A method of treating a subject afflicted with elevated triglyceride levels comprising administering to the subject a pharmaceutical composition comprising a pharmaceutical carrier and a compound that decreases KCTD17 expression in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

35. A method of treating a subject afflicted with elevated triglyceride levels comprising administering to the subject a pharmaceutical composition comprising a pharmaceutical carrier and a compound that decreases PHLPP2 phosphorylation at Serine 1119 and Serine 1210 residues in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.
36. A method of treating a subject at risk of developing elevated triglyceride levels comprising administering to the subject a pharmaceutical composition comprising a pharmaceutical carrier and a compound that decreases KCTD17 expression in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.
37. A method of treating a subject at risk of developing elevated triglyceride levels comprising administering to the subject a pharmaceutical composition comprising a pharmaceutical carrier and a compound that increases PHLPP2 phosphorylation at Serine 1119 and Serine 1210 residues in liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

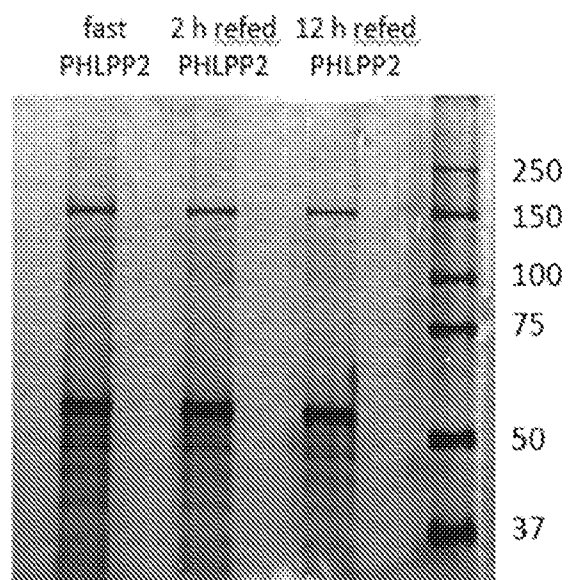


FIGURE 1

Phosphorylation sites identified in PHLPP2

Site	Fasted	2 h refed	12 h refed
S1119	+	+	+
T1123	+	-	-
S1210	+	+	+
S1188/1189	+	-	+
S1231	+	+	+

FIGURE 2

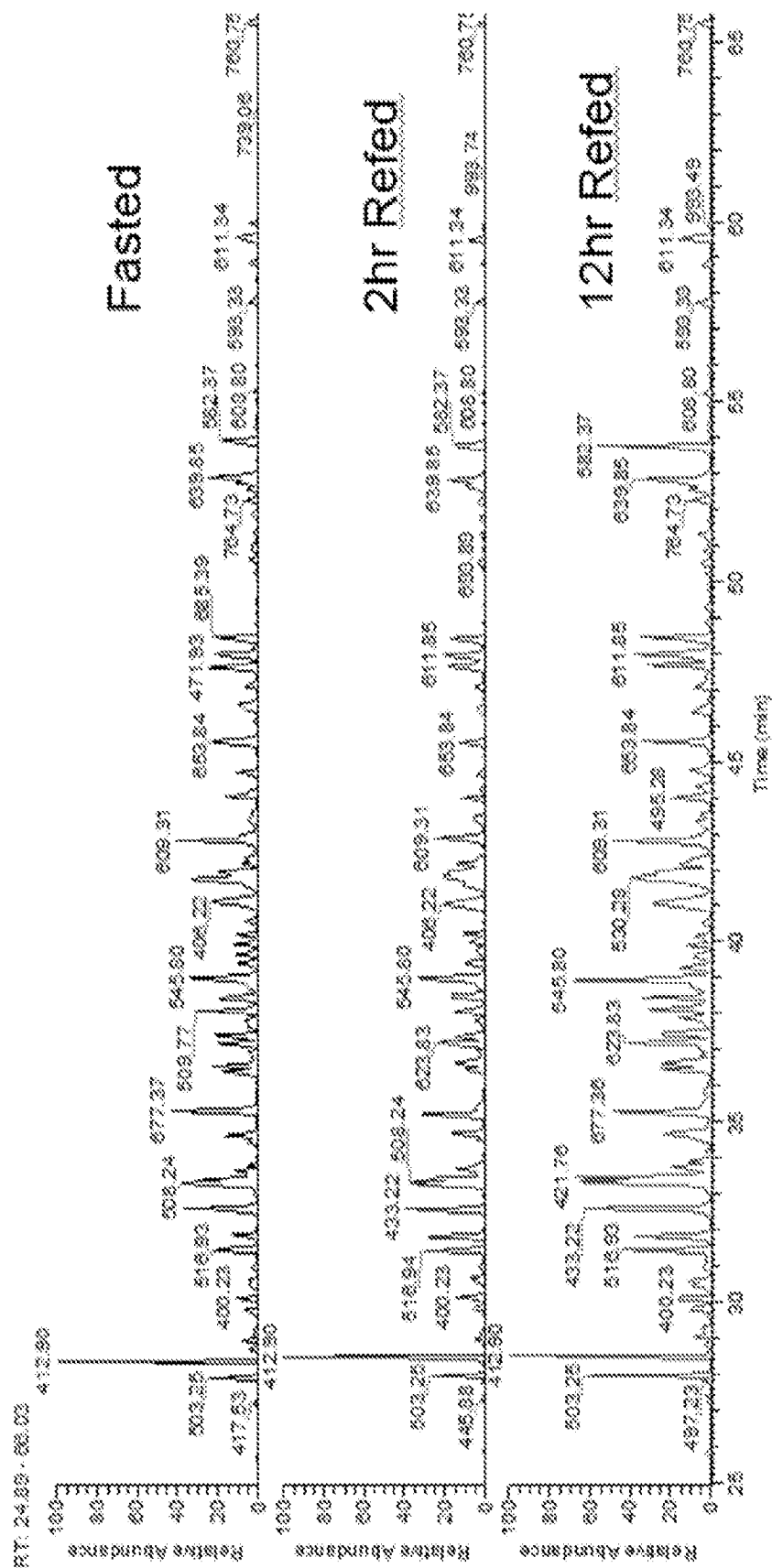


FIGURE 3

1061	Homo Sapiens	SEMSISEVS	SEVGSIASDE	HNAGSLDIAL	LEPERRCOL	HPFTSGLFC	RQPCATFSS	1140
1061	Fan troglodytes	SEMSISEVS	SEVGSIASDE	HNAGSLDIAL	LEPERRCOL	HPFTSGLFC	RQPCATFSS	1140
1061	Macaca mulatta	SEMSISEVS	SEVGSIASDE	HNAGSLDIAL	LEPERRCOL	HPFTSGLFC	RQPCATFSS	1140
1066	Canis lupus familiaris	SEMSISEVS	SEVGSIASDE	HNAGSLDIAL	LEPERRCOL	HPFTSGLFC	RQPCATFSS	1140
1077	Bos taurus	SEMSISEVS	SEVGSIASDE	HNAGSLDIAL	LEPERRCOL	HPFTSGLFC	RQPCATFSS	1140
1110	Mus musculus	SEMSISEVS	SEVGSIASDE	HNAGSLDIAL	LEPERRCOL	HPFTSGLFC	RQPCATFSS	1140
1116	Rattus norvegicus	SEMSISEVS	SEVGSIASDE	HNAGSLDIAL	LEPERRCOL	HPFTSGLFC	RQPCATFSS	1140
1176	Gallus gallus	SEMSISEVS	SEVGSIASDE	HNAGSLDIAL	LEPERRCOL	HPFTSGLFC	RQPCATFSS	1140
745	Danio rerio	SEMSISEVS	SEVGSIASDE	HNAGSLDIAL	LEPERRCOL	HPFTSGLFC	RQPCATFSS	1140
1141	Homo Sapiens	MSDNGELSD	DDPVEGVIT	NGSKVEVEVD	INCGRERDL	NSPILFESS	TCSEEHARG	1200
1141	Fan troglodytes	MSDNGELSD	DDPVEGVIT	NGSKVEVEVD	INCGRERDL	NSPILFESS	TCSEEHARG	1200
1141	Macaca mulatta	MSDNGELSD	DDPVEGVIT	NGSKVEVEVD	INCGRERDL	NSPILFESS	TCSEEHARG	1200
1146	Canis lupus familiaris	MSDNGELSD	DDPVEGVIT	NGSKVEVEVD	INCGRERDL	NSPILFESS	TCSEEHARG	1200
1197	Bos taurus	MSDNGELSD	DDPVEGVIT	NGSKVEVEVD	INCGRERDL	NSPILFESS	TCSEEHARG	1200
1179	Mus musculus	MSDNGELSD	DDPVEGVIT	NGSKVEVEVD	INCGRERDL	NSPILFESS	TCSEEHARG	1200
1176	Rattus norvegicus	MSDNGELSD	DDPVEGVIT	NGSKVEVEVD	INCGRERDL	NSPILFESS	TCSEEHARG	1200
1236	Gallus gallus	MSDNGELSD	DDPVEGVIT	NGSKVEVEVD	INCGRERDL	NSPILFESS	TCSEEHARG	1200
815	Danio rerio	MSDNGELSD	DDPVEGVIT	NGSKVEVEVD	INCGRERDL	NSPILFESS	TCSEEHARG	1200
1201	Homo Sapiens	VSFQIRKQNS	VNSGILLPNS	KDNMELQKSP	STCLYGNKL	SNCSIVPELD	SNHIEVATE	1260
1201	Fan troglodytes	VSFQIRKQNS	VNSGILLPNS	KDNMELQKSP	STCLYGNKL	SNCSIVPELD	SNHIEVATE	1260
1201	Macaca mulatta	VSFQIRKQNS	VNSGILLPNS	KDNMELQKSP	STCLYGNKL	SNCSIVPELD	SNHIEVATE	1260
1226	Canis lupus familiaris	VSFQIRKQNS	VNSGILLPNS	KDNMELQKSP	STCLYGNKL	SNCSIVPELD	SNHIEVATE	1260
1197	Bos taurus	VSFQIRKQNS	VNSGILLPNS	KDNMELQKSP	STCLYGNKL	SNCSIVPELD	SNHIEVATE	1260
1219	Mus musculus	VSFQIRKQNS	VNSGILLPNS	KDNMELQKSP	STCLYGNKL	SNCSIVPELD	SNHIEVATE	1260
1236	Rattus norvegicus	VSFQIRKQNS	VNSGILLPNS	KDNMELQKSP	STCLYGNKL	SNCSIVPELD	SNHIEVATE	1260
1296	Gallus gallus	VSFQIRKQNS	VNSGILLPNS	KDNMELQKSP	STCLYGNKL	SNCSIVPELD	SNHIEVATE	1260
873	Danio rerio	VSFQIRKQNS	VNSGILLPNS	KDNMELQKSP	STCLYGNKL	SNCSIVPELD	SNHIEVATE	1260

