Electronic supplementary material

The novel activating *GCK* variant p.Val455Leu is susceptible to allosteric activation and is conducive to weight gain and the development of diabetes

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ESM Methods

Materials

RO-28-1675 was purchased from Axon Medchem BV, Groningen, The Netherlands, and mannoheptulose from Glycoteam, Hamburg, Germany. All other chemicals were bought from Merck KGaA, Darmstadt, Germany. Restriction enzymes, oligonucleotides, Bradford assay, Dynabeads Protein G and tissue culture equipment were purchased from Thermo Fischer Scientific, MA, USA. QuikChange II Site-Directed Mutagenesis Kit was bought from Agilent, Santa Clara, CA, USA, and GKRP (GCKR, G-6) antibody (sc-74544) and GK (GCK, G-6) antibody (sc-17819) from Santa Cruz Biotechnology, Dallas, Texas, USA. The GST recombinant protein expression system was obtained from GE Healthcare, Chalfont St Giles, UK. Cell culture dishes were bought from SARSTEDT, Nümbrecht, Germany, and glass-bottomed six-well microplates from MatTek Corporation, Ashland, MA, USA. The insulin ELISA was purchased from Mercodia, Uppsala, Sweden.

Oral and intravenous glucose tolerance tests

Oral glucose tolerance tests (OGTTs) were performed in three adult family members after an overnight fast. Blood samples for glucose and insulin determinations were taken immediately before ingestion of 75 g glucose (25% (w/v) solution in H_2O) and thereafter at 30 min intervals.

Intravenous glucose tolerance tests (IVGTTs) were performed in two adult family members with an intravenous injection of 300 mg glucose per kg body weight (20% (w/v) solution in H_2O). Blood samples for glucose and insulin determinations were taken immediately before glucose injection, on completion of the injection 5 min after the first blood sample (time zero), and then after 2, 4, 6, 8, 10, 20, and 30 min. Indices of insulin resistance (HOMA-IR, QUICKI) were determined, as previously described, with reference ranges of 1.57±0.87 and 0.366±0.029, respectively [1, 2].

Hepatocyte isolation, culture, and transfection

Primary hepatocytes were isolated from male Wistar rats as described previously [3] and stored at -80°C for GKRP isolation. Primary hepatocytes isolated from male C57BL/6J mice (The Jackson Laboratory, USA; https://www.jax.org/strain/000664) were cultured and transiently transfected with jetPEI-Hepatocyte (Polyplus, Illkirch, France) as described previously [4] for intracellular localisation studies. These procedures were conducted in accordance with the German Animal Welfare Act 2006 (last amended 2014) and were approved by the State Department of Agriculture, Food Safety and Fisheries, Mecklenburg-Vorpommern (LALLF M-V).

GKRP immunoprecipitation from hepatocytes

The supernatant of 50 µl Dynabeads Protein G (Thermo Fisher Scientific) was removed. The beads were mixed with 200 µl antibody solution (5 µg anti-GKRP antibody (Santa Cruz Biotechnology) in 200 µl PBS containing 0.01% (v/v) Tween-20), incubated on a roller for 2 h at 4°C, and the supernatant was removed. Then 5×10^7 hepatocytes were resuspended in 2 ml PBS (4°C), homogenised by sonication (5 min), and insoluble material was pelleted by centrifugation (7,000 g, 10 min, 4°C). Subsequently, 150 µl hepatocyte lysate and 150 µl PBS/0.05% Tween-20 were added to the prepared beads and incubated on a roller for 2 h at

4°C. The beads were washed twice with 200 µl PBS each and were resuspended in 100 µl PBS, transferred into a fresh reaction tube, and the supernatant was removed. The precipitated GKRP protein was eluted by addition of 20 µl 50 mmol/l glycine (pH 2.8), careful mixing by pipetting, and incubation on a roller for 2 min at 4°C. The eluate was transferred into a fresh tube and 20 µl 1 M TRIS (pH 7.5) were added. These elution steps were repeated twice, and the eluates were stored at -20°C.

