A novel mutation in *KCNK16* causing a gain-of-function in the TALK-1 potassium channel: a new cause of maturity onset diabetes of the young.

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ABSTRACT

Background

Maturity-onset diabetes of the young (MODY) is a heterogeneous group of monogenic disorders of impaired glucose-stimulated insulin secretion (GSIS). Mechanisms include β-cell K_{ATP} channel dysfunction (e.g., *KCNJ11* (MODY13) or *ABCC8* (MODY12) mutations); however, no other β-cell channelopathies have been identified in MODY.

Methods

A four-generation family with autosomal dominant non-obese, non-ketotic antibody-negative diabetes, without mutations in known MODY genes, underwent exome sequencing. Whole-cell and single-channel K^+ currents, Ca^{2+} handling, and GSIS were determined in cells expressing either mutated or wild-type (WT) protein.

Results

We identified a novel non-synonymous genetic mutation in *KCNK16* (NM_001135105: c.341T>C, p.Leu114Pro) segregating with MODY. *KCNK16* is the most abundant and β-cell-restricted K^+ channel transcript and encodes the two-pore-domain K^+ channel TALK-1. Whole-cell K^+ currents in transfected HEK293 cells demonstrated drastic (312-fold increase) gain-of-function with TALK-1 Leu144Pro vs. WT, due to greater single channel activity. Glucose-stimulated cytosolic Ca^{2+} influx was inhibited in mouse islets expressing TALK-1 Leu114Pro (area under the curve [AUC] at 20mM glucose: Leu114Pro 60.1 vs. WT 89.1; *P*=0.030) and less endoplasmic reticulum calcium storage (cyclopiazonic acid-induced release AUC: Leu114Pro 17.5 vs. WT 46.8; *P*=0.008). TALK-1 Leu114Pro significantly blunted GSIS compared to TALK-1 WT in both mouse (52% decrease, *P*=0.039) and human (38% decrease, *P*=0.019) islets.

Conclusions

Our data identify a novel MODY-associated gene, *KCNK16*; with a gain-of-function mutation limiting Ca^{2+} influx and GSIS. A gain-of-function common polymorphism in *KCNK16* is associated with type 2 diabetes (T2DM); thus, our findings have therapeutic implications not only for *KCNK16*-associated MODY but also for T2DM.

INTRODUCTION

Maturity-onset diabetes of the young (MODY) is a rare monogenic cause of familial diabetes. To date, 13 MODY genes have been confirmed, all involved in pancreatic β-cell insulin secretion and all with autosomal dominant transmission[1]. 2-2.5% of pediatric diabetes cases carry pathogenic/likely pathogenic variants in MODY genes[2,3]; however, MODY is often undiagnosed, either because the diagnosis is not considered[4] or because genetic screening is limited. There are also cases with compelling clinical histories in whom, despite comprehensive screening of known MODY genes, a genetic diagnosis cannot be made[3], suggesting as-yet-unidentified genetic cause(s).

β-cell glucose-stimulated insulin secretion (GSIS) is dependent on Ca^{2+} influx, through voltage-dependent calcium channels (VDCC)[5,6]. Reduced Ca^{2+} influx decreases GSIS; thus mutations that disrupt β-cell Ca^{2+} entry can cause MODY or the closely-related condition neonatal diabetes[7,8]. For example, gain-of-function mutations in K_{ATP} channel subunits hyperpolarize the β-cell membrane potential, reducing VDCC activity, Ca^{2+} influx and GSIS[7,8]. Other β-cell K^+ channels, including two-pore domain K^+ channels (K2P), also affect VDCC activity[9]. Expression of *KCNK16*, which encodes TWIK-related alkaline pH-activated K2P (TALK-1)[10], is the most abundant and β-cell-selective of all human K^+ channel transcripts[11,12]; and TALK-1 gain-of-function mutations would be predicted to cause diabetes similarly[9].

Here we have used exome sequencing to identify the first family with MODY due to a mutation in *KCNK16*. The Leu114Pro substitution in TALK-1 affects the K^+ selectivity filter, causing a profound increase in K^+ current, altering β-cell Ca^{2+} flux, and decreasing GSIS in both human and mouse islet cells.

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METHODS

Clinical Recruitment

A four-generation family with six affected family members with autosomal dominant diabetes was identified through a non-obese proband who presented aged 15 years (Fig.1A) with elevated fasting plasma glucose (7.8mmol/L) and an abnormal oral glucose tolerance test (glucose 19.6mmol/L two hours after 75g glucose). Antibody testing (islet cell, islet antigen 2, and glutamic acid decarboxylase-65) was negative. Over two decades, the proband required minimal insulin to maintain HbA1c of 5.7-6.5%; and she did not experience ketosis or other diabetes-related complications. Sanger sequencing for mutations in *GCK*, *HNF1A* and *HNF4A* (the commonest MODY genes) was negative. Other family members manifest diabetes similarly (detailed in Supplementary Appendix: Extended Clinical Data). The study protocol was approved by the relevant human research ethics committee (approval HREC/12/QPAH/109). All living family members gave written informed consent.

