CACNB2 Is a Novel Susceptibility Gene for Diabetic Retinopathy in Type 1 Diabetes

Nadja Vuori,[1,2,3] Niina Sandholm,[1,2,3] Anmol Kumar,[1,2,3] Kustaa Hietala,[4] Anna Syreeni,[1,2,3] Carol Forsblom,[1,2,3] Kati Juuti-Uusitalo,[5] Heli Skottman,[5] Minako Imamura,[6,7,8] Shiro Maeda,[6,7,8] Paula A. Summanen,[9] Markku Lehto,[1,2,3] and Per-Henrik Groop,[1,2,3,10] on behalf of the FinnDiane Study

[1]Folkhälsan Institute of Genetics, Folkhälsan Research Center, Helsinki, Finland, [2]Abdominal Center Nephrology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland, [3]Research Program for Clinical and Molecular Metabolism, Faculty of Medicine, University of Helsinki, Helsinki, Finland, [4]Central Finland Central Hospital, Jyväskylä, Finland, [5]Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland [6]Laboratory for Endocrinology, Metabolism and Kidney Diseases, RIKEN Center for Integrative Medical Sciences, Kanagawa, Japan, [7]Department of Advanced Genomic and Laboratory Medicine, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan, 8Division of Clinical Laboratory and Blood Transfusion, University of the Ryukyus Hospital, Okinawa, Japan, [9]Ophthalmology, University of Helsinki, Helsinki University Hospital, Helsinki, Finland,[10]Department of Diabetes, Central Clinical School, Monash University, Melbourne, Victoria, Australia

Corresponding author: Per-Henrik Groop, per-henrik.groop@helsinki.fi

Diabetic retinopathy is a common diabetes complication that threatens the eyesight and may eventually lead to acquired visual impairment or blindness. While a substantial heritability has been reported for proliferative diabetic retinopathy (PDR), only a few genetic risk factors have been identified. Using genome-wide sib pair linkage analysis including 361 individuals with type 1 diabetes, we found suggestive evidence of linkage with PDR at chromosome 10p12 overlapping the *CACNB2* gene (logarithm of odds = 2.73). Evidence of association between variants in CACNB2 and PDR was also found in association analysis of 4,005 individuals with type 1 diabetes with an odds ratio of 0.83 and *P* value of 8.6 x 10^{-4} for rs11014284. Sequencing of *CACNB2* revealed two coding variants, R476C /rs202152674 and S502L/rs137886839. *CACNB2* is abundantly expressed in retinal cells and encodes the β2 subunit of the L-type calcium channel. Blocking vascular endothelial growth factor (VEGF) by intravitreous anti-VEGF injections is a promising clinical therapy to treat PDR. Our data show that L-type calcium channels regulate VEGF expression and secretion from retinal pigment epithelial cells (ARPE19) and support the role of *CACNB2* via regulation of VEGF in the pathogenesis of PDR. However, further genetic and functional studies are necessary to consolidate the findings.

Diabetic retinopathy is the leading

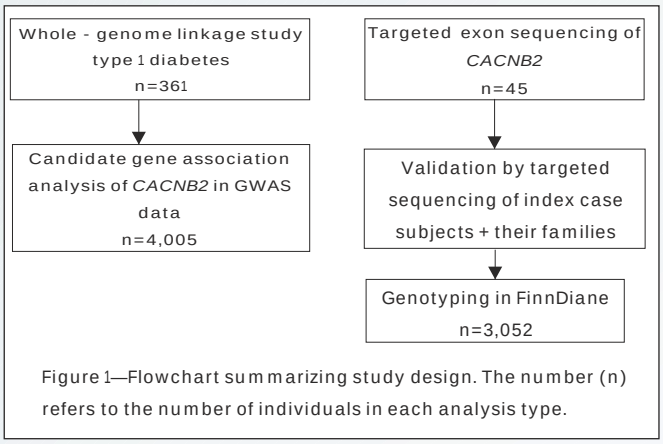

Figure 1—Flowchart summarizing study design. The number (n) refers to the number of individuals in each analysis type.

cause of vision loss in adults [1]. Diabetic retinopathy is subdivided into a milder nonproliferative form and a severe form, proliferative diabetic retinopathy (PDR). The prevalence of PDR in type 1 diabetes varies between 13% and 50% after 15–20 years of diabetes duration [2,3]. Most individuals who develop PDR would become blind within 5–10 years without treatment [4]; however, strict glycemic control and photocoagulation (or laser treatment) have been successful therapies both in the prevention and treatment of PDR [5,6]. However, panretinal photocoagulation has side effects such as peripheral visual field constraints. Injections targeting the vascular endothelial growth factor (anti-VEGF) comprise a novel treatment modality for macular edema and have also been suggested to be a promising therapy to delay PDR, although they are costly and require recurrent injections [7,8]. Several risk factors have been identified for PDR, such as poor glycemic control, long diabetes duration [2], and high blood pressure [9].

Family studies have further revealed that PDR clusters in families and our previous data suggested a significant genetic component in the pathogenesis of PDR that was as high as 52% [10]. Nevertheless, only a few genetic risk factors have been robustly identified for PDR [11–14]. Therefore, we performed linkage and association analyses in individuals with type 1 diabetes to identify novel susceptibility loci and genes predisposing to PDR and followed up the findings in retinal pigmented epithelial cells. Understanding the role of the genetic variation in the development of diabetic retinopathy may not only reveal novel molecular mechanisms but also help us discover biomarkers and ultimately novel therapies to prevent and treat the disease.

RESEARCH DESIGN AND METHODS

Overview of the Study Design

This study is part of the ongoing nationwide Finnish Diabetic Nephropathy (FinnDiane) Study, which since 1997 has studied and collected comprehensive data from individuals with type 1 diabetes in Finland. The aim of the study

Table 1—Clinical characteristics of the individuals in the linkage study

Variable	All	No PDR	PDR	*P*
Men/N (%)	199/361 (55)	124/231 (54)	68/114 (60)	0.35
Age (years)	41 (11.9)	39.4 (12.0)	44.5 (11.0)	0.0001
Age at onset (years)	16 (11.7)	18.2 (12.2)	10.47 (8.0)	<0.0001
Duration of diabetes (years)	25.4 (12.4)	21.16 (11.17)	33.99 (10.42)	<0.0001
HbA_1c (mmol/mol)	69.1 (16.4)	67.7 (16.1)	71.9 (17.3)	0.037
HbA_1c (%)	8.5 (1.5)	8.35 (1.48)	8.73 (1.58)	0.037
SBP (mmHg)	137 (19)	133 (17)	144 (20)	<0.0001
DBP (mmHg)	80 (10)	79 (10)	82 (11)	0.014
Mean arterial pressure (mmHg)	100 (12)	99 (12)	104 (13)	<0.0001
ETDRS score	43 (20–61)	35 (10–43)	75 (61–75)	

Data are means (SD) or median (interquartile range) unless otherwise indicated. All: values among all (*N*=361) patients. No PDR (*N*=231) and PDR (*N* = 114) columns include only participants with sibs after exclusion of participants without data on PDR. *P: P* value for difference between PDR groups, calculated with x^2 test for sex and with Welch two-sample *t* test for the continuous variables. DBP, diastolic blood pressure; SBP, systolic blood pressure.

is to identify risk factors for diabetes complications. The study setting has been described previously [15]. The study protocol was approved by the Ethics Committee of Helsinki and Uusimaa Health District as well as the local ethics committees of the participating centers, and the participants gave their written informed consent prior to participation. The study was conducted in accordance with the Declaration of Helsinki as revised in year 2000. The Ethics Committee of the Pirkanmaa Hospital District (Tampere, Finland) (R05116) gave approval to derive, culture, and differentiate human embryonic stem cell (hESC) lines for research.

