(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
08 August 2019 (08.08.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/150309 A1

(51) International Patent Classification:
A61K 38/00 (2006.01) *A61P 1/16* (2006.01)
A61K 39/395 (2006.01) *A61P 9/00* (2006.01)
A61K 31/713 (2006.01)

(21) International Application Number:
PCT/IB2019/050799

(22) International Filing Date:
31 January 2019 (31.01.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/625,782 02 February 2018 (02.02.2018) US

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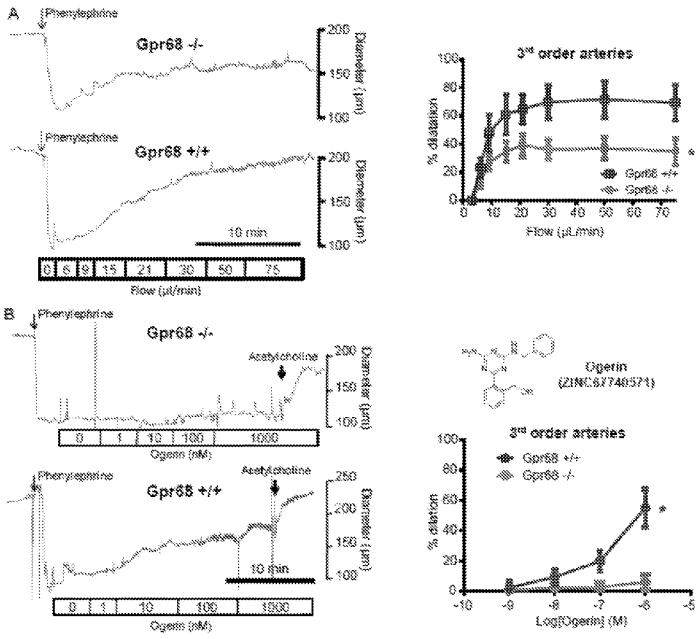
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,

WO 2019/150309 A1

(54) Title: MODULATORS OF GPR68 AND USES THEREOF FOR TREATING AND PREVENTING DISEASES

FIG. 10



(57) Abstract: This disclosure provides novel compositions, methods, and therapeutic uses related to modulators of a GPR68 gene product. Some embodiments disclosed herein provide methods of modulating flow-mediated dilation (FMD) response or flow-mediated outward remodeling (FMR) of small-diameter arteries, or reducing systemic vascular resistance (SVR) in a subject in need thereof comprising administering a modulator of a GPR68 gene product to the subject. Some embodiments disclosed herein provide methods of treating a cardiovascular disease in a subject in need thereof comprising administering a pharmaceutical composition comprising a modulator of a GPR68 gene product to the subject.

[Continued on next page]

SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

MODULATORS OF GPR68 AND USES THEREOF FOR TREATING AND PREVENTING DISEASES

[0001] This application claims the benefit of U.S. Provisional Application No. 62/625,782 filed on February 2, 2018, the content of which is hereby incorporated by reference in its entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on January 31, 2019, is named PAT058065-WO-PCT_SL.txt and is 192,981 bytes in size.

TECHNICAL FIELD

[0003] The present invention provides methods, compositions, and therapeutic uses related to modulators of a GPR68 gene product for the treatment and prevention of diseases, such as cardiavascular diseases and liver fibrosis.

BACKGROUND

[0004] Mechanotransduction is the central feature of many biological systems, including the sensing of touch and pain, hearing, cardiovascular dynamics, as well as turgor pressure sensing in bacteria. A number of membrane proteins capable of sensing acute mechanical forces have been identified, including the mechanosensitive ion channels MscL and MscS in bacteria, the DEGenerin/Epithelial Na^+ Channel (DEG/ENaC) MEC-4, MEC-10 and Transient Receptor Potential channel TRP-4 in *C.elegans*, and the transient receptor potential (TRP) ion channel NOMPC in *Drosophila* (Driscoll and Chalfie, 1991; Huang and Chalfie, 1994; Levina et al., 1999; Sukharev et al., 1994; Walker et al., 2000; Yan et al., 2013).

[0005] In vertebrates, the identity of mechanosensitive receptors has been more elusive. First identified in mammals, Piezos are a family of mechanically-activated (MA) ion channels conserved through evolution (Coste et al., 2010). Piezos are activated by disparate forms of mechanical forces, including indentation, stretch, and fluid shear stress. Consistent with this broad sensitivity to mechanical forces, Piezos play crucial roles in various physiological settings relevant to mechanotransduction. For example, Piezo1 can be activated by fluid shear stress and is required for vascular development and function in mice, while Piezo2 is essential for

somatosensation (Li et al., 2014; Lukacs et al., 2015; Nonomura et al., 2017; Ranade et al., 2014; Retailleau et al., 2015; Wang et al., 2016; Woo et al., 2014). Transmembrane channel-like (TMC) 1 and 2, as well as tetraspan membrane protein of hair cell stereocilia (TMHS) are crucial components of the mechanotransduction channel in hair cells of the mammalian inner ear (Pan et al., 2013; Xiong et al., 2012); however, whether these proteins constitute the pore-forming subunit of this complex remains unsettled. Mammalian TRP channels have diverse sensory roles, though none of them are bona fide mechanically-activated channels, with the possible exception of TRPV4. While mechanical indentation and membrane stretch does not activate TRPV4, elastomeric pillar array mediated membrane stretch leads to channel activation (Rocio Servin-Vences et al., 2017). Mechanosensitive potassium ion channels have been proposed to play a role in negatively modulating the cationic mechanotransduction signal in neurons and in mechanoprotection (Dedman et al., 2009; Hao et al., 2013). In addition, a number of G protein-coupled receptors (GPCRs) have been proposed as mechanosensors, including Angiotensin II receptor type 1 (AGTR1), bradykinin receptor B2 (BDKRB2) and parathyroid hormone 1 receptor (PTH1R) (Chachisvilis et al., 2006; Mederos y Schnitzler et al., 2008; Zhang et al., 2009). However, evidence that these GPCRs are both necessary and sufficient for acute mechanotransduction in an *in vivo* setting is lacking.

[0006] Despite recent progress, the molecular identities of various mechanosensors remain elusive. For example, the identity of high-threshold mechanically activated ion channel present in sensory neurons responsible for pain transduction is unknown. Furthermore, acute shear stress imposed by blood flow on vessel endothelial cells induces diverse downstream signaling events, most notably the phospholipase C (PLC)-dependent increase in intracellular calcium concentrations (Ishida et al., 1997; Melchior and Frangos, 2012). Piezo1 activation is shown to induce ATP release and P2y2 activation in endothelial cells, and is responsible for flow-sensitive non-selective cationic currents that depolarize the membrane (Retailleau et al., 2015; Rode et al., 2017; Wang et al., 2016). However, whether this is the only mechano-signaling system is unclear. Finally, many cell types within bone, muscle, lung, kidney, eye, and other tissues respond to mechanical stimuli through unknown mechanisms (Jaalouk and Lammerding, 2009).

SUMMARY OF THE INVENTION

[0007] Some embodiments disclosed herein provide methods of modulating flow-mediated dilation (FMD) response or flow-mediated outward remodeling (FMR) of small-diameter arteries in a subject in need thereof comprising administering a modulator of a GPR68

gene product to the subject. In some embodiments, the modulator is an antagonist or an agonist of the GPR68 gene product. In some embodiments, the subject being treated has an abnormal vessel dilation and constriction response. In some embodiments, the abnormal vessel dilation and constriction response is associated with endothelial dysfunction. In some embodiments, the endothelial dysfunction is associated with vascular disorder, peripheral arterial disease, heart failure, hypertension, hypercholesterolemia, diabetes, septic shock, Behcet's disease, exposure to smoking tobacco products, exposure to air pollution, or a combination thereof. In some embodiments, the modulator of the GPR68 gene product is selected from the group consisting of a small molecule compound, an antibody, a nucleic acid molecule, a protein, and a combination thereof. In some embodiments, the nucleic acid molecule is selected from the group consisting of an antisense oligonucleotide, a guide RNA, a ribozyme, an aptamer, and a small interfering RNA. In some embodiments, the subject is a human. In some embodiments, the methods further comprise measuring the vessel dilation response of the subject. In some embodiments, the measuring the vessel dilation response comprises brachial artery ultrasound imaging (BAUI).

[0008] Some embodiments disclosed herein provide methods of reducing systemic vascular resistance (SVR) in a subject in need thereof comprising administering a modulator of a GPR68 gene product to the subject. In some embodiments, the modulator is an antagonist or an agonist of the GPR68 gene product. In some embodiments, the subject being treated has an abnormal SVR. In some embodiments, the abnormal SVR is associated with peripheral arterial disease, heart failure, hypertension, hypercholesterolemia, diabetes, septic shock, or a combination thereof. In some embodiments, the modulator of the GPR68 gene product is selected from the group consisting of a small molecule compound, an antibody, a nucleic acid molecule, a protein, and a combination thereof. In some embodiments, the nucleic acid molecule is selected from the group consisting of an antisense oligonucleotide, a guide RNA, a ribozyme, an aptamer, and a small interfering RNA. In some embodiments, the subject is a human. In some embodiments, the methods further comprise measuring the SVR of the subject. In some embodiments, the measuring the SVR comprises measuring blood pressure (BP), heart rate (HR), stroke volume (SV), or a combination thereof. In some embodiments, the SVR of the subject is reduced by about 10% to about 50%.

[0009] Some embodiments disclosed herein provide methods of treating a cardiovascular disease in a subject in need thereof comprising administering a pharmaceutical composition comprising a modulator of a GPR68 gene product to the subject. In some embodiments, the modulator is an antagonist or an agonist of the GPR68 gene product. In some embodiments,

the cardiovascular disease is selected from the group consisting of congestive heart failure (CHF), peripheral artery disease, stroke, diabetic nephropathy, and renal hypertension. In some embodiments, the CHF comprises HF with reduced ejection fraction (aka HF due to left ventricular dysfunction) or HF with preserved ejection fraction (HFpEF) (aka diastolic HF or HF with normal ejection fraction). In some embodiments, the CHF is associated with a coronary artery disease selected from myocardial infarction (heart attack), high blood pressure, atrial fibrillation, and valvular heart disease, excess alcohol use, infection, or cardiomyopathy of an unknown cause. In some embodiments, the modulator of the GPR68 gene product reduces the SVR or the left ventricle afterload of the subject. In some embodiments, the SVR or the left ventricle afterload of the subject is reduced by about 10% to about 50%. In some embodiments, the methods further comprise diagnosing the cardiovascular disease in the subject. In some embodiments, the cardiovascular disease is diagnosed based on the history of the symptoms, physical examination, echocardiography, blood test, electrocardiography, chest radiography, or a combination thereof. In some embodiments, the modulator of the GPR68 gene product is selected from the group consisting of a small molecule compound, an antibody, a nucleic acid molecule, a protein, and a combination thereof. In some embodiments, the nucleic acid molecule is selected from the group consisting of an antisense oligonucleotide, a guide RNA, a ribozyme, an aptamer, and a small interfering RNA. In some embodiments, the subject is a human. In some embodiments, the methods further comprise administering an angiotensin converting enzyme (ACE) inhibitor, an angiotensin receptor blocker (ARB), a β-adrenergic receptor blocker, or a diuretics to the subject.

[0010] Some embodiments disclosed herein provide modulators of a GPR68 gene product for use as a medicament for the treatment of a cardiovascular disease in a subject in need thereof. In some embodiments, the modulator is an antagonist or an agonist of the GPR68 gene product. In some embodiments, the cardiovascular disease is selected from the group consisting of congestive heart failure (CHF), peripheral artery disease, stroke, diabetic nephropathy, and renal hypertension. In some embodiments, the CHF comprises HF with reduced ejection fraction (aka HF due to left ventricular dysfunction) or HF with preserved ejection fraction (HFpEF) (aka diastolic HF or HF with normal ejection fraction). In some embodiments, the CHF is associated with a coronary artery disease selected from myocardial infarction (heart attack), high blood pressure, atrial fibrillation, and valvular heart disease, excess alcohol use, infection, or cardiomyopathy of an unknown cause. In some embodiments, the modulator of the GPR68 gene product reduces the SVR or the left ventricle afterload of the subject. In some embodiments, the SVR or the left ventricle afterload of the subject is reduced

by about 10% to about 50%. In some embodiments, the treatment further comprises diagnosing the cardiovascular disease in the subject. In some embodiments, the cardiovascular disease is diagnosed based on the history of the symptoms, physical examination, echocardiography, blood test, electrocardiography, chest radiography, or a combination thereof. In some embodiments, the modulator of the GPR68 gene product is selected from the group consisting of a small molecule compound, an antibody, a nucleic acid molecule, a protein, and a combination thereof. In some embodiments, the nucleic acid molecule is selected from the group consisting of an antisense oligonucleotide, a guide RNA, a ribozyme, an aptamer, and a small interfering RNA. In some embodiments, the subject is a human. In some embodiments, the treatment further comprises administering an angiotensin converting enzyme (ACE) inhibitor, an angiotensin receptor blocker (ARB), a β-adrenergic receptor blocker, or a diuretics to the subject.

[0011] Some embodiments disclosed herein provide modulators of a GPR68 gene product in the manufacture of a medicament for the treatment of a cardiovascular disease in a subject in need thereof. In some embodiments, the modulator is an antagonist or an agonist of the GPR68 gene product. In some embodiments, the cardiovascular disease is selected from the group consisting of congestive heart failure (CHF), peripheral artery disease, stroke, diabetic nephropathy, and renal hypertension. In some embodiments, the CHF comprises HF with reduced ejection fraction (aka HF due to left ventricular dysfunction) or HF with preserved ejection fraction (HFpEF) (aka diastolic HF or HF with normal ejection fraction). In some embodiments, the CHF is associated with a coronary artery disease selected from myocardial infarction (heart attack), high blood pressure, atrial fibrillation, and valvular heart disease, excess alcohol use, infection, or cardiomyopathy of an unknown cause. In some embodiments, the modulator of the GPR68 gene product reduces the SVR or the left ventricle afterload of the subject. In some embodiments, the SVR or the left ventricle afterload of the subject is reduced by about 10% to about 50%. In some embodiments, the treatment further comprises diagnosing the cardiovascular disease in the subject. In some embodiments, the cardiovascular disease is diagnosed based on the history of the symptoms, physical examination, echocardiography, blood test, electrocardiography, chest radiography, or a combination thereof. In some embodiments, the modulator of the GPR68 gene product is selected from the group consisting of a small molecule compound, an antibody, a nucleic acid molecule, a protein, and a combination thereof. In some embodiments, the nucleic acid molecule is selected from the group consisting of an antisense oligonucleotide, a guide RNA, a ribozyme, an aptamer, and a small interfering RNA. In some embodiments, the subject is a human. In some embodiments,

the treatment further comprises administering an angiotensin converting enzyme (ACE) inhibitor, an angiotensin receptor blocker (ARB), a β-adrenergic receptor blocker, or a diuretics to the subject.

[0012] Some embodiments disclosed herein provide methods of treating liver fibrosis in a subject in need thereof comprising administering a pharmaceutical composition comprising an antagonist of a GPR68 gene product to the subject. In some embodiments, the antagonist of the GPR68 gene product is selected from the group consisting of a small molecule compound, an antibody, a nucleic acid molecule, a protein, and a combination thereof. In some embodiments, the nucleic acid molecule is selected from the group consisting of an antisense oligonucleotide, a guide RNA, a ribozyme, an aptamer, and a small interfering RNA. In some embodiments, the subject is a human.

[0013] Some embodiments disclosed herein provide methods of identifying a modulator of a GPR68 gene product comprising: providing a population of cells expressing a GPR68 gene product; adding a library of candidate molecules to the population of cells; applying a shear stress or an acidic shock to the population of cells; measuring the calcium transient in the population of cells; and identifying a candidate molecule in a cell that shows enhanced/reduced calcium transient in the cell. In some embodiments, the population of cells comprises endothelial cells, such as mouse primary brain microvascular endothelial cells, or human microvascular endothelial cells from brain, lung, bladder, or skin. In some embodiments, the library of candidate molecules comprises a small molecule compound, an antibody, a nucleic acid molecule, or a protein. In some embodiments, the shear stress comprises disturbed flow or laminar flow. In some embodiments, the acidic shock comprises extracellular proton at pH 6.5.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] **FIGs. 1A-1G** show construction and validation of high-throughput shear stress stimulation system. **FIG. 1A** is a schematic representation of the disturbed flow stimulation system. **FIG. 1B** is an exemplary drawing depicts the major components of the 384-well format high-throughput disturbed flow stimulation system (left) and the bubble-relief geometry of the stimulator pin (right). **FIG. 1C** shows a top view of HT disturbed flow system showing the acoustic transducer assembly. **FIG. 1D** shows a bottom view of the HT system showing the 384-pin array. **FIG. 1E** shows exemplary intracellular calcium levels of HeLa cells measured by FLIPR, under 6.5 Pa shear stress stimulation in the presence of 2.5 mM EGTA or untreated control. The viscosity of the assay buffer was increased to 8.37×10^{-3} Pa·s by adding PVP to 2% (w/w). Disturbed flow (0.2 s on, 2 s off) was applied at 60 Hz for 40 s. Data was average of 48

wells from 3 trials for each condition, plotted as mean ± SEM. **FIG. 1F** shows exemplary intracellular calcium levels of HeLa cells under the same disturbed flow stimulation after the treatment of scrambled siRNA or hPIEZO1 siRNA. Data is average of 48 wells from 3 trials for each condition, plotted as mean ± SEM. **FIG. 1G** shows exemplary relative mRNA level of PIEZO1 in HUVECs transfected with PIEZO1 siRNA compared with cells treated with scrambled siRNA (n=3).

[0015] **FIGs. 2A-2D** show that MDA-MB-231 cells show PIEZO1- and PIEZO2-independent shear stress-induced calcium transients. **FIG. 2A** shows exemplary responses of various human cancer cell lines to shear stress induced by the high throughput disturbed flow system. Shear stress was applied at 2 Pa, 60 Hz, with 0.2 s on, 2s off for 40 s. ** $p<0.01$ **FIG. 2B** shows exemplary response of MDA-MB-231 cells to shear stress at 2 Pa, measured by FLIPR. The shear stress was applied for 4 s at 60 Hz by the HT disturbed flow system. EGTA was added to the cells 2 min prior to the onset of shear stress. Thapsigargin was incubated with cells for 15 min before the shear stress stimulation. **FIG. 2C** shows quantification of the MDA-MB-231 cells' response to shear stress in the presence of the EGTA and Thapsigargin. ** $p<0.01$. **FIG. 2D** shows quantification of the MDA-MB-231 cells' response to shear stress 72 h after transfection of PIEZO1 and PIEZO2 siRNA. n.s., not siginificant. All results are plotted as mean ± SEM.

[0016] **FIG. 3** shows that transfecting human cell lines with PIEZO1 or PIEZO2 siRNA significantly knocks down their respective mRNA levels. Relative mRNA level of PIEZO1 and PIEZO2 in MDA-MB-231 cells transfected with PIEZO1 siRNA or PIEZO2 siRNA compared with cells transfected with scrambled siRNA (mean ± SEM from 4 trials).

[0017] **FIGs. 4A-4E** show that GPR68 is necessary for shear stress-induced calcium transients in MDA-MB-231 cells. **FIG. 4A** shows exemplary response of MDA-MB-231 cells after treatment of siRNA against the indicated candidates. ** $p<0.01$ **FIG. 4B** shows quantification of the MDA-MB-231 cells' response to shear stress after treatment with smartpool siRNA oligos and the individual siRNA oligos against GPR68. ** $p<0.01$. **FIG. 4C** shows exemplary response of MDA-MB-231 cells to proton stimulation at various final pH. The cells were incubated with HBSS assay buffer at pH7.4 before the addition of the HBSS with various pH values. Three trials. **FIG. 4D** shows exemplary response of MDA-MB-231 cells to proton stimulation (final pH 6.5) after knocking down GPR68 by siRNA smartpool. **FIG. 4E** shows exemplary response of MDA-MB-231 cells to shear stress stimulation in buffers with various pH. The cells were incubated with assay buffer at pH7.4. The pH was then changed by adding

buffer with various acidity 3 min prior to the onset of shear stress stimulation by the HT disturbed flow system. All results are plotted as mean ± SEM.

[0018] **FIGs. 5A-5D** show that GPR68 histidine mutant loses both acid sensitivity and shear stress sensitivity. **FIG. 5A** shows that HEK-293T cells were transiently transfected with pcDNA5, GPR68 WT or GPR68 histidine mutant constructs. Acid or shear stress-induced calcium transients were assayed 48h after transfection. For acid stimulation, the final pH was pH6.5. For shear stress stimulation, a 4s pulse of 60 Hz disturbed flow at 2 Pa was applied to the cells. Results were plotted as mean ± SEM from 3 trials. **FIGs. 5B-5C** show assessment of pH-sensitive fluorescent dye BCECF for measurement of extracellular and intracellular pH. Upon addition of acid or base to cells to shift the buffer pH to various final pH (from pH6.5 to pH8.3), BCECF fluorescence intensity changed accordingly, demonstrating that the dye is sensitive to distinguish minute changes in pH. **FIG. 5D** shows that upon application of shear stress, there's no measurable change in BCECF fluorescence, indicating no changes in either extracellular or intracellular pH. Traces are plotted as mean ± SEM from two trials, 16 repeats for each condition.

[0019] **FIGs. 6A-6K** show that GPR68 is sensitive to shear stress imposed by both disturbed and laminar flow in HEK-293T cells. **FIG. 6A** shows exemplary disturbed flow shear stress-induced calcium transients in HEK-293T cells. HEK-293T cells were transfected with human GPR68 or vector control 48 h before the assay. Disturbed flow was applied by the HT system at 60 Hz for 4s (arrow indicates the onset of the flow). **FIG. 6B** shows quantification of the amplitude of the calcium transients evoked by disturbed shear stress of increasing intensities. n=3 wells per intensity tested. **FIG. 6C** shows quantification of the response of HEK-293T cells to pH 6.5 stimulation and 2 Pa disturbed shear stress. ** $p<0.01$. **FIG. 6D** shows quantification of the response of HEK-293T cells to 2 Pa disturbed shear stress in the presence of 20 μM Cu^{2+} and 10 μM U73122. Cu^{2+} and U73122was added to the assay buffer 2 min prior to the start of disturbed flow. n.s., not significant. ** $p<0.01$. **FIG. 6E** shows exemplary shear stress induced responses of HEK-293T cells transiently-transfected with various human and mouse GPCRs. ** $p<0.01$. **FIG. 6F** shows exemplary agonist response of HEK-293T cells transiently-transfected with various human and mouse GPCRs. ** $p<0.01$. The chemical activators used were: pH 6.5 for human and murine GPR68, 0.5 μM Angiotensin II for AGTR1, 0.5 μM [Arg^8]-Vasopressin for AVPR1A, 0.5 μM Bradykinin for BDKRB2, 0.5 μM Acetylcholine Chloride for CHRM5, 0.5 μM Endothelin I for EDNRA, 0.5 μM Histamine Dihydrochloride for HRH1, 20 μM Parathyroid Hormone (1-34) for PTHR1, and 100 mM lactate for GPR132. **FIG. 6G** shows representative traces of intracellular calcium levels in HEK-293T cells transfected

with mouse Gpr68-IRES-eGFP upon pulsatile laminar flow stimulation. Cells with GFP signal were considered transfected and the GFP-negative ones were untransfected. Pulsatile laminar flow was applied at 3.4 Pa, 1 Hz for 120 s. **FIG. 6H** shows quantification of the response of HEK cells to pulsatile laminar flow. n=172 for MmGpr68 transfected and 165 for untransfected. **FIG. 6I** shows representative traces of intracellular calcium levels in HEK-293T cells transfected with mouse Gpr68-IRES-eGFP upon steady laminar flow stimulation. Steady laminar flow was applied at 3.4 Pa for 120 s. **FIG. 6J** shows quantification of the response of HEK cells to steady laminar flow. n=162 for MmGpr68 transfected and 183 for untransfected. **FIG. 6K** shows exemplary responses of HEK-293T cells transfected with various putative mechanosensors to steady laminar flow at 3.4 Pa. ** $p<0.01$. All results are plotted as mean ± SEM.

[0020] **FIGs. 7A-7F** show that GPR68 expression is detected in endothelial cells of small-diameter vessels. **FIG. 7A** shows an exemplary murine Gpr68 mRNA expression profile determined by qRT-PCR. Gapdh was used as the reference gene. The expression levels were normalized to spleen. Data was plotted as mean ± SEM (n=2~3). **FIG. 7B** shows representative images of colorimetric RNAscope *in situ* hybridization for Gpr68 vascular endothelial cells of small diameter blood vessels in brain, pancreas and liver. Scale bars: 25 μm. Arrows indicate cells with positive signal. **FIG. 7C** shows a schematic diagram of the BAC transgenic construct that is integrated in the genome of Gpr68 eGFP reporter mice. The blue A-box is the homologous sequence that was used to guide recombination and insert eGFP after the start codon of Gpr68. Poly-A sequence following the eGFP blocks the transcription of the downstream genes within the construct (including Gpr68). pA: poly-A sequence; AmpR, amplicilin-resistance genes; R6Kγ, origin of DNA replication (AmpR and R6Kγ are used in the initial selection of BAC constructs). **FIG. 7D** shows an exemplary FACS plot of primary endothelial cells isolated from the bladder of Gpr68 eGFP reporter mice. Cells that are positive for CD31 and negative for CD45 are shown, and grouped in the GFP+ and GFP- populations. **FIG. 7E** shows exemplary normalized RNA levels (RPKM) of Gpr68 and Piezo1 from the RNAseq data of GFP+ and GFP- endothelial cells. Bladder cells from 3 batches of isolation and sorting (10 mice in total) were pooled and subjected to RNAseq. **FIG. 7F** shows representative images of GFP antibody staining in arteries of 1st order (1º), 2nd order (2º), 3rd order (3º) superior mesenteric vessels, and the vessels in the wall of small intestine (w). Scale bars: 50 μm for 1º vessels and 2º vessels, 25 μm for 3º vessels, and 10 μm for vessels in the small intestine wall. 8 groups of vessels from 4 mice were examined.

[0021] **FIGs. 8A-8E** show that GFP immunoreactivity is detected in small diameter arteries in various tissues of GPR68 eGFP reporter mice. **FIG. 8A** shows an exemplary flow

cytometry analysis of GFP intensity in various populations of leukocytes isolated from the spleen of Gpr68 eGFP reporter mice. Results were from two eGFP reporter mice (light and dark green lines) and two C57B/L6 mice (grey and black lines). **FIG. 8B** shows exemplary relative mRNA level of Gpr68 in various populations of leukocytes isolated from the spleen of C57B/L6 mice. Results are presented as mean ± SEM from 3 trials. **FIG. 8C** shows representative images of antibody staining against GFP and CD31 in blood vessel of pancreas isolated from Gpr68 eGFP reporter mice. Scale bar: 25 µm. **FIG. 8D** shows representative images of antibody staining against GFP and CD31 in blood vessel of liver. **a**, artery; **b**, bile duct; **v**, portal vein. Scale bar: 25 µm. **FIG. 8E** shows representative images of antibody staining against GFP and CD31 in blood vessel of bladder. **a**, artery; **v**, vein. Scale bar: 50 µm. tissues from 4 mice were examined.

[0022] **FIGs. 9A-9D** show that Gpr68 is necessary for laminar flow-induced calcium transients in murine primary microvascular endothelial cells. **FIG. 9A** shows exemplary responses of mouse primary microvascular endothelial cells (MVECs) to shear stress imposed by pulsatile laminar flow with increasing amplitudes. The MVECs were isolated from cerebrum of the Gpr68 eGFP reporter mice. Pulsatile laminar flow stimulation of different amplitudes are applied at 1 Hz for 180s. n=148 for GFP+ and 155 for GFP-. Data is pooled from 3 trials. **FIG. 9B** shows exemplary responses of GFP+ and GFP- MVECs to pulsatile laminar shear stress at 4 Pa. The MVECs were infected with lentivirus containing non-targeting shRNA and Gpr68 shRNA constructs. They were cultured in endothelial growth medium with 2 µg/mL puromycin for 96 h before the shear stress test. The pulsatile flow is applied at 1 Hz for 180 s. n=166 for non-targeting and 173 for Gpr68 shRNA treated group. Data pooled from 3 trials. **FIG. 9C** shows exemplary calcium transients in primary MVECs induced by to 50 µM ATP. n=223 for non-targeting and 198 for Gpr68 shRNA treated group. 3 trials. **FIG. 9D** shows quantification of the knockdown efficiency of lentiviral shRNA against Gpr68. n=3. All results are plotted as mean ± SEM.

[0023] **FIGs. 10A-10F** show that Gpr68 is required for flow-mediated dilation and outward remodeling in third order mesenteric arteries. **FIG. 10A** shows exemplary flow-mediated dilation (FMD) response of third order mesenteric arteries (MAs) isolated from Gpr68 -/- mice and WT littermates. Vessels were cannulated and pre-constricted using 1µM phenylephrine, and subjected to stepwise increases in flow rates. Left, representative recordings of vessel diameter change. Scale bar: 10 minutes. Right, quantification of FMD response in 3rd order MAs. p=0.044, two way ANOVA, n=9 for Gpr68 KO and n=7 for WT. **FIG. 10B** shows exemplary dilation responses of third order MAs to Ogerin. Vessels were cannulated and pre-constricted

using 1 μM phenylephrine, and subjected to Ogerin with increasing concentration. Left, representative recordings of vessel diameter change. Scale bar: 10 minutes. Right, quantification of Ogerin-induced dilation 3rd order MAs. Vessels from KO and 6 WT mice were tested. P=0.018, two way ANOVA. Inset, structure of Ogerin. **FIG. 10C** shows a schematic representation of the surgery applied to mesenteric resistance arteries in vivo in order to increase blood flow locally. Second order arteries were ligated (marked by X) on 2 branches of the mesenteric arterial bed. The arteries located between the ligated branches were thus submitted to a chronic increase in blood flow. These arteries were designed as high flow (HF) arteries. Similar mesenteric arteries located at a distance were used as control or normal flow (NF) arteries. Arteries were collected 2 weeks after surgery in order to determine their diameter and thus the remodeling induced by the chronic increase in blood flow. Vessels were cannulated in an arteriograph and subjected to stepwise increases in pressure before diameter measurement. **FIGs. 10D-10E** shows exemplary flow-mediated remodeling (FMR) of 3rd order mesenteric arteries isolated from WT (P=0.0045) and Gpr68 KO mice (P=0.95). **FIG. 10F** shows the extent of outward remodeling as indicated by the percentage of vessel diameter increase (*P=0.0309). *p<0.05, two way ANOVA for repeated measures, n=5 for Gpr68 -/- and n=7 for WT. All results are plotted as mean ± SEM.

[0024] **FIGs. 11A-11D** show that Gpr68 -/- 1st and 2nd order mesenteric vessels have normal response to flow and vessels of all sizes have normal response to chemical stimulations. **FIGs. 11A-11B** show exemplary FMD in first and second order MAs from Gpr68 -/- mice and WT littermates. n=7 Gpr68 -/- and n=7 WT for 2nd order arteries, and n=4 Gpr68 -/- and n=5 WT for 1st order arteries. **FIG. 11C** shows exemplary FMD response in 3rd order MAs in the presence of 100 μM L-N^G-Nitroarginine methyl ester (L-NAME). Vessels from 6 KO and 6 WT mice were tested. **FIG. 11D** shows exemplary dilation response of various orders of MAs to 1 μM acetylcholine and 80 mM of KCl. For acetylcholine experiment, 3rd order MAs, n=5 for WT and n=8 for KO. 2nd order MAs, n=8 for WT and n=7 for KO. 1st order MAs, n=5 for WT and n=5 for KO. For KCl experiment, 3rd order MAs, n=8 for WT and n=11 for KO. 2nd order MAs, n=8 for WT and n=10 for KO. 1st order MAs, n=5 for WT and n=5 for KO. All results were plotted as mean ± SEM.

[0025] **FIG. 12** shows an exemplary dose response of Ogerin, which is a mouse Gpr68 agonist. HEK-293T cells were transiently transfected with mouse Gpr68. Relative fluorescence from calcium indicators were acquired for 10 s, then Ogerin was added to the cells in various concentrations, while measurement continued for 180 s at 1 data point per second. Maximum

fluorescence was plotted as mean ± SEM from 3 trials. Dose response curve fitting yielded an EC50 of 0.17 µM.

[0026] **FIGs. 13A-13D** show characterization of hemodynamic and cardiac parameters and assessment of mesenteric vessels of Gpr68 -/- mice. **FIG. 13A** shows exemplary echocardiogram assessment of Gpr68 -/- and WT heart. All the structural parameters were not significantly different between KO and WT mice. **FIG. 13B** shows exemplary blood pressure radiotelemetry recording of baseline hemodynamic parameters of Gpr68 -/- mice and WT littermates. Each dot represents one animal. **FIG. 13C** shows exemplary myogenic tone of MAs from various orders. 3rd order MAs, n=8 for WT and n=10 for KO. 2nd order MAs, n=7 for WT and n=7 for KO. 1st order MAs, n=3 for WT and n=5 for KO. **FIG. 13D** shows exemplary dilation response of MAs to SNP at various dosages. For 3rd order MAs, 5 WT and 7 KO vessels were examined. 2nd order MAs, n=4 for WT and n=6 for KO. 1st order MAs, n=4 for WT and n=5 for KO. All results were plotted as mean ± SEM.

[0027] **FIGs. 14A-14C** show exemplary assessment of flow-mediated outward remodeling of Gpr68 -/- mesenteric vessels. **FIGs. 14A-14B** show exemplary passive diameter distension curve at increasing pressures for WT and KO 1st order mesenteric arteries exposed to HF or NF. Both showed significant diameter increase in response to chronic HF (WT: P=0.017 and KO: P=0.0315). **FIG. 14C** shows the extent of remodeling of 1st order mesenteric vessels from WT and KO are similar. All results are plotted as mean ± SEM. *p<0.05, two way ANOVA for repeated measures, n=5 for Gpr68 KO and n=6 for WT littermates. n.s., not significant.

[0028] **FIGs. 15A-15B** show induction of GPR68 mRNA by TGFβ.

DETAILED DESCRIPTION

[0029] The present invention is based, in part, on the discovery that GPR68 is a necessary component for sheer stress response. GPR68 is expressed in the endothelial cells of resistance arteries (<100 µm), but not that of veins of any size. GPR68 mediates shear stress-induced calcium transients in primary microvascular endothelial cells and controls flow-mediated dilation (FMD) response and flow-mediated outward remodeling (FMR) of small-diameter mesenteric arteries.

[0030] Therefore, provided herein are modulators of a GPR68 gene product and compositions, methods and uses thereof for treating cardiovascular diseases and liver fibrosis.

DEFINITIONS

[0031] The term “small molecule” refers to compounds, preferably organic compounds, with a size comparable to those organic molecules generally used in pharmaceuticals. The term excludes biological macromolecules (e.g., proteins, nucleic acids, etc.). Preferred small organic molecules range in size up to about 5000 Da, e.g., up to about 4000, preferably up to 3000 Da, more preferably up to 2000 Da, even more preferably up to about 1000 Da, e.g., up to about 900, 800, 700, 600 or up to about 500 Da.

[0032] The term “inhibit,” as used herein, means to suppress an activity or function, for example, about ten percent relative to a control value. Preferably, the activity is suppressed y 50% compared to a control value, more preferably by 75%, and even more preferably by 95%. “Inhibit,” as used herein, also means to reduce the level of a molecule, a reaction, an interaction, a gene, an mRNA, and/or a protein's expression, stability, function or activity by a measurable amount or to prevent entirely. Inhibitors are compounds that, e.g., bind to, partially or totally inhibit activity, decrease, prevent, delay activation, inactivate, desensitize, or down regulate a protein, a gene, and an mRNA stability, expression, function and activity, e.g., antagonists.

[0033] The terms “modulator” and “modulation” of a molecule of interest, as used herein in its various forms, is intended to encompass antagonism, agonism, partial antagonism and/or partial agonism of an activity associated with a GPR68 gene product. In various embodiments, “modulators” may inhibit or stimulate GPR68 expression or activity. Such modulators include small molecules agonists and antagonists of GPR68, antisense molecules, ribozymes, triplex molecules, and RNAi polynucleotides, and others.

[0034] G protein coupled receptor 68 (GPR68, also known as Ovarian cancer G-protein coupled receptor 1 (OGR1), GPR12A, AI2A6) is a $G_{q/11}$-coupled receptor, and GPR68 activation may lead to the cleavage of PIP_2 into IP_3 and DAG by PLC, and induce calcium release from the store. In endothelial cells, activation of GPR68 may trigger several acute signaling pathways, such as activation of Ca^{2+}-gated channels (Orai1, $K_{Ca}2.3$, K_{Ca} 3.1) or synthesis of NO by NOS or release of EDHF. Activation of GPR68 may also activate KCNK channels and lead to the hyperpolarization of the cells. Activation of GPR68 may lead to hyperpolarization of smooth muscles surrounding the vessels, causing them to relax and therefore dilate the vessels. GPR69 mRNA was also found to be induced by pro-fibrotic stimuli (e.g. TGFβ) in primary hepatic stellate cells from rat and human. A “GPR68 gene product” may be an RNA, such as an mRNA, or a polypeptide, of a GPR68 gene. The human GPR68 protein is found in GENBANK™

Accession No: NP_001171147.1, and has the amino acid sequence set forth below (SEQ ID NO: 1):

1 MGNITADNSS MSCTIDHTIH QTLAPVVYVT VLVVGFPANC LSLYFGYLQI KARNELGVYL
61 CNLTVADLFY ICSLPFWLQY VLQHDNWSHG DLSCQVCGIL LYENIYISVG FLCCISVDRY
121 LAVAHPFRFH QFRTLKAAVG VSVVIWAKEL LTSIYFLMHE EVIEDENQHR VCFEHYPIQA
181 WQRAINYYRF LVGFLFPICL LLASYQGILR AVRRSHGTQK SRKDQIQRLV LSTVVIFLAC
241 FLPYHVLLLV RSVWEASCDF AKGVFNAYHF SLLLTSFNCV ADPVLYCFVS ETTHRDLARL
301 RGACLAFLTC SRTGRAREAY PLGAPEASGK SGAQGEEPEL LTKLHPAFQT PNSPGSGGFP
361 TGRLA

[0035] The mouse GPR68 protein is found in GENBANK™ Accession No: NP_780702.1, and has the amino acid sequence set forth below (SEQ ID NO: 2):

1 MGNITTENSS LSCPIDHTIH QTLAPVVYVT VLVVGFPANC LSLYFGYLQI KARNELGVYL
61 CNLTIADLFY ICSLPFWLQY VLQHDDWSHG DLSCQVCGIL LYENIYISVG FLCCISIDRY
121 LAVAHPFRFH QFRTLKAAVG VSVLIWAKEL LTSIYFLNHK EVIEDEDQHR VCFEHYPIQA
181 WQRSINYYRF LVGFLFPICL LLASYQGILR AVRRSHGTQK SRKDQIQRLV LSTVVIFLAC
241 FLPYHVLLLV RSLWERNCEF AKSIFNVYHF SLLLTSFNCV ADPVLYCFVS ETTHRDLARL
301 RGACLAVLTC SRTSRAREAY PLGAPEASGK SGAQGEEPEL LTKLHSAFQT PSSLGVGGPS
361 TVGLA

[0036] The rat GPR68 protein is found in GENBANK™ Accession No: NP_001101519.1, and has the amino acid sequence set forth below (SEQ ID NO: 3):

1 MRSKAPSGPK MGNITTENSS LPCPIDHTIH QTLAPVVYVT VLVVGFPANC LSLYFGYLQI
61 KARNELGVYL CNLTIADLFY ICSLPFWLQY VLQHDNWSHG DLSCQVCGIL LYENIYISVG
121 FLCCISIDRY LAVAHPFRFH QFRTLKAAVG VSVLIWAKEL LTSIYFLKHK EVIEDEDRHR
181 VCFEHYPIQA WQRGINYYRF LVGFLFPICL LLASYQGILR AVRRSHGTQK SRKDQIQRLV
241 LSTVVIFLAC FLPYHVLLLV RSLWESSCDF AKSIFNIYHF SLLLTSFNCV ADPVLYCFVS
301 ETTHRDLARL RGACLAFLTC SRTSRAREAY PLGAPEASGK SGAQGEEPEL LTKLHSAFQT
361 PNSLGLGGPP TVGLA

[0037] The cynomolgus monkey GPR68 protein is found in GENBANK™ Accession No: XP_005562082.1, and has the amino acid sequence set forth below (SEQ ID NO: 4):

1 MRSVAPSGPK MGNITADNSS MSCTIDHTIH QTLAPVVYVT VLVVGFPANC LSLYFGYLQI
61 KARNELGVYL CNLTVADLFY ICSLPFWLQY VLQHDNWSHG DLSCQVCGIL LYENIYISVG
121 FLCCISVDRY LAVAHPFRFH QFRTLKAAVG VSVVIWAKEL LTSIYFLMHE EVIEDEDQHR
181 VCFEHYPIQA WQRAINYYRF LVGFLFPICL LLASYQGILR AVRRSHGTQK SRKDQIQRLV
241 LSTVVIFLAC FLPYHVLLLV RSVWEASCDF AKGVFNAYHF SLLLTSFNCV ADPVLYCFVS

301 ETTHRDLARL RGACLAFLTC SRTGRAREAY PLGAPEASGK SGAQGEEPEL LTKLHPAFQT
361 PNSPGMVGSA TGGLA

[0038] The human GPR68 gene is found on chromosome 14 (14q32.11) in GENBANK™ Accession No: NG_052988.1.

[0039] The transcript variant 1 of human GPR68 mRNA is found in GENBANK™ Accession No: NM_001177676.1, and has the nucleotide sequence set forth below (SEQ ID NO: 5):

[0040] 1 gctcctcacc tggttgcaga gaccacactg ccttcctggg gccctgaggc tctaggagaa
[0041] 61 gctggaaagc acaggtgggg gtccatccag ctgtttccaa actgcgggtt gtgacccatt
[0042] 121 catgggtcag taaatcaact tcatagctca tgacctgcat ttaaaaaatt gaaatagaat
[0043] 181 agaaatgatc agagtgcgtg gcaagaagta ggggtcttgc ccaccaccag cgatgcccag
[0044] 241 cccttggtag agcttgaacc accttctata aacaggatgg cggtggagag acaggcccag
[0045] 301 tccctgagcc catgaggagt gtggcccctt caggcccaaa gatggggaac atcactgcag
[0046] 361 acaactcctc gatgagctgt accatcgacc ataccatcca ccagacgctg gccccggtgg
[0047] 421 tctatgttac cgtgctggtg gtgggcttcc cggccaactg cctgtccctc tacttcggct
[0048] 481 acctgcagat caaggcccgg aacgagctgg gcgtgtacct gtgcaacctg acggtggccg
[0049] 541 acctcttcta catctgctcg ctgcccttct ggctgcagta cgtgctgcag cacgacaact
[0050] 601 ggtctcacgg cgacctgtcc tgccaggtgt gcggcatcct cctgtacgag aacatctaca
[0051] 661 tcagcgtggg cttcctctgc tgcatctccg tggaccgcta cctggctgtg gcccatccct
[0052] 721 tccgcttcca ccagttccgg accctgaagg cggccgtcgg cgtcagcgtg gtcatctggg
[0053] 781 ccaaggagct gctgaccagc atctacttcc tgatgcacga ggaggtcatc gaggacgaga
[0054] 841 accagcaccg cgtgtgcttt gagcactacc ccatccaggc atggcagcgc gccatcaact
[0055] 901 actaccgctt cctggtgggc ttcctcttcc ccatctgcct gctgctggcg tcctaccagg
[0056] 961 gcatcctgcg cgccgtgcgc cggagccacg gcacccagaa gagccgcaag gaccagatcc
[0057] 1021 agcggctggt gctcagcacc gtggtcatct tcctggcctg cttcctgccc taccacgtgt
[0058] 1081 tgctgctggt gcgcagcgtc tgggaggcca gctgcgactt cgccaagggc gttttcaacg
[0059] 1141 cctaccactt ctccctcctg ctcaccagct tcaactgcgt cgccgacccc gtgctctact
[0060] 1201 gcttcgtcag cgagaccacc caccgggacc tggcccgcct ccgcggggcc tgcctggcct
[0061] 1261 tcctcacctg ctccaggacc ggccgggcca gggaggccta cccgctgggt gcccccgagg
[0062] 1321 cctccgggaa aagcggggcc cagggtgagg agcccgagct gttgaccaag ctccacccgg
[0063] 1381 ccttccagac ccctaactcg ccagggtcgg gcgggttccc cacgggcagg ttggcctagc
[0064] 1441 ctgggtcctc cgcgggtggc tccacgtgag gcctgagcct tcagcccacg ggcctcaggg
[0065] 1501 cctgccgcct cctgcttccc tcgctgcgga ggcagggaag cccctgtaac tccggaagcc
[0066] 1561 tgctctcgct tgctgagccc gctgggaccg ccgagggtgg gaataagccc cggttggctc

[0067] 1621 gtgggaataa gccgtgtcct ctgccgcggc tgcgatgtgg ccaggctggg gctgctggtc

[0068] 1681 gggggaagac agtgaactgc gttccctggc ctgcttcctg cagagtttgt gcatggggag

[0069] 1741 tgtgaggaca tggagggtgg gaggctgggc gttcagctgc cagggccttc caatgccacc

[0070] 1801 gttgtcacag acaatgcctg tccaaacgtc ccggtgggat cagcactgca gcccgcccac

[0071] 1861 aacagggtgg gaagggaaga ctggaggggg aaggaaggca ggaggggggaa ggaagaaaga

[0072] 1921 gggggaagga aagaaagagg ggggaaggaa aggaggaagg ggagaaggaa gggaggagga

[0073] 1981 aagaaggaag gtagggggtg ttgggttggg agaattgagg gagttatagg cagaggagga

[0074] 2041 tcctagctcc agccataaga actgggagag ccgcctgctg ctccagaaga cctgcccacc

[0075] 2101 tccacagaaa tgctagccct tcagaccttc tggtcagtga actgggacca gctctgacta

[0076] 2161 aggatgcctt ccatggcctg ggactcgagt tcttcagaac caagaggcta actgggcttc

[0077] 2221 ctccaagaag ggaccagggg ctagaagcag aagttggcat cagcaaacat ttctggaatc

[0078] 2281 taccaaaggg aggcccaggc agaacccctt cctcagtctc ccttcccctc ttccccccag

[0079] 2341 ccttgaagag gttgatcctt tgtgctgtgt ctcttagccc tttcatcggg gatgcccaga

[0080] 2401 ggcaacagcc caggctggag gtgccccagg gaagggtgct cacagtggtc ccagggctac

[0081] 2461 acttggcttt gactgcacag cccctgctca gatccttgtg gagggtgtcc gatgacttgg

[0082] 2521 ggaggttctc ttccccttcc tagaggagga agtgaccaag tttgaaggcg gcagaaatgg

[0083] 2581 ccacacagcc aagaagagcc catgcaactc agtccaggat tttactgggt cagtgacatt

[0084] 2641 ggtggaagcc ttcatgcctc ctcattccac agcacttcct cctatgttga ccttaaacac

[0085] 2701 tggcttccca ctgatacaga ctgggatgag ctgagggcag tctcatttac tgtcaagagt

[0086] 2761 tgtatttttg tattgtcctt tttgccaagt ctacatgtgt tgactctgta atggatttat

[0087] 2821 gtagcccact tcagtctgca aataaagcaa agtaactgga aaaaaaaaaa aaaaaaaaaa

[0088] The transcript variant 2 of human GPR68 mRNA is found in GENBANK™ Accession No: NM_003485.3, and has the nucleotide sequence set forth below (SEQ ID NO: 6):