3/31

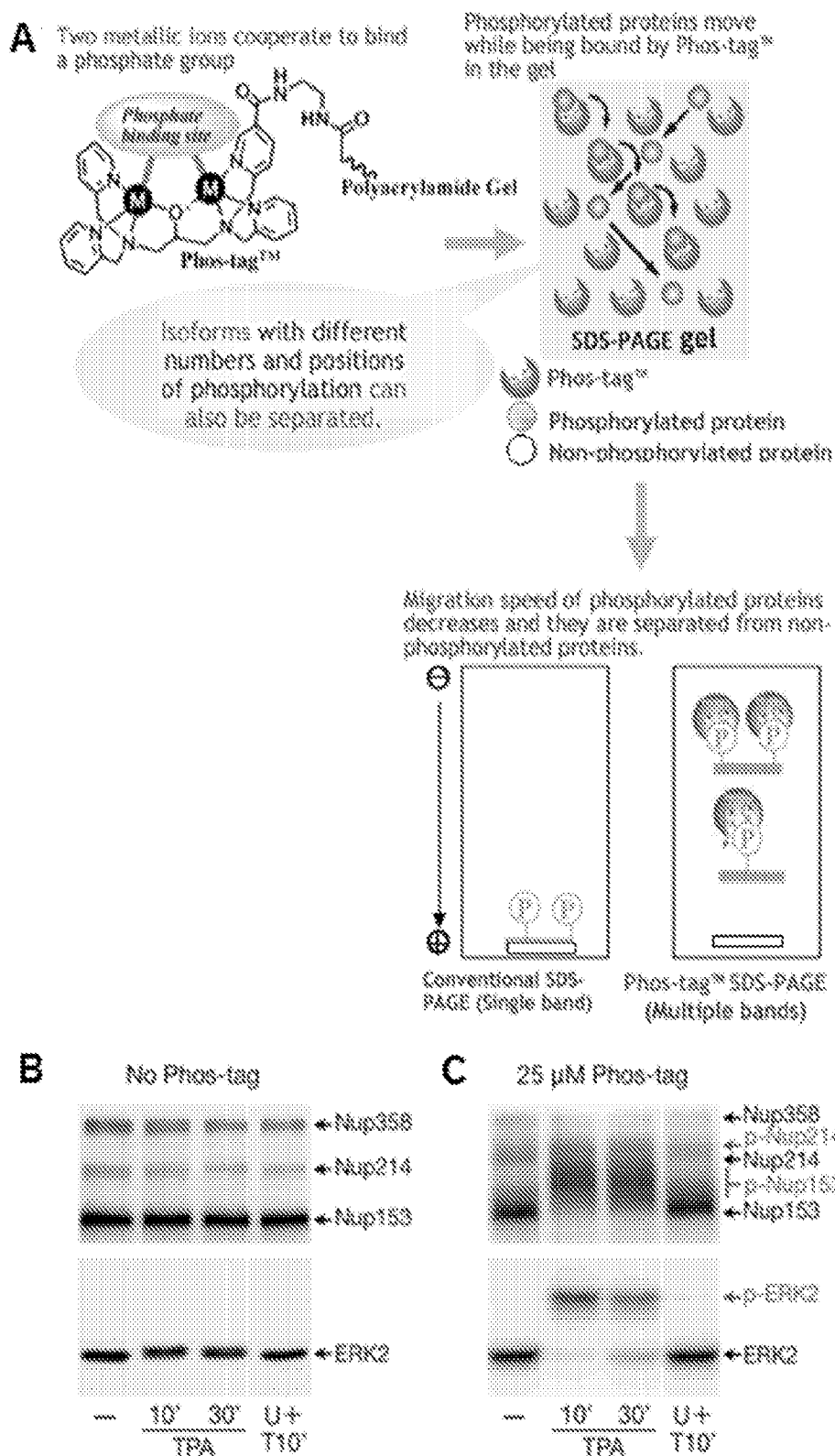


FIG. 5A-C

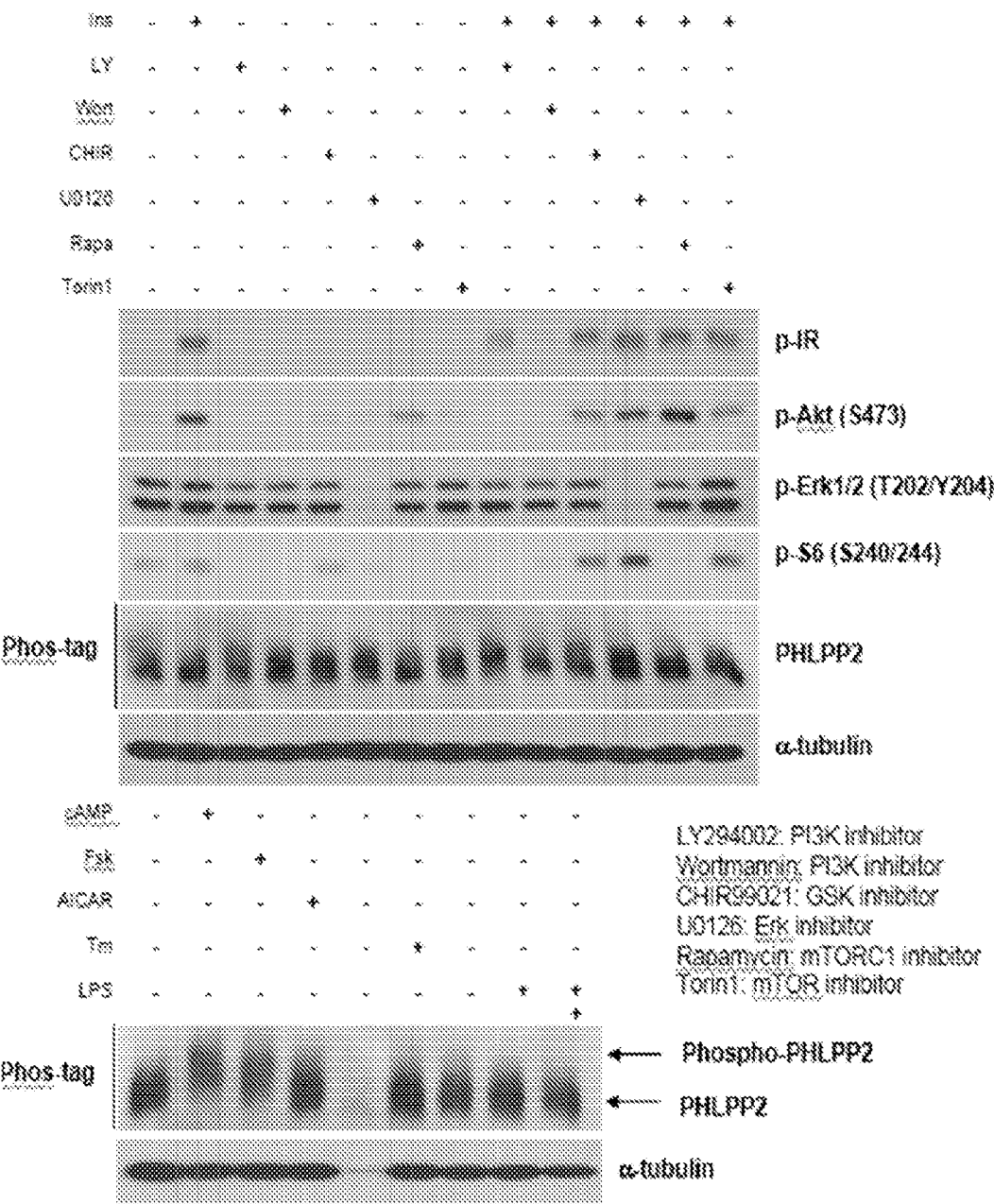


FIGURE 6

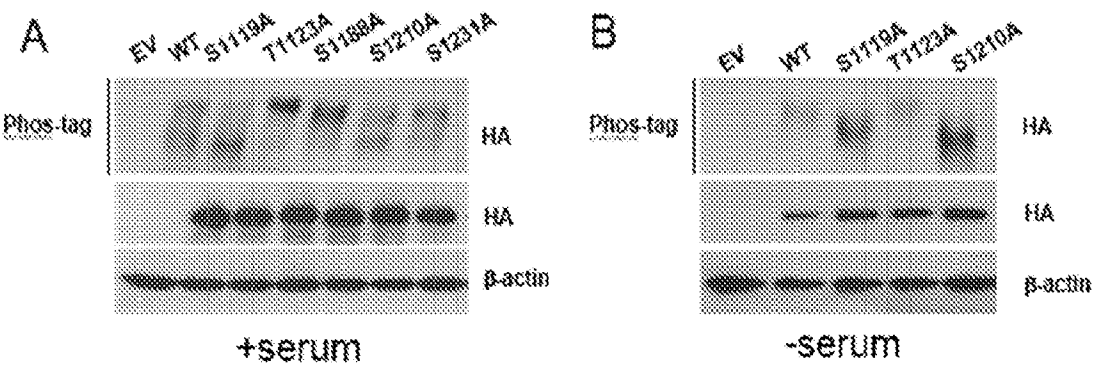


FIG. 7A-B

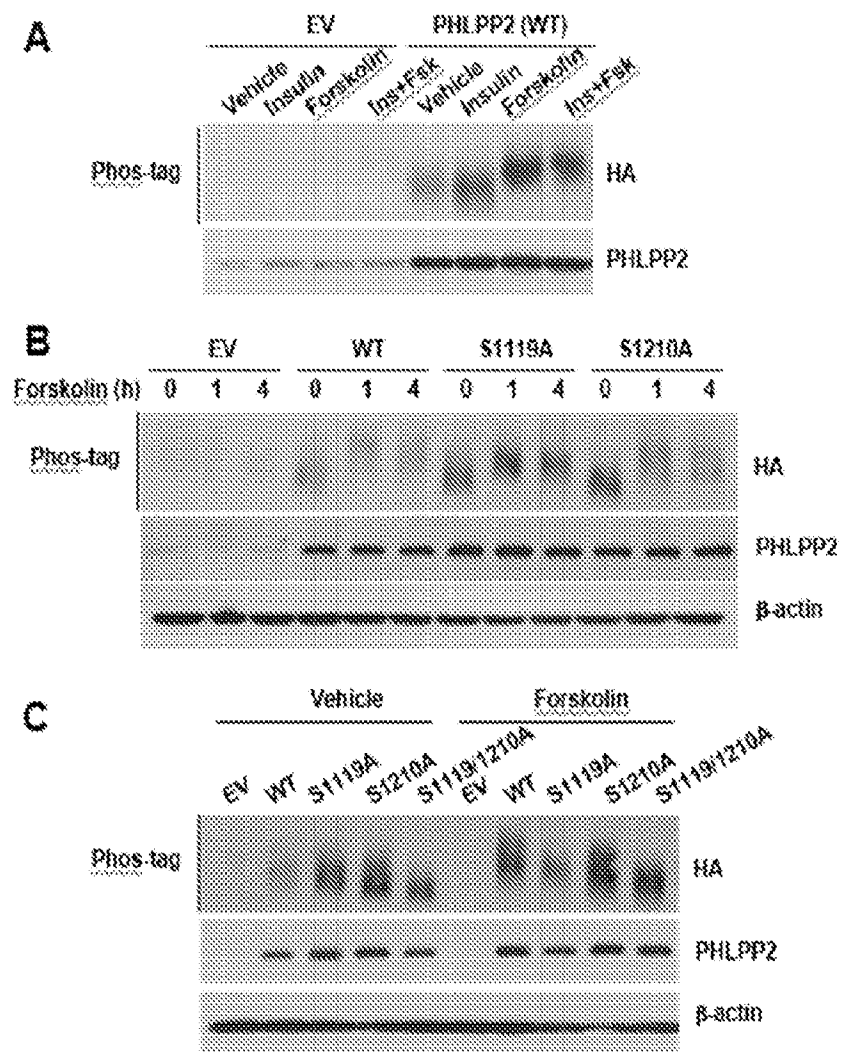


FIG. 8A-C

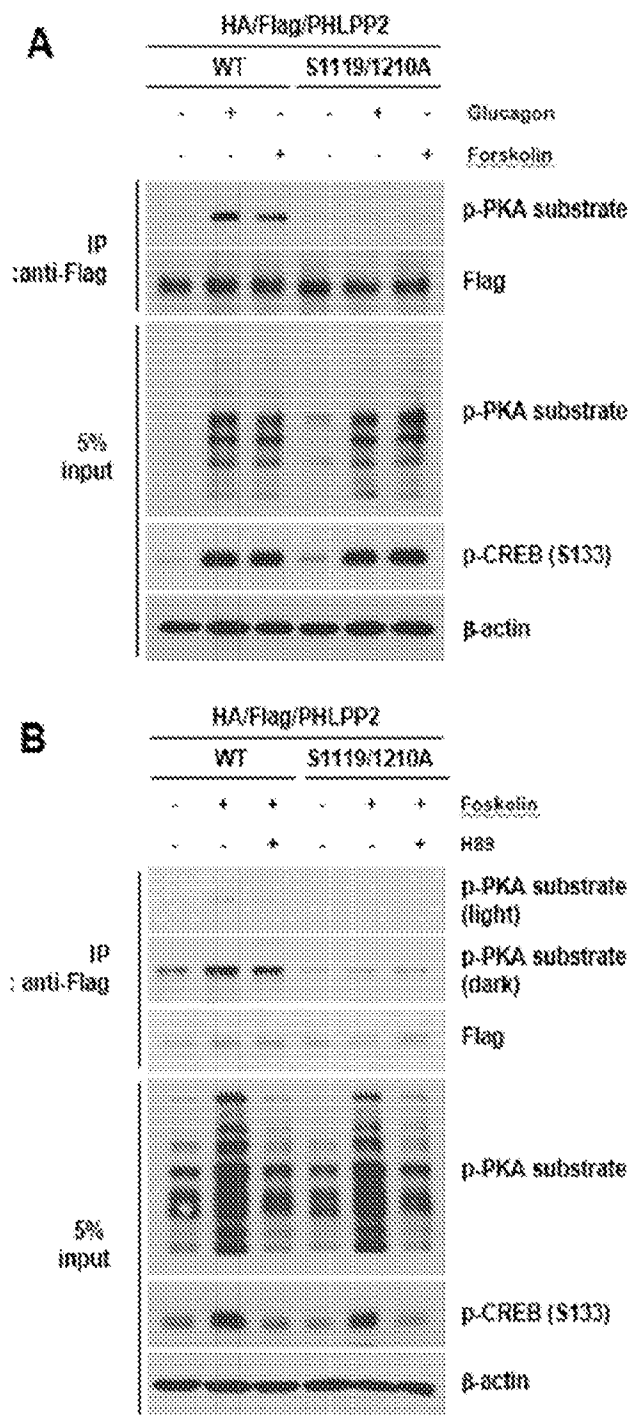


FIG. 9A-B

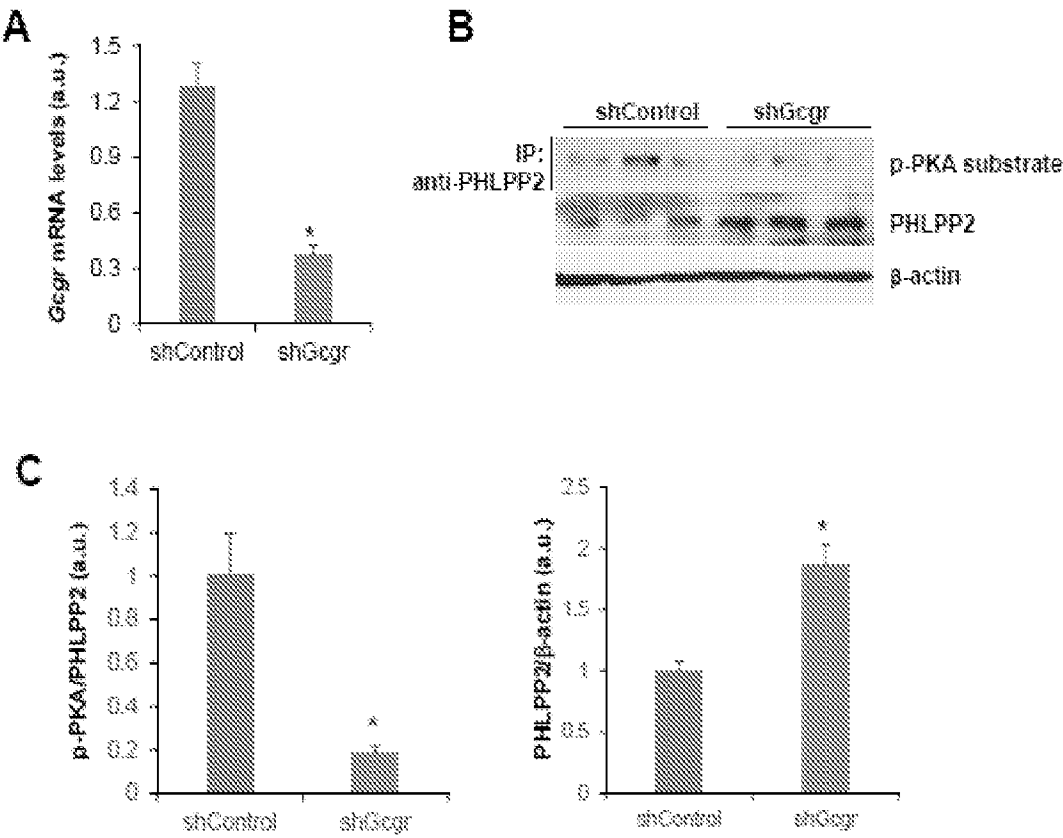


FIG. 10A-C

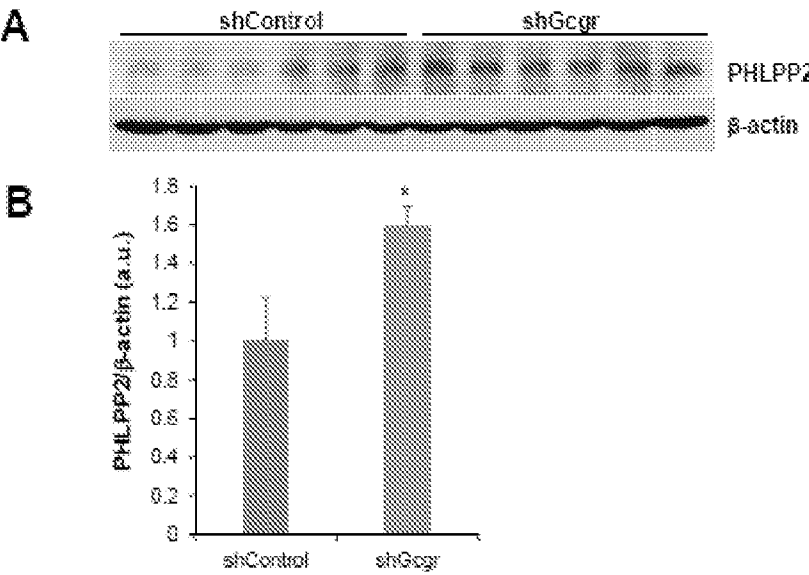


FIG. 11A-B

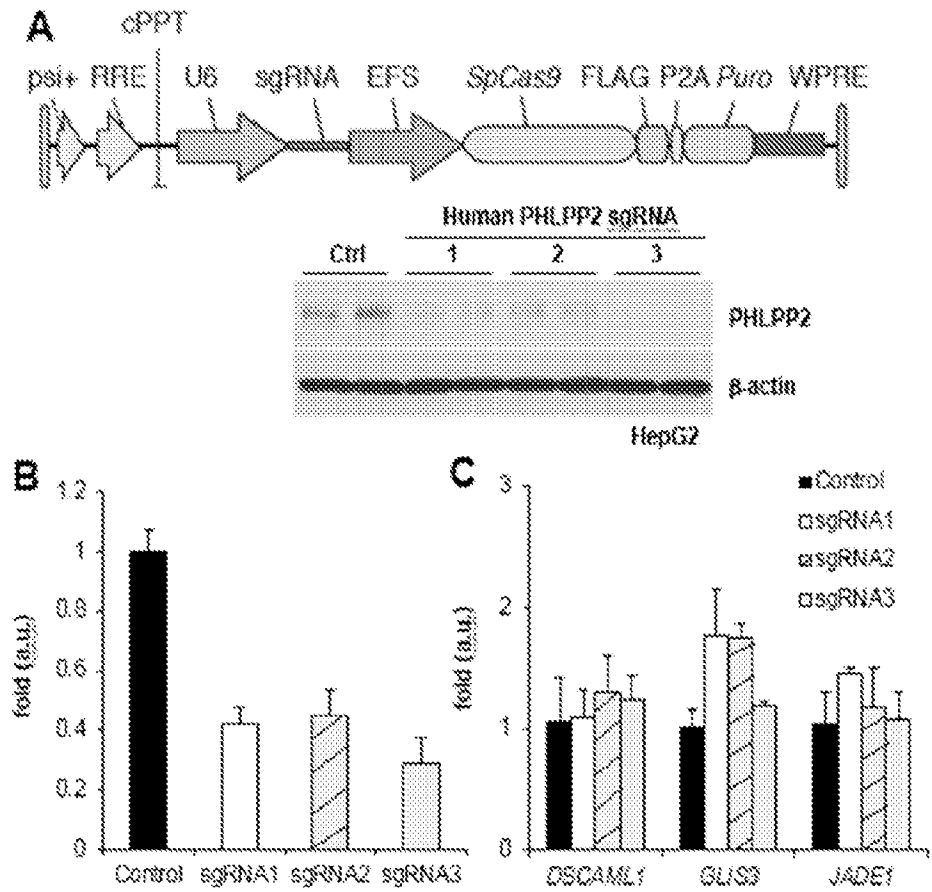


FIG. 12A-C

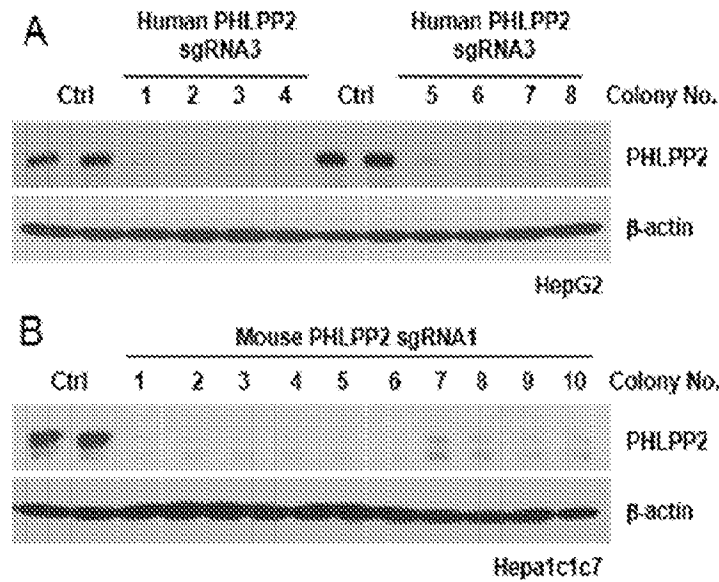


FIG. 13A-B

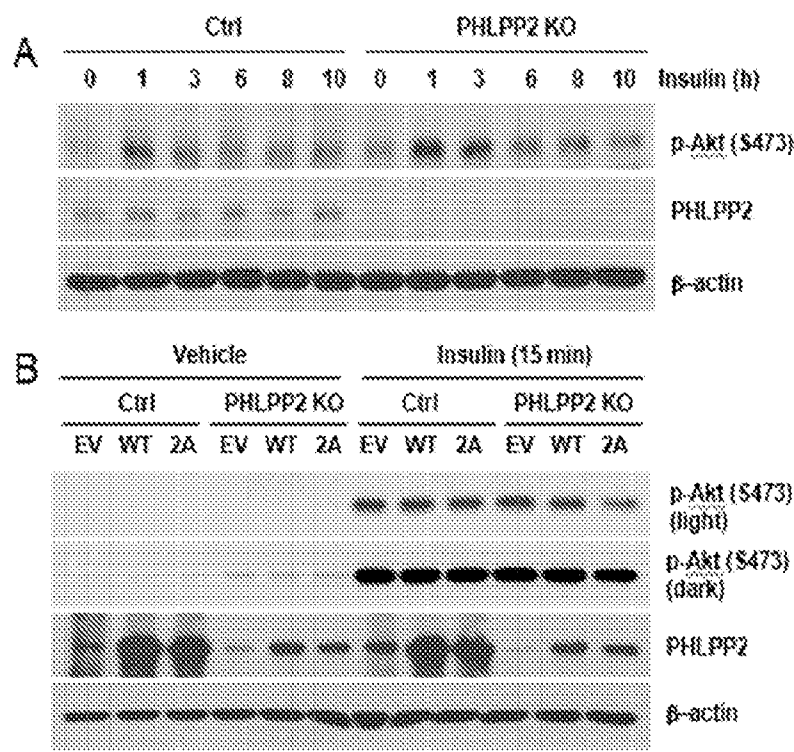


FIG. 14A-B

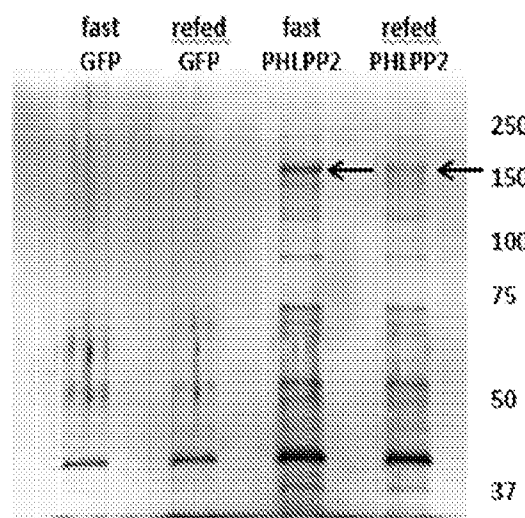


FIGURE 15

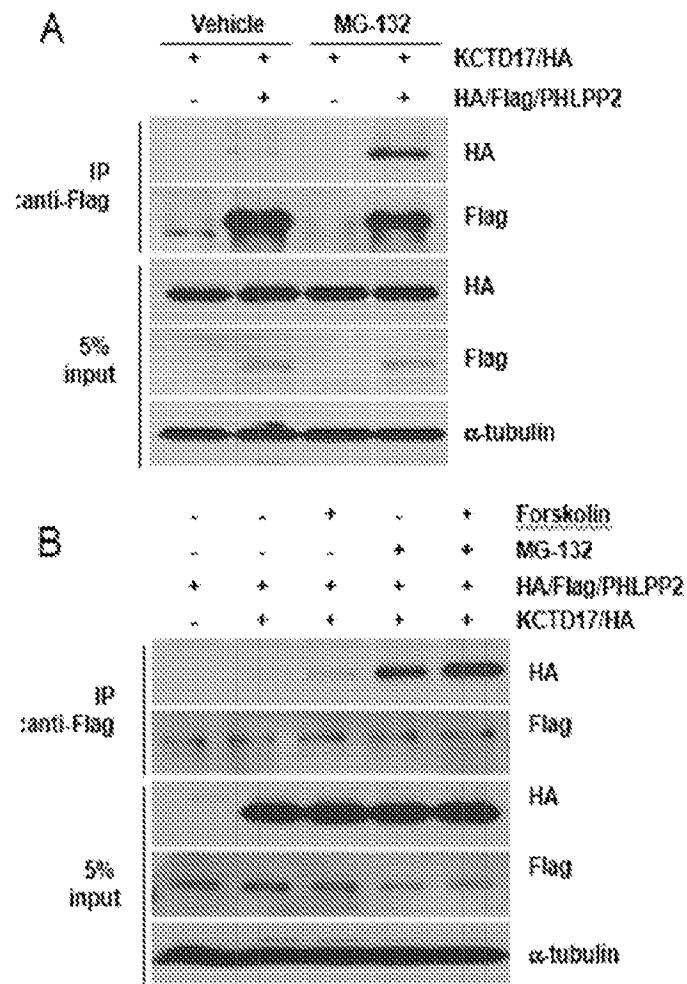


FIG. 16A-B

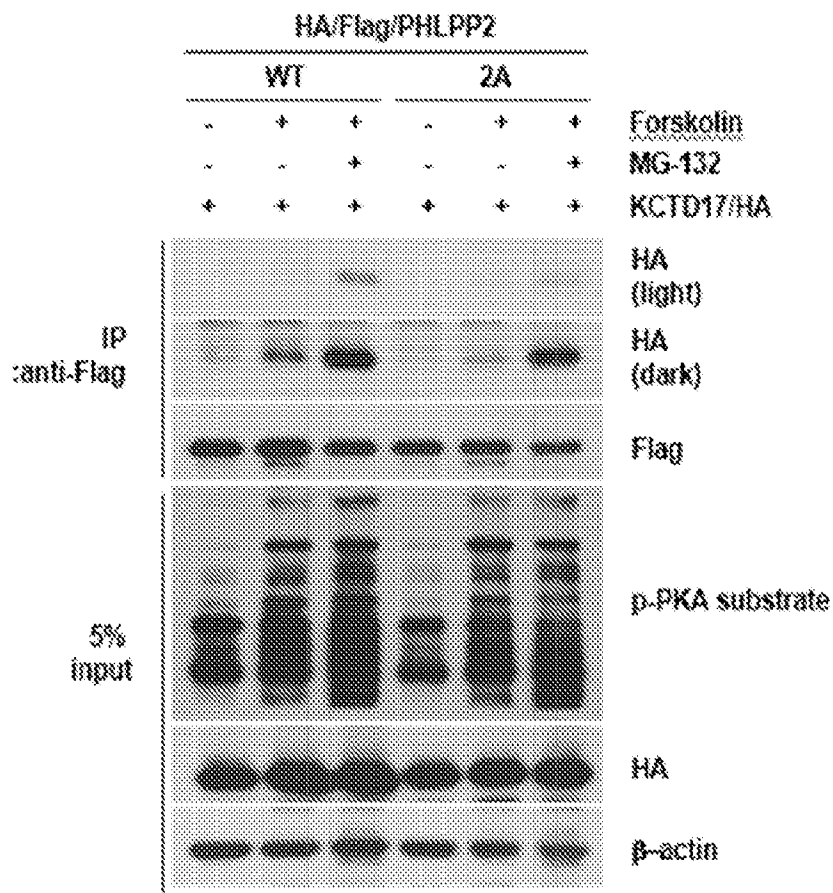


FIGURE 17

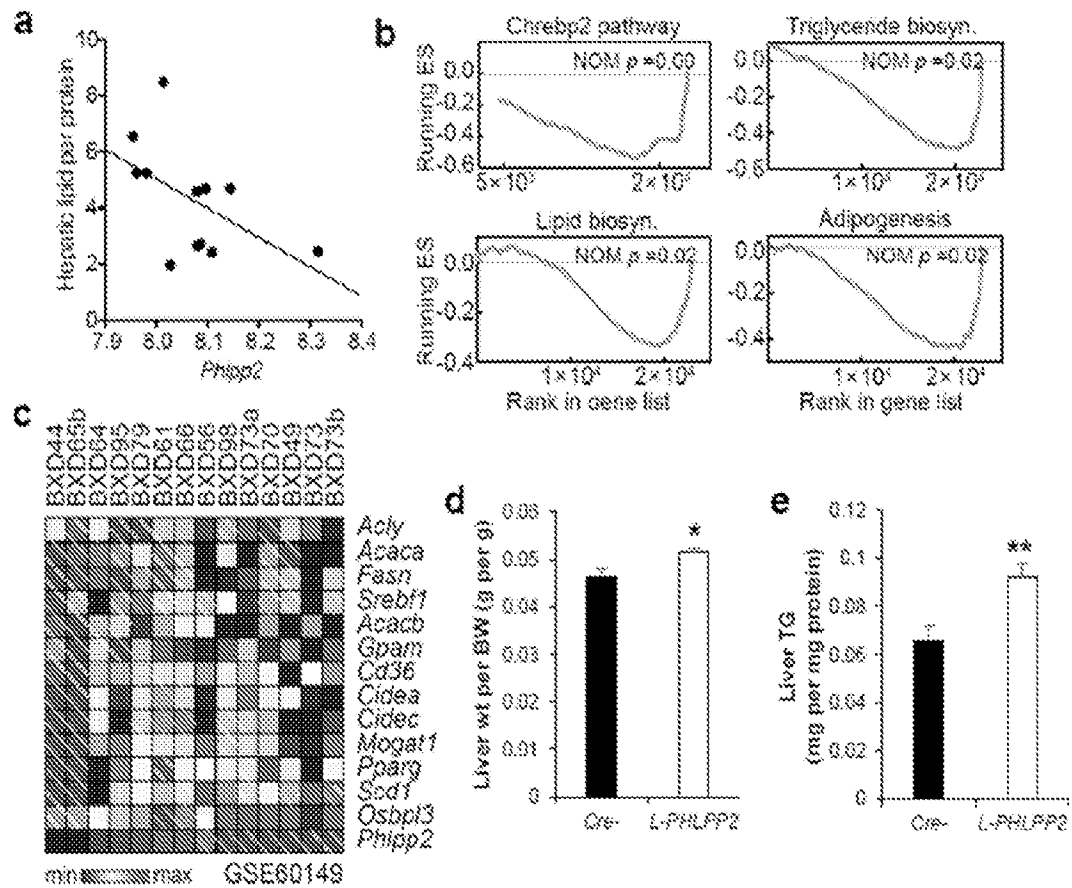


Fig. 18A-E

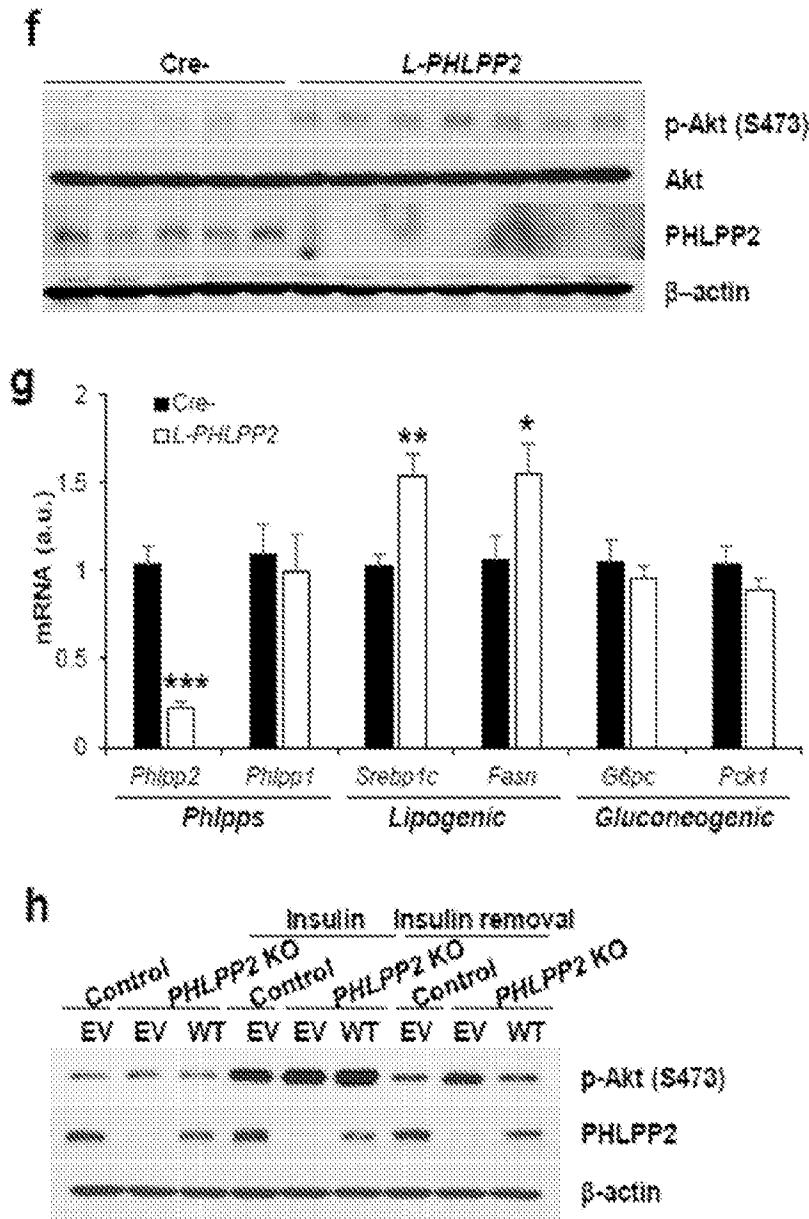


Fig. 18F-H

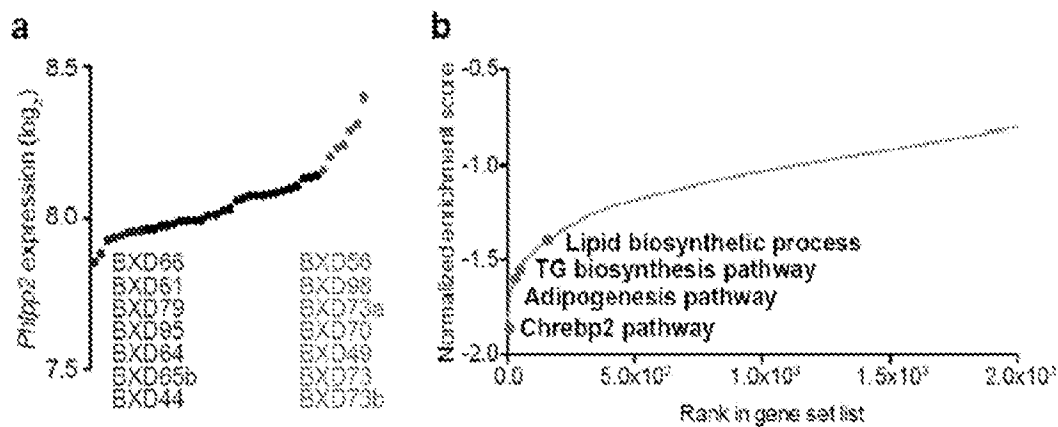


Fig. 19A-B

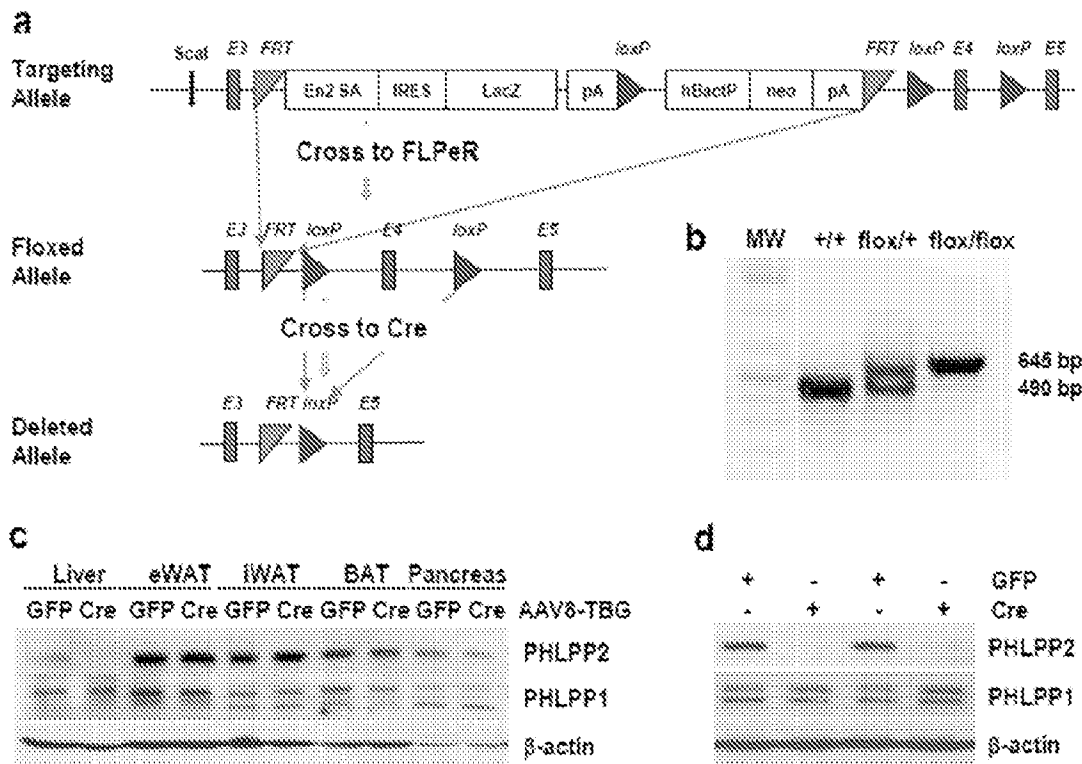


Fig. 20A-D

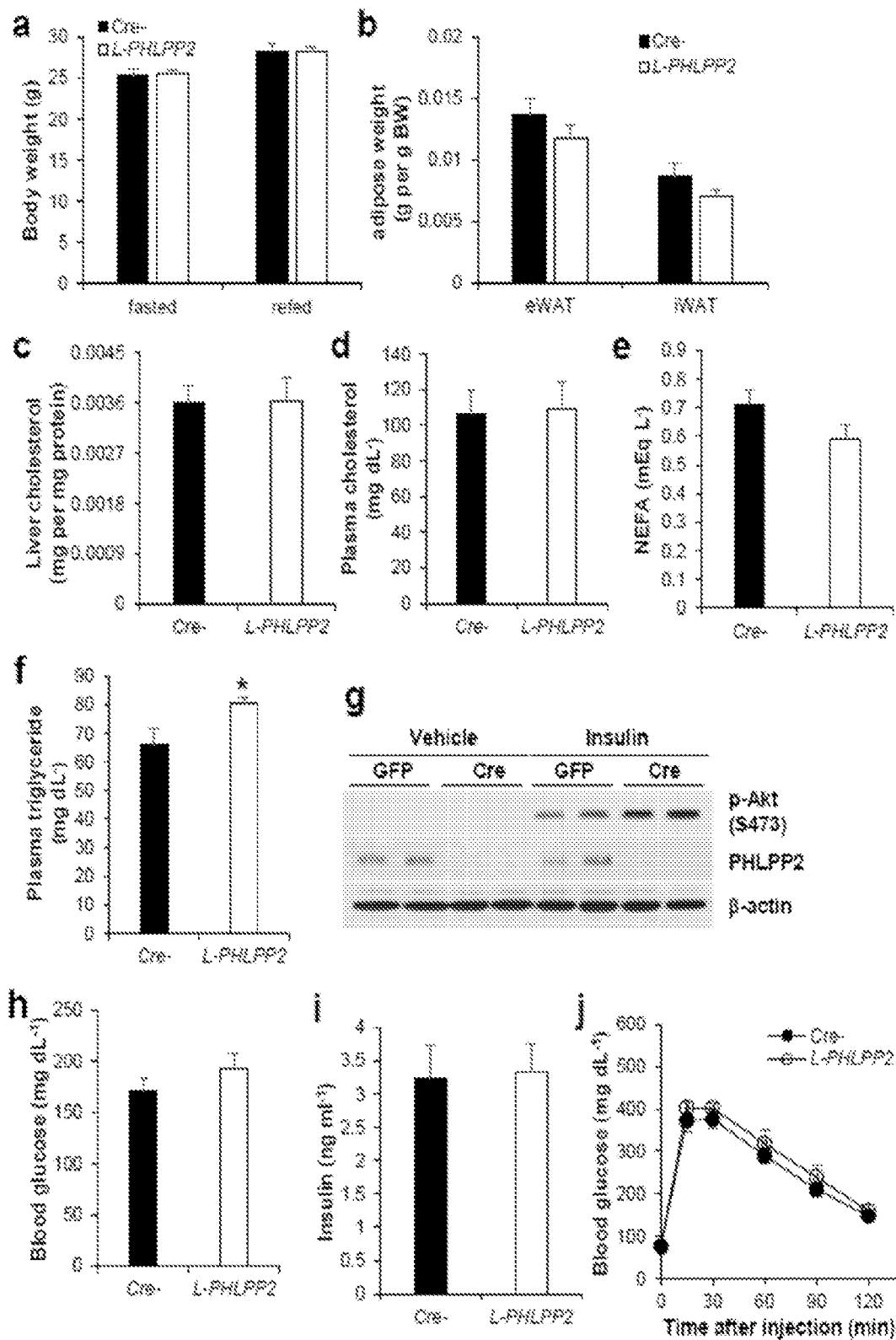


Fig. 21A-J

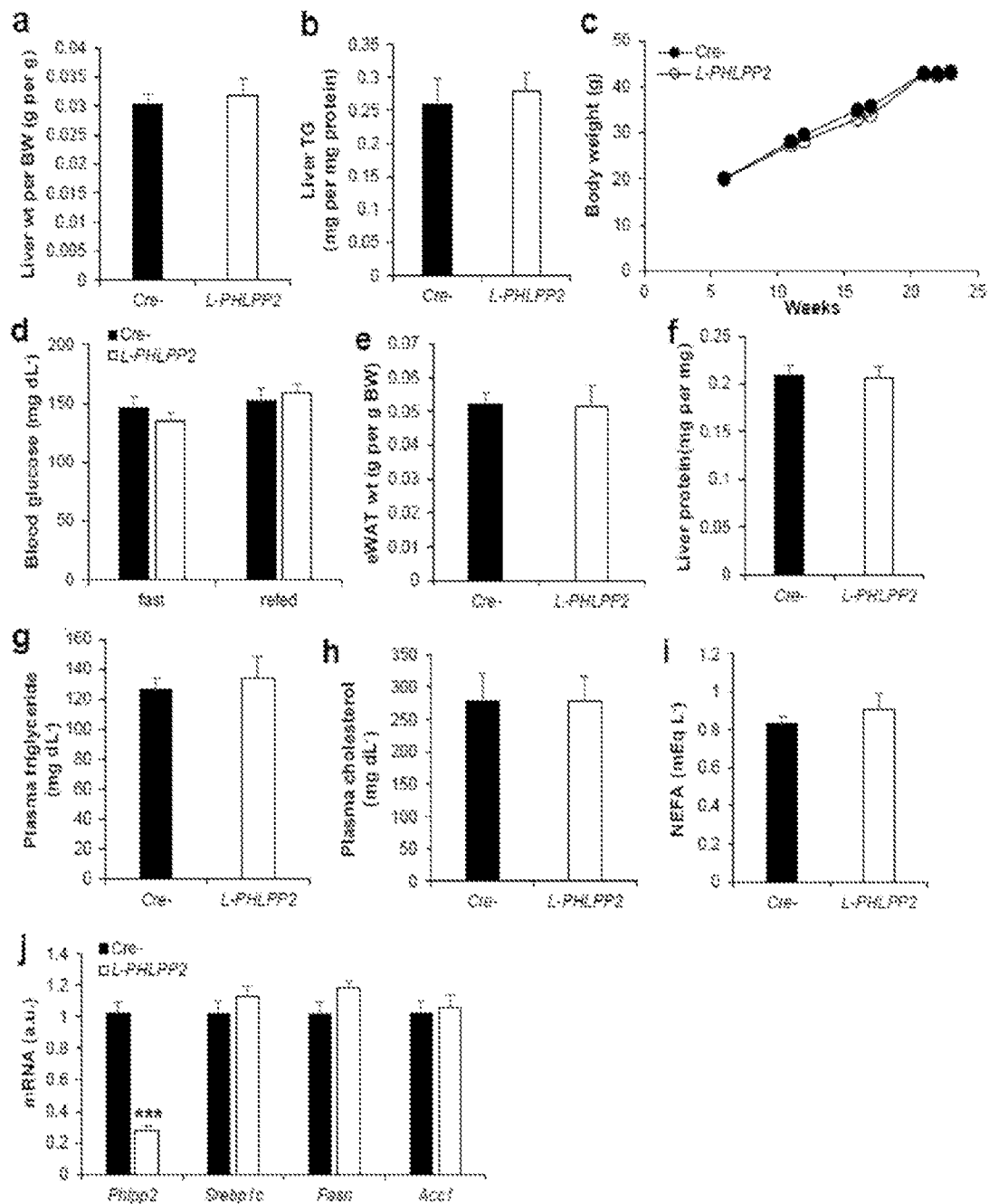


Fig. 22A-J