First, a whole-genome sib pair linkage study in individuals with type 1 diabetes was performed. This was followed by a candidate gene association analysis of *CACNB2* in genome-wide association study (GWAS) data to search for association between *CACNB2* and PDR

in a large case control setting. Thereafter, targeted sequencing was performed with the aim to find causal variants in the *CACNB2* gene region that was identified by the sib pair linkage study (Fig. 1).

Study Participants

Whole-Genome Linkage Study

The linkage study included 180 families with at least two siblings with type 1 diabetes. Altogether 361 individuals formed 202 sib pairs (Fig. 1 and Table 1). Ophthalmic records and/or fundus photographs were obtained for 94% of the individuals and used to score the severity of retinopathy. The Early Treatment of Diabetic Retinopathy Study (ETDRS) grading scale was used, where 10 represents no retinopathy and $61 PDR [16]. Unaffected control subjects were defined as those with ETDRS of 10–53E. The eye with the more severe retinopathy served to assess severity. After exclusion of individuals without data on retinopathy, 345 individuals with type 1 diabetes remained in 162 sibships of two or more siblings. Nine individuals with diabetes were included despite having an age at onset of diabetes. 40 years (up to 53.5 years). Sib pairs comprised both affected sib pairs (i.e., both with type 1 diabetes and PDR), discordant sib pairs (both with type 1 diabetes but only one with PDR), and unaffected sib pairs (both with type 1 diabetes but neither with PDR).

Table 2—Summary of the nonparametric linkage result for PDR on the regions showing significant or suggestive evidence of linkage (LOD score >1.75)

			LOD	
Chromosome	Marker	Map position (cM)	Single point	Multipoint
10	*	41.2	*	2.05
10	D10S548	43.4	2.72	1.85
19	*	106.7	*	2.69
19	D19S210	108.6	0.31	3.01

LOD scores were calculated from empirical P values. *Interval between adjacent markers.

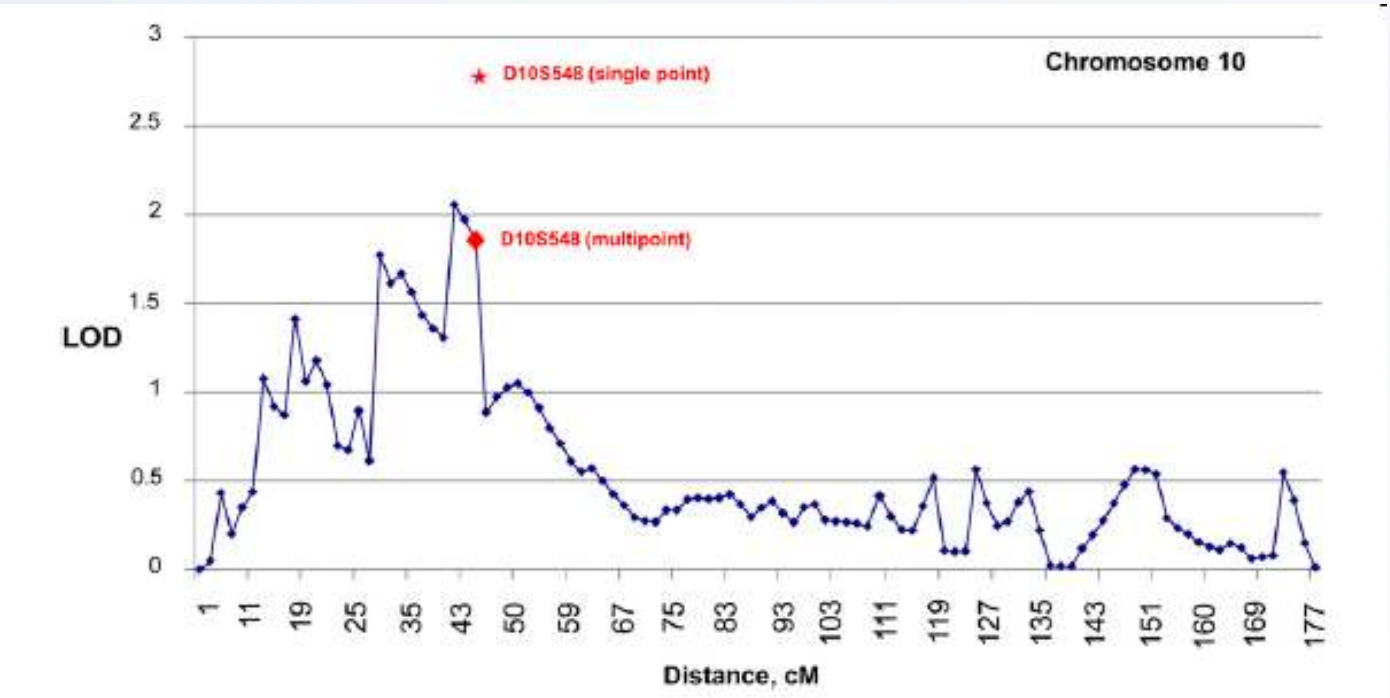

Figure 2—The results of multipoint genome-wide linkage study on chromosome 10. The genetic distance (cM) is plotted on the x-axis against the LOD score on the y-axis. Diamonds indicate the LOD scores from the multipoint analysis; the star indicates the single-point LOD score of 2.73 for the microsatellite D10S548 at 43.4 cM.

Sequencing

The sequencing of the CACNB2 gene included altogether 16 familial PDR cases (with a sibling with PDR) and 29 sporadic cases (with an unaffected sibling and no known family history of PDR) from the families participating in the linkage study. Only one of the two siblings was chosen if both siblings in a pair had PDR and shared the same risk alleles.

Candidate Gene Association Analysis

PDR was defined as laser-treated diabetic retinopathy based on a patient questionnaire. The analysis included altogether 4,005 individuals with type 1 diabetes with an onset of diabetes before the age of 40 years and insulin treatment initiated within 1 year of the diagnosis of diabetes and with complete data on PDR and covariates (sex, age, and diabetes duration) available: 1,997 case subjects with PDR and 2,008 control subjects without PDR and at least 15 years' duration of diabetes.