GK enzyme activity

For GKRP-inhibition studies 2 µg Dendra2-GK protein were incubated in assay buffer for 25 min in the absence or presence of 10 µg isolated GKRP + 1 mmol/l fructose-6-phosphate (Merck KGaA, Darmstadt, Germany) prior to glucose addition. For determination of EC_{50} values of RO-28-1675 1 µg of recombinant Dendra2-GK wild-type, -M455, or -L455 enzymes were incubated in reaction buffer for 25 min at 37°C with increasing concentrations (0.3, 0.5, 1, 3, 5, 10, 20, 30 µmol/l) of RO-28-1675 [5] prior to measurement at 2 mmol/l glucose. Finally, EC_{50} values were calculated using [Agonist] vs. response (three parameters) dose-response stimulation model of the GraphPad Prism 8.1.1 analysis program (GraphPad Software, San Diego, CA, USA).

Western blot analyses

5 µg of purified recombinant pDendra2-GK wild-type, -M455, -E455 or -L455 protein were separated by SDS-PAGE and blotted onto Roti Fluoro PVDF membrane (Roth, Karlsruhe, Germany). All steps (including dilution of the antibodies) were carried out in Odyssey blocking buffer (LI-COR, Lincoln, NE, USA) diluted 1:3 in PBS. Membranes were incubated for 1 h at room temperature with the GK-antibody (1:500). Immunoreactive bands were visualized using the anti-mouse fluorescence-labelled secondary antibody IRDye 680 CW (1:5000) via the Odyssey imaging system (LI-COR).

Structural analysis – modelling of amino acid mutations

Modelling of the structures of GK variants p.Val455Met, p.Val455Glu, and p.Val455Leu was conducted with PyMOL Mutagenesis Wizard. The side chain orientation with minimal steric clashing within the protein structure was selected from the "Backbone Dependent Rotamers" library [6]. Subsequently, the PyMOL clean command was used to move atoms within a radius of 5 Å including residue 455 to their positions of lowest local energy. Beginning from this calculated structure, another rotamer with minimal steric clashing was selected and another clean-up performed to consider various possibilities for the prediction.

References ESM Methods

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ESM Table 1

Variant	References
V62L	Gloyn et al., 2005
V62M*	Gloyn et al., 2005; Zelent et al., 2011
S64F	Pal & Miller, 2009
S64P	Pal & Miller, 2009; Zelent et al., 2011
S64Y	Christesen, Tribble, et al., 2008; Zelent et al., 2011; Langer et al., 2019
T65I	Gloyn et al., 2003; Heredia, Carlson, et al., 2006; Christesen, Tribble, et al., 2008; Cullen et al., 2011; Zelent et al., 2011; Martinez et al., 2016; Martinez et al., 2017
G68K	Zelent et al., 2011
G68V	Wabitsch et al., 2007‡; Zelent et al., 2011; Langer et al., 2019
S69P	Pal & Miller, 2009
D73E	Pal & Miller, 2009
K90R	Ping et al., 2019
V91L	Pal & Miller, 2009; Kassem et al., 2010; Zelent et al., 2011; Martinez et al., 2016; Martinez et al., 2017; Lu et al., 2019
W99C	Zelent et al., 2008; Martinez et al., 2016; Martinez et al., 2017
W99L	Sayed et al., 2009; Snider et al., 2013; Tornovsky-Babeay et al., 2014
W99R	Gloyn et al., 2003; Heredia, Carlson, et al., 2006; Christesen, Tribble, et al., 2008; Zelent et al., 2008; Cullen et al., 2011; Snider et al., 2013
T103S	Beer et al., 2011
D158A	Davis et al., 1999
N166R	Moukil et al., 2000
N180D	Pal & Miller, 2009; Jannin et al., 2018‡
M197A	Sayed et al., 2009
M197I	Sayed et al., 2009; Zelent et al., 2011; Snider et al., 2013
M197L	Sayed et al., 2009; Zelent et al., 2011
M197T	Sayed et al., 2009; Morishita et al., 2017
M197V	Pal & Miller, 2009; Sayed et al., 2009; Ping et al., 2019‡
I211F†	Pal & Miller, 2009; Henquin et al., 2013
Y214A	Moukil et al., 2000; Heredia, Carlson, et al., 2006; Zelent et al., 2011
Y214C	Cuesta-Munoz et al., 2004; Pedelini et al., 2005; Heredia, Carlson, et al., 2006; Heredia, Thomson, et al., 2006; Cullen et al., 2011; Zelent et al., 2011; Snider et al., 2013; Tornovsky-Babeay et al., 2014
Y215A	Pedelini et al., 2005; Heredia, Carlson, et al., 2006; Zelent et al., 2011
E216D	Pal & Miller, 2009
W257F	Zelent et al., 2008
K296M	Moukil et al., 2000
V389L	Beer et al., 2011; Zelent et al., 2011; Snider et al., 2013; Challis et al., 2014‡
E442K	Barbetti et al., 2009; Zelent et al., 2011; Martinez et al., 2016; Martinez et al., 2017
G446S	Pal & Miller, 2009
V452L	Cuesta-Munoz et al., 2008; Meissner et al., 2009; Zelent et al., 2011; Ajala et al., 2016‡
S453A	Pal & Miller, 2009
ins454A	Sayed et al., 2009; Zelent et al., 2011; Snider et al., 2013; Tornovsky-Babeay et al., 2014; Tornovsky-Babeay et al., 2021
V455L	Hillebrand et al., 2009; Lange et al., 2011
V455M	Glaser et al., 1998‡; Burke et al., 1999; Davis et al., 1999; Heredia, Carlson, et al., 2006; Cullen et al., 2011; Zelent et al., 2011; Tornovsky-Babeay et al., 2014‡; Maiorana et al., 2015‡
A456V	Christesen et al., 2002‡; Dullaart et al., 2004‡; Pedelini et al., 2005; Heredia, Carlson, et al., 2006; Pino et al., 2007; Christesen, Brusgaard, et al., 2008‡; Christesen, Tribble, et al., 2008; Cullen et al., 2011; Vidal-Alabro et al., 2011; Zelent et al., 2011
A460R	Pedelini et al., 2005