Exome sequencing.

Exome sequencing, pipeline processing, quality control and variant curation was performed as previously described[13] (detailed in Supplementary Appendix: Methods). Exome data from the proband was analysed for good-quality likely damaging rare variants in known MODY genes[1], using a conservative minor allele frequency (MAF) threshold of <0.001, based on: (a) prevalence of paediatric diabetes of 0.2%[14]; and (b) prevalence of MODY mutations in 2% of a pediatric diabetes population[2]; further, most MODY mutations are private. Exome sequence data from the pedigree was analysed for novel and rare (MAF<0.001) good quality variants, of potentially damaging consequence, affecting highly conserved bases with appropriate segregation (i.e., heterozygous in affected individuals, absent in unaffected individuals).

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Plasmids and Transient Expression

Human TALK-1 wildtype (WT) and TALK-1 Leu114Pro constructs were created by site-directed mutagenesis and then cloned into a vector containing a P2A cleavage site followed by mCherry (Supplementary Appendix: Fig. S1). HEK293 cells, which have no endogenous TALK-1 expression, were transfected with 2μg DNA using Lipofectamine 3000 (Life Technologies). Transfection efficacy was assessed and quantified using mCherry Fluorescence. (Supplementary Appendix: Methods and Fig. S2).

Lentivirus Production

HEK293 cells were transfected with lentiviral-producing plasmids; the plasmids used included the packaging plasmid (pCMV-dR7.74psPAX2), envelope plasmid (pMD2.G), and an expression plasmid (detailed in Supplementary Appendix: Methods and Fig. S1). Lentiviral-containing supernatants were collected three days after transfection and used for transduction of primary beta-β-cells; after transduction, equal TALK-1 expression was confirmed by equivalent mCherry expression (detailed in Supplementary Appendix: Methods and Fig. S3).

Electrophysiological Recordings

TALK-1 channel currents were recorded in HEK293 cells using a whole-cell voltage-clamp technique with an Axopatch 200B amplifier and pCLAMP10 software (Molecular Devices), as previously described[9]; single-channel current recordings of TALK-1 were recorded with an cell-attached voltage-clamp technique also as previously described[15] (detailed in Supplementary Appendix: Methods and Fig. S4).

Islet and β-Cell Isolation

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Islets were isolated from mouse pancreata as previously described[9]. Human islets from non-diabetic adult donors were provided by isolation centers of the Integrated Islet Distribution Program (donor information, Supplementary Appendix: Table S1). Some islets were dispersed into cell clusters and then cultured for 12–18 hours[9]. Cells were maintained in RPMI 1640 with 15% FBS, 100IU/mL penicillin, and 100mg/mL streptomycin in a humidified incubator at 37°C with an atmosphere of 95% air and 5% CO_2.

Calcium handling measurements

Islets were incubated for 25min in RPMI supplemented with Fura-2, AM (Molecular Probes), followed by incubation in Krebs-Ringer–HEPES buffer with 2mmol/L glucose for 20min[9]. For cytoplasmic Ca^{2+} [Ca^{2+}_{cyto}]: Ca^{2+} imaging was performed as previously described[9], switching from 2mM glucose to 20mM glucose. For endoplasmic reticulum (ER) Ca^{2+} [Ca^{2+}_{ER}]: Islets were perfused in Krebs-Ringer–HEPES buffer without extracellular Ca^{2+} and 100 μM diazoxide and monitored for Ca^{2+}_{ER} release mediated through blockade of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) with 50 μM cyclopiazonic acid (CPA, Alomone labs), as previously described[16].

Insulin Secretion Measurements

Islets were transduced with lentiviruses containing a RIP promoter[17] upstream of either TALK-1 WT or TALK-1 Leu114Pro followed by a P2A cleavage site and NanoLuc-proinsulin (Supplementary Appendix: Fig. S1)[18,19]. Importantly, NanoLuc is co-secreted with insulin, enabling measurement of insulin secretion specifically from cells expressing either the TALK-1 WT or TALK-1 Leu114Pro construct[18]. Insulin secretion was measured as previously described[18] (detailed in the Supplementary Appendix: Methods).

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Statistical Analyses

Functional data were analyzed using pCLAMP10 or Microsoft Excel and presented as mean ± SEM. Statistical significance was determined using Student's *t*-test; a two-sided *P*-value ≤0.05 was considered statistically significant.