Replication

Replication was sought in GWAS data for 11,097 individuals with type 2 diabetes from the BioBank Japan Hondo cluster [17], genotyped with the OmniExpressExome (*N*= 8,880: 4,839 case subjects with any diabetic

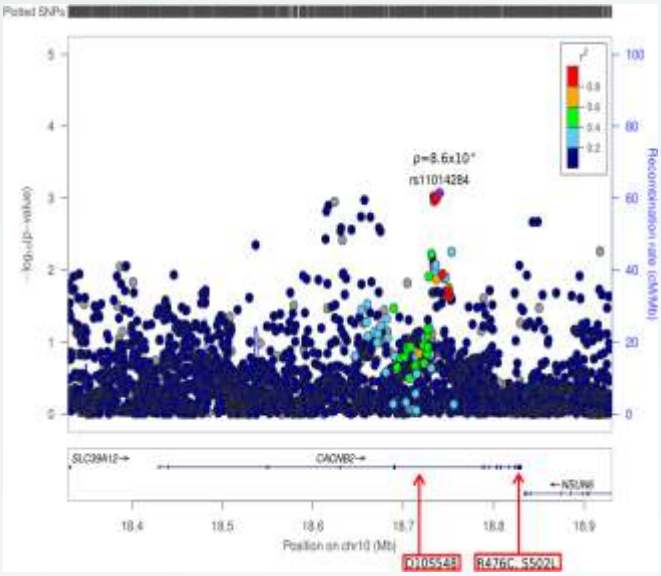

Figure 3—Regional summary of the association, linkage, and sequencing findings for PDR on chromosome 10p12 *CACNB2* locus. The LocusZoom plot (54) with the -$\log_{10}$ (*P* values) on the *y*-axis corresponds to the association analysis *P* value. The SNP with the lowest *P* value ($P = 8.6 \times 10^{-4}$) is depicted in lilac, and the r^2 color coding illustrates the linkage disequilibrium with this SNP. Arrows indicate the locations of the D10S548 microsatellite and of the identified missense mutations. Recombination rates can be seen in blue. Chromosome positions are based on hg19/1000 Genomes November 2015 EUR.

retinopathy and 4,041 control subjects without diabetic retinopathy) and Illumina 610K array (*N* = 2,217: 693 case subjects with any diabetic retinopathy and 1,524 control subjects without diabetic retinopathy). Genotype imputation with 1000 Genomes Asian phase 1 reference panel resulted in 7,521,072 single nucleotide polymorphisms (SNPs).

Marker Design and Genotyping

Sib Pair Linkage Analysis

In the sib pair linkage analysis, genomic DNA was extracted from whole blood using the PUREGENE DNA Purification Kit (Gentra Systems, Minneapolis, MN). The DNA samples were genotyped using an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA) with ABI PRISM Linkage Mapping Set MD-10 V2.5 (Généthon map) at the Institute for Molecular Medicine Finland (FIMM), Helsinki, Finland. In total, there were 367 autosomal markers with a mean (SD) interval of 9.6 (4.1) cM and 77.7% heterozygosity.

Candidate Gene Association Analysis

The candidate gene association analysis included GWAS data of 6,171 individuals. SNP genotyping was performed in three batches by using HumanCoreExome Bead arrays 12-1.0, 12-1.1, and 24-1.0. Variants were called with zCall [18]. Standard quality control procedures were applied as previously described [19], resulting in 316,899 SNPs and 6,019 individuals passing the quality control. Relatedness was then calculated (KING 1.30), and genotype imputation was performed with Minimac3/Minimac3

Table 3—The genotyping results showed that 7 individuals were found with the R476C mutation and 15 individuals with the S502L mutation

	R476C, rs202152674	S502L, rs137886839
Alleles (minor/major)	T/C	T/C
n (heterozygotes)	7	15
MAF, %	0.1	0.2
Male sex	3 (43)	5 (33)
Fundus photographs or ophthalmic records available	3 (43)	14 (93)
Laser treatment	2 (29)	4 (27)
PDR	2 (29)	5 (33)
Duration of diabetes to PDR (years)*	15.8 (13.7–17.9)	16.7 (13.0–20.0)
Nonproliferative diabetic retinopathy	1 (14)	3 (20)
No diabetic retinopathy, duration ≥15 years	0	3 (20)
No diabetic retinopathy, duration <15 years	0	3 (20)
Diabetes duration at the time of latest ophthalmic information (years)	30.3 (19.8–40.8)	20.7 (10.7–40.3)

Data are median (range) or n (%) unless otherwise indicated. *Duration of diabetes to PDR is calculated among the participants with PDR.

-omp v1.0.14 [20] using 1000 Genomes as reference population. We excluded 331 parents, 395 individuals with age at diabetes onset >40 years, diabetes type other than type 1 diabetes, or no data on diabetes onset year; 278 individuals with no data on laser treatment available; and 1,010 control subjects with diabetes duration <15 years, resulting in 4,005 individuals in the analysis. Finally, we extracted SNPs within the *CACNB2* gene (chromosome 10p12) or 100 kb upstream and downstream of the gene.

Sequencing

The sequencing of the *CACNB2* gene (chromosome 10, base pairs 18660956–18880694) (Human Mar. 2006 Assembly [hg18]) was performed with the NimbleGen Sequence Capture (http://www.nimblegen.com/products/seqcap/) sequencing technology. This area was chosen based on the linkage finding, starting 100 kb upstream of D10S548 and ending 10 kb downstream of the end of *CACNB2* (at 18870694). Our primary goal was to identify variants in the coding exons. For the exons 1–4 outside of the targeted NimbleGen sequencing area, PCR and sequencing were performed with standard procedures, and the