1 tgcccgacgt gggtgggggg ggcgtggaat cggagcacaa cttggccctg cgttcccagg

61 aagggacccg aagacctccc caggccaccc cgccatctgc gtgcgcgctg gcaagaggag

121 gggcgcgggg taatggtggc cggcgccaca gccccgcgcc gcgcagggtc ttgcccacca

181 ccagcgatgc ccagcccttg gtagagcttg aaccaccttc tataaacagg atggcggtgg

241 agagacaggc ccagtccctg agcccatgag gagtgtggcc ccttcaggcc caaagatggg

301 gaacatcact gcagacaact cctcgatgag ctgtaccatc gaccatacca tccaccagac

361 gctggccccg gtggtctatg ttaccgtgct ggtggtgggc ttcccggcca actgcctgtc

421 cctctacttc ggctacctgc agatcaaggc ccggaacgag ctgggcgtgt acctgtgcaa

481 cctgacggtg gccgacctct tctacatctg ctcgctgccc ttctggctgc agtacgtgct

541 gcagcacgac aactggtctc acggcgacct gtcctgccag gtgtgcggca tcctcctgta
601 cgagaacatc tacatcagcg tgggcttcct ctgctgcatc tccgtggacc gctacctggc
661 tgtggcccat cccttccgct tccaccagtt ccggaccctg aaggcggccg tcggcgtcag
721 cgtggtcatc tgggccaagg agctgctgac cagcatctac ttcctgatgc acgaggaggt
781 catcgaggac gagaaccagc accgcgtgtg ctttgagcac taccccatcc aggcatggca
841 gcgcgccatc aactactacc gcttcctggt gggcttcctc ttccccatct gcctgctgct
901 ggcgtcctac cagggcatcc tgcgcgccgt gcgccggagc cacggcaccc agaagagccg
961 caaggaccag atccagcggc tggtgctcag caccgtggtc atcttcctgg cctgcttcct
1021 gccctaccac gtgttgctgc tggtgcgcag cgtctgggag gccagctgcg acttcgccaa
1081 gggcgttttc aacgcctacc acttctccct cctgctcacc agcttcaact gcgtcgccga
1141 ccccgtgctc tactgcttcg tcagcgagac cacccaccgg gacctggccc gcctccgcgg
1201 ggcctgcctg gccttcctca cctgctccag gaccggccgg gccagggagg cctacccgct
1261 gggtgccccc gaggcctccg ggaaaagcgg ggcccagggt gaggagcccg agctgttgac
1321 caagctccac ccggccttcc agacccctaa ctcgccaggg tcgggcgggt tccccacggg
1381 caggttggcc tagcctgggt cctccgcggg tggctccacg tgaggcctga gccttcagcc
1441 cacgggcctc agggcctgcc gcctcctgct tccctcgctg cggaggcagg gaagcccctg
1501 taactccgga agcctgctct cgcttgctga gcccgctggg accgccgagg gtgggaataa
1561 gccccggttg gctcgtggga ataagccgtg tcctctgccg cggctgcgat gtggccaggc
1621 tggggctgct ggtcggggga agacagtgaa ctgcgttccc tggcctgctt cctgcagagt
1681 ttgtgcatgg ggagtgtgag gacatggagg gtgggaggct gggcgttcag ctgccagggc
1741 cttccaatgc caccgttgtc acagacaatg cctgtccaaa cgtcccggtg ggatcagcac
1801 tgcagcccgc ccacaacagg gtgggaaggg aagactggag ggggaaggaa ggcaggaggg
1861 ggaaggaaga aagaggggga aggaaagaaa gagggggaa ggaaaggagg aaggggagaa
1921 ggaagggagg aggaaagaag gaaggtaggg ggtgttgggt tgggagaatt gagggagtta
1981 taggcagagg aggatcctag ctccagccat aagaactggg agagccgcct gctgctccag
2041 aagacctgcc cacctccaca gaaatgctag cccttcagac cttctggtca gtgaactggg
2101 accagctctg actaaggatg ccttccatgg cctgggactc gagttcttca gaaccaagag
2161 gctaactggg cttcctccaa gaagggacca ggggctagaa gcagaagttg gcatcagcaa
2221 acatttctgg aatctaccaa agggaggccc aggcagaacc ccttcctcag tctcccttcc
2281 cctcttcccc ccagccttga agaggttgat cctttgtgct gtgtctctta gcccttcat
2341 cggggatgcc cagaggcaac agcccaggct ggaggtgccc cagggaaggg tgctcacagt
2401 ggtcccaggg ctacacttgg ctttgactgc acagcccctg ctcagatcct tgtggagggt
2461 gtccgatgac ttggggaggt tctcttcccc ttcctagagg aggaagtgac caagtttgaa
2521 ggcggcagaa atggccacac agccaagaag agcccatgca actcagtcca ggattttact

2581 gggtcagtga cattggtgga agccttcatg cctcctcatt ccacagcact tcctcctatg
2641 ttgaccttaa acactggctt cccactgata cagactggga tgagctgagg gcagtctcat
2701 ttactgtcaa gagttgtatt tttgtattgt ccttttgcc aagtctacat gtgttgactc
2761 tgtaatggat ttatgtagcc cacttcagtc tgcaaataaa gcaaagtaac tggaaaaaaa
2821 aaaaaaaaaa aaaa

[0089] The transcript variant 3 of human GPR68 mRNA is found in GENBANK™ Accession No: NM_001348437.1, and has the nucleotide sequence set forth below (SEQ ID NO: 7):

1 tgcccgacgt gggtgggggg ggcgtggaat cggagcacaa cttggccctg cgttcccagg
61 aagggacccg aagacctccc caggccaccc cgccatctgc gtgcgcgctg gcaagaggag
121 gggcgcgggg taatggtggc cggcgccaca gccccgcgcc gcgcagaagc aactccctcc
181 accaccacca ctggaattct aggccagtgc cctgaagagg atggaagagg tgagtcagag
241 actgggagtc cctgttttct gggaactgag aggttgagag ctgagtctca ggcagagact
301 gtacgtggca gggatgtgga gcaaggagac ctggattcta gcagcaacgc ttccaacctc
361 aggctggctt cagggtcttg cccaccacca gcgatgccca gcccttggta gagcttgaac
421 caccttctat aaacaggatg gcggtggaga gacaggccca gtccctgagc ccatgaggag
481 tgtggcccct tcaggcccaa agatggggaa catcactgca gacaactcct cgatgagctg
541 taccatcgac cataccatcc accagacgct ggccccggtg gtctatgtta ccgtgctggt
601 ggtgggcttc ccggccaact gcctgtccct ctacttcggc tacctgcaga tcaaggcccg
661 gaacgagctg ggcgtgtacc tgtgcaacct gacggtggcc gacctcttct acatctgctc
721 gctgcccttc tggctgcagt acgtgctgca gcacgacaac tggtctcacg gcgacctgtc
781 ctgccaggtg tgcggcatcc tcctgtacga gaacatctac atcagcgtgg gcttcctctg
841 ctgcatctcc gtggaccgct acctggctgt ggcccatccc ttccgcttcc accagttccg
901 gaccctgaag gcggccgtcg gcgtcagcgt ggtcatctgg gccaaggagc tgctgaccag
961 catctacttc ctgatgcacg aggaggtcat cgaggacgag aaccagcacc gcgtgtgctt
1021 tgagcactac cccatccagg catggcagcg cgccatcaac tactaccgct tcctggtggg
1081 cttcctcttc cccatctgcc tgctgctggc gtcctaccag ggcatcctgc gcgccgtgcg
1141 ccggagccac ggcacccaga agagccgcaa ggaccagatc cagcggctgg tgctcagcac
1201 cgtggtcatc ttcctggcct gcttcctgcc ctaccacgtg ttgctgctgg tgcgcagcgt
1261 ctgggaggcc agctgcgact tcgccaaggg cgttttcaac gcctaccact tctccctcct
1321 gctcaccagc ttcaactgcg tcgccgaccc cgtgctctac tgcttcgtca gcgagaccac
1381 ccaccgggac ctggcccgcc tccgcggggc ctgcctggcc ttcctcacct gctccaggac
1441 cggccgggcc agggaggcct acccgctggg tgcccccgag gcctccggga aaagcggggc
1501 ccagggtgag gagcccgagc tgttgaccaa gctccacccg gccttccaga cccctaactc

1561 gccagggtcg ggcgggttcc ccacgggcag gttggcctag cctgggtcct ccgcgggtgg
1621 ctccacgtga ggcctgagcc ttcagcccac gggcctcagg gcctgccgcc tcctgcttcc
1681 ctcgctgcgg aggcagggaa gcccctgtaa ctccggaagc ctgctctcgc ttgctgagcc
1741 cgctgggacc gccgagggtg ggaataagcc ccggttggct cgtgggaata agccgtgtcc
1801 tctgccgcgg ctgcgatgtg gccaggctgg ggctgctggt cgggggaaga cagtgaactg
1861 cgttccctgg cctgcttcct gcagagtttg tgcatgggga gtgtgaggac atggagggtg
1921 ggaggctggg cgttcagctg ccagggcctt ccaatgccac cgttgtcaca gacaatgcct
1981 gtccaaacgt cccggtggga tcagcactgc agcccgccca caacagggtg ggaagggaag
2041 actggagggg gaaggaaggc aggaggggga aggaagaaag agggggaagg aaagaaagag
2101 gggggaagga aaggaggaag gggagaagga agggaggagg aaagaaggaa ggtagggggt
2161 gttgggttgg gagaattgag ggagttatag gcagaggagg atcctagctc cagccataag
2221 aactgggaga gccgcctgct gctccagaag acctgcccac ctccacagaa atgctagccc
2281 ttcagacctt ctggtcagtg aactgggacc agctctgact aaggatgcct tccatggcct
2341 gggactcgag ttcttcagaa ccaagaggct aactgggctt cctccaagaa gggaccaggg
2401 gctagaagca gaagttggca tcagcaaaca tttctggaat ctaccaaagg gaggcccagg
2461 cagaacccct tcctcagtct cccttcccct cttcccccca gccttgaaga ggttgatcct
2521 ttgtgctgtg tctcttagcc ctttcatcgg ggatgcccag aggcaacagc ccaggctgga
2581 ggtgccccag ggaagggtgc tcacagtggt cccagggcta cacttggctt tgactgcaca
2641 gcccctgctc agatccttgt ggagggtgtc cgatgacttg gggaggttct cttccccttc
2701 ctagaggagg aagtgaccaa gtttgaaggc ggcagaaatg gccacacagc caagaagagc
2761 ccatgcaact cagtccagga ttttactggg tcagtgacat tggtggaagc cttcatgcct
2821 cctcattcca cagcacttcc tcctatgttg accttaaaca ctggcttccc actgatacag
2881 actgggatga gctgagggca gtctcattta ctgtcaagag ttgtattttt gtattgtcct
2941 ttttgccaag tctacatgtg ttgactctgt aatggattta tgtagcccac ttcagtctgc
3001 aaataaagca aagtaactgg a

[0090] The transcript variant 1 of mouse GPR68 mRNA is found in GENBANK™ Accession No: NM_175493.4, and has the nucleotide sequence set forth below (SEQ ID NO: 8):

1 actcaggtcc ggtcggtgcg ggtggatgcc atgcctccat cggacctgcg ccacgccctc
61 gccttgcgag ctgcgtgcct tccgtgccca ccagagagag gattgagaga gggagagctc
121 gtccgcgtgc agcgttgtcc agacactgcc aagttgagct gtaggagtac agctggcgga
181 ggactgaggg taagagccta gccggaaggg ctgaatcaga ggactcttgc taggttctct
241 cgcagagaac tctcagggct gcaggggcg gacctcactg ctgcctcccc atttcctcct
301 caactgcctg tggactcctc actgccttcc tttgccctac caggaggctc tagaaaaagc
361 caatcagtgt gggtatgggg ctggatagtt tccaaattga cctacctgtg ggacagaaat

421 caatttaatt gatcctgttc ttaaaagaaa tgaaatagaa gagacaacac cagtgtgaaa
481 aacaagcatc agggggtccg cctaccacca gtgatgccta gatcctgata catttgcatc
541 accttctata aacaagatgg caagagaggg attggctcca tctccaagcc catgaggaat
601 aaggctcctt ctggcccaaa gatggggaac atcactacag aaaactcctc actatcttgc
661 cccatcgacc acaccatcca ccagacacta gccccagtgg tctatgtgac cgtgctggtg
721 gtgggcttcc cagccaactg cctgtccctc tacttcgggt acttgcagat caaggcccgg
781 aatgagctgg gagtgtacct gtgtaacctg accattgcag acctgttcta tatctgttca
841 cttcccttct ggctgcagta cgtgcttcag cacgacgact ggtcccatgg tgacctatcc
901 tgccaggtgt gtggcatcct cctctatgag aacatttaca tcagcgtggg cttcctctgc
961 tgcatctcca tcgaccgcta cctggctgtg gcccaccct tccgcttcca ccagttccgc
1021 accctgaagg cagccgtggg tgtcagtgtg ctcatctggg ccaaggagct gctgaccagc
1081 atctacttcc tcaatcacaa ggaggtcatt gaggacgagg accagcaccg agtctgcttt
1141 gagcattacc ctatccaggc ctggcagcgt agcatcaact actaccgctt cctggtgggc
1201 tttctcttcc ccatctgcct gctgctggcc tcctaccagg gcatcctgcg ggctgtgcgc
1261 cgcagccacg gcacacagaa gagccgcaag gaccagattc agcggctggt gctcagcacc
1321 gtggtcatct tcctggcttg ctttctaccc taccacgtgc tgctgctggt acgcagcctc
1381 tgggagagaa actgtgagtt tgccaagagc atcttcaacg tctatcactt ctccctcctc
1441 ctcaccagct tcaactgtgt agctgacccg gtgctgtact gctttgtcag tgagaccact
1501 cacagggacc tagcccgcct ccgaggagcc tgcctagctg tccttacctg ctctaggaca
1561 agcagggcca gggaggccta ccctctgggt gcccctgagg cctctgggaa aagtggggcc
1621 cagggcgagg aacctgaatt gttaaccaag ctccactcag ccttccagac ccctagctca
1681 ctgggagtgg gagggccctc cacagtgggg ttggcctagc ttgagtcacg tgtatgaggc
1741 taagttaggc cctgagcttt caccaacagg ccttgtggtc gggagcttgc atggtggctg
1801 tctcccactt taccatgtac gggtgcctct ctcctcagga aggaaccatg gtttgggcca
1861 cagccaggtc cctctggccc ctggaaacct gctccgtgtc ctgagcctgg gcactgctga
1921 ctgtgtgaat gagccctgtc tcctcccatg ttcttggtgg ccgcctgggc tgcagatggg
1981 agggagtcgg catcgtcact tccaggagat ttgcaaagag cagcaggcct ggggtgcgga
2041 gaggagggta ggaccctgcg tggtcacctg ccaagccttc cagtgcccct gttgtcacag
2101 acgataccag cccaagtgtg ctatggtctt gtgtgacaca ggaagctata tagccaccat
2161 cagtatttga gcttgcctct aaactagtct aggccagagg actggttaag gtgaagaaca
2221 ggggcggggt tggtggctag ggataacagc agagaggcag tcagggaaga aaccccagca
2281 tcaggcacgt ctcactttac agaacttatg gaacagggcc ccacccccaa gggctgatgg
2341 caaagaaacc ccagggatgc cggagagatg tcaggggccc tcaaaacatc gtactaaagc
2401 ttaataagaa gacttaggcc taaaactgcg tccttagcca agttcctggc acacagggtt

2461 tctctgtgta gccctggctg tcttggaact cactctgtag accaggctgg cttcgaactc
2521 agaaatccgc ctgcctctgc ctcccaagtg ttaggattaa aggcgtgcgc caccactgcc
2581 cagccctaat tagctttttg tgtccgtcta gtctaagtgt cagacctgag agcctaaggg
2641 agaagaaaga gggagaatat aaaaaaaatg gcagggtgtc catggcaact gtatgccaag
2701 acattgatac taactaggat catgtatctg ttgtcagtaa ttcctaccct tctccccatg
2761 caccatagag catccagcag cttttgagag gagtgtgaca gtgacagttt cccagagttc
2821 ctaagtgcaa tgaactaacc acacacacac acacacacac acacacacac aatcatgtct
2881 cgtgaccaca ggtttggctt aagctgcatt ttggtatagg agagttggca gagatggctg
2941 catgccagaa agacctacac cttccccagc ctcggtcctt acaggccaat ggtgatagct
3001 aagtgttctg tgcctcctca tcccacagta tttcccctgt ttgacctgaa attctggatt
3061 ctcactggct tggactggag caagctaagc ccagctcccc ttactaagga gtgctgtatt
3121 tttgtattgt tttctctctt atgccaagta tccctgtgtt ggactgtgag gtgggtttat
3181 gtagtgactt cagtcagcaa ataaaacaaa gtagccaatc ataaaaaaaa aaaaaaaaaa
3241 a

[0091] The transcript variant 2 of mouse GPR68 mRNA is found in GENBANK™ Accession No: NM_001177673.1, and has the nucleotide sequence set forth below (SEQ ID NO: 9):

1 actcaggtcc ggtcggtgcg ggtggatgcc atgcctccat cggacctgcg ccacgccctc
61 gccttgcgag ctgcgtgcct tccgtgccca ccagagagag gattgagaga gggagagctc
121 gtccgcgtgc agcgttgtcc agacactgcc aagttgagct gtaggagtac agctggcgga
181 ggactgaggg gggtccgcct accaccagtg atgcctagat cctgatacat ttgcatcacc
241 ttctataaac aagatggcaa gagagggatt ggctccatct ccaagcccat gaggaataag
301 gctccttctg gcccaaagat ggggaacatc actacagaaa actcctcact atcttgcccc
361 atcgaccaca ccatccacca gacactagcc ccagtggtct atgtgaccgt gctggtggtg
421 ggcttcccag ccaactgcct gtccctctac ttcgggtact tgcagatcaa ggcccggaat
481 gagctgggag tgtacctgtg taacctgacc attgcagacc tgttctatat ctgttcactt
541 cccttctggc tgcagtacgt gcttcagcac gacgactggt cccatggtga cctatcctgc
601 caggtgtgtg gcatcctcct ctatgagaac atttacatca gcgtgggctt cctctgctgc
661 atctccatcg accgctacct ggctgtggcc cacccttcc gcttccacca gttccgcacc
721 ctgaaggcag ccgtgggtgt cagtgtgctc atctgggcca aggagctgct gaccagcatc
781 tacttcctca atcacaagga ggtcattgag gacgaggacc agcaccgagt ctgctttgag
841 cattacccta tccaggcctg gcagcgtagc atcaactact accgcttcct ggtgggcttt
901 ctcttcccca tctgcctgct gctggcctcc taccagggca tcctgcgggc tgtgcgccgc
961 agccacggca cacagaagag ccgcaaggac cagattcagc ggctggtgct cagcaccgtg

1021 gtcatcttcc tggcttgctt tctaccctac cacgtgctgc tgctggtacg cagcctctgg
1081 gagagaaact gtgagtttgc caagagcatc ttcaacgtct atcacttctc cctcctcctc
1141 accagcttca actgtgtagc tgacccggtg ctgtactgct ttgtcagtga gaccactcac
1201 agggacctag cccgcctccg aggagcctgc ctagctgtcc ttacctgctc taggacaagc
1261 agggccaggg aggcctaccc tctgggtgcc cctgaggcct ctgggaaaag tggggcccag
1321 ggcgaggaac ctgaattgtt aaccaagctc cactcagcct tccagacccc tagctcactg
1381 ggagtgggag ggccctccac agtggggttg gcctagcttg agtcacgtgt atgaggctaa
1441 gttaggccct gagctttcac caacaggcct tgtggtcggg agcttgcatg gtggctgtct
1501 cccactttac catgtacggg tgcctctctc ctcaggaagg aaccatggtt tgggccacag
1561 ccaggtccct ctggcccctg gaaacctgct ccgtgtcctg agcctgggca ctgctgactg
1621 tgtgaatgag ccctgtctcc tcccatgttc ttggtggccg cctgggctgc agatgggagg
1681 gagtcggcat cgtcacttcc aggagatttg caaagagcag caggcctggg gtgcggagag
1741 gagggtagga ccctgcgtgg tcacctgcca agccttccag tgcccctgtt gtcacagacg
1801 ataccagccc aagtgtgcta tggtcttgtg tgacacagga agctatatag ccaccatcag
1861 tatttgagct tgcctctaaa ctagtctagg ccagaggact ggttaaggtg aagaacaggg
1921 gcggggttgg tggctaggga taacagcaga gaggcagtca gggaagaaac cccagcatca
1981 ggcacgtctc actttacaga acttatggaa cagggcccca cccccaaggg ctgatggcaa
2041 agaaacccca gggatgccgg agagatgtca ggggccctca aaacatcgta ctaaagctta
2101 ataagaagac ttaggcctaa aactgcgtcc ttagccaagt tcctggcaca cagggtttct
2161 ctgtgtagcc ctggctgtct tggaactcac tctgtagacc aggctggctt cgaactcaga
2221 aatccgcctg cctctgcctc ccaagtgtta ggattaaagg cgtgcgccac cactgcccag
2281 ccctaattag ctttttgtgt ccgtctagtc taagtgtcag acctgagagc ctaagggaga
2341 agaaagaggg agaatataaa aaaaatggca gggtgtccat ggcaactgta tgccaagaca
2401 ttgatactaa ctaggatcat gtatctgttg tcagtaattc ctacccttct ccccatgcac
2461 catagagcat ccagcagctt ttgagaggag tgtgacagtg acagtttccc agagttccta
2521 agtgcaatga actaaccaca cacacacaca cacacacaca cacacacaat catgtctcgt
2581 gaccacaggt ttggcttaag ctgcattttg gtataggaga gttggcagag atggctgcat
2641 gccagaaaga cctacacctt ccccagcctc ggtccttaca ggccaatggt gatagctaag
2701 tgttctgtgc ctcctcatcc cacagtattt cccctgtttg acctgaaatt ctggattctc
2761 actggcttgg actggagcaa gctaagccca gctcccctta ctaaggagtg ctgtatttttt
2821 gtattgtttt ctctcttatg ccaagtatcc ctgtgttgga ctgtgaggtg ggtttatgta
2881 gtgacttcag tcagcaaata aaacaaagta gccaatcata aaaaaaaaaa aaaaaaaa

[0092] The transcript variant 3 of mouse GPR68 mRNA is found in GENBANK™ Accession No: NM_001177674.1, and has the nucleotide sequence set forth below (SEQ ID NO: 10):

1 gggggtgctg cacctccggg accggcgagc gctcaggcca cctcggccct tctagcaggc
61 gcctcatctg tggcttgcgg tcagtgtgag ctgcagggcg tggaccgaca ggtctggctg
121 tgcctgacta gggttgggag cgggaggaat ctcgcacaac gtggcccaga gtggctagga
181 gaaagggacc tgcgtgcctt gcaggagtcc ccagcctttc caggaggcgc acgcactgca
241 ccagaggaca tcaggcccgc gtcccagact gtcgctgcac aggattgaga gagggagagc
301 tcgtccgcgt gcagcgttgt ccagacactg ccaagttgag ctgtaggagt acagctggcg
361 gaggactgag gggggtccgc ctaccaccag tgatgcctag atcctgatac atttgcatca
421 ccttctataa acaagatggc aagagaggga ttggctccat ctccaagccc atgaggaata
481 aggctccttc tggcccaaag atggggaaca tcactacaga aaactcctca ctatcttgcc
541 ccatcgacca caccatccac cagacactag ccccagtggt ctatgtgacc gtgctggtgg
601 tgggcttccc agccaactgc ctgtccctct acttcgggta cttgcagatc aaggcccgga
661 atgagctggg agtgtacctg tgtaacctga ccattgcaga cctgttctat atctgttcac
721 ttcccttctg gctgcagtac gtgcttcagc acgacgactg gtcccatggt gacctatcct
781 gccaggtgtg tggcatcctc ctctatgaga acatttacat cagcgtgggc ttcctctgct
841 gcatctccat cgaccgctac ctggctgtgg cccacccctt ccgcttccac cagttccgca
901 ccctgaaggc agccgtgggt gtcagtgtgc tcatctgggc caaggagctg ctgaccagca
961 tctacttcct caatcacaag gaggtcattg aggacgagga ccagcaccga gtctgctttg
1021 agcattaccc tatccaggcc tggcagcgta gcatcaacta ctaccgcttc ctggtgggct
1081 ttctcttccc catctgcctg ctgctggcct cctaccaggg catcctgcgg gctgtgcgcc
1141 gcagccacgg cacacagaag agccgcaagg accagattca gcggctggtg ctcagcaccg
1201 tggtcatctt cctggcttgc tttctaccct accacgtgct gctgctggta cgcagcctct
1261 gggagagaaa ctgtgagttt gccaagagca tcttcaacgt ctatcacttc tccctcctcc
1321 tcaccagctt caactgtgta gctgacccgg tgctgtactg ctttgtcagt gagaccactc
1381 acagggacct agcccgcctc cgaggagcct gcctagctgt ccttacctgc tctaggacaa
1441 gcagggccag ggaggcctac cctctgggtg cccctgaggc ctctgggaaa agtggggccc
1501 agggcgagga acctgaattg ttaaccaagc tccactcagc cttccagacc cctagctcac
1561 tgggagtggg agggccctcc acagtggggt tggcctagct tgagtcacgt gtatgaggct
1621 aagttaggcc ctgagctttc accaacaggc cttgtggtcg ggagcttgca tggtggctgt
1681 ctcccacttt accatgtacg ggtgcctctc tcctcaggaa ggaaccatgg tttgggccac
1741 agccaggtcc ctctggcccc tggaaacctg ctccgtgtcc tgagcctggg cactgctgac
1801 tgtgtgaatg agccctgtct cctcccatgt tcttggtggc cgcctgggct gcagatggga

1861 gggagtcggc atcgtcactt ccaggagatt tgcaaagagc agcaggcctg gggtgcggag
1921 aggagggtag gaccctgcgt ggtcacctgc caagccttcc agtgcccctg ttgtcacaga
1981 cgataccagc ccaagtgtgc tatggtcttg tgtgacacag gaagctatat agccaccatc
2041 agtatttgag cttgcctcta aactagtcta ggccagagga ctggttaagg tgaagaacag
2101 gggcggggtt ggtggctagg gataacagca gagaggcagt cagggaagaa accccagcat
2161 caggcacgtc tcactttaca gaacttatgg aacagggccc caccccaag ggctgatggc
2221 aaagaaaccc cagggatgcc ggagagatgt caggggccct caaaacatcg tactaaagct
2281 taataagaag acttaggcct aaaactgcgt ccttagccaa gttcctggca cacagggttt
2341 ctctgtgtag ccctggctgt cttggaactc actctgtaga ccaggctggc ttcgaactca
2401 gaaatccgcc tgcctctgcc tcccaagtgt taggattaaa ggcgtgcgcc accactgccc
2461 agccctaatt agctttttgt gtccgtctag tctaagtgtc agacctgaga gcctaaggga
2521 gaagaaagag ggagaatata aaaaaaatgg cagggtgtcc atggcaactg tatgccaaga
2581 cattgatact aactaggatc atgtatctgt tgtcagtaat tcctaccctt ctccccatgc
2641 accatagagc atccagcagc ttttgagagg agtgtgacag tgacagtttc ccagagttcc
2701 taagtgcaat gaactaacca cacacacaca cacacacaca cacacacaca atcatgtctc
2761 gtgaccacag gtttggctta agctgcattt tggtatagga gagttggcag agatggctgc
2821 atgccagaaa gacctacacc ttccccagcc tcggtcctta caggccaatg gtgatagcta
2881 agtgttctgt gcctcctcat cccacagtat ttcccctgtt tgacctgaaa ttctggattc
2941 tcactggctt ggactggagc aagctaagcc cagctcccct tactaaggag tgctgtattt
3001 ttgtattgtt ttctctctta tgccaagtat ccctgtgttg gactgtgagg tgggtttatg
3061 tagtgacttc agtcagcaaa taaaacaaag tagccaatca taaaaaaaaa aaaaaaaaaa

[0093] The rat GPR68 mRNA is found in GENBANK™ Accession No: NM_001108049.1, and has the nucleotide sequence set forth below (SEQ ID NO: 11):

1 aggtggatgc caagcctcca tcatacctgt gccacgccct cgccttgcga gctgcgtgcc
61 ttccgtgtcc accagagaga ggagtcccca gcctctcctg gccgctcacg caccgcaccg
121 gaggacatta ggccgtcgtc ccagactgtc gcggcgcagg aattgagaga gggagagctt
181 gtctgtgtgc agggtcgtcc aggcactgtc aagttgagct gtaggagtcc agggctggag
241 gtttgagggt agagcctggc tggatgggtt gaaccggagt cgactctttc taggttctcc
301 cacagagaac tctcagggct gctgagggtg gacctcactg ctggttcccc atttcctcct
361 caactggctg tggactcctc gctgccttcc tttgccttcc caggaggctc tagaagaagc
421 caatcggtgt aggtatgggg ctggatagtt tccaaactga cccacttgcg ggacagaaat
481 caatttaata gcttaagacc tgttttcaaa agaaatgaga tagaagagac aatgccagca
541 tgaaaaacaa gcagcagggg tccacctacc accgatgatg tctagatcct gatacatttg
601 caccaccttc tataaacaag atggcaagag agagattggc ttcatctcca agcccatgag

661 gagtaaggcc ccttctggcc caaagatggg gaacatcact acagaaaact cctcactacc
721 ttgccccatt gaccacacca tccaccagac actggcccca gtggtctatg tgaccgtgct
781 ggtggtgggc ttcccggcca actgcctatc cctctacttt gggtacttgc agatcaaggc
841 ccgaaatgag ctgggagtgt acctgtgtaa cctgaccatt gcagacctat tctatatctg
901 ttcgctcccc ttctggctgc agtacgtact ccagcatgac aactggtccc acggtgacct
961 atcctgccag gtgtgtggca tcctcctcta tgagaacatt tacatcagcg tgggcttcct
1021 ctgctgtatc tccatcgacc gctacctggc tgtggcccac cccttccgct tccaccagtt
1081 ccgcaccctg aaggcagccg tgggtgtcag tgtgctcatc tgggccaagg agctgctgac
1141 cagcatctac ttcctcaagc acaaggaggt catcgaagac gaggaccggc accgagtctg
1201 ctttgagcat taccctatcc aggcgtggca gcgtggcatc aactactacc gtttcctggt
1261 gggctttctc tttcccatct gcctgctgct ggcctcctac cagggcatcc tgcgggctgt
1321 gcgccgcagc catggcaccc agaagagccg caaggaccag attcagcggc tggtgctcag
1381 caccgtggtc atcttcctgg cttgcttcct gccctaccac gtgctgctgc tggtgcgcag
1441 cctctgggag agcagctgtg actttgccaa gagcatcttc aacatctatc acttctccct
1501 cctcctcacc agcttcaact gtgtagctga cccggtgctg tactgctttg tcagtgagac
1561 cactcacagg gacctagccc gcctccgagg agcctgccta gccttcctta cctgctctag
1621 gacaagcagg gccagggagg cctaccctct gggtgcccct gaggcctctg ggaaaagtgg
1681 ggcccagggc gaggagcctg aattgttaac caagctccac tcagccttcc agacccctaa
1741 ctcactggga ttgggagggc cccccacagt tggcttggcc tagcttgagt catatgtgtg
1801 tgagcttcca ccgacaggcc ttgtggtctg gagcttgcat ggtggctgtc tcccattttg
1861 tcatgaatag gtgcctcgct cttcaggaag gaaccatggt cttgggcccc agccaggtca
1921 ctctggccca ctggaagttt gctctgtgtg ctgaacctgg gcactgctga ctgcatgagt
1981 gagccctgtc tccctccatg ttctctgcag ctgcctgggc tacagatggg tgggagtcag
2041 aattgtcact tccaggaggt ttgcaaagag cagcgggcct gggggtgtgg agaggagtgt
2101 agggacctgg gtgctcgttc acctgcaagc tttccctgtt gtcacagatg ataccagccc
2161 aaatatgctg tggtcttgtg tgacatagga agctgtgttg tgaccatcag tactgagctc
2221 gcctctaaac tagtgtggac cagaagactg gttaaggtga agactagggc ggggttgggg
2281 caccgatatt ggtgacccag ggataagagc agagaggcag tcagggaaga agccccagcg
2341 ccagacacat ctcactttac agggcttatg gaacagattc cccaacccct aagggctgat
2401 gggaaaaaat ccccagggct cctcggagag atgccagggg ccctttaaga tgccatacta
2461 aagcttaata aaaaggctta ggcctgaagc tgtgtcccta gccaaattcc tagcacaggc
2521 ttttcctaat tagcttttt tgtgtgtcca tctggccaaa gtgcgggacc cgagagcctg
2581 agggaaaaga gagagggaga atatgaaaat gacagagtgt ccaggacaac tgtgtgctaa
2641 gacactgatg ccagggtcat gtgactgctg ccagtaaccc ctacccttct ccacatgcac

2701 cttcaagagg agtaaacagt ttcccagaat tcctagttcc aataaactaa caacacacat
2761 acacatgact gcgtctcttg actgcaggtt tggctcaagc tccattttgg tgtaggagag
2821 ttggcaggaa taactgcatg ccagagagac ctacacctac cccagcctag atccgtgtgg
2881 ggcaatggta atagctaagt gttctgtacc tctttgtccc acagtatttc cccgtttgac
2941 ctgaaattct ggattcgcac tggcttggag tggatcaagc taagcccagc tccccttacc
3001 aaggagagct gtatttttgt gttgtcttct ctcttacgcc aagtatccct gtgttgcact
3061 gtgagatggg tttatgtagt gacttcaatc tgccaataaa acaaagtaac caatc

[0094] The transcript variant X1 of cynomolgus monkey GPR68 mRNA is found in GENBANK™ Accession No: XM_005562026.2, and has the nucleotide sequence set forth below (SEQ ID NO: 12):

1 tgcgcctggc tggaaatagt ttttaaaagg cttgctatgt ggtagcattt gcttggaacg
61 cccaggctcc actgaagggg atttccttag acccccctacc gcagagcaag gggagtggag
121 ccaaggctct tggcatgaat aagatgggca tgttttaaac tgtgttagtc cccagatcag
181 ggacctagca cagggctgtg gccgcttccg gcaggaagga ctcgcgcccc cagtggcccc
241 atgcaccgtg tgtctgttgg cttcctggct ctggcattgt agcttataat tagcttccaa
301 ggagcctgcc ttggagggag gtggttacgc caaaatgctg ggcctttcca tcctctgtgt
361 tatgtgtgtg agggagttta gtaagtgctc ctgagagcca gaaggccccc cgcggccaca
421 gccccgggct tggttcctgc tcaggggact gacacagttg caaacattga tgcccaccct
481 atccttactc atgcccagag ccttggctcc atggccttca ctctgctgct ctgccaaaca
541 ggatcctacc tgcttcacca catccatggc tccccacctt cactgcactt tagaacagtc
601 cagggagctt taaagccatt gatgctggca cctcacttgg gagcatgatt taaccagcct
661 tgggtggggc tcgggcattg ttttttttttt ataggctccc aggtatgttc tcatgcacgt
721 ggccagggtt gagccccagt gacccagatg ttgacagctt cccccgccct tgcctggatc
781 ctggtatcct gtaggtgctg aaaggtggct agaattagcc cacccctcc tcctgtatcc
841 ccccaccgca gtcctgaggg cacccgcccc tagctcaggc tttcacattc ccggtgaggc
901 tgctcactga ctgggttgcg tggagagcag aggaggatgc gtggagagag ccattccgcc
961 gccctcagcc tctggctggc gagtggttcc tcggcaaccg cccagcctga caccagattg
1021 gagttcacat gattctgcct cagctcggga atgggcagcc tccggctggg gattttctag
1081 ccggagctgg gaggttcagg ctgcgggaag tcgctggagg gagagctggg gtcgtggttg
1141 caagggtgcc caggctcctg cagagcctct gcaggaagcc cagttgtttg aggtttgagg
1201 gcagagccca gctgaggcag gcagagtcag agaggactcg tttcagactc ccccctcaga
1261 ggactccttc cgtcctgatt tcagcagctg tctgctcctc acctgtcttc agagaccaca
1321 ccgccttcct ggggccctga ggctctagga gaagctggaa agcacaggtg ggggtccatc
1381 aagctgtttc caaactgcgg gttgtaaccc attcatgggt cagtaaatca acttcgtaac

1441 tcatgacctg cattttaaaa attgaaatag aatagaaatg atcagagtgc gtggcaagaa
1501 gtaggggtct tgcccaccac cagcgatgcc cagcccttgg tagaacttga accaccttct
1561 ataaacagga tggcggtgga gagacaggcc cagtccctga gcccatgagg agtgtggccc
1621 cgtcaggccc aaagatgggg aacatcactg cagacaactc ctccatgagc tgcaccatcg
1681 accacaccat ccaccagacg ctggccccgg tggtctatgt taccgtgctg gtggtaggct
1741 tcccggccaa ctgcctgtcc ctctacttcg gctacctgca gatcaaggcc cggaacgagc
1801 tgggcgtgta cctgtgcaac ctgacggtgg ccgacctctt ctacatctgc tcgctgccct
1861 tctggctgca gtacgtgctg cagcacgaca actggtctca cggcgacctg tcctgtcagg
1921 tgtgcggcat cctcctctac gagaacatct acatcagcgt gggcttcctc tgctgcatct
1981 ctgtggaccg ctacctggct gtggcccatc ccttccgctt ccaccagttc cggaccctga
2041 aggcggccgt cggcgtcagc gtggtcatct gggccaagga gctgctgacc agcatctact
2101 tcctgatgca cgaggaggtc atcgaggacg aggaccagca ccgcgtgtgc tttgagcact
2161 accccatcca ggcatggcag cgcgccatca actactaccg cttcctggtg ggcttcctct
2221 tccccatctg cctgctgctg gcgtcctacc agggcatcct gcgcgccgtg cgccggagcc
2281 acggcaccca gaagagccgc aaggaccaga tccagcggct ggtgctcagc accgtggtca
2341 tcttcctggc ctgcttcctg ccctaccacg tgctgctgct ggtgcgcagc gtctgggagg
2401 ccagctgcga cttcgccaag ggcgtcttca acgcctacca cttctccctc ctgctcacca
2461 gcttcaactg cgtcgccgac ccggtgctct actgcttcgt cagtgagacc acccaccggg
2521 acctggcccg cctccgcggg gcctgcctgg ccttcctcac ctgctctagg accggccggg
2581 cccgggaggc ctacccgctg ggtgcccccg aggcctccgg gaaaagtggg gcccagggcg
2641 aggagcccga gctgttgacc aagctccacc cggccttcca gacccctaac tcgccaggga
2701 tggtagggtc tgccacgggt gggttggcct agcctgggtc ccccgcgggt ggggccctgt
2761 gaggcctgag ccttcagccc atgggcctca gggccggctg cctcctgctt cccccagcac
2821 cagtgccgct ttctgcggag gcagcgaggc cccgtgactc cagaagtctg ctgtcccttg
2881 ctgagcccgc tgggacggcc gagggcggga ataagccccc gttggttcat ggtgtcctct
2941 gctgcggctg cgatgtggcc aggctggggc tgctggtggg gggaagacag tgagctgcgc
3001 tccctggcct gcttcccgca gagtttgtgc atagggattg tgaggacttg gcgggtggga
3061 ggctgggcgt tcacttgcca gggccttcca atgccaccgt tgtcacagac aatgcctgtc
3121 caggtgtccc ggtgggacta gcgctggagc cccgcctaca acagggtggg aagagaagac
3181 tggaggggga aggaagggag gaggggagaa ggaagggagg aggggagaag gaagggaggg
3241 ggtgttgggt tgggggagtg ggggtagtta taggcagaga agggccctgg ctccagccac
3301 aagaacaggg agagccacct gccgctccag aagacctgcc acctccacag aaatgctggc
3361 tcttcagacc ttctggtcag tgggctggga ccagctctga ctggggatgt cctccaaggc
3421 ctgggactcc agttcctcag aaccaagagg ctacctgggc ttcctccaag aagggaccag

3481 tggctaggag cagaagttgg catcagtaaa tatttctgga atctaccaaa gggaggccca
3541 ggtagaaccc cttcctcagt ctcccttccc ctcttccccc cagcttttaa gaggttgatc
3601 ctttgtgctg tgtctcttag ccctttcatc ggggatgccc agaggcagac agcccaggct
3661 ggaggtgccc cagggaaggg tgctcacagt ggtcccaggg atacacttgg cttcgactgc
3721 acagcccctg atcagatcct tgtggagggt gtccgatgac ttggggaggt tctcttcccc
3781 ttcctagagg aggaagtgac caagtttgaa ggcagcagaa atgaccacac agccaagagc
3841 ccatgcagct ccgcccagga tcttactggg tcagtgacat tggtggaagc ctccatgcct
3901 cctcattcca cagcacctcc tcctatgttg accttaaaca ccggctcccc actgacacag
3961 actgggacga gctgagggca ctctcattta ctatcaagag ttgtattttt gtattgtcct
4021 ctcttttgc caagtctaca tgtgttgact gtgcaatgga tttatgtagc caacttcagt
4081 ctgcaaataa agcaaagtaa ctgga

[0095] The transcript variant X2 of cynomolgus monkey GPR68 mRNA is found in GENBANK™ Accession No: XM_005562025.2, and has the nucleotide sequence set forth below (SEQ ID NO: 13):

1 tgcgcctggc tggaaatagt ttttaaaagg cttgctatgt ggtagcattt gcttggaacg
61 cccaggctcc actgaagggg atttccttag acccccctacc gcagagcaag gggagtggag
121 ccaaggctct tggcatgaat aagatgggca tgttttaaac tgtgttagtc cccagatcag
181 ggacctagca cagggctgtg gccgcttccg gcaggaagga ctcgcgcccc cagtggcccc
241 atgcaccgtg tgtctgttgg cttcctggct ctggcattgt agcttataat tagcttccaa
301 ggagcctgcc ttggagggag gtggttacgc caaaatgctg ggcctttcca tcctctgtgt
361 tatgtgtgtg agggagttta gtaagtgctc ctgagagcca gaaggccccc cgcggccaca
421 gccccgggct tggttcctgc tcaggggact gacacagttg caaacattga tgcccaccct
481 atccttactc atgcccagag ccttggctcc atggccttca ctctgctgct ctgccaaaca
541 ggatcctacc tgcttcacca catccatggc tccccacctt cactgcactt tagaacagtc
601 cagggagctt taaagccatt gatgctggca cctcacttgg gagcatgatt taaccagcct
661 tgggtggggc tcgggcattg ttttttttt ataggctccc aggtatgttc tcatgcacgt
721 ggccagggtt gagccccagt gacccagatg ttgacagctt cccccgccct tgcctggatc
781 ctggtatcct gtaggtgctg aaaggtggct agaattagcc cacccctcc tcctgtatcc
841 ccccaccgca gtcctgaggg cacccgcccc tagctcaggc tttcacattc ccggtgaggc
901 tgctcactga ctgggttgcg tggagagcag aggaggatgc gtggagagag ccattccgcc
961 gccctcagcc tctggctggc gagtggttcc tcggcaaccg cccagcctga caccagattg
1021 gagttcacat gattctgcct cagctcggga atgggcagcc tccggctggg gattttctag
1081 ccggagctgg gaggttcagg ctgcgggaag tcgctggagg gagagctggg gtcgtggttg
1141 caagggtgcc caggctcctg cagagcctct gcaggaagcc cagttgtttg aggtttgagg

1201 gcagagccca gctgaggcag gcagagtcag agaggactcg tttcagactc ccccctcaga
1261 ggactccttc cgtcctgatt tcagcagctg tctgctcctc acctgtcttc agagaccaca
1321 ccgccttcct ggggccctga ggctctagga gaagctggaa agcacagggt cttgcccacc
1381 accagcgatg cccagccctt ggtagaactt gaaccacctt ctataaacag gatggcggtg
1441 gagagacagg cccagtccct gagcccatga ggagtgtggc cccgtcaggc ccaaagatgg
1501 ggaacatcac tgcagacaac tcctccatga gctgcaccat cgaccacacc atccaccaga
1561 cgctggcccc ggtggtctat gttaccgtgc tggtggtagg cttcccggcc aactgcctgt
1621 ccctctactt cggctacctg cagatcaagg cccggaacga gctgggcgtg tacctgtgca
1681 acctgacggt ggccgacctc ttctacatct gctcgctgcc cttctggctg cagtacgtgc
1741 tgcagcacga caactggtct cacggcgacc tgtcctgtca ggtgtgcggc atcctcctct
1801 acgagaacat ctacatcagc gtgggcttcc tctgctgcat ctctgtggac cgctacctgg
1861 ctgtggccca tcccttccgc ttccaccagt tccggaccct gaaggcggcc gtcggcgtca
1921 gcgtggtcat ctgggccaag gagctgctga ccagcatcta cttcctgatg cacgaggagg
1981 tcatcgagga cgaggaccag caccgcgtgt gctttgagca ctaccccatc caggcatggc
2041 agcgcgccat caactactac cgcttcctgg tgggcttcct cttccccatc tgcctgctgc
2101 tggcgtccta ccagggcatc ctgcgcgccg tgcgccggag ccacggcacc cagaagagcc
2161 gcaaggacca gatccagcgg ctggtgctca gcaccgtggt catcttcctg gcctgcttcc
2221 tgccctacca cgtgctgctg ctggtgcgca gcgtctggga ggccagctgc gacttcgcca
2281 agggcgtctt caacgcctac cacttctccc tcctgctcac cagcttcaac tgcgtcgccg
2341 acccggtgct ctactgcttc gtcagtgaga ccacccaccg ggacctggcc cgcctccgcg
2401 gggcctgcct ggccttcctc acctgctcta ggaccggccg ggcccgggag gcctacccgc
2461 tgggtgcccc cgaggcctcc gggaaaagtg gggcccaggg cgaggagccc gagctgttga
2521 ccaagctcca cccggccttc cagaccccta actcgccagg gatggtaggg tctgccacgg
2581 gtgggttggc ctagcctggg tcccccgcgg gtggggccct gtgaggcctg agccttcagc
2641 ccatgggcct cagggccggc tgcctcctgc ttcccccagc accagtgccg ctttctgcgg
2701 aggcagcgag gccccgtgac tccagaagtc tgctgtccct tgctgagccc gctgggacgg
2761 ccgagggcgg gaataagccc ccgttggttc atggtgtcct ctgctgcggc tgcgatgtgg
2821 ccaggctggg gctgctggtg gggggaagac agtgagctgc gctccctggc ctgcttcccg
2881 cagagtttgt gcatagggat tgtgaggact tggcgggtgg gaggctgggc gttcacttgc
2941 cagggccttc caatgccacc gttgtcacag acaatgcctg tccaggtgtc ccggtgggac
3001 tagcgctgga gccccgccta caacagggtg ggaagagaag actggagggg gaaggaaggg
3061 aggaggggag aaggaaggga ggaggggaga aggaagggag ggggtgttgg gttgggggag
3121 tgggggtagt tataggcaga gaagggccct ggctccagcc acaagaacag ggagagccac
3181 ctgccgctcc agaagacctg ccacctccac agaaatgctg gctcttcaga ccttctggtc

3241 agtgggctgg gaccagctct gactggggat gtcctccaag gcctgggact ccagttcctc
3301 agaaccaaga ggctacctgg gcttcctcca agaagggacc agtggctagg agcagaagtt
3361 ggcatcagta aatatttctg gaatctacca aagggaggcc caggtagaac cccttcctca
3421 gtctcccttc ccctcttccc cccagctttt aagaggttga tcctttgtgc tgtgtctctt
3481 agccctttca tcggggatgc ccagaggcag acagcccagg ctggaggtgc cccagggaag
3541 ggtgctcaca gtggtcccag ggatacactt ggcttcgact gcacagcccc tgatcagatc
3601 cttgtggagg gtgtccgatg acttggggag gttctcttcc ccttcctaga ggaggaagtg
3661 accaagtttg aaggcagcag aaatgaccac acagccaaga gcccatgcag ctccgcccag
3721 gatcttactg ggtcagtgac attggtggaa gcctccatgc ctcctcattc cacagcacct
3781 cctcctatgt tgaccttaaa caccggctcc ccactgacac agactgggac gagctgaggg
3841 cactctcatt tactatcaag agttgtattt ttgtattgtc ctctcttttt gccaagtcta
3901 catgtgttga ctgtgcaatg gatttatgta gccaacttca gtctgcaaat aaagcaaagt
3961 aactgga

[0096] The transcript variant X3 of cynomolgus monkey GPR68 mRNA is found in GENBANK™ Accession No: XM_005562028.2, and has the nucleotide sequence set forth below (SEQ ID NO: 14):

1 tgcgcctggc tggaaatagt ttttaaaagg cttgctatgt ggtagcattt gcttggaacg
61 cccaggctcc actgaagggg atttccttag acccccctacc gcagagcaag gggagtggag
121 ccaaggctct tggcatgaat aagatgggca tgttttaaac tgtgttagtc cccagatcag
181 ggacctagca cagggctgtg gccgcttccg gcaggaagga ctcgcgcccc cagtggcccc
241 atgcaccgtg tgtctgttgg cttcctggct ctggcattgt agcttataat tagcttccaa
301 ggagcctgcc ttggagggag gtggttacgc caaaatgctg ggcctttcca tcctctgtgt
361 tatgtgtgtg agggagttta gtaagtgctc ctgagagcca gaaggccccc cgcggccaca
421 gccccgggct tggttcctgc tcaggggact gacacagttg caaacattga tgcccaccct
481 atccttactc atgcccagag ccttggctcc atggccttca ctctgctgct ctgccaaaca
541 ggatcctacc tgcttcacca catccatggc tccccacctt cactgcactt tagaacagtc
601 cagggagctt taaagccatt gatgctggca cctcacttgg gagcatgatt taaccagcct
661 tgggtggggc tcgggcattg ttttttttt ataggctccc aggtatgttc tcatgcacgt
721 ggccagggtt gagccccagt gacccagatg ttgacagctt cccccgccct tgcctggatc
781 ctggtatcct gtaggtgctg aaaggtggct agaattagcc caccccctcc tcctgtatcc
841 ccccaccgca gtcctgaggg cacccgcccc tagctcaggc tttcacattc ccggtgaggc
901 tgctcactga ctgggttgcg tggagagcag aggaggatgc gtggagagag ccattccgcc
961 gccctcagcc tctggctggc gagtggttcc tcggcaaccg cccagcctga caccagattg
1021 gagttcacat gattctgcct cagctcggga atgggcagcc tccggctggg gattttctag