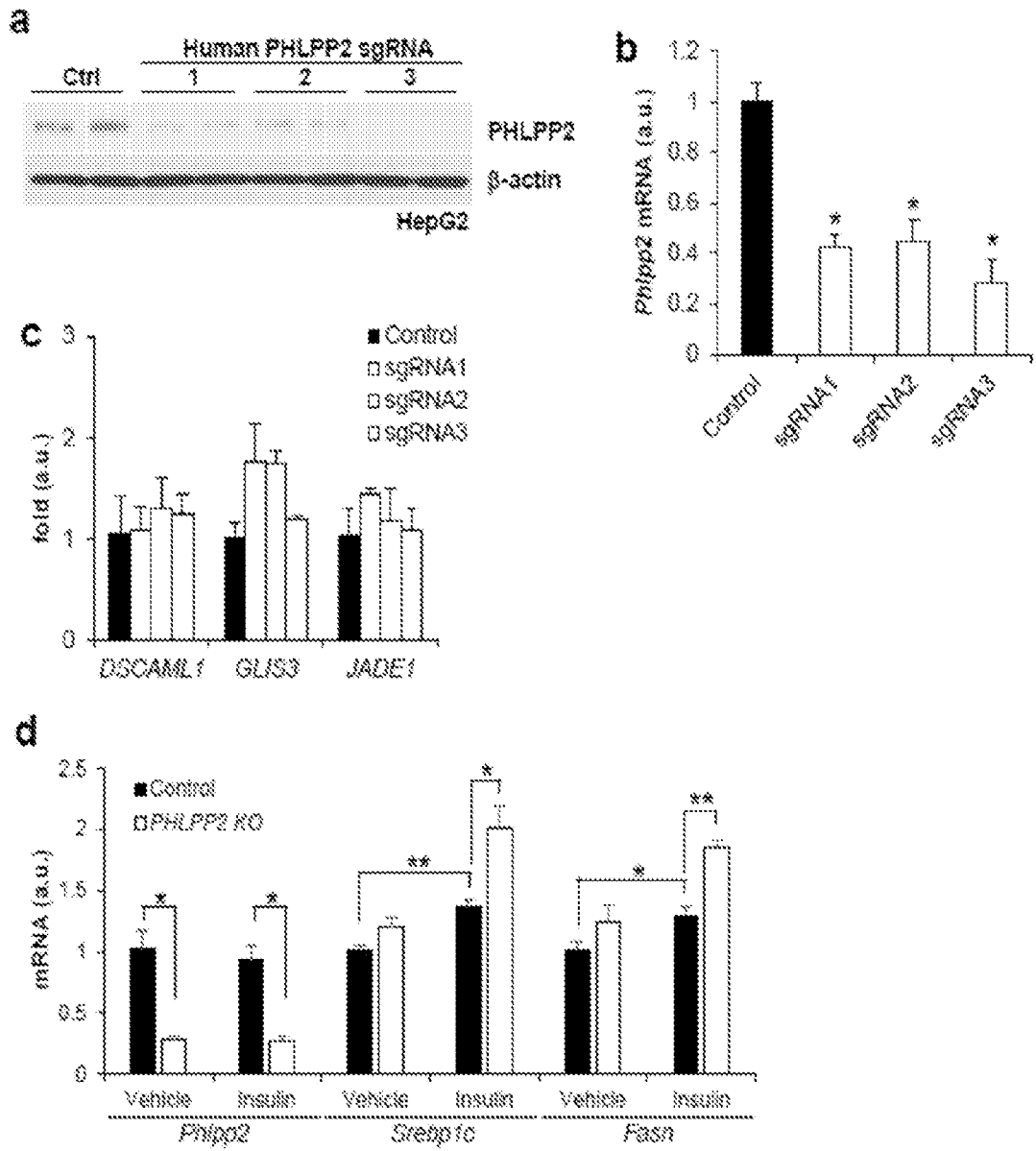


Fig. 23A-D

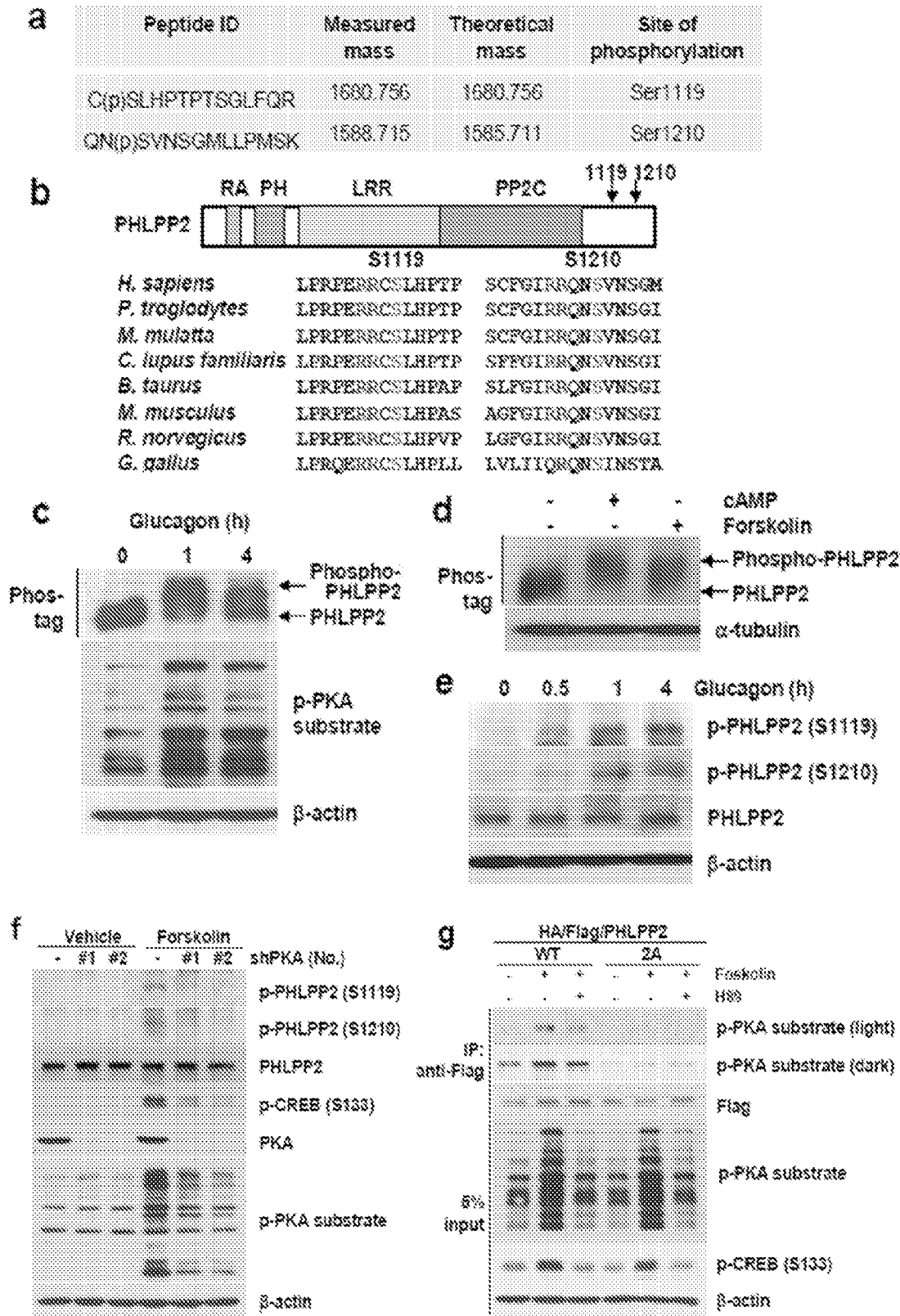


Fig. 24A-G

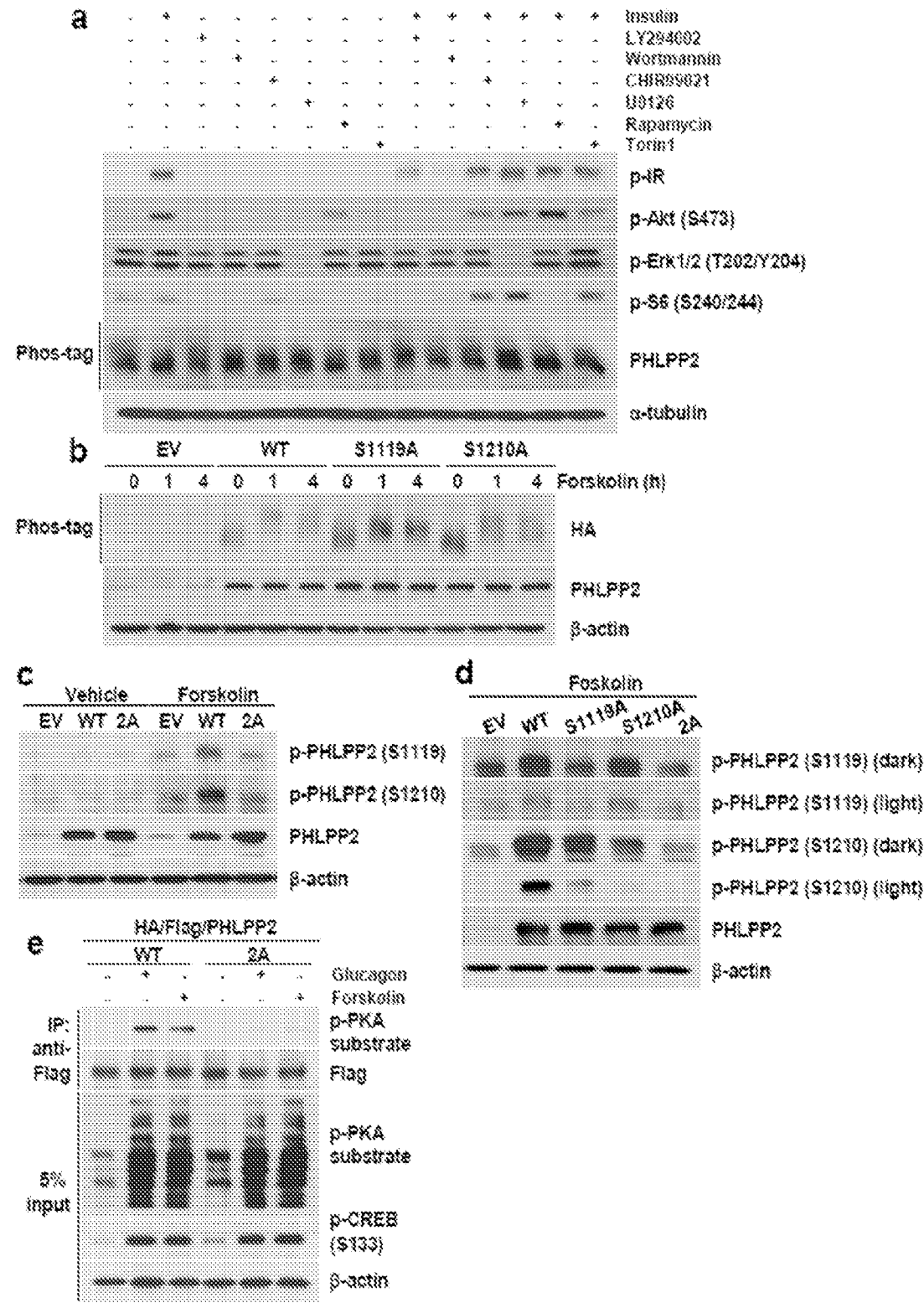


Fig. 25A-E

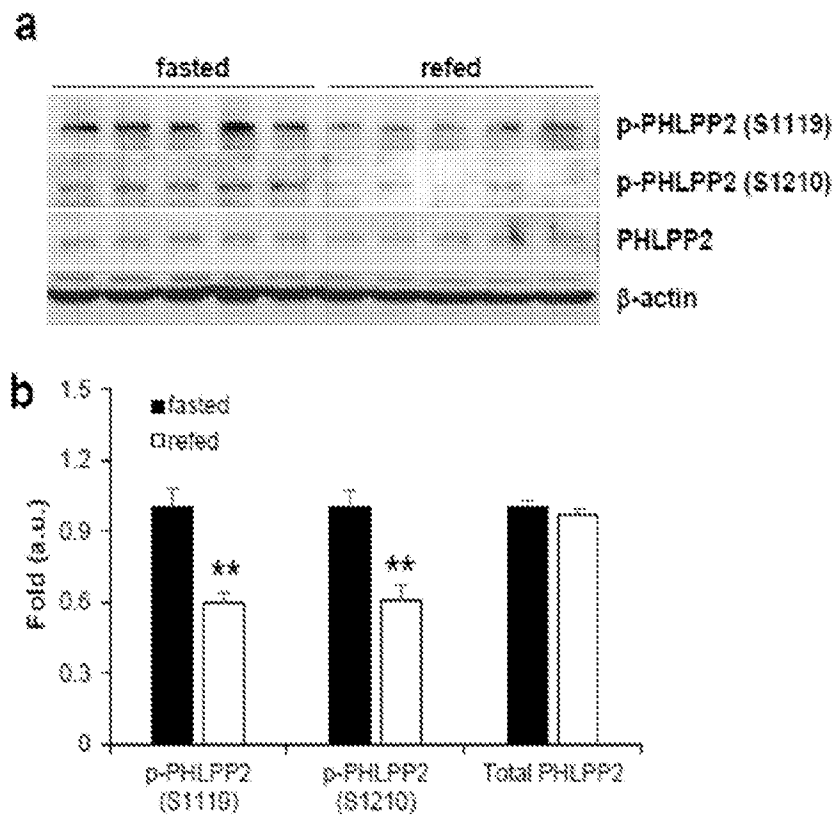


Fig. 26A-B

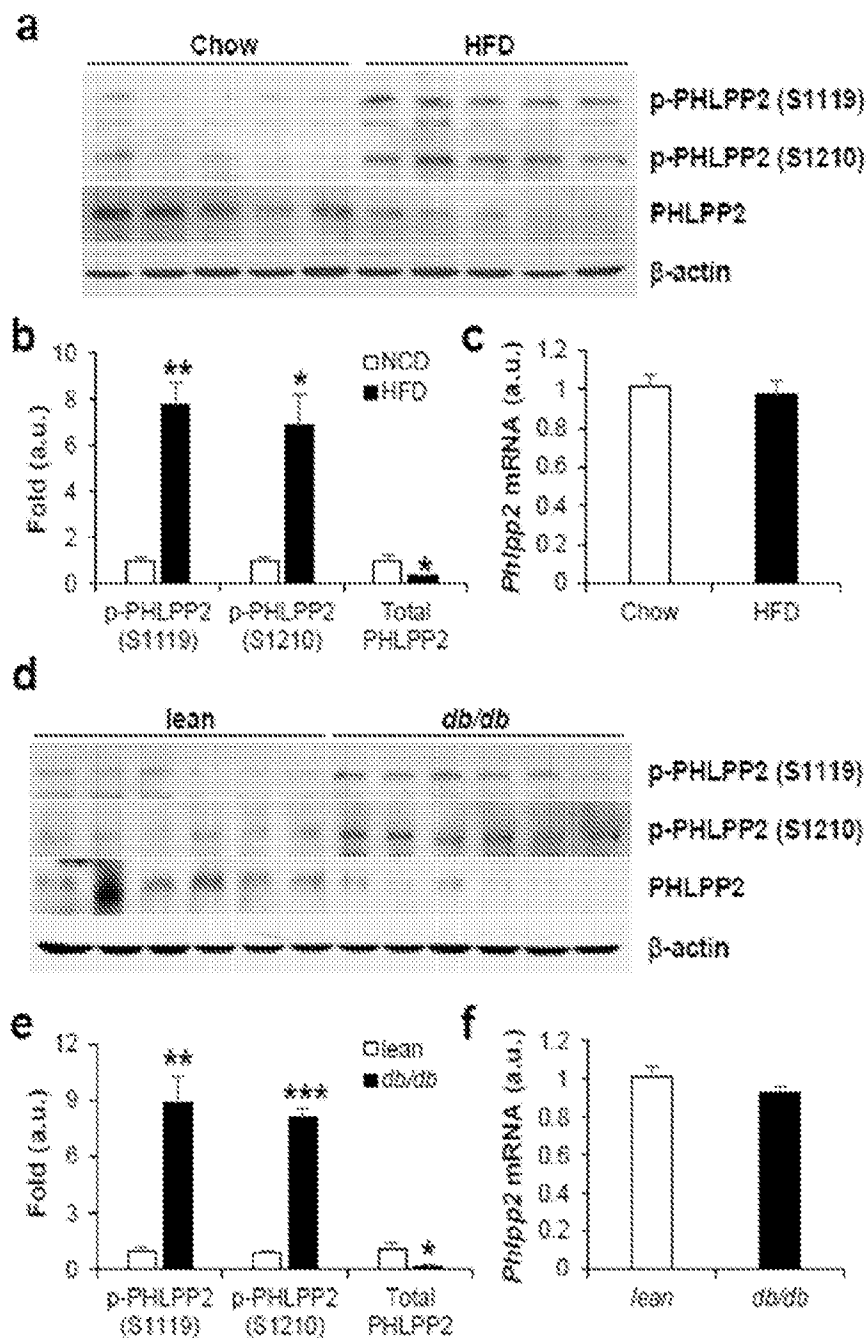


Fig. 27A-F

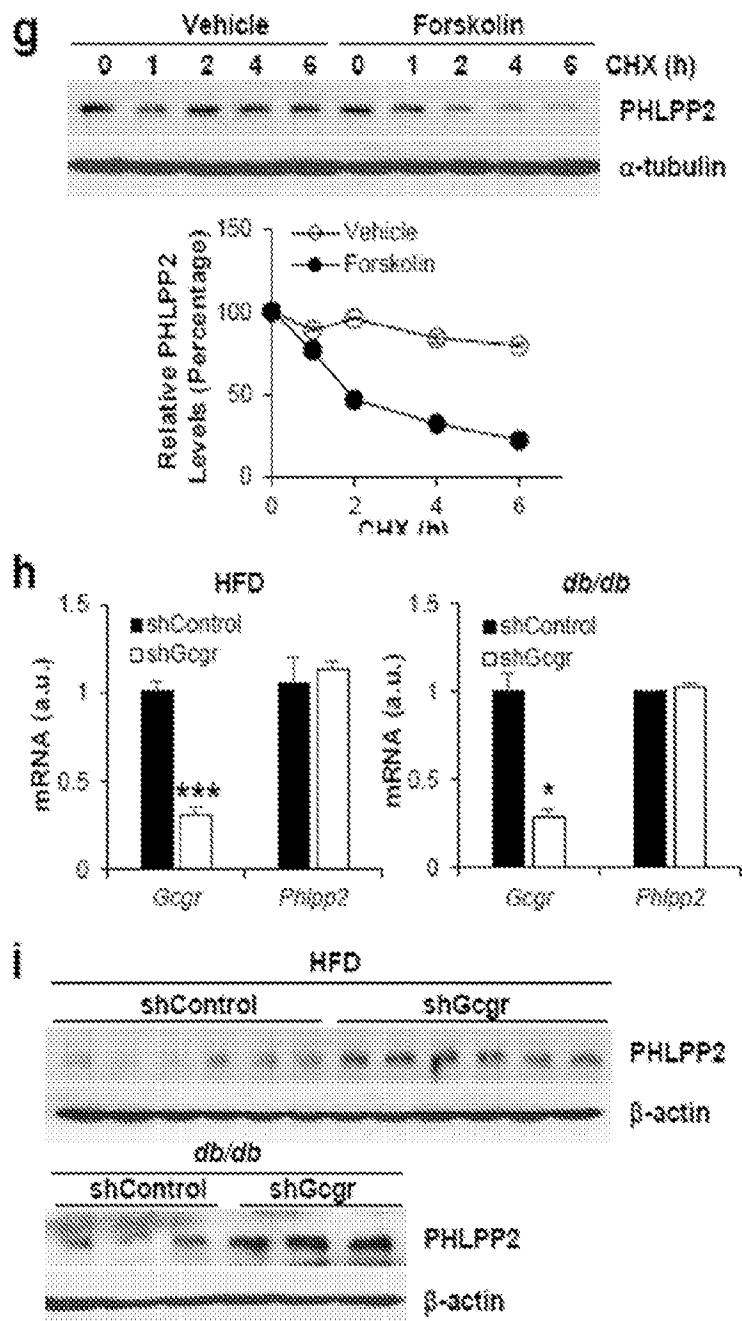


Fig. 27G-I

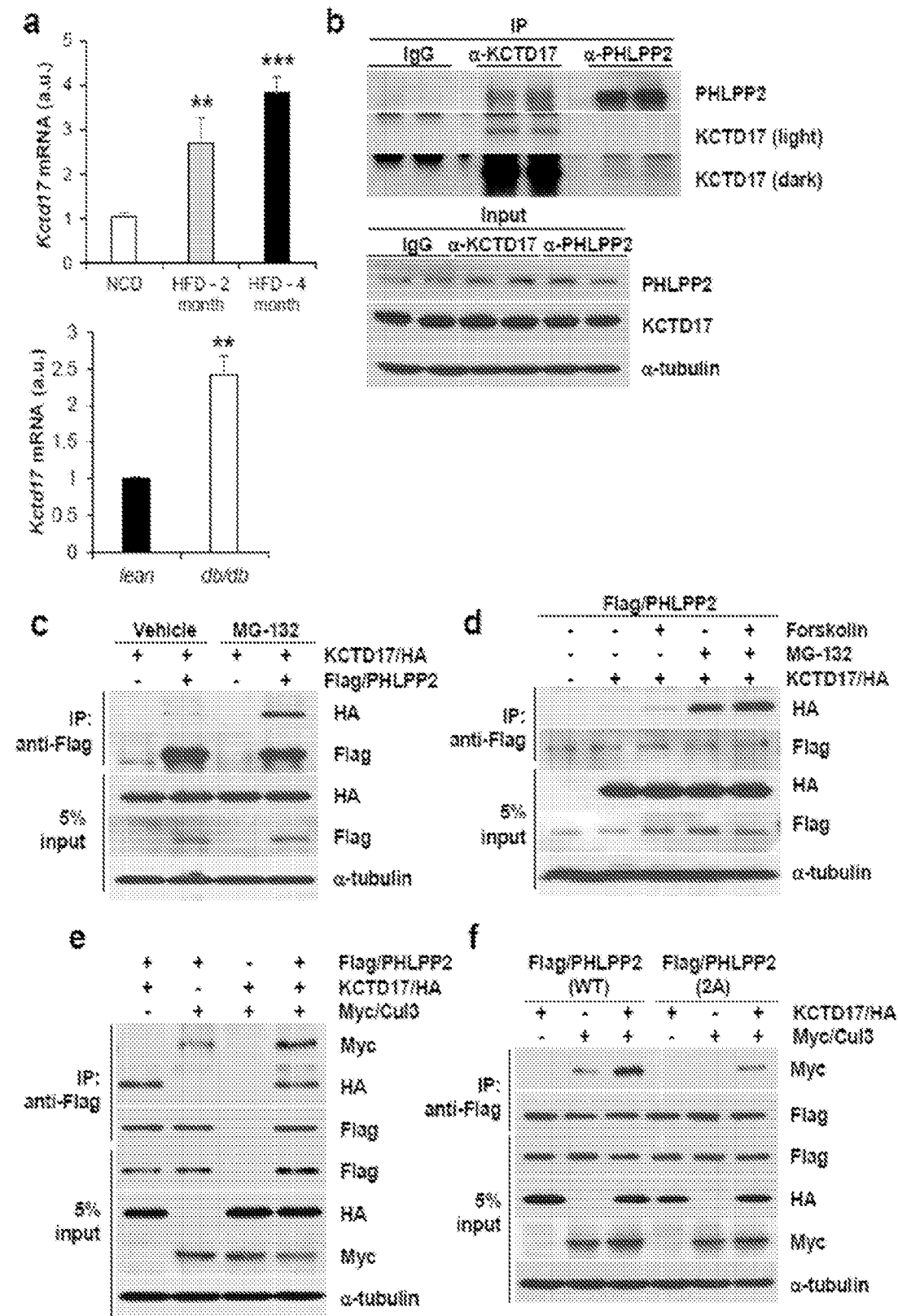


Fig. 28A-F

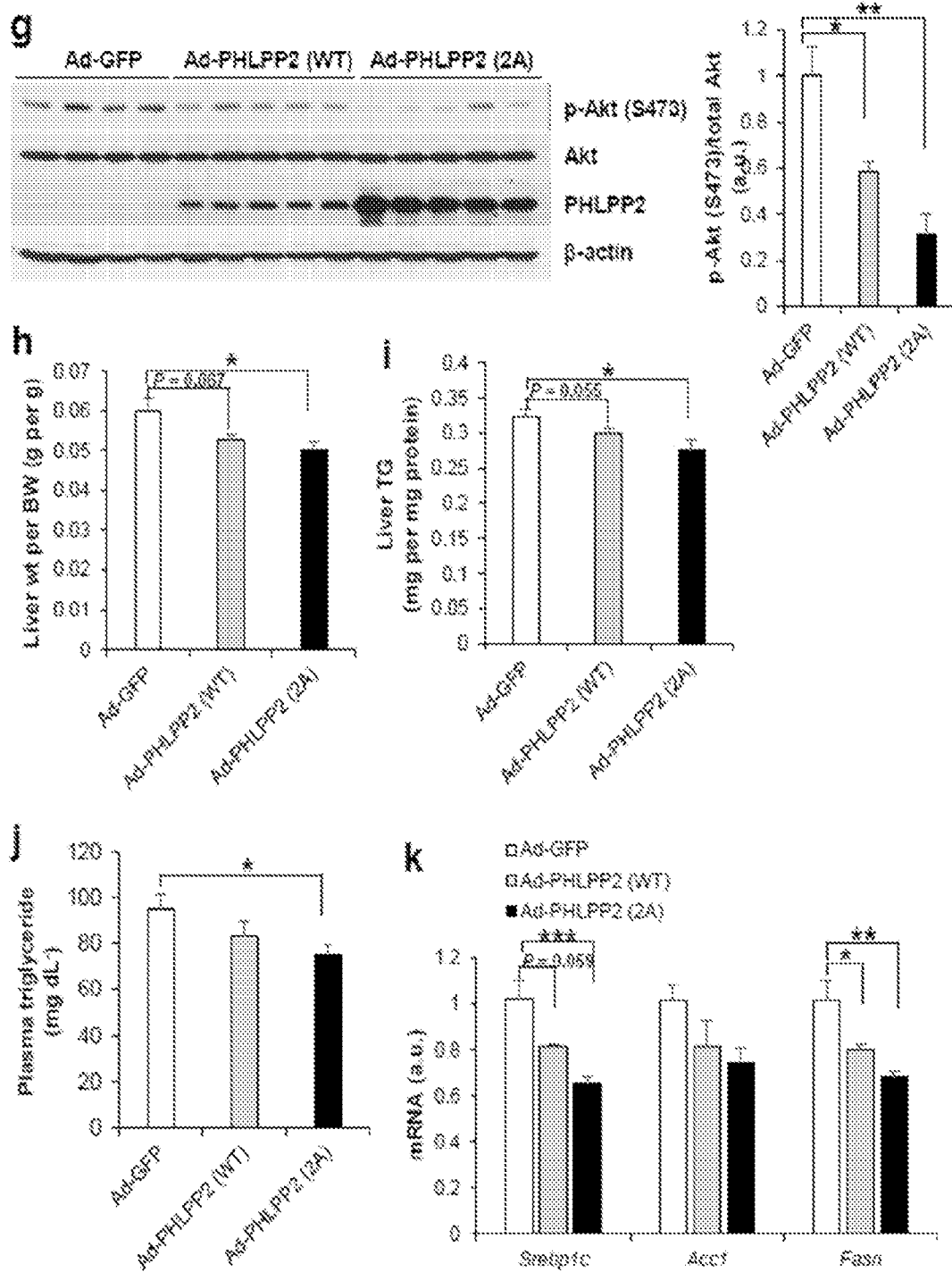


Fig. 28G-K

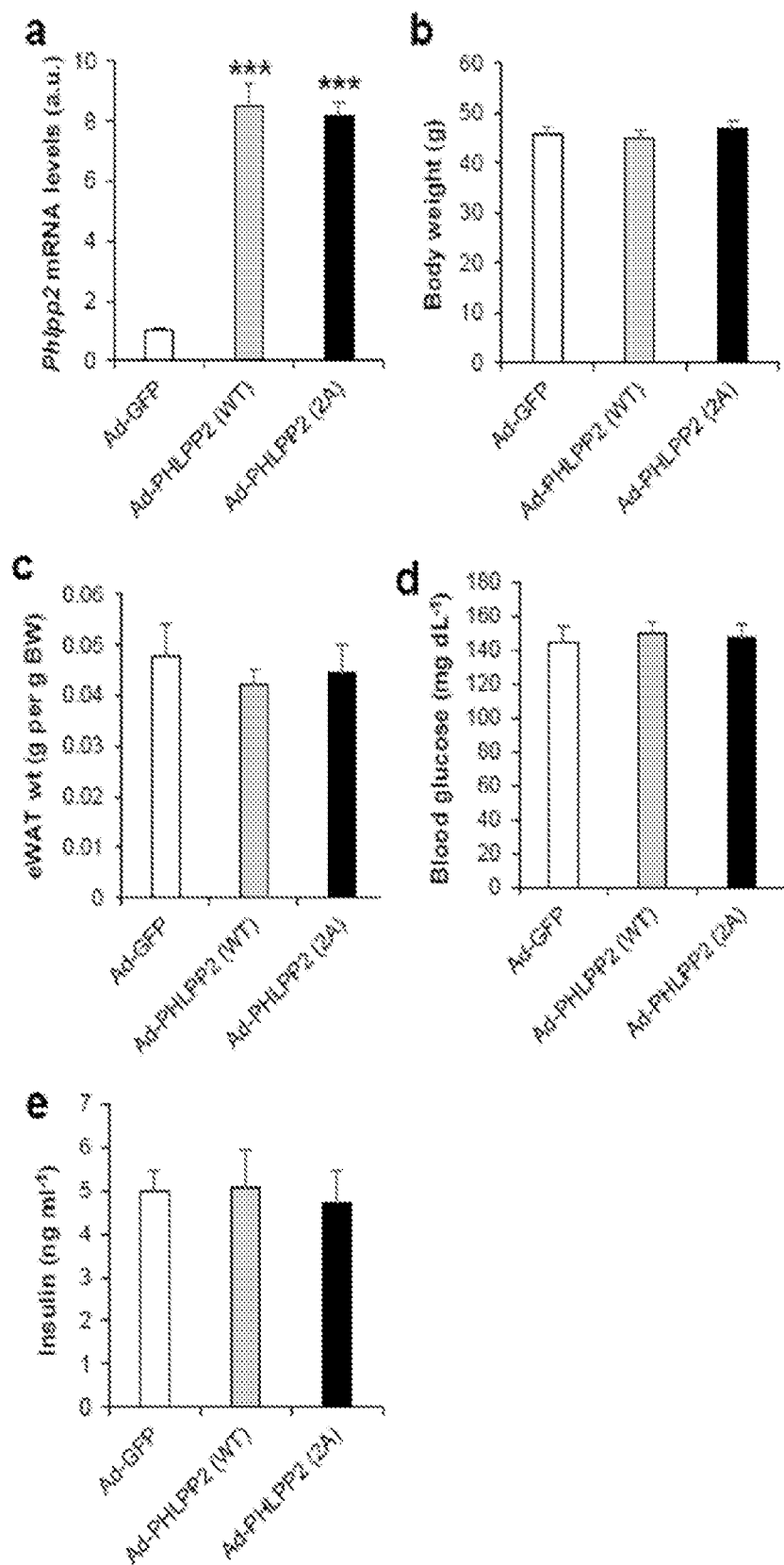


Fig. 29A-E

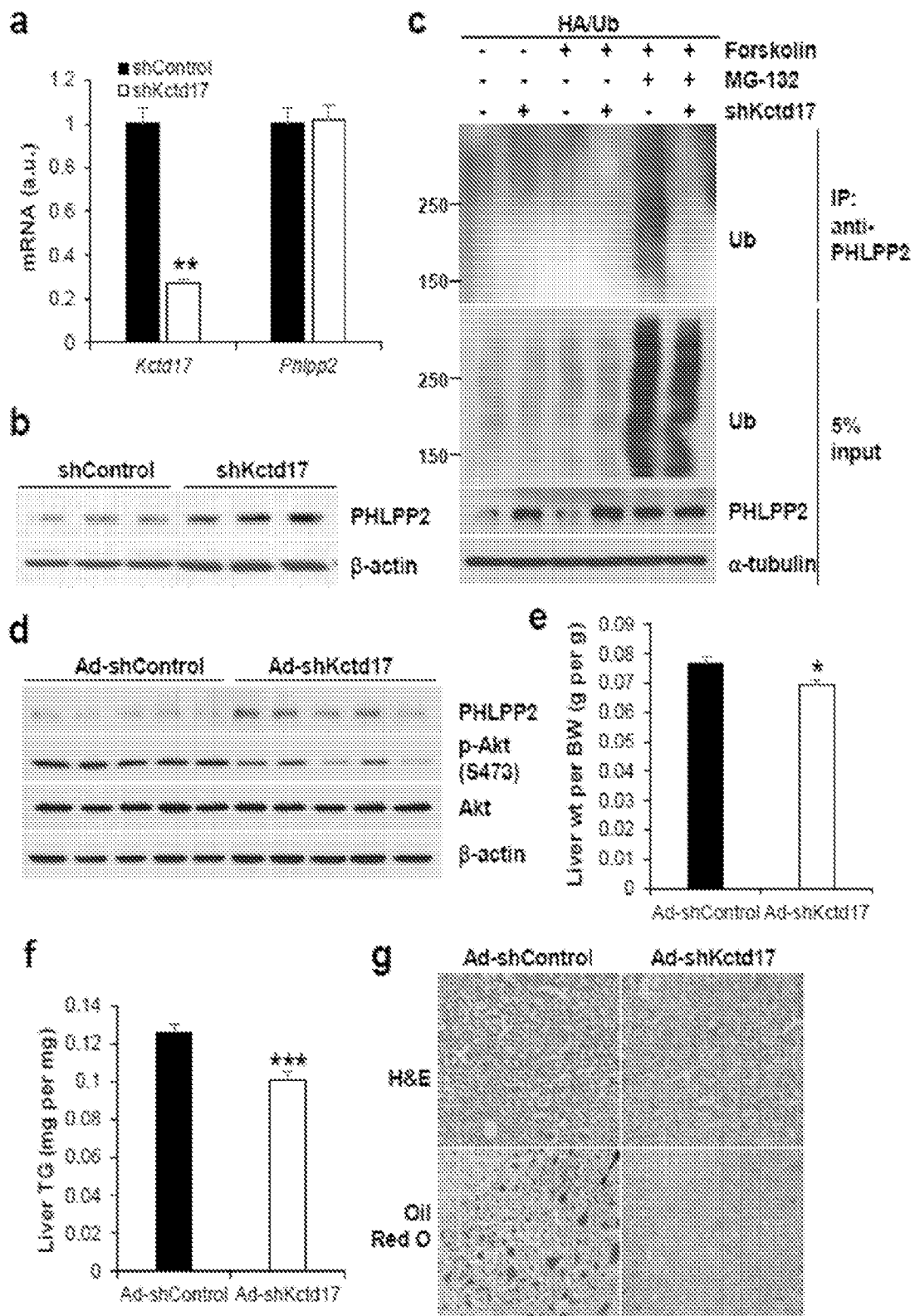


Fig. 30A-G

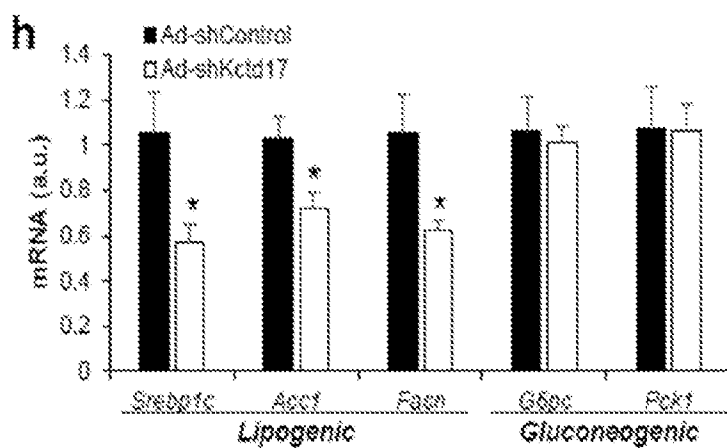


Fig. 30H

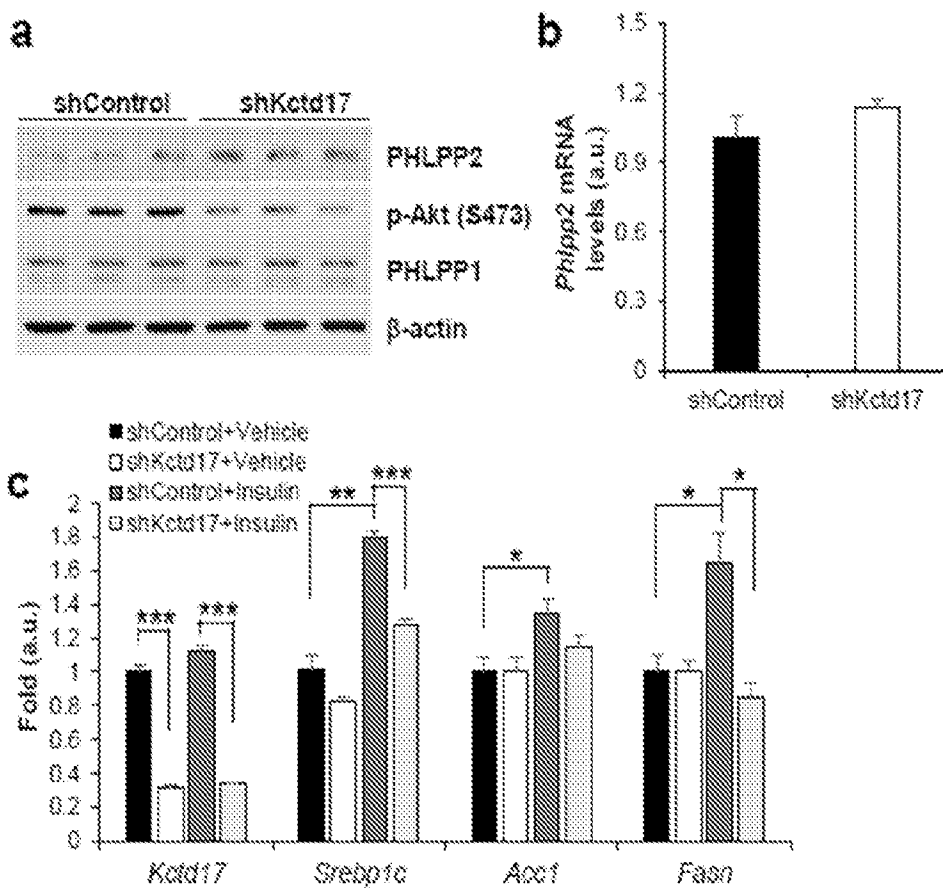


Fig. 31A-C

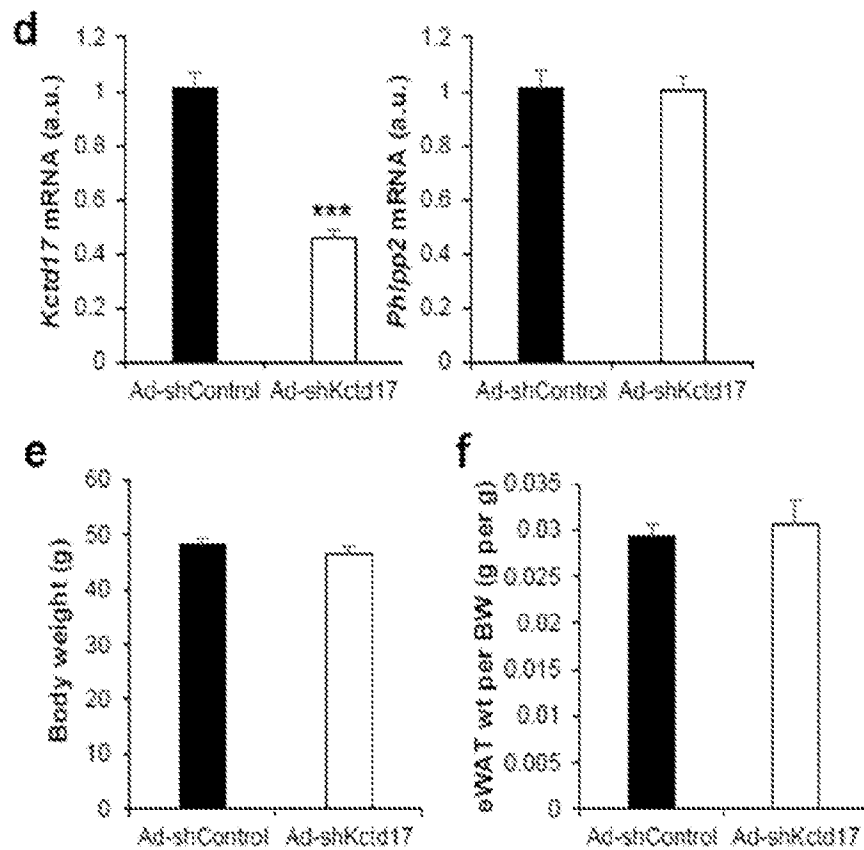


Fig. 31D-F

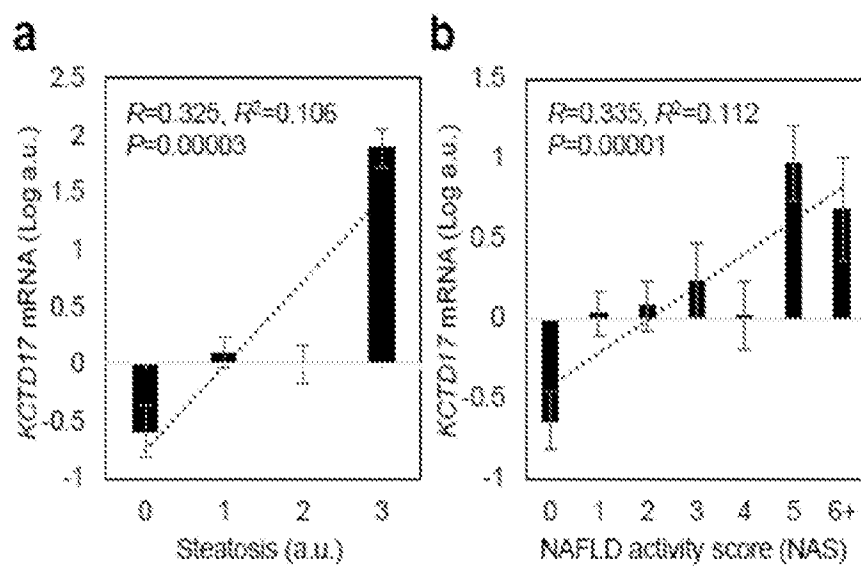


Fig. 32A-B

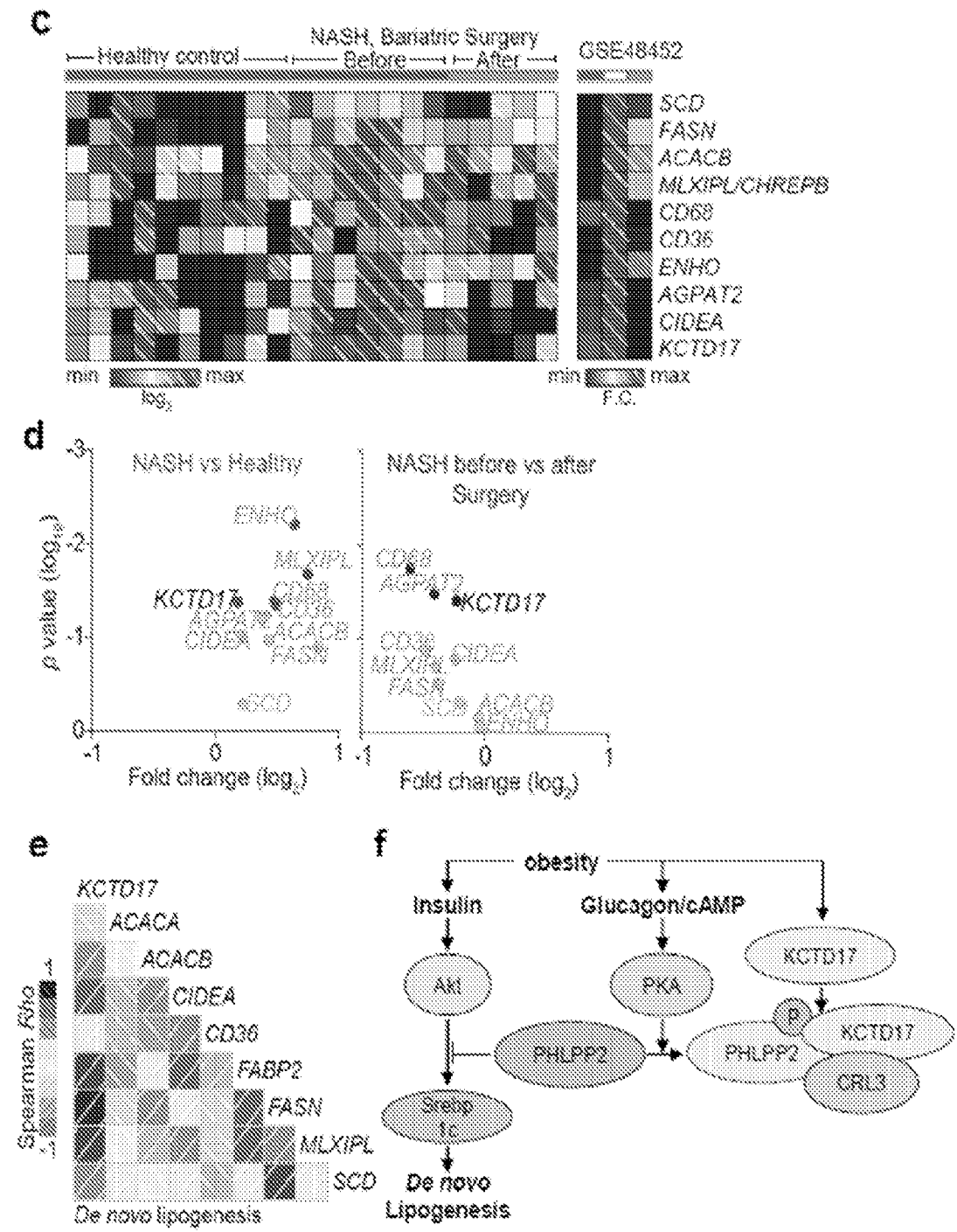


Fig. 32C-F

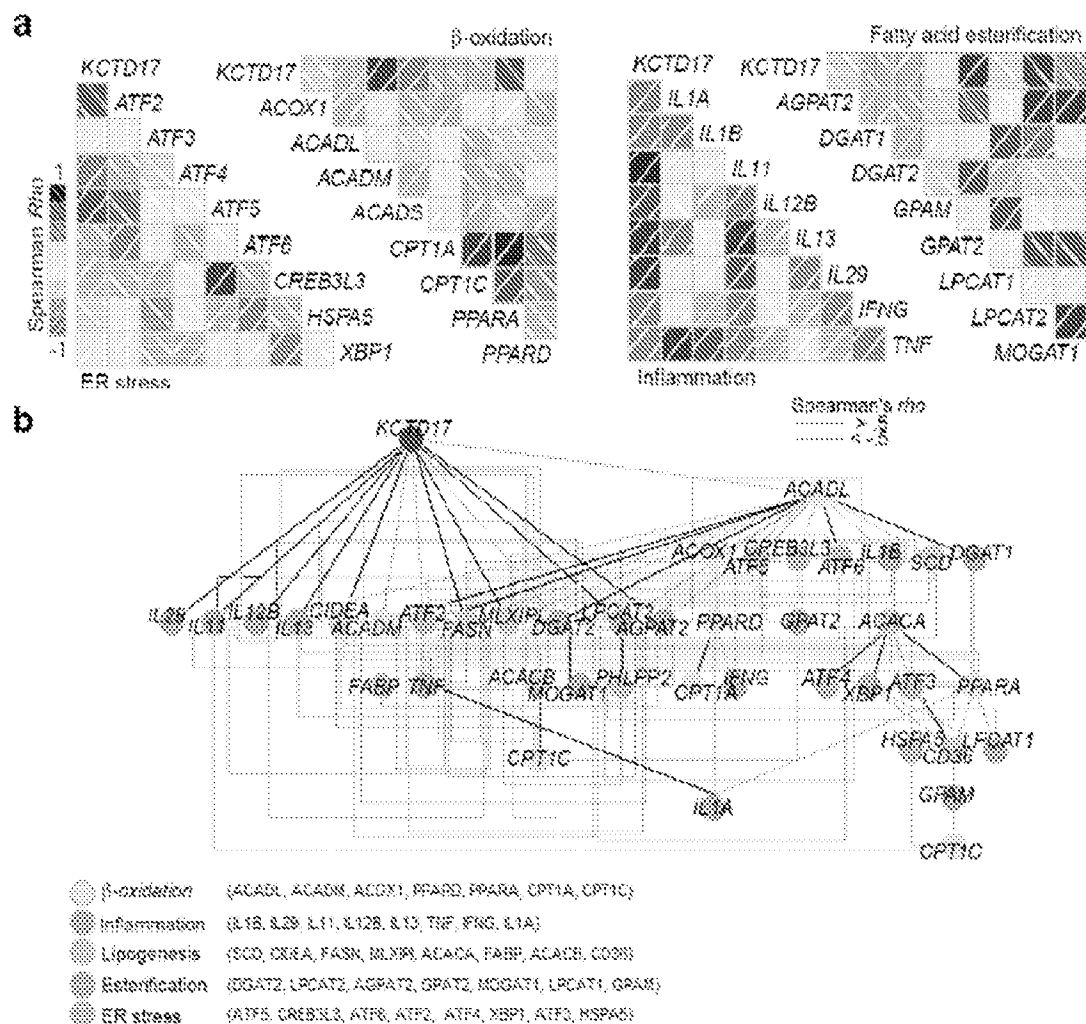


Fig. 33A-B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/30102

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 38/22, 38/28, 38/31, 31/7088, 39/395; A61P 3/06, 9/10; C12N 15/113 (2017.01)