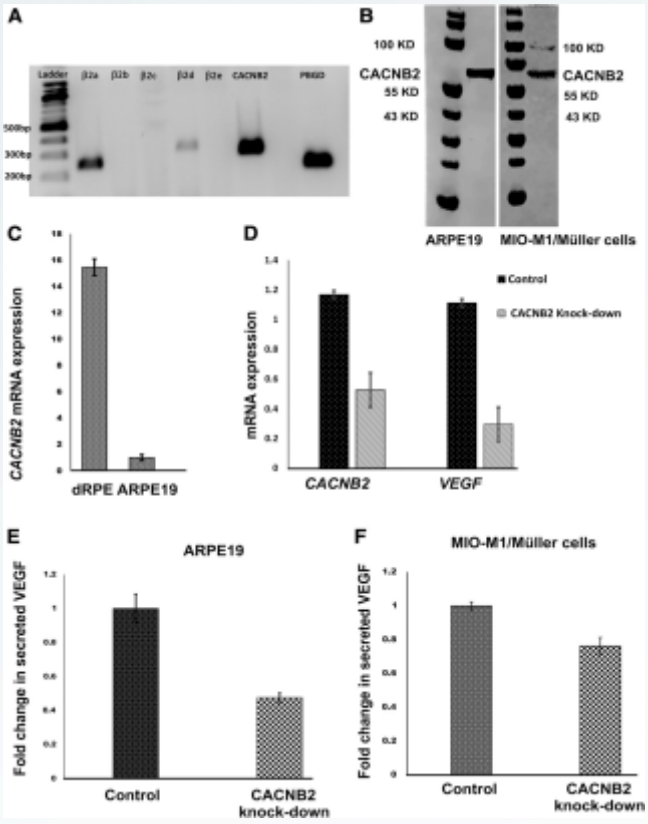

Figure 4—In vitro expression and effect of CACNB2 knockdown. A: Semiquantitative RT -PCR to show endogenous expression of different splice variants (β2a, β2b, β2c, β2d, β2e) of *CACNB2* in ARPE19 cells. Last exon is common in all variants. Total *CACNB2* expression was detected by primers designed to amplify a region in last exon. Porphobilinogen deaminase (PBGD) was used as housekeeper in the same expression range as *CACNB2*. bp, base pairs. B: Western blot showing expression of endogenous CACNB2 protein in ARPE19 and MIO-M1 cells. KD, kilodalton. C: Quantitative RT-PCR to show expression of *CACNB2* mRNA in human stem cell–derived differentiated mature retinal pigmented cells (dRPEs) compared with undifferentiated ARPE19 cells. *N* = 3. *D*: Change in *CACNB2* and *VEGF* mRNA after knocking down *CACNB2* using siRNAs against *CACNB2* compared with nontargeting siRNA control in ARPE19 cells. We observe statistically significant downregulation of *CACNB2* mRNA impacting level of *VEGF mRNA* (*P* value < 0.01). *N* = 3. *E* and *F*: VEGF ELISA shows significantly less secreted VEGF in ARPE19 and MIO-M1 cell medium (*P* value < 0.01). Differences in siRNA knockdown levels are attributed to varied transfection efficiencies between cell lines. *N* = 3.

primers used are described in the Supplementary Table 1. Mutation Taster was used to evaluate the impact of putative variants [21].

Targeted Sequencing and Genotyping

The two nonsynonymous missense mutations found in the sequencing data were verified with targeted sequencing, and all available family members were sequenced for these variants. Thereafter, we genotyped these two variants in 3,052 individuals with type 1 diabetes from FinnDiane, most of whom were also included in the GWAS, with TaqMan technology. Predesigned TaqMan assays were ordered from Life Technologies (Life Technologies, Foster City, CA). ABI PRISM 7900HT Sequence Detection System and SDS 2.3 software (Life Technologies) were used for genotyping and genotype calling. Genotyping success rates were 96.9% for R476C (rs202152674) and 96.4% for S502L (rs137886839). New heterozygotes found from the FinnDiane cohort were verified with PCR and sequencing.

Molecular Biology and Cell Culture Techniques

Retinal pigmented epithelial cell line ARPE19 was obtained from ATCC (ATCC

CRL-2302). ARPE19 cells were grown in DMEM-F12 (D6421; Sigma-Aldrich) supplemented with 10% FBS (10270106; Gibco/Life Technologies), penicillin - streptomycin (15140122; Gibco/Life Technologies), GlutaMAX Supplement (35050061; Gibco/Life Technologies), and Normocin (ant-nr-1; InvivoGen). MIO-M1 cells (Müller glial cell lines derived from adult human retina) were obtained from Limb's laboratory [22] and grown in DMEM (1196509 2; Thermo Fisher Scientific), otherwise similarly to ARPE19 cells. We divided cells 1:4 once per week and used cells below passage number 30 for experiments. At 24 h before transfection, 0.2 3 106 cells/sixwell (CLS3516-50EA; Corning) were plated. siGenome human CACNB2 (783) siRNA-SMART pool (M-008741-01-005) and siGenome nontargeting siRNA pool (10 mmol/L) were transfected twice at 48-h intervals with Lipofectamine RNAiMAX Transfection Reagent (13778075; Invitrogen) as per the manufacturer's recommendations for potent knockdown. Cell medium was collected 48 h after the second transfection and spin 3,000 rpm for 5 min to remove cell debris and stored in 280°C for future VEGF measurement by human VEGF quantikine ELISA kit (DVE00; R&D systems) as per the manufacturer's recommendations. Cells were lysed in TRIzol reagent (15596026; Invitrogen), and RNAs were extracted per the manufacturer's protocol. cDNAs were synthesized using Super-Script III Reverse Transcriptase (18080093; Invitrogen). Semiquantitative PCR was done with AmpliTaq Gold (4486226; Applied Biosystem) and quantitative PCR using SsoAdvanced Universal SYBR Green Supermix (1725271; Bio-Rad). Western blot to detect *CACNB2* protein in ARPE19 cells was done by blocking polyvinylidene fluoride (PVDF) membrane (Trans-Blot Turbo Mini PVDF Transfer Packs, 1704156; Bio-Rad) in 5% fat-free milk in 1X TBS-0.05% Tween (P9416; Sigma-Aldrich) overnight at 14°C. Blocked PVDF membrane was later incubated with the primary *CACNB2* antibody (6C4) (H00000783-M05; Novus Biologicals) for 1 h at room temperature diluted 1:1,000 in blocking buffer and incubated for 1 h in antimouse IgG, HRP-linked secondary antibody 1:3,000 dilution in the blocking buffer. The mRNA was extracted from hESC-derived retinal pigmented epithelium (hESC-RPE) cultured as previously described [23].

Statistical Analyses

Sib Pair Linkage Analysis

In the sib pair linkage analysis, allele frequencies, Mendelian inconsistencies, and relationships were checked with S.A.G.E. software with the FREQ, PEDCHECK, and RELTEST options [24]. One sib pair was reclassified as half sibs. The GENIBD program in S.A.G.E. generated single-point and multipoint identity-by-descent estimates.

In order to pool information from multiple markers, we performed multipoint, nonparametric linkage analysis with SIBPAL (sib pair linkage program) in S.A.G.E. with modified Haseman-Elston regression [25] of full-sib pairs and included duration of diabetes as a covariate. The binary PDR status was treated as a continuous trait. Empirical P values by up to 106 permutations were converted to pointwise logarithm of odds (LOD) scores [26]. A LOD score of >2.2 is generally considered suggestive linkage and an LOD score of >3.6 significant linkage [27]. In our study, LOD scores >1.75 represent one false per scan for experiments involving 400 markers and were regarded as promising.

Candidate Gene Association Analysis

We estimated the effect of the selected SNPs with minor allele frequency (MAF) ≥ 0.1% around the main linkage peak by logistic regression corrected for sex, age, and duration of diabetes, and genotyping batch using RvTests [28], and limited the variants to those with imputation quality ≥0.6. The effective number of independent SNPs was estimated with Genetic type 1 error calculator (GEC) [29].

Sequencing

For each individual with a mutation found in the sequencing, we matched four control subjects by age, sex, and diabetes duration and compared the clinical characteristics between the groups using t test in R.