1081 ccggagctgg gaggttcagg ctgcgggaag tcgctggagg gagagctggg gtcgtggttg
1141 caagggtgcc caggctcctg cagagcctct gcaggaagcc cagttgtttg aggtttgagg
1201 gcagagccca gctgaggcag gcagagtcag agaggactcg tttcagactc ccccctcaga
1261 ggactccttc cgtcctgatt tcagcagctg tctgctcctc acctgtcttc agagaccaca
1321 ccgccttcct ggggccctga ggctctagga gaagctggaa agcacaggtg ggggtccatc
1381 aagctgtttc caaactgcgg gttgtaaccc attcatgggg tcttgcccac caccagcgat
1441 gcccagccct tggtagaact tgaaccacct tctataaaca ggatggcggt ggagagacag
1501 gcccagtccc tgagcccatg aggagtgtgg ccccgtcagg cccaaagatg gggaacatca
1561 ctgcagacaa ctcctccatg agctgcacca tcgaccacac catccaccag acgctggccc
1621 cggtggtcta tgttaccgtg ctggtggtag gcttcccggc caactgcctg tccctctact
1681 tcggctacct gcagatcaag gcccggaacg agctgggcgt gtacctgtgc aacctgacgg
1741 tggccgacct cttctacatc tgctcgctgc ccttctggct gcagtacgtg ctgcagcacg
1801 acaactggtc tcacggcgac ctgtcctgtc aggtgtgcgg catcctcctc tacgagaaca
1861 tctacatcag cgtgggcttc ctctgctgca tctctgtgga ccgctacctg gctgtggccc
1921 atcccttccg cttccaccag ttccggaccc tgaaggcggc cgtcggcgtc agcgtggtca
1981 tctgggccaa ggagctgctg accagcatct acttcctgat gcacgaggag gtcatcgagg
2041 acgaggacca gcaccgcgtg tgctttgagc actaccccat ccaggcatgg cagcgcgcca
2101 tcaactacta ccgcttcctg gtgggcttcc tcttccccat ctgcctgctg ctggcgtcct
2161 accagggcat cctgcgcgcc gtgcgccgga gccacggcac ccagaagagc cgcaaggacc
2221 agatccagcg gctggtgctc agcaccgtgg tcatcttcct ggcctgcttc ctgccctacc
2281 acgtgctgct gctggtgcgc agcgtctggg aggccagctg cgacttcgcc aagggcgtct
2341 tcaacgccta ccacttctcc ctcctgctca ccagcttcaa ctgcgtcgcc gacccggtgc
2401 tctactgctt cgtcagtgag accacccacc gggacctggc ccgcctccgc ggggcctgcc
2461 tggccttcct cacctgctct aggaccggcc gggcccggga ggcctacccg ctgggtgccc
2521 ccgaggcctc cgggaaaagt ggggcccagg gcgaggagcc cgagctgttg accaagctcc
2581 acccggcctt ccagacccct aactcgccag ggatggtagg gtctgccacg ggtgggttgg
2641 cctagcctgg gtcccccgcg ggtggggccc tgtgaggcct gagccttcag cccatgggcc
2701 tcagggccgg ctgcctcctg cttcccccag caccagtgcc gctttctgcg gaggcagcga
2761 ggccccgtga ctccagaagt ctgctgtccc ttgctgagcc cgctgggacg gccgagggcg
2821 ggaataagcc cccgttggtt catggtgtcc tctgctgcgg ctgcgatgtg gccaggctgg
2881 ggctgctggt gggggggaaga cagtgagctg cgctccctgg cctgcttccc gcagagtttg
2941 tgcataggga ttgtgaggac ttggcgggtg ggaggctggg cgttcacttg ccagggcctt
3001 ccaatgccac cgttgtcaca gacaatgcct gtccaggtgt cccggtggga ctagcgctgg
3061 agccccgcct acaacagggt gggaagagaa gactggaggg ggaaggaagg gaggagggga

3121 gaaggaaggg aggaggggag aaggaaggga gggggtgttg ggttggggga gtgggggtag
3181 ttataggcag agaagggccc tggctccagc cacaagaaca gggagagcca cctgccgctc
3241 cagaagacct gccacctcca cagaaatgct ggctcttcag accttctggt cagtgggctg
3301 ggaccagctc tgactgggga tgtcctccaa ggcctgggac tccagttcct cagaaccaag
3361 aggctacctg ggcttcctcc aagaagggac cagtggctag gagcagaagt tggcatcagt
3421 aaatatttct ggaatctacc aaagggaggc ccaggtagaa ccccttcctc agtctccctt
3481 cccctcttcc ccccagcttt taagaggttg atcctttgtg ctgtgtctct tagcccttc
3541 atcggggatg cccagaggca gacagcccag gctggaggtg ccccagggaa gggtgctcac
3601 agtggtccca gggatacact tggcttcgac tgcacagccc ctgatcagat ccttgtggag
3661 ggtgtccgat gacttgggga ggttctcttc cccttcctag aggaggaagt gaccaagttt
3721 gaaggcagca gaaatgacca cacagccaag agcccatgca gctccgccca ggatcttact
3781 gggtcagtga cattggtgga agcctccatg cctcctcatt ccacagcacc tcctcctatg
3841 ttgaccttaa acaccggctc cccactgaca cagactggga cgagctgagg gcactctcat
3901 ttactatcaa gagttgtatt tttgtattgt cctctctttt tgccaagtct acatgtgttg
3961 actgtgcaat ggatttatgt agccaacttc agtctgcaaa taaagcaaag taactgga

[0097] The transcript variant X4 of cynomolgus monkey GPR68 mRNA is found in GENBANK™ Accession No: XM_015454130.1, and has the nucleotide sequence set forth below (SEQ ID NO: 15):

1 aaaaaaaagt ggctaaaatg gcaaatgtta ttttacatgt attttaccac aacaaaaaca
61 agaagcacct ccctccacca ccatcactgg aattctaggc cagtgccctg aagaggacgg
121 aagagggtct tgcccaccac cagcgatgcc cagcccttgg tagaacttga accaccttct
181 ataaacagga tggcggtgga gagacaggcc cagtccctga gcccatgagg agtgtggccc
241 cgtcaggccc aaagatgggg aacatcactg cagacaactc ctccatgagc tgcaccatcg
301 accacaccat ccaccagacg ctggccccgg tggtctatgt taccgtgctg gtggtaggct
361 tcccggccaa ctgcctgtcc ctctacttcg gctacctgca gatcaaggcc cggaacgagc
421 tgggcgtgta cctgtgcaac ctgacggtgg ccgacctctt ctacatctgc tcgctgccct
481 tctggctgca gtacgtgctg cagcacgaca actggtctca cggcgacctg tcctgtcagg
541 tgtgcggcat cctcctctac gagaacatct acatcagcgt gggcttcctc tgctgcatct
601 ctgtggaccg ctacctggct gtggcccatc ccttccgctt ccaccagttc cggaccctga
661 aggcggccgt cggcgtcagc gtggtcatct gggccaagga gctgctgacc agcatctact
721 tcctgatgca cgaggaggtc atcgaggacg aggaccagca ccgcgtgtgc tttgagcact
781 accccatcca ggcatggcag cgcgccatca actactaccg cttcctggtg ggcttcctct
841 tccccatctg cctgctgctg gcgtcctacc agggcatcct gcgcgccgtg cgccggagcc
901 acggcaccca gaagagccgc aaggaccaga tccagcggct ggtgctcagc accgtggtca

961 tcttcctggc ctgcttcctg ccctaccacg tgctgctgct ggtgcgcagc gtctgggagg
1021 ccagctgcga cttcgccaag ggcgtcttca acgcctacca cttctccctc ctgctcacca
1081 gcttcaactg cgtcgccgac ccggtgctct actgcttcgt cagtgagacc acccaccggg
1141 acctggcccg cctccgcggg gcctgcctgg ccttcctcac ctgctctagg accggccggg
1201 cccgggaggc ctacccgctg ggtgcccccg aggcctccgg gaaaagtggg gcccagggcg
1261 aggagcccga gctgttgacc aagctccacc cggccttcca gacccctaac tcgccaggga
1321 tggtagggtc tgccacgggt gggttggcct agcctgggtc ccccgcgggt ggggccctgt
1381 gaggcctgag ccttcagccc atgggcctca gggccggctg cctcctgctt cccccagcac
1441 cagtgccgct ttctgcggag gcagcgaggc cccgtgactc cagaagtctg ctgtcccttg
1501 ctgagcccgc tgggacggcc gagggcggga ataagccccc gttggttcat ggtgtcctct
1561 gctgcggctg cgatgtggcc aggctggggc tgctggtggg gggaagacag tgagctgcgc
1621 tccctggcct gcttcccgca gagtttgtgc atagggattg tgaggacttg gcgggtggga
1681 ggctgggcgt tcacttgcca gggccttcca atgccaccgt tgtcacagac aatgcctgtc
1741 caggtgtccc ggtgggacta gcgctggagc cccgcctaca acagggtggg aagagaagac
1801 tggaggggga aggaagggag gaggggagaa ggaagggagg aggggagaag gaagggaggg
1861 ggtgttgggt tgggggagtg ggggtagtta taggcagaga agggccctgg ctccagccac
1921 aagaacaggg agagccacct gccgctccag aagacctgcc acctccacag aaatgctggc
1981 tcttcagacc ttctggtcag tgggctggga ccagctctga ctggggatgt cctccaaggc
2041 ctgggactcc agttcctcag aaccaagagg ctacctgggc ttcctccaag aagggaccag
2101 tggctaggag cagaagttgg catcagtaaa tatttctgga atctaccaaa gggaggccca
2161 ggtagaaccc cttcctcagt ctcccttccc ctcttccccc cagcttttaa gaggttgatc
2221 ctttgtgctg tgtctcttag ccctttcatc ggggatgccc agaggcagac agcccaggct
2281 ggaggtgccc cagggaaggg tgctcacagt ggtcccaggg atacacttgg cttcgactgc
2341 acagcccctg atcagatcct tgtggagggt gtccgatgac ttggggaggt tctcttcccc
2401 ttcctagagg aggaagtgac caagtttgaa ggcagcagaa atgaccacac agccaagagc
2461 ccatgcagct ccgcccagga tcttactggg tcagtgacat tggtggaagc ctccatgcct
2521 cctcattcca cagcacctcc tcctatgttg accttaaaca ccggctcccc actgacacag
2581 actgggacga gctgagggca ctctcattta ctatcaagag ttgtattttt gtattgtcct
2641 ctcttttgc caagtctaca tgtgttgact gtgcaatgga tttatgtagc caacttcagt
2701 ctgcaaataa agcaaagtaa ctgga

[0098] The term “isolated,” when applied to a nucleic acid or protein, denotes that the nucleic acid or protein is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state. It can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry

techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein that is the predominant species present in a preparation is substantially purified. In particular, an isolated gene is separated from open reading frames that flank the gene and encode a protein other than the gene of interest. The term "purified" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

[0099] The term “nucleic acid” or “polynucleotide” refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); and Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)).

[00100] The terms “polypeptide,” “peptide,” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

[00101] The term “amino acid” refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refer to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α-carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical

compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

[00102] "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG, and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in each described sequence.

[00103] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles. The following eight groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M) (*see*, *e.g.*, Creighton, *Proteins* (1984)).

[00104] "Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (*e.g.*, a polypeptide), which does not comprise additions or

deletions, for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

[00105] The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same sequences. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*i.e.*, at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity over a specified region, or, when not specified, over the entire sequence of a reference sequence), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. The disclosure provides polypeptides or polynucleotides that are substantially identical to the polypeptides or polynucleotides, respectively, exemplified herein. The identity exists over a region that is at least about 15, 25 or 50 nucleotides in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides in length, or over the full length of the reference sequence. With respect to amino acid sequences, identity or substantial identity can exist over a region that is at least 5, 10, 15 or 20 amino acids in length, optionally at least about 25, 30, 35, 40, 50, 75 or 100 amino acids in length, optionally at least about 150, 200 or 250 amino acids in length, or over the full length of the reference sequence. With respect to shorter amino acid sequences, *e.g.*, amino acid sequences of 20 or fewer amino acids, substantial identity exists when one or two amino acid residues are conservatively substituted, according to the conservative substitutions defined herein.

[00106] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[00107] A "comparison window," as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600,

usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman (1970) *Adv. Appl. Math.* 2:482c, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Nat'l. Acad. Sci. USA* 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g.,* Ausubel *et al.*, *Current Protocols in Molecular Biology* (1995 supplement)).

[00108] Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.* (1977) *Nuc. Acids Res.* 25:3389-3402, and Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-410, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62

scoring matrix (*see* Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

[00109] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5787). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[00110] An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below. Yet another indication that two nucleic acid sequences are substantially identical is that the same primers can be used to amplify the sequence.

[00111] The terms “subject,” “patient,” and “individual” interchangeably refer to a mammal, for example, a human or a non-human primate mammal. The mammal can also be a laboratory mammal, *e.g.*, mouse, rat, rabbit, hamster. In some embodiments, the mammal can be an agricultural mammal (e.g., equine, ovine, bovine, porcine, camelid) or domestic mammal (e.g., canine, feline).

[00112] As used herein, the terms “treat,” “treating,” or “treatment” of any disease or disorder refer in one embodiment, to ameliorating the disease or disorder (*i.e.*, slowing or arresting or reducing the development of the disease or at least one of the clinical symptoms thereof). In another embodiment, “treat,” “treating,” or “treatment” refers to alleviating or ameliorating at least one physical parameter including those which may not be discernible by the patient. In yet another embodiment, “treat,” “treating,” or “treatment” refers to modulating the disease or disorder, either physically, (*e.g.*, stabilization of a discernible symptom), physiologically, (*e.g.*, stabilization of a physical parameter), or both. In yet another embodiment, “treat,” “treating,” or “treatment” refers to preventing or delaying the onset or development or progression of a disease or disorder.

[00113] The terms “therapeutically acceptable amount” or “therapeutically effective dose” interchangeably refer to an amount sufficient to effect the desired result (*i.e.*, a reduction in inflammation, inhibition of pain, prevention of inflammation, inhibition or prevention of inflammatory response). In some embodiments, a therapeutically acceptable amount does not induce or cause undesirable side effects. A therapeutically acceptable amount can be determined by first administering a low dose, and then incrementally increasing that dose until the desired effect is achieved. In some embodiments, a “prophylactically effective dosage,” and a “therapeutically effective dosage,” of a GPR68 modulator can prevent the onset of, or result in a decrease in severity of, respectively, disease symptoms, including symptoms associated with a cardiovascular disease or liver fibrosis.

[00114] As used herein, the phrase “consisting essentially of” refers to the genera or species of active pharmaceutical agents included in a method or composition, as well as any inactive carrier or excipients for the intended purpose of the methods or compositions. In some embodiments, the phrase “consisting essentially of” expressly excludes the inclusion of one or more additional active agents other than a GPR68 modulator. In some embodiments, the phrase “consisting essentially of” expressly excludes the inclusion of more additional active agents other than a GPR68 modulator and a second co-administered agent.

[00115] The terms "a," "an," and "the" include plural referents, unless the context clearly indicates otherwise.

GPR68 Modulators

[00116] Some embodiments of the present invention provides a modulator, e.g., an antagonist or an agonist, of a GPR68 gene product.

[00117] The term “a GPR68 polypeptide” is used to refer collectively to all naturally occurring isoforms of a GPR68 protein of any species, or a variant thereof. For example, a “GPR68 polypeptide” can be a human GPR68 polypeptide, a mouse GPR68 popypeptide, a rat GPR68 polypeptide, a cyno GPR68 polypeptide, or a variant thereof. In some embodiments, a GPR68 polypeptide can be a protein having the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or a variant thereof.

[00118] A GPR68 variant can differ from a naturally occurring GPR68 protein by, for example, a modification (e.g., substitution, deletion, or insertion) of one or more amino acid residues in the naturally occurring GPR68 protein, but retains the biological activities of GPR68, e.g., endothelial shear stress sensor or mechanosensor activity. The GPR68 variant can have one or more conservative or nonconservative amino acid substitution. A “conservative amino

acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains(e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

[00119] In some embodiments, the GPR68 variant includes one or more mutations (e.g., substitutions (e.g., conservative substitutions or substitutions), insertions, or deletions) of non-essential amino acids relative to a naturally occurring GPR68 protein. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of GPR68 protein without abolishing or more preferably, without substantially altering a biological activity, such as endothelial shear stress sensor or mechanosensor activity, whereas changing an "essential" amino acid residue results in a substantial loss of biological activity.

[00120] A GPR68 variant may have at least one, two, three, or four, and no more than 10, 9, 8, 7, 6, or 5 mutations (e.g., substitutions (e.g., conservative substitutions or substitutions of non-essential amino acids), insertions, or deletions) relative to a naturally occurring GPR68 protein. Whether or not a particular substitution will be tolerated, i.e., will not adversely affect biological properties, such as fusogenic activity, can be predicted, e.g., by evaluating whether the mutation is conservative or by an activity assay.

[00121] In some embodiments, a GPR68 variant can have about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher sequence identity to a naturally occurring GPR68 protein. For example, a human GPR68 variant can have about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher sequence identity to SEQ ID NO: 1. A mouse GPR68 variant can have about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher sequence identity to SEQ ID NO: 2. A rat GPR68 variant can have about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher sequence identity to SEQ ID NO: 3. A cyno GPR68 variant can have about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher sequence identity to SEQ ID NO: 4.

[00122] In some embodiments, the modulator of a GPR68 gene product may decrease or increase the level of a GPR68 gene product. It will be understood by one skilled in the art, based upon the disclosure provided herein, that a decrease or increase in the level of a GPR68 gene product encompasses the decrease or increase in the expression, including DNA

transcription, mRNA translation, mRNA stability, protein stability or any all of their combinations. The skilled artisan will also appreciate, once armed with the teachings of the present invention, that a decrease or increase in the level of a GPR68 gene product includes a decrease or increase in the activity of GPR68, e.g., endothelial shear stress sensor or mechanosensor activity. Thus, decrease or increase in the level or activity of GPR68 includes, but is not limited to, decreasing or increasing the amount of polypeptide of GPR68, and decreasing or increasing transcription, translation, or both, of a nucleic acid encoding GPR68; and it also includes decreasing or increasing any activity of GPR68, e.g., endothelial shear stress sensor or mechanosensor activity.

[00123] In some embodiments, the modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, may reduce flow-mediated dilation (FMD) response and/or flow-mediated outward remodeling (FMR) of small-diameter arteries, also known as resistance arteries, in a subject. For example, the modulatoer of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, may reduce the FMR response and/or FMR of small-diameter arteries in a subject by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or more.

[00124] In some embodiments, the modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, may reduce the systemic vascular resistance (SVR) and/or the left ventricle afterload of a subject. For example, the modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, may reduce the SVR and/or the left ventricle afterload of a subject by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or more.

Small Molecule GPR68 Modulators

[00125] In various embodiments, the GPR68 modulator is a small molecule. When the GPR68 modulator is a small molecule, a small molecule may be obtained using standard methods known to the skilled artisan. Such methods include chemical organic synthesis or biological means. Biological means include purification from a biological source, recombinant synthesis and in vitro translation systems, using methods well known in the art. In one embodiment, a small molecule modulator of the invention comprises an organic molecule, inorganic molecule, biomolecule, synthetic molecule, and the like.

[00126] Combinatorial libraries of molecularly diverse chemical compounds potentially useful in treating a variety of diseases and conditions are well known in the art as are methods of making the libraries. The methods may use a variety of techniques well-known to the skilled

artisan including solid phase synthesis, solution methods, parallel synthesis of single compounds, synthesis of chemical mixtures, rigid core structures, flexible linear sequences, deconvolution strategies, tagging techniques, and generating unbiased molecular landscapes for lead discovery vs. biased structures for lead development.

[00127] In a general method for small library synthesis, an activated core molecule is condensed with a number of building blocks, resulting in a combinatorial library of covalently linked, core-building block ensembles. The shape and rigidity of the core determines the orientation of the building blocks in shape space. The libraries can be biased by changing the core, linkage, or building blocks to target a characterized biological structure ("focused libraries") or synthesized with less structural bias using flexible cores.

[00128] When the modulator is a small molecule, a small molecule antagonist or agonist may be obtained using standard methods known to the skilled artisan. Such methods include chemical organic synthesis or biological means. Biological means include purification from a biological source, recombinant synthesis and in vitro translation systems, using methods well known in the art. In one embodiment, the GPR68 modulator is a small molecule compound named Ogerin.

GPR68 RNAi Molecules

[00129] In various embodiments, the expression of GPR68 may be inhibited by an RNA interference (RNAi) molecule, e.g., siRNA, shRNA, etc. In some embodiments, the RNAi molecules comprise an antisense strand and a sense strand. In some embodiments, the antisense and sense strand can be two physically separated strands, or can be components of a single strand or molecule, e.g., they are linked a loop of nucleotides or other linker. A non-limiting example of the former is a siRNA; a non-limiting example of the latter is a shRNA. The can also, optionally, exist single-stranded nicks in the sense strand, or one or more mismatches between the antisense and sense strands. Some RNAi agents for GPR68 are available commercially, e.g., GPR68 TRC lentiviral shRNA from Dharmacon.

[00130] Alternatively, RNA interference molecules can be determined using computer software programs and the gene of GPR68.

[00131] Public access software programs and methods of predicting and selecting antisense oligonucleotides and siRNA are known in the art and are also found on the world wide web sites of GENSCRIPT™, AMBION®, DHARMACON™, OLIGOENGINE™, Wadsworth Bioinformatics Center, Whitehead Institute at the Massachusetts Institute of Technology and are also described in U.S. Pat. No. 6,060,248. After selecting the antisense oligonucleotides and

siRNA sequences, these molecules can be produced biologically using an expression vector carrying the polynucleotides that encode the siRNA or antisense RNA. General molecular biological methods known in the art can be used to clone these sequences into the expression vectors. Examples of such are described herein.

[00132] As used herein, the term “RNAi agent,” "RNAi molecule," "GPR68 RNAi molecule", "siRNA to GPR68", "GPR68 targeting siRNA" and the like refer to an siRNA (short inhibitory RNA), shRNA (short or small hairpin RNA), iRNA (interference RNA) agent, RNAi (RNA interference) agent, dsRNA (double-stranded RNA), microRNA, and the like, which specifically binds to the GPR68 mRNA and which mediates the targeted cleavage of the RNA transcript via an RNA-induced silencing complex (RISC) pathway. In one embodiment, the RNAi agent is an oligonucleotide composition that activates the RISC complex/pathway. In another embodiment, the RNAi agent comprises an antisense strand sequence (antisense oligonucleotide). In one embodiment, the RNAi comprises a single strand. This single-stranded RNAi agent oligonucleotide or polynucleotide can comprise the sense or antisense strand, as described by Sioud 2005 J. Mol. Bioi. 348:1079-1090, and references therein. Thus the disclosure encompasses RNAi agents with a single strand comprising either the sense or antisense strand of an RNAi agent described herein. The use of the RNAi agent to GPR68 results in a decrease of GPR68 post-translational modification, production, expression, level, stability and/or activity, e.g., a "knock-down" or "knock-out" of the GPR68 target gene or target sequence. In some embodiments, the GPR68 antagonist is molecule capable of mediating RNA interference against a GPR68 mRNA selected from the group consisting of SEQ ID NOs: 5-15.

[00133] RNA interference-inducing molecules include but are not limited to siRNA, dsRNA, stRNA, shRNA, microRNAi (mRNAi)/microRNA (miRNA), antisense oligonucleotides etc. and modified versions thereof, where the RNA interference molecule silences the gene expression of GPR68. An anti-sense oligonucleic acid, or a nucleic acid analogue, for example but are not limited to DNA, RNA, peptide-nucleic acid (PNA), pseudo-complementary PNA (pc-PNA), or locked nucleic acid (LNA) and the like.

[00134] RNA interference (RNAi) is an evolutionally conserved process whereby the expression or introduction of RNA of a sequence that is identical or highly similar to a target gene results in the sequence specific degradation or specific post-transcriptional gene silencing (PTGS) of messenger RNA (mRNA) transcribed from that targeted gene (see Coburn, G. and Cullen, B. (2002) J. of Virology 76(18):9225), thereby inhibiting expression of the target gene. In one embodiment, the RNA is double stranded RNA (dsRNA). This process has been described in plants, invertebrates, and mammalian cells. In nature, RNAi is initiated by the dsRNA-specific

endonuclease Dicer, which promotes processive cleavage of long dsRNA into double-stranded fragments termed siRNAs. siRNAs are incorporated into a protein complex (termed "RNA induced silencing complex," or "RISC") that recognizes and cleaves target mRNAs. RNAi can also be initiated by introducing nucleic acid molecules, e.g., synthetic siRNAs or RNA interfering agents, to inhibit or silence the expression of target genes. As used herein, "inhibition of target gene expression" includes any decrease in expression or protein activity or level of the target gene or protein encoded by the target gene as compared to a situation wherein no RNA interference has been induced. The decrease can be of at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% or more as compared to the expression of a target gene or the activity or level of the protein encoded by a target gene which has not been targeted by an RNA interfering agent.

[00135] "Short interfering RNA" (siRNA), also referred to herein as "small interfering RNA" is defined as an agent which functions to inhibit expression of a target gene, e.g., by RNAi. A siRNA can be chemically synthesized, can be produced by in vitro transcription, or can be produced within a host cell. In one embodiment, siRNA is a double stranded RNA (dsRNA) molecule of about 15 to about 40 nucleotides in length, preferably about 15 to about 28 nucleotides, more preferably about 19 to about 25 nucleotides in length, and more preferably about 19, 20, 21, 22, or 23 nucleotides in length, and can contain a 3′ and/or 5′ overhang on each strand having a length of about 0, 1, 2, 3, 4, or 5 nucleotides. The length of the overhang is independent between the two strands, i.e., the length of the overhang on one strand is not dependent on the length of the overhang on the second strand. Preferably the siRNA is capable of promoting RNA interference through degradation or specific post-transcriptional gene silencing (PTGS) of the target messenger RNA (mRNA).

[00136] siRNAs also include small hairpin (also called stem loop) RNAs (shRNAs). In one embodiment, these shRNAs are composed of a short (e.g., about 19 to about 25 nucleotide) antisense strand, followed by a nucleotide loop of about 5 to about 9 nucleotides, and the analogous sense strand. Alternatively, the sense strand can precede the nucleotide loop structure and the antisense strand can follow. These shRNAs can be contained in plasmids, retroviruses, and lentiviruses and expressed from, for example, the pol III U6 promoter, or another promoter (see, e.g., Stewart, et al. (2003) RNA April; 9(4):493-501, incorporated by reference herein in its entirety).

[00137] The target gene or sequence of the RNA interfering agent can be a cellular gene or genomic sequence, e.g. a GPR68 gene. An siRNA can be substantially homologous to the target gene or genomic sequence, or a fragment thereof. As used in this context, the term

"homologous" is defined as being substantially identical, sufficiently complementary, or similar to the target mRNA, or a fragment thereof, to effect RNA interference of the target. In addition to native RNA molecules, RNA suitable for inhibiting or interfering with the expression of a target sequence includes RNA derivatives and analogs. Preferably, the siRNA is identical to its target.

[00138] The siRNA preferably targets only one sequence. Each of the RNA interfering agents, such as siRNAs, can be screened for potential off-target effects by, for example, expression profiling. Such methods are known to one skilled in the art and are described, for example, in Jackson et al, Nature Biotechnology 6:635-637, 2003. In addition to expression profiling, one can also screen the potential target sequences for similar sequences in the sequence databases to identify potential sequences which can have off-target effects. For example, according to Jackson et al. (Id.) 15, or perhaps as few as 11 contiguous nucleotides of sequence identity are sufficient to direct silencing of non-targeted transcripts. Therefore, one can initially screen the proposed siRNAs to avoid potential off-target silencing using the sequence identity analysis by any known sequence comparison methods, such as BLAST.

[00139] siRNA molecules need not be limited to those molecules containing only RNA, but, for example, further encompasses chemically modified nucleotides and non-nucleotides, and also include molecules wherein a ribose sugar molecule is substituted for another sugar molecule or a molecule which performs a similar function. Moreover, a non-natural linkage between nucleotide residues can be used, such as a phosphorothioate linkage. For example, siRNA containing D-arabinofuranosyl structures in place of the naturally-occurring D-ribonucleosides found in RNA can be used in RNAi molecules according to the present invention (U.S. Pat. No. 5,177,196). Other examples include RNA molecules containing the o-linkage between the sugar and the heterocyclic base of the nucleoside, which confers nuclease resistance and tight complementary strand binding to the oligonucleotides molecules similar to the oligonucleotides containing 2′-O-methyl ribose, arabinose and particularly D-arabinose (U.S. Pat. No. 5,177,196).

[00140] The RNA strand can be derivatized with a reactive functional group of a reporter group, such as a fluorophore. Particularly useful derivatives are modified at a terminus or termini of an RNA strand, typically the 3′ terminus of the sense strand. For example, the 2′-hydroxyl at the 3′ terminus can be readily and selectively derivatized with a variety of groups.

[00141] siRNA and miRNA molecules having various "tails" covalently attached to either their 3′- or to their 5′-ends, or to both, are also known in the art and can be used to stabilize the siRNA and miRNA molecules delivered using the methods of the present invention. Generally speaking, intercalating groups, various kinds of reporter groups and lipophilic groups attached to

the 3′ or 5′ ends of the RNA molecules are well known to one skilled in the art and are useful according to the methods of the present invention. Descriptions of syntheses of 3′-cholesterol or 3′-acridine modified oligonucleotides applicable to preparation of modified RNA molecules useful according to the present invention can be found, for example, in the articles: Gamper, H. B., Reed, M. W., Cox, T., Virosco, J. S., Adams, A. D., Gall, A., Scholler, J. K., and Meyer, R. B. (1993) Facile Preparation and Exonuclease Stability of 3′-Modified Oligodeoxynucleotides. Nucleic Acids Res. 21 145-150; and Reed, M. W., Adams, A. D., Nelson, J. S., and Meyer, R. B., Jr. (1991) Acridine and Cholesterol-Derivatized Solid Supports for Improved Synthesis of 3′-Modified Oligonucleotides. Bioconjugate Chem. 2 217-225 (1993).

[00142] siRNAs useful for the methods described herein include siRNA molecules of about 15 to about 40 or about 15 to about 28 nucleotides in length, which are homologous to the GPR68 gene. Preferably, the GPR68 targeting siRNA molecules have a length of about 19 to about 25 nucleotides. More preferably, the targeting siRNA molecules have a length of about 19, 20, 21, or 22 nucleotides. The targeting siRNA molecules can also comprise a 3′ hydroxyl group. The targeting siRNA molecules can be single-stranded or double stranded; such molecules can be blunt ended or comprise overhanging ends (e.g., 5′, 3′). In specific embodiments, the RNA molecule is double stranded and either blunt ended or comprises overhanging ends.

[00143] In one embodiment, at least one strand of the GPR68 targeting RNA molecule has a 3′ overhang from about 0 to about 6 nucleotides (e.g., pyrimidine nucleotides, purine nucleotides) in length. In other embodiments, the 3′ overhang is from about 1 to about 5 nucleotides, from about 1 to about 3 nucleotides and from about 2 to about 4 nucleotides in length. In one embodiment the targeting RNA molecule is double stranded—one strand has a 3′ overhang and the other strand can be blunt-ended or have an overhang. In the embodiment in which the targeting RNA molecule is double stranded and both strands comprise an overhang, the length of the overhangs can be the same or different for each strand. In a particular embodiment, the RNA of the present invention comprises about 19, 20, 21, or 22 nucleotides which are paired and which have overhangs of from about 1 to about 3, particularly about 2, nucleotides on both 3′ ends of the RNA. In one embodiment, the 3′ overhangs can be stabilized against degradation. In a preferred embodiment, the RNA is stabilized by including purine nucleotides, such as adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, e.g., substitution of uridine 2 nucleotide 3′ overhangs by 2′-deoxythymidine is tolerated and does not affect the efficiency of RNAi. The

absence of a 2′ hydroxyl significantly enhances the nuclease resistance of the overhang in tissue culture medium.

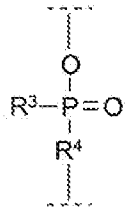
[00144] In one embodiment, the RNAi agent comprises a modification that causes the RNAi agent to have increased stability in a biological sample or environment.

[00145] In one embodiment, the RNAi agent comprises at least one sugar backbone modification (e.g., phosphorothioate linkage) or at least one 2' -modified nucleotide.

[00146] In one embodiment, the RNAi agent comprises: at least one 5'-uridine-adenine-3' (5'-ua-3') dinucleotide, wherein the uridine is a 2'-modified nucleotide; at least one 5'-uridine-5 guanine-3' (5' -ug-3') dinucleotide, wherein the 5'-uridine is a 2' -modified nucleotide; at least one 5'-cytidine-adenine-3' (5'-ca-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide; or at least one 5'-uridine-uridine-3' (5' -uu-3 ') dinucleotide, wherein the 5' -uridine is a 2'-modified nucleotide. These dinucleotide motifs are particularly prone to serum nuclease degradation (e.g. RNase A). Chemical modification at the 2'-position of the first pyrimidine nucleotide in the motif prevents or slows down such cleavage. This modification recipe is also known under the term 'endo light'.

[00147] In one embodiment, the RNAi agent comprises a 2'-modification selected from the group consisting of: 2'-deoxy, 2'-deoxy-2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), and 2'-O-N-methylacetamido (2'-O-NMA). In one embodiment, all pyrimidines (uridine and cytidine) are 2'-O-methyl-modified nucleosides. In some embodiments, one or more nucleotides can be modified, or substituted with DNA, or a nucleotide substitute such as a peptide nucleic acid (PNA), locked nucleic acid (LNA), morpholino nucleotide, threose nucleic acid (TNA), glycol nucleic acid (GNA), arabinose nucleic acid (ANA), 2´-fluoroarabinose nucleic acid (FANA), cyclohexene nucleic acid (CeNA), anhydrohexitol nucleic acid (HNA), unlocked nucleic acid (UNA).

[00148] In some embodiments, the sense and/or antisense strand can terminate at the 3' end with a phosphate or modified internucleoside linker, and further comprise, in 5' to 3' order: a spacer, a second phosphate or modified internucleoside linker, and a 3' end cap. In some embodiments, modified internucleoside linker is selected from phosphorothioate, phosphorodithioate, phosphoramidate, boranophosphonoate, an amide linker, and a compound



of formula (I): (I), where R^3 is selected from O-, S-, NH_2, BH_3, CH_3, C_{1-6} alkyl, C_{6-10} aryl, C_{1-6} alkoxy and C_{6-10} aryl-oxy, wherein C_{1-6} alkyl and C_{6-10} aryl are unsubstituted or optionally independently substituted with 1 to 3 groups independently selected from halo, hydroxyl and NH_2; and R^4 is selected from O, S, NH, and CH_2. In some embodiments, the spacer can be a sugar, alkyl, cycloakyl, ribitol or other type of abasic nucleotide, 2'-deoxy-ribitol, diribitol, 2'-methoxyethoxy-ribitol (ribitol with 2'-MOE), C_{3-6} alkyl, or 4-methoxybutane-1,3-diol (5300). In some embodiments, the 3' end cap can be selected from any of various 3' end caps described herein or known in the art. In some embodiments, one or more phosphates can be replaced by a modified internucleoside linker.

[00149] In one embodiment, the RNAi agent comprises at least one blunt end.

[00150] In one embodiment, the RNAi agent comprises an overhang having 1 nt to 4 nt.

[00151] In one embodiment, the RNAi agent comprises an overhang at the 3'-end of the antisense strand of the RNAi agent.

[00152] In one embodiment, the RNAi agent is ligated to one or more diagnostic compound, reporter group, cross-linking agent, nuclease-resistance conferring moiety, natural or unusual nucleobase, lipophilic molecule, cholesterol, lipid, lectin, steroid, uvaol, hecigenin, diosgenin, terpene, triterpene, sarsasapogenin, Friedelin, epifriedelanol-derivatized lithocholic acid, vitamin, carbohydrate, dextran, pullulan, chitin, chitosan, synthetic carbohydrate, oligo lactate 15-mer, natural polymer, low- or medium-molecular weight polymer, inulin, cyclodextrin, hyaluronic acid, protein, protein-binding agent, integrin-targeting molecule, polycationic, peptide, polyamine, peptide mimic, and/or transferrin.

[00153] In one embodiment, the composition further comprises a second RNAi agent to a GPR68 gene product.

[00154] Specific RNAi agents include: the shRNAs to a GPR68 gene product disclosed herein (particularly those having a target sequence of any of SEQ ID NOs: 5-15, and the complementary sequence thereof, or a target sequence comprising 15 contiguous nt of a GPR68 target sequence thereof). Additional RNAi agents to GPR68 can be prepared, or are known in the art. It is noted that in the present disclosure a RNAi agent to GPR68 may be recited to target a particular GPR68 sequence, indicating that the recited sequence may be comprised in the sequence of the sense or anti-sense strand of the RNAi agent; or, in some

cases, a sequence of at least 15 contiguous nt of this sequence may be comprised in the sequence of the sense or anti-sense strand. It is also understood that some of the target sequences are presented as DNA, but the RNAi agents targeting these sequences can be RNA, or any nucleotide, modified nucleotide or substitute disclosed herein.

[00155] In some embodiments, the RNAi agent to GPR68 includes any shRNA used in the experiments described herein, namely GPR68 sh1, sh2, and sh3 (shRNA1, shRNA2 and shRNA3), whose GPR68 target sequences are presented below:

GPR68 sh1:

TGAAGCACGTACTGCAGCC [SEQ ID NO: 16]

GPR68 sh2:

TACACTCCCAGCTCATTCC [SEQ ID NO: 17]

GPR68 sh3:

ACTCAAGCTAGGCCAACCC [SEQ ID NO: 18]

[00156] In some embodiments, the RNAi agent to GPR68 includes shRNAs that are commercially available, e.g., SHCLNG-NM_003485, SHCLND-NM_003485, SHCLNV-NM_003485, SHCLNG-NM_175493, SHCLND-NM_175493, SHCLNV-NM_175493 (Sigma-Aldrich); sc-75186-SH, sc-75186-V (Santa Cruz); VGH5518-200225097, VGH5518-200225475, VGH5518-200226483, VGH5518-200227207, VGH5518-200228644, VGH5518-200228799, VGH5518-200229262, VGH5518-200230237, VGH5518-200260949 (Dharmacon). In some embodiments, the RNAi agent to GPR68 includes siRNAs that are commercially available, e.g., NM_003485, NM_001108049, NM_175493 (Sigma-Aldrich); sc-75186 (Santa Cruz); E-005591-00-0005, EQ-005591-00-0002, A-005591-15-0005, A-005591-16-0005, A-005591-17-0005, A-005591-18-0005, EU-005591-00-0002, L-005591-00-0005, LQ-005591-00-0002, J-005591-07-0002, J-005591-08-0002, J-005591-097-0002, J-005591-10-0002, LU-005591-00-0002, M-005591-02-0005, MQ-005591-02-0002, D-005591-01-0002, D-005591-02-0002, D-005591-05-0002, D-005591-06-0002, MU-005591-02-0002 (Dharmacon).

[00157] RNAi agents of the present invention can be delivered or introduced (e.g., to a cell in vitro or to a subject) by any means known in the art.

[00158] "Introducing into a cell," when referring to an iRNA, means facilitating or effecting uptake or absorption into the cell, as is understood by those skilled in the art. Absorption or uptake of an iRNA can occur through unaided diffusive or active cellular processes, or by auxiliary agents or devices. The meaning of this term is not limited to cells in vitro; an iRNA may also be "introduced into a cell," wherein the cell is part of a living organism. In such an instance,

introduction into the cell will include the delivery to the organism. For example, for in vivo delivery, iRNA can be injected into a tissue site or administered systemically. In vivo delivery can also be by a beta-glucan delivery system, such as those described in U.S. Patent Nos. 5,032,401 and 5,607,677, and U.S. Publication No. 2005/0281781 which are hereby incorporated by reference in their entirety. In vitro introduction into a cell includes methods known in the art including, but not limited to, electroporation and lipofection. Further approaches are described below or known in the art.

[00159] Delivery of RNAi agent to tissue is a problem both because the material must reach the target organ and must also enter the cytoplasm of target cells. RNA cannot penetrate cellular membranes, so systemic delivery of naked RNAi agent is unlikely to be successful. RNA is quickly degraded by RNAse activity in serum. For these reasons, other mechanisms to deliver RNAi agent to target cells has been devised. Methods known in the art include but are not limited to: viral delivery (retrovirus, adenovirus, lentivirus, baculovirus, AAV); liposomes (Lipofectamine, cationic DOTAP, neutral DOPC) or nanoparticles (cationic polymer, PEI), bacterial delivery (tkRNAi), and also chemical modification (LNA) of siRNA to improve stability. Xia et al. 2002 Nat. Biotechnol. 20 and Devroe et al. 2002. BMC Biotechnol. 21: 15, disclose incorporation of siRNA into a viral vector. Other systems for delivery of RNAi agents are contemplated, and the RNAi agents of the present invention can be delivered by various methods yet to be found and/or approved by the FDA or other regulatory authorities.

[00160] Liposomes have been used previously for drug delivery (e.g., delivery of a chemotherapeutic). Liposomes (e.g., cationic liposomes) are described in PCT publications W002/100435A1, W003/015757A1, and W004029213A2; U.S. Pat. Nos. 5,962,016; 5,030,453; and 6,680,068; and U.S. Patent Application 2004/0208921. A process of making liposomes is also described in W004/002453Al. Furthermore, neutral lipids have been incorporated into cationic liposomes (e.g., Farhood et al. 1995). Cationic liposomes have been used to deliver RNAi agent to various cell types (Sioud and Sorensen 2003; U.S. Patent Application 2004/0204377; Duxbury et al., 2004; Donze and Picard, 2002). Use of neutral liposomes disclosed in Miller et al. 1998, and U.S. Publ. 2003/0012812.

[00161] As used herein, the term "SNALP" refers to a stable nucleic acid-lipid particle. A SNALP represents a vesicle of lipids coating a reduced aqueous interior comprising a nucleic acid such as an iRNA or a plasmid from which an iRNA is transcribed. SNALPs are described, e.g., in U.S. Patent Application Publication Nos. 20060240093, 20070135372, and in International Application No. WO 2009082817. These applications are incorporated herein by reference in their entirety.

[00162] Chemical transfection using lipid-based, amine-based and polymer-based techniques, is disclosed in products from Ambion Inc., Austin, Tex.; and Novagen, EMD Biosciences, Inc, an Affiliate of Merck KGaA, Darmstadt, Germany); Ovcharenko D (2003) "Efficient delivery of siRNAs to human primary cells." Ambion TechNotes 10 (5): 15-16). Additionally, Song et al. (Nat Med. published online (Fete l 0, 2003) doi: 10.1038/nm828) and others (Caplen et al. 2001 Proc. Natl. Acad. Sci. (USA), 98: 9742-9747; and McCaffrey et al. Nature 414: 34-39) disclose that liver cells can be efficiently transfected by injection of the siRNA into a mammal's circulatory system.

[00163] A variety of molecules have been used for cell-specific RNAi agent delivery. For example, the nucleic acid-condensing property of protamine has been combined with specific antibodies to deliver siRNAs (Song et al. 2005 Nat Biotch. 23: 709-717). The self-assembly PEGylated polycation polyethylenimine has also been used to condense and protect siRNAs (Schiffelers et al. 2004 Nucl. Acids Res. 32: 49, 141-110).

[00164] The siRNA-containing nanoparticles were then successfully delivered to integrin overexpressing tumor neovasculature (Hu-Lieskovan et al. 2005 Cancer Res. 65: 8984-8992).

[00165] The RNAi agents of the present invention can be delivered via, for example, Lipid nanoparticles (LNP); neutral liposomes (NL); polymer nanoparticles; double-stranded RNA binding motifs (dsRBMs); or via modification of the RNAi agent (e.g., covalent attachment to the dsRNA).

[00166] Lipid nanoparticles (LNP) are self-assembling cationic lipid based systems. These can comprise, for example, a neutral lipid (the liposome base); a cationic lipid (for siRNA loading); cholesterol (for stabilizing the liposomes); and PEG-lipid (for stabilizing the formulation, charge shielding and extended circulation in the bloodstream). The cationic lipid can comprise, for example, a headgroup, a linker, a tail and a cholesterol tail. The LNP can have, for example, good tumor delivery, extended circulation in the blood, small particles (e.g., less than 100 nm), and stability in the tumor microenvironment (which has low pH and is hypoxic).

[00167] Neutral liposomes (NL) are non-cationic lipid based particles.

[00168] Polymer nanoparticles are self-assembling polymer-based particles.

[00169] Double-stranded RNA binding motifs (dsRBMs) are self-assembling RNA binding proteins, which will need modifications.

GPR68 Aptamers

[00170] In one embodiment, the modulator of a GPR68 gene product may be an aptamer, including for example a protein aptamer or a polynucleotidal aptamer. In one embodiment, the aptamer inhibits or enhances the expression, activity, or both of a GPR68 gene product.

[00171] In one embodiment, an apatmer is a nucleic acid or oligonucleotide molecule that binds to a specific molecular target, such as a GPR68 gene product comprising a sequence set forth in SEQ ID NOs: 1-15. In one embodiment, aptamers are obtained from an in vitro evolutionary process known as SELEX (Systematic Evolution of Ligands by EXponential Enrichment), which selects target-specific aptamer sequences from combinatorial libraries of single stranded oligonucleotide templates comprising randomized sequences. In some embodiments, aptamer compositions are double-stranded or single-stranded, and in various embodiments include deoxyribonucleotides, ribonucleotides, nucleotide derivatives, or other nucleotide-like molecules. In some embodiments, the nucleotide components of an aptamer include modified or non-natural nucleotides, for example nucleotides that have modified sugar groups (e.g., the 2′-OH group of a ribonucleotide is replaced by 2′-F or 2′-NH2), which in some instances, improves a desired property, e.g., resistance to nucleases or longer lifetime in blood.

[00172] In some instances, individual aptamers having the same nucleotide sequence differ in their secondary structure. In some embodiments, the aptamers of the invention are conjugated to other molecules, e.g., a high molecular weight carrier to slow clearance of the aptamer from the circulatory system. In some instances, aptamers are specifically cross-linked to their cognate ligands, e.g., by photo-activation of a cross-linker. (Brody, E. N. and L. Gold (2000) J. Biotechnol. 74:5-13).

[00173] A method for the in vitro evolution of nucleic acid molecules with high affinity binding to target molecules is known to those of skill in the art and is described in U.S. Pat. No. 5,270,163. The method, known as SELEX (Selective Evolution of Ligands by EXponential Enrichment) involves selection from a mixture of candidate oligonucleotides from a library comprising a large sequence variations (e.g. about 1015) and step-wise iterations of binding, partitioning and amplification, using the same general selection theme, to achieve virtually any desired criterion of binding affinity and selectivity.

[00174] Starting from a mixture of nucleic acids, preferably comprising a segment of randomized sequence, the SELEX method includes the steps of contacting the mixture with the desired target, partitioning unbound nucleic acids from those nucleic acids which have bound to the target molecule, dissociating the nucleic acid-target complexes, amplifying the nucleic acids

dissociated from the nucleic acid-target complexes to yield a ligand-enriched mixture of nucleic acids, then reiterating the steps of binding, partitioning, dissociating and amplifying through as many cycles as desired to yield high affinity nucleic acid ligands to the target molecule.

CRISPR to Modulate GPR68

[00175] By "CRISPR" or "CRISPR to GPR68" or "CRISPR to modulate GPR68" and the like is meant a set of clustered regularly interspaced short palindromic repeats, or a system comprising such a set of repeats. By "Cas", as used herein, is meant a CRISPR-associated protein. By "CRISPR/Cas" system is meant a system derived from CRISPR and Cas which can be used to silence, enhance or mutate the GPR68 gene.

[00176] Naturally-occurring CRISPR/Cas systems are found in approximately 40% of sequenced eubacteria genomes and 90% of sequenced archaea (Grissa et al. 2007. BMC Bioinformatics 8: 172). This system is a type of prokaryotic immune system that confers resistance to foreign genetic elements such as plasmids and phages and provides a form of acquired immunity (Barrangou et al. 2007. Science 315: 1709-1712; Marragini et al. 2008 Science 322: 1843-1845).

[00177] The CRISPR/Cas system has been modified for use in gene editing (silencing, enhancing or changing specific genes) in eukaryotes such as mice or primates (Wiedenheft et al. 2012. Nature 482: 331-8). This is accomplished by introducing into the eukaryotic cell a plasmid containing a specifically designed CRISPR and one or more appropriate Cas.

[00178] The CRISPR sequence, sometimes called a CRISPR locus, comprises alternating repeats and spacers. In a naturally-occurring CRISPR, the spacers usually comprise sequences foreign to the bacterium such as a plasmid or phage sequence; in the GPR68 CRISPR/Cas system, the spacers are derived from the GPR68 gene sequence. The repeats generally show some dyad symmetry, and may form a secondary structure such as a hairpin, and may or may not be palindromic.

[00179] RNA from the CRISPR locus is constitutively expressed and processed by Cas proteins into small RNAs. These processed RNAs comprise a spacer flanked by a repeat sequence. The RNAs guide other Cas proteins to silence exogenous genetic elements at the RNA or DNA level (Horvath et al. 2010. Science 327: 167-170; Makarova et al. 2006 Biology Direct 1: 7). The spacers thus serve as templates for RNA molecules, analogously to siRNAs (Pennisi 2013. Science 341: 833-836).

[00180] As these naturally occur in many different types of bacteria, the exact arrangements of the CRISPR and structure, function and number of Cas genes and their

product differ somewhat from species to species (Haft et al. 2005 PLoS Comput. Biol. 1: e60; Kunin et al. 2007. Genome Biol. 8: R61; Mojica et al. 2005. J. Mol. Evol. 60: 174-182; Bolotin et al. 2005. Microbiol. 151: 2551-2561; Pourcel et al. 2005. Microbiol. 151: 653-663; and Stern et al. 2010. Trends. Genet. 28: 335-340). For example, the Cse (Cas subtype, E. coli) proteins (e.g., CasA) form a functional complex, Cascade, that processes CRISPR RNA transcripts into spacer-repeat units that Cascade retains (Brouns et al. 2008. Science 321: 960-964). In other prokaryotes, Cas6 processes the CRISPR transcript. The CRISPR-based phage inactivation in E. coli requires Cascade and Cas3, but not Cas1 or Cas2. The Cmr (Cas RAMP module) proteins in Pyrococcus furiosus and other prokaryotes form a functional complex with small CRISPR RNAs that recognizes and cleaves complementary target RNAs. A simpler CRISPR system relies on the protein Cas9, which is a nuclease with two active cutting sites, one for each strand of the double helix. Combining Cas9 and modified CRISPR locus RNA can be used in a system for gene editing (Pennisi 2013. Science 341: 833-836).

[00181] The CRISPR/Cas system can thus be used to edit the GPR68 gene (adding or deleting a basepair), e.g., repairing a damaged GPR68 gene (e.g., if the damage to GPR68 results in high or low post-translational modification, production, expression, level, stability or activity of GPR68), or introducing a premature stop which thus decreases expression of an over-expressed GPR68. The CRISPR/Cas system can alternatively be used like RNA interference, turning off the GPR68 gene in a reversible fashion. In a mammalian cell, for example, the RNA can guide the Cas protein to the GPR68 promoter, sterically blocking RNA polymerases.