CPC - A61K 38/22, 38/28, 38/31, 31/7088, 39/395; C12N 15/113

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2010/0297105 A1 (GEARY, R.S. et al.) November 25, 2010; paragraphs [0009], [0027], [0083], [0150], [0170], [0174], [0245];	1, 34, 36
A	US 2013/0142861 A1 (NATIONAL YANG MING UNIVERSITY) June 6, 2013; abstract; paragraph [0102], Table 3; column 18; claim 53	1, 31-32, 34, 36
A	KASAHARA et al. 'Ubiquitin-Proteasome System Controls Ciliogenesis At The Initial Step Of Axoneme Extension' Nature Communications, 2014; Vol. 5; pp 1-10; abstract; DOI: 10.1038/ncomms6081	1, 31-32, 34, 36
A	US 2015/0284801 A1 (THE CHINESE UNIVERSITY OF HONG KONG) October 8, 2015; claims 1, 2	31-32

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

07 September 2017 (07.09.2017)

Date of mailing of the international search report

03 OCT 2017

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/30102

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7, 9-30, 33
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

---Please See Supplemental Page---

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1, 31-32, 34, 36

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/US17/30102

-***-Continued from Box No. III Observations where unity of invention is lacking: -***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, Claims 1, 31, 32, 34 and 36 are directed toward a method of treating a subject afflicted with elevated triglyceride levels, and reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a compound that decreases KCTD17 expression in liver cells; and processes for determining the amount of KCTD17 expression in a subject's liver, and diagnosing whether a subject is afflicted with increased KCTD17 expression, based thereon.

Group II, Claims 2, 3, 35 and 37 are directed toward a method of treating a subject afflicted with elevated triglyceride levels and reducing a subject's hepatic and plasma triglyceride levels comprising a compound that prevents PHLPP2 degradation in liver cells or a compound that decreases PHLPP2 phosphorylation at Serine 1119 and Serine 1210 residues in liver cells.