Data and Resource Availability

The single-point linkage study results and the significant summary statistics from the *CACNB2* candidate gene association study are available in Supplementary Tables 2 and 3. The ethics statement and the informed consent do not allow sharing of individual-level data.

RESULTS

Linkage Analysis in Sib Pairs

The mean number of generations in the whole-genome linkage study was 2.1 (2 [93.3%], 3 [5.6%], or 4 [0.6%]), the

mean pedigree size was 5.0 (4 [52.8%], 5 [25.0%], and 6 [9.4%]), and the mean (SD) number of siblings in each family was 2.6 (1.0). Participants with PDR were older, had longer duration of diabetes, and higher systolic and diastolic blood pressure (Table 1). We performed linkage analysis in sib pairs to identify chromosomal regions linked to PDR and identified one microsatellite (D10S548) in the *CACNB2* gene on chromosome 10 with a suggestive single-point LOD score of 2.73 ($P = 1.96 \times 10^{-4}$) and a multipoint LOD score of 1.85 ($P = 0.0017$) (Table 2 and Fig. 2). Evidence of linkage was also found on chromosome 19 with a multipoint LOD score of 2.69 and 3.01 (D19S210), but the single-point LOD score was nonsignificant (Supplementary Table 2).

Candidate Gene Association Analysis of *CACNB2*

The candidate gene association study was performed in order to examine whether the area under the linkage peak on chromosome 10 would also show association with PDR in the GWAS data. The logistic regression showed that 197 SNPs out of 3,528 SNPs with MAF ≥0.1% had a *P* value < 0.05 and 33 SNPs a *P* value < 0.01 (Supplementary Table 3). The SNP with the lowest *P* value of 8.6×10^{-4} for association with PDR was a common SNP, rs11014284, with an odds ratio of 0.83 (95% CI 0.74– 0.92) (MAF = 27.8%) (Fig. 3). The effective number of independent SNPs with MAF ≥0.1% was estimated as 1,578, resulting in a significance threshold of *P* value < 3.17×10^{-5} after correction for multiple testing.

Targeted Sequencing of the *CACNB2* Gene and Validation by Genotyping the Mutations

The sequencing analysis of CACNB2 exons in 45 case subjects with PDR identified two missense mutations at the COOH-terminal half of the protein in the last exon of the *CACNB2* gene. We identified a point mutation of C to T resulting in a substitution of arginine for cysteine (rs202152674/R476C) and another point mutation of C to T resulting in a substitution of serine for leucine (rs137886839/S502L) in *CACNB2* (ENST00000396576), with both mutations identified once. MutationTaster [21] predicted both mutations to have an impact on the protein function (disease causing); PolyPhen predicted both variants to probably be damaging, while SIFT predicted R476C to be deleterious (with low confidence) and S502L as tolerated.

In the genome aggregation (GnomAD) database (gnomad.broadinstitute.org), the rs202152674/R476C and rs137886839/S502L variants showed 0.02% and 0.3% allele frequency in the Finnish and 0.009% and 0.02% in the non-Finnish Europeans, respectively. The allele frequencies of both variants were the highest in the East Asian populations, 0.2% and 1%, respectively. Therefore, we sought in silico replication of these variants in 11,097 Japanese individuals with type 2 diabetes[17].

However, no copies of the variants were identified. We genotyped the identified missense variants in 3,052 subjects with type 1 diabetes. Heterozygous genotypes for R476C and S502L of one sib pair already sequenced with NimbleGen sequencing were verified, and a total of seven individuals were heterozygous for the R476C mutation and 15 individuals heterozygous for the S502L mutation (Table 3). Targeted sequencing verified the genotypes for R476C and S502L mutation carriers. Approximately 30% of both R476C and S502L carriers had PDR. While very sparse ophthalmic data were available for the other R476C carriers, 20% of the S502L carriers had only mild retinopathy, and 20% had no retinopathy despite long duration (≥ 15 years) of diabetes.

For each individual with a mutation, four control subjects were matched for age, sex, and duration, but no differences occurred between the case and the control subjects except for higher total cholesterol values in those with the S502L mutation (5.41 mmol/L) compared with the matched control subjects (4.79 mmol/L) (P = 0.047) (Supplementary Table 4). Interestingly, the mean duration of diabetes to PDR was ~16–17 years in the S502L and R476C carriers, while the average (SD) in the FinnDiane population is 21.4 (7.6) years, suggesting that PDR develops faster in the mutation carriers. However, formal survival analysis was not calculated because of the small number of observations.

CACNB2 gene expression was detected in multiple tissues, including retina, in the Functional Annotation of Mammalian Genomes (FANTOM5) data [30]. *CACNB2* encodes the β2 subunit of the L-type calcium channel. While the channel can have one of the β1, β2, β3, or β4 subunits, the β2 subunit has the highest mRNA expression in retina (37.8 tags per million [tpm] vs. β1, 6.9 tpm; β3, 9.3 tpm; and β4, 8.5 tpm).

Functional Role of the *CACNB2* Gene

in Retinal Pigmented Cell

To explore the role of the *CACNB2* gene for the function of the L-type Ca21 channels, we tested the expression of the *CACNB2* gene in undifferentiated retinal origin cells, ARPE19 and MIO-M1 cells. *CACNB2* was abundantly expressed at the mRNA level in ARPE19 cells (Fig. 4A) and at the protein level in ARPE19 and MIO-M1 cells (Fig. 4B). Additionally, we found a 12-fold higher expression of *CACNB2* mRNA in the differentiated hESC-RPE compared with undifferentiated ARPE19 cells (Fig. 4C). Based on the findings from a previous study, where the authors showed a role of L-type Ca21 channels for the regulation of VEGF secretion in normal retinal pigmented epithelium (RPE) cells [31], we knocked down the *CACNB2* by using RNA interference, which led to a significant decrease in the *VEGF* mRNA levels (Fig. 4D) and almost twofold decrease in the VEGF secretion by ARPE19 cells in culture medium (Fig. 4E). Furthermore, we knocked down *CACNB2* in the Müller cell line (MIO-M1), as Müller cell– derived VEGF has been shown to play a crucial role in diabetes-induced inflammation and vascular leakage [32,33]. We observed ~30% reduction in the secreted VEGF protein in cell culture medium of

subunit of the L-type voltage-dependent calcium channel. Interestingly, only the L-type voltage-dependent calcium channels are sensitive to calcium channel blockers that are used as antihypertensive treatment [31]. *CACNB2* may be involved in the pathogenesis of PDR through a pathway by which calcium channels regulate vascular endothelial growth factor (VEGF) expression and release in the retinal pigment epithelium [31,42]; in particular, previous work shows that the L-type Ca^{2+} channels participate in the regulation of VEGF secretion in hESC-RPE cells [43]. Furthermore, our *CACNB2* knockdown experiments in ARPE19 and MIO-M1 cell lines show its role in VEGF regulation in these cells. VEGF, in turn, plays an essential role in angiogenesis and the development of diabetic retinal neovascularization by increasing delivery of oxygen and energy substrates [44–46] and is thereby involved in stimulating microaneurysm formation, capillary occlusion, and enhancement of vascular permeability at the early stages of diabetic retinopathy [47]. Inhibition of VEGF prevents ocular neovascularization in animal models. From the clinical point of view, it is of note that anti-VEGF treatment is used to treat macular edema in humans and is also considered

a potential treatment for PDR [48]. Long-term results are, however, still lacking.