RESULTS

Exome sequencing in a family with MODY identifies a novel variant in KCNK16

Exome sequencing and analysis of known MODY genes in the proband identified a splice site mutation in *ABCC8* (NM_000352 c.1332+4 delC); however, this variant was not predicted to affect splicing[20] and did not segregate appropriately in the pedigree.

Exome sequencing and analysis of the extended pedigree identified novel good-quality coding variants in two genes, *KCNK16* and *USP42*, with appropriate segregation (Fig. 1A and 1B; coverage statistics for exome sequencing, Supplementary Appendix: Table S2; exome data filtering, Supplementary Appendix: Table S3). *USP42* (*Ubiquitin-specific peptidase 42*) is involved in spermatogenesis[21] and is not expressed in the pancreas; and was considered an unlikely MODY candidate. However, *KCNK16 (Potassium channel, subfamily K, member 16)* encodes for TALK-1, with its established role in GSIS[9]. Further, the *KCNK16*-containing locus is associated with T2DM[9,22,23].

The *KCNK16* variant *(*NM_001135105: c.341T>C) has not previously been reported in ExAC (http://exac.broadinstitute.org), 1000 Genomes (http://www.1000genomes.org), or dbSNP137 (http://www.ncbi.nlm.nih.gov/projects/SNP/) databases. It affects a highly conserved base (GERP score 5.65) with the resultant amino acid change (p.Leu114Pro) predicted to involve the pore domain one of TALK-1, immediately downstream of the GYG K$^+$ selectivity filter (Fig. 1C). The GYG motif, and leucine 114 specifically, shows strong sequence homology with other K2P channels (Fig. 1D). As the crystal structure of TALK-1 is unpublished, TREK-2 was used to model the p.Leu114Pro mutation which demonstrated a conformational shift in both the GYG motif and pore domain (Fig. 1E), strongly suggesting that TALK-1 Leu114Pro would significantly affect K$^+$ permeability.

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TALK-1 Leu114Pro results in a gain-of-function

K^+ currents recorded using HEK293 cells transfected with either TALK-1 WT or TALK-1 Leu114Pro demonstrated that TALK-1 Leu114Pro caused a massive (312-fold) increase in whole-cell K^+ currents compared to TALK-1 WT (current at −40mV: TALK-1 Leu114Pro 774.16 ±218.75 pA vs. TALK-1 WT 2.48 ± 1.86 pA; Fig. 2A, 2B, Supplementary Appendix: Fig. S4). Individual TALK-1 channel activity showed a 3.6-fold increase in current amplitude and a 2.9-fold increase in open probability at 100mV for TALK-1 Leu114Pro compared to TALK-1 WT (Fig. 2C, 2D, 2E, 2F)[15]. Thus, TALK-1 Leu114Pro is a gain-of-function mutation, predicted to cause hyperpolarization of the β-cell membrane potential.

TALK-1 Leu114Pro reduces β−cell Ca^{2+} influx and ER Ca^{2+} stores

Ca^{2+} handling was monitored in mouse β-cells following transduction of either TALK-1 WT or TALK-1 Leu114Pro. Glucose-stimulated (20mM) β-cell Ca^{2+} influx was abolished by expression of TALK-1 Leu114Pro (Fig. 3A, 3C, and 3D). TALK-1 has been previously shown to modulate Ca^{2+}_{ER} homeostasis by providing a countercurrent for Ca^{2+}_{ER} release[16]; thus TALK-1 Leu114Pro control of Ca^{2+}_{ER} storage was also examined. Inhibition of SERCAs with CPA resulted in significantly less Ca^{2+}_{ER} release in β-cells expressing TALK-1 Leu114Pro compared to TALK-1 WT (62.6% decrease; Fig. 3E, 3F), suggesting reduced Ca^{2+}_{ER} storage with TALK-1 Leu114Pro[16]. β-cells expressing TALK-1 Leu114Pro also showed elevated basal $[Ca^{2+}_{cyto}]$ compared to β-cells expressing TALK-1 WT (28.8% increase in AUC; Fig. 3A, 3B). Taken together, this suggests that under basal conditions, TALK-1 Leu114Pro enhances Ca^{2+}_{ER} leak, thereby increasing basal $[Ca^{2+}_{cyto}]$. Furthermore, the usual transient drop in β-cell $[Ca^{2+}_{cyto}]$ following glucose stimulation of Ca^{2+}_{ER} uptake (termed phase-0) was amplified with TALK-1 Leu114Pro compared to TALK-1 WT[24]. These changes would be predicted to diminish GSIS.

TALK-1 Leu114Pro reduces Glucose-Stimulated Insulin Secretion

β-cells expressing TALK-1 Leu114Pro showed comparable basal (5mM glucose) insulin secretion but reduced GSIS (14mM glucose) compared to TALK-1 WT, in both mouse (52% decrease in GSIS) and human (38% decrease in GSIS) islets (Fig. 4).