Group III, Claims 4-6 and 8 are directed toward a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a compound that inhibits Glucagon signaling in liver cells.

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include KCTD17, not present in either of Groups II or III; the special technical features of Group II include PHLPP2, not present in either of Groups I or III; the special technical features of Group III include a compound that inhibits glucagon signaling, not present in either of Groups I or II.

Groups I-III share the technical features including: a method of reducing a subject's hepatic and plasma triglyceride levels comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound to liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels. Groups I and II share the technical features including: a method of treating a subject afflicted with elevated triglyceride levels, or at risk of developing elevated triglyceride levels, the method comprising administering to the subject a pharmaceutical composition comprising a pharmaceutical carrier and a compound to liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels.

However, these shared technical features are previously disclosed by US 2010/0297105 A1 to Geary, R.S. et al. (hereinafter 'Geary').

Geary discloses a method of reducing a subject's hepatic and plasma triglyceride levels (a method of reducing a subject's liver (hepatic) and serum (plasma) triglyceride levels; paragraphs [0009], [0027], [0083], [0245]) comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and a compound (comprising administering to a subject in need thereof a pharmaceutical composition comprising a pharmaceutical carrier and an antisense oligonucleotide (a compound); paragraphs [0009], [0170]) to liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels (to liver cells in an amount effective to reduce the subject's liver (hepatic) and serum (plasma) triglyceride levels; paragraphs [0027], [0083], [0150], [0174], [0245]; wherein intravenous administration provides, via circulation, administration to liver cells); a method of treating a subject afflicted with elevated triglyceride levels (a method of treating a subject afflicted with elevated triglyceride levels; paragraphs [0009], [0016]), the method comprising administering to the subject a pharmaceutical composition comprising a pharmaceutical carrier (the method comprising administering to the subject a pharmaceutical composition comprising a pharmaceutical carrier; paragraphs [0009], [0170]) and a compound (and an antisense oligonucleotide (and a compound); paragraph [0009]) to liver cells in an amount effective to reduce the subject's hepatic and plasma triglyceride levels (to liver cells in an amount effective to reduce the subject's liver (hepatic) and serum (plasma) triglyceride levels; paragraphs [0027], [0083], [0150], [0174], [0245]; wherein intravenous administration provides, via circulation, administration to liver cells).

Since none of the special technical features of the Groups I-III inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the Geary reference, unity of invention is lacking.