ESM Table 1. Overview of published GK variants that display enhanced enzyme activity, including in vitro kinetic studies. To date, 22 of these variants have been described in individuals diagnosed with hyperinsulinaemic hypoglycaemia. These are listed in bold and the reference publications are underlined. *Mutant V62M activates in vitro, but paradoxically results in a MODY2 phenotype; †mutant I211F was somatic, described in one individual in pathological islets only; ‡these publications reported overweight in affected individuals. The reference details are as follows:

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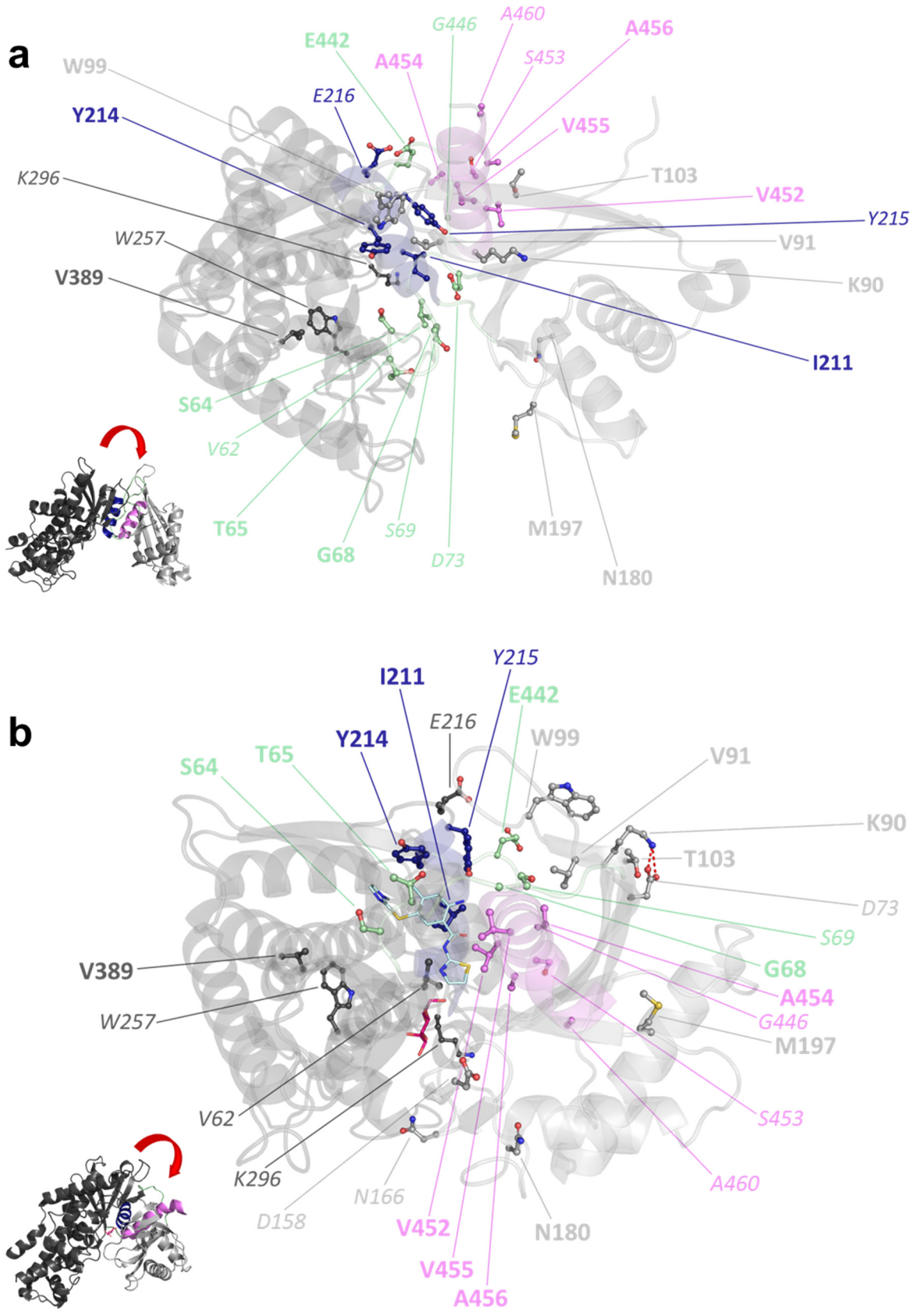
ESM Table 2