DISCUSSION

We have identified the first family with MODY due to a mutation in *KCNK16*. The novel TALK-1 gain-of-function p.Leu114Pro mutation increases β-cell K^+ efflux resulting in membrane hyperpolarization, altering β-cell Ca^{2+} handling and decreasing GSIS; and highlight the critical role of TALK-1 in β−cell physiology. Unlike the only other MODY-associated K^+ channelopathy (i.e., K_{ATP} channel dysfunction), TALK-1 is unresponsive to sulfonylureas[9]. Thus our data suggest not only a novel therapeutic target for *KCNK16*-associated MODY but for other forms of diabetes also.

TALK-1 belongs to the K2P channel family characterized by constitutive K^+ flux, which serve critical roles setting the membrane potential of electrically excitable cells. The *KCNK16* transcript encoding TALK-1 is the most abundant K^+ channel transcript in the human β-cell[11,12]; and *KCNK16* shows the most islet-selective expression of all ion channel transcripts[10,25]. Similar to other K^+ channels, such as K_{ATP}[26], TALK-1-mediated hyperpolarization of mouse and human β-cell membrane potential limits VDCC activity, Ca^{2+} entry and GSIS[9]. However, the K_{ATP} K^+ conductance is significantly greater than the small constitutive conductance of TALK-1[9,10,15,26]. Thus, TALK-1 mainly regulates the β-cell membrane potential following glucose stimulation, when K_{ATP} channels close: their activity limits islet Ca^{2+} oscillation frequency and hence GSIS[9]. A gain-of-function TALK-1 mutation would be predicted to affect glucose tolerance adversely resulting in hyperglycemia, as demonstrated here.

The *KCNK16*-containing locus is strongly associated with T2DM, in multiple genome-wide association studies including populations with differing ethnicities[22,23,27-29], with strongest association ($p<2\times10^{-8}$) observed with the common nonsynonymous polymorphism rs1535500 (minor allele frequency 0.41, ExAC database, non-Finnish European descent subjects). The protein change (p.Ala277Glu) affects the C-

terminal tail of TALK-1 and causes a modest (1.4-fold) increase in TALK-1 channel current, with both enhanced open probability and increased cell surface localization[9]. The risk haplotype is also associated with increased expression of the adjacent gene *KCNK17* which encodes another K2P channel, TALK-2[30]. TALK-2 is also expressed in islet cells with high specificity, though lower than TALK-1 (islet expression specificity index for *KCNK16*=0.98 and for *KCNK17*=0.76)[30]. These data suggest that the association of this locus with T2DM is driven by more than one mechanism, with combined effects from overactive TALK-1 and overexpression of TALK-2, each of which potentially contributing to hyperpolarization of the β-cell membrane potential, reducing glucose-stimulated Ca^{2+} influx and GSIS. That association is seen in T2DM with a common variant in the same gene in which we are now reporting a novel mutation causing MODY indicates that the encoded protein (i.e., TALK-1) is not functionally redundant; rather, that it is likely relevant to a high proportion of T2DM cases. These properties increase the potential of TALK-1 as a therapeutic target for T2DM.

Mutations in K2P channels causing dramatic changes in K^+ channel currents typically affect the pore domains of these channels[31-33]. For example, loss-of-function mutations in the first or second pore domains of *KCNK3* (respectively, p.Gly97Arg and p.Gly203Asp) cause pulmonary hypertension[31]. Similarly, a loss-of-function mutation in the first pore domain of TASK-2 (p.Thr108Pro) causes Balkan Endemic Nephropathy[32]. A gain-of-function mutation (p.Gly88Arg) in the first pore domain of TALK-2, coded by *KCNK17*, causes a severe cardiac arrhythmia[33]; and is the only previously identified disease-associated mutation in TALK channels.

Gain-of-function mutations in K_{ATP} significantly increase β-cell K^+ flux, resulting in neonatal diabetes and MODY[7,8]. In contrast, TALK-1 p.Leu114Pro results in a more modest diabetes phenotype despite the 300-

fold increase in whole-cell TALK-1 activity. This may because TALK-1 activation shows voltage-dependence[9,10]. Unlike K_{ATP}, which is active at all voltages, TALK-1 is an outward rectifying channel that shows increased activation during depolarization[9,10]. Therefore, a gain-of-function in TALK-1 would be most active post-β-cell depolarization – limiting, but not abrogating, insulin secretion. The p.Leu114Pro mutation does increase TALK-1 current near the resting membrane potential **(Fig. 2A, and Supplementary Appendix Fig. S4)**; however, this current is still less than the total β-cell K_{ATP} conductance under euglycemic conditions. These data concord with the proband's clinical phenotype, with dramatic glucose elevation after an oral glucose load but only a modest increase in fasting plasma glucose.