[00182] Artificial CRISPR systems can be generated which inhibit GPR68, using technology known in the art, e.g., that described in U.S. Patent App. No. 13/842859 (published as US 20140068797). Such GPR68-inhibitory CRISPR system can include a guide RNA (gRNA) comprising a GPR68-targeting domain, i.e., a nucleotide sequence that is complementary to a GPR68 DNA strand, and a second domain that interacts with an RNA-directed nuclease, e.g., cpf1 or Cas molecule, e.g., Cas9 molecule.

[00183] In some embodiments, the ability of an RNA-directed nuclease, e.g., cpf1 or Cas molecule, e.g., Cas9 molecule, to interact with and cleave a target nucleic acid is Protospacer Adjacent Motif (PAM) sequence dependent. A PAM sequence is a sequence in the target nucleic acid. In some embodiments, cleavage of the target nucleic acid occurs upstream from the PAM sequence. RNA-directed nuclease molecules, e.g., cpf1 or Cas molecules, e.g., Cas9 molecules, from different bacterial species can recognize different sequence motifs (e.g., PAM sequences). In addition to recognizing different PAM sequences, RNA-directed nucleases, e.g.,

cpf1 or Cas molecules, e.g., Cas9 molecules, from different species may be directed to different target sequences (e.g., target sequences adjacent, e.g., immediately upstream, to the PAM sequence) by gRNA molecules comprising targeting domains capable of hybridizing to said target sequences and a tracr sequence that binds to said RNA-directed nuclease, e.g., cpf1 or Cas molecule, e.g., Cas9 molecule.

[00184] In some embodiments, the CRISPR system comprises a gRNA molecule and a Cas9 molecule from S. pyogenes. A Cas9 molecule of S. pyogenes recognizes the sequence motif NGG and directs cleavage of a target nucleic acid sequence 1 to 10, e.g., 3 to 5, base pairs upstream from that sequence. A gRNA molecule useful with S. pyogenes-based CRISPR systems may include a GPR68-targeting sequence described in Table 1, e.g., any of SEQ ID NOs: 21-678, and a tracr sequence known to interact with S. Pyogenes (see, e.g., Mali el ai, SCIENCE 2013; 339(6121): 823- 826).

[00185] In some embodiments, the CRISPR system comprises a gRNA molecule and a Cas9 molecule from S. thermophilus. A Cas9 molecule of S. thermophilus recognizes the sequence motif NGGNG and NNAGAAW (W = A or T) and directs cleavage of a core target nucleic acid sequence 1 to 10, e.g., 3 to 5, base pairs upstream from these sequences. A gRNA molecule useful with S. thermophilus-based CRISPR systems may include a GPR68-targeting sequence, and a tracr sequence known to interact with S. thermophilus (see, e.g., Horvath et al., SCIENCE 2010; 327(5962): 167- 170, and Deveau et al., J BACTERIOL 2008; 190(4): 1390- 1400).

[00186] In some embodiments, the CRISPR system comprises a gRNA molecule and a Cas9 molecule from S. aureus. A Cas9 molecule of S. aureus recognizes the sequence motif NNGRR (R = A or G) and directs cleavage of a target nucleic acid sequence 1 to 10, e.g., 3 to 5, base pairs upstream from that sequence. A gRNA molecule useful with S. aureus-based CRISPR systems may include a GPR68-targeting sequence, and a tracr sequence known to interact with S. aureus (see, e.g., Ran F. et al., NATURE, vol. 520, 2015, pp. 186-191).

[00187] In some embodiments, the CRISPR system comprises a gRNA molecule and a RNA-directed nuclease, e.g., cpf1 molecule, e.g., a cpf1 molecule from Lachnospiraceae bacterium or a cpf1 molecule from Acidaminococcus sp. A cpf1 molecule, e.g., a cpf1 molecule from Lachnospiraceae bacterium or a cpf1 molecule from Acidaminococcus sp., recognizes the sequence motive of TTN (where N = A, T, G or C) or preferably TTTN (where N = A, T, G or C), and directs cleavage of a target nucleic acid sequence 1-25 base pairs upstream of the PAM sequence, e.g., 18-19 base pairs upstream from the PAM sequence on the same strand as the PAM and 23 base pairs upstream of the PAM sequence on the opposite strand as the PAM,

creating a sticky end break. A gRNA molecule useful with cpf1-based CRISPR systems (e.g., those utilizing cpf1 molecules from Lachnospiraceae bacterium or Acidaminococcus sp.) may include a GPR68-targeting sequence, and a tracr sequence which interacts with cpf1 (see, e.g., Zetsche B. et al., CELL, vol. 163:3, Oct. 2015, 759-771).

Table 1. GPR68-targeting sequences

ID	STRAND	GENOMIC LOCATION (hg38)	gRNA SEQUENCE	SEQ ID NO
8111_3::chr14:91232511-91235199_3	+	chr14:91232538-91232563	ACUUUGCUUUAUUUGCAGACUGAAG	21
8111_3::chr14:91232511-91235199_4	+	chr14:91232539-91232564	CUUUGCUUUAUUUGCAGACUGAAGU	22
8111_3::chr14:91232511-91235199_13	+	chr14:91232579-91232604	AUUACAGAGUCAACACAUGUAGACU	23
8111_3::chr14:91232511-91235199_16	+	chr14:91232587-91232612	GUCAACACAUGUAGACUUGGCAAAA	24
8111_3::chr14:91232511-91235199_21	+	chr14:91232656-91232681	CUCAGCUCAUCCCAGUCUGUAUCAG	25
8111_3::chr14:91232511-91235199_23	+	chr14:91232657-91232682	UCAGCUCAUCCCAGUCUGUAUCAGU	26
8111_3::chr14:91232511-91235199_25	+	chr14:91232673-91232698	UGUAUCAGUGGGAAGCCAGUGUUUA	27
8111_3::chr14:91232511-91235199_27	+	chr14:91232683-91232708	GGAAGCCAGUGUUUAAGGUCAACAU	28
8111_3::chr14:91232511-91235199_30	+	chr14:91232686-91232711	AGCCAGUGUUUAAGGUCAACAUAGG	29
8111_3::chr14:91232511-91235199_35	+	chr14:91232697-91232722	AAGGUCAACAUAGGAGGAAGUGCUG	30
8111_3::chr14:91232511-91235199_39	+	chr14:91232704-91232729	ACAUAGGAGGAAGUGCUGUGGAAUG	31
8111_3::chr14:91232511-91235199_41	+	chr14:91232707-91232732	UAGGAGGAAGUGCUGUGGAAUGAGG	32
8111_3::chr14:91232511-91235199_43	+	chr14:91232715-91232740	AGUGCUGUGGAAUGAGGAGGCAUGA	33
8111_3::chr14:91232511-91235199_46	+	chr14:91232750-91232775	CAAUGUCACUGACCCAGUAAAAUCC	34
8111_3::chr14:91232511-91235199_49	+	chr14:91232764-91232789	CAGUAAAAUCCUGGACUGAGUUGCA	35
8111_3::chr14:91232511-91235199_50	+	chr14:91232765-91232790	AGUAAAAUCCUGGACUGAGUUGCAU	36
8111_3::chr14:91232511-91235199_51	+	chr14:91232775-91232800	UGGACUGAGUUGCAUGGGCUCUUCU	37
8111_3::chr14:91232511-91235199_52	+	chr14:91232783-91232808	GUUGCAUGGGCUCUUCUUGGCUGUG	38
8111_3::chr14:91232511-91235199_56	+	chr14:91232808-91232833	UGGCCAUUUCUGCCGCCUUCAAACU	39
8111_3::chr14:91232511-91235199_61	+	chr14:91232825-91232850	UUCAAACUUGGUCACUUCCUCCUCU	40
8111_3::chr14:91232511-91235199_64	+	chr14:91232829-91232854	AACUUGGUCACUUCCUCCUCUAGGA	41
8111_3::chr14:91232511-91235199_66	+	chr14:91232830-91232855	ACUUGGUCACUUCCUCCUCUAGGAA	42

8111_3::chr14:91232511-91235199_68	+	chr14:91232831-91232856	CUUGGUCACUUCCUCCUCUAGGAAG	43
8111_3::chr14:91232511-91235199_75	+	chr14:91232855-91232880	GGGGAAGAGAACCUCCCCAAGUCAU	44
8111_3::chr14:91232511-91235199_77	+	chr14:91232870-91232895	CCCAAGUCAUCGGACACCCUCCACA	45
8111_3::chr14:91232511-91235199_81	+	chr14:91232881-91232906	GGACACCCUCCACAAGGAUCUGAGC	46
8111_3::chr14:91232511-91235199_82	+	chr14:91232882-91232907	GACACCCUCCACAAGGAUCUGAGCA	47
8111_3::chr14:91232511-91235199_83	+	chr14:91232883-91232908	ACACCCUCCACAAGGAUCUGAGCAG	48
8111_3::chr14:91232511-91235199_85	+	chr14:91232913-91232938	GUGCAGUCAAAGCCAAGUGUAGCCC	49
8111_3::chr14:91232511-91235199_87	+	chr14:91232914-91232939	UGCAGUCAAAGCCAAGUGUAGCCCU	50
8111_3::chr14:91232511-91235199_91	+	chr14:91232938-91232963	UGGGACCACUGUGAGCACCCUUCCC	51
8111_3::chr14:91232511-91235199_92	+	chr14:91232939-91232964	GGGACCACUGUGAGCACCCUUCCCU	52
8111_3::chr14:91232511-91235199_93	+	chr14:91232940-91232965	GGACCACUGUGAGCACCCUUCCCUG	53
8111_3::chr14:91232511-91235199_95	+	chr14:91232954-91232979	ACCCUUCCCUGGGGCACCUCCAGCC	54
8111_3::chr14:91232511-91235199_96	+	chr14:91232955-91232980	CCCUUCCCUGGGGCACCUCCAGCCU	55
8111_3::chr14:91232511-91235199_99	+	chr14:91232968-91232993	CACCUCCAGCCUGGGCUGUUGCCUC	56
8111_3::chr14:91232511-91235199_100	+	chr14:91232969-91232994	ACCUCCAGCCUGGGCUGUUGCCUCU	57
8111_3::chr14:91232511-91235199_103	+	chr14:91232985-91233010	GUUGCCUCUGGGCAUCCCCGAUGAA	58
8111_3::chr14:91232511-91235199_104	+	chr14:91232986-91233011	UUGCCUCUGGGCAUCCCCGAUGAAA	59
8111_3::chr14:91232511-91235199_108	+	chr14:91233007-91233032	GAAAGGGCUAAGAGACACAGCACAA	60
8111_3::chr14:91232511-91235199_109	+	chr14:91233023-91233048	ACAGCACAAAGGAUCAACCUCUUCA	61
8111_3::chr14:91232511-91235199_112	+	chr14:91233027-91233052	CACAAAGGAUCAACCUCUUCAAGGC	62
8111_3::chr14:91232511-91235199_113	+	chr14:91233028-91233053	ACAAAGGAUCAACCUCUUCAAGGCU	63
8111_3::chr14:91232511-91235199_116	+	chr14:91233029-91233054	CAAAGGAUCAACCUCUUCAAGGCUG	64
8111_3::chr14:91232511-91235199_117	+	chr14:91233030-91233055	AAAGGAUCAACCUCUUCAAGGCUGG	65
8111_3::chr14:91232511-91235199_120	+	chr14:91233031-91233056	AAGGAUCAACCUCUUCAAGGCUGGG	66
8111_3::chr14:91232511-91235199_124	+	chr14:91233037-91233062	CAACCUCUUCAAGGCUGGGGGGAAG	67
8111_3::chr14:91232511-91235199_125	+	chr14:91233038-91233063	AACCUCUUCAAGGCUGGGGGGAAGA	68
8111_3::chr14:91232511-91235199_127	+	chr14:91233039-91233064	ACCUCUUCAAGGCUGGGGGGAAGAG	69
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8111_3::chr14:91232511-91235199_141	+	chr14:91233057-91233082	GGAAGAGGGGAAGGGAGACUGAGGA	73
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8111_3::chr14:91232511-91235199_145	+	chr14:91233069-91233094	GGGAGACUGAGGAAGGGGUUCUGCC	76
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8111_3::chr14:91232511-91235199_172	+	chr14:91233187-91233212	CUUGGUUCUGAAGAACUCGAGUCCC	83
8111_3::chr14:91232511-91235199_176	+	chr14:91233193-91233218	UCUGAAGAACUCGAGUCCCAGGCCA	84
8111_3::chr14:91232511-91235199_178	+	chr14:91233197-91233222	AAGAACUCGAGUCCCAGGCCAUGGA	85
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8111_3::chr14:91232511-91235199_283	+	chr14:91233754-91233779	ACACGGCUUAUUCCCACGAGCCAAC	122
8111_3::chr14:91232511-91235199_284	+	chr14:91233755-91233780	CACGGCUUAUUCCCACGAGCCAACC	123
8111_3::chr14:91232511-91235199_285	+	chr14:91233756-91233781	ACGGCUUAUUCCCACGAGCCAACCG	124
8111_3::chr14:91232511-91235199_288	+	chr14:91233773-91233798	GCCAACCGGGGCUUAUUCCCACCCU	125
8111_3::chr14:91232511-91235199_289	+	chr14:91233776-91233801	AACCGGGGCUUAUUCCCACCCUCGG	126

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8111_3::chr14:91232511-91235199_320	+	chr14:91233863-91233888	CGCAGCGAGGGAAGCAGGAGGCGGC	139
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8111_3::chr14:91232511-91235199_327	+	chr14:91233886-91233911	GCAGGCCCUGAGGCCCGUGGGCUGA	143
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8111_3::chr14:91232511-91235199_336	+	chr14:91233917-91233942	AGGCCUCACGUGGAGCCACCCGCGG	147
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8111_3::chr14:91232511-91235199_340	+	chr14:91233944-91233969	GACCCAGGCUAGGCCAACCUGCCCG	150
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8111_3::chr14:91232511-91235199_346	+	chr14:91233963-91233988	UGCCCGUGGGGAACCCGCCCGACCC	153
8111_3::chr14:91232511-91235199_349	+	chr14:91233972-91233997	GGAACCCGCCCGACCCUGGCGAGUU	154

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8111_3::chr14:91232511-91235199_352	+	chr14:91233974-91233999	AACCCGCCCGACCCUGGCGAGUUAG	156
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8111_3::chr14:91232511-91235199_1112	-	chr14:91233979-91234004	GACCCCUAACUCGCCAGGGUCGGGC	487
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8111_3::chr14:91232511-91235199_1116	-	chr14:91233983-91234008	UCCAGACCCCUAACUCGCCAGGGUC	489
8111_3::chr14:91232511-91235199_1117	-	chr14:91233984-91234009	UUCCAGACCCCUAACUCGCCAGGGU	490

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8111_3::chr14:91232511-91235199_1123	-	chr14:91234014-91234039	CCCGAGCUGUUGACCAAGCUCCACC	493
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8111_3::chr14:91232511-91235199_1129	-	chr14:91234049-91234074	AGGCCUCCGGGAAAAGCGGGGCCCA	495
8111_3::chr14:91232511-91235199_1130	-	chr14:91234050-91234075	GAGGCCUCCGGGAAAAGCGGGGCCC	496
8111_3::chr14:91232511-91235199_1132	-	chr14:91234056-91234081	GCCCCCGAGGCCUCCGGGAAAAGCG	497
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8111_3::chr14:91232511-91235199_1137	-	chr14:91234066-91234091	CCCGCUGGGUGCCCCCGAGGCCUCC	500
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8111_3::chr14:91232511-91235199_1161	-	chr14:91234146-91234171	CACCGGGACCUGGCCCGCCUCCGCG	513
8111_3::chr14:91232511-91235199_1162	-	chr14:91234147-91234172	CCACCGGGACCUGGCCCGCCUCCGC	514
8111_3::chr14:91232511-91235199_1164	-	chr14:91234148-91234173	CCCACCGGGACCUGGCCCGCCUCCG	515
8111_3::chr14:91232511-91235199_1166	-	chr14:91234161-91234186	GUCAGCGAGACCACCCACCGGGACC	516
8111_3::chr14:91232511-91235199_1168	-	chr14:91234167-91234192	UGCUUCGUCAGCGAGACCACCCACC	517
8111_3::chr14:91232511-91235199_1170	-	chr14:91234168-91234193	CUGCUUCGUCAGCGAGACCACCCAC	518

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8111_3::chr14:91232511-91235199_1184	-	chr14:91234290-91234315	GUGUUGCUGCUGGUGCGCAGCGUCU	522
8111_3::chr14:91232511-91235199_1186	-	chr14:91234291-91234316	CGUGUUGCUGCUGGUGCGCAGCGUC	523
8111_3::chr14:91232511-91235199_1190	-	chr14:91234305-91234330	UUCCUGCCCUACCACGUGUUGCUGC	524
8111_3::chr14:91232511-91235199_1192	-	chr14:91234338-91234363	GUGCUCAGCACCGUGGUCAUCUUCC	525
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8111_3::chr14:91232511-91235199_1206	-	chr14:91234434-91234459	AUCUGCCUGCUGCUGGCGUCCUACC	533
8111_3::chr14:91232511-91235199_1210	-	chr14:91234446-91234471	UUCCUCUUCCCCAUCUGCCUGCUGC	534
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8111_3::chr14:91232511-91235199_1233	-	chr14:91234608-91234633	GGCGUCAGCGUGGUCAUCUGGGCCA	543
8111_3::chr14:91232511-91235199_1236	-	chr14:91234614-91234639	GCCGUCGGCGUCAGCGUGGUCAUCU	544
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8111_3::chr14:91232511-91235199_1239	-	chr14:91234623-91234648	CUGAAGGCGGCCGUCGGCGUCAGCG	546

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8111_3::chr14:91232511-91235199_1247	-	chr14:91234654-91234679	CCAUCCCUUCCGCUUCCACCAGUUC	550
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8111_3::chr14:91232511-91235199_1269	-	chr14:91234883-91234908	UGCAGAUCAAGGCCCGGAACGAGCU	563
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8111_3::chr14:91232511-91235199_1274	-	chr14:91234894-91234919	CUUCGGCUACCUGCAGAUCAAGGCC	565
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8111_3::chr14:91232511-91235199_1282	-	chr14:91234949-91234974	UGGUCUAUGUUACCGUGCUGGUGGU	569
8111_3::chr14:91232511-91235199_1283	-	chr14:91234950-91234975	GUGGUCUAUGUUACCGUGCUGGUGG	570
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8111_3::chr14:91232511-91235199_1288	-	chr14:91234977-91235002	CAUACCAUCCACCAGACGCUGGCCC	574

8111_3::chr14:91232511-91235199_1289	-	chr14:91234983-91235008	AUCGACCAUACCAUCCACCAGACGC	575
8111_3::chr14:91232511-91235199_1293	-	chr14:91235047-91235072	UGUGGCCCCUUCAGGCCCAAAGAUG	576
8111_3::chr14:91232511-91235199_1295	-	chr14:91235048-91235073	GUGUGGCCCCUUCAGGCCCAAAGAU	577
8111_3::chr14:91232511-91235199_1296	-	chr14:91235049-91235074	AGUGUGGCCCCUUCAGGCCCAAAGA	578
8111_3::chr14:91232511-91235199_1299	-	chr14:91235060-91235085	AGCCCAUGAGGAGUGUGGCCCCUUC	579
8111_3::chr14:91232511-91235199_1300	-	chr14:91235070-91235095	CCAGUCCCUGAGCCCAUGAGGAGUG	580
8111_3::chr14:91232511-91235199_1302	-	chr14:91235077-91235102	GACAGGCCCAGUCCCUGAGCCCAUG	581
8111_3::chr14:91232511-91235199_1306	-	chr14:91235099-91235124	AUAAACAGGAUGGCGGUGGAGAGAC	582
8111_3::chr14:91232511-91235199_1309	-	chr14:91235108-91235133	CCACCUUCUAUAAACAGGAUGGCGG	583
8111_3::chr14:91232511-91235199_1312	-	chr14:91235111-91235136	GAACCACCUUCUAUAAACAGGAUGG	584
8111_3::chr14:91232511-91235199_1314	-	chr14:91235114-91235139	CUUGAACCACCUUCUAUAAACAGGA	585
8111_3::chr14:91232511-91235199_1315	-	chr14:91235118-91235143	AGAGCUUGAACCACCUUCUAUAAAC	586
8111_3::chr14:91232511-91235199_1320	-	chr14:91235147-91235172	CCCACCACCAGCGAUGCCCAGCCCU	587
8111_2::chr14:91244276-91244528_7	+	chr14:91244327-91244352	UAUUCUAUUUCAAUUUUUUAAAUGC	588
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8111_2::chr14:91244276-91244528_21	+	chr14:91244366-91244391	GAAGUUGAUUUACUGACCCAUGAAU	590
8111_2::chr14:91244276-91244528_27	+	chr14:91244384-91244409	CAUGAAUGGGUCACAACCCGCAGUU	591
8111_2::chr14:91244276-91244528_29	+	chr14:91244394-91244419	UCACAACCCGCAGUUUGGAAACAGC	592
8111_2::chr14:91244276-91244528_31	+	chr14:91244398-91244423	AACCCGCAGUUUGGAAACAGCUGGA	593
8111_2::chr14:91244276-91244528_36	+	chr14:91244438-91244463	GCUUUCCAGCUUCUCCUAGAGCCUC	594
8111_2::chr14:91244276-91244528_37	+	chr14:91244439-91244464	CUUUCCAGCUUCUCCUAGAGCCUCA	595
8111_2::chr14:91244276-91244528_42	+	chr14:91244446-91244471	GCUUCUCCUAGAGCCUCAGGGCCCC	596
8111_2::chr14:91244276-91244528_43	+	chr14:91244450-91244475	CUCCUAGAGCCUCAGGGCCCCAGGA	597
8111_2::chr14:91244276-91244528_45	+	chr14:91244458-91244483	GCCUCAGGGCCCCAGGAAGGCAGUG	598
8111_2::chr14:91244276-91244528_46	+	chr14:91244472-91244497	GGAAGGCAGUGUGGUCUCUGCAACC	599
8111_2::chr14:91244276-91244528_49	+	chr14:91244477-91244502	GCAGUGUGGUCUCUGCAACCAGGUG	600
8111_2::chr14:91244276-91244528_51	+	chr14:91244483-91244508	UGGUCUCUGCAACCAGGUGAGGAGC	601
8111_2::chr14:91244276-91244528_53	+	chr14:91244500-91244525	UGAGGAGCAGGCAGCUGCUGAAAUC	602

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8111_2::chr14:91244276-91244528_66	-	chr14:91244310-91244335	UAUCUUAUCUUUACUAGUCUCACGC	608
8111_2::chr14:91244276-91244528_77	-	chr14:91244385-91244410	UUUGACGCCCAACACUGGGUAAGUA	609
8111_2::chr14:91244276-91244528_78	-	chr14:91244386-91244411	GUUUGACGCCCAACACUGGGUAAGU	610
8111_2::chr14:91244276-91244528_82	-	chr14:91244403-91244428	GUCCAUCCAGCUGAAAGGUUUGACG	611
8111_2::chr14:91244276-91244528_83	-	chr14:91244404-91244429	GGUCCAUCCAGCUGAAAGGUUUGAC	612
8111_2::chr14:91244276-91244528_85	-	chr14:91244430-91244455	AGGAGAAGCUGGAAAGCACAGGUGG	613
8111_2::chr14:91244276-91244528_86	-	chr14:91244431-91244456	UAGGAGAAGCUGGAAAGCACAGGUG	614
8111_2::chr14:91244276-91244528_87	-	chr14:91244432-91244457	CUAGGAGAAGCUGGAAAGCACAGGU	615
8111_2::chr14:91244276-91244528_89	-	chr14:91244433-91244458	UCUAGGAGAAGCUGGAAAGCACAGG	616
8111_2::chr14:91244276-91244528_92	-	chr14:91244436-91244461	GGCUCUAGGAGAAGCUGGAAAGCAC	617
8111_2::chr14:91244276-91244528_93	-	chr14:91244446-91244471	GGGGCCCUGAGGCUCUAGGAGAAGC	618
8111_2::chr14:91244276-91244528_99	-	chr14:91244455-91244480	UGCCUUCCUGGGGCCCUGAGGCUCU	619
8111_2::chr14:91244276-91244528_102	-	chr14:91244462-91244487	ACCACACUGCCUUCCUGGGGCCCUG	620
8111_2::chr14:91244276-91244528_105	-	chr14:91244470-91244495	UUGCAGAGACCACACUGCCUUCCUG	621
8111_2::chr14:91244276-91244528_106	-	chr14:91244471-91244496	GUUGCAGAGACCACACUGCCUUCCU	622
8111_2::chr14:91244276-91244528_107	-	chr14:91244472-91244497	GGUUGCAGAGACCACACUGCCUUCC	623
8111_2::chr14:91244276-91244528_113	-	chr14:91244498-91244523	UUUCAGCAGCUGCCUGCUCCUCACC	624
8111_1::chr14:91253694-91253900_1	+	chr14:91253694-91253719	UGGCUCGCGGGGACACCUACCUGCG	625
8111_1::chr14:91253694-91253900_4	+	chr14:91253699-91253724	CGCGGGGACACCUACCUGCGCGGCG	626
8111_1::chr14:91253694-91253900_5	+	chr14:91253700-91253725	GCGGGGACACCUACCUGCGCGGCGC	627
8111_1::chr14:91253694-91253900_6	+	chr14:91253701-91253726	CGGGGACACCUACCUGCGCGGCGCG	628
8111_1::chr14:91253694-91253900_7	+	chr14:91253707-91253732	CACCUACCUGCGCGGCGCGGGGCUG	629
8111_1::chr14:91253694-91253900_8	+	chr14:91253713-91253738	CCUGCGCGGCGCGGGGCUGUGGCGC	630

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8111_1::chr14:91253694-91253900_12	+	chr14:91253763-91253788	CUCUUGCCAGCGCGCACGCAGAUGG	632
8111_1::chr14:91253694-91253900_14	+	chr14:91253764-91253789	UCUUGCCAGCGCGCACGCAGAUGGC	633
8111_1::chr14:91253694-91253900_15	+	chr14:91253765-91253790	CUUGCCAGCGCGCACGCAGAUGGCG	634
8111_1::chr14:91253694-91253900_16	+	chr14:91253768-91253793	GCCAGCGCGCACGCAGAUGGCGGGG	635
8111_1::chr14:91253694-91253900_20	+	chr14:91253773-91253798	CGCGCACGCAGAUGGCGGGGUGGCC	636
8111_1::chr14:91253694-91253900_21	+	chr14:91253774-91253799	GCGCACGCAGAUGGCGGGGUGGCCU	637
8111_1::chr14:91253694-91253900_23	+	chr14:91253775-91253800	CGCACGCAGAUGGCGGGGUGGCCUG	638
8111_1::chr14:91253694-91253900_25	+	chr14:91253778-91253803	ACGCAGAUGGCGGGGUGGCCUGGGG	639
8111_1::chr14:91253694-91253900_27	+	chr14:91253785-91253810	UGGCGGGGUGGCCUGGGGAGGUCUU	640
8111_1::chr14:91253694-91253900_28	+	chr14:91253786-91253811	GGCGGGGUGGCCUGGGGAGGUCUUC	641
8111_1::chr14:91253694-91253900_30	+	chr14:91253797-91253822	CUGGGGAGGUCUUCGGGUCCCUUCC	642
8111_1::chr14:91253694-91253900_33	+	chr14:91253798-91253823	UGGGGAGGUCUUCGGGUCCCUUCCU	643
8111_1::chr14:91253694-91253900_35	+	chr14:91253806-91253831	UCUUCGGGUCCCUUCCUGGGAACGC	644
8111_1::chr14:91253694-91253900_36	+	chr14:91253807-91253832	CUUCGGGUCCCUUCCUGGGAACGCA	645
8111_1::chr14:91253694-91253900_41	+	chr14:91253849-91253874	GAUUCCACGCCCCCCCCACCCACGU	646
8111_1::chr14:91253694-91253900_42	+	chr14:91253850-91253875	AUUCCACGCCCCCCCCACCCACGUC	647
8111_1::chr14:91253694-91253900_47	+	chr14:91253871-91253896	CGUCGGGCACACGCAGCCCUGAGUG	648
8111_1::chr14:91253694-91253900_48	+	chr14:91253872-91253897	GUCGGGCACACGCAGCCCUGAGUGA	649
8111_1::chr14:91253694-91253900_50	-	chr14:91253712-91253737	CGCCACAGCCCCGCGCCGCGCAGGU	650
8111_1::chr14:91253694-91253900_51	-	chr14:91253716-91253741	CCGGCGCCACAGCCCCGCGCCGCGC	651
8111_1::chr14:91253694-91253900_52	-	chr14:91253740-91253765	AGGAGGGGCGCGGGGUAAUGGUGGC	652
8111_1::chr14:91253694-91253900_53	-	chr14:91253744-91253769	CAAGAGGAGGGGCGCGGGGUAAUGG	653
8111_1::chr14:91253694-91253900_54	-	chr14:91253747-91253772	UGGCAAGAGGAGGGGCGCGGGGUAA	654
8111_1::chr14:91253694-91253900_55	-	chr14:91253753-91253778	GCGCGCUGGCAAGAGGAGGGGCGCG	655
8111_1::chr14:91253694-91253900_56	-	chr14:91253754-91253779	UGCGCGCUGGCAAGAGGAGGGGCGC	656
8111_1::chr14:91253694-91253900_57	-	chr14:91253755-91253780	GUGCGCGCUGGCAAGAGGAGGGGCG	657
8111_1::chr14:91253694-91253900_60	-	chr14:91253760-91253785	UCUGCGUGCGCGCUGGCAAGAGGAG	658

8111_1::chr14:91253694-91253900_61	-	chr14:91253761-91253786	AUCUGCGUGCGCGCUGGCAAGAGGA	659
8111_1::chr14:91253694-91253900_63	-	chr14:91253762-91253787	CAUCUGCGUGCGCGCUGGCAAGAGG	660
8111_1::chr14:91253694-91253900_66	-	chr14:91253765-91253790	CGCCAUCUGCGUGCGCGCUGGCAAG	661
8111_1::chr14:91253694-91253900_69	-	chr14:91253772-91253797	GCCACCCCGCCAUCUGCGUGCGCGC	662
8111_1::chr14:91253694-91253900_70	-	chr14:91253799-91253824	CAGGAAGGGACCCGAAGACCUCCCC	663
8111_1::chr14:91253694-91253900_74	-	chr14:91253818-91253843	CAACUUGGCCCUGCGUUCCCAGGAA	664
8111_1::chr14:91253694-91253900_76	-	chr14:91253819-91253844	ACAACUUGGCCCUGCGUUCCCAGGA	665
8111_1::chr14:91253694-91253900_78	-	chr14:91253823-91253848	GAGCACAACUUGGCCCUGCGUUCCC	666
8111_1::chr14:91253694-91253900_81	-	chr14:91253838-91253863	GGGGGCGUGGAAUCGGAGCACAACU	667
8111_1::chr14:91253694-91253900_83	-	chr14:91253850-91253875	GACGUGGGUGGGGGGGGCGUGGAAU	668
8111_1::chr14:91253694-91253900_86	-	chr14:91253856-91253881	GUGCCCGACGUGGGUGGGGGGGGCG	669
8111_1::chr14:91253694-91253900_88	-	chr14:91253861-91253886	UGCGUGUGCCCGACGUGGGUGGGGG	670
8111_1::chr14:91253694-91253900_89	-	chr14:91253862-91253887	CUGCGUGUGCCCGACGUGGGUGGGG	671
8111_1::chr14:91253694-91253900_91	-	chr14:91253863-91253888	GCUGCGUGUGCCCGACGUGGGUGGG	672
8111_1::chr14:91253694-91253900_92	-	chr14:91253864-91253889	GGCUGCGUGUGCCCGACGUGGGUGG	673
8111_1::chr14:91253694-91253900_94	-	chr14:91253865-91253890	GGGCUGCGUGUGCCCGACGUGGGUG	674
8111_1::chr14:91253694-91253900_96	-	chr14:91253866-91253891	AGGGCUGCGUGUGCCCGACGUGGGU	675
8111_1::chr14:91253694-91253900_99	-	chr14:91253867-91253892	CAGGGCUGCGUGUGCCCGACGUGGG	676
8111_1::chr14:91253694-91253900_101	-	chr14:91253870-91253895	ACUCAGGGCUGCGUGUGCCCGACGU	677
8111_1::chr14:91253694-91253900_102	-	chr14:91253871-91253896	CACUCAGGGCUGCGUGUGCCCGACG	678

TALEN to Inhibit GPR68

[00188] By "TALEN" or "TALEN to GPR68" or "TALEN to inhibit GPR68" and the like is meant a transcription activator-like effector nuclease, an artificial nuclease which can be used to edit a GPR68 gene.

[00189] TALENs are produced artificially by fusing a TAL effector DNA binding domain to a DNA cleavage domain. Transcription activator-like effects (TALEs) can be engineered to bind any desired DNA sequence, including a portion of the GPR68 gene. By combining an engineered TALE with a DNA cleavage domain, a restriction enzyme can be produced which is specific to any desired DNA sequence, including a GPR68 sequence. These can then be

introduced into a cell, wherein they can be used for genome editing (Boch 2011 Nature Biotech. 29: 135-6; and Boch et al. 2009 Science 326: 1509-12; Moscou et al. 2009 Science 326: 3501).

[00190] TALEs are proteins secreted by Xanthomonas bacteria. The DNA binding domain contains a repeated, highly conserved 33-34 amino acid sequence, with the exception of the 12th and 13th amino acids. These two positions are highly variable, showing a strong correlation with specific nucleotide recognition. They can thus be engineered to bind to a desired DNA sequence.

[00191] To produce a TALEN, a TALE protein is fused to a nuclease (N), which is a wild-type or mutated FokI endonuclease. Several mutations to FokI have been made for its use in TALENs; these, for example, improve cleavage specificity or activity (Cermak et al. 2011 Nucl. Acids Res. 39: e82; Miller et al. 2011 Nature Biotech. 29: 143-8; Hockemeyer et al. 2011 Nature Biotech. 29: 731-734; Wood et al. 2011 Science 333: 307; Doyon et al. 2010 Nature Methods 8: 74-79; Szczepek et al. 2007 Nature Biotech. 25: 786-793; and Guo et al. 2010 J. Mol. Biol. 200: 96).

[00192] The FokI domain functions as a dimer, requiring two constructs with unique DNA binding domains for sites in the target genome with proper orientation and spacing. Both the number of amino acid residues between the TALE DNA binding domain and the FokI cleavage domain and the number of bases between the two individual TALEN binding sites appear to be important parameters for achieving high levels of activity (Miller et al. 2011 Nature Biotech. 29: 143-8).

[00193] A GPR68 TALEN can be used inside a cell to produce a double-stranded break (DSB). A mutation can be introduced at the break site if the repair mechanisms improperly repair the break via non-homologous end joining. For example, improper repair may introduce a frame shift mutation. Alternatively, foreign DNA can be introduced into the cell along with the TALEN; depending on the sequences of the foreign DNA and chromosomal sequence, this process can be used to correct a defect in the GPR68 gene or introduce such a defect into a wt GPR68 gene, thus decreasing expression of GPR68.

[00194] TALENs specific to sequences in GPR68 can be constructed using any method known in the art, including various schemes using modular components (Zhang et al. 2011 Nature Biotech. 29: 149-53; Geibler et al. 2011 PLoS ONE 6: e19509).

Zinc Finger Nuclease to Inhibit GPR68

[00195] By "ZFN" or "Zinc Finger Nuclease" or "ZFN to GPR68" or "ZFN to inhibit GPR68" and the like is meant a zinc finger nuclease, an artificial nuclease which can be used to edit a GPR68 gene.

[00196] Like a TALEN, a ZFN comprises a FokI nuclease domain (or derivative thereof) fused to a DNA-binding domain. In the case of a ZFN, the DNA-binding domain comprises one or more zinc fingers (Carroll et al. 2011. Genetics Society of America 188: 773-782; and Kim et al. Proc. Natl. Acad. Sci. USA 93: 1156-1160).

[00197] A zinc finger is a small protein structural motif stabilized by one or more zinc ions. A zinc finger can comprise, for example, Cys2His2, and can recognize an approximately 3-bp sequence. Various zinc fingers of known specificity can be combined to produce multi-finger polypeptides which recognize about 6, 9, 12, 15 or 18-bp sequences. Various selection and modular assembly techniques are available to generate zinc fingers (and combinations thereof) recognizing specific sequences, including phage display, yeast one-hybrid systems, bacterial one-hybrid and two-hybrid systems, and mammalian cells.

[00198] Like a TALEN, a ZFN must dimerize to cleave DNA. Thus, a pair of ZFNs are required to target non-palindromic DNA sites. The two individual ZFNs must bind opposite strands of the DNA with their nucleases properly spaced apart (Bitinaite et al. 1998 Proc. Natl. Acad. Sci. USA 95: 10570-5).

[00199] Also like a TALEN, a ZFN can create a double-stranded break in the DNA, which can create a frame-shift mutation if improperly repaired, leading to a decrease in the expression and level and/or activity of GPR68 in a cell. ZFNs can also be used with homologous recombination to mutate, or repair defects, in the GPR68 gene.

[00200] ZFNs specific to sequences in GPR68 can be constructed using any method known in the art (Cathomen et al. Mol. Ther. 16: 1200-7; and Guo et al. 2010. J. Mol. Biol. 400: 96).

Anti-GPR68 Antibodies

[00201] Some embodiments of the present disclosure provide anti-GPR68 antibodies as GPR68 modulators. An anti-GPR68 antibody of the invention binds at least one specified epitope specific to a GPR68 protein, subunit, fragment, portion of the invention, or any combination thereof. The epitope can comprise an antibody binding region that comprises at least one portion of the amino acid sequence of GPR68 (e.g., SEQ ID NOs: 1-4), which epitope is preferably comprised of at least 1-5 amino acids of the sequences. The antibody can include or be derived from any mammal, such as, but not limited to, a human, a mouse, a rabbit, a rat, a rodent, a primate, or any combination thereof, and the like.

[00202] An anti-GPR68 antibody, as described herein, has at least one activity, such as, but not limited to inhibiting or enhancing GPR68 activity, such as GPR68-dependent increase in intracellular calcium levels. An anti-GPR68 antibody can thus be screened for a corresponding activity according to known methods, such as but not limited to, at least one biological activity of human GPR68.

[00203] As used herein, an "anti-GPR68 antibody," "anti-GPR68 antibody portion," or "anti-GPR68 antibody fragment" and/or "anti-GPR68 antibody variant" and the like include any protein or polypeptide containing molecule that comprises at least a portion of an immunoglobulin molecule, such as but not limited to, at least one complementarity determining region (CDR) of a heavy or light chain or a ligand binding portion thereof, a heavy chain or light chain variable region, a heavy chain or light chain constant region, a framework region, or any portion thereof. Such antibody optionally further affects a specific ligand, such as but not limited to, where such antibody modulates, decreases, increases, antagonizes, angonizes, mitigates, alleviates, blocks, inhibits, abrogates and/or interferes with at least one GPR68 activity or binding, or with GPR68 ligand activity or binding, in vitro, in situ and/or in vivo. As a non-limiting example, a suitable anti-GPR68 antibody, specified portion or variant of the present invention can bind at least one GPR68 protein or polypeptide of the invention, or specified portions, variants or domains thereof. A suitable anti-GPR68 antibody, specified portion, or variant can also optionally affect at least one of GPR68 activity or function, such as but not limited to, RNA, DNA or protein synthesis, GPR68 release, GPR68 signaling, GPR68 activity, GPR68 production and/or synthesis.

[00204] An "antibody" refers to a molecule of the immunoglobulin family comprising a tetrameric structural unit. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" chain (about 25 kD) and one "heavy" chain (about 50-70

kD), connected through a disulfide bond. Recognized immunoglobulin genes include the κ, λ, α, γ, δ, ε, and μ constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either κ or λ. Heavy chains are classified as γ, μ, α, δ, or ε, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD, and IgE, respectively. Antibodies can be of any isotype/class (e.g., IgG, IgM, IgA, IgD, and IgE), or any subclass (e.g., IgG1, IgG2, IgG3, IgG4, IgA1, IgA2).

[00205] The term "antibody" is further intended to encompass antibodies, digestion fragments, specified portions and variants thereof, including antibody mimetics or portions of antibodies that mimic the structure and/or function of an antibody or specified fragment or portion thereof, including single chain antibodies and fragments thereof. Functional fragments include antigen-binding fragments that bind to a mammalian GPR68. For example, antibody fragments capable of binding to GPR68 or portions thereof, including, but not limited to, Fab (e.g., by papain digestion), Fab′ (e.g., by pepsin digestion and partial reduction) and F(ab′)2 (e.g., by pepsin digestion), facb (e.g., by plasmin digestion), pFc′ (e.g., by pepsin or plasmin digestion), Fd (e.g., by pepsin digestion, partial reduction and reaggregation), Fv or scFv (e.g., by molecular biology techniques) fragments, are encompassed by the invention (see, e.g., Colligan, Immunology, supra).

[00206] Both the light and heavy chains are divided into regions of structural and functional homology. The terms "constant" and "variable" are used structurally and functionally. The N-terminus of each chain defines a variable (V) region or domain of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these regions of light and heavy chains respectively. The pairing of a V_H and V_L together forms a single antigen-binding site. In addition to V regions, both heavy chains and light chains contain a constant (C) region or domain. A secreted form of an immunoglobulin C region is made up of three C domains, CH1, CH2, CH3, optionally CH4 (Cμ), and a hinge region. A membrane-bound form of an immunoglobulin C region also has membrane and intracellular domains. Each light chain has a V_L at the N-terminus followed by a constant domain (C) at its other end. The constant domains of the light chain (CL) and the heavy chain (CH1, CH2 or CH3) confer important biological properties such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like. By convention, the numbering of the constant region domains increases as they become more distal from the antigen binding site or amino-terminus of the antibody. The N-terminus is a variable region and at the C-terminus is a constant region; the CH3 and CL domains actually comprise the carboxy-terminal domains of the heavy and light chain, respectively.The VL is aligned with the VH and

the CL is aligned with the first constant domain of the heavy chain (CH1). As used herein, an "antibody" encompasses conventional antibody structures and variations of antibodies. Thus, within the scope of this concept are full length antibodies, chimeric antibodies, humanized antibodies, human antibodies, and fragments thereof.

[00207] Antibodies exist as intact immunoglobulin chains or as a number of well-characterized antibody fragments produced by digestion with various peptidases. The term "antibody fragment," as used herein, refers to one or more portions of an antibody that retain the ability to specifically interact with (*e.g.*, by binding, steric hindrance, stabilizing/destabilizing, spatial distribution) an epitope of an antigen. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce $F(ab)'_2$, a dimer of Fab' which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The $F(ab)'_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the $F(ab)'_2$ dimer into an Fab' monomer. The Fab' monomer is essentially Fab with part of the hinge region (Paul, *Fundamental Immunology* 3d ed. (1993)). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by using recombinant DNA methodology. As used herein, an "antibody fragment" refers to one or more portions of an antibody, either produced by the modification of whole antibodies, or those synthesized *de novo* using recombinant DNA methodologies, that retain binding specificity and functional activity. Examples of antibody fragments include Fv fragments, single chain antibodies (ScFv), Fab, Fab', Fd (Vh and CH1 domains), dAb (Vh and an isolated CDR); and multimeric versions of these fragments (*e.g.*, $F(ab')_{2,}$) with the same binding specificity.

[00208] Such fragments can be produced by enzymatic cleavage, synthetic or recombinant techniques, as known in the art and/or as described herein. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons have been introduced upstream of the natural stop site. For example, a combination gene encoding a F(ab')2 heavy chain portion can be designed to include DNA sequences encoding the CH1 domain and/or hinge region of the heavy chain. The various portions of antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques.

[00209] A "Fab" domain as used in the context comprises a heavy chain variable domain, a constant region CH1 domain, a light chain variable domain, and a light chain constant region CL domain. The interaction of the domains is stabilized by a disulfide bond between the CH1 and CL domains. In some embodiments, the heavy chain domains of the Fab are in the order, from

N-terminus to C-terminus, VH-CH and the light chain domains of a Fab are in the order, from N-terminus to C-terminus, VL-CL. In some embodiments, the heavy chain domains of the Fab are in the order, from N-terminus to C-terminus, CH-VH and the light chain domains of the Fab are in the order CL-VL. Although the Fab fragment was historically identified by papain digestion of an intact immunoglobulin, in the context of this disclosure, a "Fab" is typically produced recombinantly by any method. Each Fab fragment is monovalent with respect to antigen binding, i.e., it has a single antigen-binding site.

[00210] "Complementarity-determining domains" or "complementarity-determining regions" ("CDRs") interchangeably refer to the hypervariable regions of V_L and V_H. CDRs are the target protein-binding site of antibody chains that harbors specificity for such target protein. There are three CDRs (CDR1-3, numbered sequentially from the N-terminus) in each human V_L or V_H, constituting about 15-20% of the variable domains. CDRs are structurally complementary to the epitope of the target protein and are thus directly responsible for the binding specificity. The remaining stretches of the V_L or V_H, the so-called framework regions (FR), exhibit less variation in amino acid sequence (Kuby, Immunology, 4th ed., Chapter 4. W.H. Freeman & Co., New York, 2000).

[00211] Positions of CDRs and framework regions can be determined using various well known definitions in the art, *e.g.*, Kabat, Chothia, and AbM (see, *e.g.*, Kabat et al. 1991 Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, Johnson *et al.*, Nucleic Acids Res., 29:205-206 (2001); Chothia and Lesk, J. Mol. Biol., 196:901-917 (1987); Chothia *et al.*, Nature, 342:877-883 (1989); Chothia *et al.*, J. Mol. Biol., 227:799-817 (1992); Al-Lazikani *et al.*, J.Mol.Biol., 273:927-748 (1997)). Definitions of antigen combining sites are also described in the following: Ruiz *et al.*, Nucleic Acids Res., 28:219–221 (2000); and Lefranc, M.P., Nucleic Acids Res., 29:207-209 (2001); (ImMunoGenTics (IMGT) numbering) Lefranc, M.-P., The Immunologist, 7, 132-136 (1999); Lefranc, M.-P. *et al.*, Dev. Comp. Immunol., 27, 55-77 (2003); MacCallum *et al.*, J. Mol. Biol., 262:732-745 (1996); and Martin *et al.*, Proc. Natl. Acad. Sci. USA, 86:9268–9272 (1989); Martin *et al.*, Methods Enzymol., 203:121–153 (1991); and Rees *et al.*, In Sternberg M.J.E. (ed.), Protein Structure Prediction, Oxford University Press, Oxford, 141–172 (1996).

[00212] Under Kabat, CDR amino acid residues in the V_H are numbered 31-35 (HCDR1), 50-65 (HCDR2), and 95-102 (HCDR3); and the CDR amino acid residues in the V_L are numbered 24-34 (LCDR1), 50-56 (LCDR2), and 89-97 (LCDR3). Under Chothia, CDR amino acids in the V_H are numbered 26-32 (HCDR1), 52-56 (HCDR2), and 95-102 (HCDR3); and the amino acid residues in V_L are numbered 26-32 (LCDR1), 50-52 (LCDR2), and 91-96 (LCDR3).

By combining the CDR definitions of both Kabat and Chothia, the CDRs consist of amino acid residues 26-35 (HCDR1), 50-65 (HCDR2), and 95-102 (HCDR3) in human VH and amino acid residues 24-34 (LCDR1), 50-56 (LCDR2), and 89-97 (LCDR3) in human VL.

[00213] An “antibody variable light chain” or an “antibody variable heavy chain” as used herein refers to a polypeptide comprising the V_L or V_H, respectively. The endogenous V_L is encoded by the gene segments V (variable) and J (junctional), and the endogenous V_H by V, D (diversity), and J. Each of V_L or V_H includes the CDRs as well as the framework regions (FR). The term “variable region” or “V-region” interchangeably refer to a heavy or light chain comprising FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. A V-region can be naturally occurring, recombinant or synthetic. In this application, antibody light chains and/or antibody heavy chains may, from time to time, be collectively referred to as “antibody chains.”

[00214] The C-terminal portion of an immunoglobulin heavy chain herein, comprising, e.g., CH2 and CH3 domains, is the "Fc" domain. An "Fc region" as used herein refers to the constant region of an antibody excluding the first constant region (CH1) immunoglobulin domain. Fc refers to the last two constant region immunoglobulin domains of IgA, IgD, and IgG, and the last three constant region immunoglobulin domains of IgE and IgM, and the flexible hinge N-terminal to these domains. For IgA and IgM Fc may include the J chain. For IgG, Fc comprises immunoglobulin domains Cγ2 and Cγ3 and the hinge between Cγ1 and Cγ. It is understood in the art that boundaries of the Fc region may vary, however, the human IgG heavy chain Fc region is usually defined to comprise residues C226 or P230 to its carboxyl-terminus, using the numbering is according to the EU index as in Kabat et al. (1991, NIH Publication 91-3242, National Technical Information Service, Springfield, Va.). "Fc region" may refer to this region in isolation or this region in the context of an antibody or antibody fragment. "Fc region" includes naturally occurring allelic variants of the Fc region, *e.g.,* in the CH2 and CH3 region, including, e.g., modifications that modulate effector function. Fc regions also include variants that don't result in alterations to biological function. For example, one or more amino acids are deleted from the N-terminus or C-terminus of the Fc region of an immunoglobulin without substantial loss of biological function. For example, in certain embodiments a C-terminal lysine is modified replaced or removed. In particular embodiments one or more C-terminal residues in the Fc region is altered or removed. In certain embodiments one or more C-terminal residues in the Fc (e.g., a terminal lysine) is deleted. In certain other embodiments one or more C-terminal residues in the Fc is substituted with an alternate amino acid (e.g., a terminal lysine is replaced). Such variants are selected according to general rules known in the art so as to have minimal effect on activity (see, e.g., Bowie, et al., Science 247:306-1310, 1990). The Fc domain is the

portion of the immunoglobulin (Ig) recognized by cell receptors, such as the FcR, and to which the complement-activating protein, C1 q, binds. The lower hinge region, which is encoded in the 5' portion of the CH2 exon, provides flexibility within the antibody for binding to FcR receptors.