While the highest linkage peak was located within the *CACNB2* gene, it should be noted that the 10p12 region contains 48 additional protein-coding genes; variants near two of these genes, PLXDC2 and MALRD1, located 500 kb from *CACNB2*, have been suggestively associated with diabetic retinopathy in individuals with type 2 diabetes in GWAS studies [49,50]. PLXDC2 is involved in endothelial cell angiogenesis and may thus play a role in mediating the development and progression of diabetic retinopathy similarly as VEGF [51,52]. Altogether, GWAS have identified only a very few loci for PDR despite substantial reported heritability [10,11]. Of note, GWAS on PDR are limited in number of participants at the discovery stage—at the most, a few thousand, very few GWAS meta-analyses have so far been published, and only a few genome-wide significant findings have been successfully replicated in other studies [12,14].

The major strengths of this study are the large number of individuals, a comprehensive phenotypic characterization of the individuals with type 1 diabetes, and the availability of both linkage and GWAS data. The sample size is crucial in association *CACNB2* knockdown cells compared with control scrambled siRNAs.

DISCUSSION

In linkage analysis of type 1 diabetes sib pairs, we detected evidence of linkage (LOD score 2.73) between PDR and chromosome 10p12 (D10S548 in *CACNB2)*. Subsequent candidate gene association analysis showed the lowest *P* value for PDR at rs11014284 ($P = 8.6 \times 10^{-4}$) in the vicinity of D10S548. By next-generation sequencing, we discovered two missense mutations (R476C/rs20 2152674 and S502L/rs137886839) predicted to have an impact on the protein function and located in the same region as the linkage and the association findings (Fig. 3). Even though the two *CACNB2* variants do not seem to have any major influence on PDR, as the mutations occurred only in a few individuals, we nevertheless speculate that the variants may play a role in the pathogenesis of PDR: the individuals with the mutation had indeed a shorter mean duration of diabetes until PDR (16–17 years) compared with the rest of the FinnDiane population (21 years), despite a similar PDR prevalence (33% of 1,117 FinnDiane individuals [34]). The

R476C mutation is of particular interest, since introduction of an additional cysteine residue may disturb the usual pairing of cysteine residues and lead to the formation of unnatural disulfide bonds within the multimers.

Many tissues express *CACNB2* in the inner surface of the cell membrane, and gene expression was detected in the retinal tissue; furthermore, *CACNB2* was detected in the ARPE19 cell line, iPSC-derived RPE cells, and MIO-M1 cell line. It is of note that alternatively spliced variants of the gene have been identified [35]. Interestingly, the *CACNB2* knockout is associated with night blindness and altered retinal morphology in mice, while knockout of the alternative β1, β3, or β4 subunits did not show any effect [36]. *CACNB2* has also been linked to the Brugada syndrome (i.e., abnormal electrical activity within the heart) [37], sudden cardiac death syndrome with arrhythmia, hypertension [38], Alzheimer disease, and migraine in man [39,40]. In addition, GWAS showed association between a number of psychiatric disorders (autism spectrum disorder, attention deficit-hyperactivity disorder, major depressive disorder, bipolar disorder, and schizophrenia) and CACNB2 [41].

The *CACNB2* gene encodes the β2 studies because the statistical power is enhanced with larger sample sizes. We had a reasonably large sample size in comparison with other genetic studies on PDR [11], considering that both the PDR case and the control subjects had to have type 1 diabetes. By using both family-based and case-control approaches, we covered both genetic linkage and association based on linkage disequilibrium. What makes the findings of this study interesting is that the linkage finding suggests a rare variant with high penetrance, but it also replicates as an association with a common variant. The common variant, however, is probably not directly responsible for the disease susceptibility; it is more likely that it is in linkage disequilibrium with the truly functional variant. Further functional studies are required to understand the role of these variants in the susceptibility to DR.

A weakness of this study is the lack of replication of the two identified missense mutations in other studies. While they are rare in the European population, both were identified with a slightly higher frequency in East Asian populations (MAF 0.2% and 1% for rs202152674/R476C and rs137886839/S502L, respectively). However, neither variant was found in the in silico

replication data in >11,000 Japanese individuals with type 2 diabetes. Another limitation of this study is that the classification of diabetic retinopathy is based on the presence or absence of laser-treated retinopathy. Laser treatment correlates with PDR, but laser treatment can be given already at earlier stages for severe nonproliferative retinopathy or macular edema. We have previously shown that in individuals with type 1 diabetes, the majority (>80%) of laser treatment is due to PDR [15]. Furthermore, the reason for laser treatment was confirmed to be PDR in the sib pair analysis. Control subjects used in the candidate gene association analysis were required to have at least 15 years' duration of diabetes without PDR. This limit was chosen because the incidence peak of PDR may occur already at 15–20 years after the onset of diabetes, as shown in the Wisconsin study [2]. However, the incidence reported in these older studies may not reflect the PDR incidence today, as there seems to be a declining trend in the cumulative incidence of PDR [53].

In summary, we found evidence of linkage and association between PDR and a novel locus on 10p12 in the *CACNB2* gene and a role of *CACNB2* in VEGF secretion in cell cultures. In addition, two missense mutations were identified in the same locus. While the role of *CACNB2* has previously been described for retinal phenotypes in mouse knockout models, this is the first report linking genetic variation in *CACNB2* to human PDR. As calcium channel blockers targeting the L-type calcium channels are already in clinical use to treat hypertension, and calcium channels regulate VEGF, these findings on *CACNB2* open up novel translational possibilities for treatment of human PDR. Additional functional studies are being carried out in our laboratory to further understand the role of these *CACNB2* mutations in the pathogenesis of PDR.

Acknowledgments. The skilled technical assistance of Maikki Parkkonen, Hanna Olanne, Anna Sandelin, Mira Korolainen, and Jaana Tuomikangas (Folkhälsan Research Center, Helsinki, Finland) and Outi Melin and Hanna Pekkanen (Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland) is gratefully acknowledged. The authors also acknowledge all the physicians and nurses at each center participating in the collection of patient data (Supplementary Table 5).