TALK-1 is expressed on both the β-cell plasma membrane and the ER membrane[16]. Ca^{2+}_{ER} release is balanced by negative charge on the luminal ER membrane; this charge is dissipated by ER TALK-1 K^+ influx leading to enhanced Ca^{2+}_{ER} release[16]. Thus overactive TALK-1 channels (e.g., TALK-1 Ala277Glu) increase Ca^{2+}_{ER} release, whereas TALK-1 ablation reduces Ca^{2+}_{ER} release[16]. Importantly, enhanced β-cell Ca^{2+}_{ER} release under hyperglycemic conditions results in ER-stress, contributing to β-cell dysfunction[34]. TALK-1 Leu114Pro may contribute to β-cell dysfunction via ER-stress, as observed in some MODY subtypes (e.g., *INS* mutations in MODY-10[35]); however, this remains speculative. Additionally, although highly β-cell specific, TALK-1 is also expressed in human pancreatic δ-cells where it negatively regulates somatostatin release [36]. TALK-1 KO mice show increased somatostatin secretion under low and high glucose conditions, due to enhanced Ca^{2+}_{ER} release[36]; thus, a gain-of-function mutation in TALK-1 may reduce δ-cell somatostatin secretion. The glycemic effects of this would be complex given the inhibitory effect of somatostatin on both insulin and glucagon secretion[36]; and require future investigation.

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Some MODY subtypes (e.g., *ABCC8*-, *KCNJ11*-, *HNF1α*- and *HNF4α* MODY) are manageable through K_{ATP} inhibition[7,37,38] – i.e., sulfonylurea use. Although β-cell membrane potential depolarization with sulfonylureas may allow greater VDCC activity, potentially increasing insulin secretion in affected individuals in this family, TALK-1 itself is not sensitive to sulfonylureas[9]. Further, and as detailed above, TALK-1 primarily modulates β-cell membrane potential during active insulin secretion when K_{ATP} is closed (i.e., during hyperglycemic conditions)[9]. Thus, K_{ATP} inhibition may not completely normalize β-cell membrane potential or insulin secretion in individuals with TALK-1 gain-of-function MODY. This raises the possibility of TALK-1 inhibition as a druggable target. Genetic evidence whether from rare (e.g., MODY) or common (e.g., T2DM[22,23,27-29]) human disease is a strong predictor of future successful drug development[39]. Thus our data have important therapeutic implications not only for TALK-1 MODY but also for the far more common form of diabetes T2DM.

In conclusion, we have identified a novel mutation in *KCNK16* causing a gain-of-function in TALK-1, reducing glucose-stimulated Ca^{2+} influx, Ca^{2+}_{ER} storage, and GSIS; and resulting in MODY. TALK-1 is the first ion channel linked to MODY after K_{ATP}; and is expressed more selectively in islet cells compared to K_{ATP}. The *KCNK16* locus is associated with T2DM risk in the general population. Our data suggest TALK-1 as an efficacious and islet-selective therapeutic target for both *KCNK16*-associated MODY and T2DM.

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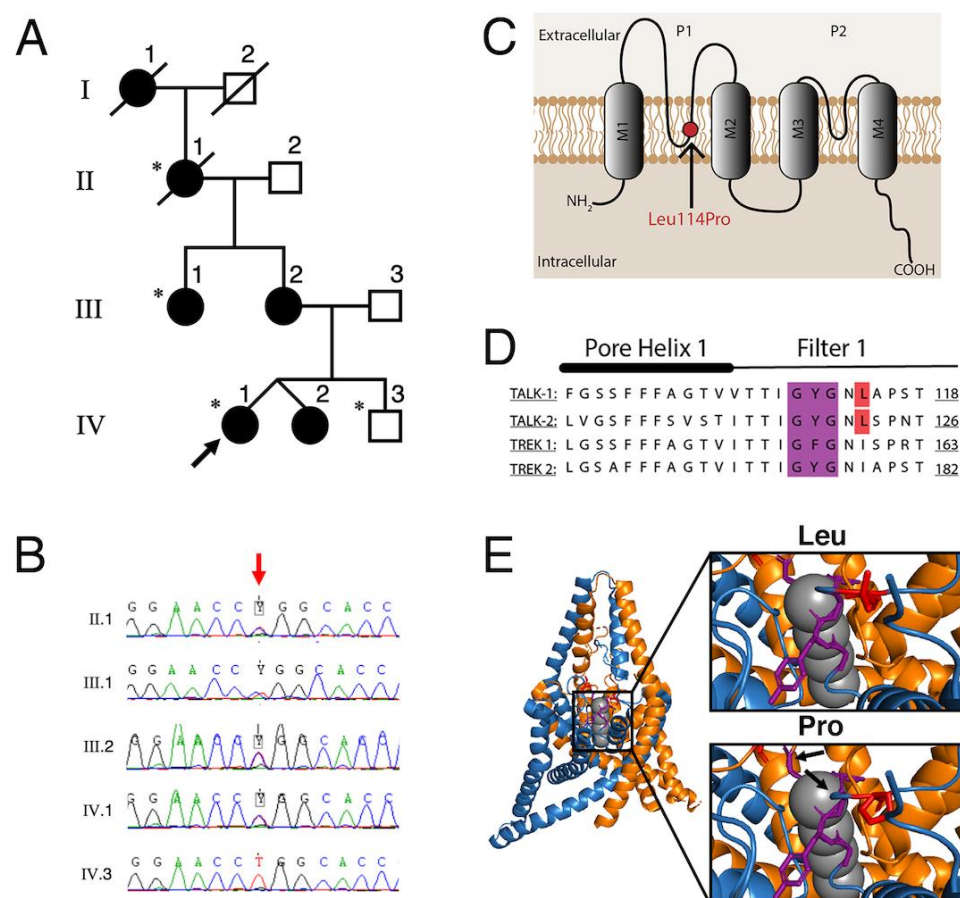
Figures