	Patient II-2	Patient II-5	Patient III-4
Triglycerides (mmol/l) [0.80-2.05]	2.45	0.67	0.24
Total cholesterol (mmol/l) [3.90-6.24]	5.46	4.03	4.11

ESM Table 2. Fasting serum triglyceride and cholesterol levels in patients II-2, II-5, and III-4.

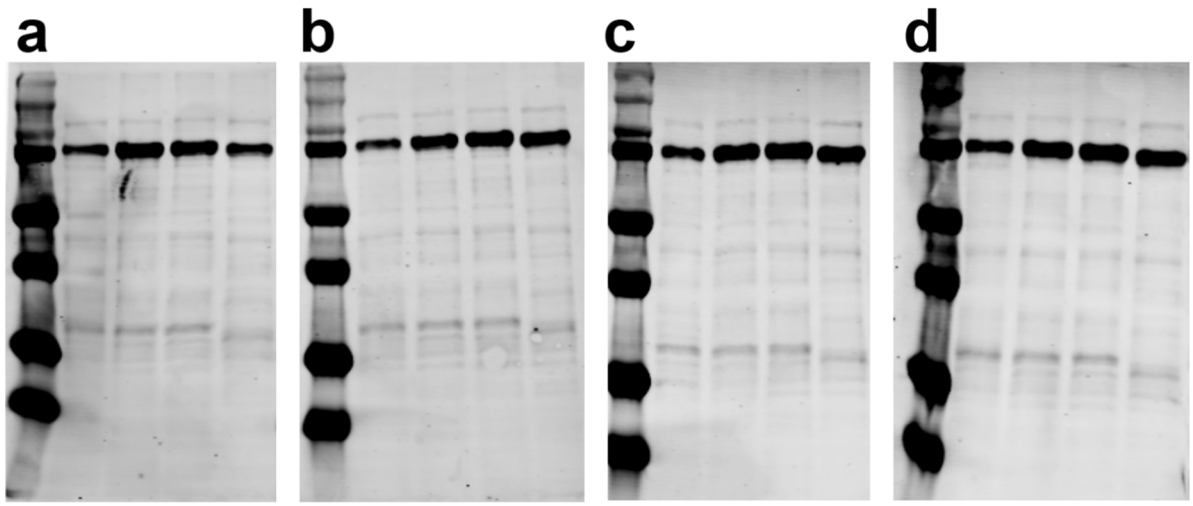
ESM Figure 1


a
W99
E442
G446
A460
A456
A454
S453
E216
Y214
V455
K296
T103
V452
Y215
W257
V91
V389
K90
I211
S64
V62
T65
S69
M197
G68
D73
N180
b
I211
Y215
E442
E216
Y214
W99
T65
S64
V91
K90
T103
D73
S69
V389
G68
A454
W257
G446
M197
S453
V62
A460
K296
N166
D158
V452
N180
V455
A456