Funding. This study was supported by grants from Academy of Finland (275614, 299200, and 316664), Novo Nordisk Foundation (NNF Oc0013659), Folkhälsan Research Foundation, Wilhelm and Else Stockmann Foundation, Liv och Hälsa Society, the Helsinki University Hospital Research Funds (EVO), Päivikki and Sakari Sohlberg Foundation, European Foundation for the Study of Diabetes Young Investigator Research Award funds, Diabetes Research Foundation, Diabetes Wellness Finland, Eye Foundation, Finland, and Mary and Georg C. Ehrnrooth Foundation. Genotyping of the GWAS data was funded by JDRF within the Diabetic Nephropathy Collaborative Research Initiative (grant 17-2013-7), with GWAS quality control and imputation performed at University of Virginia. Japanese GWAS was supported by a grant from the Tailor-Made Medical Treatment Program (the BioBank Japan Project) of the Ministry of Education, Culture, Sports, Science, and Technology and from the Japan Agency for Medical Research and Development (18km040 5202h0803).

Funding agencies did not contribute to the study design, the conduct of the study, data analysis, interpretation of findings, writing of the manuscript, or the decision to submit the manuscript for publication.

Duality of Interest. P.-H.G. has received research grants from Eli Lilly and Roche; is an advisory board member for AbbVie, Astellas, AstraZeneca, Boehringer Ingelheim, Cebix, Eli Lilly, Janssen, Merck Sharp & Dohme (MSD), Medscape, Mundipharma, Nestlé, Novartis, Novo Nordisk, and Sanofi; and has received lecture fees from AstraZeneca, Boehringer Ingelheim, Eli Lilly, Elo Water, Genzyme, MSD, Mundipharma, Novartis, Novo Nordisk, PeerVoice, and Sanofi. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. N.V. had the main responsibility for analysis and interpretation of the data and writing the manuscript. N.S., A.K., K.H., and A.S. contributed to data analysis. N.S., A.K., K.H., C.F., M.L., and P.-H.G. designed the study. N.S., K.H., C.F., P.A.S., M.L., and P.-H.G. contributed to acquisition of data. A.S., K.J.-U., and H.S. contributed to producing research material. M.I. and S.M. contributed to in silico replication. N.S., A.K., K.H., M.L., and P-H.G. contributed to interpretation of data and editing the manuscript. All authors revised the manuscript critically for important intellectual content. All authors approved the final

version of the manuscript to be published. P.-H.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented in abstract form at the 48th Annual Meeting of the European Association for the Study of Diabetes, Berlin, Germany, 1–5 October 2012.

References

1. Cheung N,Mitchell P,Wong TY. Diabetic retinopathy. Lancet 2010; 376:124–136
2. Klein R. The epidemiology of diabetic retinopathy: findings from the Wisconsin Epidemiologic Study of Diabetic Retinopathy. Int Ophthalmol Clin 1987; 27:230–238
3. Rossing K, Jacobsen P, Rossing P, Lauritzen E, Lund-Andersen H, Parving HH. Improved visual function in IDDM patients with unchanged cumulative incidence of sight-threatening diabetic retinopathy. Diabetes Care 1998;21:2007–2015
4. Deckert T, Simonsen SE, Poulsen JE. Prognosis of proliferative retinopathy in juvenile diabetics. Diabetes 1967; 16: 728–733
5. Nathan DM, Genuth S, Lachin J, et al.; Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 1993; 329:977–986
6. Kollias AN, Ulbig MW. Diabetic retinopathy: early diagnosis and effective treatment. Dtsch Arztebl Int 2010;107:75–83
7. Hutton DW, Stein JD, Bressler NM, Jampol LM, Browning D, Glassman AR; Diabetic Retinopathy Clinical Research Network. Cost-effectiveness of intravitreous ranibizumab compared with panretinal photocoagulation for proliferative diabetic retinopathy: secondary analysis from a Diabetic Retinopathy Clinical Research Network randomized clinical trial. JAMA Ophthalmol 2017;135:576–584
8. Zhao Y, Singh RP. The role of anti-vascular endothelial growth factor (anti-VEGF) in the management of proliferative diabetic retinopathy. Drugs Context 2018;7:212532
9. Klein R, Knudtson MD, Lee KE, Gangnon R, Klein BE. The Wisconsin Epidemiologic Study of Diabetic Retinopathy: XXII the twenty-five-year progression of retinopathy in persons with type 1 diabetes. Ophthalmology 2008; 115:

1859–1868

10. Hietala K, Forsblom C, Summanen P, Groop PH; FinnDiane Study Group. Heritability of proliferative diabetic retinopathy. Diabetes 2008;57:2176 – 2180
11. Dahlström E, Sandholm N. Progress in defining the genetic basis of diabetic complications. Curr Diab Rep 2017;17:8 0
12. Burdon KP, Fogarty RD, Shen W, et al. Genome-wide association study for sight-threatening diabetic retinopathy reveals association with genetic variation near the GRB2 gene. Diabetologia 2015; 58: 2288– 2297
13. Pollack S, Igo RP Jr, Jensen RA, et al.; Family Investigation of Nephropathy and Diabetes-Eye Research Group, DCCT/EDIC Research Group. Multiethnic genome-wide association study of diabetic retinopathy using liability threshold modeling of duration of diabetes and glycemic control. Diabetes 2019;68: 441–456
14. Meng W, Shah KP, Pollack S, et al.; Wellcome Trust Case Control Consortium 2 (WTCCC2), Surrogate markers for Micro- and Macro-vascular hard endpoints for Innovative diabetes Tools (SUMMIT) Study Group. A genome-wide association study suggests new evidence for an association of the NADPH Oxidase 4 (NOX4) gene with severe diabetic retinopathy in type 2 diabetes. Acta Ophthalmol 2018; 96: e811– e819
15. Hietala K, Wadén J, Forsblom C, et al.; FinnDiane Study Group. HbA1c variability is associated with an increased risk of retinopathy requiring laser treatment in type 1 diabetes. Diabetologia 2013;56: 737–745
16. Davis MD, Fisher MR, Gangnon RE, et al. Risk factors for high-risk proliferative diabetic retinopathy and severe visual loss: Early Treatment Diabetic Retinopathy Study report #18. Invest Ophthalmol Vis Sci 1998;39: 233–252
17. Taira M, Imamura M, Takahashi A, et al.; SUMMIT Consortium. A variant within the FTO confers susceptibility to diabetic nephropathy in Japanese patients with type 2 diabetes. PLoS One 2018;13: e0208654
18. Goldstein JI, Crenshaw A, Carey J, et al.; Swedish Schizophrenia Consortium; ARRA Autism Sequencing Consortium. zCall: a rare variant caller for array-based genotyping: genetics and population analysis. Bioinformatics 2012;28:2543– 2545
19. Syreeni A, Sandholm N, Cao J, et al.; DCCT/EDIC Research Group; FinnDiane Study Group. Genetic determinants of glycated hemoglobin in type 1 diabetes. Diabetes 2019;68: 858–

867

20.Das S, Forer L, Schönherr S, et al. Next-generation genotype imputation service and methods. Nat Genet 2016;48:1284–1287

21. Schwarz JM, Cooper DN, Schuelke M, Seelow D. Mutation Taster2: mutation prediction for the deep-sequencing age. Nat Methods 2014;11:361–362