Figure 1: A novel *KCNK16* mutation co-segregates with MODY in a four-generation family and is predicted to affect the K⁺-selectivity channel of TALK-1.

Family pedigree (Panel A: asterisks indicate individuals undergoing WES; filled-in shapes indicate individuals with diabetes; arrow indicates proband), with chromatogram of *KCNK16* variant (c.341T>C) (Panel B; red arrow indicates variant). Location of the predicted protein change (p.Leu114Pro), within the first pore domain and K⁺-selectivity channel of TALK-1 (Panel C), with alignment of Pore Helix 1 and Filter 1 amino acid sequences of *KCNK16* with other KCNK channels (Panel D; mutation position indicated in red; selectivity filter indicated in purple). Predicted conformational shifts (indicated by the arrows) in the K⁺ selectivity filter, modelled using TREK2 crystalline structure (Panel E).
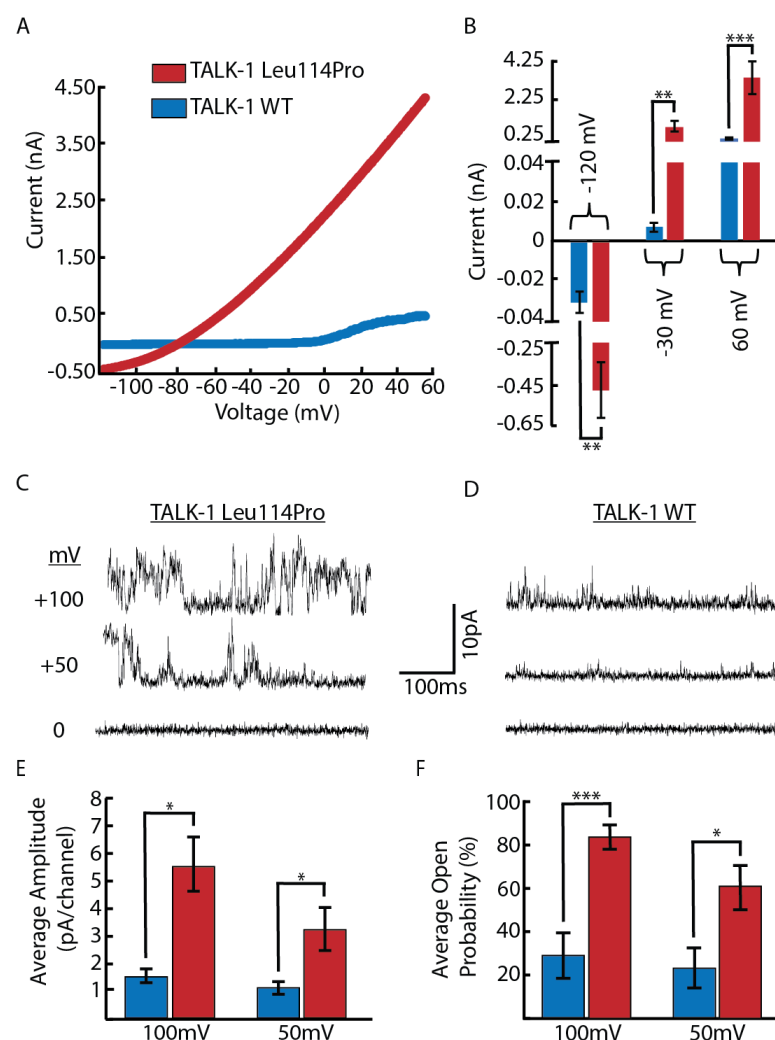