[00215] A "chimeric antibody" is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, *e.g.*, an enzyme, toxin, hormone, growth factor, and drug; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

[00216] A "humanized" antibody is an antibody that retains the reactivity (e.g., binding specificity, activity) of a non-human antibody while being less immunogenic in humans. This can be achieved, for instance, by retaining non-human CDR regions and replacing remaining parts of an antibody with human counterparts (*see, e.g.*, Morrison *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984); Morrison and Oi, *Adv. Immunol.*, 44:65-92 (1988); Verhoeyen *et al.*, *Science*, 239:1534-1536 (1988); Padlan, *Molec. Immun.*, 28:489-498 (1991); Padlan, *Molec. Immun.*, 31(3):169-217 (1994)).

[00217] A "human antibody" includes antibodies having variable regions in which both the framework and CDR regions are derived from sequences of human origin. Furthermore, if an antibody contains a constant region, the constant region also is derived from such human sequences, *e.g.*, human germline sequences, or mutated versions of human germline sequences or antibody containing consensus framework sequences derived from human framework sequences analysis, for example, as described in Knappik *et al.*, J. Mol. Biol. 296:57-86, 2000. Human antibodies may include amino acid residues not encoded by human sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo, or a conservative substitution to promote stability or manufacturing).

[00218] The term "corresponding human germline sequence" refers to a nucleic acid sequence encoding a human variable region amino acid sequence or subsequence that shares the highest determined amino acid sequence identity with a reference variable region amino acid sequence or subsequence in comparison to all other all other known variable region amino acid sequences encoded by human germline immunoglobulin variable region sequences. A corresponding human germline sequence can also refer to the human variable region amino acid sequence or subsequence with the highest amino acid sequence identity with a reference variable region amino acid sequence or subsequence in comparison to all other evaluated

variable region amino acid sequences. A corresponding human germline sequence can be framework regions only, complementary determining regions only, framework and complementary determining regions, a variable segment (as defined above), or other combinations of sequences or sub-sequences that comprise a variable region. Sequence identity can be determined using the methods described herein, for example, aligning two sequences using BLAST, ALIGN, or another alignment algorithm known in the art. The corresponding human germline nucleic acid or amino acid sequence can have at least about 90%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with the reference variable region nucleic acid or amino acid sequence.

[00219] The term “valency” as used herein refers to the number of potential target binding sites in a polypeptide. Each target binding site specifically binds one target molecule or a specific site on a target molecule. When a polypeptide comprises more than one target binding site, each target binding site may specifically bind the same or different molecules (*e.g.*, may bind to different molecules, *e.g.*, different antigens, or different epitopes on the same molecule). A conventional antibody, for example, has two binding sites and is bivalent; "trivalent" and "tetravalent" refer to the presence of three binding sites and four binding sites, respectively, in an antibody molecule.

[00220] The phrase “specifically binds” when used in the context of describing the interaction between a target (*e.g.*, a protein) and an antibody, refers to a binding reaction that is determinative of the presence of the target in a heterogeneous population of proteins and other biologics, *e.g.*, in a biological sample, *e.g.*, a blood, serum, plasma or tissue sample. Thus, under certain designated conditions, an antibody with a particular binding specificity binds to a particular target at least two times the background and do not substantially bind in a significant amount to other targets present in the sample. In one embodiment, under designated conditions, an antibody with a particular binding specificity binds to a particular antigen at least ten (10) times the background and does not substantially bind in a significant amount to other targets present in the sample. Specific binding to an antibody under such conditions may require an antibody to have been selected for its specificity for a particular protein. As used herein, specific binding includes antibodies that selectively bind to GPR68 and do not include antibodies that cross-react with, *e.g.*, other GPCRs. A variety of formats may be used to select antibodies that are specifically reactive with a particular target antigen protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (see, *e.g.*, Harlow & Lane, Using Antibodies, A Laboratory Manual (1998), for a description of immunoassay formats and conditions that can be used to

determine specific immunoreactivity). Typically a specific or selective binding reaction will produce a signal at least twice over the background signal and more typically at least than 10 to 100 times over the background.

[00221] The term "equilibrium dissociation constant (K_D, M)" refers to the dissociation rate constant (k_d, time^{-1}) divided by the association rate constant (k_a, time^{-1}, M^{-1}). Equilibrium dissociation constants can be measured using any known method in the art. The antibody generally will have an equilibrium dissociation constant of less than about 10^{-7} or 10^{-8} M, for example, less than about 10^{-9} M or 10^{-10} M, in some embodiments, less than about 10^{-11} M, 10^{-12} M or 10^{-13} M.

[00222] At least one antibody of the invention binds at least one specified epitope specific to at least one GPR68 protein, subunit, fragment, portion or any combination thereof, as described herein (e.g., SEQ ID NOs: 1-4). The at least one epitope can comprise at least one antibody binding region that comprises at least one portion of the protein sequences corresponding to the peptide sequences from the receptor binding region of human GPR68, as described herein, which epitope is preferably comprised of at least one extracellular, soluble, hydrophilic, external or cytoplasmic portion of the protein.

[00223] Generally, the human antibody or antigen-binding fragment of the present invention will comprise an antigen-binding region that comprises at least one human complementarity determining region (CDR1, CDR2 and CDR3) or variant of at least one heavy chain variable region and at least one human complementarity determining region (CDR1, CDR2 and CDR3) or variant of at least one light chain variable region.

[00224] Anti-GPR68 antibodies useful in the methods and compositions of the present invention can optionally be characterized by high affinity binding to a GPR68 protein and, optionally and preferably, having low toxicity. In particular, an antibody, specified fragment or variant of the invention, where the individual components, such as the variable region, constant region and framework, individually and/or collectively, optionally and preferably, possess low immunogenicity, is useful in the present invention. The antibodies that can be used in the invention are optionally characterized by their ability to treat patients for extended periods with measurable alleviation of symptoms and low and/or acceptable toxicity. Low or acceptable immunogenicity and/or high affinity, as well as other suitable properties, can contribute to the therapeutic results achieved. "Low immunogenicity" is defined herein as raising significant HAHA, HACA or HAMA responses in less than about 75%, or preferably less than about 50% of the patients treated and/or raising low titres in the patient treated (less than about 300,

preferably, less than about 100 measured with a double antigen enzyme immunoassay) (Elliott et al., Lancet 344:1125-1127 (1994), entirely incorporated herein by reference).

[00225] In another aspect, the invention relates to human antibodies and antigen-binding fragments, as described herein, which are modified by the covalent attachment of an organic moiety. Such modification can produce an antibody or antigen-binding fragment with improved pharmacokinetic properties (e.g., increased in vivo serum half-life). The organic moiety can be a linear or branched hydrophilic polymeric group, fatty acid group, or fatty acid ester group. Lipid molecules such as a disteroylphosphatidyl ethanolamine moiety, either alone or covalently bonded to a hydrophilic polymer, are useful. In particular embodiments, the hydrophilic polymeric group can have a molecular weight of about 800 to about 120,000 Daltons and can be a polyalkane glycol (e.g., polyethylene glycol (PEG), polypropylene glycol (PPG)), carbohydrate polymer, amino acid polymer or polyvinyl pyrolidone, and the fatty acid or fatty acid ester group can comprise from about eight to about forty carbon atoms.

[00226] The modified antibodies and antigen-binding fragments of the invention can comprise one or more organic moieties that are covalently bonded, directly or indirectly, to the antibody. Each organic moiety that is bonded to an antibody or antigen-binding fragment of the invention can independently be a hydrophilic polymeric group, a fatty acid group or a fatty acid ester group. As used herein, the term “fatty acid” encompasses mono-carboxylic acids and di-carboxylic acids. A “hydrophilic polymeric group,” as the term is used herein, refers to an organic polymer that is more soluble in water than in octane. For example, polylysine is more soluble in water than in octane. Thus, an antibody modified by the covalent attachment of polylysine is encompassed by the invention. Hydrophilic polymers suitable for modifying antibodies of the invention can be linear or branched and include, for example, polyalkane glycols (e.g., PEG, monomethoxy-polyethylene glycol (mPEG), PPG and the like), carbohydrates (e.g., dextran, cellulose, oligosaccharides, polysaccharides and the like), polymers of hydrophilic amino acids (e.g., polylysine, polyarginine, polyaspartate and the like), polyalkane oxides (e.g., polyethylene oxide, polypropylene oxide and the like) and polyvinyl pyrolidone. Preferably, the hydrophilic polymer that modifies the antibody of the invention has a molecular weight of about 800 to about 150,000 Daltons as a separate molecular entity. For example, PEG5000 and PEG20,000, wherein the subscript is the average molecular weight of the polymer in Daltons, can be used. The hydrophilic polymeric group can be substituted with one to about six alkyl, fatty acid or fatty acid ester groups. Hydrophilic polymers that are substituted with a fatty acid or fatty acid ester group can be prepared by employing suitable methods. For example, a polymer comprising an amine group can be coupled to a carboxylate

of the fatty acid or fatty acid ester, and an activated carboxylate (e.g., activated with N,N-carbonyl diimidazole) on a fatty acid or fatty acid ester can be coupled to a hydroxyl group on a polymer.

[00227] Fatty acids and fatty acid esters suitable for modifying antibodies of the invention can be saturated or can contain one or more units of unsaturation. Fatty acids that are suitable for modifying antibodies of the invention include, for example, n-dodecanoate (C12, laurate), n-tetradecanoate (C14, myristate), n-octadecanoate (C18, stearate), n-eicosanoate (C20, arachidate), n-docosanoate (C22, behenate), n-triacontanoate (C30), n-tetracontanoate (C40), cis-Δ9-octadecanoate (C18, oleate), all cis-Δ5,8,11,14-eicosatetraenoate (C20, arachidonate), octanedioic acid, tetradecanedioic acid, octadecanedioic acid, docosanedioic acid, and the like. Suitable fatty acid esters include mono-esters of dicarboxylic acids that comprise a linear or branched lower alkyl group. The lower alkyl group can comprise from one to about twelve, preferably, one to about six, carbon atoms.

[00228] The modified human antibodies and antigen-binding fragments can be prepared using suitable methods, such as by reaction with one or more modifying agents. A "modifying agent" as the term is used herein, refers to a suitable organic group (e.g., hydrophilic polymer, a fatty acid, a fatty acid ester) that comprises an activating group. An "activating group" is a chemical moiety or functional group that can, under appropriate conditions, react with a second chemical group thereby forming a covalent bond between the modifying agent and the second chemical group. For example, amine-reactive activating groups include electrophilic groups such as tosylate, mesylate, halo (chloro, bromo, fluoro, iodo), N-hydroxysuccinimidyl esters (HS), and the like. Activating groups that can react with thiols include, for example, maleimide, iodoacetyl, acrylolyl, pyridyl disulfides, 5-thiol-2-nitrobenzoic acid thiol (TNB-thiol), and the like. An aldehyde functional group can be coupled to amine- or hydrazide-containing molecules, and an azide group can react with a trivalent phosphorous group to form phosphoramidate or phosphorimide linkages. Suitable methods to introduce activating groups into molecules are known in the art (see for example, Hermanson, G. T., Bioconjugate Techniques, Academic Press: San Diego, Calif. (1996)). An activating group can be bonded directly to the organic group (e.g., hydrophilic polymer, fatty acid, fatty acid ester), or through a linker moiety, for example, a divalent C1-C12 group wherein one or more carbon atoms can be replaced by a heteroatom such as oxygen, nitrogen or sulfur. Suitable linker moieties include, for example, tetraethylene glycol, —$(CH_2)_3$—, —NH—$(CH_2)_6$—NH—, —$(CH_2)_2$—NH— and —CH_2—O—CH_2—CH_2—O—CH_2—CH_2—O—CH—NH—. Modifying agents that comprise a linker moiety can be produced, for example, by reacting a mono-Boc-alkyldiamine (e.g., mono-Boc-

ethylenediamine, mono-Boc-diaminohexane) with a fatty acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to form an amide bond between the free amine and the fatty acid carboxylate. The Boc protecting group can be removed from the product by treatment with trifluoroacetic acid (TFA) to expose a primary amine that can be coupled to another carboxylate as described, or can be reacted with maleic anhydride and the resulting product cyclized to produce an activated maleimido derivative of the fatty acid. (See, for example, Thompson, et al., WO 92/16221, the entire teachings of which are incorporated herein by reference.)

[00229] The modified antibodies of the invention can be produced by reacting a human antibody or antigen-binding fragment with a modifying agent. For example, the organic moieties can be bonded to the antibody in a non-site specific manner by employing an amine-reactive modifying agent, for example, a NHS ester of PEG. Modified human antibodies or antigen-binding fragments can also be prepared by reducing disulfide bonds (e.g., intra-chain disulfide bonds) of an antibody or antigen-binding fragment. The reduced antibody or antigen-binding fragment can then be reacted with a thiol-reactive modifying agent to produce the modified antibody of the invention. Modified human antibodies and antigen-binding fragments comprising an organic moiety that is bonded to specific sites of an antibody of the present invention can be prepared using suitable methods, such as reverse proteolysis (Fisch et al., Bioconjugate Chem., 3:147-153 (1992); Werlen et al., Bioconjugate Chem., 5:411-417 (1994); Kumaran et al., Protein Sci. 6(10):2233-2241 (1997); Itoh et al., Bioorg. Chem., 24(1): 59-68 (1996); Capellas et al., Biotechnol. Bioeng., 56(4):456-463 (1997)), and the methods described in Hermanson, G. T., Bioconjugate Techniques, Academic Press: San Diego, Calif. (1996).

[00230] Monoclonal antibodies of this invention may be raised by traditional immunization and hybridoma technology. After immunization of mice with human GPR68 antigenic compositions comprising the polypeptides of the invention, spleen cells or lymphocytes from lymph node tissue from immunized animals are recovered and immortalized by fusion with myeloma cells or by Epstein-Barr (EB)-virus transformation. Monoclonal antibodies are obtained by screening for clones expressing the desired antibody. While mice are frequently employed as the test model, it is contemplated that any mammalian subject, including human subjects or antibody-producing cells, can be manipulated according to the processes of this invention to serve as the basis for production of mammalian, including human and hybrid cell lines. Techniques for cloning recombinant DNA of antibody molecule directly from an antibody-expressing B cell are within the scope of this invention. Such B cells can be isolated by the fluorescence activated cell sorter.

[00231] While routinely, mouse monoclonal antibodies are generated, the invention is not so limited. For therapeutic applications, human or humanized antibodies are desired. Such antibodies can be obtained by using human hybridomas or by generating humanized antibodies. Humanized antibodies can be developed by replacing the specific segments of a non-human antibody with corresponding segments of a human antibody gene. This process retains most or all of CDR regions of the light and heavy chain variable regions of parental antibody and largely replaces the framework regions with human sequences (EP Patent No. 184187; EP Patent No. 171496; EP Patent No. 173494 and WO Patent No. 86/01533). Human monoclonal antibodies are also generated in transgenic mice that contain genes or gene segments encoding human antibodies in their genome (U.S. Pat. No. 6,162,963; WO Patent No. 93/12227; U.S. Pat. No. 5,877,5397; U.S. Pat. No. 5,874,299; U.S. Pat. No. 5,814,318; U.S. Pat. No. 5,789,650; U.S. Pat. No. 5,770,429; U.S. Pat. No. 5,661,016; U.S. Pat. No. 5,625,126; U.S. Pat. No. 5,569,825; U.S. Pat. No. 5,545,806; and WO Patent No. 91/10741).

[00232] Human monoclonal antibodies are also obtained from recombinant antibody libraries, generated in vitro or in vivo, using phage display, ribosome display, or related screening or selection techniques. Examples of procedures for generating antibody libraries, primarily of human origin, are disclosed by A. Knappik and others (U.S. Pat. No. 6,291,158; U.S. Pat. No. 6,291,159; U.S. Pat. No. 6,291,160 and U.S. Pat. No. 6,291,161). Examples of methods for selections of human antibodies to specific antigen targets from such libraries are disclosed by B. Krebs and others (U.S. Pat. No. 5,955,341; U.S. Pat. No. 5,759,817; U.S. Pat. No. 5,658,727; U.S. Pat. No. 6,235,469; U.S. Pat. No. 5,969,108; U.S. Pat. No. 5,886,793)].

[00233] At least one anti-GPR68 antibody of the present invention can be optionally produced by a cell line, a mixed cell line, an immortalized cell or clonal population of immortalized cells, as well known in the art. See, e.g., Ausubel, et al., ed., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., NY, N.Y. (1987-2001); Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor, N.Y. (1989); Harlow and Lane, antibodies, a Laboratory Manual, Cold Spring Harbor, N.Y. (1989); Colligan, et al., eds., Current Protocols in Immunology, John Wiley & Sons, Inc., NY (1994-2001); Colligan et al., Current Protocols in Protein Science, John Wiley & Sons, NY, N.Y., (1997-2001), each entirely incorporated herein by reference.

[00234] Human antibodies that are specific for human GPR68 (as described herein), variants, or fragments thereof can be raised against an appropriate immunogenic antigen as described herein, such as the isolated and/or GPR68, variants, or portions thereof (including synthetic molecules, such as synthetic polypeptides) as described herein. Other specific or

general mammalian antibodies can be similarly raised. Preparation of immunogenic antigens, and monoclonal antibody production can be performed using any suitable technique.

[00235] In one approach, a hybridoma is produced by fusing a suitable immortal cell line, e.g., a myeloma cell line such as, but not limited to, Sp2/0, Sp2/0-AG14, NSO, NS1, NS2, AE-1, L.5, >243, P3X63Ag8.653, Sp2 SA3, Sp2 MAI, Sp2 SS1, Sp2 SA5, U937, MLA 144, ACT IV, MOLT4, DA-1, JURKAT, WEHI, K-562, COS, RAJI, NIH 3T3, HL-60, MLA 144, NAMAIWA, NEURO 2A, or the like, heteromylomas, fusion products thereof, any cell or fusion cell derived therefrom, or any other suitable cell line as known in the art, which can be found, for example at ATCC, with antibody producing cells, such as, but not limited to, isolated or cloned spleen, peripheral blood, lymph, tonsil, or other immune or B cell containing cells, or any other cells expressing heavy or light chain constant or variable or framework or CDR sequences, either as endogenous or heterologous nucleic acid molecule, as recombinant or endogenous, viral, bacterial, algal, prokaryotic, amphibian, insect, reptilian, fish, mammalian, rodent, equine, ovine, goat, sheep, primate, eukaryotic, genomic DNA, cDNA, rDNA, mitochondrial DNA or RNA, chloroplast DNA or RNA, hnRNA, mRNA, tRNA, single, double or triple stranded, hybridized, and the like or any combination thereof. See, e.g., Ausubel, *supra*, and Colligan, Immunology, supra, chapter 2, entirely incorporated herein by reference.

[00236] Antibody producing cells can also be obtained from the peripheral blood or, preferably the spleen or lymph nodes, of humans or other suitable animals that have been immunized with the antigen of interest. Any other suitable host cell can also be used for expressing heterologous or endogenous nucleic acid encoding an antibody, specified fragment or variant thereof, of the present invention. The fused cells (hybridomas) or recombinant cells can be isolated using selective culture conditions or other suitable known methods, and cloned by limiting dilution or cell sorting, or other known methods. Cells that produce antibodies with the desired specificity can be selected by a suitable assay (e.g., ELISA).

[00237] Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, but not limited to, methods that select recombinant antibody from a polypeptide or protein display library (e.g., but not limited to, a bacteriophage, ribosome, oligonucleotide, RNA, cDNA, or the like, display library; e.g., as available from Cambridge antibody Technologies, Cambridgeshire, UK; MorphoSys, Martinsreid/Planegg, Del.; Biovation, Aberdeen, Scotland, UK; BioInvent, Lund, Sweden; Dyax Corp., Enzon, Affymax/Biosite; Xoma, Berkeley, Calif.; Ixsys. See, e.g., EP 368,684, PCT/GB91/01134; PCT/GB92/01755; PCT/GB92/002240; PCT/GB92/00883; PCT/GB93/00605; U.S. Ser. No. 08/350,260(May 12, 1994); PCT/GB94/01422; PCT/GB94/02662; PCT/GB97/01835; (CAT/MRC); WO90/14443;

WO90/14424; WO90/14430; PCT/US94/1234; WO92/18619; WO96/07754; (Scripps); WO96/13583, WO97/08320 (MorphoSys); WO95/16027 (BioInvent); WO88/06630; WO90/3809 (Dyax); U.S. Pat. No. 4,704,692 (Enzon); PCT/US91/02989 (Affymax); WO89/06283; EP 371 998; EP 550 400; (Xoma); EP 229 046; PCT/US91/07149 (Ixsys); or stochastically generated peptides or proteins—U.S. Pat. Nos. 5,723,323, 5,763,192, 5,814,476, 5,817,483, 5,824,514, 5,976,862, WO 86/05803, EP 590 689 (Ixsys, now Applied Molecular Evolution (AME), each entirely incorporated herein by reference) or that rely upon immunization of transgenic animals (e.g., SCID mice, Nguyen et al., Microbiol. Immunol. 41:901-907 (1997); Sandhu et al., Crit. Rev. Biotechnol. 16:95-118 (1996); Eren et al., Immunol. 93:154-161 (1998), each entirely incorporated by reference as well as related patents and applications) that are capable of producing a repertoire of human antibodies, as known in the art and/or as described herein. Such techniques, include, but are not limited to, ribosome display (Hanes et al., Proc. Natl. Acad. Sci. USA, 94:4937-4942 (May 1997); Hanes et al., Proc. Natl. Acad. Sci. USA, 95:14130-14135 (November 1998)); single cell antibody producing technologies (e.g., selected lymphocyte antibody method ("SLAM") (U.S. Pat. No. 5,627,052, Wen et al., J. Immunol. 17:887-892 (1987); Babcook et al., Proc. Natl. Acad. Sci. USA 93:7843-7848 (1996)); gel microdroplet and flow cytometry (Powell et al., Biotechnol. 8:333-337 (1990); One Cell Systems, Cambridge, Mass.; Gray et al., J. Imm. Meth. 182:155-163 (1995); Kenny et al., Bio/Technol. 13:787-790 (1995)); B-cell selection (Steenbakkers et al., Molec. Biol. Reports 19:125-134 (1994); Jonak et al., Progress Biotech, Vol. 5, In Vitro Immunization in Hybridoma Technology, Borrebaeck, ed., Elsevier Science Publishers B.V., Amsterdam, Netherlands (1988)).

[00238] Methods for engineering or humanizing non-human or human antibodies can also be used and are well known in the art. Generally, a humanized or engineered antibody has one or more amino acid residues from a source that is non-human, e.g., but not limited to, mouse, rat, rabbit, non-human primate or other mammal. These human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable, constant or other domain of a known human sequence. Known human Ig sequences are disclosed, e.g., at www.ncbi.nlm.nih.gov/entrez/query.fcgi; www.atcc.org/phage/hdb.html; www.sciquest.com/; www.abcam.com/; www.antibodyresource.com/onlinecomp.html; www.public.iastate.edu/~pedro/research_tools.html; www.mgen.uni-heidelberg.de/SD/IT/IT.html; www.whfreeman.com/immunology/CH05/kuby05.htm; www.library.thinkquest.org/12429/Immune/Antibody.html; www.hhmi.org/grants/lectures/1996/vlab/; www.path.cam.ac.uk/~mrc7/mikeimages.html; www.antibodyresource.com/;

mcb.harvard.edu/BioLinks/Immunology.html.www.immunologylink.com; pathbox.wustl.edu/~hcenter/index.html; www.biotech.ufl.edu/~hcl/; www.pebio.com/pa/340913/340913.html; www.nal.usda.gov/awic/pubs/antibody/; www.m.ehime-u.ac.jp/~yasuhito/Elisa.html; www.biodesign.com/table.asp; www.icnet.uk/axp/facs/davies/links.html; www.biotech.ufl.edu/~fccl/protocol.html; www.isac-net.org/sites_geo.html; aximtl.imt.uni-marburg.de/~rek/AEPStart.html; baserv.uci.kun.nl/~jraats/linksl.html; www.recab.uni-hd.de/immuno.bme.nwu.edu/; www.mrc-cpe.cam. ac.uk/imt-doc/public/INTRO.html; www.ibt.unam.mx/vir/V_mice.html; imgt.cnusc.fr:8104/; www.biochem.ucl.ac.uk/~martin/abs/index.html; antibody.bath.ac.uk/; abgen.cvm.tamu.edu/lab/wwwabgen.html; www.unizh.ch/~honegger/AHOseminar/Slide01.html; www.cryst.bbk.ac.uk/~ubcg07s/; www.nimr.mrc.ac.uk/CC/ccaewg/ccaewg.htm; www.path.cam.ac.uk/~mrc7/humanisation/TAHHP.html; www.ibt.unam.mx/vir/structure/stat_aim.html; www.biosci.missouri. edu/smithgp/index.html; www.cryst.bioc.cam.ac.uk/~fmolina/Web-pages/Pept/spottech.html; www.jerini.de/fr_products.htm; www.patents.ibm.com/ibm.html.Kabat et al., Sequences of Proteins of Immunological Interest, U.S. Dept. Health (1983), each entirely incorporated herein by reference.

[00239] Such imported sequences can be used to reduce immunogenicity or reduce, enhance or modify binding, affinity, on-rate, off-rate, avidity, specificity, half-life, or any other suitable characteristic, as known in the art. Generally part or all of the non-human or human CDR sequences are maintained while the non-human sequences of the variable and constant regions are replaced with human or other amino acids. Antibodies can also optionally be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, humanized antibodies can be optionally prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues

are directly and most substantially involved in influencing antigen binding. Humanization or engineering of antibodies of the present invention can be performed using any known method, such as but not limited to, those described in, Winter (Jones et al., Nature 321:522 (1986); Riechmann et al., Nature 332:323 (1988); Verhoeyen et al., Science 239:1534 (1988)), Sims et al., J. Immunol. 151: 2296 (1993); Chothia and Lesk, J. Mol. Biol. 196:901 (1987), Carter et al., Proc. Natl. Acad. Sci. U.S.A. 89:4285 (1992); Presta et al., J. Immunol. 151:2623 (1993), U.S. Pat. Nos. 5,723,323, 5,976,862, 5,824,514, 5,817,483, 5,814,476, 5,763,192, 5,723,323, 5,766,886, 5,714,352, 6,204,023, 6,180,370, 5,693,762, 5,530,101, 5,585,089, 5,225,539; and 4,816,567, PCT/: US98/16280, US96/18978, US91/09630, US91/05939, US94/01234, GB89/01334, GB91/01134, GB92/01755; WO90/14443, WO90/14424, WO90/14430, and EP 229246, each entirely incorporated herein by reference, included references cited therein.

[00240] The anti-GPR68 antibody can also be optionally generated by immunization of a transgenic animal (e.g., mouse, rat, hamster, non-human primate, and the like) capable of producing a repertoire of human antibodies, as described herein and/or as known in the art. Cells that produce a human anti-GPR68 antibody can be isolated from such animals and immortalized using suitable methods, such as the methods described herein.

[00241] Transgenic mice that can produce a repertoire of human antibodies that bind to human antigens can be produced by known methods (e.g., but not limited to, U.S. Pat. Nos. 5,770,428, 5,569,825, 5,545,806, 5,625,126, 5,625,825, 5,633,425, 5,661,016 and 5,789,650 issued to Lonberg et al.; Jakobovits et al. WO 98/50433, Jakobovits et al. WO 98/24893, Lonberg et al. WO 98/24884, Lonberg et al. WO 97/13852, Lonberg et al. WO 94/25585, Kucherlapate et al. WO 96/34096, Kucherlapate et al. EP 0463 151 B1, Kucherlapate et al. EP 0710 719 A1, Surani et al. U.S. Pat. No. 5,545,807, Bruggemann et al. WO 90/04036, Bruggemann et al. EP 0438 474 B1, Lonberg et al. EP 0814 259 A2, Lonberg et al. GB 2 272 440 A, Lonberg et al. Nature 368:856-859 (1994), Taylor et al., Int. Immunol. 6(4)579-591 (1994), Green et al, Nature Genetics 7:13-21 (1994), Mendez et al., Nature Genetics 15:146-156 (1997), Taylor et al., Nucleic Acids Research 20(23):6287-6295 (1992), Tuaillon et al., Proc Natl Acad Sci USA 90(8)3720-3724 (1993), Lonberg et al., Int Rev Immunol 13(1):65-93 (1995) and Fishwald et al., Nat Biotechnol 14(7):845-851 (1996), which are each entirely incorporated herein by reference). Generally, these mice comprise at least one transgene comprising DNA from at least one human immunoglobulin locus that is functionally rearranged, or which can undergo functional rearrangement. The endogenous immunoglobulin loci in such mice can be disrupted or deleted to eliminate the capacity of the animal to produce antibodies encoded by endogenous genes.

[00242] Screening antibodies for specific binding to similar proteins or fragments can be conveniently achieved using peptide display libraries. This method involves the screening of large collections of peptides for individual members having the desired function or structure. Antibody screening of peptide display libraries is well known in the art. The displayed peptide sequences can be from 3 to 5,000 or more amino acids in length, frequently from 5-100 amino acids long, and often from about 8 to 25 amino acids long. In addition to direct chemical synthetic methods for generating peptide libraries, several recombinant DNA methods have been described. One type involves the display of a peptide sequence on the surface of a bacteriophage or cell. Each bacteriophage or cell contains the nucleotide sequence encoding the particular displayed peptide sequence. Such methods are described in PCT Patent Publication Nos. 91/17271, 91/18980, 91/19818, and 93/08278. Other systems for generating libraries of peptides have aspects of both in vitro chemical synthesis and recombinant methods. See, PCT Patent Publication Nos. 92/05258, 92/14843, and 96/19256. See also, U.S. Pat. Nos. 5,658,754 and 5,643,768. Peptide display libraries, vector, and screening kits are commercially available from such suppliers as Invitrogen (Carlsbad, Calif.), and Cambridge antibody Technologies (Cambridgeshire, UK). See, e.g., U.S. Pat. Nos. 4,704,692, 4,939,666, 4,946,778, 5,260,203, 5,455,030, 5,518,889, 5,534,621, 5,656,730, 5,763,733, 5,767,260, 5,856,456, assigned to Enzon; 5,223,409, 5,403,484, 5,571,698, 5,837,500, assigned to Dyax, 5,427,908, 5,580,717, assigned to Affymax; 5,885,793, assigned to Cambridge antibody Technologies; 5,750,373, assigned to Genentech, 5,618,920, 5,595,898, 5,576,195, 5,698,435, 5,693,493, and 5,698,417, assigned to Xoma, Colligan, supra; Ausubel, supra; or Sambrook, supra, each of the above patents and publications entirely incorporated herein by reference.

[00243] Antibodies of the present invention can also be prepared in milk by administering at least one anti-GPR68 antibody encoding nucleic acid to transgenic animals or mammals, such as goats, cows, horses, sheep, and the like, that produce antibodies in their milk. Such animals can be provided using known methods. See, e.g., but not limited to, U.S. Pat. Nos. 5,827,690; 5,849,992; 4,873,316; 5,849,992; 5,994,616; 5,565,362; 5,304,489, and the like, each of which is entirely incorporated herein by reference.

[00244] Antibodies of the present invention can additionally be prepared using at least one anti-GPR68 antibody encoding nucleic acid to provide transgenic plants and cultured plant cells (e.g., but not limited to, tobacco and maize) that produce such antibodies, specified portions or variants in the plant parts or in cells cultured therefrom. As a non-limiting example, transgenic tobacco leaves expressing recombinant proteins have been successfully used to provide large amounts of recombinant proteins, e.g., using an inducible promoter. See, e.g., Cramer et al.,

Curr. Top. Microbol. Immunol. 240:95-118 (1999) and references cited therein. Also, transgenic maize has been used to express mammalian proteins at commercial production levels, with biological activities equivalent to those produced in other recombinant systems or purified from natural sources. See, e.g., Hood et al., Adv. Exp. Med. Biol. 464:127-147 (1999) and references cited therein. Antibodies have also been produced in large amounts from transgenic plant seeds including antibody fragments, such as single chain antibodies (scFv's), including tobacco seeds and potato tubers. See, e.g., Conrad et al., Plant Mol. Biol. 38:101-109 (1998) and reference cited therein. Thus, antibodies of the present invention can also be produced using transgenic plants, according to known methods. See also, e.g., Fischer et al., Biotechnol. Appl. Biochem. 30:99-108 (October, 1999), Ma et al., Trends Biotechnol. 13:522-7 (1995); Ma et al., Plant Physiol. 109:341-6 (1995); Whitelam et al., Biochem. Soc. Trans. 22:940-944 (1994); and references cited therein. Each of the above references is entirely incorporated herein by reference.

[00245] The antibodies of the invention can bind human GPR68 with a wide range of affinities (KD). In a preferred embodiment, at least one human mAb of the present invention can optionally bind human GPR68 with high affinity. For example, a human mAb can bind human GPR68 with a KD equal to or less than about 10^{-7} M, such as but not limited to, 0.1-9.9 (or any range or value therein) X 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}, 10^{-13} or any range or value therein.

[00246] The affinity or avidity of an antibody for an antigen can be determined experimentally using any suitable method. (See, for example, Berzofsky, et al., “Antibody-Antigen Interactions,” In Fundamental Immunology, Paul, W. E., Ed., Raven Press: New York, N.Y. (1984); Kuby, Janis Immunology, W. H. Freeman and Company: New York, N.Y. (1992); and methods described herein). The measured affinity of a particular antibody-antigen interaction can vary if measured under different conditions (e.g., salt concentration, pH). Thus, measurements of affinity and other antigen-binding parameters (e.g., KD, Ka, Kd) are preferably made with standardized solutions of antibody and antigen, and a standardized buffer, such as the buffer described herein.

[00247] An anti-GPR68 antibody can be recovered and purified from recombinant cell cultures by well-known methods including, but not limited to, protein A purification, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. High performance liquid chromatography (“HPLC”) can also be employed for purification. See, e.g., Colligan, Current Protocols in Immunology, or Current Protocols in Protein Science, John Wiley

& Sons, NY, N.Y., (1997-2001), e.g., Chapters 1, 4, 6, 8, 9, 10, each entirely incorporated herein by reference.

[00248] Antibodies of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a eukaryotic host, including, for example, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the antibody of the present invention can be glycosylated or can be non-glycosylated, with glycosylated preferred. Such methods are described in many standard laboratory manuals, such as Sambrook, supra, Sections 17.37-17.42; Ausubel, supra, Chapters 10, 12, 13, 16, 18 and 20, Colligan, Protein Science, supra, Chapters 12-14, all entirely incorporated herein by reference.

[00249] A typical mammalian expression vector contains at least one promoter element, which mediates the initiation of transcription of mRNA, the antibody coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences, and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRS) from Retroviruses, e.g., GAS-6, HTLVI, HIVI and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter). Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pIRES1neo, pRetro-Off, pRetro-On, PLXSN, or pLNCX (Clonetech Labs, Palo Alto, Calif.), pcDNA3.1 (+/–), pcDNA/Zeo (+/–) or pcDNA3.1/Hygro (+/–) (Invitrogen), PSVL and PMSG (Pharmacia, Uppsala, Sweden), pGAS-6cat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC 67109). Mammalian host cells that could be used include human Hela 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV 1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

[00250] Alternatively, the gene can be expressed in stable cell lines that contain the gene integrated into a chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, or hygromycin allows the identification and isolation of the transfected cells.

[00251] The transfected gene can also be amplified to express large amounts of the encoded antibody. The DHFR (dihydrofolate reductase) marker is useful to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy, et al., Biochem. J. 227:277-279 (1991); Bebbington, et al., Bio/Technology 10:169-175 (1992)). Using these markers, the mammalian cells are grown in selective medium and the cells with the highest

resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of antibodies.

[00252] The expression vectors pC1 and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen, et al., Molec. Cell. Biol. 5:438-447 (1985)) plus a fragment of the CMV-enhancer (Boshart, et al., Cell 41:521-530 (1985)). Multiple cloning sites, e.g., with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vectors contain in addition the 3′ intron, the polyadenylation and termination signal of the rat preproinsulin gene.

[00253] Various anti-GPR68 antibodies include, but are not limited to, those known in the art.

[00254] A novel GPR68 modulator which is an antibody can be prepared; alternatively, many GPR68 antibodies are known in the art.

[00255] For example, Yang et al. demonstrated an inhibitory anti-GPR68 antibody which reduced osteoclast differentiation in a dose-dependent manner (Yang et al., J Biol Chem. 2006 Aug 18;281(33):23598-605).

[00256] Anti-GPR68 antibodies are also available commercially. These are available from, for example:

OriGene Technologies (AP05165PU-N);
Aviva Systems Biology (OAEG00956);
Novus Biologicals (NLS1194; NLS1195; NBP1-02402; NB100-58990; NBP2-32747);
Abcam (ab133818; ab72500);
ProSci, Inc. (48-237; 48-238); and
ABclonal (A7348).

[00257] All references to GPR68 antibodies cited immediately above are hereby incorporated by reference in their entirety.

[00258] Any inhibitory anti-GPR68 antibody or fragment thereof can be used with any method disclosed herein.

[00259] All the documents listed herein describing a GPR68 modulator, including, but not limited to, an antibody, a RNAi agent, a low molecular weight compound, or any other GPR68 modulator, are hereby incorporated in their entirety by reference.

[00260] Any anti-GPR68 antibody described herein or known in the art can be used in the methods described herein. For example, any of the anti-GPR68 antibodies described herein

can be used in a method of treating a cardiovascular disease in a subject in need thereof, the method comprising the step of administering to the subject a GPR68 modulator.

[00261] The various GPR68 modulators disclosed herein exhibit valuable pharmacological properties, e.g. GPR68 modulating properties, e.g. as indicated in in vitro and/or in vivo tests as provided in the next sections, and are therefore indicated for therapy in treating a disorder which may be treated by modulating GPR68, such as those described below.

Methods of Modulating FMD Response and/or FMR of Small-Diameter Arteries

[00262] In various aspects, the present disclosure provides methods of modulating flow-mediated dilation (FMD) response and/or flow-mediated outward remodeling (FMR) of small-diameter arteries, also known as resistance arteries, in a subject in need thereof comprising administering a modulator of a GPR68 gene product to the subject, in an amount that is effective to modulate FMD response or FMR of small-diameter arteries in the subject. According to the present invention, a modulator of a GPR68 gene product includes, but is not limited to, a low molecular weight compound, an RNA inhibitor (e.g., a RNAi agent), an aptamer, a CRISPR, a TALEN, a zinc finger nuclease, an mRNA, or an antibody or derivative thereof.

[00263] In some embodiments, the subject being treated has an abnormal vessel dilation and/or constriction response. An abnormal vessel dilation and/or constriction response may be caused by a variety of diseases or conditions, such as endothial dysfunction. Endothelial dysfunction may be associated with a variety of diseases or conditions, such as vascular disorders, peripheral arterial disease, heart failure, hypertension, hypercholesterolemia, diabetes, septic shock, Behcet's disease, exposure to smoking tobacco products, exposure to air pollution, or a combination thereof. In some embodiments, the methods may further comprise measuring the vessel dilation and/or constriction response of the subject. For example, the vessel dilation and/or constriction response of the subject may be measured by brachial artery ultrasound imaging (BAUI).

[00264] In some embodiments, the modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, may increase flow-mediated dilation (FMD) response and/or flow-mediated outward remodeling (FMR) of small-diameter arteries, also known as resistance arteries, in a subject. For example, the modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, may increase the FMR response and/or FMR of small-diameter arteries in a subject by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least

200%, at least 300%, at least 400%, at least 500%, at least 6 times, at least 7 times, at least 8 times, at least 9 times, at least 10 times, or more.

Methods of Reducing SVR

[00265] The present disclosure further provides methods of reducing systemic vascular resistance (SVR) in a subject comprising administering a modulator of a GPR68 gene product to the subject, in an amount that is effective to reduce SVR in the subject. According to the present invention, a modulator of a GPR68 gene product includes, but is not limited to, a low molecular weight compound, an RNA inhibitor (e.g., a RNAi agent), an aptamer, a CRISPR, a TALEN, a zinc finger nuclease, an mRNA, or an antibody or derivative thereof. In some embodiments, the subject being treated has an abnormal SVR. An abnormal SVR may be associated with a variety of diseases or conditions, such as peripheral arterial disease, heart failure, hypertension, hypercholesterolemia, diabetes, septic shock, etc.

[00266] In some embodiments, the subject being treated has an abnormal SVR. An abnormal SVR may be associated with a variety of diseases or conditions, such as peripheral arterial disease, heart failure, hypertension, hypercholesterolemia, diabetes, septic shock, or a combination thereof. In some embodiments, the methods may further comprise measuring the SVR of the subject. For example, measuring the SVR of the subject may be performed by measuring blood pressure (BP), heart rate (HR) and/or stroke volume (SV) of the subject.

[00267] In some embodiments, the modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, may reduce the systemic vascular resistance (SVR) and/or the left ventricle afterload of a subject. For example, the modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, may reduce the SVR and/or the left ventricle afterload of a subject by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, or more.

Methods of Treating or Preventing Cardiovascular Diseases

[00268] The present disclosure further provides methods of treating, amiorating and preventing a cardiovascular disease using a modulator of a GPR68 gene product, which comprise the step of administering a modulator of a GPR68 gene product to a subject in need thereof, in an amount that is effective to reduce FMD response or FMR of small-diameter arteries, SVR, and/or the left ventricle afterload in the subject. According to the present invention, a modulator of a GPR68 gene product includes, but is not limited to, a low molecular

weight compound, an RNA inhibitor (e.g., a RNAi agent), an aptamer, a CRISPR, a TALEN, a zinc finger nuclease, an mRNA, or an antibody or derivative thereof.

[00269] The present disclosure further provides use of a modulator of a GPR68 gene product, such as a low molecular weight compound, an RNA inhibitor (e.g., a RNAi agent), an aptamer, a CRISPR, a TALEN, a zinc finger nuclease, an mRNA, or an antibody or derivative thereof, for the treatment or prevention of a cadiovascular disease. Also provided is use of a modulator of a GPR68 gene product, such as a low molecular weight compound, an RNA inhibitor (e.g., a RNAi agent), an aptamer, a CRISPR, a TALEN, a zinc finger nuclease, an mRNA, or an antibody or derivative thereof, for the manufacture of a medicament for treating or preventing a cadiovascular disease.

[00270] The present disclosure further provides methods of treating, ameliorating and preventing a cardiovascular disease using a pharmaceutical composition comprising a modulator of a GPR68 gene product, which comprise the step of administering a pharmaceutical composition comprising a modulator of a GPR68 gene product to a subject in need thereof, in an amount that is effective to reduce FMD response or FMR of small-diameter arteries, SVR, and/or the left ventricle afterload in the subject. According to the present invention, a modulator of a GPR68 gene product includes, but is not limited to, a low molecular weight compound, an RNA inhibitor (e.g., a RNAi agent), an aptamer, a CRISPR, a TALEN, a zinc finger nuclease, an mRNA, or an antibody or derivative thereof.

[00271] In some embodiments, the modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, may increase the flow-mediated dilation (FMD) response and/or flow-mediated outward remodeling (FMR) of small-diameter arteries, also known as resistance arteries, in a subject. For example, the modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, may increase the FMR response and/or FMR of small-diameter arteries in a subject by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 200%, at least 300%, at least 400%, at least 500%, at least 6 times, at least 7 times, at least 8 times, at least 9 times, at least 10 times, or more.

[00272] In some embodiments, the modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, may reduce the systemic vascular resistance (SVR) and/or the left ventricle afterload of a subject. For example, the modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, may reduce the SVR and/or the left ventricle afterload of a subject by at least 10%, at least 20%, at least 30%, at least 40%,

at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, or more.

[00273] In some embodiments, the subject may be further treated with an angiotensin converting enzyme (ACE) inhibitor, an angiotensin receptor blocker (ARB), a β-adrenergic receptor blocker, or a diuretics, such as sacubitril/valsartan (ENTRESTO®).

[00274] As used herein, the term "cardiovascular disease" or "CVD," generally refers to heart and blood vessel diseases, including atherosclerosis, coronary heartdisease, cerebrovascular disease, and peripheral vascular disease. Cardiovascular disorders are acute manifestations of CVD and include myocardial infarction, stroke, angina pectoris, transient ischemic attacks, and congestive heart failure.

[00275] Cardiovascular disease, including atherosclerosis, usually results from the build-up of cholesterol, inflammatory cells, extracellular matrix and plaque. The term "cardiovascular disease" also includes indications caused by oxidative stress by reactive oxygen species, and includes but is not limited to angina pectoris, coronary heart disease, hypertension, endothelial dysfunction, atherosclerosis and the like.

[00276] The term "cardiac dysfunction" refers to a pathological decline in cardiac performance. Cardiac dysfunction may be manifested through one or more parameters or indicies including changes to stroke volume, ejection fraction, end diastolic fraction, stroke work, arterial elastance (defined as the ratio of left ventricular (LV) end-systolic pressure and stroke volume), or an increase in heart weight to body weight ratio. Unless otherwise noted, cardiac dysfunctions encompass any cardiac disorders or aberrant conditions that are associated with or induced by the various cardiomyopathies, cardiomyocyte hypertrophy, cardiac fibrosis, or other cardiac injuries described herein. Specific examples of cardiac dysfunction include cardiac remodeling, cardiac hypertrophy, and heart failure.

[00277] As used herein, the terms "congestive heart failure (CHF)," "chronic heart failure," "acute heart failure," and "heart failure" are used interchangeably, and refer to any condition in which the heart is unable to pump blood at an adequate rate or to do so only in the presence of increased left ventricular filling pressures. When the heart is unable to adequately pump blood to the rest of the body at normal filling left ventricular pressures, blood can back up into the lungs, causing the lungs to become congested with fluid. Typical symptoms of heart failure include shortness of breath (dyspnea), fatigue, weakness, difficulty breathing when lying flat, and swelling of the legs, ankles or abdomen (edema). Causes of heart failure are related to various disorders including coronary artery disease, systemic hypertension, cardiomyopathy or myocarditis, congenital heart disease, abnormal heart valves or valvular heart disease, severe

lung disease, diabetes, severe anemia hyperthyroidism, arrhythmia or dysrhythmia and myocardial infarction. Heart failure can occur in the presence of a normal (>50%) or a reduced (<50%) left ventricular ejection fraction. There is increased recognition that these two conditions represent two different disease states, rather than a continuum (Borlaug B A, Redfield M M. Circulation. 2011 May 10; 123(18):2006-13).

[00278] As used herein, the term "coronary heart disease" or "CHD" refers to atherosclerosis in the arteries of the heart causing a heart attack or other clinical manifestation such as unstable angina.

[00279] The terms "heart failure", "acute heart failure" and "chronic heart failure" as used herein carry their respective art-established meanings. By means of further guidance, the term "heart failure" as used herein broadly refers to pathological conditions characterised by an impaired diastolic or systolic blood flow rate and thus insufficient blood flow from the ventricle to peripheral organs.

[00280] "Acute heart failure" or also termed "acute decompensated heart failure" may be defined as the rapid onset of symptoms and signs secondary to abnormal cardiac function, resulting in the need for urgent therapy. AHF can present itself acute de novo (new onset of acute heart failure in a patient without previously known cardiac dysfunction) or as acute decompensation of CHF.

[00281] The cardiac dysfunction may be related to systolic or diastolic dysfunction, to abnormalities in cardiac rhythm, or to preload and afterload mismatch. It is often life threatening and requires urgent treatment. According to established classification, AHF includes several distinct clinical conditions of presenting patients: (I) acute decompensated congestive heart failure, (II) AHF with hypertension/hypertensive crisis, (III) AHF with pulmonary edema, (IVa) cardiogenic shock/low output syndrome, (IVb) severe cardiogenic shock, (V) high output failure, and (VI) right-sided acute heart failure. For detailed clinical description, classification and diagnosis of AHF, and for summary of further AHF classification systems including the Killip classification, the Forrester classification and the 'clinical severity' classification, refer inter alia to Nieminen et al. 2005 ("Executive summary of the guidelines on the diagnosis and treatment of acute heart failure: the Task Force on Acute Heart Failureof the European Society of Cardiology". Eur Heart J 26: 384-416) and references therein.

[00282] Left ventricular hypertrophy (LVH) generally encompasses the thickening of the myocardium of the left ventricle of the heart. LVH may represent a pathological reaction to cardiovascular diseases that increase the afterload (e.g., aortic stenosis or aortic insufficiency) or high blood pressure. LVH may also represent primary hypertrophic cardiomyopathy. LVH

diagnosis may be made inter alia using echocardiography, using criteria known per se such as the Sokolow-Lyon index, the Cornell voltage criteria, the Romhilt-Estes point score system or other voltage-based criteria.

[00283] Cardiac fibrosis generally encompasses abnormal thickening of the heart valves due to inappropriate proliferation of cardiac fibroblasts and the concomitant excessive production of matrix proteins.

[00284] A cardiovascular disease is generally understood to refer to a disease, condition, or disorder involving the heart or blood vessels. Non-limiting examples of cardiovascular diseases include but are not limited to atherosclerosis, atherosclerosis-associated diseases, peripheral arterial occlusive disease, congestive heart failure (CHF), stroke, diabetic nephropathy, renal hypertension, hypertension, cerebrovascular disease, dyslipidemia, and vasospastic disorders, including Raynaud's disease. In some embodiments, the CHF may be HF with reduced ejection fraction (aka HF due to left ventricular dysfunction) or HF with preserved ejection fraction (HFpEF) (aka diastolic HF or HF with normal ejection fraction). In some embodiments, the CHF may be associated with a coronary artery disease selected from myocardial infarction (heart attack), high blood pressure, atrial fibrillation, and valvular heart disease, excess alcohol use, infection, or cardiomyopathy of an unknown cause.