22. Lawrence JM, Singhal S, Bhatia B, et al. MIO-M1 cells and similar muller glial cell lines derived from adult human retina exhibit neural stem cell characteristics. Stem Cells 2007; 25:2033–2043

23.Vaajasaari H, Ilmarinen T, Juuti-Uusitalo K, et al. Toward the defined and xeno-free differentiation of functional human pluripotent stem cell-derived retinal pigment epithelial cells. Mol Vis 2011; 17:558–575

24.S.A.G.E. 6.3 [2012]. statistical analysis for genetic epidemiology. 2012, 2016

25.Haseman JK, Elston RC. The investigation of linkage between a quantitative trait and a marker locus. Behav Genet 1972;2:3–19

26. Nyholt DR. All LODs are not created equal. Am J Hum Genet 2000;67: 282– 288

27.Lander E, Kruglyak L. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat Genet 1995;11: 241–247

28.Zhan X, Hu Y, Li B, Abecasis GR, Liu DJ. RVTESTS: an efficient and comprehensive tool for rare variant association analysis using sequence data. Bioinformatics 2016;32:1423–1426

29.Li MX, Yeung JM, Cherny SS, Sham PC. Evaluating the effective numbers of independent tests and significant p-value thresholds in commercial genotyping arrays and public imputation reference datasets. Hum Genet 2012;131: 747– 756

30.Takahashi H, Lassmann T, Murata M, Carninci P. 59 end-centered expression profiling using cap-analysis gene expression and next-generation sequencing. Nat Protoc 2012;7:542–561

31. Rosenthal R, Heimann H, Agostini H, Martin G, Hansen LL, Strauss O. Ca21 channels in retinal pigment epithelial cells regulate vascular endothelial growth factor secretion rates in health and disease. Mol Vis 2007;13: 443–456

32. Wang J, Xu X, Elliott MH, Zhu M, Le YZ. Müller cell–derived VEGF is essential for diabetes-induced retinal inflammation and vascular leakage. Diabetes 2010;59:2297 –2305

33. Fu S, Dong S, Zhu M, et al. Müller glia are a major cellular source of

survival signals for retinal neurons in diabetes. Diabetes 2015;64:3554–3563

34. Hietala K, Harjutsalo V, Forsblom C, Summanen P, Groop PH; FinnDiane Study Group. Age at onset and the risk of proliferative retinopathy in type 1 diabetes. Diabetes Care 2010; 33:1315–1319

35. Takahashi SX, Mittman S, Colecraft HM. Distinctive modulatory effects of five human auxiliary β2 subunit splice variants on L-type calcium channel gating. Biophys J 2003;84: 3007–3021

36. Ball SL, Powers PA, Shin HS, Morgans CW, Peachey NS, Gregg RG. Role of the beta(2) subunit of voltage-dependent calcium channels in the retinal outer plexiform layer. Invest Ophthalmol Vis Sci 2002;43:1595–1603

37. Brugada P, Brugada J. Right bundle branch block, persistent ST segment elevation and sudden cardiac death: a distinct clinical and electrocardiographic syndrome. A multicenter report. J Am Coll Cardiol 1992;20:1391–1396

38. Levy D, Ehret GB, Rice K, et al. Genome-wide association study of blood pressure and hypertension. Nat Genet 2009; 41:677–687

39. Liang X, Slifer M, Martin ER, et al. Genomic convergence to identify candidate genes for Alzheimer disease on chromosome 10. Hum Mutat 2009;30:463–471

40. Nyholt DR, LaForge KS, Kallela M, et al. A high-density association screen of 155 ion transport genes for involvement with common migraine. Hum Mol Genet 2008;17:3318–3331

41. Cross-Disorder Group of the Psychiatric Genomics Consortium. Identification of risk loci with shared effects on five major psychiatric disorders: a genome-wide analysis. Lancet 2013;381: 1371–1379

42. Capiod T. Cell proliferation, calcium influx and calcium channels. Biochimie 2011;93: 2075–2079

43. Korkka I, Viheriälä T, Juuti-Uusitalo K, et al. Functional voltage-gated calcium channels are present in human embryonic stem cell-derived retinal pigment epithelium. Stem Cells Transl Med 2019;8:179–193

44. Miller JW, Adamis AP, Aiello LP. Vascular endothelial growth factor in ocular neovascularization and proliferative diabetic retinopathy. Diabetes Metab Rev 1997;13:37–50

45. Yang Y, Hayden MR, Sowers S, Bagree SV, Sowers JR. Retinal redox stress and remodeling in cardiometabolic syndrome and diabetes. Oxid Med Cell Longev

2010;3:392–403

46.Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA. Vascular endothelial growth factor and angiogenesis. Pharmacol Rev 2004; 56:549–580

47.Chakrabarti S, Cukiernik M, Hileeto D, Evans T, Chen S. Role of vasoactive factors in the pathogenesis of early changes in diabetic retinopathy. Diabetes Metab Res Rev 2000;16: 393– 407

48.Gross JG, Glassman AR, Liu D, et al.; Diabetic Retinopathy Clinical Research Network. Five-year outcomes of panretinal photocoagulation vs intravitreous ranibizumab for proliferative diabetic retinopathy: a randomized clinical trial. JAMA Ophthalmol 2018;136:1138–1148

49.Huang YC, Lin JM, Lin HJ, et al. Genome-wide association study of diabetic retinopathy in a Taiwanese population. Ophthalmology 2011;118: 642– 648

50.Grassi MA, Tikhomirov A, Ramalingam S, Below JE, Cox NJ, Nicolae DL. Genome-wide meta-analysis for severe diabetic retinopathy. Hum Mol Genet 2011;20:2472–2481

51. Awata T, Inoue K, Kurihara S, et al. A common polymorphism in the 59-untranslated region of the VEGF gene is associated with diabetic retinopathy in type 2 diabetes. Diabetes 2002;51: 1635 –1639

52.Aiello LP, Avery RL, Arrigg PG, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med 1994;331: 1480–1487

53.Kytö JP, Harjutsalo V, Forsblom C, Hietala K, Summanen PA, Groop PH; FinnDiane Study Group. Decline in the cumulative incidence of severe diabetic retinopathy in patients with type 1 diabetes. Diabetes Care 2011;34:2005–2007

54.Pruim RJ, Welch RP, Sanna S, et al. LocusZoom: regional visualization of genome-wide association scan results. Bioinformatics 2010;26: 2336–2337

Credits: CACNB2 Is a Novel Susceptibility Gene for Diabetic Retinopathy in Type 1 Diabetes Nadja Vuori, Niina Sandholm, Anmol Kumar, Kustaa Hietala, Anna Syreeni, Carol Forsblom, Kati Juuti-Uusitalo, Heli Skottman, Minako Imamura, Shiro Maeda, Paula A. Summanen, Markku Lehto, Per-Henrik Groop Diabetes Nov 2019, 68 (11) 2165-2174; DOI: 10.2337/db19-0130