Figure 2: TALK-1 Leu114Pro causes a drastic gain-of-function in TALK-1 K⁺ current. K⁺ currents monitored from TALK-1 WT or TALK-1 Leu114Pro with whole-cell voltage clamp recordings, in response to a voltage ramp from -120mV to 60mV (Panel A); mean ± SEM; N= 11 control cells; N= 10 TALK-1 Leu114Pro cells (Panel B). Single-channel plasma membrane K⁺ currents monitored through TALK-1 Leu114Pro or TALK-1 WT with attached patch voltage clamp recordings, in response to the indicated voltage steps (Panel C and Panel D). Single channel recordings were analyzed for current amplitude (Panel E) and channel open probability (Panel F); mean ± SEM; N= 8 TALK-1 WT cells; N= 11 TALK-1 Leu114Pro cells *$P<0.05$, **$P<0.01$, ***$P<0.001$**.
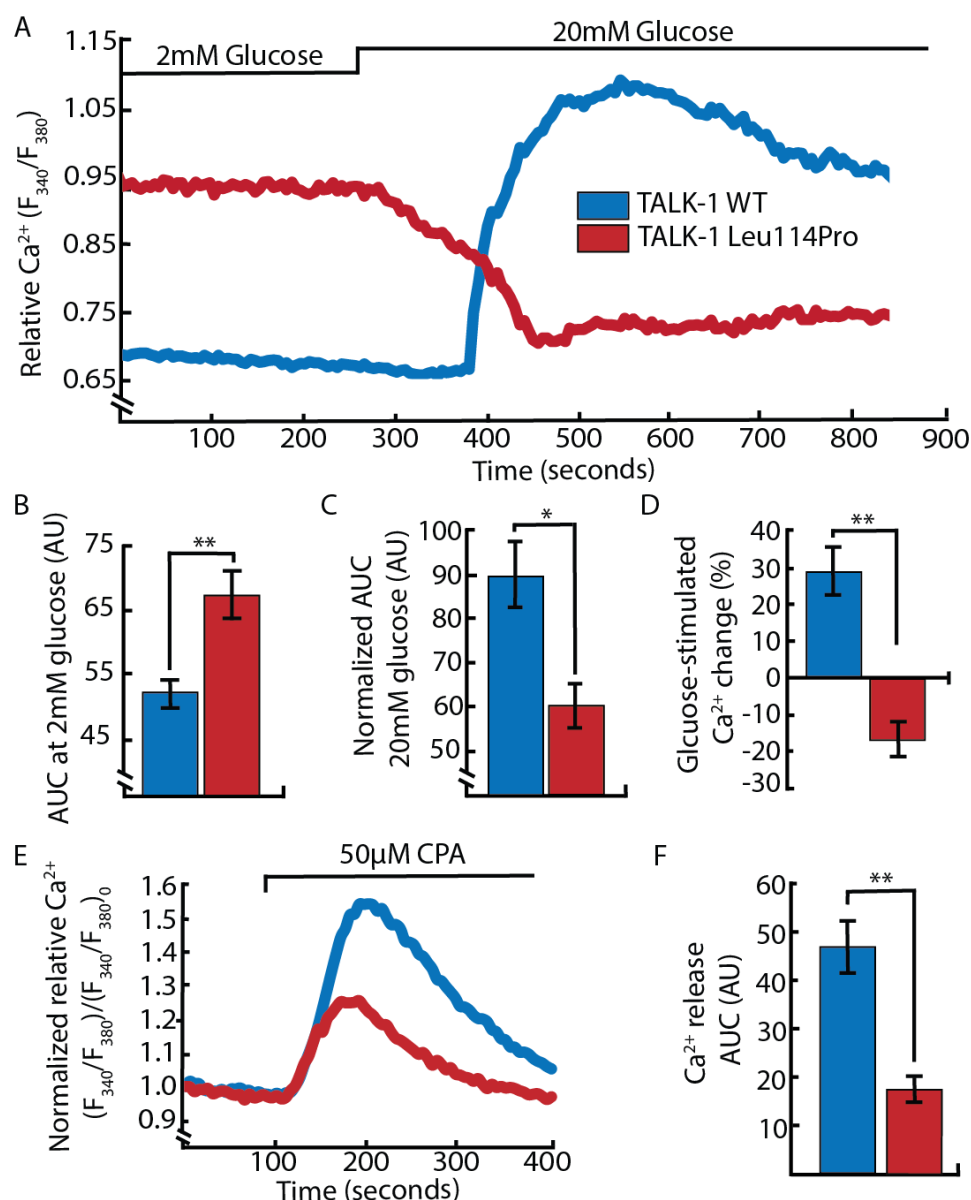
Figure 3: TALK-1 Leu114Pro modulates β-cell Ca²⁺ homeostasis. Representative β-cell Ca²⁺ measurements in response to 2mM and 20mM glucose (Panel A). Area under the curve (AUC) analysis of β-cell Ca²⁺ under low (2mM) glucose (Panel B) and high (20mM) glucose conditions (Panel C). AUC percent change