[00285] The subject treated using the methods and compositions of the present invention can also be at an increased risk of developing a cardiovascular disease. This can include (but is not limited to) individuals with hypertension (systemic or pulmonary), obesity, endocrine disease (including diabetes, thyroid disease, adrenal disease, dysregulation of homocysteine metabolism), iron storage disease, amyolidosis, renal disease, connective tissue disease, infectious diseases, thromboembolic disease, immune diseases, hematologic diseases.

[00286] Provided herein are methods of increasing or enhancing the chances of survival of a subject with a cardiovascular disease, comprising administering to a subject in need thereof an effective amount of a modulator of a GPR68 gene product of the invention, thereby increasing or enhancing the chances of survival of the subject treated by a certain period of time, for example, by at least 10 days, 1 month, 3 months, 6 months, 1 year, 1.5 years, 2 years, 3 years, 4 years, 5 years, 8 years, or 10 years. The increase in survival of a subject can be defined, for example, as the increase in survival of a preclinical animal model by a certain period of time, for example, by at least 10 days, 1 month, 3 months, 6 months, or 1 year, or at least 2 times, 3 times, 4 times, 5 times, 8 times, or 10 times, more than a control animal model (that has the same type of disease) without the treatment with the inventive method. Optionally, the increase in survival of a mammal can also be defined, for example, as the increase in survival of

a subject with heart disease by a certain period of time, for example, by at least 10 days, 1 month, 3 months, 6 months, 1 year, 1.5 years, 2 years, 3 years, 4 years, 5 years, 8 years, or 10 years more than a subject with the same type of heart disease but without the treatment with the inventive method. The control subject may be on a placebo or treated with supportive standard care such as chemical therapy, biologics and/or radiation that do not include the inventive method as a part of the therapy.

Methods of Treating or Preventing Liver Fibrosis

[00287] GPR68 has also been shown to be induced in rat and human primary hepatic stellate cells by pro-fibrotic stimuli (e.g., TGFβ).

[00288] Therefore, the present disclosure further provides methods of treating, amiorating and preventing liver fibrosis using an antagonist of a GPR68 gene product, which comprise the step of administering an antagonist of a GPR68 gene product to a subject in need thereof, in an amount that is effective to reduce the level and/or activity of the GPR68 gene product in the subject. According to the present invention, an antagonist of a GPR68 gene product includes, but is not limited to, a low molecular weight compound, an RNA inhibitor (e.g., a RNAi agent), an aptamer, a CRISPR, a TALEN, a zinc finger nuclease, an mRNA, or an antibody or derivative thereof.

[00289] The present disclosure further provides use of an antagonist of a GPR68 gene product, such as a low molecular weight compound, an RNA inhibitor (e.g., a RNAi agent), an aptamer, a CRISPR, a TALEN, a zinc finger nuclease, an mRNA, or an antibody or derivative thereof, for the treatment or prevention of liver fibrosis. Also provided is use of an antagonist of a GPR68 gene product, such as a low molecular weight compound, an RNA inhibitor (e.g., a RNAi agent), an aptamer, a CRISPR, a TALEN, a zinc finger nuclease, an mRNA, or an antibody or derivative thereof, for the manufacture of a medicament for treating or preventing liver fibrosis.

[00290] In some embodiments, the antagonist of a GPR68 gene product may reduce the level and/or activity of the GPR68 gene product of a subject. For example, the antagonist of a GPR68 gene product, may reduce the level and/or activity of the GPR68 gene product in a subject by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, or more.

[00291] As used herein,"liver fibrosis" includes liver fibrosis due to any cause, including but not limited to virally-induced liver fibrosis such as that due to hepatitis B and C; exposure to alcohol (alcoholic liver disease), pharmaceutical compounds, oxidative stress, cancer radiation

therapy or industrial chemicals; and diseases such as primary biliary cirrhosis, fatty liver, obesity, non-alcoholic steatohepatitis, cystic fibrosis, hemochromatosis, and auto-immune hepatitis.

[00292] In one embodiment, the antagonists of a GPR68 gene product disclosed herein and pharmaceutical compositions thereof may be used for treating a liver disease or a gastrointestinal disease, including but not limited to liver diseases selected from intrahepatic cholestasis, estrogen-induced cholestasis, drug-induced cholestasis, cholestasis of pregnancy, parenteral nutrition-associated cholestasis, progressive familiar cholestasis (PFIC), Alagille syndrome, primary biliary cirrhosis (PBC), primary sclerosing cholangitis, ductopenic liver transplant rejection, liver transplant associated graft versus host disease, cystic fibrosis liver disease, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), alcoholic liver disease, and parenteral nutrition-associated liver disease; and gastrointestinal diseases selected from bile acid malabsorption (including primary bile acid diarrhea and secondary bile acid diarrhea), bile reflux gastritis, and inflammatory bowel disease such as Crohn's disease, ulcerative colitis, collagenous colitis, lymphocytic colitis, diversion colitis, indeterminate colitis and Behçet's disease.

[00293] In one embodiment, said antagonists of a GPR68 gene product disclosed herein and pharmaceutical compositions thereof may be used for the preparation of a medicament for the treatment of chronic intrahepatic and some forms of extrahepatic cholestatic conditions, such as primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), progressive familiar cholestasis (PFIC), alcohol- induced cirrhosis and associated cholestasis, or liver fibrosis resulting from chronic cholestatic conditions or acute intraheptic cholestatic conditions such as estrogen or drug induced cholestasis.

[00294] Furthermore, conditions and diseases which result from chronic fatty and fibrotic degeneration of organs due to enforced lipid and specifically triglyceride accumulation and subsequent activation of profibrotic pathways may also be treated by applying the modulators of a GPR68 gene product disclosed herein or pharmaceutical compositions thereof. Such conditions and diseases encompass Non-Alcoholic Steatohepatitis (NASH) and chronic cholestatic conditions in the liver, Glomerulosclerosis and Diabetic Nephropathy in the kidney, Macula Degeneration and Diabetic Retinopathy in the eye and Neurodegenerative diseases such as Alzheimer's Disease in the brain or Diabetic Neuropathies in the peripheral nervous system.

[00295] Provided herein are methods of increasing or enhancing the chances of survival of a subject with liver fibrosis, comprising administering to a subject in need thereof an effective

amount of an antagonist of a GPR68 gene product of the invention, thereby increasing or enhancing the chances of survival of the subject treated by a certain period of time, for example, by at least 10 days, 1 month, 3 months, 6 months, 1 year, 1.5 years, 2 years, 3 years, 4 years, 5 years, 8 years, or 10 years. The increase in survival of a subject can be defined, for example, as the increase in survival of a preclinical animal model by a certain period of time, for example, by at least 10 days, 1 month, 3 months, 6 months, or 1 year, or at least 2 times, 3 times, 4 times, 5 times, 8 times, or 10 times, more than a control animal model (that has the same type of disease) without the treatment with the inventive method. Optionally, the increase in survival of a mammal can also be defined, for example, as the increase in survival of a subject with heart disease by a certain period of time, for example, by at least 10 days, 1 month, 3 months, 6 months, 1 year, 1.5 years, 2 years, 3 years, 4 years, 5 years, 8 years, or 10 years more than a subject with the same type of heart disease but without the treatment with the inventive method. The control subject may be on a placebo or treated with supportive standard care such as chemical therapy, biologics and/or radiation that do not include the inventive method as a part of the therapy.

Methods of Diagnosis

[00296] In some embodiments, the methods disclosed herein may further comprise diagnosing the subject with a cardiovascular disease or liver fibrosis, or at risk of developing a cardiovascular disease or liver fibrosis. For example, the subject may be diagnosed based on the history of the symptoms, physical examination, echocardiography, blood test, electrocardiography, chest radiography, or a combination thereof. In some embodiments, the subject may be diagnosed based on risk factors, family history, genetic testing, or a combination thereof.

[00297] Risk factors for cardiovascular diseases include, but are not limited to, a coronary artery disease selected from myocardial infarction (heart attack), high blood pressure, atrial fibrillation, and valvular heart disease, excess alcohol use, infection, or cardiomyopathy of an unknown cause. Risk factors for liver fibrosis include, but are not limtied to, hepatitis B and C; exposure to alcohol (alcoholic liver disease), pharmaceutical compounds, oxidative stress, cancer radiation therapy or industrial chemicals; and diseases such as primary biliary cirrhosis, fatty liver, obesity, non-alcoholic steatohepatitis, cystic fibrosis, hemochromatosis, and auto-immune hepatitis.

Pharmaceutical Compositions

[00298] Some embodiments disclosed herein provide pharmaceutical compositions comprising a modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, formulated together with a pharmaceutically acceptable carrier. Optionally, pharmaceutical compositions additionally contain other therapeutic agents that are suitable for treating or preventing a given disorder. Pharmaceutically acceptable carriers enhance or stabilize the composition, or facilitate preparation of the composition. Pharmaceutically acceptable carriers include solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible.

[00299] A pharmaceutical composition of the present disclosure can be administered by a variety of methods known in the art. Route and/or mode of administration vary depending upon the desired results. It is preferred that administration be by parenteral administration (e.g., selected from any of intravenous, intramuscular, intraperitoneal, intrathecal, intraarterial, or subcutaneous), or administered proximal to the site of the target. A pharmaceutically acceptable carrier is suitable for administration by any one or more of intravenous, intramuscular, intraperitoneal, intrathecal, intraarterial, subcutaneous, intranasal, inhalational, spinal or epidermal administration (e.g., by injection). Depending on the route of administration, active compound, e.g., a modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound. In some embodiments the pharmaceutical composition is formulated for intravenous administration. In some embodiments the pharmaceutical composition is formulation for subcutaneous administration.

[00300] A modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, alone or in combination with other suitable components, can be made into aerosol formulations (i.e., they can be “nebulized”) to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

[00301] In some embodiments, a pharmaceutical composition is sterile and fluid. Proper fluidity can be maintained, for example, by use of coating such as lecithin, by maintenance of required particle size in the case of dispersion and by use of surfactants. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol or sorbitol, and sodium chloride in the composition. Long-term absorption of the injectable

compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate or gelatin. In certain embodiments compositions can be prepared for storage in a lyophilized form using appropriate excipients (e.g., sucrose).

[00302] Pharmaceutical compositions can be prepared in accordance with methods well known and routinely practiced in the art. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions. Applicable methods for formulating a GPR68 modulator and determining appropriate dosing and scheduling can be found, for example, in Remington: The Science and Practice of Pharmacy, 21st Ed., University of the Sciences in Philadelphia, Eds., Lippincott Williams & Wilkins (2005); and in Martindale: The Complete Drug Reference, Sweetman, 2005, London: Pharmaceutical Press., and in Martindale, Martindale: The Extra Pharmacopoeia, 31st Edition., 1996, Amer Pharmaceutical Assn, and Sustained and Controlled Release Drug Delivery Systems, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978. Pharmaceutical compositions are preferably manufactured under GMP conditions. Typically, a therapeutically effective dose or efficacious dose of a GPR68 modulator is employed in the pharmaceutical compositions. A GPR68 modulator is formulated into pharmaceutically acceptable dosage form by conventional methods known to those of skill in the art. Dosage regimens are adjusted to provide the desired response (e.g., a therapeutic response). In determining a therapeutically or prophylactically effective dose, a low dose can be administered and then incrementally increased until a desired response is achieved with minimal or no undesired side effects. For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier.

[00303] Actual dosage levels of active ingredients in the pharmaceutical compositions can be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level depends upon a variety of

pharmacokinetic factors including the activity of the particular compositions employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors.

Articles of Manufacture/Kits

[00304] In some aspects a modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, is provided in an article of manufacture (i.e., a kit). A provided modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, is generally in a vial or a container. Thus, an article of manufacture comprises a container and a label or package insert, on or associated with the container. Suitable containers include, for example, a bottle, vial, syringe, solution bag, etc. As appropriate, the modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, can be in liquid or dried (e.g., lyophilized) form. The container holds a composition which, by itself or combined with another composition, is effective for preparing a composition for treating, preventing and/or ameliorating a cardiovascular disease or liver fibrosis. The label or package insert indicates the composition is used for treating, preventing and/or ameliorating a cardiovascular disease or liver fibrosis. Articles of manufacture (kits) comprising a modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, as described herein, optionally contain one or more additional agent. In some embodiments, an article of manufacture (kit) contains a modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, and a pharmaceutically acceptable diluent. In some embodiments a modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, is provided in an article of manufacture (kit) with one or more additional active agent in the same formulation (e.g., as mixtures). In some embodiments a modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, is provided in an article of manufacture (kit) with a second or third agent in separate formulations (e.g., in separate containers). In certain embodiments an article of manufacture (kit) contains aliquots of the modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, wherein the aliquot provides for one or more doses. In some embodiments aliquots for multiple administrations are provided, wherein doses are uniform or varied. In particular embodiments varied dosing regimens are escalating or decreasing, as appropriate. In some embodiments

dosages of a modulator of a GPR68 gene product, e.g., an agonist or antagonist of a GPR68 gene product, and a second agent are independently uniform or independently varying. In certain embodiments, an article of manufacture (kit) comprises an additional agent such as an anti-heart failure agent. Selection of one or more additional agent will depend on the dosage, delivery, and disease condition to be treated.

Administration of GPR68 Modulators

[00305] A physician or veterinarian can start doses of a modulator of a GPR68 gene product employed in the pharmaceutical composition at levels lower than that required to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, effective doses of the compositions vary depending upon many different factors, including the specific disease or condition to be treated, means of administration, target site, physiological state of the patient, whether a patient is human or an animal, other medications administered, and whether treatment is prophylactic or therapeutic. Treatment dosages typically require titration to optimize safety and efficacy. For administration with a modulator of a GPR68 gene product, dosage ranges from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg, of the host body weight. For example dosages can be 1 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 mg/kg. Dosing can be daily, weekly, bi-weekly, monthly, or more or less often, as needed or desired. An exemplary treatment regime entails administration once weekly, once per every two weeks or once a month or once every 3 to 6 months.

[00306] The modulator of a GPR68 gene product can be administered in single or divided doses. A modulator of a GPR68 gene product is usually administered on multiple occasions. Intervals between single dosages can be weekly, bi-weekly, monthly or yearly, as needed or desired. Intervals can also be irregular as indicated by measuring blood levels of modulator of a GPR68 gene product in the patient. In some methods, dosage is adjusted to achieve a plasma concentration of 1–1000 µg/ml and in some methods 25-300 µg/ml of the modulator of a GPR68 gene product. Alternatively, a modulator of a GPR68 gene product can be administered as a sustained release formulation, in which case less frequent administration is required. Dosage and frequency vary depending on the half-life of the modulator of a GPR68 gene product in the patient. Dosage and frequency of administration can vary depending on whether treatment is prophylactic or therapeutic. In general for prophylactic applications, a relatively low dosage is administered at relatively infrequent intervals over a long period of time. Some patients continue to receive treatment for the duration of their lives. In general for therapeutic

applications, a relatively high dosage in relatively short intervals is sometimes required until progression of the disease is reduced or terminated, and preferably until the patient shows partial or complete amelioration of symptoms of disease. Thereafter, a patient may be administered a prophylactic regime.

Co-Administration with a Second Agent

[00307] The term "combination therapy" refers to the administration of two or more therapeutic agents to treat a therapeutic condition or disorder described in the present disclosure. Such administration encompasses co-administration of these therapeutic agents in a substantially simultaneous manner, such as in a single capsule having a fixed ratio of active ingredients. Alternatively, such administration encompasses co-administration in multiple, or in separate containers (e.g., capsules, powders, and liquids) for each active ingredient. Powders and/or liquids may be reconstituted or diluted to a desired dose prior to administration. In addition, such administration also encompasses use of each type of therapeutic agent in a sequential manner, either at approximately the same time or at different times. In either case, the treatment regimen will provide beneficial effects of the drug combination in treating the conditions or disorders described herein.

[00308] The combination therapy can provide "synergy" and prove "synergistic", i.e., the effect achieved when the active ingredients used together is greater than the sum of the effects that results from using the compounds separately. A synergistic effect can be attained when the active ingredients are: (1) co-formulated and administered or delivered simultaneously in a combined, unit dosage formulation; (2) delivered by alternation or in parallel as separate formulations; or (3) by some other regimen. When delivered in alternation therapy, a synergistic effect can be attained when the compounds are administered or delivered sequentially, e.g., by different injections in separate syringes. In general, during alternation therapy, an effective dosage of each active ingredient is administered sequentially, i.e., serially, whereas in combination therapy, effective dosages of two or more active ingredients are administered together.

[00309] In one aspect, the present disclosure provides a method of treating, ameliorating or preventing a cardiovascular disease by administering to a subject in need thereof a modulator of a GPR68 gene product in combination with one or more of the following catagories: a) ACE inhibitor/NEP inhibitor combination: Sacubitril/Valsartan (Entresto); b) ACE inhibitors: captopril (Capoten), enalapril (Vasotec), lisinopril (Zestril, Prinivil), benazepril (Lotensin), ramipril (Altace); c) beta blockers: carvedilol (Coreg), metoprolol (Toprol XL); and d) digoxin (Lanoxin).

[00310] In one aspect, the present disclosure provides a method of treating, ameliorating or preventing liver fibrosis by administering to a subject in need thereof a modulator of a GPR68 gene product in combination with Tropifexor.

Identification of a Modulator of a GPR68 Gene Product

[00311] Some embodiments of the present disclosure provide methods of identifying a modulator of a GPR68 gene product comprising: a. Providing a population of cells expressing a GPR68 gene product; b. Adding a library of candidate molecules to the population of cells; c. Applying a shear stress or an acidic shock to the population of cells; d. Measuring the calcium transient in the populationof cells; and e. Identifying a candidate molecule in a cell that shows enhanced or reduced calcium transients in the cell. In some embodiments, the population of cells comprises endothelial cells, such as mouse or human microvascular endothelial cells from brain, lung, bladder, skin. In some embodiments, the library of candidate molecules comprises a small molecule compound, an antibody, a nucleic acid molecule, or a protein. In some embodiments, the shear stress comprises disturbed flow or laminar flow. In some embodiments, the acidic shock comprises extracellular proton at pH 6.5.

EXAMPLES

[00312] The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Example 1. GPR68 is a sensor of shear stress in endothelial cells of small-diameter arteries and regulates flow-mediated dilation and remodeling

[00313] We previously conducted a functional genomic small interfering RNA (siRNA) screen to identify Piezo1 using membrane indentation and electrophysiological recording (Coste et al., 2010). It was a low-throughput screen that required a year to identify Piezo1 after screening 71 other candidate genes. Here we aimed to accelerate genomic screens relevant to mechanotransduction by designing a novel high-throughput (HT) mechanical stimulation system.

[00314] Mechanotransduction, the conversion of mechanical force into biochemical signals, is the basis for many biological processes, including the sense of touch, pain, hearing, as well as blood flow regulation. The molecules responsible for many forms of mechanotransduction remain unknown. We designed a 384-well screening system that applies shear stress on

cultured cells. We identified a mechanosensitive cell line that exhibits shear stress-activated calcium transients, screened a focused RNAi library, and identified GPR68 as a necessary component for shear stress response. Overexpressing GPR68 in HEK-293T cells induced robust shear stress-activated responses. GPR68 is expressed in endothelial cells of small-diameter blood vessels. Knocking down Gpr68 in primary mouse microvascular endothelial cells abolished shear stress-activated calcium transients. Gpr68-deficient mice display markedly impaired flow-mediated acute dilation and flow-mediated chronic outward remodeling in third order mesenteric arteries. Our findings demonstrate that GPR68 is an endothelial shear stress sensor that controls flow-mediated dilation and outward remodeling.

Experimental Procedures

[00315] Additional information regarding the construction of the shear stress stimulation systems, estimation of the shear stress intensities, qRT-PCR are available in the Extended Experimental Procedures.

DNA constructs, compounds and mouse lines

[00316] Expression constructs of human GPR68, GPR132, AGTR1, AVPR1A, BDKRB2, CHRM5, EDNRA, HRH1, PTHR1 and mouse Gpr68 were sourced from GNF collection and Missouri S&T cDNA Resource Center. Constructs of mouse Piezo1, rat TrpV4 were described previously (Coste et al., 2010; Liedtke et al., 2000). Agonists for the human GPCRs listed above and the PLC inhibitor U73122 were purchased from Tocris Bioscience. Ogerin was synthesized by WuXi AppTek. Gpr68 KO mice breeders (C57BL/6J background) were provided by Dr. Yan Xu (Indiana University School of Medicine). Gpr68-eGFP reporter mice were purchased from GenSat Project from The Rockefeller University. All procedures involving animal handling were approved by Institutional Animal Care and Use Committees (IACUC) of Genomics Institute of the Novartis Research Foundation, The Scripps Research Institute. In addition, FMD and FMR experiments were performed in agreement with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC): Committee on the Ethics of Animal Experiments of “Pays de la Loire” (CEEA, permit # CEEA PdL 2012.141).

Cell line culture, plasmid DNA and siRNA transfection

[00317] Cell lines were maintained with vendor recommended reagents and standard protocols. For DNA plasmid transfection with HEK-293T cells in poly-D-Lysine coated 384-well assay plates (Greiner Bio-One), we use 65ng plasmid, 0.195 µl Fugene 6 (Roche) and 1×104 cells in 40 µl standard growth medium for each well. When transfecting HEK-293T cells on poly-

D-Lysine glass cover slips (BD Biosciences), we use 500 ng of plasmids, 1.5 µl Fugene 6 and 1×105 cells in 600 µl growth medium. For laminar flow chamber experiments, cells were first transfected in 24-well plate in the same condition. 24h later they were trypsinized and plated in µ-slides I 0.4 flow chambers (Ibidi). Cells were assayed 48h post-transfection.

[00318] For siRNA transfection in 384-well plates, we mixed 1 pmol siRNA with 20 µl Opti-MEM and 0.15 µl Lipofectamine RNAiMax (Life Technologies)in each well, incubated for 20 min at room temperature, then added 20 µl cells (6×10^3 cells per well for MDA-MB-231, 1×10^4 cells per well for HeLa). When transfecting siRNA on glass coverslips, 20 pmol siRNA, 1 µl RNAiMax and 100 µl Opti-MEM were mixed and incubated for 20 min, then mixed with 500 µl cells (1×10^5 cells per well for HeLa, 2×10^5 cells per well for early passage HUVECs). SiRNAs were from Dhamacon. Cells were assayed 72h post-transfection.

Calcium imaging

[00319] Fura-2 single cell calcium imaging was performed as previous described (Ranade et al., 2014). Briefly, cells were seeded onto poly-D-Lysine cover slips (BD Biosciences). We loaded the cells with the ratiometric Ca2+ indicator Fura-2 (Life Technologies) in imaging buffer (1x Hanks Balanced Salt Solution (HBSS) and 10 mM HEPES, pH adjusted to 7.4 by NaOH). After 30 minutes of loading at room temperature, cover slips were washed with imaging buffer and then mounted onto the disturbed shear stress imaging chamber and assayed at room temperature. The intracellular Ca2+ concentration was expressed as the 340/380 ratio.

FLIPR experiments

[00320] FLIPR experiments were performed as previous described (Ranade et al., 2014). Before loading, the cells were washed with assay buffer (1x HBSS with calcium and 10 mM HEPES, pH adjusted to 7.4) using ELx405 CW plate washer (BioTek). Cells were loaded with Fluo-3 for 60 min (4 µM Fluo-3, 0.04% F-127 in assay buffer) at 37 ºC. Cells were washed again with assay buffer and the plates were centrifuged at 15 g for 5 s before assayed on FLIPR Tetra (Molecular Devices). The data was analyzed on Screenworks 3.1 (Molecular Devices).

Disturbed shear stress stimulation

[00321] When applying shear stress with high-throughput disturbed flow system, we first thoroughly dry the pin array with compressed air then mounted the 384-well plated on the system. Two stimulation protocols were used. The short protocol applies stimulation for 4 s at 60 Hz, and the long protocol applies 0.2 s pulses at 60 Hz every 2 s for 40 s. After each assay, the pin array was washed thoroughly with distilled water and air dried before mounting onto the next plate. Whenever compounds were used, to avoid carry-over, the array was first thoroughly washed with 70% EtOH, then with distilled water and dried before assaying the next plate.

High-throughput siRNA screen

[00322] To assemble a set of arrayed siRNAs targeting genes encoding multi-pass transmembrane (MPTM) proteins, we subjected 34,226 non-identical protein sequences extracted from the August 21, 2011 version of Human Refseq to sequence-based membrane topology and signal peptide prediction using the Phobius algorithm and website, identifying 2,907 human genes encoding one or more MPTM proteins (Kall et al., 2004, 2007). An unconstrained prediction model was employed, signal peptides annotated where predicted, and all two-pass or more predictions collapsed from protein to gene space to ensure that no siRNAs targeting non-MPTM protein isoforms were excluded. The siRNA library was picked from a genome-wide siRNA library and arrayed in 384-well poly-D-Lysine coated assay plate with one single oligo in each well (63 plates in total). MDA-MB-231 cells were transfected with the siRNA using a robotic liquid handler and incubated in a robot-assisted incubator (GNF Systems). Cells were assayed after 72h with HT disturbed shear stress system. Wells with calcium signal 3× standard deviations below the plate mean were considered as hit wells. Genes with more than 3 hit wells were considered hits. We then crosschecked the bioinformatics results and the literature to filter out the hits that have known functions unrelated to acute (second-scale) calcium signaling. We picked the siRNA against hit genes from primary screen from the original genome-wide library and carried out a reconfirmation screen. For final screen, we purchased smartpool siRNA (Dharmacon OTP siRNAs, GE Healthcare) against the hits from the reconfirmation screen.

Real-time pH measurement

[00323] 2',7'-Bis(3-carboxypropyl)-5(6)-carboxyfluorescein (BCEFC) was selected as pH indicator based on its dynamic range (pH6~pH8), which fits GPR68 activation range best among all pH-sensitive dyes. BCECF increase fluorescence when pH increases. Free BCECF was used to monitor extracellular pH and its cell-permeable acetoxymethyl (AM) ester version was used for monitoring intracellular pH. Cells in assay plates were first washed with HBSS-pH7.4 on an ELx405 CW plate washer (BioTek). BCECF was then incubated with cells for 1 h at 37 ℃. When measuring intracellular pH, cells were then washed with HBSS-pH7.4 again. When measuring extracelluar pH, no wash step was added. Fluorescence was read on FLIPR for 10s and acid or base were added to the buffer to shift pH to various final values from pH6.1 to pH8.3, while fluorescence was continuously monitoring for additional 150 s. For shear stress stimulation, fluorescence was recorded for 10s and disturbed flow was applied for 60 s. Data was analyzed by ScreenWorks 3.0 (Molecular Devices).

Laminar shear stress stimulation

[00324] HEK-293T cells were plated in the Collagen IV-coated µ-slide I 0.4 laminar flow chambers (Ibidi) 24 h prior to experiments. After loading Fura-2, the chambers were mounted onto the microscope and connected to the Ibidi perfusion pump. Cells were given pulsatile (1 Hz) or continuous laminar flow for 120 s.

Immunostaining

[00325] For GFP immunostaining experiments, tissues from various organs were collected from 7-week-old wild type and Gpr68 eGFP reporter mice after perfusion with 4% PFA. Tissues were briefly fixed in 4% PFA and were dehydrated through an ethanol series/xylene and embedded in paraffin. 5µm sections were cut and incubated with blocking solution containing 1x PBS, 10% blocking serum, 3% BSA and 0.4% TritonX 100 for 1hr at room temperature. Slides were stained overnight with a goat anti-GFP antibody (Lifespan Biosciences). Slides were treated with Omni-Map anti -goat secondary antibody conjugated with HRP (Ventana) followed by development using the Chromo Map DAB kit (Ventana). Slides were mounted with Cytoseal and scanned using the Nanozoomer 2.0 HT (Hamamatsu).

[00326] We tested murine Gpr68 antibodies from Lifespan Biosciences (LS-A3968 and LS-A1194) and antibodies custom generated at Pierce Biotechnologies (peptide sequences: RTSRAREAYPLGAPEASGK (SEQ ID NO: 19) and EEPELLTKLHSAFQTPSSLG (SEQ ID NO: 20)). We determined that none of the above can specifically detect Gpr68 protein in HEK-293T cells overexpressing murine Gpr68 in our immunohistochemistry experiments.

In situ hybridization

[00327] Tissues from C57BL/6J mice were isolated after perfusion and fixed in 10% formalin overnight. The tissues were dehydrated through an ethanol series/xylene and embedded in paraffin. 10µm sections were cut and in situ hybridization was carried out using the RNAscope assay (Advanced Cell Diagnostics). Development of signal was done using the RNAscope 2.0 HD brown detection kit. Probes for mGpr68 (cat. #: 319321) and DapB negative control (cat #: 310043) were purchased from Advanced Cell Diagnostics. Slides were mounted with Cytoseal and scanned using the Nanozoomer 2.0 HT (Hamamatsu).

Fluorescence-activated Cell Sorting

[00328] Spleens from Gpr68 eGFP reporter mice were isolated and dissociated in FACS buffer (PBS containing 2% FBS and 2 mM EDTA) and red blood cells were lysed using RBC Lysis Buffer (eBioscience). Samples were washed once in FACS buffer, filtered through 100 µm diameter nylon mesh, and incubated with FcBlock (BD Biosciences) for 5 minutes at room temperature. They were then stained with BV421 CD4 (Biolegend), BV650 CD3 (Biolegend),

APC-Cy7 CD19 (Biolegend), PerCP-Cy5.5 CD8 (BD Biosciences), and APC NK1.1 (eBioscience) for 20 min at 4 ºC in the dark. The cells were washed once then resuspended in FACS buffer. Data was acquired using a LSRII flow cytometer (BD Biosciences) and was analyzed using Flowjo (Treestar). Populations were defined as follows: CD4T cells: CD3+ CD4+ CD8-, CD8T cells: CD3+ CD8+ CD4-, B cells: CD3- NK1.1- CD19+, NK cells: CD3- NK1.1+ CD19-. For cell sorting, spleens were processed and stained as above, but were sorted using a FACSAria II Cell sorter (BD Bioscences) into Trizol LS (Life Technologies) and processed as previously described.

RNA sequencing of mouse bladder MVECs

[00329] The Gpr68 eGFP reporter mice were sacrificed with CO_2 and the bladders were dissected out and cut into small pieces with micro scissors in PBS. The tissues were digested with 12.5 mg/mL collagenase IV (Gibco) and 10 U/mL papain (Worthington) at 37 ºC for 1 h. The suspension was triturated with fire-polished glass pipettes and then spun down to collect the cell pellet. After washing the pellet twice with PBS, the cells were suspended in FACS buffer. The cells were stained with CD31-APC and CD45-APC-Cy7 (BD Biosciences) for 20 min at 4 ºC in the dark, and stained with PI before sorting. We sorted the cells that are CD31+ CD45- GFP+ and CD31+ CD45- GFP- into two 1.5 mL tubes each containing 1ml Trizol (Life Technologies). Total RNA was extracted and column purified using RNeasy kit (Qiagen). For each condition, 10ng of total RNA was used to produce the cDNA libraries using Ovation RNA-Seq v2 System (Nugen). The cDNA was then fragmented to ~200bp size range by Covaris S220. cDNA was prepared for RNA Sequencing by Ovation Ultralow DR Multiplex System (Nugen) and the quality validated Bioanalyzer using High Sensitivity DNA Chip (Agilent Technologies). cDNA libraries were run on a HiSeq 1000 (Illumina) with 50 bp single-end reads. Reads were de-multiplexed with the Illumina pipeline. Raw reads were aligned to a reference FASTA file which contains a curated list of mouse transcripts, plus mitochondrial sequence, and mitochondrial transcripts, and Illumina adaptor sequences. Alignment was done with BWA (Burrows-Wheeler Aligner) (Li and Durbin, 2009) and RPKM values were created from the SAM output file (Li et al., 2009a). All samples contained at least 20 million mapped reads. Messenger RNAs less than 200 bp were eliminated from analysis.

Culturing of mouse primary cerebral MVECs

[00330] Whole cerebrum from Gpr68-eGFP reporter mice were isolated in cold PBS and homogenized in buffer containing 1x HBSS, 15mM HEPES and 1% dextran sulphate on ice. Equal volumes of 26% dextran sulphate was mixed well with the homogenized samples and centrifuged at 5800g at 4 ºC for 10 min. The pellet was resuspended in 0.625mg/mL Liberase

enzyme mix (Roche) and digested at 37 ºC for 1hr (triturate once every 30 min using fire-polished glass pipette). Digestion was then stopped with cold isolation buffer and spun down. The pellet was resuspended in 25% BSA and spun for 15 minutes at 1500rpm at room temperature. The final pellet was resuspended in Endo-PM media (Navone et al., 2013) and plated onto laminin / fibronectin coated culture plates. Cells were cultured for at 37 ºC, 5% CO_2 until confluent with media changes every 2 days.

Lentiviral shRNA knockdown and laminar flow stimulation of MVECs

[00331] Dharmacon TRC shRNA constructs for murine Gpr68 were purchased from GE Healthcare. Gpr68 shRNA constructs were co-transfected with first generation packaging vectors using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions into HEK293T cells. Medium was changed after 24hrs and the supernatant was collected 48hrs after transfection. We centrifuged the supernatant at 500 g for 10 min and added over cultured primary endothelial cells (400 µL of supernatant per well on 24-well plate). 24hrs after infection medium was replaced with Endo-PM containing 2 µg/mL puromycin. 48h post infection, the MVECs were gently dissociated using Trysin (Lonza) and plated in µ-slide I 0.2 laminar flow chamber (Ibidi) and kept in Endo-PM with 2 µg/mL puromycin. 72 h later, cells were loaded with Fura-2 and calcium imaging experiments were conducted with pulsatile laminar flow (1Hz) stimulation.

Mesenteric arteries cannulation

[00332] 1st, 2nd and 3rd arterial segments were isolated from the mesenteric circulation and cannulated at both ends on glass micro-cannulae and mounted in a video-monitored perfusion system (Living System, LSI, Burlington, VT). Arterial segments were bathed in a 5 ml organ bath containing a physiological salt solution (PSS) of the following composition (in mM): 135.0, NaCl, 15.0, $NaHCO_3$, 4.6 KCl, 1.5, $CaCl_2$, 1.2, $MgSO_4$, 11.0, glucose, 10.0, N-2-hydroxyethylpiperazine-N-2-ethylsulfonic acid. The PSS was maintained at pH7.4, PO_2 160 mmHg, PCO_2 37 mmHg. Perfusion of arterial segments was obtained with 2 peristaltic pumps, one controlling flow rate and one under the control of a pressure-servo control system. Pressure at both ends of the arterial segment was monitored using pressure transducers. Arterial contractility was assessed with KCl (80 mM) and phenylephrine 1 µmol/L. Endothelium integrity was assessed with acetylcholine (1 µM) after precontraction with phenylephrine (50% of maximum contraction). Pressure was then set at 75mmHg and flow was increased by step (3 to 50 µl per min) through the distal pipette with a peristaltic pump. Arteries were then bathed in a Ca^{2+}-free PSS containing ethylene-bis-(oxyethylenenitrolo) tetra-acetic acid (2 mmol/L) and

sodium nitroprusside (10 µmol/L). Pressure was then increased by step from 10 to 125 mmHg, in the absence of flow, in order to determine passive arterial diameter (Henrion et al., 1997).

Flow-mediated outward remodeling of resistance arteries

[00333] 4-5-month old GPR68 KO and WT mice were submitted to surgery in order to increase blood flow in mesenteric artery as previously described (Caillon et al., 2016). Briefly, 3 consecutive first-order mesenteric arteries were used. Ligatures were applied to second-order branches as shown on Figure 10C. The artery located between the two ligated arteries was designed as high flow (HF) artery. Arteries located at distance of the ligated arteries were used as control (normal flow, NF). In this protocol, animals were anesthetized with isoflurane (2.5%). They were treated with buprenorphine (Temgesic; 0.1 mg/kg, s.c.) before and after surgery.

[00334] Mice were sacrificed after 14 days before collection of HF and NF first order arteries and of the corresponding HF and NF third order mesenteric arteries. Their passive diameter was then measured using pressure arteriography as describe above.

Blood pressure radiotelemetry

[00335] Pressure radio transmitters (HD-X11, Data Science International) were implanted into left carotid artery according to manufacturer's recommended procedure. Blood pressure and heart rate were recorded continuously and exported as 5-minute segments. Baseline blood pressure and heart rate data was averaged from last day of baseline measurements of all cohorts (usually 3rd or 4th day after the start of recording to obtain consistently stable measurements).

Ultrasound echocardiography

[00336] Mice were continuously anesthetized by 0.8-1% isoflurane (mixed with 1 L/min 100% O2) inhalation and immobilized on a heating platform to maintain the body temperature at 37°C ± 0.5°C. Heart rate (HR) and respiratory physiology were continuously monitored by ECG electrodes. They were analyzed by B-mode and M-mode echocardiography using a Vevo3100 instrument with a 40 MHz transducer (VisualSonics). All M-mode measurements were performed in end-diastole and end-systole. End-diastolic and end-systolic measurements were obtained at the time of maximal internal chamber dimensions and at the minimal internal chamber dimensions, respectively. The LV structural parameters measured from short axis view in M-mode were used in the calculation of LV ejection fraction (EF) and LV fractional shortening (FS).

Data Analysis

[00337] Unless otherwise noted, statistical significance was evaluated using unpaired two-tailed Students's t test when comparing the difference between two samples, and one-way

ANOVA was employed when comparing the samples among groups with more than two samples.

Construction of the high-throughput shear stress stimulation system

[00338] The high-throughput disturbed flow system utilizes a 384-pin array printed by Projet 3500 (3D Systems) using UV-cured Visijet Crystal resin (3D Systems). The circular face of the pin is 2.8mm in diameter. A square diaphragm neodymium subwoofer driver (W3-1750S, Tang Band) is used as the driver. The length of the cylindrical pin is parallel to the direction of the driver displacement when activated. A miniature 3-axis accelerometer (ADXL326, Analog Devices) affixed to array monitors the motion of the array. The assembly sits on a machined aluminum assay plate guide. Shims and set screws are employed to accurately align the array with the assay plate. A custom interface is written using LabView (National Instruments) which allows the user to set frequency, amplitude and duty cycle of the stimulation. The signal is sent to an amplifier (D-150A II, Crown) to drive the pin and create fluid motion. The accelerometer signal is acquired by a data acquisition card (USB-6341, National Instruments) and instantaneously displayed in LabView for the user to monitor the motion of the pin array.

Estimation of the shear stress intensity

[00339] The particle image velocimetery method used for estimating the shear stress intensity on the bottom of the assay well was described previously (Ranade et al., 2014). Briefly, we dispensed a suspension of 6 μm-diameter Texas Red fluorescent beads (Life Technologies) into the imaging chamber and let them settle to the bottom. We applied sine wave stimulations at 60Hz and imaged the motion of the beads at 500 frames per second using ORCA-Flash 4.0 CMOS camera (Hamamatsu). Images were analyzed using NIS-Elements (Nikon) and velocity values of ~60 beads were averaged for each input power setting. We calculated shear stress intensity at 3 μm above the surface of the cover slip as an approximation of that experienced by the cells cultured on the surface of the cover slip.

Quantitative Reverse Transcriptase PCR

[00340] For tissue qPCR, DRG were freshly isolated from adult C57BL/6J wild type mice and snap frozen on dry ice and total RNA was isolated using Trizol/choloform method. Total RNA from all other tissues was purchased from Zyagen. For qPCR using cultured cells, samples were lysed with Trizol and total RNA was isolated using chloroform/isopropanol precipitation. 100-200ng of total RNA was used to generate cDNA using the Quantitect Reverse transcript kit (Qiagen). Real time Taqman PCR assays for mouse and human Gpr68 were purchased from Life Technologies (assay id: Mm 00558545_s1 and Hs 00268858_s1) and IDT

(Mm.PT.58.42531755 and Hs.PT.58.27651490.g) with a FAM reporter dye and a non-fluorescent quencher.

[00341] We used FastStart Universal probe master mix (Rox) (Roche). The reaction was run in the 7900HT fast real time system (ABI) using 0.5ul of the cDNA in a 10ul reaction according to the manufacturer's instructions in triplicate. Calibrations and normalizations were done using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), where $\Delta\Delta CT$ = ((CT (target gene) - CT (reference gene)) - (CT (calibrator) - CT (reference gene). Gapdh and b-actin were used as the reference genes.

Results

Design and construction of a 384-well mechanical stimulation assay

[00342] Among different types of mechanical forces, fluid shear stress is perhaps the most amenable to a HT assay format. We aimed to develop a high-throughput compatible mechanical stimulation system capable of generating physiological levels of fluid shear stress. Our design concept featured a flat-headed piston driven by an acoustic transducer controlled by a signal generator (Figure 1A). During operation, the piston was immersed in buffer in a transparent-bottom welled plate, moving up and down at commanded frequency and amplitude to create disturbed fluid motion with an oscillatory fashion. The moving fluid imposes shear stress on the cells cultured at the bottom of the chamber. The response of the cells can be imaged by a detector below the plate (Figure 1A). After extensive validation, we scaled up the system to 384-well format. Briefly, a 384-pin stimulation array was constructed using 3D-printing technique and then affixed to an acoustic transducer. (Figure 1B). We optimized the shape of the pins to avoid trapping air bubbles when mounting the plate (Figure 1B, right). A customized user interface was created in LabView to control the frequency, amplitude, duty cycle, and duration of the stimulus. The system was compact enough to fit inside the Molecular Devices Fluorescent Image Plate Reader (FLIPR Tetra) (Figure 1C, D). We measured the intensity of the shear stress generated by the HT system using particle image velocimetry and found that it is between 0.1~2 Pa (Ranade et al., 2014). When desired, we also can increase the intensity of the shear stress up to ~16.7 Pa by increasing the viscosity of the buffer using viscosity modifier such as polyvinylpyrrolidone (PVP), a bio-compatible, water-soluble polymer.

[00343] Next, we asked if Human Umbilical Vein Endothelial Cells (HUVECs) could be acutely activated by the shear stress imposed by the HT system using intracellular calcium levels as readout. HUVECs express endogenous PIEZO1 and show PIEZO1-dependent

alignment to the direction of flow when subjected to shear stress stimulation (Li et al., 2014; Ranade et al., 2014). Indeed, we observed robust transient increase in intracellular calcium levels in HUVECs when stimulated with shear stress at 6.5 Pa (with increased viscosity by PVP) and above (Figure 1E). In the presence of 2.5 mM EGTA, the shear stress-induced calcium transients were completely abolished, suggesting that the signal was caused by extracellular calcium entering the cells (Figure 1E). The calcium signals were greatly attenuated in cells treated with PIEZO1 siRNA, indicating that the signal was indeed PIEZO1-dependent (Figure 1F, G). These results demonstrate that our high-throughput mechanical stimulation system is capable of applying physiologically relevant shear stress in a precise, quantitative, and reproducible fashion, paving the way for its application in siRNA screens to identify novel mechanically activated receptors.

MDA-MB-231 breast cancer cells express an unknown shear stress sensor

[00344] We had three criteria for a cell line suitable for the high-throughput siRNA screen of novel mechanosensitive receptors: a) the cell line must respond to the shear stress generated by our HT stimulation system by displaying calcium transients, b) the cell line must be transfectable by siRNA with satisfactory knockdown efficiency, and c) the response to shear stress should not depend on PIEZO1 or PIEZO2 (this third criteria was added after several new mechanosensitive cell lines identified turned out to respond to shear stress via PIEZO1). We compiled a list of candidate cell lines based on the National Cancer Institute 60 human tumor cell line collection (NCI-60). We selected cell lines that have low levels of RNA transcripts of PIEZO1 and PIEZO2 based on the data obtained from BioGPS.org. The candidate cell lines were subjected to shear stress at 2 Pa, and their intracellular calcium levels were recorded. Out of the 25 cell lines tested, the lung cancer cell line A549 and the breast cancer cell line MDA-MB-231 showed shear stress-evoked calcium transients (Figure 2A). The MDA-MB-231 responded to disturbed shear stress of 2 Pa with robust calcium transients, while those of A549 were relatively modest (Figure 2A). The shear stress-induced calcium signal in MDA-MB-231 cells was almost completely abolished when the calcium stores of the cells were depleted by 2 μM thapsigargin. Removing extracellular Ca2+ by incubating the cells with 2 mM EGTA prior to the onset of shear stress greatly reduced but did not abolish the response (Figure 2B, C). This suggests that the calcium transients are store-dependent; however, the signal also partly depends on the entry of calcium from the intracellular space. We therefore carried out siRNA experiments to see if the calcium transients are dependent on the low levels of PIEZO1 and PIEZO2. Transfecting MDA-MB-231 cells with siRNA against PIEZO1 or PIEZO2 achieved 86% and 78% knockdown at the transcript level, respectively, suggesting that the cells are

transfectable with high efficiency (Figure 3). Knocking down PIEZO1 and PIEZO2 did not affect the shear stress-induced calcium transients in MDA-MB-231 cells, suggesting an unknown molecule other than the PIEZOs is responsible for sensing shear stress (Figure 2D).

GPR68 is required for shear stress responses in MDA-MB-231 cells

[00345] We focused on membrane-integrated proteins as the most probable candidates for novel mechanosensors. We therefore constructed a limited library of siRNAs against genes encoding proteins with two or more predicted transmembrane domains (2+TM), a characteristic trait shared by ion channels and GPCRs. The library contains 21,925 unique siRNAs, targeting 3,175 unique reference sequences encoded by 2,765 unique genes, arranged in a singlet configuration, i.e., each well of the 384-well plate contains one siRNA oligo. On average, each gene is represented by 8 siRNAs across the library, most of them located on different plates. We transfected the MDA-MB-231 cells with the siRNA library and carried out the screen using the HT shear stress system. The primary screen yielded 56 hits, of which 26 were reconfirmed in a secondary screen (for hitpick criteria, see EXPERIMENTAL PROCEDURES). We then used independent reagents to silence the 26 candidate genes by testing siRNA pools (4 pooled siRNA oligos targeting a single gene) in a re-confirmation screen. For controls, in addition to PIEZO1 siRNA, we also included siRNA against a SERCA Ca2+-ATPase (ATP2A2) to control for the effect of intracelluar calcium store. Of the 26 genes, GPR68 was the only one to show significantly reduced calcium transients caused by disturbed shear stress in MDA-MB-231 cells upon knockdown (Figure 4A). siRNAs targeting different regions of GPR68 (a combination of four siRNA (Dharmacon catalog # L-056512-00-00) targeting ORFs of NM_175493 (SEQ ID NO: 8), propietery sequences) were all effective in inhibiting the shear stress-induced calcium transients, suggesting that attenuation is not likely to be due to off-target effects (Figure 4B).

[00346] GPR68 was described to be a proton-activated GPCR. It is fully activated at pH 6.8 and is mostly inactive at pH 7.8(Ludwig et al., 2003). We measured intracellular calcium levels in MDA-MB-231 cells when stimulated with different concentrations of extracellular protons. We found that MDA-MB-231 cells showed little intracellular calcium increase to pH 7.4 and above, while the calcium response was maximal to pH 6.5 and below (Figure 4C). Knocking down GPR68 by siRNA significantly reduced proton-induced intracellular calcium increase (Figure 4D), another line of evidence that siRNAs that we used are on-target. We next tested if the shear stress response we observed in MDA-MB-231 cells can occur at physiological pH levels. Indeed, we observed a bell-shaped curve such that shear stress is most effective in evoking calcium transients at pH levels of 7.4-6.9 (normal arterial blood pH is ~7.4, and 6.9 represents severe acidosis) (Figure 4E) (Schwaderer and Schwartz, 2004). This suggests that

GPR68 requires the presence of protons to sense shear stress. Ludwig et al. demonstrated the importance of extracellular histidine residues for activation of the receptor (Ludwig et al., 2003). Based on a structural model of GPR68, they suggest that protonation leads to loss of bonding and probably repulsion between residues, allowing the receptor to adopt an active conformation at physiological pH. At slightly alkaline pH, GPR68 is stabilized in an inactive state by hydrogen bonding involving histidines, and inhibit receptor activity. We tested a mutant of GPR68 where histidine 17, 20, 84, 169 and 269 were changed to phenylalanine, and found that it loses both pH sensitivity and shear stress sensitivity (Figure 5A), further supporting the notion that appropriate levels of proton must be present for the receptor to be activated. Hence, all follow-up experiments were carried out at pH 7.4. Taken together, these data suggest that GPR68 is necessary for the shear stress-induced calcium transients in MDA-MB-231 cells within the physiological pH range.

GPR68 is activated by shear stress imposed by disturbed as well as laminar flow

[00347] To test if GPR68 activation is sufficient to confer mechanosensitivity, we transiently expressed GPR68 in HEK-293T cells and applied shear stress generated by the HT system. HEK cells transfected with human GPR68 showed an increase in intracellular calcium levels in response to 2-Pa shear stress, while cells transfected with vector were not responsive (Figure 6A). The amplitude of the calcium signals was dependent on the intensity of shear stress applied (Figure 6B). The maximum amplitude of the shear stress response that we observe is ~70% of the response when stimulated with extracellular proton at pH 6.5 (Figure 6C). This implies that shear stress responses are overall comparable to the pH responses. The slightly lesser response compared to pH may be due to the fact that not all GPR68 proteins on the cell membrane experience shear stress as they would experience changes in pH. Notably, the calcium transients were completely abolished by 10 μM U73122, a PLC inhibitor, suggesting that signal is indeed dependent on the activity of GPR68, a $G_{q/11}$-coupled receptor (Figure 6D). Proton-induced GPR68 activity is acutely blocked by Cu^{2+} (Ludwig et al., 2003), and 20 μM Cu^{2+} also blocked the shear stress-induced calcium signal (Figure 6D). This acute blockage of shear stress-dependent calcium transients argues against the possibility that overexpression of GPR68 causes alterations in HEK cells (e.g., changes in membrane or cytoskeleton property) that indirectly induce mechanosensitivity. One possible mechanism of flow activation of GPR68 is that shear stress causes a decrease in pH, which in turn activates GPR68 indirectly. We measured both extracellular and intracellular pH real time by fluorescence imaging and found no change in either with the application of shear stress (Figure 5B-D), arguing against this

possibility. Together, our data provide evidence for a necessary and sufficient role of GPR68 in the shear stress-induced release of calcium from stores.

