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 $8.6 \times 10^{-4}$ 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 intravitreal 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.
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 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 $\geq 61$ 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).

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 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 (Génethon 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-omp v1.0.14 (20) using 1000 Genomes as reference population. We excluded 331 parents, 395 individuals with no data on diabetes onset year; 278 individuals with no data on laser treatment available; and 1,010 control subjects with diabetes type other than type 1 diabetes, or no data on diabetes onset year; 1,010 control subjects with diabetes duration 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/seqcapt/) 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 primers used are described in the Supplementary Table 1. MutationTaster 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 (11965092; 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 × 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 μmol/L) were transfected twice at 48-h intervals with Lipofectamine RNAiMAX Transfection Reagent (13778075;
In our study, LOD scores were calculated from empirical P values. *Interval between adjacent markers.*

### 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 × 10^{-8}) 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 non-significant (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^{-8} for association with PDR was a common SNP, rs11014284, with an odds ratio of 0.83 (95% CI 0.74–0.92) (MAF = 0.1%).

### 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. The candidate gene association study is available in Supplementary Tables 2 and 3.

#### Candidate Gene Association Analysis of CACNB2

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).
27.8%) (Fig. 3). The effective number of independent SNPs with MAF $\geq 0.1\%$ was estimated as 1,578, resulting in a significance threshold of $P$ value $3.17 \times 10^{-5}$ 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 $\sim 16\text{–}17$ 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 $\beta 2$ subunit of the L-type calcium channel. While the channel can have one of the $\beta 1$, $\beta 2$, $\beta 3$, or $\beta 4$ subunits, the $\beta 2$ subunit has the highest mRNA expression in retina ($37.8\text{ tags per million [tpm]}$ vs. $\beta 1$, 6.9 tpm; $\beta 3$, 9.3 tpm; and $\beta 4$, 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 $Ca^{2+}$ 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 Ca\textsuperscript{2+} 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

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

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

Data are median (range) or n (%) unless otherwise indicated. *Duration of diabetes to PDR is calculated among the participants with PDR.
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.
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 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 × 10^{-5}) in the vicinity of D10S548. By next-generation sequencing, we discovered two missense mutations (R476C/rs202152674 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 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 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 rs13786839/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.

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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.

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