from low glucose to high glucose (Panel D). Representative β-cell Ca²⁺ measurements in response to [Ca$^{2+}_{ER}$] depletion by CPA (Panel E) and the AUC analysis of the CPA response (Panel F). mean ± SEM; N= 3 animals for TALK-1 WT and TALK-1 Leu114Pro Ca²⁺ experiments except the for the 2mM condition that included N=9 animals. *$P<0.05$, **$P<0.01$, ***$P<0.001$.
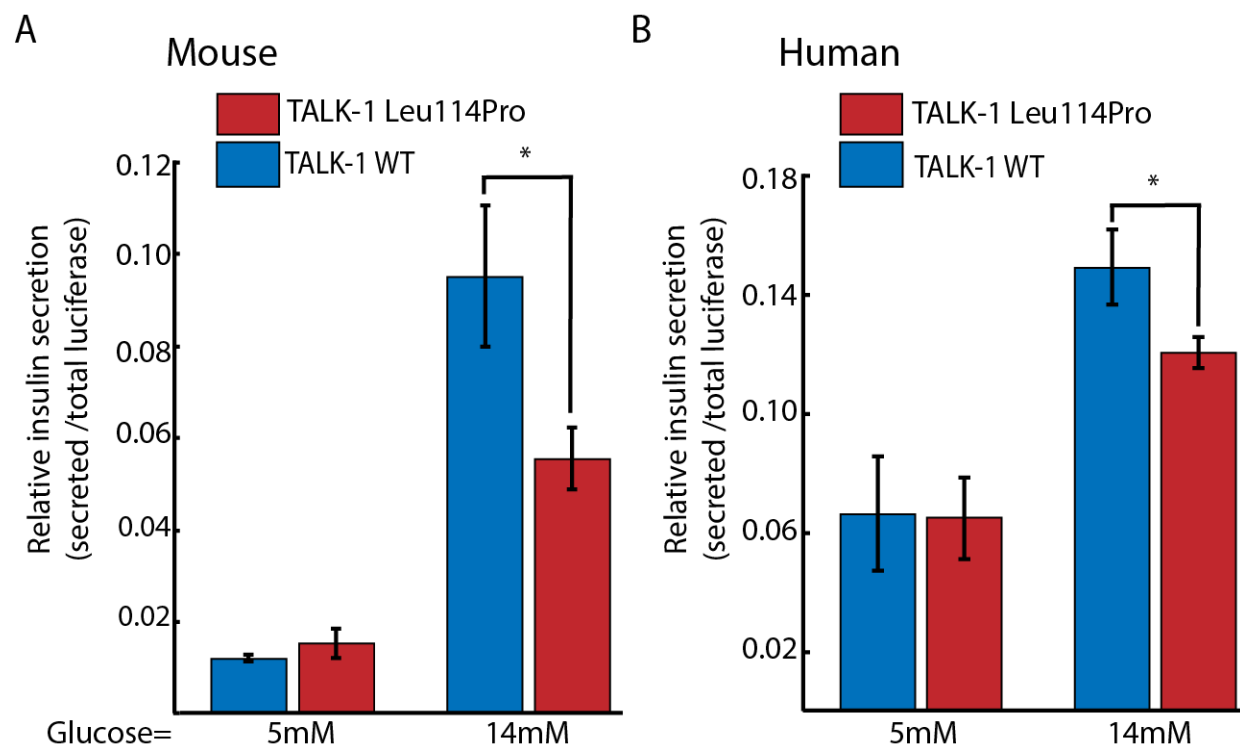
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Figure 4: TALK-1 Leu114Pro reduces glucose-stimulated insulin secretion. Mouse (Panel A) and human (Panel B) islets transduced with viruses selectively expressing either TALK-1 WT or TALK-1 Leu114Pro and the NanoLuc-proinsulin luciferase insulin reporter. Islets were monitored for total secreted luciferase following exposure to 5mM or 14mM glucose; mean ± SEM; N=6 animals (14mM glucose, Panel A), N=3 animals (5mM glucose, Panel A); N=8 Human Donors (14mM glucose, Panel B), N=5 Human Donors (5mM glucose, Panel B). *P<0.05