[00348] Many GPCRs have been proposed to be mechanosensitive, although direct activation by mechanical forces (on the order of seconds) has not been demonstrated using intracellular calcium as a readout (Storch et al., 2012). To test if sensitivity to shear stress of GPR68 is unique among GPCRs, we selected human GPCR clones that are considered mechanosensitive, including angiotensin II receptor type 1 (AGTR1), arginine vasopressin receptor 1A (AVPR1A), bradykinin receptor B2 (BDKRB2), muscarinic cholinergic receptor 5 (CHRM5), endothelin receptor type A (EDNRA), histamine receptor H1 (HRH1) and parathyroid hormone 1 receptor (PTHR1). These are $G_{q/11}$–coupled receptors that cause an increase in the intracellular calcium levels through the mobilization of calcium from intracellular stores upon activation. We transiently transfected HEK-293T cells with these GPCRs and subjected them to disturbed shear stress at 2 Pa generated by the HT system. HEK cells expressing human GPR68 and mouse Gpr68 showed robust calcium transients when stimulated (Figure 6E). In contrast, we did not detect significant increase in intracellular calcium levels upon application of disturbed shear stress at 2 Pa in cells transfected with the other GPCRs. We demonstrated functional expression of these receptors by evaluating their response to known agonists (Figure 6F). Finally, we tested GPR132, the closest family member of GPR68, and also a $G_{q/11}$-coupled receptor, and found that it is not responsive to the same level of shear stress, while it did respond normally to its agonist lactate (Figure 6E, F)(Chen et al., 2017). These results highlight the unique sensitivity of GPR68 to disturbed shear stress.

[00349] Fluid flow that cells experience *in vivo* can be laminar or disturbed, with unique functions described to each form. In laminar flow, the fluid flows in parallel layers in an orderly fashion (Chiu and Chien, 2011). Laminar flow can be pulsatile (with regular pulsatility) or steady (without pulsatility), both having a definite direction. In contrast, disturbed flow has irregular fluctuations with time and no clear direction. We first tested if GPR68 is able to display calcium transients upon shear stress imposed by pulsatile laminar flow. HEK-293T cells were transiently transfected with mouse Gpr68 and plated in a parallel plate flow chamber. Upon application of 1 Hz pulsatile laminar flow at 3.4 Pa, Gpr68-transfected cells showed a robust increase of intracellular calcium levels. In most cells, the intracellular calcium level returned to normal within minutes after the termination of the flow. Non-transfected cells in the same chamber did not show significant change in calcium levels (Figure 6G, H). There were large, periodic fluctuations in calcium signals in both Gpr68-transfected cells and untransfected cells synchronous with the flow pulsatility. These are artifacts caused by the cyclic shift in optical focus as a result of the

expansion and contraction of the flow chamber in response to the pulsatile flow. We also assayed the cells' response to steady laminar flow. Robust calcium transients were also observed upon application of steady laminar flow in the transfected cells, but not in the non-transfected cells (Figure 6I, J).

[00350] There are several proteins that are implicated as sensors of laminar flow, including transient receptor potential channel TRPV4, PIEZO1, and $G_{q/11}$-coupled receptors AGTR1 and BDKRB2 (Chachisvilis et al., 2006; Li et al., 2014; Mendoza et al., 2010; Ramkhelawon et al., 2013). Neither Piezo1- nor TrpV4-transfected HEK cells showed any significant increase in calcium levels with the same flow stimulation (Figure 6K) (See DISCUSSION). Finally, human AGTR1 and BDKRB2 were not responsive to steady laminar shear stress at 3.4 Pa (Figure 6K). Taken together, these results indicate that GPR68 is a unique GPCR that is sensitive to shear stress imposed by disturbed flow, pulsatile and steady laminar flow when heterologously-expressed, suggesting that it may be an essential component of shear stress sensing mechanism.

GPR68 is expressed in endothelial cells of small-diameter arteries

[00351] GPR68 is evolutionally conserved among vertebrates. No orthologues are found in bacteria, fungi, plants or invertebrates. In mouse, Gpr68 mRNA is detected in a variety of tissues as assayed by quantitative RT-PCR (Figure 7A). The expression level is highest in the spleen, consistent with previous reports that Gpr68 transcripts are enriched in immune cells (Yan et al., 2014). We tested a number of commercially available antibodies against Gpr68 and found that none of them can specifically detect the heterologously expressed Gpr68 (listed in Experimental Procedures). Therefore we employed in situ hybridization to detect Gpr68 RNA transcripts in tissue sections. Interestingly, we found that Gpr68 mRNA is detected in small diameter blood vessels across a number of tissues, including pancreas, liver and brain (Figure 7B). Based on morphology, the Gpr68+ cells appear to be endothelial cells. In order to better determine the identity of the Gpr68-expressing cells, we obtained a reporter line of Gpr68 generated by the GenSat Project. This BAC transgenic line contains an eGFP reporter construct for Gpr68 that is integrated in the genome (Gong et al., 2003). In this construct, the coding sequence of the eGFP was inserted immediately after the start codon of Gpr68. It is followed by a polyA sequence and selection markers, thereby preventing the transcription of Gpr68. The construct includes 50~100 kb of promoter and putative regulatory sequences flanking the 5' and 3' of the GFP-polyA-Amp-R6Kγ-Gpr68 (Figure 7C). We first tested if the eGFP reporter faithfully represents endogenous Gpr68 expression. We isolated immune cells from the spleen of the reporter mice and wild-type C57 B/L6 mice, and subjected them to fluorescence-activated cell

sorting (FACS). GFP signal was detected in most CD8+ T cells, NK cells and a subset of CD4+ cells. No GFP signal was detected in B cells (Figure 8A). qRT-PCR of the RNA isolated from sorted cells of wild-type mice showed that Gpr68 transcript levels are high in CD8+ T cells and NK cells, intermediate in CD4+ cells, and absent in B cells (Figure 8B). These results indicate that the GFP signal accurately represents endogenous Gpr68 transcription. Next, we tested if GFP signal is detected in endothelial cells. We isolated primary vascular endothelial cells from the mouse bladder and sorted cells that are positive for CD31 (marker for endothelial cells) and negative for CD45 (marker for leukocytes). We observed both GFP+ and GFP- populations of endothelial cells (Figure 5D, green and grey population, respectively). We then conducted RNASeq experiments using RNA isolated from GFP+ and GFP- endothelial cells. The results showed that Gpr68 transcripts are enriched nearly 8 fold in GFP+ cells compared to that of the GFP- cells. In comparison, Piezo1 is expressed at similar levels across the two populations. These results validate that GFP levels accurately represent endogenous Gpr68 expression in endothelial cells. Thus, the Gpr68 eGFP reporter line not only provides a sensitive way to identify Gpr68-expressing cells in fixed tissues, but is also a marker for Gpr68 expression in living cells.

[00352] We next evaluated Gpr68 expression pattern by staining a panel of tissue sections from eGFP reporter mice using a GFP antibody. The GFP antibody stains endothelial cells in pancreas, liver and bladder (Figure 8C, D, E). Consistent with the endothelial FACS result, we detected GFP only in a subset of endothelial cells. This is especially evident in sections from liver and bladder (Figure 8D, E). Interestingly, based on the morphology, the GFP+ cells appear to be endothelial cells of arterioles, the small diameter arteries surrounded by a layer of relatively thick smooth muscles. The venules, which have a less prominent smooth muscle layer, are mostly negative for GFP staining (Figure 8D, E). This is further confirmed by staining sections of mesenteric vessels. Mouse superior mesenteric vessels that supply blood to the small intestine were dissected out. The vessels were divided into groups containing 1st, 2nd, 3rd order vessels and vessels that attach to the wall of the small intestine (Figure 7F). The small diameter arteries are major sites of regulation of resistance to blood flow through shear stress-dependent vessel dilation and pressure-dependent constriction (Davies, 1995). The GFP signal was not detected in 1st and 2nd order arteries. The endothelial cells of 3rd order arterioles and the arterioles in the wall of small intestine showed the strongest GFP antibody staining (Figure 7F). These results indicate that Gpr68 is preferentially expressed in small-diameter resistance arteries, suggesting that Gpr68 may play a role in flow-mediated vasodilation.

GPR68 is required for laminar shear stress responses of mouse primary microvascular endothelial cells

[00353] We have shown that GPR68 is a shear stress sensor in cell lines. Next, we asked if GPR68 is required for shear stress in a physiologically relevant setting. The striking expression pattern of Gpr68 predicts that the GFP-positive endothelial cells should respond to shear stress with increased calcium transients, and that their mechanosensitivity should be at least partly dependent on Gpr68. To test this, we set up a culture system of mouse primary microvascular endothelial cells (MVECs) from the cerebrum of homozygous Gpr68-eGFP reporter mice and assayed their response to shear stress in the flow chamber. 1 Hz pulsatile laminar flow was applied with shear stress of increasing intensity of 2 Pa, 4 Pa and 6 Pa to cultured cells (physiologically relevant levels of shear stress experienced by small arteries). Notably, the GFP+ endothelial cells responded to the flow by increasing intracellular calcium levels, while the GFP- cells showed significantly lower responses (Figure 9A). To test if the flow-induced calcium increase is dependent on Gpr68, we carried out knockdown experiments by infecting the endothelial cells with viruses containing Gpr68 small hairpin RNAs (shRNA) (a combination of three viruses, target sequences: TGAAGCACGTACTGCAGCC (SEQ ID NO: 16), TACACTCCCAGCTCATTCC (SEQ ID NO: 17) and ACTCAAGCTAGGCCAACCC (SEQ ID NO: 18)) and non-targeting shRNA (since siRNA transfection of these primary cells was not successful). Upon stimulation with pulsatile flow at 4 Pa, the GFP+ cells infected with non-targeting shRNA virus showed an increase in intracellular calcium levels. Remarkably, however, cells infected with Gpr68 shRNA virus showed no appreciable responses to flow stimulation (Figure 9B). As expected, GFP- cells did not show significant responses to shear stress whether treated with non-targeting shRNA or Gpr68 shRNA (Figure 9B). Importantly, GFP+ endothelial cells infected with either virus showed similar response to 50 μM ATP, demonstrating that Gpr68 shRNA infected cells are healthy and able to respond to chemical stimuli (Figure 9C). These results show that the endogenous Gpr68 in primary MVECs are necessary for flow-induced calcium transients. qRT-PCR demonstrated that the RNA level of Gpr68 was knocked down by 73% in the Gpr68 shRNA- treated endothelial cells, compared to the non-targeting shRNA-treated cells (Figure 9D). Collectively, our results support the notion that Gpr68 is an essential component of shear stress-sensing mechanism in small diameter blood vessel endothelial cells.

GPR68 is required for flow-mediated dilation responses of mouse small-diameter mesenteric arteries *ex vivo*

[00354] The endothelial cells in resistance arteries sense increase in blood flow and signal to smooth muscles via Nitric Oxide (NO), Endothelium-derived hyperpolarizing Factor (EDHF) and other mechanisms to cause their relaxation (Niebauer and Cooke, 1996). This process results in an increase in the diameter of the vessel and is called flow-mediated dilation (FMD). To investigate whether GPR68 is responsible for FMD in this setting, we isolated mesenteric arteries (MAs) from the Gpr68 -/- mice and performed *ex vivo* cannulation experiments. We found that, compared to WT littermates, Gpr68 -/- mice had significantly lower FMD response in 3rd order MAs (Figure 10A). Interestingly, in 1st and 2nd order MAs, there are no differences in FMD responses between Gpr68 KO and WT (Figure 11A, B). This matches the expression pattern of Gpr68 in the mesenteric vessel bed, where there was no or little expression in 1st and 2nd order, but prominent expression in 3rd order or smaller arteries (Figure 7F). Furthermore, application of the nitric oxide synthase (NOS) inhibitor L-NAME almost completely abolished FMD response in third order MAs of both Gpr68 KO and WT animals, indicating that Gpr68 likely functions upstream of the NO pathway (Figure 11C). Notably, in 3rd order MAs, dilation responses to acetylcholine and constriction responses to KCl were similar between Gpr68 KO mice and WT littermates (Figure 11D), indicating that the lack of FMD response in KO vessels is not due to structural defects. These data demonstrate that loss of Gpr68 activity specifically affects the mechanical aspect of the dilation response in 3rd order arteries.

[00355] Since loss of Gpr68 receptor activity in Gpr68 -/- vessels leads to impaired FMD response, we predicted that activating Gpr68 by chemical means (i.e., without flow) would lead to vessel dilation. We opted against using acid as a stimulus since existence of other acid receptors in vessels renders it difficult to interpret the results; furthermore, lowering pH in arterioles causes severe vasospasm, making this experiment impractical. Instead, we synthesized an allosteric modulator of Gpr68 named Ogerin, which was shown to specifically modulate human and mouse GPR68 activity (Huang et al., 2015). We found that Ogerin activates Gpr68 expressed in HEK-293T cells, with an EC50 around 0.17 μM (Figure 12). Consistent with our prediction, adding Ogerin to 3rd order WT MAs induced a dose-dependent dilation (Figure 10B). Importantly, similar vessels from Gpr68 -/- mice displayed no dilation response to Ogerin across a concentration range from 1 nM to 1 μM (Figure 10B). Taken together, Gpr68 is both necessary and sufficient for flow sensing in small-diameter MAs. Our findings demonstrate that GPR68 is an important endothelial shear stress sensor that underlies flow-mediated dilation.

[00356] Gpr68 -/- mice were reported to be grossly normal (Yan et al., 2014). Our echocardiography study showed that they have similar cardiac parameters compared to WT littermates (Figure 13A). We also measured hemodynamic parameters in Gpr68 -/- mice by radiotelemetry and found slightly lower systolic pressure, while diastolic pressure, mean arterial pressure and heart rate were indistinguishable from WT mice (Figure 13B). This is consistent with the notion that FMD in resistance arteries are more involved in the control of local blood flow to tissues, and changes in resistance arteries tone in a specific tissue may not be extrapolated to changes in systemic blood pressure (see DISCUSSION). The marginally lower systolic blood pressure phenotype could be due to compensatory mechanisms, which might cause a lowering of the baseline vascular tone or an increase in the effectiveness of alternative dilation mechanisms. Indeed, we found that the myogenic tone was lower in 3rd order arteries from Gpr68-/- mice compared to their WT littermates (Figure 13C). In addition, the dilation response to nitric oxide donor sodium nitroprusside (SNP) is higher in KO vessels, suggesting that KO mice could be more sensitive to NO-induced vasodilation (Figure 13D).

GPR68 is required for flow-mediated outward remodeling of mouse small-diameter mesenteric arteries *in vivo*

[00357] We demonstrated above using an *ex vivo* preparation that FMD is disrupted in Gpr68-deficient mice. To show if this observation is relevant in an *in vivo* pathological setting, we explored the role of GPR68 in arterial remodeling in response to chronically increased shear stress. Indeed, a chronic increase in flow induces a diameter enlargement as seen in collateral arteries growth in ischemic tissues. This flow-mediated outward remodeling (FMR), driven by the rise in shear stress, has a major role in post-ischemic revascularization (Henrion et al., 1997). To test if GPR68 is required for FMR, we performed ligation of second order mesenteric vessels to create a branch of arteries with high flow (HF, Figure 10C). We found that FMR was absent in third order mesenteric arteries isolated from GPR68 KO mice, compared to WT mice where the increase in flow induced a significant diameter enlargement (Figure 10D-F). Importantly, FMR was significant in first order arteries isolated of GPR68-/- mice (Figure 14B) and it was not significantly different from that observed in WT mice (Figure 14A, C), suggesting GPR68's shear stress-sensing role is specific to small-diameter arteries (resistance arteries, where GPR68 is expressed). Taken together, our studies with constitutive GPR68 knockout mice demonstrate that under normal circumstances, this receptor plays a major role in flow-mediated dilation, without having a profound cardiovascular performance. However, when placed in a stressed state caused by occlusion events, etc., GPR68 plays a critical role in vessel remodeling to increase flow capacity to meet the perfusion demand of affected tissues.

Discussion

Criteria for a mechanosensor and the case for GPR68

[00358] Many membrane proteins, including ion channels and GPCRs, have been postulated to be mechanosensitive. To categorize a protein as a mechanosensor, several criteria should ideally be met: expression in the correct cells, essential for the immediate signaling response of cells to the relevant force, and activation by the relevant mechanical force when expressed in heterologous cells or reconstituted in lipid bilayers (Ernstrom and Chalfie, 2002). In some cases, the third requirement cannot be met for technical reasons, and other forms of evidence, such as altering ion selectivity of the endogenous mechanically activated currents by inducing a point mutation in the pore of a candidate ion channel, are invoked instead. A few ion channels satisfy these criteria, including bacterial MscS and MscL, C. elegans MEC-4 and MEC-10, Drosophila NOMPC, mammalian TREK1/TRAAK, and PIEZO ion channels (Coste et al., 2010; Dedman et al., 2009; Driscoll and Chalfie, 1991; Huang and Chalfie, 1994; Levina et al., 1999; Sukharev et al., 1994; Yan et al., 2013).

[00359] GPCRs are sensory molecules responsible for vision, smell, and taste, among other functions. In addition, many GPCRs have been proposed to be involved in mechanotransduction (Storch et al., 2012). Here we provide evidence that GPR68, a Class A Rhodopsin-like GPCR, satisfies all three criteria for a mechanosensor. We show that Gpr68 is expressed in a subset of vascular endothelial cells, specifically in the cells that have a significant response to shear stress. Knocking down Gpr68 abolishes shear stress-induced calcium transients in primary MVECs, indicating that it is required for shear stress-sensitivity of endothelial cells. Importantly, overexpressing human or murine Gpr68 in HEK-293T cells induces sensitivity to shear stress in response to various types of flow stimulations. The shear stress-induced calcium signaling in heterologous cells is acute (onset within seconds), and these kinetics of activation is on a similar time scale as the activation in endothelial cells.

[00360] We compared mechanosensitivity of GPR68 to other GPCRs implicated in mechanosensation and found that only GPR68 overexpression gives rise to detectable levels of calcium transients in response to the HT disturbed shear stress assay as well as the laminar shear stress assay used here. The results are technically valid as all the other GPCRs are Gq/11-coupled, and calcium signals were observed when these receptors were activated by known agonists. It is possible that the other GPCRs tested are mechanosensitive; however, GPR68 is more sensitive (lower threshold), and that higher levels of shear stress or other forms

of mechanical forces are required to activate those GPCRs. Nevertheless, we could not find evidence in the literature that direct mechanical stimulation, rather than osmotic stress, can activate any of these other GPCRs to induce immediate downstream events (e.g., PIP2 deletion, change in intracellular calcium levels, etc.). However, AGTR1 and other GPCRs are proposed to be sensitive to membrane stretch when assayed indirectly by electrophysiological recording from HEK cells co-expressing TRPC6 (Mederos y Schnitzler et al., 2008). Evidence that TRPV4 is necessary and sufficient for sensing shear stress is lacking.

[00361] There are reports of bradykinin B2 GPCR activation by shear stress (Chachisvilis et al., 2006). By using time-resolved fluorescence microscopy and GPCR conformation-sensitive FRET, conformational dynamics of the receptor was detected by monitoring redistribution of GPCRs between inactive and active forms upon application of fluid shear stress. This suggests that GPCRs, although lacking obvious flow sensing domains, can indeed undergo conformational change under fluid shear stress. GPR68 may work in a similar way but have higher signaling efficiency. It is also possible that an unknown upstream mechanosensor can activate GPR68; however, this factor would be expressed quite ubiquitously. For Piezo1 ion channels, we also raised this alternative hypothesis as a possibility till we demonstrated that Piezo1 ion channels alone in bilayers can be activated by mechanical force by testing its sensitivity to membrane perturbations without any other cellular component (Syeda et al., 2016).

Role of GPR68 in endothelial cells

[00362] It is interesting that both Piezo1 and GPR68 are expressed in endothelial cells. Piezo1 can be activated by fluid shear stress when overexpressed in HEK cells as assayed by whole-cell patch clamp recording. However, we did not detect Piezo1 activation by calcium imaging upon laminar flow (Figure 6K), although Piezo1-dependent calcium transients were recorded in response to disturbed shear stress. This difference could be due to the fast inactivation of Piezo1 when heterologously expressed. Piezo1 opens within 1 ms after a mechanical force is applied, and mostly closes within tens of milliseconds. During this brief channel opening period, the amount of calcium ions that enter through Piezo1 is challenging to detect via Fura-2 calcium imaging technique. We can imagine that Piezo1 is less susceptible to inactivation in response to disturbed shear stress. Regardless, any Piezo1-dependent Ca2+ signal is likely to rely on downstream amplification steps. Unlike Piezo1, however, activation of Gpr68 causes a robust calcium release from intracellular stores in response to both laminar and disturbed flows. Importantly, we show that Gpr68-dependent mechanoresponses are present in MDA-MB-231, even when Piezo1 is knocked down, indicating that the two signaling molecules can function independently (Figure 2D). In this sense, Gpr68 signaling is distinct from Piezo1,

and the two sensors could play different or complementary roles in vivo. Indeed, Piezo1 is required for embryonic arterial remodeling and its constitutive deletion causes embryonic lethality. Gpr68-deficient mice survive to adulthood and are grossly normal (Li et al., 2009b), suggesting that Gpr68 is not crucial for vasculature development, and as shown here, plays a role in flow-mediated dilation (FMD) in the adult. Given that Piezo1 has also been recently shown to play a role in FMD (Wang et al., 2016), as well as flow-stimulated vasoconstriction through endothelium-smooth muscle coupling (Rode et al., 2017), it is possible that both Piezo1 and Gpr68 contribute to sensing shear stress within endothelial cells. For example, Piezo1 may act in larger mesenteric arteries (2nd order) to induce vasoconstriction during exercising whereas Gpr68 in smaller arteries may allow the maintenance of a baseline blood flow thus preventing ischemia. Analysis of double knockout mice could shed light on whether Piezo1 and Gpr68 play additive/overlapping roles in endothelial cells.

[00363] GPR68 can also be activated by protons. It is fully activated at pH 6.8 and is inactive at pH 7.8 (Ludwig et al., 2003). Low pH and shear stress are both relevant stimuli that endothelial cells experience in vivo. Moreover, both acidosis (blood pH~7.0) and shear stress cause acute vasodilation (Lindauer et al., 2003; Niebauer and Cooke, 1996), raising the possibility that GPR68 could be a poly-modal receptor for the two distinct but functionally related stimuli. We found that the activation of GPR68 by shear stress is modulated by extracellular proton concentration. The shear stress response is robust at physiological pH, and diminishes when pH is below 6.9 or above 7.4 (Figure 4E). At pH<6.9, it is likely that most GPR68 are already active or desensitized, therefore shear stress stimulation causes little additional calcium signal. The attenuated response at pH>7.4 suggest that GPR68 requires protons to be activated by shear stress. This is reminiscent of coincidence detection of TRPM8 in response to icillin only in the presence of calcium (Chuang et al., 2004). Coincidence activation of GPR68 by proton and shear stress could endow GPR68 with the capacity to generate complex responses to these two physiologically relevant stimuli. For example, shear stress would induce intracellular calcium increase (and consequently vasodilation) only when blood pH is at physiological level. This is important in acute alkalosis conditions (pH>7.45), where the removal of local CO_2 by blood flow exceeds the production by metabolic tissues (Schwaderer and Schwartz, 2004). Vasodilation due to shear stress could be counter-productive by increasing flow and removing even more CO_2 from local tissues, therefore exacerbating the condition. Low proton concentration may serve as a checkpoint to prevent vasodilation in this case. This intriguing biophysical design greatly increases the response profile of GPR68, enabling it to react properly to multiple scenarios in vivo.

[00364] What are the downstream consequences of GPR68 activation in endothelial cells? As a Gq/11-coupled receptor, GPR68 activation is expected to lead to the cleavage of PIP2 into IP3 and DAG by PLC, and induces calcium release from the store. In endothelial cells, this could trigger several acute signaling pathways. Increase in cytosolic calcium levels could lead to the activation of Ca2+-gated channels (Orai1, K_{Ca}2.3, K_{Ca} 3.1) or synthesis of NO by NOS or release of EDHF (Grgic et al., 2009). Depletion of membrane-bound PIP2 may also activate KCNK channels and lead to the hyperpolarization of the cells (Pathan and Rusch, 2011). The release of NO, EDHF and activation of KCNK channels in turn can lead to hyperpolarization of smooth muscles surrounding the vessels, causing them to relax and therefore dilate the vessels. We have shown that NO is likely to be an essential downstream pathway of GPR68 and leads to vasodilation (Figure 11C). Future studies might reveal additional pathways that could be activated by GPR68.

Vascular resistance and blood pressure regulation by GPR68

[00365] We have shown that in isolated resistance arteries, acute activation of the Gpr68 by agonist causes dilation, and that loss of Gpr68 activity leads to impaired FMD. FMD is an acute dilatory response of the endothelium involved in tissue perfusion when the blood flow rate rises with increase in metabolic demand, together with pressure-induced myogenic tone which prevents excessive rise in capillary pressure (Joyner and Casey, 2015; Levy et al., 2008). In several mouse models with reduced FMD due to lack of vimentin (Henrion et al., 1997) or dystrophin (Loufrani et al., 2001), FMD is selectively and strongly reduced with little change in blood pressure. Recently, it was shown that deleting flow-sensitive Piezo1 in endothelial cells causes no changes in resting blood pressure in adult mice (Rode et al., 2017). In humans, a reduction in FMD is the hallmark of endothelial dysfunction as observed in a variety of diseases such as hypertension, obesity and type 2 diabetes, whereas a reduced FMD is also found in subjects with risk factors not necessarily associated with high blood pressure such as tobacco consumption, menopause, aging, high blood cholesterol, chronic inflammatory diseases (Deanfield et al., 2007; Moroni et al., 2017; Poredos and Jezovnik, 2013). Here, we found that the systolic pressure in Gpr68 constitutive KO mice is actually slightly lower, while diastolic pressure was unaffected (Figure 13A). It is possible that compensatory mechanisms in response to chronic reduced FMD have caused this phenotype. We found evidence of reduced baseline myogenic tone in third order arteries from KO mice (Figure 13C). These mice also have a more effective dilation response to exogenous NO donor (Figure 13D). Both these parameters can potentially cause the observed reduced systolic pressure. In addition, it is important to note that systemic blood pressure is affected by many factors, including

sympathetic/parasympathetic nerve activities, differential regulation of dilation/constriction of the vessels from different tissues (i.e., brain vs. skeletal muscle). Therefore, our results with the cannulated 3rd order mesenteric arteries in Gpr68 knockout mice clearly establish a specific role of Gpr68 in sensing shear stress within blood vessel endothelial cells, and in regulating small blood vessel dilation.

Pathophysiological significance in ischemic disorders

[00366] A chronic increase in blood flow in arteries stimulates the endothelium and induces an increase in arterial diameter. This process, called flow-mediated outward remodeling (FMR), is governed by the rise in shear stress exerted on the endothelial cells and leads to the outward growth of the vascular wall so that shear stress is normalized within a few days. FMR plays a major role in collateral artery growth, or arteriogenesis, which is crucial to improve collateral circulation and tissue perfusion in patients with ischemic diseases. FMR becomes progressively reduced in aging (Dumont et al., 2008), is decreased even earlier in rat models of hypertension (Tuttle et al., 2002) and diabetes (Freidja et al., 2012), and has a strong prognostic significance in patients (Lynch et al., 2012). New therapeutics that improve FMR would potentially bring significant benefit to patients suffering severe ischemia as this would accelerate collateral artery growth and thus reperfusion (Silvestre et al., 2013).

Role of GPR68 in other tissues

[00367] GPR68 is also expressed in a few other cell types, including neurons in the brain and DRG, and in leukocytes. We detected murine Gpr68 RNA in a subset of DRG neurons by in situ hybridization (data not shown). However, it is unlikely that Gpr68 is the sensor underlying the MA currents observed in DRG neurons. First, it is not clear if shear stress is a relevant mechanical stimulus for DRG neurons. Furthermore, all MA currents in DRG neurons have extraordinarily fast sub-millisecond onset (Delmas et al., 2011). In comparison, the fastest-signaling GPCR described to date are the ones involved in photosensing, which require tens of millisecond to activate and to couple to downstream ion channels (Burns and Pugh, 2010). We are investigating the possibility that Gpr68 serves as a modulator of other ion channels in DRG, similar to muscarinic acetylcholine receptors that modulate inward-rectifying K+ channels (Chuang et al., 1997). Gpr68 is also highly expressed in immune cells (Figure 8A, B). It has been suggested that the expression of Gpr68 in myeloid-derived cells is required for prostate cancer cell-induced immunosuppression (Yan et al., 2014). GPR68 mice also have impaired T cell responses associated with a reduced frequency and number of dendritic cells (DCs), higher production of nitric oxide by macrophages, and defect in DCs migration to draining lymph nodes

(Aoki et al., 2013; D'Souza et al., 2016). There's also evidence suggesting that GPR68 could be involved in recall of fear conditioning in mice (Huang et al., 2015). Interestingly, it's been suggested that GPR68 as a proton sensor is required for proper enamel formation in humans (Parry et al., 2016). However, the mechanism of these effects, and whether mechanotransduction is involved, especially in the setting of immune cells, is not clear.

Significance of the high throughput mechanical assay system

[00368] Our novel high throughput shear stress stimulation system vastly improves the throughput of mechanotransduction assays. This enabling technology has made unbiased, genome-wide screen for novel mechanosensitive proteins and pathways feasible. This system could also greatly impact the discovery of small molecules that modulate mechanosensory proteins. The identification of GPR68 as a novel mechanosensor validates the value and potential of this novel high throughput system.

Example 2: GPR68 is induced in rat and human hepatic stellate cells by TGFβ treatment

[00369] Rat hepatic stellate cells were isolated using enzyme perfusion followed by density gradient centrifugation. Cultured rat hepatic stellate cells were treated with TGF-β or vehicle, and mRNA was isolated and subject to RNAseq to enable transcriptome analysis, and quantitative PCR to assess GPR68 transcript levels.

[00370] Cultured human primary hepatic stellate cells were treated with TGF-β or vehicle, and mRNA was isolated and subject to RNAseq to enable transcriptome analysis, and quantitative PCR to assess GPR68 transcript levels.

[00371] GPR68 mRNA was induced in both rat and human hepatic stellate cells by treatment with TGFβ (Figure 15).

Example 3: Treating CHF in a Mouse Model

[00372] CHF in mice is induced by one of the following means: a) pressure overload by transverse aortic constriction surgery, or b) myocardial infarction by left anterior descending artery ligation surgery. A GPR68 modulator is administered prior-to, during or after the induction of CHF. Cardiac function is assessed by echocardiography, left ventrical hypertrophy levels are determined by histology, and fibrosis level is assessed by Masson's trichrome staining and Pico Sirus Red staining.

Example 4: Treating Stroke in a Mouse Model

[00373] Stroke in mice is induced by one of the following means: a) permanent occlusion of the middle cerebral artery (MCA), or b) transient occlusion of the MCA followed by reperfusion, via the intraluminal suture technique. A GPR68 modulator is administered prior-to, during or after the induction of stroke. Efficacy is assessed by infarct size measured by magnetic resonance imaging, cerebral blood flow recovery is determined by laser Doppler imaging, and histology is assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining.

Example 5: Treating peripheral arterial disease (PAD) in a Mouse Model

[00374] Mouse models of PAD, including but not limited to: a) surgical ligation of either iliac artery proximal to the internal branch, or femoral artery proximal to the superficial epigastric artery, b) critical limb ischemia, c) diabetic neuropathy are established. A GPR68 modulator is administered prior-to, during or after the induction of PAD. Efficacy is assessd by blood flow recovery measured by laser Doppler imaging, magnetic resonance imaging or computed tomography, biomarker assessment and histological analysis are perforemed by haematoxylin and eosin staining.

Example 6: Treating Liver Fibrosis in a Mouse Model

[00375] Liver fibrosis in mice is induced by one of the following means: a) administration of chemicals including carbon tetrachloride (CCl4), thioacetamide (TAA), alcohol or dimethylnitrosamine (DMN) and diethylnitrosamine (DEN); b) special diet including high-fat, high-cholestrol, high-fructose diet, methionine-deficient and choline-deficient diet and choline-deficient L-amino acid defined diet; and c) bile-duct ligation surgery. A GPR68 modulator is administered prior-to, during or after the induction of liver fibrosis. Liver enzyme levels (aspartate transaminase (AST) and alanine transaminase (ALT)) in the blood are determined and fibrosis score is determined by histology (Masson's trichrome staining and Pico Sirus Red staining).

[00376] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

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We Claim:

1. A method of modulating flow-mediated dilation (FMD) response or flow-mediated outward remodeling (FMR) of small-diameter arteries in a subject in need thereof comprising administering a modulator of a GPR68 gene product to the subject.
2. The method of claim 1, wherein the modulator is an antagonist or an agonist of the GPR68 gene product.
3. The method of claim 1 or 2, wherein the subject being treated has an abnormal vessel dilation and constriction response.
4. The method of claim 3, wherein the abnormal vessel dilation and constriction response is associated with endothelial dysfunction.
5. The method of claim 4, wherein the endothelial dysfunction is associated with vascular disorder, peripheral arterial disease, heart failure, hypertension, hypercholesterolemia, diabetes, septic shock, Behcet's disease, exposure to smoking tobacco products, exposure to air pollution, or a combination thereof.
6. The method of any one of claims 1-5, wherein the modulator of the GPR68 gene product is selected from the group consisting of a small molecule compound, an antibody, a nucleic acid molecule, a protein, and a combination thereof.
7. The method of claim 6, wherein the nucleic acid molecule is selected from the group consisting of an antisense oligonucleotide, a guide RNA, a ribozyme, an aptamer, and a small interfering RNA.
8. The method of any one of claims 1-7, wherein the subject is a human.
9. The method of any one of claims 1-8, further comprising measuring the vessel dilation response of the subject.
10. The method of claim 9, wherein the measuring the vessel dilation response comprises brachial artery ultrasound imaging (BAUI).
11. A method of reducing systemic vascular resistance (SVR) in a subject in need thereof comprising administering a modulator of a GPR68 gene product to the subject.
12. The method of claim 11, wherein the modulator is an antagonist or an agonist of the GPR68 gene product.
13. The method of claim 11 or 12, wherein the subject being treated has an abnormal SVR.
14. The method of claim 13, wherein the abnormal SVR is associated with peripheral arterial disease, heart failure, hypertension, hypercholesterolemia, diabetes, septic shock, or a combination thereof.

15. The method of any one of claims 11-14, wherein the modulator of the GPR68 gene product is selected from the group consisting of a small molecule compound, an antibody, a nucleic acid molecule, a protein, and a combination thereof.
16. The method of claim 15, wherein the nucleic acid molecule is selected from the group consisting of an antisense oligonucleotide, a guide RNA, a ribozyme, an aptamer, and a small interfering RNA.
17. The method of any one of claims 11-16, wherein the subject is a human.
18. The method of any one of claims 11-17, further comprising measuring the SVR of the subject.
19. The method of claim 18, wherein the measuring the SVR comprises measuring blood pressure (BP), heart rate (HR), stroke volume (SV), or a combination thereof.
20. The method of any one of claims 11-18, wherein the SVR of the subject is reduced by about 10% to about 50%.
21. A method of treating a cardiovascular disease in a subject in need thereof comprising administering a pharmaceutical composition comprising a modulator of a GPR68 gene product to the subject.
22. The method of claim 21, wherein the modulator is an antagonist or an agonist of the GPR68 gene product.
23. The method of claim 21 or 22, wherein the cardiovascular disease is selected from the group consisting of congestive heart failure (CHF), peripheral artery disease, stroke, diabetic nephropathy, and renal hypertension.
24. The method of claim 23, wherein the CHF comprises HF with reduced ejection fraction (aka HF due to left ventricular dysfunction) or HF with preserved ejection fraction (HFpEF) (aka diastolic HF or HF with normal ejection fraction).
25. The method of claim 23 or 24, wherein the CHF is associated with a coronary artery disease selected from myocardial infarction (heart attack), high blood pressure, atrial fibrillation, and valvular heart disease, excess alcohol use, infection, or cardiomyopathy of an unknown cause.
26. The method of any one of claims 21-25, wherein the modulator of the GPR68 gene product reduces the SVR or the left ventricle afterload of the subject.
27. The method of claim 26, wherein the SVR or the left ventricle afterload of the subject is reduced by about 10% to about 50%.
28. The method of any one of claims 21-27, further comprising diagnosing the cardiovascular disease in the subject.

29. The method of claim 28, wherein the cardiovascular disease is diagnosed based on the history of the symptoms, physical examination, echocardiography, blood test, electrocardiography, chest radiography, or a combination thereof.
30. The method of any one of claims 22-29, wherein the modulator of the GPR68 gene product is selected from the group consisting of a small molecule compound, an antibody, a nucleic acid molecule, a protein, and a combination thereof.
31. The method of claim 30, wherein the nucleic acid molecule is selected from the group consisting of an antisense oligonucleotide, a guide RNA, a ribozyme, an aptamer, and a small interfering RNA.
32. The method of any one of claims 21-31, wherein the subject is a human.
33. The method of any one of claims 21-32, further comprising administering an angiotensin converting enzyme (ACE) inhibitor, an angiotensin receptor blocker (ARB), a β-adrenergic receptor blocker, or a diuretics to the subject.
34. A modulator of a GPR68 gene product for use as a medicament for the treatment of a cardiovascular disease in a subject in need thereof.
35. A modulator of a GPR68 gene product in the manufacture of a medicament for the treatment of a cardiovascular disease in a subject in need thereof.
36. A method of treating liver fibrosis in a subject in need thereof comprising administering a pharmaceutical composition comprising an antagonist of a GPR68 gene product to the subject.
37. The method of claim 36, wherein the antagonist of the GPR68 gene product is selected from the group consisting of a small molecule compound, an antibody, a nucleic acid molecule, a protein, and a combination thereof.
38. The method of claim 37, wherein the nucleic acid molecule is selected from the group consisting of an antisense oligonucleotide, a guide RNA, a ribozyme, an aptamer, and a small interfering RNA.
39. The method of any one of claims 36-38, wherein the subject is a human.
40. A method of identifying a modulator of a GPR68 gene product comprising:
    a. Providing a population of cells expressing a GPR68 gene product;
    b. Adding a library of candidate molecules to the population of cells;
    c. Applying a shear stress or an acidic shock to the population of cells;
    d. Measuring the calcium transient in the population of cells; and
    e. Identifying a candidate molecule in a cell that shows enhanced/reduced calcium transient in the cell.

41. The method of claim 40, wherein the population of cells comprises endothelial cells, such as mouse primary brain microvascular endothelial cells, or human microvascular endothelial cells from brain, lung, bladder, or skin.
42. The method of claim 40 or 41, wherein the library of candidate molecules comprises a small molecule compound, an antibody, a nucleic acid molecule, or a protein.
43. The method of any one of claims 40-42, wherein the shear stress comprises disturbed flow or laminar flow.
44. The method of any one of claims 40-42, wherein the acidic shock comprises extracellular proton at pH 6.5.

FIG. 1

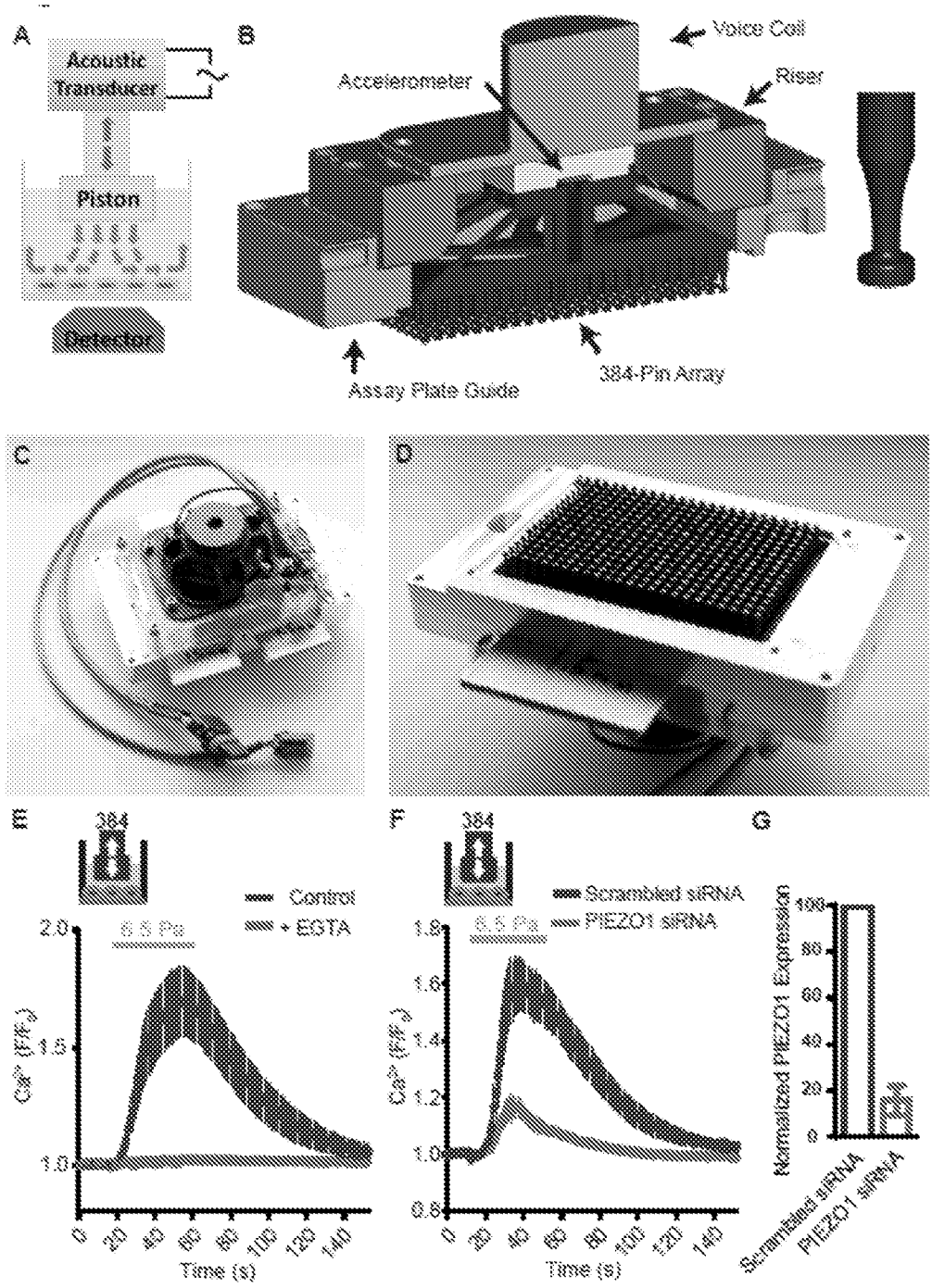

A
Acoustic Transducer
Piston
Deflector
B
Voice Coil
Riser
Accelerometer
Assay Plate Guide
384-Pin Array
C
D
E
384
Control
+ EGTA
6.5 Pa
Ca2+ (F/F0)
Time (s)
F
384
Scrambled siRNA
PIEZO1 siRNA
6.5 Pa
Ca2+ (F/F0)
Time (s)
G
Normalized PIEZO1 Expression
Scrambled siRNA
PIEZO1 siRNA

FIG. 2

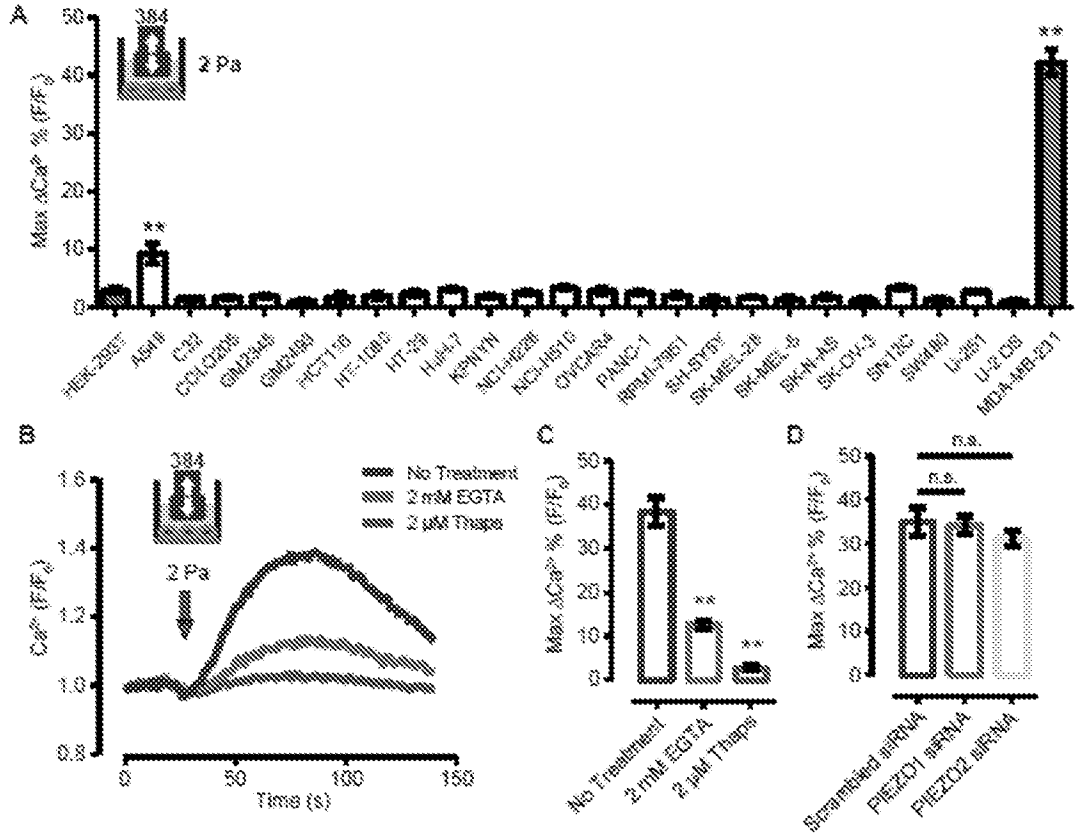

A
384
2 Pa
**
Max ΔCa²⁺ % (F/F0)
HEK-293T
A549
C-33
COLO205
GM2345
GM2680
HCT116
HT-1080
HT-29
HuH-7
KPNTN
MCI-H226
NCI-H510
OVCAR4
PANC-1
RPMI-7951
SH-SY5Y
SK-MEL-28
SK-MEL-5
SK-N-AS
SK-OV-3
SK-UC
SW480
U-251
U-2 OS
MDA-MB-231
B
384
2 Pa
No Treatment
2 mM EGTA
2 μM Thaps
Ca²⁺ (F/F0)
Time (s)
C
Max ΔCa²⁺ % (F/F0)
No Treatment
2 mM EGTA
2 μM Thaps
D
n.s.
n.s.
Max ΔCa²⁺ % (F/F0)
Scrambled siRNA
PIEZO1 siRNA
PIEZO2 siRNA

FIG. 3

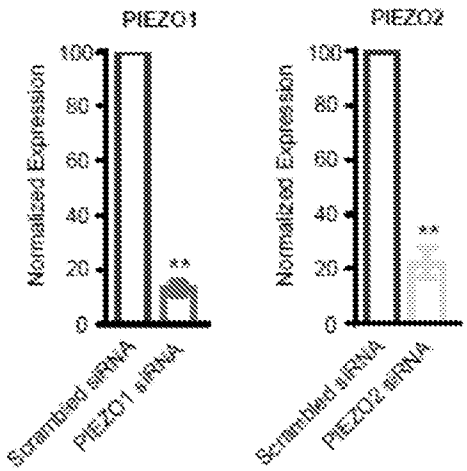

PIEZO1
PIEZO2
Normalized Expression
**
Scrambled siRNA
PIEZO1 siRNA
PIEZO2 siRNA

FIG. 4

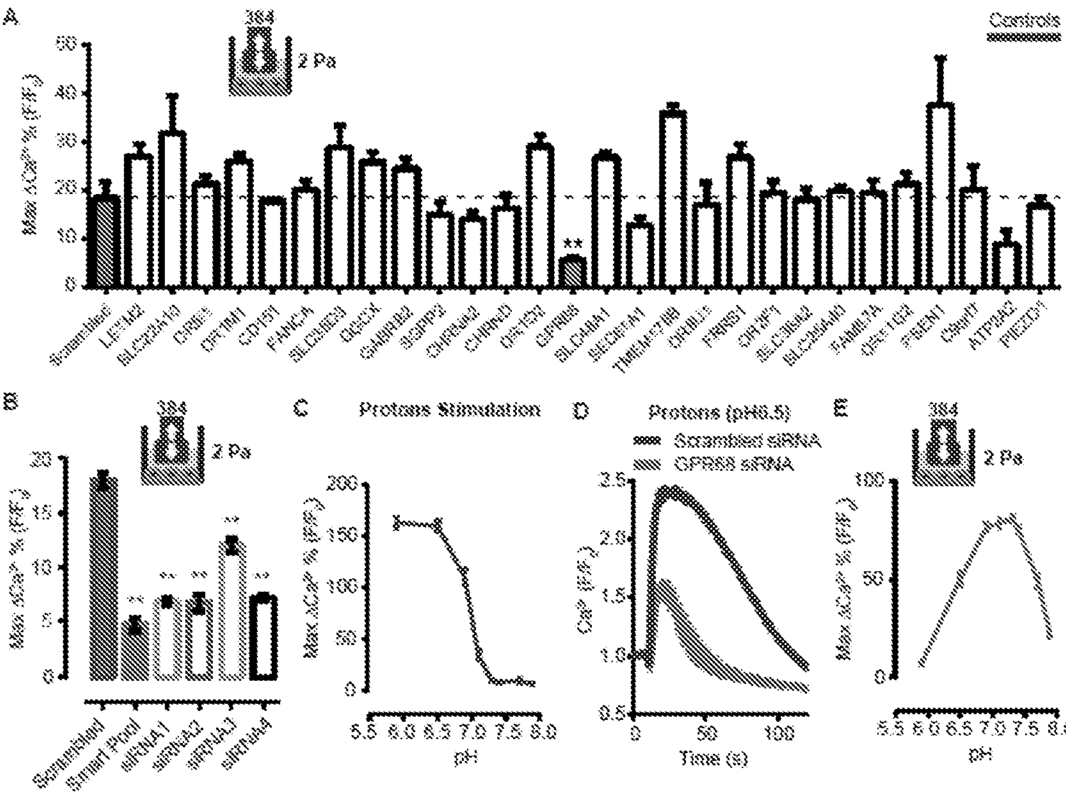

A
384
2 Pa
Controls
Max ΔCa²⁺ % (F/F₀)
**
B
384
2 Pa
Scrambled
Smart Pool
siRNA1
siRNA2
siRNA3
siRNA4
C
Protons Stimulation
pH
D
Protons (pH6.5)
Scrambled siRNA
GPR68 siRNA
Time (s)
E
384
2 Pa
pH