ESM Figure 1. Intramolecular locations of GK amino acids for which activating mutations have been described. The 29 residues of wild-type GK, as listed in ESM Table 1, are highlighted as ball-and-stick models (alpha-carbons plus side chains, with oxygen red, nitrogen blue, sulphur yellow) within the structures of the super-open (a, 1v4t [1]) and the closed conformation of GK (b, 1v4s [1]). The molecule view shows the allosteric site, with the large domain in dark grey and the small domain in light grey, the interconnecting regions I-III in pale green, the C-terminal helix α13 in violet, and helix α5 in dark blue. The labelling of the highlighted residues also follows this colour scheme. Italic labelling indicates that activating mutations of the particular amino acid have not to date been found in humans. In (b), glucose is shown in magenta, the GKA compound A (2-amino-4-fluoro-5-[(1-methyl-1*H*-imidazol-2-yl)sulfanyl]-*N*-(1,3-thiazol-2-yl)benzamide) in cyan, and a hydrogen bond between residues Asp-73 and Lys-90 – suggesting a similar mechanism of activation for the variants D73E and K90R – as red dotted lines. Note that residues Asp-158 and Asn-166 are not displayed in the super-open GK conformation (a) because they lie within a mobile loop structure of the small domain that is disordered in the unliganded state. Also note that the secondary structure of the protein varies slightly between the super-open and the closed enzyme conformation: for example, residue Gly-446 is part of interconnecting region III in (a) but part of helix α13 in (b).

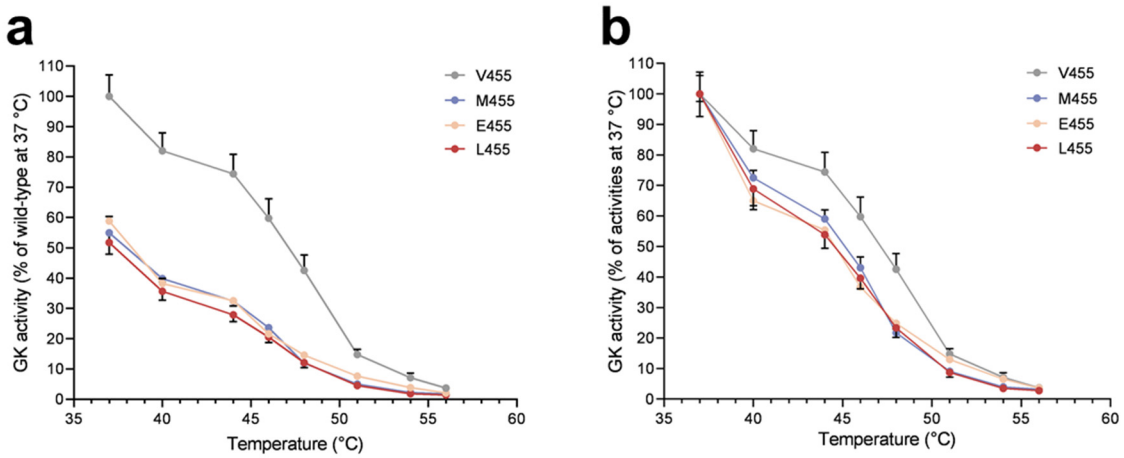
[1] Kamata K, Mitsuya M, Nishimura T, Eiki J, Nagata Y (2004) Structural basis for allosteric regulation of the monomeric allosteric enzyme human glucokinase. Structure 12(3): 429-438. 10.1016/j.str.2004.02.005

ESM Figure 2



ESM Figure 2. GK Western blot analyses of recombinant Dendra2-GK wild-type, M455, E455, and L455 enzymes. 5 µg of recombinant protein of each preparation was loaded in a 10% polyacrylamide gel. Representative blots (a – d) of the experiments are shown. Molecular weight marker 20, 25, 37, 50, 75 kDa (lane 1), Dendra2-GK wild-type (lane 2), Dendra2-GK M455 (lane 3), Dendra2-GK E455 (lane 4), and Dendra2-GK L455 (lane 5).

ESM Figure 3



ESM Figure 3. Thermostability of recombinant Dendra2-GK wild-type, -M455, -E455, and -L455 enzymes. Glucose-phosphorylating activities of 1 μg recombinant fusion proteins were measured after 30 min heating at the temperature indicated prior to glucose addition (100 mmol/l). Data are shown as means ± SEM from seven independent experiments, expressed as (a) percentage of wild-type activity after incubation at 37°C or as (b) percentage relative to the respective activities at 37°C.