FIG. 5

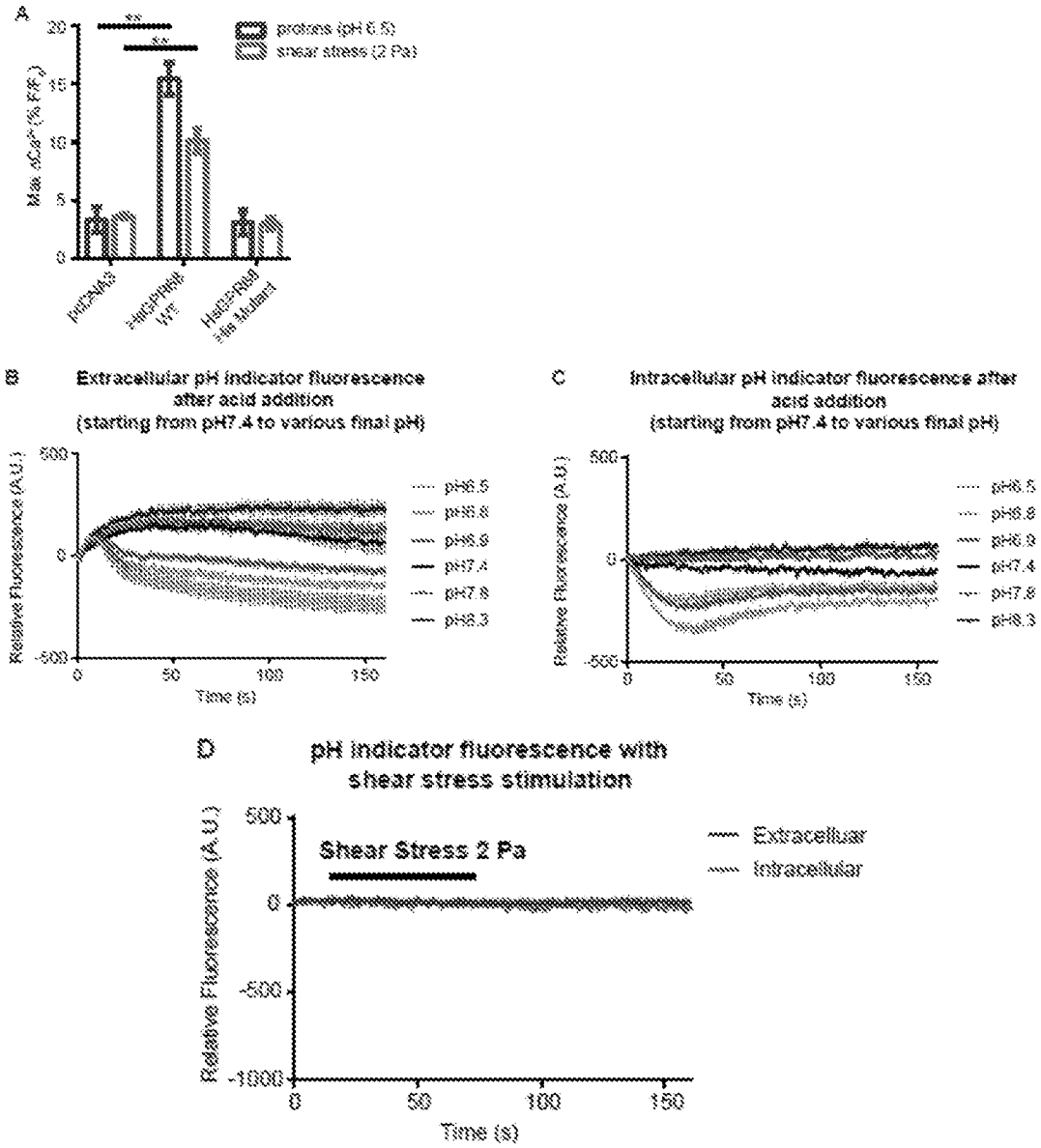

A
protons (pH 6.5)
shear stress (2 Pa)
B
Extracellular pH indicator fluorescence after acid addition (starting from pH7.4 to various final pH)
Relative Fluorescence (A.U.)
Time (s)
pH6.5
pH6.8
pH6.9
pH7.4
pH7.8
pH8.3
C
Intracellular pH indicator fluorescence after acid addition (starting from pH7.4 to various final pH)
Relative Fluorescence (A.U.)
Time (s)
D
pH indicator fluorescence with shear stress stimulation
Shear Stress 2 Pa
Extracellular
Intracellular
Relative Fluorescence (A.U.)
Time (s)

FIG. 6

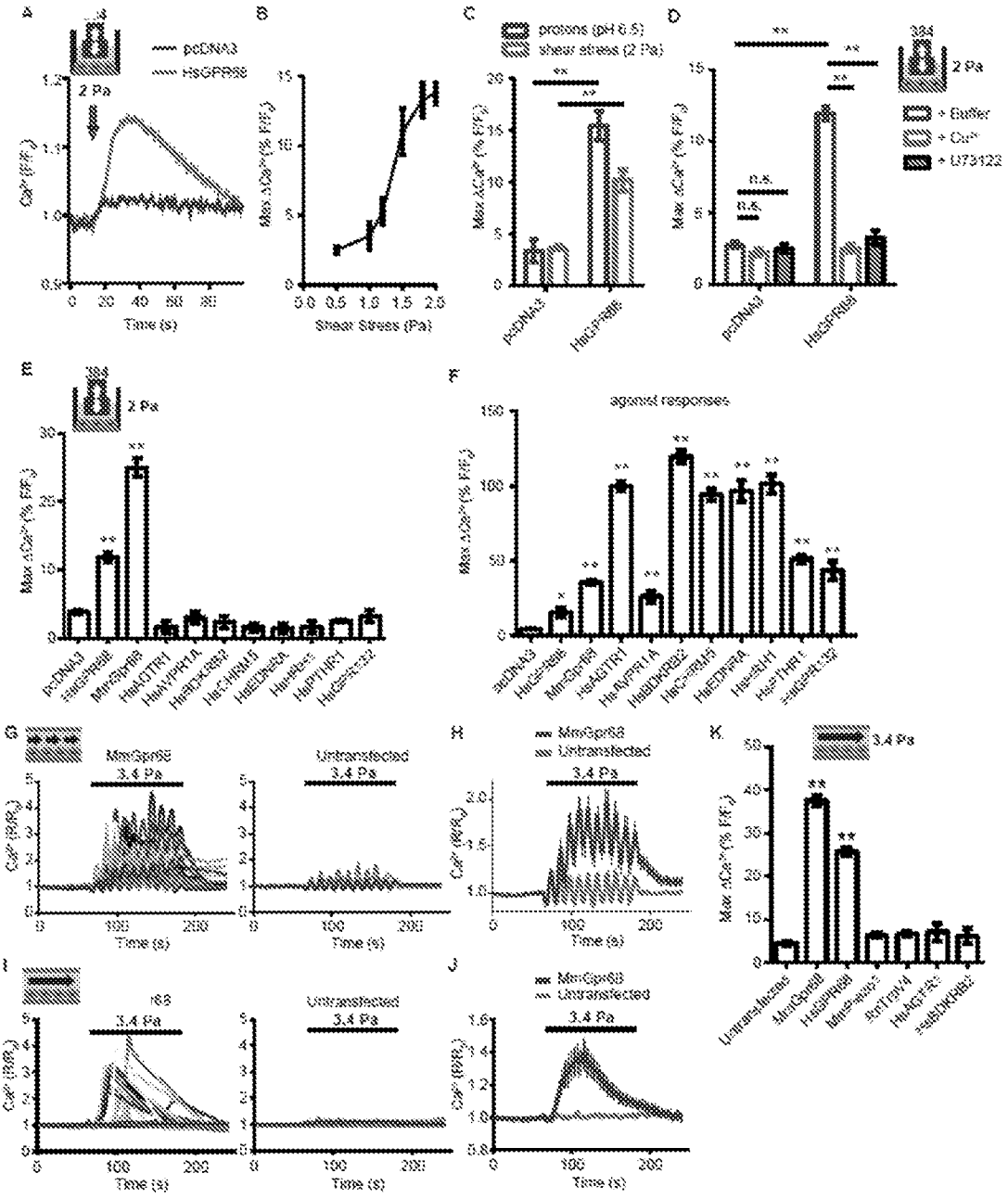

A
pcDNA3
HsGPR68
2 Pa
Time (s)
B
Shear Stress (Pa)
C
protons (pH 6.5)
shear stress (2 Pa)
pcDNA3
HsGPR68
D
+ Buffer
+ Ca2+
+ U73122
n.s.
E
2 Pa
F
agonist responses
G
MmGpr68
3.4 Pa
Untransfected
H
MmGpr68
Untransfected
I
J
K
3.4 Pa

FIG. 7

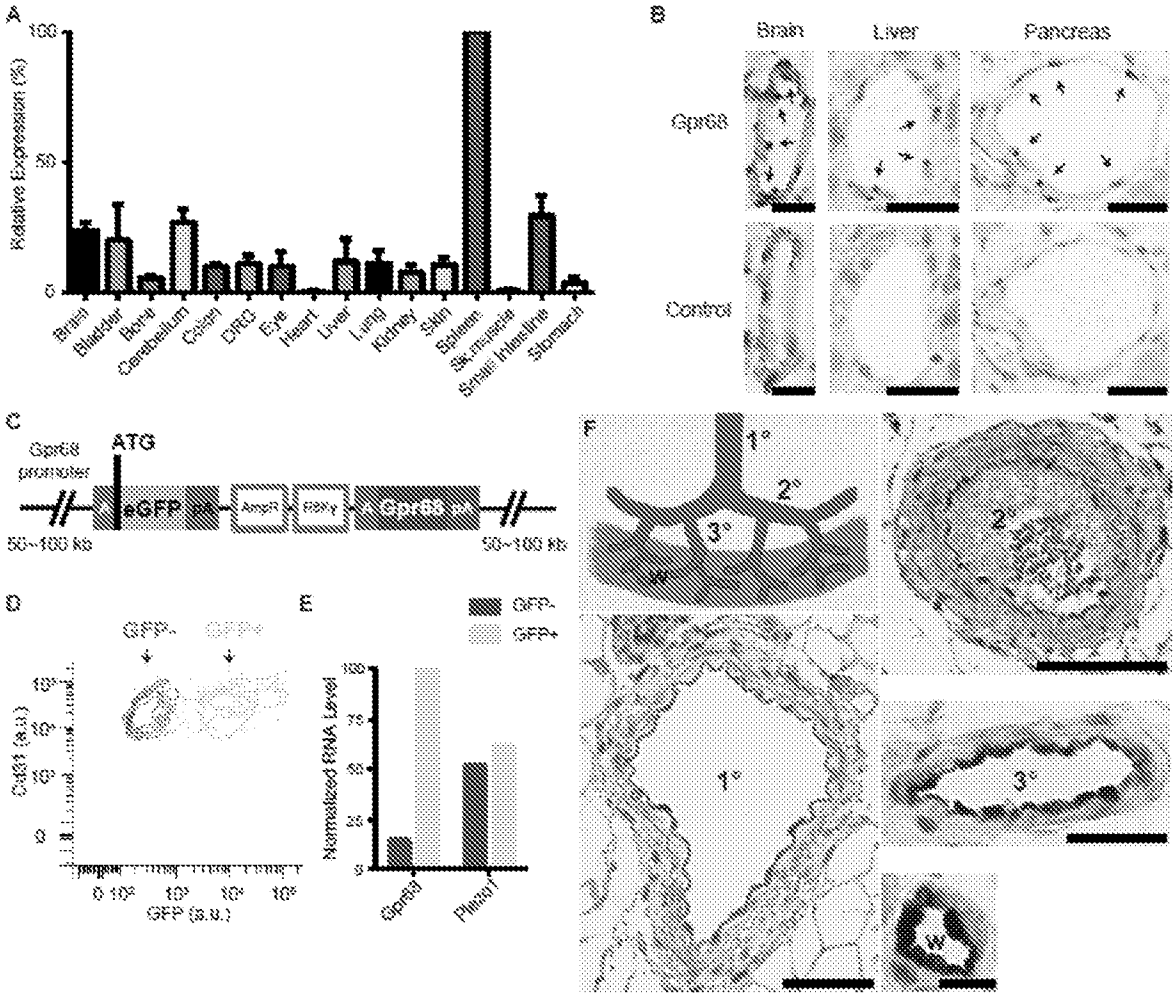

A
Relative Expression (%)
100
50
0
Brain
Bladder
Bone
Cerebellum
Colon
DRG
Eye
Heart
Liver
Lung
Kidney
Skin
Spleen
Skeletal muscle
Small Intestine
Stomach
B
Brain
Liver
Pancreas
Gpr68
Control
C
Gpr68 promoter
ATG
eGFP pA
Amp
Poly
Gpr68 pA
50~100 kb
50~100 kb
D
GFP-
GFP+
Cd31 (a.u.)
GFP (a.u.)
E
GFP-
GFP+
Normalized mRNA Level
Gpr68
Plexin1
F
1°
2°
3°
2°
1°
3°

FIG. 8

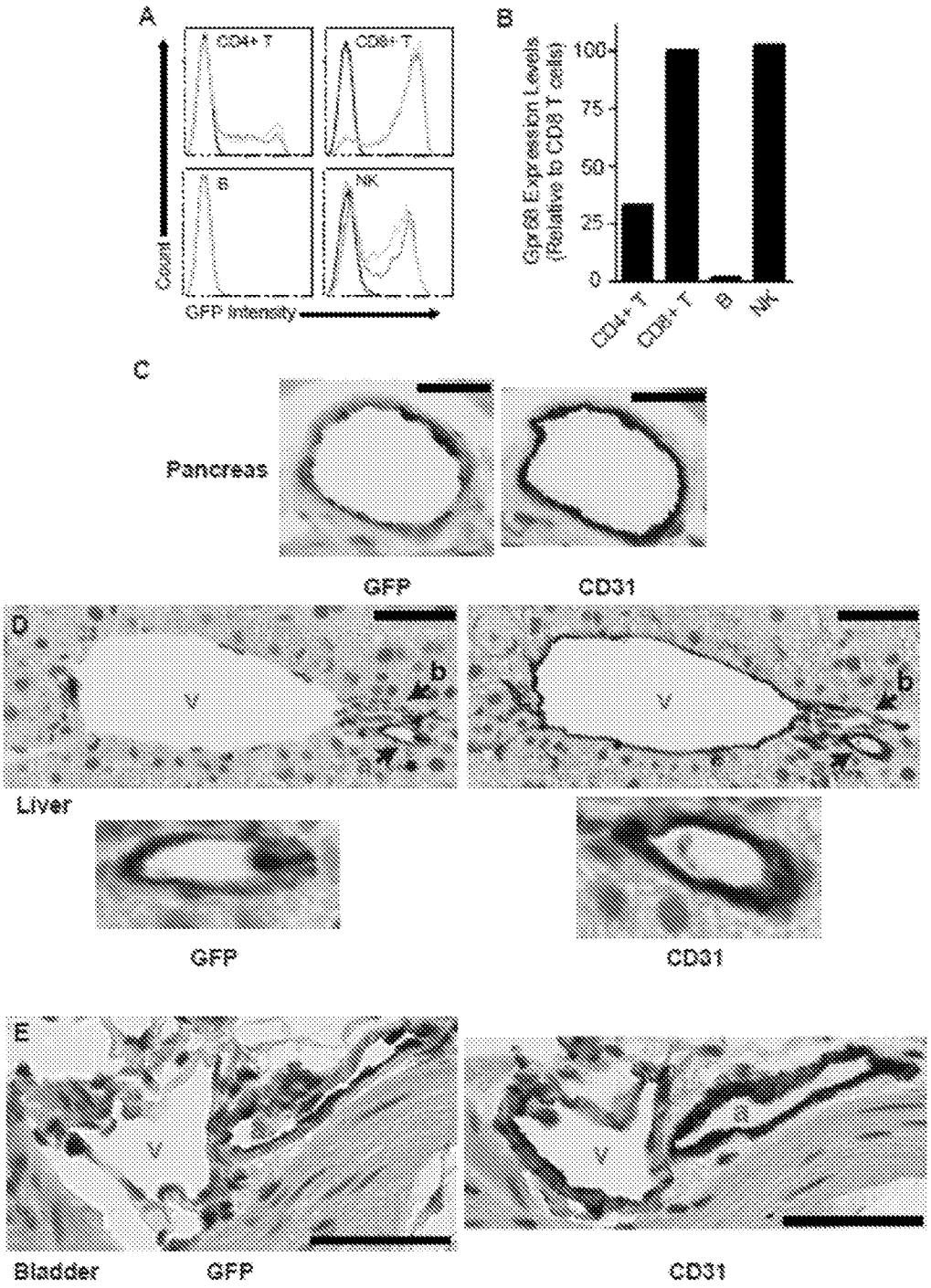

A
CD4+ T
CD8+ T
B
NK
Count
GFP Intensity
B
Gpr68 Expression Levels (Relative to CD8 T cells)
100
75
50
25
0
CD4+ T
CD8+ T
B
NK
C
Pancreas
GFP
CD31
D
V
b
Liver
GFP
CD31
E
V
Bladder
GFP
CD31

FIG. 9

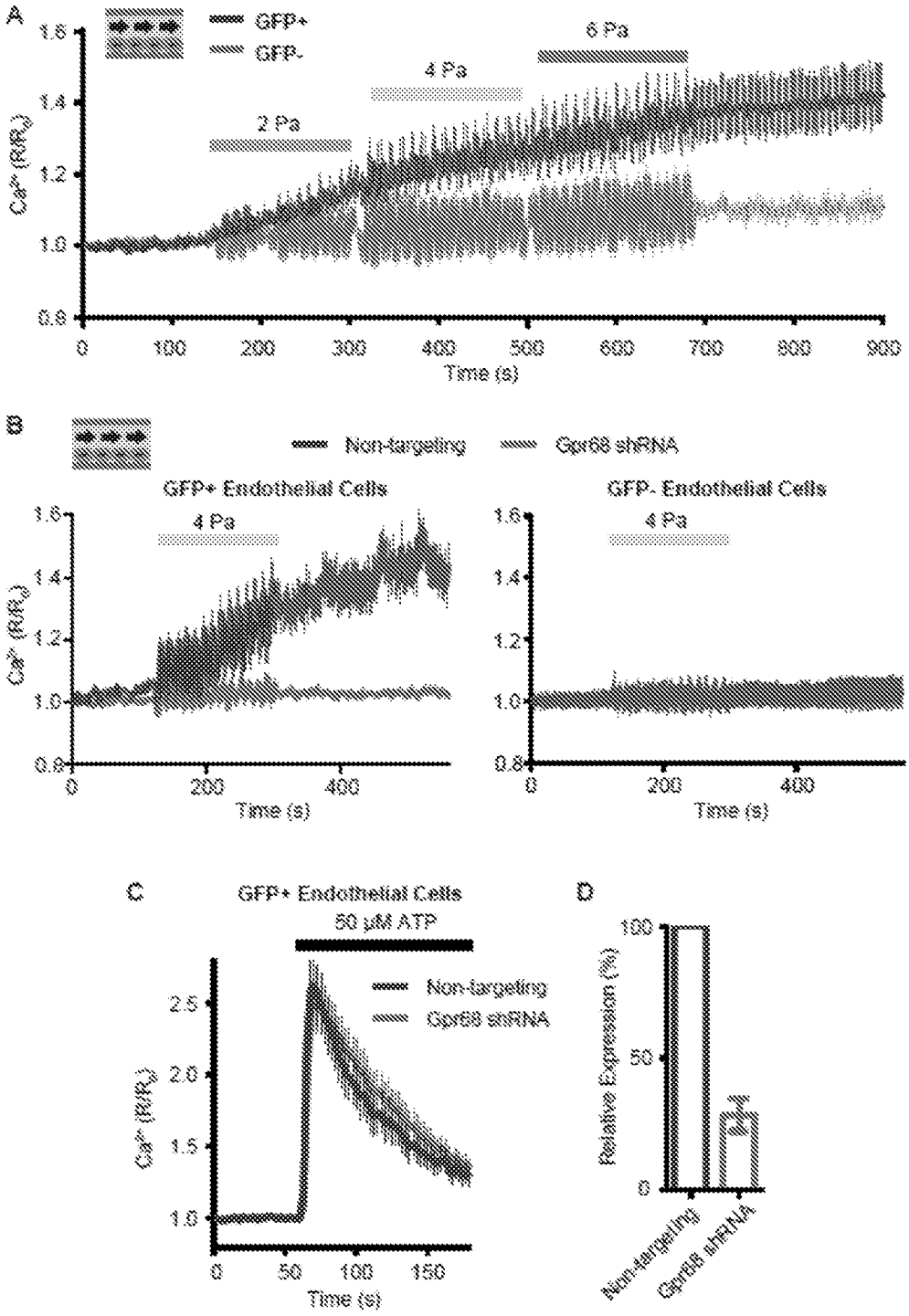

A
GFP+
GFP-
2 Pa
4 Pa
6 Pa
Ca2+ (R/R0)
Time (s)
B
Non-targeting
Gpr68 shRNA
GFP+ Endothelial Cells
GFP- Endothelial Cells
4 Pa
Ca2+ (R/R0)
Time (s)
C
GFP+ Endothelial Cells
50 µM ATP
Non-targeting
Gpr68 shRNA
Ca2+ (R/R0)
Time (s)
D
Relative Expression (%)
Non-targeting
Gpr68 shRNA

FIG. 10

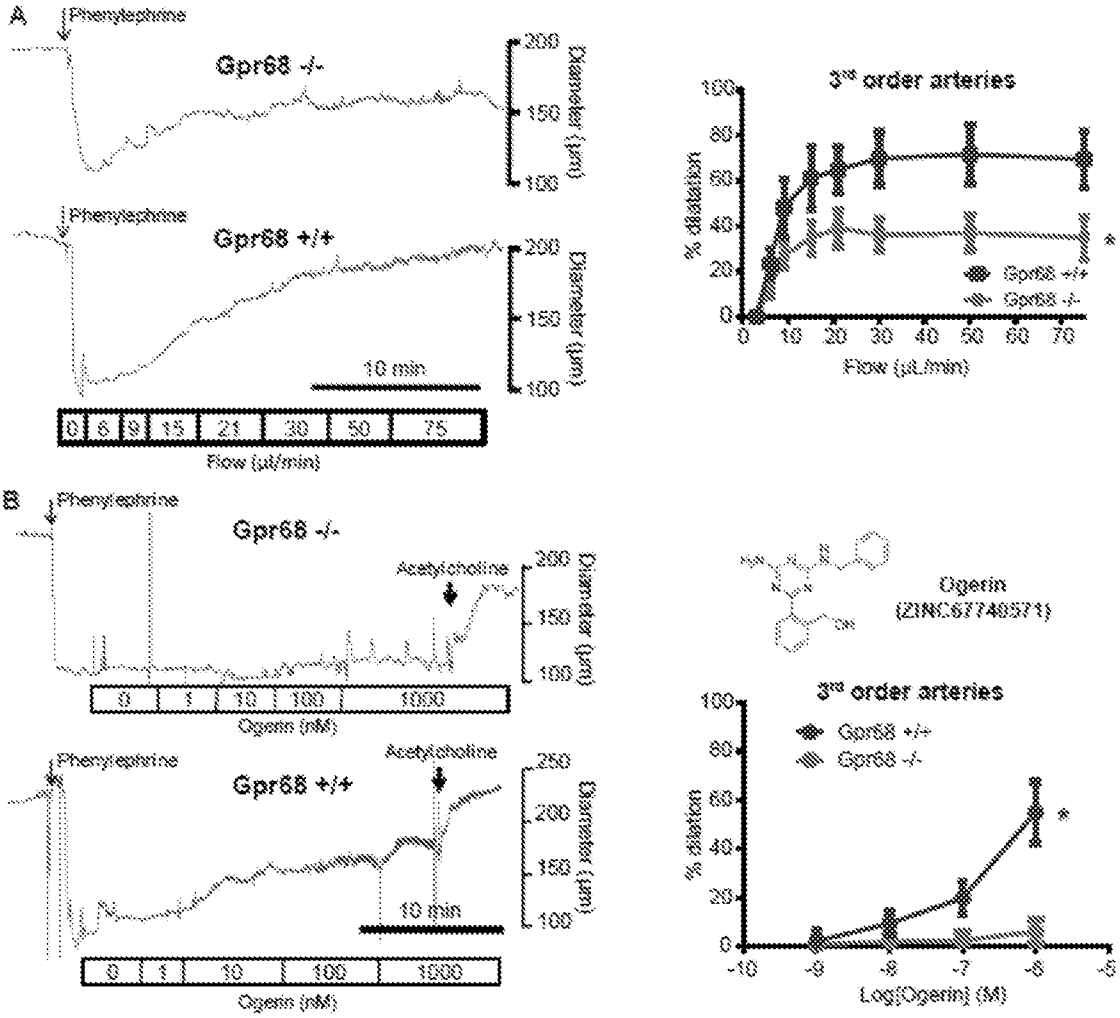

A
Phenylephrine
Gpr68 -/-
Diameter (µm)
200
150
100
Phenylephrine
Gpr68 +/+
10 min
0 6 9 15 21 30 50 75
Flow (µl/min)
3rd order arteries
% dilatation
Gpr68 +/+
Gpr68 -/-
Flow (µL/min)
B
Phenylephrine
Gpr68 -/-
Acetylcholine
0 1 10 100 1000
Ogerin (nM)
Gpr68 +/+
250
10 min
Ogerin
(ZINC67740571)
Log[Ogerin] (M)

FIG. 10 (cont.)

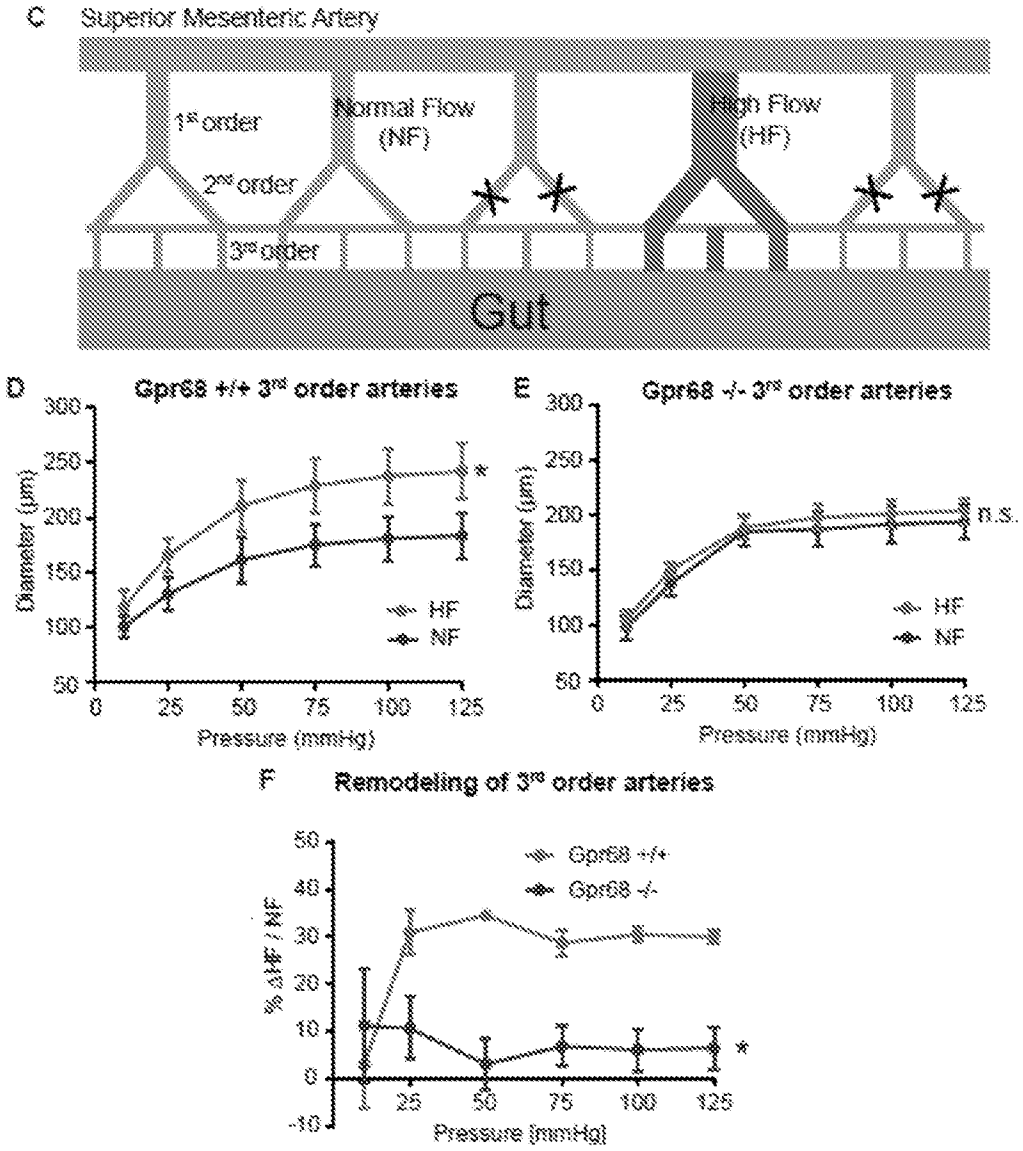

C
Superior Mesenteric Artery
1st order
2nd order
3rd order
Normal Flow (NF)
High Flow (HF)
Gut
D
Gpr68 +/+ 3rd order arteries
Diameter (µm)
Pressure (mmHg)
HF
NF
*
E
Gpr68 -/- 3rd order arteries
n.s.
F
Remodeling of 3rd order arteries
Gpr68 +/+
Gpr68 -/-
% ΔHF / NF
Pressure (mmHg)

FIG. 11

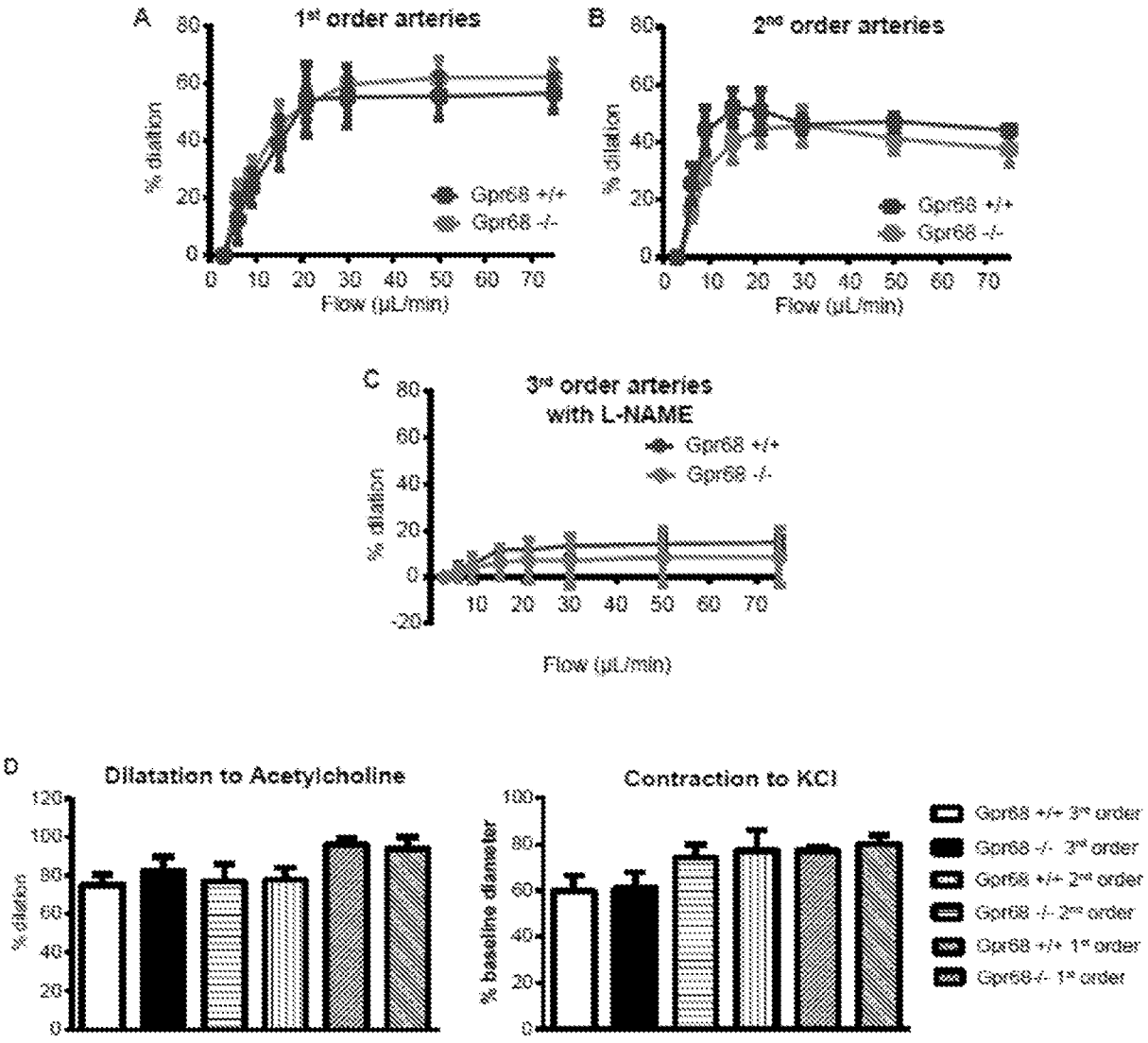

A
1st order arteries
% dilation
Flow (µL/min)
Gpr68 +/+
Gpr68 -/-
B
2nd order arteries
% dilation
Flow (µL/min)
Gpr68 +/+
Gpr68 -/-
C
3rd order arteries
with L-NAME
Gpr68 +/+
Gpr68 -/-
% dilation
Flow (µL/min)
D
Dilatation to Acetylcholine
% dilation
Contraction to KCl
% baseline diameter
Gpr68 +/+ 3rd order
Gpr68 -/- 3rd order
Gpr68 +/+ 2nd order
Gpr68 -/- 2nd order
Gpr68 +/+ 1st order
Gpr68 -/- 1st order

FIG. 12

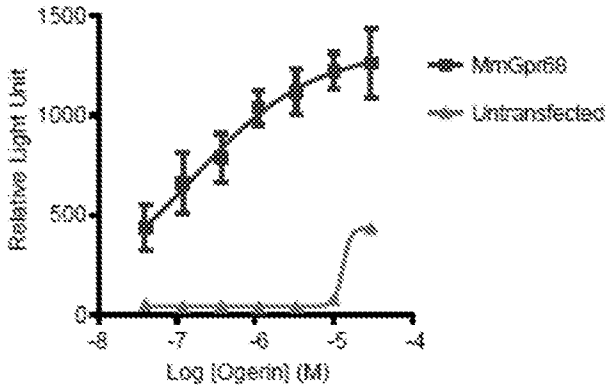

1500
1000
500
0
Relative Light Unit
-8
-7
-6
-5
-4
Log [Ogerin] (M)
MmGpr68
Untransfected

FIG. 13

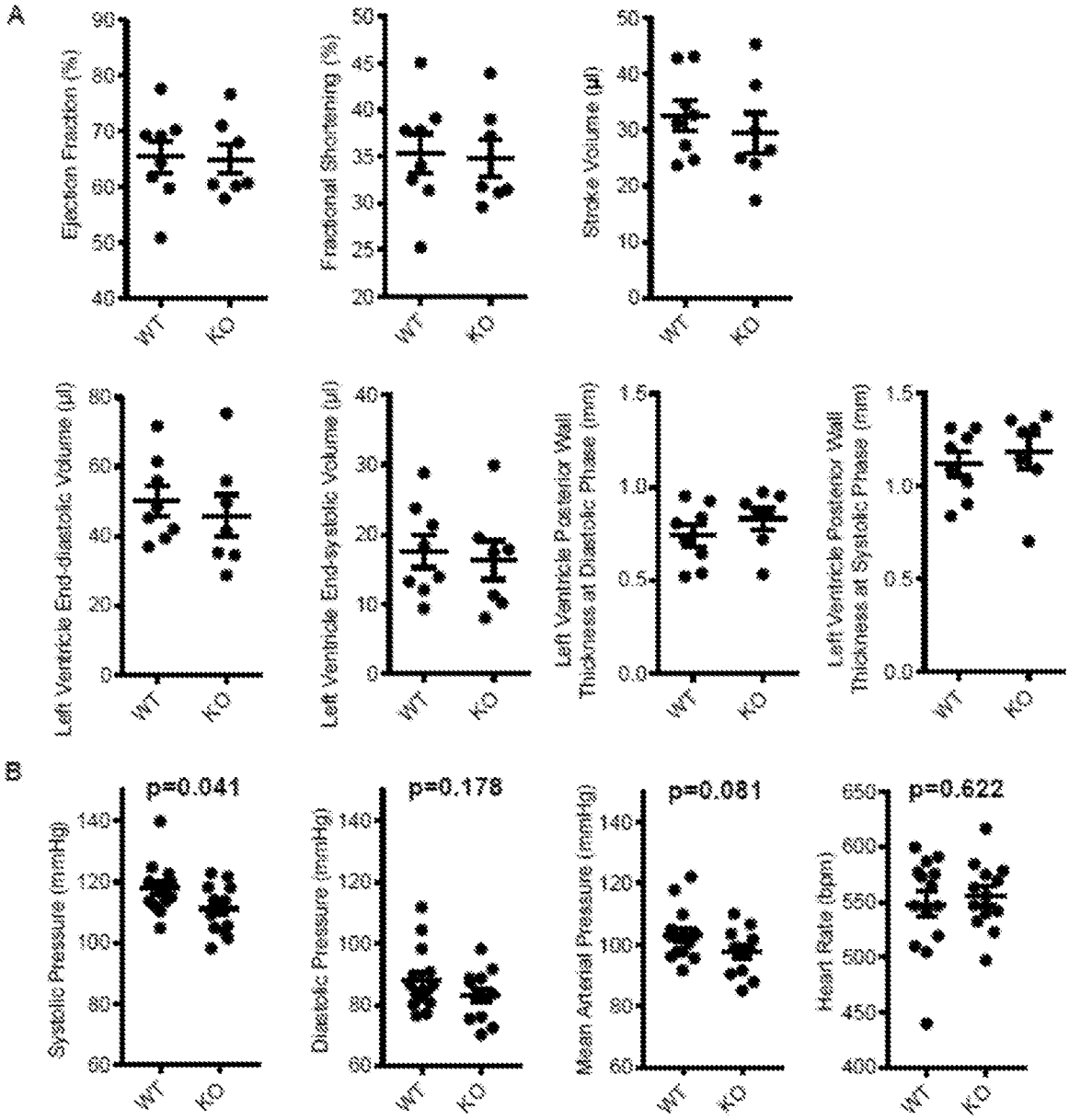

A
Ejection Fraction (%)
Fractional Shortening (%)
Stroke Volume (µl)
Left Ventricle End-diastolic Volume (µl)
Left Ventricle End-systolic Volume (µl)
Left Ventricle Posterior Wall Thickness at Diastolic Phase (mm)
Left Ventricle Posterior Wall Thickness at Systolic Phase (mm)
WT
KO
B
Systolic Pressure (mmHg)
p=0.041
Diastolic Pressure (mmHg)
p=0.178
Mean Arterial Pressure (mmHg)
p=0.081
Heart Rate (bpm)
p=0.622

FIG. 13 (cont.)

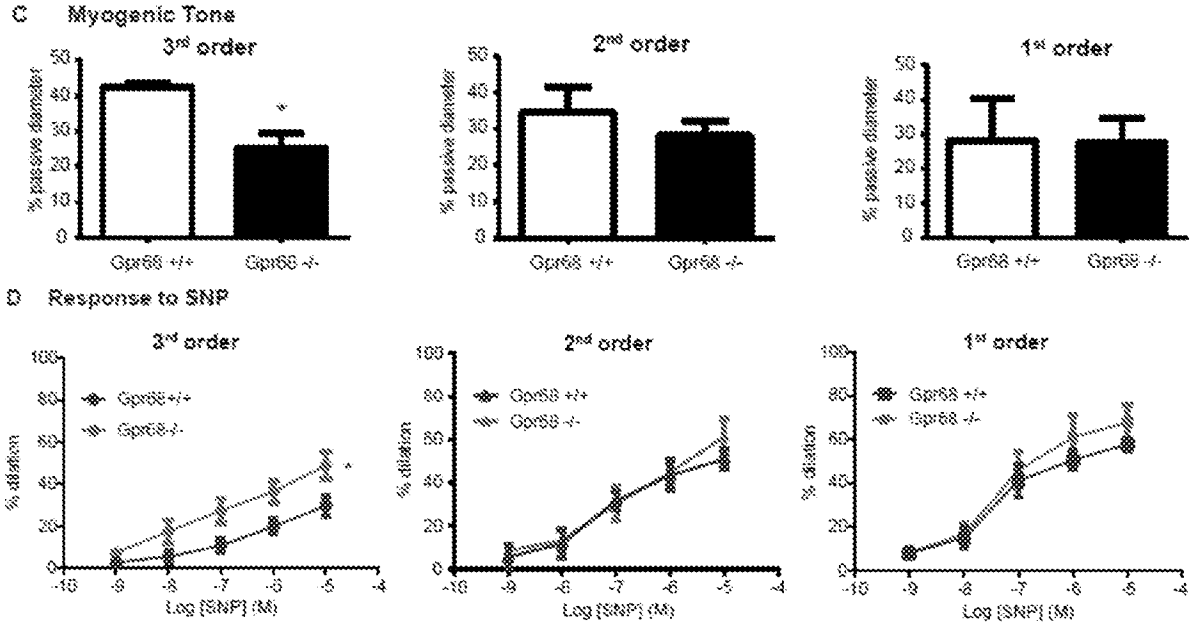


FIG. 14

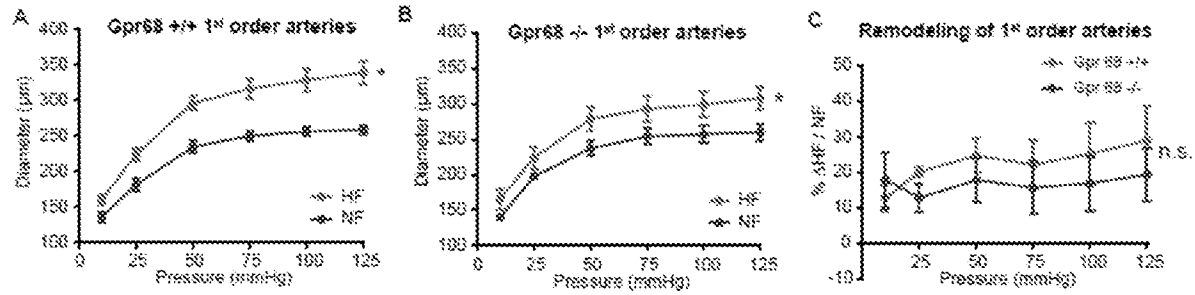


FIG. 15A

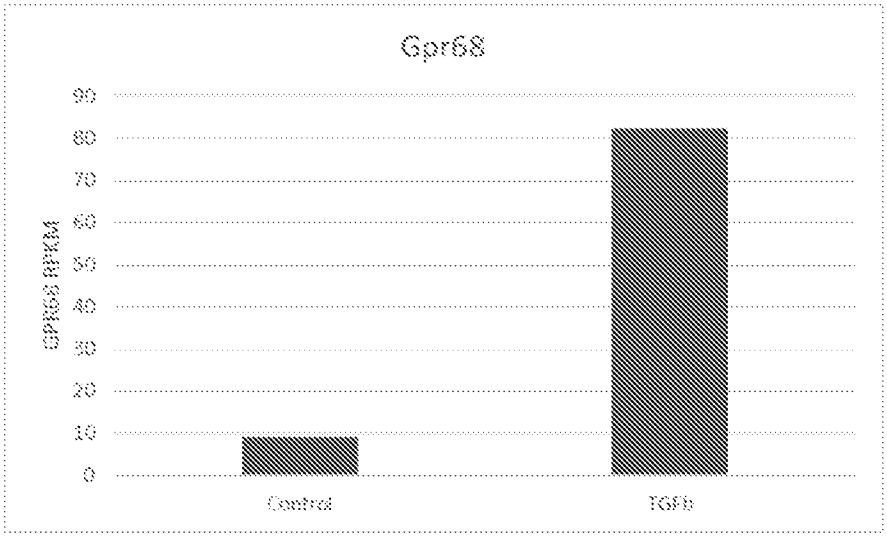
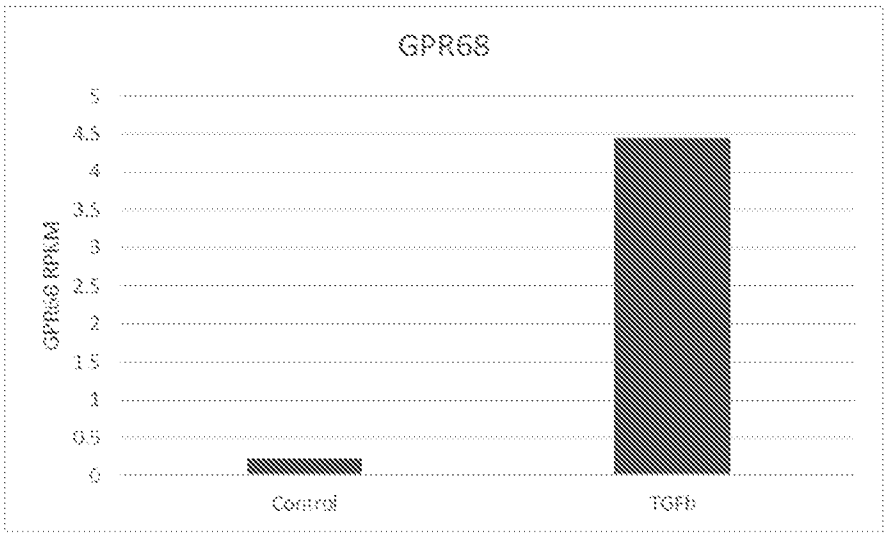


FIG. 15B



INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2019/050799

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/00 A61K39/395 A61K31/713 A61P1/16 A61P9/00
ADD.

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)
A61K A61P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	JIE XU ET AL: "GPR68 Senses Flow and Is Essential for Vascular Physiology", CELL, vol. 173, no. 3, 1 April 2018 (2018-04-01) , pages 762-775.e16, XP55584454, AMSTERDAM, NL ISSN: 0092-8674, DOI: 10.1016/j.cell.2018.03.076 the whole document ----- -/--	1-44

[X] Further documents are listed in the continuation of Box C.

[X] 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)

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"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

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Date of the actual completion of the international search	Date of mailing of the international search report
30 April 2019	21/05/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schnack, Anne

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JIN-PENG LIU ET AL: "Ovarian cancer G protein-coupled receptor 1-dependent and -independent vascular actions to acidic pH in human aortic smooth muscle cells", AMERICAN JOURNAL OF PHYSIOLOGY: HEART AND CIRCULATORY PHYSIOLOGY, vol. 299, no. 3, 1 September 2010 (2010-09-01), pages H731-H742, XP55584478, US ISSN: 0363-6135, DOI: 10.1152/ajpheart.00977.2009 the whole document	1-44
A	H SAXENA ET AL: "The GPCR OGR1 (GPR68) mediates diverse signalling and contraction of airway smooth muscle in response to small reductions in extracellular pH : Acid-induced contraction of smooth muscle by OGR1", BRITISH JOURNAL OF PHARMACOLOGY, vol. 166, no. 3, 1 June 2012 (2012-06-01), pages 981-990, XP55584473, UK ISSN: 0007-1188, DOI: 10.1111/j.1476-5381.2011.01807.x the whole document	1-44
X	CHERYL DE VALLIÈRE ET AL: "The pH-sensing receptor OGR1 improves barrier function of epithelial cells and inhibits migration in an acidic environment", AMERICAN JOURNAL OF PHYSIOLOGY - GASTROINTESTINAL AND LIVER PHYSIOLOGY, vol. 309, no. 6, 15 September 2015 (2015-09-15), pages G475-G490, XP55584461, US ISSN: 0193-1857, DOI: 10.1152/ajpgi.00408.2014	40-42,44
A	page G475, column 2, paragraph 3 full	1-39,43
X	LUDWIG M-G ET AL: "Proton-sensing G-protein-coupled receptors", NATURE, MACMILLAN JOURNALS LTD, LONDON, vol. 425, no. 6953, 4 September 2003 (2003-09-04), pages 93-98, XP002303706, ISSN: 0028-0836, DOI: 10.1038/NATURE01905	40-42,44
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A	EP 2 765 197 A1 (ETH ZUERICH [CH]) 13 August 2014 (2014-08-13) claims 1-14	1-44

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2019/050799

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2016/340674 A1 (JIN ZHENG-GEN [US]) 24 November 2016 (2016-11-24) the whole document -----	1-44

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2019/050799

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